Cloning the *Cercospora zeina Ecp2* effector gene for *Agrobacterium*-mediated transient transformation

by

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Declaration of Originality

I, ... declare that the thesis, which I hereby submit for the degree ..... at the University of Pretoria, is my own work and has not previously been submitted by me for a degree at this or any other tertiary institution.

SIGNATURE: .....

DATE: .....

Abstract

Maize (*Zea mays*) is a staple crop in Africa that is under severe threat to disease by pathogenic organisms. Grey leaf spot (GLS) caused by *Cercospora zeina* drastically limits the yield and quality of maize produced. Not enough is understood about how *C. zeina* causes GLS, but it is known that it is a maize-specific hemibiotrophic fungus. Proteins called effectors are essential for the virulence of pathogens such as *Cladosporium fulvum.* Extracellular protein 2 (Ecp2), an effector identified in some Dothideomycete fungi, has an unknown function but has been shown to play a role in the virulence of the fungi. The aim of this study was to clone *C. zeina Ecp2* (*CzEcp2*) into a binary vector for agroinfiltration of *Nicotiana* spp. The *C. zeina* genome and RNA sequence data (*in planta* and *in vitro*) were searched for a candidate *Ecp2* gene. The complete *CzEcp2* sequence (with the fungal signal peptide) and the mature sequence (lacking the fungal signal peptide) were cloned into the pTRAkc-ERH binary vector. pTRAkc-ERHCzEcp2 EB (fungal signal peptide) and pTRAkc-ERHCzEcp2 NB (LPH signal peptide) were respectively transformed into *Agrobacterium tumefaciens* GV3101 (pSOUP+pMP90). Phytophthora *infestans* INF1 was used as the positive control for HR expression. Untransformed *Agrobacterium* and the pTRAkc-ERH empty vector were used as negative controls. *Nicotiana benthamiana, Nicotiana tabacum* cv. Petit Havana and *Nicotiana tabacum* cv. LA Burley were then transiently agroinfiltrated. The plants were monitored for a hypersensitive response (HR) for 10 days. *CzEcp2* expression did not result in HR for the three *Nicotiana* spp., but chlorosis was observed. INF1 caused a HR in all three *Nicotiana* spp. and the negative controls did not cause any changes. The lack of HR where *CzEcp2* was expressed, may be due to lack of CzECP2 transport and recognition, a delayed HR or that the T-DNA was not adequately transferred into the host cells.

Preface

The research presented in the following thesis was conducted in the laboratories of the Molecular Plant-Pathogen Interactions research group at the University of Pretoria, South Africa.

Grey leaf spot is known to cause extensive losses of maize yield. In South Africa, this disease is caused by *Cercospora zeina*. The infection strategy of this fungus is unknown, therefore more research into understanding the molecular mechanisms it uses during host invasion is essential. Maize being a staple crop in Africa emphasises the importance of studying and understanding the pathogens and diseases that impact its productivity. To meet the United Nations Sustainable Development Goals, food production must be increased so it can reach all parts of the world, including Africa amongst others.

Recent studies have shown that the effector biology of a pathogen plays a role in how it causes infection in hosts. Many effector proteins have been identified to date, especially in the tomato pathogen, *Cladosporium fulvum*. They have been shown to be essential for host invasion, fungal growth during invasion and for evasion of host detection. The presence or absence of specific effectors can have profound effects on a pathogens ability to cause disease. Therefore, studying which effectors maize pathogens have can shed light onto how they attack the host and reduce productivity. In future, effector biology can be used as a basis for breeding resistant crop varieties, and ultimately reduce the amount of harmful chemicals currently being used for pathogen control.

The **first aim of this research** was to confirm the presence of the extracellular protein 2 (*Ecp2*) effector gene in the *C. zeina* genome. The **second aim** was to clone the effector into a binary vector for *Agrobacterium*-mediated transient infiltration of *Nicotiana* spp. The hypothesis of this study was that *CzEcp2* will cause a hypersensitive response in *Nicotiana* spp.

Chapter 1 is a literature review which provides background on the pathogen of interest, *C. zeina* and its host plant, maize. The chapter gives a brief overview on food security worldwide and global maize production according to recent statistics from the Food and Agriculture Organisation (FAO) of the United Nations (UN). *C. zeina*, its characteristic disease symptoms and its life cycle are discussed next. Insight is then given into the effector biology of various pathogens, especially those discovered in Dothideomycete fungi, and how it influences pathogen virulence and pathogenicity. Lastly the use of *Agrobacterium tumefaciens*-mediated transformation of plants, especially *Nicotiana* spp. is discussed.

Chapter 2 describes the research done in this study and the results obtained from identifying and cloning *Ecp2* into a binary vector for transformation studies. A homolog of *C. fulvum Ecp2* was found in the *C. zeina* genome and transcriptome. The effector gene was cloned into a binary vector, pTRAkc-ERH with and without its signal peptide. The two constructs carrying the genes of interest

were transformed into *Agrobacterium tumefaciens* GV3101 (pSOUP+pMP90). Of the two constructs, only the one carrying the complete *CzEcp2* sequence with the signal peptide was transiently transformed into *Nicotiana* spp. The plants were monitored for a hypersensitive response for 10 days.

Chapter 3 rounds of this thesis by discussing some conclusions drawn from the findings of this research and how they can be applied to future work. Some suggestions for future work include how the study can be improved and which methods can alternatively be used. The chapter also suggests and describes and what kind of biotechnology innovations can be created based on *C. zeina* effector biology.

The following outputs have been generated from this research to date:

Segal CC, Berger DK (2019) Cloning a *Cercospora zeina Ecp2* effector gene for *Agrobacterium*-mediated transient transformation

- 30th SANSOR (South African National Seed Organisation) Congress;
    - Abstract submission
    - Poster presentation
    - Winner of the Best MSc Research Poster presentation
- 2019 IS-MPMI XVII{ Congress;
    - Abstract submission
    - Poster presentation
    - 2019 Ko Shimamoto Travel Awardee
- 2019 Department of Plant and Soil Sciences Postgraduate Symposium;
    - Research presentation

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I'd like to extend my appreciation to the National Research Foundation (NRF) and Bayer (Monsanto) for granting me scholarships from 2018-2019 to complete my master's degree fulltime.

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Thank you to the members of the Molecular Plant-Pathogen Interactions (MPPI) research group at the University of Pretoria for their invaluable input and discussions centred around my project. Thank you to the Forestry and Agricultural Biotechnology Institute (FABI) and the Department of Plant and Soil Sciences at UP for creating a research environment where students can learn and grow. The seminars and conferences have enriched my skills immensely.

Thank you to all my loved ones, especially my parents, Siya and Kelly for your consistent support and prayers throughout my academic career. Thank you all for sacrificing your time, money and energy and for investing in my growth above your own at times. I especially thank you Siya ☾, for staying up with me in the lab and while writing up, for encouraging me to be strong and reminding me that I am worth it. My degree would have been unbearable if I had to go through it without any of you.

Lastly, to God be the glory!

"Be strong and courageous. Do not be afraid; do not be discouraged, for the Lord your God will be with you wherever you go" Joshua 1:9.

List of Abbreviations

°C	Degrees Celsius
µl	Microliter
µM	Micromolar
µg/ml	Microgram per litre
A. tumefaciens	*Agrobacterium tumefaciens*
Avr	Avirulence
BAK1	BRASSINOSTEROID INSENSITIVE-1 ASSOCIATED KINASE 1
BLAST	Basic Local Alignment Search Tool
Bp	Base pairs
$CaCl_2$	Calcium chloride
CERK1	CHITIN ELICITOR RECEPTOR KINASE 1
cDNA	Complementary DNA
CRISPR	Clustered Regularly Interspaced Short Palindromic Repeats
CTB	Cercosporin toxin biosynthesis
C. zeina	*Cercospora zeina*
C. heterostrophus	*Cochliobolus heterostrophus*
CfEcp2	*Cladosporium fulvum* extracellular protein 2
CzEcp2	*Cercospora zeina* extracellular protein 2
ddH_2O	Double distilled water
DNA	Deoxyribonucleic acid
E. coli	*Escherichia coli*
EtBr	Ethidium bromide
Ecp2	Extracellular protein 2
ETI	Effector triggered immunity
ETD	Effector triggered defence
ETS	Effector triggered susceptibility
E. turcicum	*Exserohilum turcicum*
FAO	Food and Agriculture Organisation
gDNA	Genomic DNA
g	Grams
GLS	Grey Leaf Spot
GFP	Green fluorescent protein
GM	Genetic modification
hr	Hours
HR	Hypersensitive response
L	Litre
LB	Left border
LB	Luria-Bertani media
MfEcp2	*Mycosphaerella fijiensis Ecp2*
mg/l	Miligram per litre
min	Minute
ml	Mililiter
mM	Milimolar
mRNA	Messenger RNA
ng/µl	Nanogram per microliter
NCBI	National Centre for Biotechnology Information
NCLB	Northern Corn Leaf Blight
OD600	Optical density at wavelength 600 nm
PAMPs	Pathogen associated molecular patterns
PCR	Polymerase Chain Reaction
PDA	Potato Dextrose Agar
PRRs	Pattern recognition receptors

PTI	PAMP triggered immunity
QTL	Quantitative trait loci
RB	Right border
RNA	Ribonucleic acid
ROS	Reactive oxygen species
RPM	Revolutions per minute
R-protein	Resistance protein
RT-PCR	Reverse transcription PCR
SDG	Sustainable development goals
SLB	Southern leaf blight
T-DNA	Transfer deoxyribonucleic acid
Ti	Tumour inducing
UN	United Nations
UV	Ultraviolet
V	Volts
UTR	Untranslated region
vir genes	Virulence genes
WHO	World Health Organisation

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Chapter 1

Literature Review: Molecular Plant-Pathogen Interactions

1.1 Introduction

Plants are sessile, and therefore cannot move to escape disease or pathogens. They are naturally adapted to fight off attack from microbes that could be potential pathogens (Kettles et al. 2017). Plants have two ways of responding to microorganisms, either by using pattern recognition receptors (PRRs) (transmembrane proteins) that recognise pathogen associated-molecular patterns (PAMPs) or by using R (resistance) proteins that recognise effectors (Jones and Dangl 2006). PAMPs are molecular patterns released by microbes, for example chitin in a fungal cell wall or bacterial flagellin. PRRs recognise PAMPs and trigger the first line of plant defence known as PAMP-triggered immunity (PTI). (Kettles et al. 2017; Lo Presti et al. 2015; Vleeshouwers and Oliver 2014).

For fungal pathogens to establish infection in host plants, they must avoid eliciting PTI, suppress it or cope with it. They can achieve this by releasing fungal effectors or secondary metabolites that kill the host plant cells (Lo Presti et al. 2015). Vleeshouwers and Oliver (2014) defined effectors as pathogen-produced molecules that have a distinct effect on one or more genotypes of host or non-host plants. Effector proteins are recognised by cognate R-proteins within host plants after which they are called avirulence (Avr) proteins. Effector triggered immunity (ETI), a stronger version of PTI, occurs when effectors are recognised by host plants. It causes a hypersensitive response (HR), or localised cell death which limits the spread of the pathogen and therefore reduces the severity of infection (Rovenich et al. 2014; Jones and Dangl 2006).

Cercospora zeina is a fungal pathogen of maize (*Zea mays*) that causes a foliar disease known as grey leaf spot (GLS). In South Africa, this pathogen was first sighted in the Kwa-Zulu Natal province and has since spread to the North-West, Mpumalanga and Free State provinces respectively (Ward et al. 1999). GLS is characterised by rectangular mature lesions that are grey to tan in colour (Ward et al. 1999). Meisel et al. (2009) proved that GLS is caused only by *C. zeina* in South Africa, whereas in the USA and other countries this same disease is also produced by its sister species, *Cercospora zeae-maydis* (Crous et al. 2006; Dunkle and Levy 2000; Ward et al. 1999). Despite the economic importance of this pathogen, little is known about the molecular mechanisms it uses to cause GLS. With the fast spread and prevalence of this disease in many countries, researchers have taken to studying the *C. zeina* infection strategy to come up with improved solutions for GLS management (Berger et al. 2014; Meyer et al. 2017).

According to the Food and Agricultural Organisation (FAO) of the United Nations (UN), one in nine people in the world today do not have access to adequate food sources and are undernourished (World Health Organization 2018). The highest prevalence of food insecurity is estimated to be in Africa because many people go hungry every other day. Many factors such as a lack of income and natural disasters have contributed toward this problem. As part of the UN initiative to reach zero hunger in the world by 2030, innovations in biotechnology are required (World Health Organization

2018). Smallholder farmers face the greatest threat by diseases that infect the crops they plant, which results in people being hungry because of smaller income and food production. Therefore, more effort should be invested towards understanding crop pathogens and eradicating diseases like GLS amongst others, in an environmentally and economically friendly manner.

At present, there is neither a maize line that has total resistance against GLS, nor a fungicide that completely eradicates the causal agent, *C. zeina*. The aim of this study was to isolate the *C. zeina* extracellular protein 2 (*Ecp2*) effector gene from the genome and clone it into a binary vector for *Agrobacterium tumefaciens*-mediated transient transformation of tobacco plants. The hypothesis was that a hypersensitive response will be observed where *CzEcp2* is expressed in the plants. The findings from this study could provide a basis for studying *C. zeina* effector biology and deciphering how it influences the *C. zeina* infection strategy. In the long run, the knowledge acquired from such transformation studies can be used in maize effector-breeding programs where maize R (resistance) genes can be identified, and hybrids can be created where the R genes are upregulated to improve resistance against *C. zeina*. Research into the use of eco-friendly fungicides based on effector biology will contribute towards a safer environment, free of the harmful chemicals that are currently used to control crop pathogens. It may also improve the quality of life for smallholder farmers who struggle to maintain their fields with expensive fungicides.

1.2 Literature Review

1.2.1 Maize (Zea mays) – A Food Security Staple

One of the United Nation's sustainable development goals (SDG) is to reach zero hunger in the world by 2030 (World Health Organization 2018). As reported by FAO in 2018, from 2016-2017 the number of undernourished people in the world has been increasing (10.9% of the world population). The estimated increase is from approximately 802 to 821 million people from 2016-2017 (Figure 1.1). The rapid rate at which the world population is growing is putting pressure on agricultural systems. The increased demand for food and increased use of biofuels has resulted in the need for higher crop productivity (Beddington 2010). Pest damage to crops during cultivation and postharvest dramatically reduce the amount of food produced (Waterfield and Zilberman 2012). Those tasked with feeding the growing world population therefore need to find ways to produce more food, that is high quality and accessible to everyone without further harming the environment.

Maize (*Zea mays*), a crop believed to have originated in Mexico, is an important cereal crop worldwide, especially in Africa where it is a staple (Ranum et al. 2017). Climate change is the biggest threat to food security in Africa due to the fast decline in arable land and low water availability (Ochieng et al. 2016; Jones and Thornton 2003). Models created to simulate climate change conditions in 2055 were used to determine the severity of the threat. It was hypothesised that innovations in plant breeding

and biotechnology could mitigate these issues of reduced crop productivity. Some countries may however need to import basic foods to compensate for crop losses due to loss of viable land and rainfall reduction (Jones and Thornton 2003).

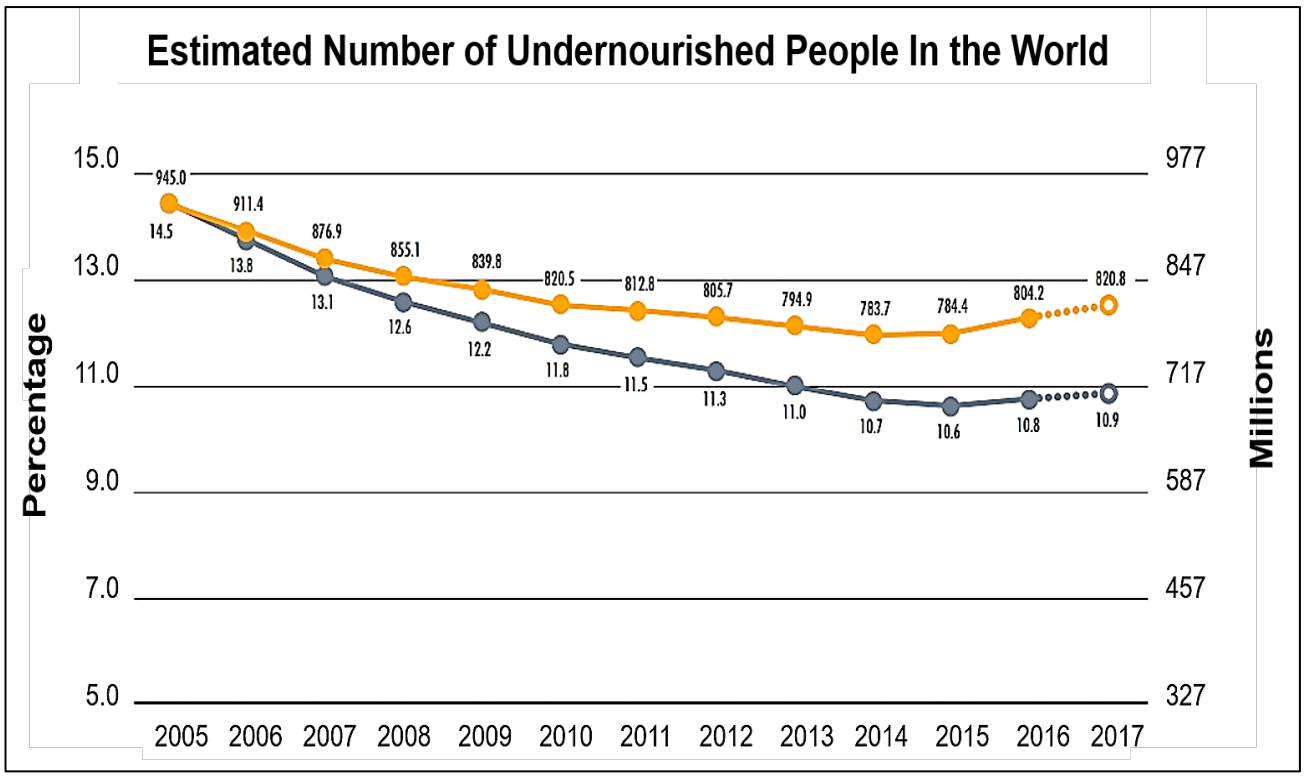


Figure 1.1: The estimated of the number of people who are undernourished in the world. *The Food and Agriculture Organisation (FAO) of the United Nations estimated that from 2016-2017, the number of undernourished people in the world increased from 802 to 821 million. The orange graph shows the amount of people in millions, while the blue graph shows the corresponding percentage of the world population from 2005-2017. The dotted lines from 2016-2017 show projected increased values from recent statistical analyses, indicating the increase in the number of people who go hungry worldwide. Adapted from: World Health Organisation 2019. Accessed on 8 June 2019 from: http://www.fao.org/faostat/en/#data/QC/visualize.*

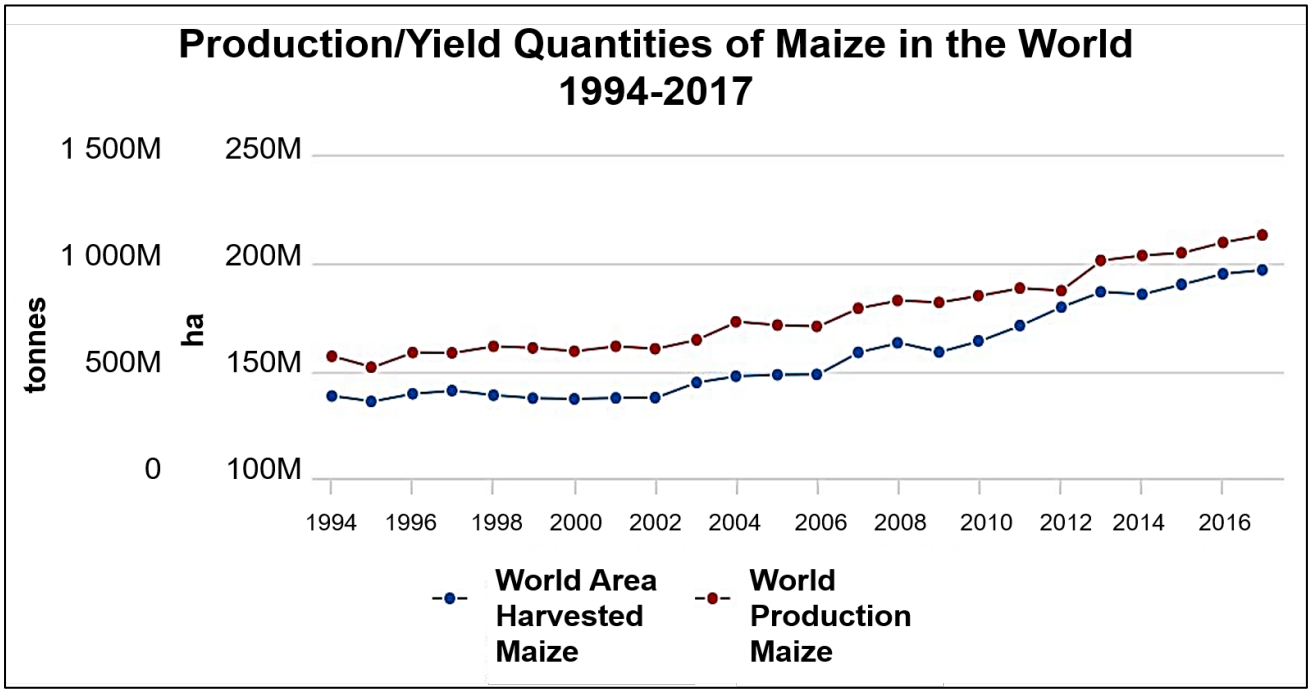
In regions of the world such as Africa and Latin America where people depend on subsistence maize farming for survival, the risk of food insecurity is high. This is due in part to farmers inability to spray expensive chemicals on their crops to control pests, poor access to water for irrigation and natural disasters that damage whole crop fields. Africa and Asia have been estimated to have the highest number of countries affected by food insecurity, with Africa (21% of the population) having the highest prevalence of undernourishment in the world (World Health Organization 2018).

Farming maize is an important source of income and subsistence for smallholder farmers in South Africa. There are various maize varieties in existence, all distinguished based on the colour of the grain produced (for example purple, yellow or white) (Ranum et al. 2017). In South Africa, white maize is the cereal of choice for human consumption and yellow maize for animal feed. Studies have shown that yellow maize is richer in micronutrients (e.g. vitamin A precursors) and that the preference for white maize consumption in SA is due to cosmetic reasons and tradition. Therefore, in countries

where white maize is eaten, biofortification of the resulting food products is essential for prevention of malnutrition (Ranum et al. 2017).

Niacin (vitamin B3), of which maize is a poor source, is an essential vitamin required for optimal human health. Given the high consumption levels of maize worldwide, steps must be taken to increase its nutrient levels to benefit even the poorest people who completely depend on this crop. In early Latin America, a process called nixtamalization was developed in which maize cobs were soaked in a calcium hydroxide solution to release consumable nutrients (such as niacin). The maize was then ground to produce masa, the paste from which tortillas were made (Caballero-Briones et al. 2000; Ranum et al. 2017). Nixtamalization can be incorporated as a maize preparation technique in Africa. It may assist in reducing malnutrition and speed up the process of achieving zero hunger by 2030 as per the SDGs (World Health Organisation 2018).

According to the FAO, overall maize production worldwide is increasing (Figure 1.2) (FAO 2019). This could be attributed to the increased use of genetically modified (GM) maize varieties and conservation agricultural practices. Genetically modified maize is grown in many countries, including South Africa, where common varieties include those bred for resistance against insects (Bt maize) and herbicide (glyphosate) tolerance (Gouse et al. 2005; Vermeulen et al. 2005; Gouse et al. 2006).



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Maize production in South Africa has increased over the years (Figure 1.3) (FAO 2019). These increases may indicate that even in the face of the negative impacts caused by climate change,

agricultural productivity is seeing positive returns. Therefore, the goal toward reducing world hunger may be attained. The planting of GM crops has been shown to dramatically reduce input costs because the need for fertilizers and pesticides is mitigated (Gouse et al. 2006). Studies have shown that smallholder farmers benefit from planting GM crops due to improved yield and reduced production costs. However, due to intellectual property rights, farmers are banned from sharing or reusing seed. Therefore, smallholder farmers do not always plant these hybrids because they are expensive. Open-pollinated varieties are often more favoured because seed can be shared, stored and planted in subsequent seasons (Walker and Schulze 2006; Fischer and Hajdu 2015). GM maize varieties are highly beneficial on a commercial scale, but for those farmers who depend on farming for subsistence, it may be unprofitable due to restrictions imposed upon purchase and the sensitivity of some hybrids toward postharvest diseases/infestations during storage (Fischer and Hajdu 2015).

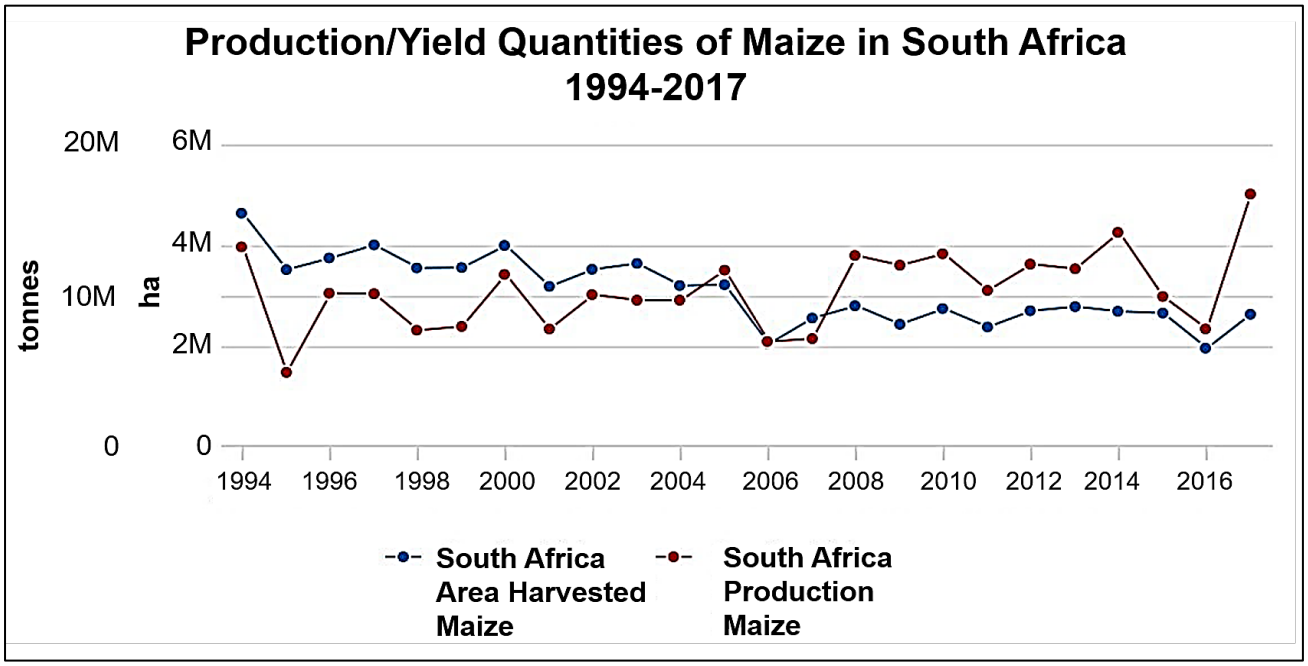

Figure 1.3: Maize production and yield in South Africa from 1994-2017 according to FAO. *Maize production has been increasing in South Africa since 1994 according to the Food and Agriculture Organisation (FAO). The blue graph shows the area of land harvested for maize in hectares (ha). The red graph shows the total amount of maize produced in tonnes until 2017. Adapted from World Health Organization 2019. Accessed on 8 June 2019 from: http://www.fao.org/faostat/en/#data/QC/visualize.*

1.2.2 Cercospora zeina – A Threat to Maize Production

Amongst the maize pathogens that have been identified as important is *Cercospora zeina*, a causal agent of grey leaf spot (GLS) foliar disease (Ward et al. 1999). *C. zeina* is the only known causal agent of GLS in South Africa, Zambia, Zimbabwe, Brazil and China (Liu and Xu 2013; Neves et al. 2015; Meisel et al. 2009). In the USA, GLS is also caused by *Cercospora zeae-maydis*, the sister species of *C. zeina* (Crous et al. 2006; Wang et al. 1998; Ward et al. 1999). The symptoms of GLS caused by these two species are indistinguishable, therefore in regions that have not yet been

surveyed to determine the causal agent of the disease, molecular identification of the fungus in the lesions may be required (Korsman et al. 2012).

GLS mature lesions are matchstick-shaped and parallel to the maize leaf veins. Lesions tend to localise within the leaf veins until they coalesce to blight whole leaf surfaces (Ward et al. 1999). In combination with other foliar diseases such as northern and southern corn leaf blight (caused by *Exserohilum turcicum* and *Cochliobolus heterostrophus* respectively), GLS causes dramatic yield losses. Farmers may lose whole fields of maize if these diseases go untreated when conditions favour their proliferation. These diseases lower the photosynthetic capacity of maize leaves due to coalescence of lesions, producing poor quality maize cobs and making the plants more susceptible to stalk rots (Mallowa et al. 2015; Belcher et al. 2012).

To an untrained eye, GLS symptoms are very similar to those of southern leaf blight (SLB) (Pioneer 2010) as shown in Figure 1.4. Identification of the causal agent of the lesions can be done using molecular biology techniques. This will allow distinction between the different foliar pathogens present in infected maize leaves and result in employment of adequate control measures. The amount of *C. zeina* present in GLS lesions can also be quantified (Korsman et al. 2012). Figure 1.4 shows three of the most common maize foliar disease symptoms including GLS, Northern corn leaf blight (NCLB) and Southern corn leaf blight (SLB) in comparison to a healthy maize leaf.

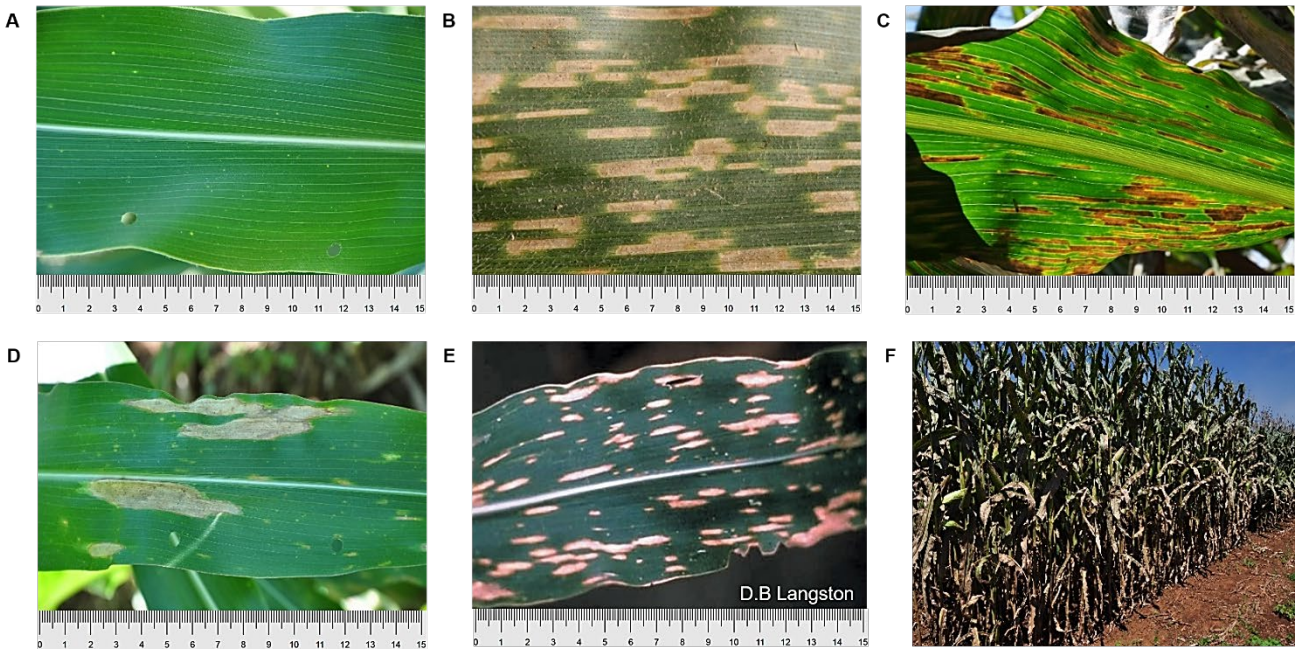

Figure 1.4: Maize foliar diseases. *Grey leaf spot (GLS), Northern corn leaf blight (NCLB) and Southern corn leaf bight (SLB) are common maize fungal diseases. They blight leaf surfaces and dramatically reduce yield when all three are present in the field. A) A healthy uninfected maize leaf. B) and C) GLS infested leaves showing characteristic rectangular matchstick-shaped lesions. D) NCLB lesions are cigar shaped and larger than GLS and SLB lesions. E) SLB has lesions like those of GLS in appearance but are more rounded and not limited within the minor leaf veins. F) When GLS and NCLB occur simultaneously, the devastation results in dramatic losses of yield and complete blighting. Scale=15 cm.* ***Photo credit: DK. Berger****.*

The increased prevalence of GLS in SA and other countries is attributed to farming practices such as conservation tillage and monoculture. *C. zeina* conidia overwinter in infected maize debris, therefore reduced tillage allows a build-up of inoculum. The conidia germinate during warm climates and high relative humidity. Disease severity therefore depends on the climate before and during the maize growing season (Ward and Nowell 1998; Wang et al. 1998; Ward et al. 1999). In situations where disease appears after grain filling, there is no loss of yield, therefore fungicide applications may not be required (Mallowa et al. 2015). Figure 1.5 shows the GLS disease cycle which describes how the fungus prepares for its next cycle of infection in the host plant.

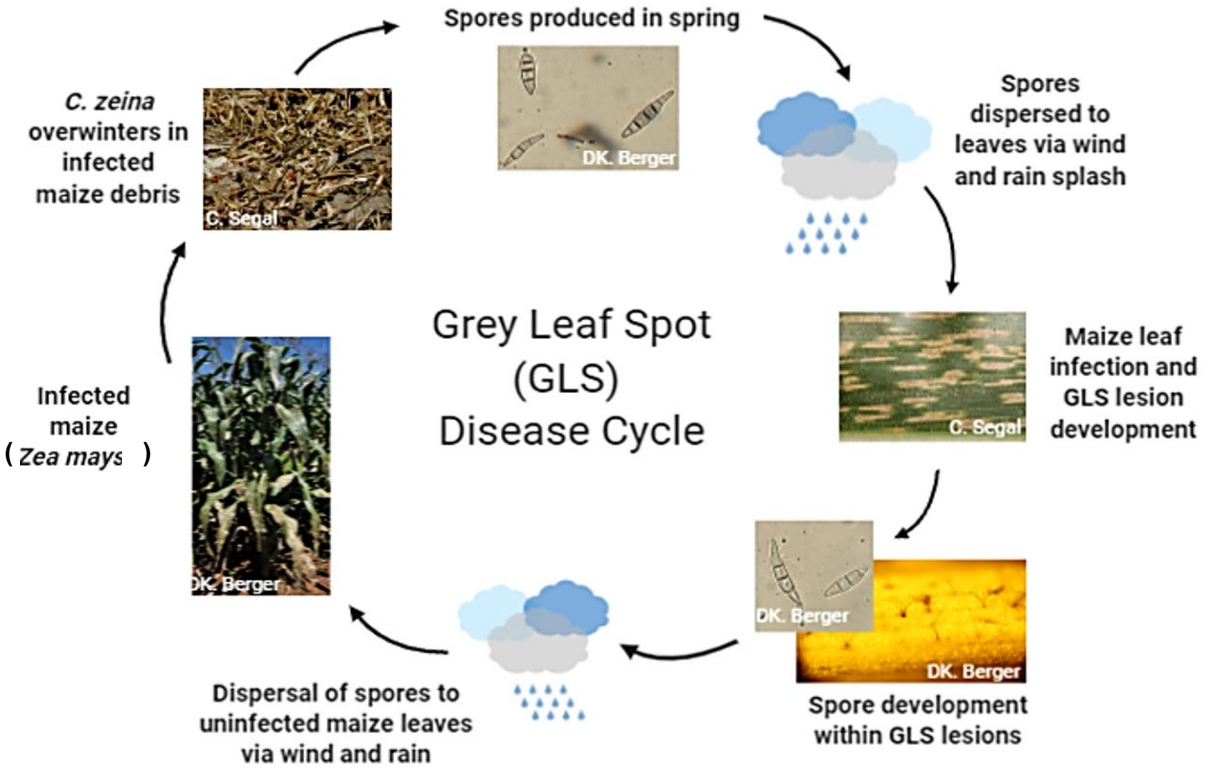

Figure 1.5: The Cercospora zeina disease and lifecycle. *C. zeina overwinters in infected maize debris. Favourable conditions in spring (high temperatures and humidity) result in spore/conidia formation. Spores are dispersed to uninfected maize plants via rain splash and wind. They germinate within the leaves and produce GLS lesions. Spores are produced within the lesions which are then dispersed with rain and wind to healthy maize plants. GLS is caused again and infected maize plants eventually die.*

Current control strategies for foliar diseases such as GLS include crop rotation, tillage and fungicide application. All these mechanisms are temporary, with the most effective being the application of fungicides. Strobilurin fungicides have been shown to be the most effective against GLS. Examples include DuPont™ Approach® Prima and Headline® (Wise 2014). These fungicides act by disrupting mitochondrial respiration through binding at the Q_0 site of cytochrome b. Electron transfer is therefore blocked and the energy cycle of the fungus is disrupted (Bartlett et al. 2002). During seasons where the climate does not favour GLS development/severity, conservation tillage may not be detrimental and fungicide application may not be required (Paul et al. 2011; Ward and Nowell 1998).

Fungicides have proven to be lethal to the environment due to their broad-spectrum functions. Some studies show that farmers are increasing their use of fungicides on maize in the absence of foliar diseases due to claims that yield is improved (Paul et al. 2011). Others however, have shown inconsistencies in this claim; therefore, yield does not increase solely due to fungicide application, but due to many contributing factors (Paul et al. 2011; Mallowa et al. 2015). The adoption of this habit increases damage to the environment caused by harmful chemicals. Alternative pathogen control and yield increase measures therefore need to be implemented.

Recent research has shown that there are genetic differences in the haplotypes of *C. zeina* found in commercial versus smallholder farms in South Africa (Nsibo et al. 2019). Commercial maize farms that grow monocultures and spray fungicides have smaller genetic varieties compared to smallholder farms that grow various varieties of maize and refrain from using fungicides (Nsibo et al. 2019). The reduced genetic variability of the pathogen in commercial farms increases the risk of fungicide-resistance development. In the case of smallholder farms where great genetic diversity has been found, challenges may arise with control of the pathogen. Farmers should therefore have diverse control strategies to prevent the evolution of fungicide-resistant *C. zeina* and to control a variety of haplotypes of the fungus (Nsibo et al. 2019).

At present, the best control against GLS and other foliar diseases is the use of resistant maize hybrids. Quantitative trait loci (QTL) are mostly responsible for GLS disease resistance. QTL have small effects that add up to provide tolerance or resistance against foliar pathogens (Sibiya et al. 2012). QTL for resistance against GLS caused specifically by *C. zeina* in South Africa have been identified (Berger et al. 2014). Resistance breeding is the most ideal yet challenging option in integrated management systems. In some cases, where resistance is improved, yield will be decreased, therefore complicating potential breeding outputs (Nelson et al. 2018). The timing of fungicide application must coincide with disease appearance for it to be economical. Even though GLS-resistant hybrids are the best option, it may be better to use them in conjunction with fungicides and tillage practices (Paul et al. 2011; Mallowa et al. 2015).

C. zeae-maydis produces a toxin called cercosporin which is crucial for the fungus to cause GLS in maize. The phytotoxin has a reddish colour *in vitro* (Figure 1.6) (Fajola 1978; Okubo et al. 1975; Swart et al. 2017). Other *Cercospora* species, such as *Cercospora beticola* (sugar beet leaf spot) and *Cercospora kikuchii* (soybean leaf spot) also produce this toxin during host infection (Weiland and Koch 2004; Daub 1982; Upchurch 1995). Studies have shown that genes in the cercosporin toxin biosynthesis (CTB) cluster are responsible for cercosporin production. In those fungal species that produce cercosporin, infection of host plants will not result if the phytotoxin is absent. Given that symptoms of GLS caused by *C. zeae-maydis* are identical to symptoms caused by *C. zeina*, it was expected that *C. zeina* has the same infection strategy. However, *C. zeina* has been shown to not

produce cercosporin *in vitro* due to a mutation of its *CTB7* gene (Figure 1.6), therefore it is unknown how it causes GLS in maize leaves (Swart et al. 2017).

Due to the lack of understanding how *C. zeina* causes GLS, effective GLS control strategies are still elusive. Various aspects of research into understanding the molecular mechanisms underlying the pathogenicity of *C. zeina* are required. Studies focusing on the metabolites the fungus produces before, during and after infection may shed light into whether it produces toxic substances to kill the host. Focusing on the effector biology of the fungus may create a foundation for understanding which effector proteins contribute towards causing GLS. It may also be valuable to study how *C. zeina* interacts with competing fungal pathogens (such as *E. turcicum*) during host attack to determine if they hinder or accelerate the development of GLS.

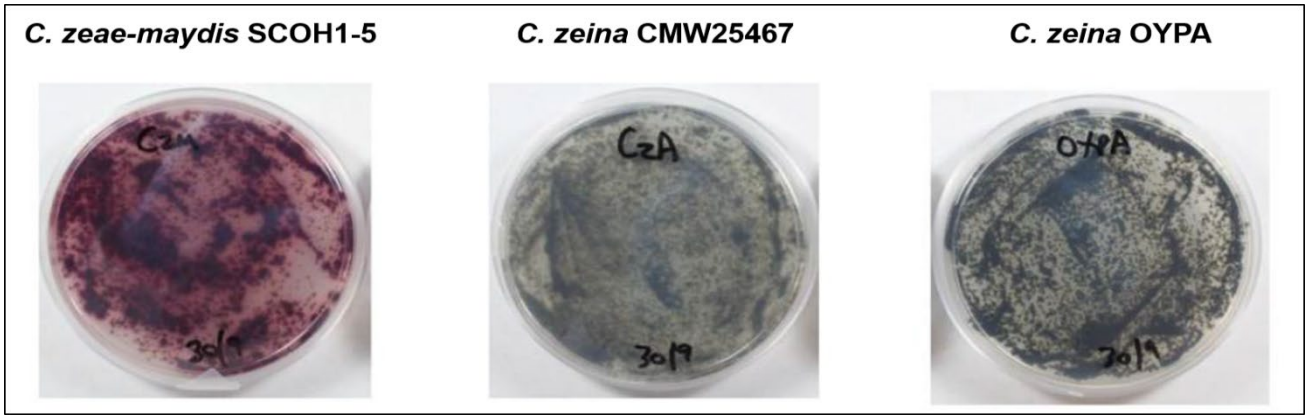

Figure 1.6: Cercospora species in vitro cercosporin production. *When cultured on 0.2X PDA media, C. zeae-maydis SCOH1-5 produces a red pigment representing cercosporin. Cercosporin is the phytotoxin required for the fungus to cause GLS in maize. C. zeina CMW25467 (African isolate) and C. zeina OYPA (American isolate) do not produce the red pigment when cultured on 0.2X PDA media. Therefore, in vitro C. zeina does not produce cercosporin and may not produce it in planta during host invasion. The greenish-grey colour represents the colour of the fungal conidia. Adapted from Swart et al. (2017).*

1.2.3 Plant Defence Responses – How Plants Fight Off Pathogen Attack

Plants protect themselves against pathogen attack through innate immunity (Zipfel et al. 2006; Dodds and Rathjen 2010). Various microorganisms inhabit the outer surfaces of plants, but for some of them to acquire nutrients, they need to invade the host plant cells. Plant pathogens range from bacteria, fungi, oomycetes and viruses to nematodes (Jones and Dangl 2006). Various pathogen infection strategies exist depending on the type of pathogen. Bacteria attack plants via wounds and stomata, fungi and oomycetes penetrate plant surfaces using hyphae and haustoria and nematodes use a stylet (Jones and Dangl 2006; Knogge 1996).

The lifestyle of a pathogen determines how it acquires its nutrients from the host plant. Biotrophic pathogens do not kill their hosts immediately, they feed off them until they completely senesce. Necrotrophic pathogens kill host cells before nutrients are acquired. Hemibiotrophic pathogens keep hosts alive (biotrophic) for a short while before killing them (necrotrophic) for nutrient acquisition

(Vleeshouwers and Oliver 2014; Dangl and Jones 2001; Lo Presti et al. 2015). As soon as fungal pathogens enter host tissues, defence responses are triggered by host recognition of pathogen secreted molecules. Pathogens that succeed in causing disease must overcome host defence responses (Jones and Dangl 2006).

Effectors are proteins produced by microbial pathogens to have a specific effect on host or non-host plants during pathogen attack. Upon recognition of these effector proteins, host immune defence responses may result (Dodds and Rathjen 2010; Vleeshouwers and Oliver 2014). Plants use two innate mechanisms to protect themselves against pathogen attack as described in the zigzag model of plant immunity proposed by Jones *et al*. (2006) (Figure 1.7). Their first mode of defence is the use of pattern recognition receptors (PRRs) to identify conserved extracellular pathogen-associated molecular patterns (PAMPs). This results in PAMP-triggered immunity (PTI). Examples of PAMPs are bacterial flagellin and fungal chitin (Jones and Dangl 2006; Thomma et al. 2011). PTI involves immune responses such as strengthening of cell walls via callose deposition and the production of reactive oxygen species (ROS). This provides basal defence against various potential pathogens, not just host specific pathogens (Jones and Dangl 2006).

Two classes of PRRs are currently known, namely transmembrane receptor-like proteins and transmembrane receptor-like kinases. Recognition of PAMPs usually occurs extracellularly because pathogens do not penetrate cell plasma membranes. For recognition to occur successfully, PRRs have an extracellular leucine rich repeat (LRR) and an intracellular kinase domain (which receptor-like proteins lack). In most cases, for PTI to be elicited successfully, PRRs have been shown to interact with BAK1 (BRASSINOSTEROID INSENSITIVE-1 ASSOCIATED KINASE 1), except in fungi where they interact with the receptor for chitin known as CERK1 (CHITIN ELICITOR RECEPTOR KINASE 1) (Dodds and Rathjen 2010; Desaki et al. 2019). It has been shown that BAK1 is essential for plant immunity and is therefore a common target for pathogen effectors (Van der Burgh et al. 2019; Dodds and Rathjen 2010).

When effectors interfere with PTI, effector triggered susceptibility (ETS) results and plant immunity is reduced (de Wit et al. 2009; Jones and Takemoto 2004; Jones and Dangl 2006). The second mode of defence occurs once pathogens overcome PTI, which results in effector-triggered immunity (ETI) (Figure 1.7). Effectors are small secreted proteins (SSPs) that are classified as apoplastic or cytoplasmic depending on where they function (Liu et al. 2019). Apoplastic effectors function at the host-pathogen interface while cytoplasmic effectors target specific intracellular proteins or DNA (Liu et al. 2019).

ETI is the cytoplasmic recognition by host cognate R-proteins of pathogen secreted effectors (Figure 1.7). Effectors become known as avirulence (Avr) proteins upon recognition because they cannot carry out their virulence functions. R-proteins are characterised by their nucleotide binding and leucine

rich repeat (NB-LRR) domains (Kanyuka and Rudd 2019). When R-proteins recognise effectors, ETI usually causes a hypersensitive response (HR) in the host, which kills the cells in the infection area and therefore inhibits further pathogen spread (Jones and Dangl 2006; Thomma et al. 2011). The gene-for-gene hypothesis explains the events that occur during ETI, and states that specific R genes exist in plants for each dominant Avr gene present in pathogens. Upon recognition of the Avr proteins, host defence responses are elicited (Flor 1971; de Wit et al. 2009).

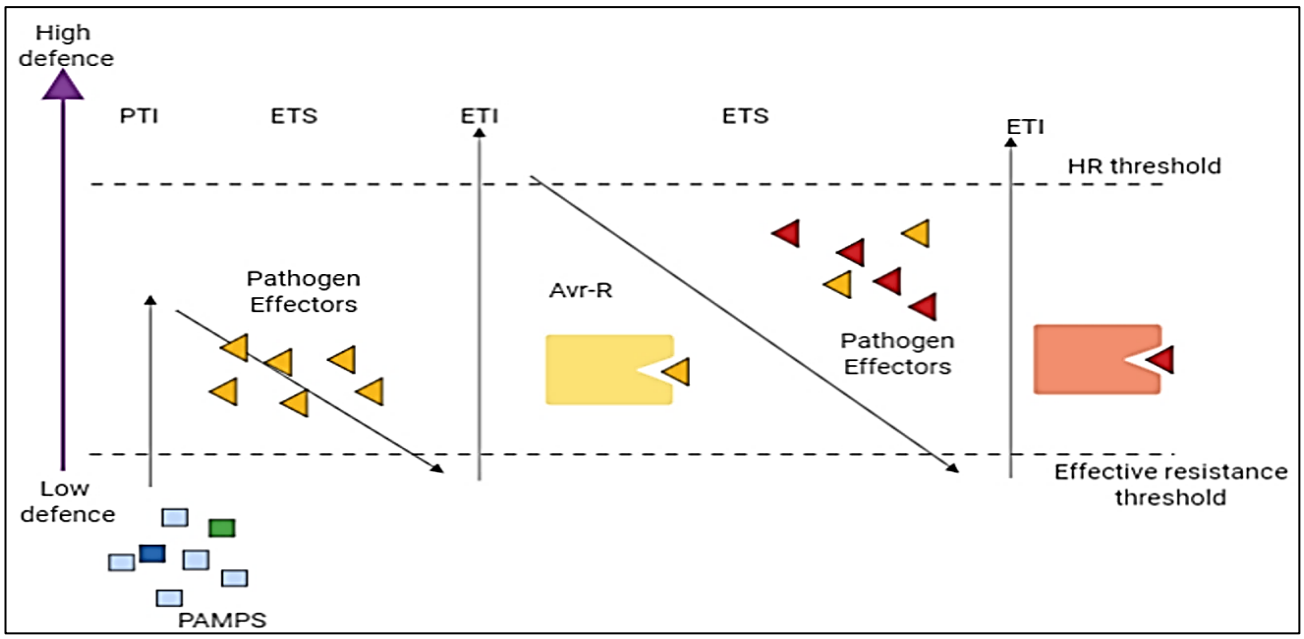

Figure 1.7: Zigzag model of plant immunity*. Two lines of innate plant immunity are activated upon pathogen attack. PTI is the first defence triggered when pathogen molecules (PAMPs) are recognised by plant proteins (PRRs). When effectors interfere with PTI, plants are susceptible to infection during ETS. Host R-proteins recognise specific effectors (Avr proteins) resulting in ETI. Pathogens evolve to overcome recognition, resulting in ETS again. When plants adapt their R-proteins by natural selection to recognise the evolved Avr proteins, the threshold for HR is reached when ETI occurs. HR kills pathogen-colonised cells to prevent the spread of infection. Adapted from Jones et al. (2006).*

It has been suggested that the zigzag model of immunity (Figure 1.7) does not fully describe plant defence against apoplastic fungal pathogens (Kanyuka and Rudd 2019; Stotz et al. 2014). Effector triggered defence (ETD) has been proposed to describe recognition of apoplastic effectors because of slow development of HR in infected plants. During ETI, the pathogen is completely killed whereas with ETD the pathogen is stopped, but not eliminated. It is not known why HR does not always manifest during ETD even though plants seemingly protect themselves against apoplastic pathogens. In cases where apoplastic effector recognition cannot be observed macroscopically (HR), stains (such as Trypan blue) can be used to observe the responses microscopically (Jones and Dangl 2006; Stotz et al. 2014).

ETD was hypothesised to occur when extracellular receptor-like proteins interact with receptor-like kinases such as SOBIR1 to initiate defence signalling. ETD therefore consolidated the views that some PRRs directly/indirectly recognise apoplastic fungal effectors and that some R genes encode

receptor-like proteins. It is important to note however that ETD is not a widely accepted model because it was found that not all RLP/SOBIR complexes trigger similar responses in various plant-pathogen systems (Kanyuka and Rudd 2019; van der Burgh and Joosten 2019; Stotz et al. 2014; Thomma et al. 2011).

Pathogenic fungi often complete their sexual cycle in the host before cell death is observed during ETD. Therefore, it may be more appropriate to suggest that some plants have defence instead of immunity against potential pathogens. Apoplastic pathogens operate intercellularly whereas obligate biotrophs form haustoria during infection. Therefore, apoplastic pathogens produce effectors that are recognised intercellularly, whereas biotrophic effectors are recognised cytoplasmically. These differences contribute towards the observation that ETD and ETI are similar yet different defence mechanisms against plant pathogens due to effector recognition in different parts of the plant (Thomma et al. 2011; Stotz et al. 2014).

An example that supports the ETD hypothesis is *Zymoseptoria tritici* (causes wheat blast disease), which enters leaves via the stomata and grows endophytically. Symptoms of plant defence have only been observed 10 days post inoculation (dpi) when the *Z. tritici* switches to necrotrophy, supporting the hypothesis of ETD (Duncan and Howard 2000; Stotz et al. 2014). In resistant oilseed rape, it has also been shown that expression of ETD is slow against *Pyrenopeziza brassicae.* Symptoms of HR were only observed 36 dpi, after which sexual reproduction occurred in senescent leaves. ETD therefore does not interfere with sporulation and spread of the pathogen, it merely slows down its proliferation (Boys et al. 2012; Stotz et al. 2014).

C. zeina enters maize leaves through stomata and causes disease while proliferating intercellularly. GLS symptoms generally appear 14 dpi (days post infection), indicating that the fungus is latent before switching to a necrotrophic lifestyle to form lesions. The fungus overwinters in dead leaf material and sporulates when conditions are favourable (Figure 1.5) (Ward et al. 1999). These similarities to the infection strategy of other apoplastic fungi may suggest that resistant maize lines protect themselves against *C. zeina* via ETD, where the fungus is slowed down, but not killed and the plant takes longer to protect itself against GLS development.

Effector function and viability is influenced by environmental cues perceived by the pathogen during infection of their host plant (Uhse and Djamei 2018). Generally, the type of effectors secreted must provide optimal support to the pathogen's lifestyle. For example, biotrophic pathogens must ensure that the host plant stays alive, therefore effectors cannot kill the plant or trigger defences that kill the pathogen. Where pathogens are hemibiotrophic, effector combinations change when the lifestyle is switched from biotrophic to necrotrophic (Uhse and Djamei 2018; Lahrmann et al. 2013).

Several categories have been suggested to classify effectors according to their functions. Some can modify their structure upon host penetration to minimize recognition. For example, LysM effectors

such as *Ecp6* and *Slp1* of *Cladosporium fulvum* and *Magnaporthe oryzae* respectively. These effectors bind to chitin molecules released during host infection to prevent pathogen recognition by host defence proteins (Mentlak et al. 2012; de Jonge et al. 2010; Uhse and Djamei 2018). Other effectors function by inhibiting host defence proteins. *Ustilago maydis* Pep1 inhibits peroxidase function during peroxide accumulation when maize first recognises the pathogen (Hemetsberger et al. 2012). Interestingly, some effectors interfere with how the pathogen deactivates host proteins, and function as activators by promoting expression of host genes (Uhse and Djamei 2018).

Models on how plant proteins perceive pathogen proteins have been proposed (Figure 1.8) (Dodds and Rathjen 2010). The receptor-ligand model assumes that host plant cognate R-proteins directly interact with fungal effectors and result in a host immune response (Stergiopoulos and Wit 2009). For example, the direct interaction of Avr-Pita from *Magnaporthe oryzae* with the rice Pi-ta R-protein (Jia et al. 2000). This model therefore implies that plants need to carry many R-proteins to recognise all the individual types of effectors produced by their pathogens (Stergiopoulos and Wit 2009).

In the guard model, most plant R-proteins interact indirectly with effector proteins by monitoring the changes in their host target and binding to accessory proteins modified by the effectors. This therefore allows a single R-protein to guard the host target so that it interacts with various unrelated effectors that interact with the host plant (Stergiopoulos and Wit 2009). An example of the guard model may occur in tomato plants under attack by *C. fulvum* when *Avr4* interacts indirectly with Cf-4 (native tomato R-protein) (van Esse et al. 2007). A third model is the bait model which proposes that an effector interacts with an accessory protein associated with an NB-LRR protein, after which the actual effector is recognised by the R-protein (Dodds and Rathjen 2010). Figure 1.8 illustrates how each model would work when the host R-protein (NB-LRR) recognises the pathogen effector protein.

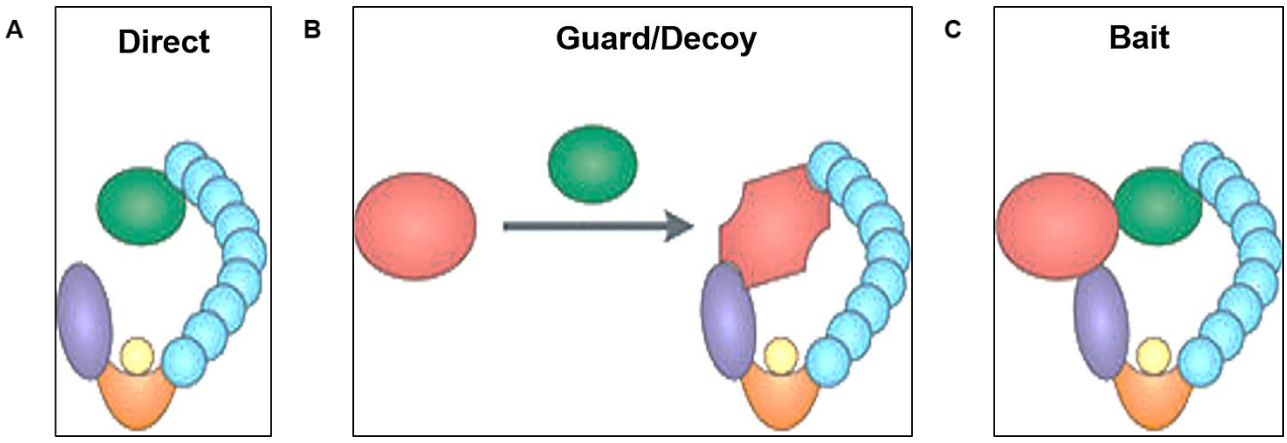

Figure 1.8: Models proposed for direct and indirect effector recognition in host plants. *Host plant (NB-LRR) receptors either use direct or indirect mechanisms to recognise pathogen effectors to elicit ETI. A) Direct recognition involves physical binding of the NB-LRR receptor (blue, orange, purple, yellow) to the effector (green) which triggers host immune signalling. B) In the guard and decoy models, an accessory protein (red) is modified by the effector (green) which may be recognised by the NB-LRR receptor. The accessory may be the structural mimic (decoy model) or the virulence target (guard model) of the effector. C) The bait model results in facilitation of direct recognition by NB-LRR receptors through effector interaction with an accessory protein. Adapted from Dodds and Rathjen (2010).*

The findings from the above-mentioned studies and others indicated that effector genes are present in many fungal plant pathogens and that they play a vital role in virulence and pathogenicity. In some cases, it was found that without certain effectors the pathogens could not cause disease, indicating a niche for further study of these proteins in these and other fungal pathogens. Understanding how a pathogen causes disease in a host plant provides an opportunity for researchers to develop resistant crops and alternative pathogen eradication strategies.

Pathogen recognition by host plants involves compatible or incompatible interactions. During compatible interactions, effectors act as virulence factors because they are not recognised by host defence proteins, which results in successful infection of host cells. Incompatible interactions result due to recognition of effectors by their cognate R-proteins in the plant. ETI is triggered (HR) to stop pathogen growth due to cell death at the infection site (Stotz et al. 2014; van Esse et al. 2007; Bolton et al. 2008; Laugé et al. 1997; Mesarich et al. 2017; Mesarich et al. 2016). Figure 1.9 illustrates the interactions of a fungal pathogen with host plant cells during compatible and incompatible reactions in the apoplast.

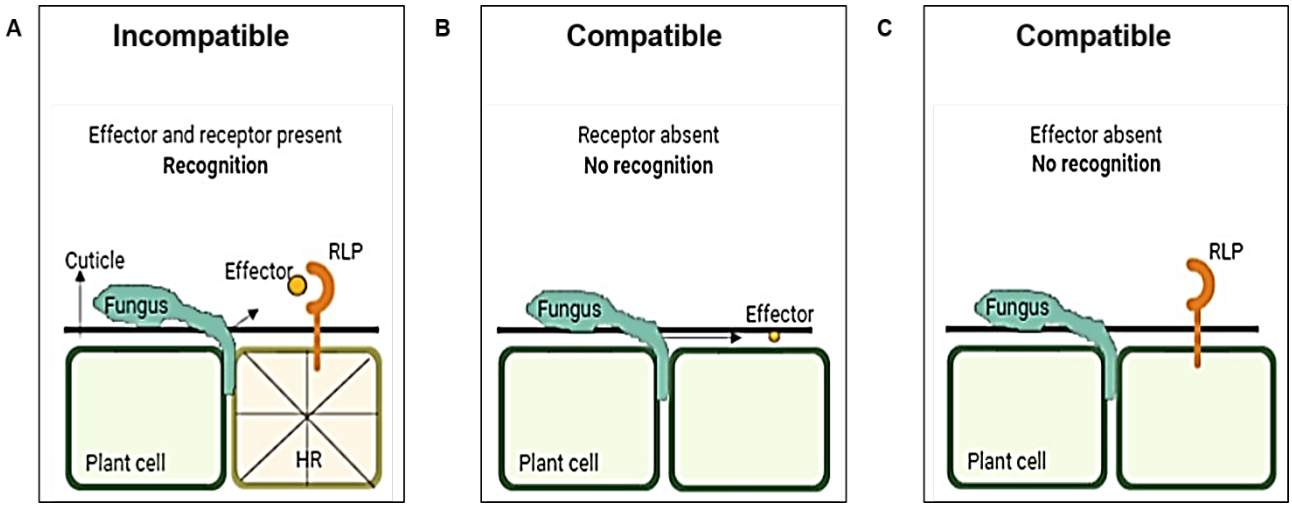

Figure 1.9: Incompatible vs. compatible interactions during pathogen attack. *A) Incompatible interactions occur when cognate host R-proteins recognise fungal effectors, causing HR to arrest fungal proliferation. B) Lack of cognate R-proteins leads to compatible interactions due to unrecognised pathogen effectors, resulting in host plant infection. C) A lack of effectors leads to compatible interactions because host R-proteins cannot detect the pathogens presence. The absence of effectors may limit fungal proliferation and therefore reduce disease severity. Adapted from Stotz et al. (2014).*

Cladosporium fulvum is a well-studied biotrophic fungal pathogen that causes tomato leaf mould disease. *C. zeina* and *C. fulvum* are distant relatives that belong to the Dothideomycete class of Ascomycete fungi. *C. fulvum* penetrates tomato leaves through open stomata but does not produce any feeding structures such as haustoria. Its infection strategy is like that of *C. zeina* (Swart et al. 2017; Ward et al. 1999; Wit 1992). Once *C. fulvum* establishes itself in its host, proliferation is limited to the apoplastic (extracellular) space of the leaves, similar to *C. zeina* (Ward et al. 1999; Van den Ackerveken et al. 1993; van Esse et al. 2007).

Cysteine-rich proteins such as *Ecp6*, *Ecp2, Avr4* and *Avr9* first identified in *C. fulvum,* have been recognised as effectors that trigger host defence responses. These effectors form disulphide bridges to increase their stability, thereby protecting themselves against protease degradation in the host apoplast (Joosten et al. 1997; Joosten and de Wit 1999; Stergiopoulos and Wit 2009; Stergiopoulos et al. 2010). The studies done on *C. fulvum* effector biology may be applied to *C. zeina* to decipher its effectors and corresponding R-proteins in maize.

Homologs of *C. fulvum* effector genes have been found in other fungal species that belong to the Dothideomycete class. An example is the presence of *Avr4* and *Ecp2* homologs in *Mycosphaerella fijiensis* (asexual form = *Psuedocercospora fijiensis* (Churchill 2011)) which causes black Sigatoka disease of banana (Stergiopoulos et al. 2010). Stergiopoulos et al. (2010) proposed that *M. fijiensis Ecp2* (*MfEcp2*) induces a strong virulence effect by interacting with virulence factors that cause necrosis in the host plant to release nutrients for the fungus. However, the essential biochemical function of *Ecp2* is still unknown. *M. fijiensis Ecp2* was able to cause necrosis in the absence of Mf-ECP2 (R-protein native to banana) in tomato plants, which contrasted the *C. fulvum Ecp2* inability to cause necrosis in the absence of the Cf-ECP2 (R-protein native to tomato). This indicated that *MfEcp2* (fungal effector) was able to interact with host target genes that resulted in necrotic lesions on the tomato leaves. A study of *C. zeina Ecp2* (*CzEcp2*) infiltration in tomato cultivars in the absence of Cz-ECP2 (R-protein potentially native to maize) could be done to determine if the effector will cause an HR in the absence of its cognate R-protein.

C. fulvum Ecp2 (*CfEcp2*) has been shown to cause non-host HR due to recognition by a dominant protein in some plants of *Nicotiana* spp. The HR was characterised as grey necrotic lesions that remained confined to the PVX::Ecp2 inoculated leaves of *Nicotiana paniculata* (Laugé et al. 2000a). This same response was later identified in PVX::Ecp2 inoculated leaves of *Nicotiana tabacum* var. Samsun NN, indicating that these species of tobacco, and possibly others specifically respond to *C. fulvum Ecp2* (Takken et al. 2000a). Some research has proposed that Cf-ECP2 (R-protein native to tomato) may be a homologue of *Cf-9* (*Hcr9*), the R-protein that recognises *C. fulvum Avr9*. Cf-ECP2 may therefore also occur at the *Cf-9* or *Cf-4* locus, facilitating recognition of effectors other than *Avr9* and *Avr4* respectively (Laugé et al. 2000a).

Non-host HR was also shown in *Nicotiana sylvestris* and *Nicotiana undulata* by De Kock et al. (2004). The pathogenesis-related protein 1a (PR1a) signal peptide was fused to *CfEcp2* in PVX transformation (PVX::PR1aCfEcp2). Much higher and earlier symptom development was observed compared to *CfEcp2* (PVX::CfEcp2) delivered into the plant cells without PR1a (or any other signal peptide). Therefore, it was concluded that *Nicotiana* spp. can express *CfEcp2* with and without a signal peptide fused to it. Recognition of *CfEcp2* in *Nicotiana* spp. may be extracellular and cytoplasmic due to observation of necrosis for *CfEcp2* expressed with and without the signal peptide respectively. Non-host resistance may also occur without any visible symptoms or show a delayed

symptom development. It has yet to be determined if non-host HR results as a function of resistance or due to random evolution of R-genes in non-host plants (De Kock et al. 2004).

In a study done by Luo et al (2018), researchers elucidated the genome sequence of the Dothideomycete *Cercospora sojina*, the causal agent of soybean frogeye leaf spot. This fungus, like *C. zeina* does not produce the cercosporin toxin responsible for pathogenicity of many *Cercospora* spp. (Goodwin et al. 2001). During this study, they also determined that the fungus might use effector proteins to cause disease within soybean host plants. Fifty putative effectors were identified, and 13 of them could strongly suppress BAX-induced apoptosis, indicating that those putative effectors may play a role in *C. sojina* virulence and pathogenicity (Luo et al. 2018).

Given the poor understanding of *C. zeina* effector biology, putative effectors may be identified using bioinformatics tools such as EffectorP and ApoplastP from RNA sequencing transcriptomic data (Sperschneider et al. 2018; Sperschneider et al. 2016; Swart et al. 2017). The candidate effectors may then be infiltrated into tobacco leaves which can be monitored for HR. Corresponding R-proteins from the *C. zeina* host (maize) have yet to be elucidated. Therefore, tobacco transformations in the absence of cognate R genes (maize specific) may be valuable in understanding the influence of effectors on *C. zeina* pathogenicity and virulence.

1.2.4 Genetic Modification of Plants – Agrobacterium tumefaciens-mediated transformation

Agrobacterium tumefaciens-mediated transient transformation is a common method used to study gene expression in plants. Studies have shown that transient transformation occurs from copies of transfer DNA (T-DNA) that are temporarily in host genome (Krenek et al. 2015). Transient expression is short lived compared stable expression that allows for inheritance of the transformed traits by progeny. Any sequence can be inserted between the left and right border of a binary vector, allowing for its delivery into a plant genome (Shiboleth and Tzfira 2012; Gelvin 2003; Krenek et al. 2015). The most commonly used bacterium for transformation is the phytopathogen *A. tumefaciens*. This bacterium is classically known for causing crown gall disease characterised by tumours in a variety of host plants (Gelvin 2003). Various strains of the bacteria exist with some of the most common for agroinfiltration being *A. tumefaciens* GV3101::pMP90, *A tumefaciens* LBA4404 and *A. tumefaciens* AGL1 (Krenek et al. 2015).

Each *Agrobacterium* typically possesses a Ti plasmid that has the T-DNA region (*cis*) and a region of virulence (*vir*) genes (*trans*). The T-DNA is flanked by short 25 bp sequences (left and right border) that delineate the start and end of the section that is transferred into plants. The virulence genes express proteins required for the excision and transfer of the T-DNA into host cells. The virulence genes themselves do not enter plants. For agroinfiltration, the Ti plasmid is disarmed by removal of the native T-DNA region. A binary vector carrying the genes of interest is then inserted into the

Agrobacterium. The *vir* genes on the remaining Ti plasmid (helper plasmid) facilitate excision and transfer of the gene of interest from the binary vector into the plant cells (Figure 1.10) (An 1987; Shiboleth and Tzfira 2012; Krenek et al. 2015).

Inoculation of plants with *Agrobacterium* carrying specific genes is a useful tool for studying genes with unknown function and for recombinant protein production (Shah et al. 2013; Sheludko 2008). Other examples of transient transformation methods that precede agroinfiltration include particle bombardment and protoplast transformation (Lörz et al. 1985; An 1987; Christou 1995; Klein et al. 1992; Sanford et al. 1987).

Most agroinfiltrations of plants, transient and stable, are done in *Nicotiana benthamiana* because the plants have a short life cycle. They are a popular choice for recombinant protein production because they are easy to genetically transform and produce plenty of seed for scaling up production. They also have the advantage of being able to take up various types of expression vectors, therefore various protein types may be produced (Shah et al. 2013; Gelvin 2003; Krenek et al. 2015; Leuzinger et al. 2013).

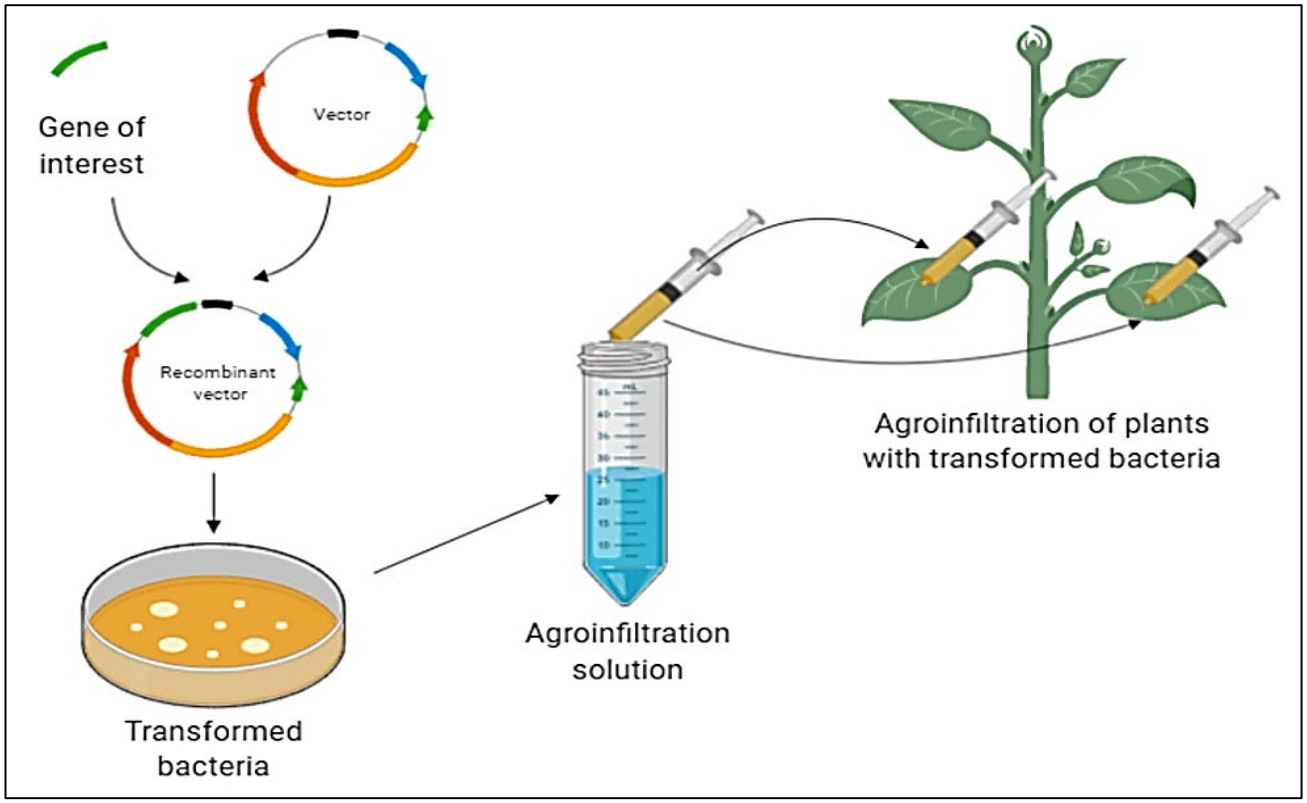

Figure 1.10: Transient agroinfiltration of Nicotiana spp. *Binary vectors are transformed with a gene of interest after which they are called recombinant vectors. Recombinant vectors are inserted into Agrobacterium cells which are grown on selection media. Transformed bacteria colonies are resuspended in infiltration media to activate virulence genes on the Ti helper plasmid. Plant leaves are infiltrated with the agroinfiltration solution using a syringe. The vir genes express proteins to excise the T-DNA from the binary vector and transport it into the plant cells where the gene of interest is transiently expressed.*

1.3 Conclusion

Plants have innate immunity against microorganisms that threaten their health. Despite this, they still need assistance to protect themselves against diseases. To control pathogen attack against crops, the molecular basis of infection must be understood. Virulent molecules such as effectors have been shown to contribute towards a pathogens ability to cause disease and influence disease severity. Functional analysis of effectors through *A. tumefaciens*-mediated transformation may help in elucidation of their role in pathogen virulence. At present the best control strategies against diseases such as grey leaf spot (caused by *C. zeina*) are the use of resistant maize hybrids and fungicides. More environmentally friendly control strategies must be explored to reduce the detrimental side effects of broad-spectrum control agents. Deciphering how *C. zeina* causes disease in its host requires the exploration of various avenues such as effector biology, secondary metabolites and fungal structures amongst others. The use of bioinformatics tools and established expression systems such as agroinfiltration of tobacco will contribute towards figuring out which genes and proteins play a role in *C. zeina* virulence and pathogenicity. The knowledge gained can contribute towards breeding resistant maize hybrids that target *C. zeina* effectors through upregulation of genes in the host plants.

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Chapter 2

Cloning the *C. zeina Ecp2* effector gene into a binary vector and *Agrobacterium tumefaciens*-mediated transient transformation of *Nicotiana* spp.

Abstract

Maize (*Zea mays*) is a staple crop in Africa that is under severe threat to disease by pathogenic organisms. Grey leaf spot (GLS) caused by *Cercospora zeina* drastically limits the yield and quality of maize produced. Not enough is understood about how *C. zeina* causes GLS, but it is known that it is a maize-specific hemibiotrophic fungus. Proteins called effectors are essential for the virulence of pathogens such as *Cladosporium fulvum.* Extracellular protein 2 (Ecp2), an effector identified in some Dothideomycete fungi, has an unknown function but has been shown to play a role in the virulence of the fungi. The aim of this study was to clone *C. zeina Ecp2* (*CzEcp2*) into a binary vector for agroinfiltration of *Nicotiana* spp. The *C. zeina* genome and RNA sequence data (*in planta* and *in vitro*) were searched for a candidate *Ecp2* gene. The complete *CzEcp2* sequence (with the fungal signal peptide) and the mature sequence (lacking the fungal signal peptide) were cloned into the pTRAkc-ERH binary vector. pTRAkc-ERHCzEcp2 EB (fungal signal peptide) and pTRAkc-ERHCzEcp2 NB (LPH signal peptide) were respectively transformed into *Agrobacterium tumefaciens* GV3101 (pSOUP+pMP90). Phytophthora *infestans* INF1 was used as the positive control for HR expression. Untransformed *Agrobacterium* and the pTRAkc-ERH empty vector were used as negative controls. *Nicotiana benthamiana, Nicotiana tabacum* cv. Petit Havana and *Nicotiana tabacum* cv. LA Burley were then transiently agroinfiltrated. The plants were monitored for a hypersensitive response (HR) for 10 days. *CzEcp2* expression did not result in HR for the three *Nicotiana* spp., but chlorosis was observed. INF1 caused a HR in all three *Nicotiana* spp. and the negative controls did not cause any changes. The lack of HR where *CzEcp2* was expressed, may be due to lack of CzECP2 transport and recognition, a delayed HR or that the T-DNA was not adequately transferred into the host cells.

2.1 Introduction

In Africa, a continent where water scarcity threatens food security, maize (*Zea mays*) is an essential staple crop. Various foliar pathogens threaten maize productivity, especially for smallholder farmers who rely on subsistence farming for their livelihood (World Health Organization 2018). Grey leaf spot (GLS), an important maize foliar disease is caused by *Cercospora zeina* in South Africa (Meisel et al. 2009; Ward et al. 1999). It produces rectangular lesions that localise within the minor veins of maize leaves. The fungus localises in the apoplastic space where it proliferates and performs its infectious function. *C. zeina* initially remains latent for up to 14 days before the first symptoms of GLS are visible as lesions (Wang et al. 1998; Korsman et al. 2012; Meisel et al. 2009; Ward et al. 1999; Ward and Nowell 1998). This suggests a hemibiotrophic lifestyle where the pathogen keeps its host alive while being in the biotrophic phase. As soon as it switches to necrotrophy, disease symptoms become visible as the host begins to senesce (Dangl and Jones 2001).

Plants and pathogens have co-evolved overtime, allowing plants to develop genetic mechanisms of defence against attack. The detection of all microbes including pathogenic and non-pathogenic strains on plant surfaces triggers plant immunity (Jones and Takemoto 2004). Pathogenic microbes release virulence factors such as effectors which cause the plants to be susceptible to pathogen attack. Before infection becomes severe, plant proteins recognise the virulence proteins resulting in effector triggered immunity (hypersensitive response). Effectors constantly evolve to evade host recognition however they are species, strain and race specific. Therefore, most effectors have specific effects on the host plants of the pathogen in question (Thomma et al. 2011).

Ecp2 (extracellular protein 2) is an effector gene first identified in the Dothideomycete *Cladosporium fulvum.* It has an unclear biological function during pathogen attack, however it has been shown to contribute to *C. fulvum* virulence during host (*Solanum lycopersicum*) invasion. ECP2 was first purified from apoplastic fluid of infected tomato leaves. It was shown to be secreted by the fungal hyphae during pathogenesis due to cleavage of a signal peptide sequence to produce a mature protein (Van den Ackerveken et al. 1993; Wubben et al. 1994). *C. fulvum* ECP2 is a 165-amino acid cysteine rich protein recognised by tomato Cf-Ecp2 R-proteins (native to tomato) (Van den Ackerveken et al. 1993; Stergiopoulos et al. 2010; Chisholm et al. 2006).

Laugé et al. (1997) hypothesised that *Ecp2* is dispensable due to *ecp2*-mutant *C. fulvum* still having the ability to cause disease in tomato plants. They also showed however that mutants lacking *Ecp2* had a drastically reduced ability to colonize plant cells due to reduced emergence of mycelia and conidia. This subsequently reduced the production of other effectors required for *C. fulvum* virulence such as *Avr4* and *Avr9*. In plants colonised with the *C. fulvum ecp2* mutants, the stomatal guard cells were more prone to collapse, indicating that *Ecp2* plays a role in *C. fulvum* pathogenicity and virulence and the development of HR (Laugé et al. 1997; Laugé et al. 1998; Marmeisse et al. 1994). This study

also showed that *ecp2*-deficient *C. fulvum* mutants triggered stronger plant defence responses, indicating that although the mutant may still cause infection, it will be outcompeted by the innate plant defences. This may support the hypothesis for a possible role of *Ecp2* in counteracting plant defence mechanisms during pathogen attack (Laugé et al. 1997).

Given that *C. fulvum Ecp2* (*CfEcp2*) has been shown to cause HR in non-host tobacco plants (Laugé et al. 2000b; Takken et al. 2000b; De Kock et al. 2004), it may be valuable to study this effector in other fungi. *C. zeina* effector biology has yet to be explored and its mechanism of infection has yet to be elucidated. Corresponding R-proteins in maize have also yet to be identified. Homologs of well-studied effectors such as *CfEcp2* can be searched for in the *C. zeina* genome, isolated and transformed into tobacco plants to functionally characterise them. This may be a starting point towards determining how effectors influence *C. zeina* virulence and pathogenicity during GLS development in maize leaves.

Genetic modification is a biotechnology innovation that was developed to manipulate the genetics of organisms to obtain a specific outcome. A common way to modify genes or study their expression is to use *Agrobacterium tumefaciens*-mediated transformation of plants (Leuzinger et al. 2013; Krenek et al. 2015). A gene of interest may be inserted into a plant genome using bacterial plasmids and a recombinant binary vector carrying the gene. Any gene of interest and binary vector may be digested with restriction enzymes and ligated to form a recombinant binary vector. A bacterial strain commonly used to deliver binary vectors is *Agrobacterium tumefaciens* GV3101::pMP90 (Koncz and Schell 1986). The resulting proteins are either stably or transiently expressed, therefore either long-term inheritance of the trait or short-term expression of the protein respectively (Krenek et al. 2015; Wroblewski et al. 2005). Once transformed into the bacteria, the T-DNA region of the recombinant plasmid is excised and transferred into the plant cells by the bacterial *vir* proteins. This method, and others such as CRISPR-Cas9 and biolistics have revolutionised the study of elucidating gene functions (Schuster et al. 2016; Christou 1995; An 1987; Gelvin 2003).

The binary vector used in this study is pTRAkc-ERH (Figure 2.1) (Maclean et al. 2007). It contains a SEKDEL sequence required for endoplasmic reticulum protein retention, a 6x his sequence for addition of histidine residues to proteins for purification, and an LPH plant signal peptide sequence for secretion of proteins of interest. Disruption of the SEKDEL sequence by cloning genes of interest into restriction sites that cut in this region, results in transport of the resulting proteins to the intercellular spaces (apoplast). For retention of the protein in the cytoplasm, the gene would have to be cloned without disrupting the SEKDEL sequence and without a signal peptide sequence. Genes can also be cloned without their native signal peptide sequences and fused to the LPH signal peptide sequence on the vector. pTRAkc-ERH is therefore a suitable binary vector to study apoplastic proteins such as the ECP2 effector (Beiss et al. 2015; Mortimer et al. 2012; Maclean et al. 2007).

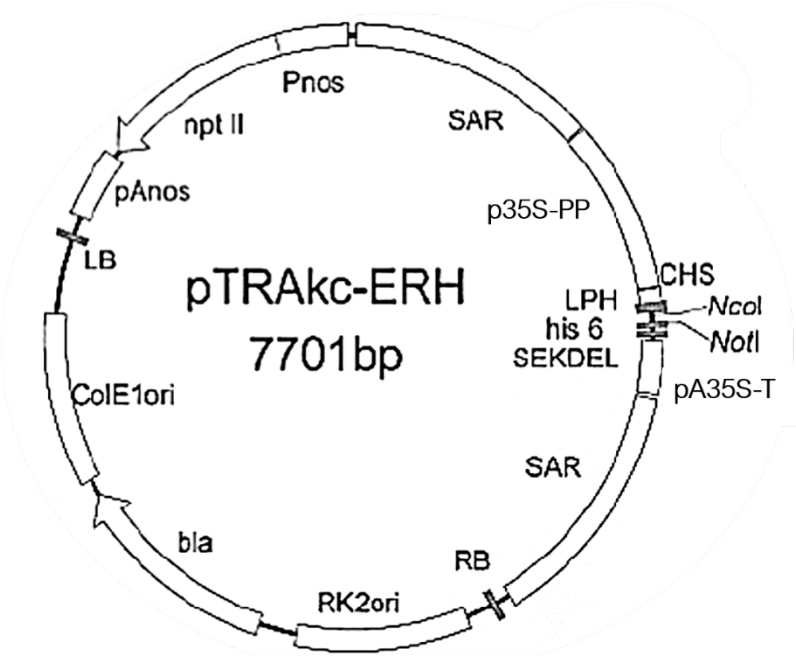

***Figure 2.1: pTRAkc-ERH Agrobacterium binary vector**. A gene of interest can be ligated into pTRAkc-ERH for Agrobacterium-mediated infiltration of plants. LB and RB, left and right borders respectively for T-DNA transfer; Pnos and pAnos, promoter and polyadenylation sequence of nopaline synthase; npt II, kanamycin resistance gene; SAR, scaffold attachment region of the tobacco Rb7 gene; p35S-PP, CaMV35S promoter with duplicated enhancer; CHS, calchone synthase 5' untranslated region; LPH, murine mAb24 heavy chain signal sequence; his 6, 6xhistidine tag; SEKDEL, endoplasmic reticulum retention sequence; pA35S-T, CaMV35S polyadenylation sequence; RK2ori, Agrobacterium origin of replication; bla, ampicillin/carbenicillin resistance gene; ColE1Ori, E. coli origin or replication. Adapted from Maclean et al. (2007).*

2.2 Materials and Methods

The reagents in this study were acquired from Sigma Aldrich (Missouri, United States of America) unless stated otherwise. All primers were synthesised at Inqaba Biotech (Pretoria, South Africa). Sanger sequencing was performed at the University of Pretoria sequencing facility (South Africa). Centrifugation was done using the table top Eppendorf Minispin® centrifuge (Hamburg, Germany) unless otherwise stated. The molecular weight marker used in all agarose gel images was the Fast DNA ladder (New England Biolabs, Ipswich, England). All PCR reactions were performed using the Applied Biosystems GeneAmp® PCR 2700 System (Foster City, California, USA).

2.2.1 Identifying the Cercospora zeina Ecp2 candidate effector

The *Cercospora zeina* CMW25467 V4A whole genome sequence is available on NCBI Genbank [Accession: MVDW01000014; Bioproject: PRJNA355276] (Wingfield et al. 2017). It was searched for homologues of the *Ecp2* (extracellular protein 2) effector gene. The *Cladosporium fulvum* (*Passalora fulva*) *Ecp2* (*CfEcp2*) nucleotide sequence [Accession: Z14024.1] (Van den Ackerveken et al. 1993) was used as a reference to search the NCBI (National Centre for Biotechnology Institute) database via BLASTx for *C. zeina* ECP2 protein homologs. Where a *C. zeina* protein accession was observed, tBLASTn was done using that accession against the *C. zeina* genome assembly [Accession: GCA_002844615.1]. A 100% identity against a nucleotide sequence on a contig from the genome assembly was expected. Using the *C. zeina* protein accession as a reference, the gene, mRNA and CDS sequences were located on the contig. These sequences were aligned using CLC Main Workbench 7 (CLCBio, Aarhus, Denmark) to check for the presence of introns and to determine the amino acid reading frame after intron excision.

The putative *C. zeina* ECP2 protein (CzECP2) accession was used in a BLASTp analysis against the GenBank database. The search was done to verify similarity against the *C. fulvum* (*P. fulva*) ECP2 protein (CfECP2) accessions [Accession: CAA78401.1 published 14 November 2006 and Accession: QDX18258.1 published 07 August 2019] and other Dothideomycete fungi. Putative CzECP2 was aligned (pairwise alignment) (using MatGat Matrix Global Alignment Tool (Campanella et al. 2003)) against the ECP2 protein sequences of other Dothideomycete fungi (*Cercospora beticola, Cercospora berteroae, Dothistroma septosporum, Pseudocercospora (Mycosphaerella) fijiensis* and *C. fulvum*) (Hyde et al. 2013; Crous and Braun 2003) to compare their similarity and identity. The signal peptide of putative CzECP2 (and the other Dothideomycete fungi) was identified using SignalP 5.0 (Almagro Armenteros et al. 2019). The CzECP2 mature protein sequence (lacking the signal peptide) was compared to those of the other fungi using the ClustalW alignment tool (Larkin et al. 2007). The alignments were imported into CLC Main Workbench 7 for editing.

Gene expression data from a previous study (Swart et al. 2017) was analysed for the presence of an accession that matched the putative CzECP2 protein accession. The data was based on the study of

C. zeina genes expressed *in planta* (maize) and *in vitro* under different conditions for annotation of the *C. zeina* genome using gene models. The *in vitro* RNA sequence data compared the expression of putative genes from *C. zeina* cultured in various growth media such as cornmeal agar, complete medium agar and PDA (potato dextrose agar). Possible expression of *CzEcp2* in the genome was deduced based on the corresponding putative protein accession being listed in the RNA sequence data (GEO Dataset: *in planta*, GSE94442; *in vitro*, GSE90705) (Swart et al. 2017). Homology of the accession to that of CfECP2 and other ECP2 protein sequences was used as motivation to select it as CzECP2.

Putative *CzEcp2* expression was compared to the expression of a stably expressed housekeeping gene, GAPDH (glyceraldehyde-3-phosphate dehydrogenase), and a previously characterised cercosporin toxin biosynthesis (*CTB1*) gene (Swart et al. 2017). The median read counts of each gene was calculated for each *in planta* and *in vitro* treatment. This was used to compare the expression of each gene against the expression of the total number of *C. zeina* genes (transcripts) in the RNA sequence data. The average expression of putative *CzEcp2*, GAPDH and *CTB1* were then obtained by dividing the read count value for each treatment (*in planta* and *in vitro*) by the median obtained for the total number of genes expressed for each treatment. The media with the highest read count of *CzEcp2* RNA transcripts was selected for *in vitro* growth of *C. zeina* CMW25467 in this study to stimulate high expression levels of candidate *Ecp2*.

2.2.2 Primer design

Gene specific primer pairs were designed using *C. zeina* cDNA (complementary DNA) as a template to isolate the *Ecp2* gene from the genome [MVDW01000239.1] (Table 2.1). The cDNA instead of gDNA (genomic DNA) sequence was used due to the presence of an intron (65 bp) in the gDNA sequence which would cause an amino acid frameshift after intron excision. Primer Designer 4 software (Scientific and Educational Software 2000) was used, and the following parameters were specified: %GC content minimum and maximum of 50 and 60 respectively; minimum and maximum melting temperatures of 55 and 80 ºC respectively; forward and reverse primer lengths of 20 nucleotides; annealing temperatures of 55 ºC and 5’ vs 3’ end stability greater than or equal to 1.2 kcal.

The forward and reverse primers flanked the start (ATG) and stop (TAG) codons respectively so that the full *CzEcp2* coding sequence could be isolated (Table 2.1). The expected product size was 575 bp (base pairs) including nucleotide bases in the 5’ and 3’ UTR. For downstream PCR reactions, *C. zeina* primers (*CzCTB10*) designed in a previous study to flank an intron (*Segal, 2017 Honours Report*) were used to screen for gDNA contamination in isolated RNA and cDNA samples.

Cloning primers were designed from the cDNA sequence to add restriction enzyme sites before and after the start and stop codons respectively of candidate *CzEcp2* (Table 2.1). NcoI (5’ CCATGG 3’)

and EcoRI (5' GAATTC 3') sites were added to the 5' ends of the respective forward primers and a BamHI (5' GGATCC 3') site was added to the 3' end of the reverse primer. Primer pair 1 (*CzEcp2*-EcoRI Forward 1 and *CzEcp2*-BamHI Reverse) was designed to amplify the complete *CzEcp2* sequence with its signal peptide. Primer pair 2 (*CzEcp2*-NcoI Forward 2 and *CzEcp2*-BamHI Reverse) was designed to amplify the mature *CzEcp2* sequence without its signal peptide. The amplification product (575 bp) from the gene specific primer pair in Table 2.1 was used as the reference sequence from which the cloning primers were designed.

Table 2.1: Primers designed to isolate CzEcp2 from the C. zeina genome and clone it into the pTRAkc-ERH binary vector.

	Sequence Direction 5'- 3' [1]			
Primer name	***Sequence***	***Restriction site [2]***	***Leader sequence [3]***	***Annealing temperature [4]***
	Gene specific			
CzEcp2 Forward	ACCACACTCCTCCACCAAGA	-	-	58
CzEcp2 Reverse	TCCACCAGCAGCGCATACTC	-	-	58
	gDNA screening			
CzCTB10 Forward	CGCCAAGCTGCAACCTGTTC	-	-	58
CzCTB10 Reverse	CGAGCAACGTGAGCTGATGA	-	-	58
	Cloning			
CzEcp2-EcoRI Forward 1	TGACGAATTC**C**ATGCTTTTCA ACGTCGCTACC	EcoRI (GAATTC)	TGAC	58
CzEcp2-NcoI Forward 2	CAGCCATGGTCCCACAGAGGA AGAA	NcoI (CCATG**G**)	CAG	58
CzEcp2-BamHI Reverse	AGTGGATCCCTAGTTCGATGG GTTGTA	BamHI (GGATCC)	AGT	58
	pJET1.2/blunt specific			
pJET Forward	CGACTCACTATAGGGAGAGCG GC	-	-	60
pJET Reverse	AAGAACATCGATTTTCCATGG CAG	-	-	60
	pTRAkc-ERH specific			
pA35S Forward	CTGACGTAAGGGATGACGCAC	-	-	60
pA35S Reverse	GATTTGTAGAGAGACTGGTG	-	-	60

1 The directionality of each sequence (primer, restriction enzyme and leader) in the table is from 5' to 3'.
2 Restriction sites were added to primers designed for the cloning of CzEcp2 into pTRAkc-ERH.
3 The primers to which restriction sites were added also had leader sequences to improve enzyme digestion.
4 Each primer had an optimal annealing temperature for polymerase chain reactions.

Leader sequences of 3-4 bp (Table 2.1) were included at the beginning of each cloning primer restriction site to facilitate adequate restriction enzyme digestion of the *CzEcp2* amplicons. The mature *CzEcp2* sequence (lacking a signal peptide) has GTC (instead of ATG) as the first codon, therefore the last G nucleotide from the NcoI restriction sequence was removed to ensure that the final protein sequence would be in the correct reading frame. An extra C nucleotide was added to the

EcoRI restriction sequence for the *CzEcp2*-EcoRI Forward 1 primer so that it could be a better match to the Kozak sequence required for protein translation (Kozak 2002).

2.2.3 Culturing Cercospora zeina

To promote growth of *C. zeina* CMW25467 V4A, conidia from a 10% glycerol stock (250 µl) were spread onto V8 juice agar media (20 g agar bacteriological, 3.49 g calcium carbonate, 200 ml V8 juice, made up to 1 L with double distilled water) (Meisel et al. 2009). The V8 agar was supplemented with cefotaxamine antibiotics [50 µg/ml]. For maintenance of conidiation, a pat-culture technique was used to transfer the *C. zeina* conidia to fresh V8 agar after 7 days of growth in complete darkness (Swart et al. 2017). Conidia were sub-cultured when they turned greenish-grey in colour and grew to cover the whole surface of the V8 agar. The *C. zeina* conidia were transferred to fresh V8 media until a total of 40 V8 agar cultures were obtained over eight consecutive weeks.

2.2.4 Harvesting Cercospora zeina conidia

C. zeina conidia were harvested from the V8 agar cultures and transferred onto cornmeal agar (17 g cornmeal; 1 L ddH_2O) supplemented with cefotaxamine [50 µg/ml] and lined with cellophane. The cultures were incubated under constant light at room temperature for 7 days (Swart et al. 2017). The conidia were transferred from V8 agar as follows: 3 ml double distilled water (ddH_2O) was pipetted onto the lawn of conidia on each V8 agar plate; a sterile glass spreader was used to dislodge the conidia, after which the suspensions from each plate were pooled into a glass beaker and kept on ice.

A Neubauer haemocytometer (Celeromics, Grobler, France) was used to measure the spore suspension concentration. Three dilutions were prepared as follows: $\frac{1}{10}$, $\frac{1}{50}$ and $\frac{1}{100}$ (conidial suspension to ddH_2O). From each dilution, 10 ul was pipetted onto the haemocytometer and analysed under a light microscope at 10X magnification. The spores were counted in the quadrants (Figure 2.2) of the haemocytometer to determine the final concentration of the suspension. Concentration calculations were done according to the Celeromics guidelines.

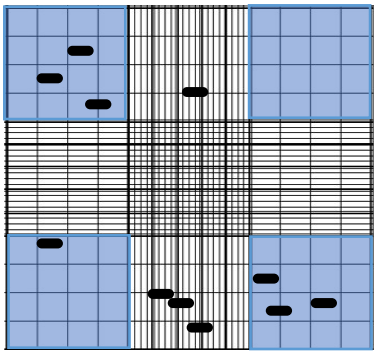

Figure 2.2: Neubauer Heamocytometer. *The counting chamber used to calculate the C. zeina conidia (black cylindrical shapes) concentration by only counting the conidia within the blue quadrants. For each dilution, conidia in all four quadrants were counted and the average calculated.*

2.2.5 Cercospora zeina RNA extraction and cDNA synthesis

RNA was extracted from *C. zeina* biological replicates using the QIAzol Lysis Reagent (Qiagen, Hilden, Germany) according to the kit guidelines. The RNeasy Plant Mini kit was used for clean-up of the RNA samples as per the kit guidelines to remove organic material. Genomic DNA (gDNA) contamination was removed by treating the isolated RNA with the Turbo DNA-free kit (Life Technologies, California, USA) according to the manual. RNA quality and concentrations were determined with the Thermo Fisher Scientific NanoDrop 2000 Spectrophotometer (Massachusetts, USA). For good quality RNA, the A_{260}/A_{280} and A_{260}/A_{230} ratios were expected to be between 1.8-2.2 and above 2.0 respectively.

To ascertain that the RNA was not degraded and did not have gDNA contamination, a 1% TAE agarose gel electrophoresis was conducted (1 g Seakem agarose (Lonza, Basel, Switzerland); 100 ml 1X TAE; 0.4 µl Ethidium bromide (EtBr) [10 mg/ml]) in 1X TAE buffer (10 mM Tris-HCl, pH 7.4; 1 mM EDTA) at 80 V for 1 hr. Agarose gels were visualised on a Molecular Imager® Gel Doc™ XR System (BIORAD, California, USA) to look for bands that represented the isolated RNA (28S, 18S and 5S rRNA). A standard PCR (polymerase chain reaction) (Table 2.2) was set up using the isolated RNA as a template and the *CzCTB10* primers (Table2.1) to check for gDNA contamination. Observation of bands would indicate contamination. Positive and negative control reactions were prepared using *C. zeina* gDNA (0.3 µg/µl) and ddH_2O respectively.

A single strand (first strand) of cDNA was synthesised from *C. zeina* RNA using the Maxima H Minus First Strand cDNA Synthesis Kit (Thermo Fisher Scientific, Massachusetts, USA) as per the kit guidelines. OligodT primers from the kit were used to amplify the cDNA in RT-PCR (reverse transcriptase PCR) reactions to obtain a double stranded template. Each cDNA sample was diluted to 10 pg/µl. The cDNA samples were stored at -70 ºC.

2.2.6 RT-PCR of candidate Cercospora zeina Ecp2

The *CzEcp2* gene specific primers (Table 2.1) were used to isolate candidate *Ecp2* from the *C. zeina* CMW25467 genome. The reactions (10 µl final volume) were prepared as follows: 0.5 µl forward primer [10 µM]; 0.5 µl reverse primer [10 µM]; 3 µl Ampliqon Taq Polymerase (Odense, Denmark); 1 µl *C. zeina* cDNA template and 5 µl nuclease-free water. Positive and negative control reactions were prepared using *C. zeina* CMW25467 gDNA [0.3 µg/µl] and nuclease-free water respectively. The Applied Biosystems GeneAmp 2700 thermal cycler (Foster City, California, United States of America) was programmed according to Table 2.2. The expected size for the *CzEcp2* PCR product was 575 bp. The amplicon was visualised in a 1% TAE agarose gel as previously described.

Table 2.2: Thermal cycler protocol used for C. zeina cDNA synthesis and CzEcp2 isolation via RT-PCR.

Steps	Temperature	Time	Cycles
Initial Denaturation	95ºC	3 minutes	1
Denaturation	95ºC	15 seconds	
Annealing	58ºC	15 seconds	30
Extension	72ºC	20 seconds	
Final Extension	72ºC	25 minutes	1
Hold	4ºC	10 minutes	1

2.2.7 Growing Nicotiana spp.

Seeds of *Nicotiana benthamiana, Nicotiana tabacum* cv. Petit Havana and *Nicotiana tabacum* cv. LA Burley were sown on rehydrated Jiffy® peat pellets (Kristiansand, Norway) which were kept under controlled conditions in a phytotron (16 hr light at 25 ºC and 8 hr dark at 20 ºC (Leuzinger et al. 2013)). Approximately 5-7 seeds were sprinkled onto the surface of each rehydrated jiffy which were placed into cat litter trays. The jiffies were watered every three days with tap water. Once a week, the tap water was supplemented with Nitrosol fertilizer (Builders Warehouse, Pretoria, South Africa). When seedlings emerged (approximately 2-3 cm after 3-4 weeks), they were transplanted into polystyrene cups that contained a 1:1 ratio of organic compost (Garden Master, Johannesburg, South Africa) and swimming pool filter sand (BlueChem, Builders Warehouse, Pretoria, South Africa). Transplanted seedlings were watered 3 days a week with tap water and once a week with tap water containing Nitrosol. The seedlings were left to grow under the same conditions for 9 weeks.

2.2.8 Bacterial strains and plasmid vectors

Competent *Escherichia coli* DH5α cells were prepared using the calcium chloride ($CaCl_2$) technique (Evans 1990). Briefly, a single colony of bacteria was picked from a culture grown for 24 hr at 37 ºC on Luria-Bertani (LB) agar. The single colony was grown overnight in LB broth at 37 ºC with shaking at 150 rpm (revolutions per minute). The overnight culture was diluted by adding 1 ml of bacteria into 50 ml LB broth and grown to an OD_{600} of 0.4 at 37 ºC and shaking at 150 rpm. The cells were cooled on ice then spun down for 30 min at 956xg at 4 ºC in the Eppendorf 5810 R centrifuge (Hamburg, Germany). The pellet was dissoled in 25 ml of ice-cold 0.1 M $CaCl_2$ (1.4702 g $CaCl_2$ in 100 ml ddH_2O). The cells were then precipitated at 956xg for 30 min at 4 ºC. The supernatant was discarded, and the cells were dissolved in 2.5 ml ice-cold $CaCl_2$ and 375 ml sterile 100% glycerol. The suspension was incubated on ice for 1 hr after which 100 µl aliquots were flash frozen in liquid nitrogen and stored at -70 ºC.

The $CaCl_2$ competent *E. coli* DH5α cells were heat shocked prior to transformation. The 100 µl aliquots were defrosted on ice before recombinant vector DNA was added to the cells, which were incubated for 30 min on ice. To test if the cells were highly competent, a positive control transformation was prepared using pUC18 plasmid DNA [150 ng/µl]. The samples were heat shocked at 42 ºC for 90

seconds followed by incubation on ice for 2 min. LB-glucose broth (900 µl) was added to the bacteria which were incubated at 37 ºC for 1 hr, shaking at 150 rpm. The transformed cells (200 µl) were cultured on LB agar plates (with antibiotics) and incubated at 37 ºC overnight to check for transformation. Untransformed competent *E. coli* DH5α cells were also heat shocked and spread onto LB agar (with antibiotics) as a negative control to test the viability of the antibiotics in the agar. No bacterial growth was expected. *E. coli* DH5α transformation was performed for replication of the recombinant vectors at a high rate. The vectors were extracted from the bacteria and used in downstream experiments.

A. tumefaciens GV3101 (pSOUP+pMP90) obtained from the James Hutton Institute (Dundee, United Kingdom) was transformed using electroporation. The pSOUP vector was not a requirement for agroinfiltration of tobacco with the gene of interest (*CzEcp2*). It carries the *repA* (pSa replicase gene) sequence essential for transfer of T-DNA from pGreen vectors because *repA* recognises the pGreen pSa origin of replication. Without pSOUP, pGreen cannot undergo replication (Hellens et al. 2000b; Hellens et al. 2000a). It was maintained using tetracycline selection for consistency throughout the study. The cells were made electrocompetent as follows: The bacteria were grown at 28 °C on YEB agar (containing 5 µg/ml tetracycline and 25 µg/ml gentamicin) for 3 days. A single colony was picked from the culture and grown at 28 °C in 100 ml YEB broth (containing 5 µg/ml tetracycline and 25 µg/ml gentamicin) with shaking at 150 rpm; the cells were centrifuged at 2 057xg for 10 min at 4 °C; the pellets were resuspended in 50 ml ddH_2O and pelleted again; the water-rinse step was repeated; the bacterial pellet was dissolved in 50 ml 10% glycerol and washed as described above; the glycerol-wash step was repeated; the pellet was resuspended in a final volume of 5 ml 10% glycerol and 100 µl aliquots were flash-frozen and stored at – 70 °C (Evans 1990).

Electroporation was performed using the Eppendorf® Electroporator 2510 machine (Hamburg, Germany). The protocol was as follows: The 100 µl aliquots of electrocompetent *Agrobacterium* cells were thawed on ice; recombinant plasmid DNA (5 µl) was added to the thawed cells and incubated for 5 min on ice; the cells were transferred to a pre-chilled electroporation cuvette; the machine was set to 1 800 V; once the cells were electroporated, 1 ml YEB-glucose was added to the cuvette then aliquoted into 2 ml Eppendorf tubes; the cells were incubated at 28 °C for 2 hr shaking at 80 rpm; the bacteria (100 µl) were spread onto YEB agar containing 5 µg/ml tetracycline, 25 µg/ml gentamicin and 150 µg/ml carbenicillin and incubated at 28 ºC for 2-5 days.

Transformation efficiency was calculated using the following formula:

$$Transformation\ efficiency\left(\frac{CFU}{\mu g}\right) =$$

$$\frac{number\ of\ single\ colonies\ (CFU)}{DNA\ concentration\ (\mu g)} \times \frac{transformation\ volume\ (\mu l)}{volume\ plated\ (\mu l)}$$

pCambia2300 (https://cambia.org/welcome-to-cambialabs/cambialabs-projects/cambialabs-projects-legacy-pcambia-vectors-pcambia-legacy-vectors-1/) plasmid DNA was used for positive control of the *Agrobacterium* transformations. The PCR cloning vector used was the Thermo Fisher Scientific pJET1.2/blunt vector (Massachusetts, USA) and the binary vector used for transfer of *CzEcp2* into the tobacco plants was pTRAck-ERH (Maclean et al. 2007).

2.2.9 PCR ligation of candidate C. zeina Ecp2 into pJET1.2/blunt

Candidate *C. zeina Ecp2* (*CzEcp2*) was ligated into the CloneJET PCR cloning vector (pJET1.2/blunt) [50 ng/µl] (Thermo Fisher Scientific) as per the kit instructions, to obtain the full-length sequence. The vector has a lethal gene for positive selection of recombinant constructs. Therefore, if *CzEcp2* was not successfully ligated into the vector, the *E. coli* DH5α bacteria would be killed. Where bacterial growth was observed, the vector and *CzEcp2* were successfully ligated. A control cloning experiment was performed using the kit control PCR product [24 ng/µl] to verify the efficiency of the blunting and ligation steps. The recombinant vector (3 µl), pJETCzEcp2 was used to transform 100 µl of calcium chloride competent *E. coli* DH5α cells cultured on LB- ampicillin [50 µg/ml] as described above. Recombinant pJET1.2/blunt carrying the control PCR product (3 µl) was also transformed into *E. coli* DH5α. pUC18 plasmid DNA [150 ng/µl] was transformed into *E. coli* DH5α as the positive control for the transformation reactions. The transformation negative control was *E. coli* DH5α lacking plasmid DNA.

Colony PCR screening of four transformed *E. coli* DH5α colonies was performed to determine the presence of pJETCzEcp2 in the bacteria. A 10 µl reaction was prepared as follows: 0.5 µl each of pJET1.2/blunt forward and reverse primers [10 µM] (Table 2.1); 3 µl Ampliqon Taq polymerase; 6 µl ddH_2O; a single colony of transformed bacteria picked as template. The thermal cycler was programmed according to Table 2.2, but the annealing temperature was altered to 60 °C and the final extension step was excluded. pJETCzEcp2 was extracted from colony #1 of the screened *E. coli* DH5α pJETCzEcp2 transformants that grew on the selection agar. The GeneJet Plasmid Midiprep Kit was used (Thermo Fisher Scientific) as per the kit instructions. The vector DNA concentrations and quality were measured using the NanoDrop 2000 Spectrophotometer (Thermo Fisher Scientific).

Sanger sequencing was performed to determine if the *CzEcp2* gene sequence in pJETCzEcp2 was correctly inserted and unmutated. The pJET1.2/blunt sequencing primers were used in standard PCR reactions with pJETCzEcp2 as the template as described above (Table 2.2). ExoSap-IT (Thermo Fisher Scientific) was used to clean the PCR products as per the kit guidelines. The PCR products were visualised in a 1% TAE agarose gel separated at 80V for 1 hr as described above. The PCR product concentrations were measured using the NanoDrop 2000 Spectrophotometer (Thermo Fisher Scientific) and were expected to be between 60-100 ng per 1000 bp. Cycle sequencing (10 µl final volume) PCR reactions were then set up according to the BigDye v3.1 (Applied Biosystems,

California, USA) protocol with separate forward and reverse primer reactions as follows: 2 µl cleaned standard PCR product; 2 µl BigDye (Applied Biosystems); 0.5 µl forward/reverse pJET1.2/blunt sequencing primer [10 µM]; 2 µl 5X sequence buffer (Applied Biosystems); 4.5 µl nuclease-free water. The thermal cycler was programmed according to Table 2.3.

The sequence data was analysed using CLC Main Workbench 7 (CLCBio, Qiagen). The sequences from the forward and reverse reactions were trimmed and aligned to obtain contigs. The contigs were then aligned to reference sequences of *CzEcp2* cDNA and gDNA obtained from the NCBI database [Accession: MVDW01000239.1]. Complete similarity was expected between the sequencing results and the reference sequences.

Table 2.3: BigDye v3.1 sequencing PCR thermal cycler protocol.

Steps	*Temperature*	*Time*	*Cycles*
Initial denaturation	96 ºC	1 minute	1
Denaturation	96 ºC	10 seconds	25
Annealing	58 ºC	5 seconds	
Extension	60 ºC	4 minutes	
Hold	4 ºC	10 minutes	1

2.2.10 Addition of restriction sites to CzEcp2 for ligation into pTRAkc-ERH

pJETCzEcp2 was used in standard PCR reactions as described above with the cloning primers in Table 2.1. The primers added respective restriction enzyme sites upstream and downstream of the *CzEcp2* gene. Primer pair 1 (*CzEcp2*-EcoRI Forward 1 and *CzEcp2*-BamHI Reverse) added an EcoRI and BamHI site to the 5' and 3' ends respectively. Primer pair 2 (*CzEcp2*-NcoI Forward 2 and *CzEcp2*-BamHI Reverse) added NcoI and BamHI to the 5' and 3' ends respectively.

Primer pair 1 amplified *CzEcp2* with the signal peptide sequence therefore isolating the complete sequence (*CzEcp2* EB). Primer pair 2 amplified *CzEcp2* without the signal peptide sequence (*CzEcp2* NB) therefore isolating the mature sequence. The amplicons were separated in a 1.2 % TAE agarose gel (1.2 g SeaKem agarose; 100 ml 1X TAE; 0.4 µl EtBr [10 µg/ml]) at 80V for 1 hr with an expected band size of 524 bp and 458 bp for *CzEcp2* EB and *CzEcp2* NB respectively. Sanger sequencing was performed as described above for both sequences to determine that the restriction sites were added to the correct part of each sequence and that the genes were unmutated.

2.2.11 Binary vector construction

The pTRAkc-ERH binary vector was obtained from the Biopharming Research Unit at the University of Cape Town (South Africa) (Maclean et al. 2007) (Appendix, Figure S8). Restriction enzyme double digests of the vector were performed as follows: 14 µl ddH_2O; 2 µl 10X FastDigest buffer; 2 µl plasmid DNA; 1 µl FastDigest enzyme 1 and 2 respectively. The reaction mixtures were incubated for 5 min at 37 °C. For ligation of *CzEcp2* EB (full sequence) into pTRAkc-ERH, both sequences were double digested with EcoRI and BamHI. For ligation of *CzEcp2* NB (mature sequence) into pTRAkc-ERH,

both sequences were double digested with NcoI and BamHI. Digested *CzEcp2* was separated in a 1.2% TAE agarose gel as described above. Digested pTRAkc-ERH was separated in a 0.8% TAE agarose gel as described above with 1 µl 6X loading dye (Fermentas). The expected product sizes were 515 bp for *CzEcp2* EB (complete sequence) and 450 bp for *CzEcp2* NB (mature sequence). For pTRAkc-ERH 1 (digested with EcoRI and BamHI) and pTRAkc-ERH 2 (digested with NcoI and BamHI), the expected product sizes were 7 499 bp and 7 608 bp respectively.

The Zymoclean Gel Recovery Kit (Zymo Research, Tustin, USA) was used to extract DNA from the bands of interest according to the kit guidelines. The recovered DNA was quantified using the NanoDrop 2000 Spectrophotometer (Thermo Fisher Scientific). The recovered DNA was separated in a 1% TAE agarose gel as described above to analyse the quality before ligation reactions. Ligation reactions were performed according to the NEB T4 DNA ligase kit (New England Biolabs, Ipswich, England) protocol using a 5:1 ratio of insert:vector DNA. pTRAkc-ERH 1 was ligated with *CzEcp2* EB to create recombinant pTRAkc-ERHCzEcp2 EB (fungal signal peptide). pTRAkc-ERH 2 was ligated with *CzEcp2* NB (fused to LPH signal peptide) to create recombinant pTRAkc-ERHCzEcp2 NB (LPH signal peptide). Control ligation reactions were prepared where insert DNA was excluded from the reactions (pTRAkc-ERH minus insert DNA including ligase; pTRAkc-ERH minus insert DNA excluding ligase).

Each ligation reaction (including controls) (3 µl) was used to respectively transform 100 µl of $CaCl_2$ competent *E. coli* DH5α cells as described above. To screen for transformed bacteria, 200 µl of each reaction was spread onto LB agar containing 50 µg/ml ampicillin. pUC18 plasmid DNA [150 ng/µl] ddH_2O were used for the positive and negative control transformations respectively. The cultures were incubated overnight at 37 °C with the expectation of colony formation on all plates except the negative control and vector minus insert DNA excluding ligase transformations. Colony PCR was performed as described above to determine if the recombinant plasmid was present in the transformed bacteria (Table 2.2). The pA35S forward primer (Table 2.1) specific to a portion of the pTRAkc-ERH promoter, and the insert-specific reverse cloning primer CzEcp2BamHI Reverse (Table 2.1) were used in the colony PCR reactions. A 1.2% agarose gel was used to visualise the amplicons as described above.

pTRAkc-ERHCzEcp2 EB (fungal signal peptide) and pTRAkc-ERHCzEcp2 NB (LPH signal peptide) recombinant plasmid DNA were extracted from transformed *E. coli* DH5α cells using the GeneJet Plasmid Midiprep Kit (Thermo Fisher Scientific). The plasmid DNA concentrations were quantified using the NanoDrop 2000. The recombinant constructs were double digested as described above with EcoRI and BamHI, and NcoI and BamHI respectively to check that the genes were inserted into the vectors and that the restriction enzyme sites were recreated. The restriction digest products were separated in a 0.8% TAE agarose gel as described above. Standard PCR reactions were performed using the respective recombinant vectors (pTRAkc-ERHCzEcp2 EB and pTRAkc-ERHCzEcp2 NB) as template and the cloning primers in Table 2.1 (*CzEcp2*-EcoRI Forward 1 + *CzEcp2*-BamHI

Reverse; *CzEcp2*-NcoI Forward 2 + *CzEcp2*-BamHI Reverse respectively) to validate the presence of *CzEcp2*. The PCR products were separated in a 1.2% TAE agarose gel as described above. Sanger sequencing was performed as described above using the pA35S forward primer and the *CzEcp2*-BamHI reverse primer. This was done to ensure that the *CzEcp2* EB (full sequence) and *CzEcp2* NB (mature sequence) sequences were inserted into pTRAkc-ERH 1 and pTRAkc-ERH 2 respectively, were not mutated and were in the correct orientation.

2.2.12 Agrobacterium tumefaciens GV3101 (pSOUP+pMP90) transformation

Agrobacterium tumefaciens GV3101 (pSOUP+pMP90) (obtained from JHI, Dundee, Scotland) was made competent using electroporation as described above. The pSOUP vector was not a requirement for agroinfiltration and was maintained for consistency throughout the study. The bacteria were transformed with 5 µl of pTRAkc-ERHCzEcp2 EB and pTRAkc-ERHCzEcp2 NB respectively. For the negative vector control, nonrecombinant pTRAkc-ERH was transformed into the bacteria. To test the bacterial competency and transformation efficiency, pCambia2300 plasmid DNA [150 ng/µl] (CAMBIA) was transformed into the bacteria as a positive control. YEB agar containing 150 µg/ml carbenicillin, 5 µg/ml tetracycline and 25 µg/ml gentamicin was used to select for transformants. Untransformed *A. tumefaciens* GV3101 (pSOUP+pMP90) was also cultured as a negative control.

Screening for the presence of empty pTRAkc-ERH within the transformed *Agrobacterium* was done using the pA35S pTRAkc-ERH specific primers in Table 2.1. The expected product size was 338 bp. pTRAkc-ERHCzEcp2 EB and pTRAkc-ERHCzEcp2 NB were located using the pA35S forward primer and the *CzEcp2*-BamHI reverse primer (Table 2.1). The expected product sizes were 612 bp and 656 bp respectively. The amplification product of pTRAkc-ERHCzEcp2 NB was expected to be larger due to amplification of *CzEcp2* NB plus the LPH sequence and some of the promoter sequence of the vector.

The accuracy of the transformed *Agrobacterium* colony PCR reactions was validated using *Agrobacterium* specific primers (MiaA5 forward primer: 5' CCGGCCCGACGGCAAGCGGC 3'; MiaA3 reverse primer: 5' CGGCTGGATGCGCGTCCAG 3'; 72 °C T_m) (Grayburn and Vick 1995). This was done in case amplification was not obtained for any of the recombinant vectors transformed into the bacteria. The colony PCR products were separated in a 1.2% TAE agarose gel as described above.

2.2.13 Nicotiana spp. transient agroinfiltration

Nine-week-old *N. benthamiana, N. tabacum* cv. Petit Havana and *N. tabacum* cv. LA Burley were transiently infiltrated with transformed *A. tumefaciens* GV3101 (pSOUP+pMP90). The bacterial strains carrying the recombinant vectors were respectively named Agro pTRAkc-ERHCzEcp2 EB (complete *CzEcp2*) and Agro pTRAkc-ERHCzEcp2 NB (mature *CzEcp2*) where Agro was short for *A. tumefaciens* GV3101 (pSOUP+pMP90). The transformed bacterial cells were prepared for infiltration as follows: single colonies carrying the recombinant plasmids were respectively inoculated

into 5 ml YEB broth containing 25 µg/ml gentamicin, 5 µg/ml tetracycline and 150 µg/ml carbenicillin; the cultures were incubated overnight at 28 °C with 150 rpm shaking; 25 ml of YEB broth containing the same antibiotics and 20 µM acetosyringone was inoculated with 1 ml of the overnight cultures; the diluted cultures were pelleted at 5000xg for 15 min at room temperature in the Eppendorf 5810 R centrifuge; the pellets were resuspended in resuspension solution (infiltration media supplemented with 100 µM acetosyringone) and the OD_{600} adjusted to 0.4; the bacterial solutions were incubated at room temperature for 2 hr prior to infiltration (Li 2011).

Infiltration media was prepared as follows: 0.9762 g 10 mM MES; 1.0165 g 10 mM $MgCl_26H_2O$; 500 ml ddH_2O; pH 5.4. Acetosyringone (200 mM) was prepared as follows: 0.392 g acetosyringone in 10 ml DMSO (Dimethyl sulfoxide). Untransformed *A. tumefaciens* GV3101 (pSOUP+pMP90) was prepared as described above for infiltration as a negative control. *A. tumefaciens* GV3101 (pSOUP+pMP90) transformed with pTRAkc-ERH only was also prepared as a negative vector control for infiltration. The positive control for infiltration was *A. tumefaciens* GV3101 (pSOUP+pMP90) carrying the *Phytophthora infestans* INF1 effector gene. The *repA* sequence on the pSOUP vector was required for pSa replication of the vector carrying INF1 (Kamoun et al. 1998).

The abaxial surface of the tobacco leaves was infiltrated with the bacteria using a 1 ml sterile needleless syringe. Three leaves on each of three plants for each *Nicotiana* cultivar were infiltrated with 0.6 ml of each bacterial solution: Agro pTRAkc-ERHCzEcp2 EB (complete *CzEcp2*); Agro pTRAkc-ERH (no gene); Agro GV3101 (pSOUP+pMP90) only and Agro GV3101 (pSOUP+pMP90) INF1). The plants were monitored for a hypersensitive response and any other morphological change for 10 days post infiltration (dpi).

2.3 Results

2.3.1 Cercospora zeina candidate Ecp2 is present in the C. zeina genome

The *C. zeina* CMW25467 (Mkushi) V4A genome was searched for a candidate *Ecp2* gene using the *C. fulvum* (*P. fulva*) *Ecp2* nucleotide sequence [Accession: Z14024.1]. The nucleotide sequence (shown to produce a 165 amino acid precursor protein [Accession: CAA78401.1]) was previously characterised (Van den Ackerveken et al. 1993) and used as a reference to identify *Ecp2* in *C. zeina* (Lombard, MSc Thesis 2015). BLASTx against GenBank was done to identify a potential protein in *C. zeina* based on the translated nucleotide sequence of *CfEcp2* (*P. fulva Ecp2).* The search found 64% identity to a hypothetical protein from *C. zeina* (167 amino acids) [Accession: PKR94769.1] (Table 2.4).

tBLASTn of PKR94769.1 was done against the *C. zeina* genome assembly [Accession: GCA_002844615.1] to locate a translated nucleotide sequence of putative *C. zeina Ecp2 (CzEcp2)*. The accession hit part of a 14 893 bp contig [Accession: MVDW01000239.1] annotated with the putative protein, mRNA and CDS (Coordinates, Exon 1: 10 134 to 10 392; Exon 2: 10 458 to 10 702; Intron: 65 bp) sequences of *CzEcp2* (Table 2.4). The identified gene and CDS sequences of putative *C. zeina Ecp2* were aligned using CLC Main Workbench. The intron sequence was identified and the amino acid translation in the first reading frame included in the alignment (Appendix, Figure S1).

Table 2.4: NCBI BLAST search for candidate C. zeina Ecp2 using the C. fulvum (P. fulva) Ecp2 gene sequence (Z14024.1).

Accession	*Query cover* [1]	*E value* [2]	*% Identity* [3]
BLASTx of C. fulvum (P. fulva) Ecp2 (Z14024.1) against NCBI			
PKR94769.1	76%	5e-33	63.75
tBLASTn of PKR94769.1 against C. zeina genome assembly			
MVDW01000239.1	100%	6e-77	100

1 Query cover shows how much of the query sequence aligned to the BLAST hit.
2 The expected value shows the statistical significance of alignments that occur by chance with a cut-off value of 1.0e-5.
3 The percentage identity shows how many bases were identical between the query and the BLAST hit.

BLASTp of PKR94769.1 (putative *C. zeina* ECP2 accession) against GenBank was done to determine similarity and identity against other ECP2 proteins (hypothetical or characterised). Table 2.5 shows the results obtained from the search. The putative CzECP2 protein (PKR94769.1) had a 55.03% match with the *P. fulva* (*C. fulvum*) ECP2 [Accession: QDX18258.1 which was an identical match to the *C. fulvum* CAA78401.1 accession previously published (14 November 2006)].

Table 2.5: BLASTp search of GenBank with putative C. zeina ECP2 (PKR94769.1).

Organism	*Accession*	*E-value*	*% Identity*	*Hypothetical protein/ECP2*
C. zeina	PKR94769.1	3e-117	100	Hypothetical protein
Cercospora beticola	XP_023457559.1	2e-67	71.86	Hypothetical protein
Cercospora berteroae	PPJ52722.1	7e-66	70.66	Hypothetical protein
Pseudocercospora (Mycoshpaerella) fijiensis	XP_007922577.1	3e-58	59.63	Hypothetical protein
Dothistroma septosporum	EME39817.1	3e-52	58.33	Extracellular protein
C. fulvum (P. fulva)	QDX18258.1	6e-50	55.03	ECP2
C. fulvum (P. fulva)	CAA78401.1	6e-50	55.03	ECP2

SignalP 5.0 was used to determine the signal peptide of the putative *C. zeina* ECP2 protein (Almagro Armenteros et al. 2019). The complete (with signal peptide) and mature peptide sequences (lacking signal peptide) of CzECP2 were aligned using ClustalW (Larkin et al. 2007) against those of the fungi listed in Table 2.5 (Appendix, Figure S2 and S3). Pairwise alignments were performed with MatGat (Campanella et al. 2003) using each fungal peptide accession (complete and mature) in Table 2.5 against the *C. zeina* ECP2 accession (PKR94769.1) to determine the percentage identity and similarity (Table 2.6). The highest identity and similarity were observed for the alignments of *C. beticola* and *C. berteroae* against *C. zeina*.

Table 2.6: Pairwise alignments of complete and mature ECP2 peptide sequences of Dothideomycete fungi against C. zeina ECP2 (PKR94769.1).

Pairwise alignment of peptides against C. zeina ECP2 (PKR94769.1)					
Organism	*Accession*	*% Similarity*		*% Identity*	
		Complete	*Mature*	*Complete*	*Mature*
Cercospora beticola	XP_023457559.1	81.4	82.3	71.9	71.4
Cercospora berteroae	PPJ52722.1	79.6	80.3	70.7	70.1
Pseudocercospora fijiensis	XP_007922577.1	71.3	75.5	58.7	62.6
Dothistroma septosporum	EME39817.1	70.1	70.7	57.6	57.5
C. fulvum (P. fulva)	QDX18258.1	70.1	71.4	54.4	56
C. fulvum (P. fulva)	CAA78401.1	70.1	71.4	54.4	56.0

In vitro and *in planta* RNA sequence expression data was analysed for the potential presence of the CzECP2 protein accession (PKR94769.1) and therefore expression of candidate *CzEcp2*. Location of the accession in the RNA transcript read data suggested expression of a candidate *CzEcp2* under different *in vitro* and *in planta* conditions (Swart et al. 2017). This was used as motivation to select PKR94769.1 as candidate CzECP2. Expression of putative CzECP2 was compared against the expression of *GAPDH* (housekeeping gene) and another *C. zeina* gene, *CTB1* (Appendix, Table S1). GAPDH expression was consistently higher for all treatments compared to the other genes as expected for housekeeping genes.

The highest expression of putative *CzEcp2* transcripts was observed for *C. zeina* grown on PDA-AP, complete medium and cornmeal agar compared to the other *in vitro* treatments and *in planta*. *In planta CzEcp2* expression was higher than expression in YPD, V8 agar, PDA broth and PDA agar. The expression of *CTB1* was lower than that of *CzEcp2 in planta* and *in vitro* for PDA-AP agar, cornmeal agar and complete medium. The average expression of each gene (*GAPDH*, *CTB1* and *CzEcp2*) relative to the total number of *C. zeina* genes in the RNA sequence data was calculated using the median of the total reads obtained for each treatment (Appendix, Table S1). Therefore, the average expression (read/median) of *CzEcp2 in planta* was higher than for all the *in vitro* treatments and compared to the average expression *in planta* of GAPDH and CTB1.

2.3.2 Cercospora zeina growth, RNA extraction and cDNA synthesis

C. zeina CMW25467 V4A from a 10% glycerol stock was cultured on V8 agar to promote conidiation of the fungus. The conidia where then transferred to cornmeal agar to promote expression of *CzEcp2* (Swart et al. 2017). After 7 days in constant darkness on V8 and constant light on cornmeal agar the fungus appeared greenish-grey in colour as expected (Figure 2.3).

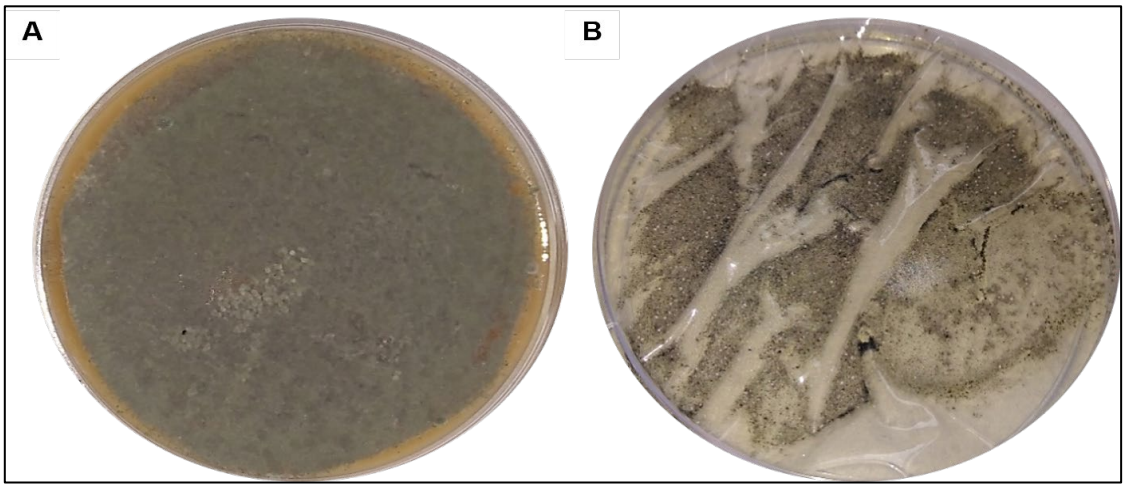

Figure 2.3: C. zeina cultures grown on V8 and cornmeal agar to stimulate Ecp2 expression. *A) Conidiation of C. zeina CMW25467 was achieved through culturing the fungus on V8 agar for 7 days in constant darkness. B) Conidia grew on cornmeal agar for Ecp2 expression in constant light for 7 days (Swart et al. 2017).*

RNA was extracted from *C. zeina* grown on cornmeal agar and cDNA was subsequently synthesised. Table 2.7 shows the RNA concentrations and quality values of each *C. zeina* replicate after isolation. The RNA was analysed in a 1% agarose gel (Figure 2.4A) where three bands were visible in each lane as expected. The top, middle and bottom bands represented 28S, 18S and 5S rRNA respectively as expected. Figure 2.4B shows that RT-PCR of *C. zeina* cDNA with the *CzEcp2* forward and reverse primers (Table 2.1) produced bands for the replicates (lanes 2-4) at the expected size of 575 bp. The *CzEcp2* gDNA sequence had an intron of 65 bp which was absent in the cDNA sequence, hence the bands in lanes 2-4 being smaller than the band in lane 1. The positive control (lane 1) amplified with the *CzEcp2* forward and reverse primers (Table 2.1) produced a band of 640 bp as expected because it included the intron. Lane 5 had no bands as expected for the negative control reaction. The PCR reactions with RNA template to test for gDNA contamination using the *CzCTB10* primers (Table 2.1) had no bands for any of the samples as expected (data not shown).

Table 2.7: C. zeina CMW25467 RNA quantification and quality analysis using the NanoDrop 2000 Spectrophotometer (Thermo Fisher Scientific).

Sample	Concentration (ng/µl)	$A_{260/280}$	$A_{260/230}$
C. zeina RNA rep 1	197.6	2.18	2.38
C. zeina RNA rep 2	168.4	2.17	2.37
C. zeina RNA rep 3	121.6	2.19	2.38

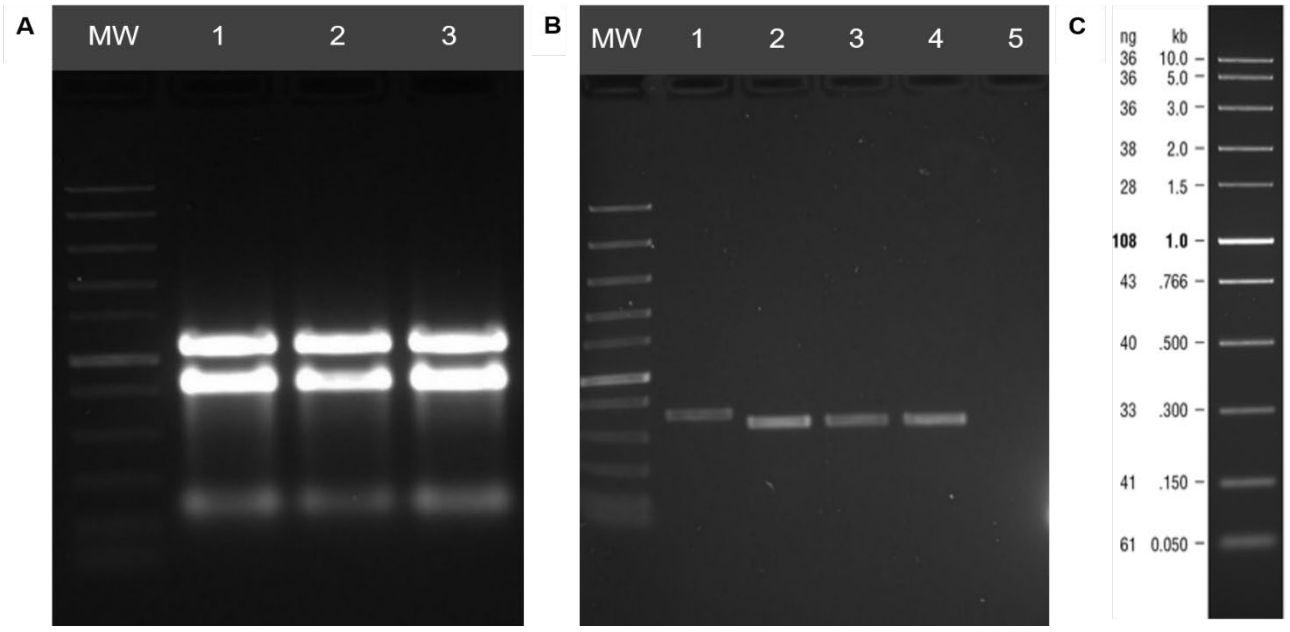

Figure 2.4: C. zeina CMW25467 RNA agarose gel and CzEcp2 amplification via RT-PCR. *A) In each lane, three rRNA bands were obtained for each sample (28S=top, 18S=middle and 5S=bottom). The RNA replicates were separated in a 1% agarose gel stained with EtBr. B) CzEcp2 was isolated from C. zeina CMW25467 cDNA in reverse transcriptase polymerase chain reactions (RT-PCR) using CzEcp2 gene specific primers (lanes 2-4, 575 bp). A positive control (640 bp, lane 1) and negative water control (lane 5) were included. The products were separated in a 1% agarose gel stained with EtBr. C) The NEB Fast DNA molecular weight marker (MW lane) was used to infer product sizes in the agarose gels.*

2.3.3 pJETCzEcp2 recombinant vector PCR and Sequencing

The full-length of *CzEcp2* was obtained by ligating the gene (575 bp RT-PCR product) into the pJET1.2/blunt PCR vector (Appendix, Figure S4). The recombinant vector, pJETCzEcp2 (3 549 bp) (Appendix, Figure S4) was transformed into chemically competent *E. coli* DH5α cells for rapid replication of the recombinant vector. The pJET1.2/blunt PCR vector contains a lethal gene in the multiple cloning site that kills bacteria that are not carrying the recombinant plasmid. The *E. coli* cells transformed with pJETCzEcp2 grew successfully on selection media as expected (150 colony forming units (CFU)), indicating insertion of *CzEcp2* into the vector and disruption of the lethal gene. Where *E. coli* was transformed with the vector carrying the control PCR product (ligation control) from the kit, colonies were observed as expected (130 CFU). *E. coli* transformed with pUC18 as a positive control for transformation also produced colonies as expected (200 CFU). This indicated successful ligation and transformation due to disruption of the lethal gene. No colonies were observed for the *E. coli* only negative control transformation as expected.

Colony PCR was performed (pJET specific primers, Table 2.1) to verify that pJETCzEcp2 was present in the transformed *E. coli* DH5α pJETCzEcp2 colonies. In lanes 6-9 of Figure 2.4A, the top bands (in red box) show the expected product of 623 bp for *CzEcp2*. The bottom bands were not expected and may be an indication of nonspecific binding of the pJET1.2/blunt primers or the need for an increase in stringency of the PCR protocol. No bands were observed for the negative control (lane 10) as expected. Single bands (1 095 bp) were obtained in lanes 1-4 for the pJET1.2/blunt control PCR ligation product and no bands for the negative control (lane 4) as expected. Figure 2.5B shows the expected product size of 575 bp (red box) for *CzEcp2* amplified from pJETCzEcp2 plasmid DNA with gene specific primers (*CzEcp2* Forward and Reverse, Table 2.1). The three bands above the band of interest show the native conformations of the vector DNA (top=open-circular, middle=linear and bottom=supercoiled). Figure 2.5C shows the NEB molecular weight size marker (New England Biolabs) used to infer band sizes on all the agarose gels of Figure 2.5.

Of the four colonies screened for pJETCzEcp2, the recombinant plasmid was extracted from colony number 1 (lane 6, Figure 2.5A). The plasmid DNA concentration was 145 ng/µl and the quality values were 1.86 and 2.2 for $A_{260/280}$ and $A_{260/230}$ respectively. To determine if *CzEcp2* was successfully cloned into pJETCzEcp2, Sanger sequencing was performed as described above using the pJET primers (Table 2.1). Good quality sequence was obtained where *CzEcp2* was shown to be within the recombinant plasmid an unmutated (Appendix, Figure S5). Conservation was observed between the sequenced product (488 bp) and *CzEcp2* (575 bp) from the in-silico reference recombinant plasmid. The sequence coverage was shorter than expected, but adequate to infer the presence of the gene within the vector.

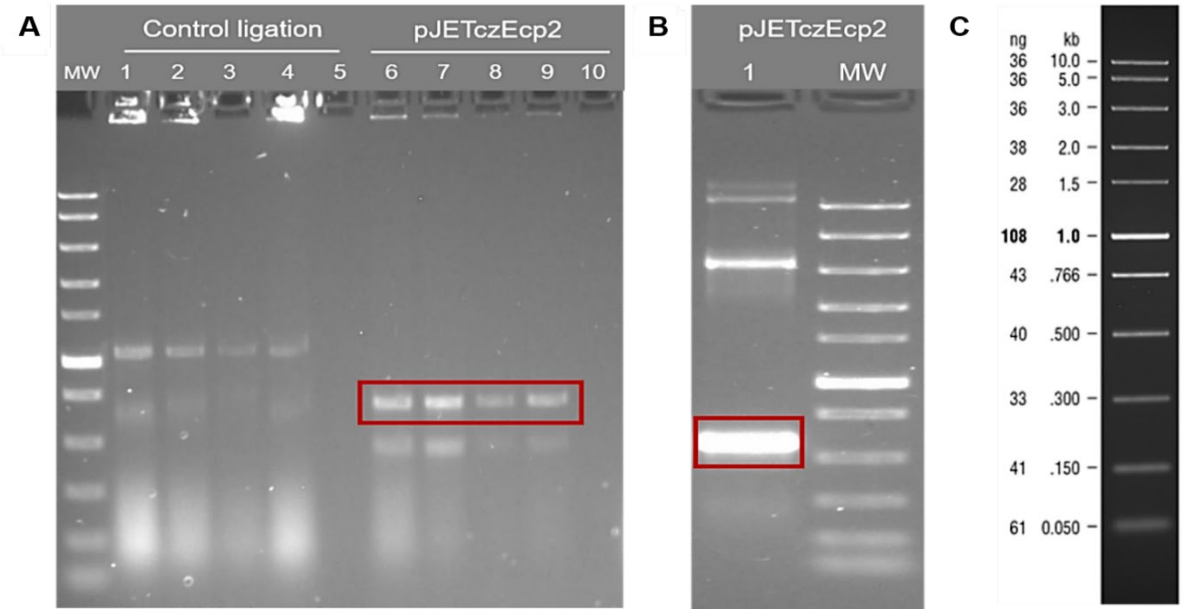

Figure 2.5: Colony PCR of E. coli DH5α pJETCzEcp2. *PCR products were visualised in a 1.2% agarose gel stained with EtBr. A) The PCR product from the CloneJET PCR cloning kit (Thermo Fisher Scientific) was ligated into pJET1.2/blunt as a positive control (lanes 1-4, 1095 bp). Full-length CzEcp2 was cloned into pJET1.2/blunt to obtain pJETCzEcp2 (lanes 6-9, 623 bp) (red box). Negative water control reactions (lanes 5 and 10) were included. B) CzEcp2 was isolated from pJETCzEcp2 using the CzEcp2 gene specific primers (575 bp, lane 1, red box). The bands above the box were the plasmid DNA native conformations (open-circular, linear and supercoiled). C) The NEB Fast DNA ladder was used to infer product sizes (MW lanes in each gel).*

2.3.4 pTRAkc-ERHCzEcp2 recombinant binary vector construction

The cloning primers in Table 2.1 were used to isolate *CzEcp2* from pJETCzEcp2 with and without its fungal signal peptide sequence. The primers added restriction digestion sites to the gene of interest. EcoRI and BamHI were added to the 5' and 3' ends respectively of *CzEcp2* EB (fungal signal peptide) using primer pair 1 (Table 2.1). NcoI and BamHI were added to the 5' and 3' ends respectively of *CzEcp2* NB (LPH signal peptide) using primer pair 2 (Table 2.1). Figure 2.6A shows the *CzEcp2* EB amplicons (524 bp) in lanes 1 and 2. The *CzEcp2* NB amplicons (458 bp) are shown in lanes 4 and 5. The negative controls (lanes 3 and 6) have no bands as expected. *CzEcp2* EB and *CzEcp2* NB PCR products were double digested with EcoRI and BamHI and NcoI and BamHI respectively. The digested products were separated in an agarose gel and excised to purify the DNA from the bands of interest. The concentrations and product sizes are shown in Table 2.6.

Sanger sequencing was done to confirm that *CzEcp2* was isolated from pJETCzEcp2 with and without its signal peptide sequence. It was also for confirmation that the restriction sites were present on each gene. The sequenced *CzEcp2* EB and *CzEcp2* NB products were aligned against respective in silico references of the complete and mature *CzEcp2* sequence within pJETCzEcp2. There was 100% conservation between the aligned nucleotides. The sequence coverage of both genes was less than expected (422 bp instead of 542 bp for *CzEcp2* EB and 355 bp instead of 458 bp for *CzEcp2* NB), however enough of the nucleotides matched to conclude the presence of *CzEcp2* within the recombinant vectors (Appendix, Figure S6 and S7).

Figure 2.6B shows the digestion products of pTRAkc-ERH [94.4 ng/µl]. pTRAkc-ERH 1 was digested with EcoRI and BamHI, and pTRAkc-ERH 2 was digested with NcoI and BamHI. Lane 1 shows three bands of different conformations (top=open-circular, middle=linear, bottom=supercoiled) of uncut pTRAkc-ERH (Chancham and Hughes 2001). Lanes 2-4 show the single digest (EcoRI, NcoI and BamHI respectively) products of pTRAkc-ERH. Lanes 5 and 6 show the pTRAkc-ERH double digest products of NcoI and BamHI as well as EcoRI and BamHI respectively. Two bands were expected for the double digested vector. However, because of how small the second fragments for each digestion were, they likely ran off the gel. Agarose gel extraction was performed to purify the digested plasmid DNA from the bands of interest in Figure 2.7B. The product sizes and concentrations are shown in Table 2.8.

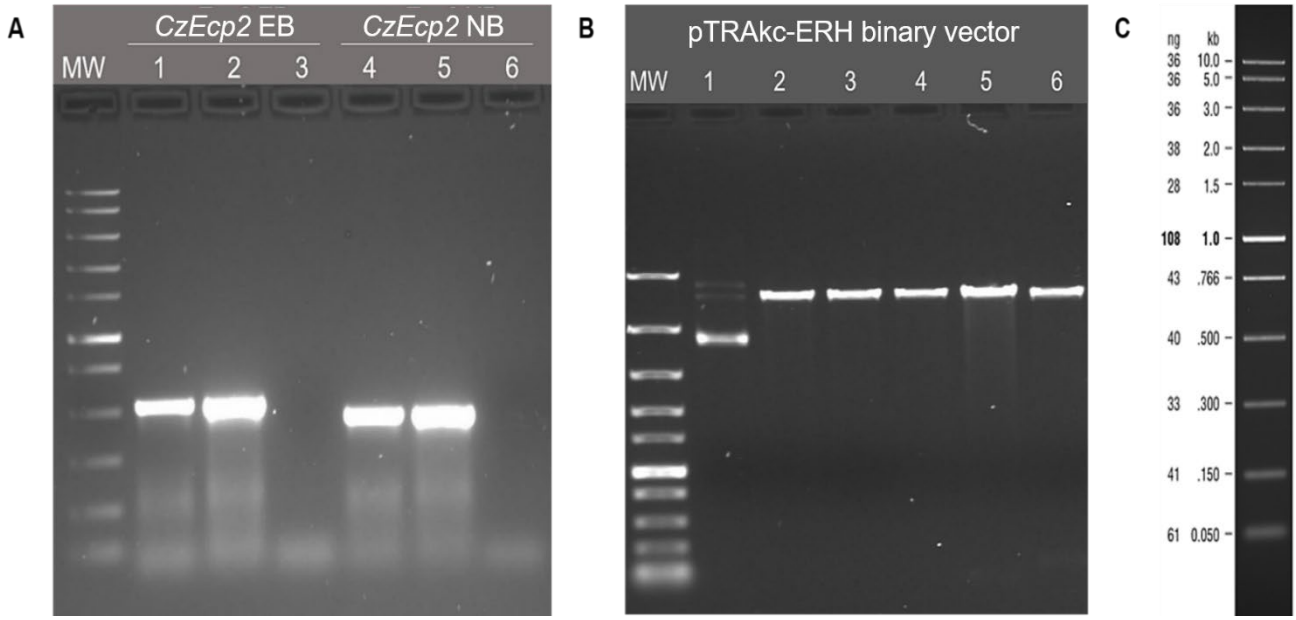

Figure 2.6: CzEcp2 PCR isolation from pJETCzEcp2 with and without its signal peptide and pTRAkc-ERH restriction enzyme digestion. *A) Complete CzEcp2 EB (524 bp, lanes 1-2) (signal peptide) was isolated from pJETCzEcp2 using cloning primers. EcoRI and BamHI were added to the 5' and 3' ends respectively. Mature CzEcp2 NB was isolated from pJETCzEcp2 (458 bp, lanes 4-5). NcoI and BamHI were added to the 5' and 3' ends. Negative water controls were included (lanes 3 and 6). Amplicons were separated in a 1.2% agarose gel stained with EtBr. B) Uncut pTRAkc-ERH had three conformations: open-circular, linear and supercoiled respectively (lane 1). pTRAkc-ERH cut with NcoI, EcoRI and BamHI respectively produced single bands of the same size (7 701 bp, lanes 2-4). Digestion with NcoI and BamHI produced a band of 7 608 bp (lane 5). Digestion with EcoRI and BamHI produced a band of 7 499 bp (lane 6). The products were separated in a 0.8% agarose gel stained with EtBr. C) The Fast DNA ladder (New England Biolabs) (lane MW) was used to infer product sizes.*

Table 2.8: Gel extraction of pTRAkc-ERH and CzEcp2 digested with EcoRI and BamHI, and NcoI and BamHI respectively.

Sample name	Restriction enzymes	Product size (bp)	Concentration (ng/µl)
pTRAkc-ERH 1 (EB)	EcoRI and BamHI	7 513	29.4
pTRAkc-ERH 2 (NB)	NcoI and BamHI	7 622	25.3
***CzEcp2* EB**	EcoRI and BamHI	515	11.5
***CzEcp2* NB**	NcoI and BamHI	450	33.7

Ligation reactions were performed to create recombinant pTRAkc-ERHCzEcp2 EB (signal peptide) and pTRAkc-ERHCzEcp2 NB (LPH signal peptide) respectively. *CzEcp2* EB was ligated to pTRAkc-ERH 1 (EB) and *CzEcp2* NB was ligated into pTRAkc-ERH 2 (NB). The ligation products (Appendix, Figure S8) were transformed into chemically competent *E. coli* DH5α cells which were selected on LB-ampicillin agar. The number of single colonies for each ligation sample is shown in Table 2.9.

Table 2.9: Transformed E. coli DH5α single colony growth on selection media.

Plasmid constructs	Number of single colonies (CFU)	Transformation efficiency (CFU/µg)
Negative water control	0	0
pUC18 positive control	200	$6.69x10^3$
Vector DNA plus insert DNA		
pTRAkc-ERHCzEcp2 EB	170	-
pTRAkc-ERHCzEcp2 NB	134	-
Vector DNA minus insert DNA		
pTRAkc-ERH 1 (EB) (no ligase)	7	-
pTRAkc-ERH 2 (NB) (no ligase)	2	-
pTRAkc-ERH 1 (EB) (ligase)	45	-
pTRAkc-ERH 2 (NB) (ligase)	39	

Colony PCR was done for *E. coli* DH5α pTRAkc-ERHCzEcp2 EB and *E. coli* DH5α pTRAkc-ERHCzEcp2 NB transformants. The PCR reactions amplified a portion of the recombinant plasmids that included *CzEcp2* using the cloning primers in Table 2.1. Figure 2.7A shows single bands for each *E. coli* DH5α pTRAkc-ERHCzEcp2 EB colony (lanes 1-5, 524 bp) which matched the positive control (lane 6). No bands were observed for the negative control (lane 7) as expected. Figure 2.7B shows the expected products of *E. coli* DH5α pTRAkc-ERHCzEcp2 NB colonies (lanes 1-4, 458 bp). The amplicons matched the positive control (lane 5) and the negative control had no bands (lane 6) as expected. The recombinant plasmid constructs were extracted from the *E. coli* DH5α transformants, and the concentrations were measured using the NanoDrop 2000 spectrophotometer. The concentration and absorbance readings are shown in Table 2.10.

Restriction enzyme double digestion was done on pTRAkc-ERHCzEcp2 EB using EcoRI and BamHI. Digestion of pTRAkc-ERHCzEcp2 NB was done using NcoI and BamHI. Figure 2.8A shows uncut

pTRAkc-ERHCzEcp2 EB where three bands of different plasmid conformations (open-circular, linear and supercoiled respectively) are faintly visible (lane 1). The cut plasmid (lane 2) shows two bands of vector and insert DNA (top=7 509 bp; bottom=511 bp) respectively. In Figure 2.8B, double digestion of pTRAkc-ERHCzEcp2 NB (lane 1) only shows a single band which was smaller than expected (6 219 bp). Two bands were expected of vector (7 618 bp) and insert DNA (446 bp) respectively. Uncut pTRAkc-ERHCzEcp2 NB (lane 2) produced three bands of native plasmid conformations (top=open-circular; middle=linear; bottom=supercoiled).

Sanger sequencing was done to confirm that *CzEcp2* was present in the recombinant vectors and that the sequences on the backbone were not rearranged. The sequenced *CzEcp2* EB and *CzEcp2* NB products were aligned against respective in silico references of the complete and mature *CzEcp2* sequences. There was 100% conservation between the aligned nucleotides as expected. The sequence coverage of both genes was less than expected (456 bp instead of 542 bp for *CzEcp2* EB and 394 bp instead of 458 bp for *CzEcp2* NB), however enough of the nucleotides matched to conclude the presence of *CzEcp2* within the recombinant vectors (Appendix, Figure S9 and S10).

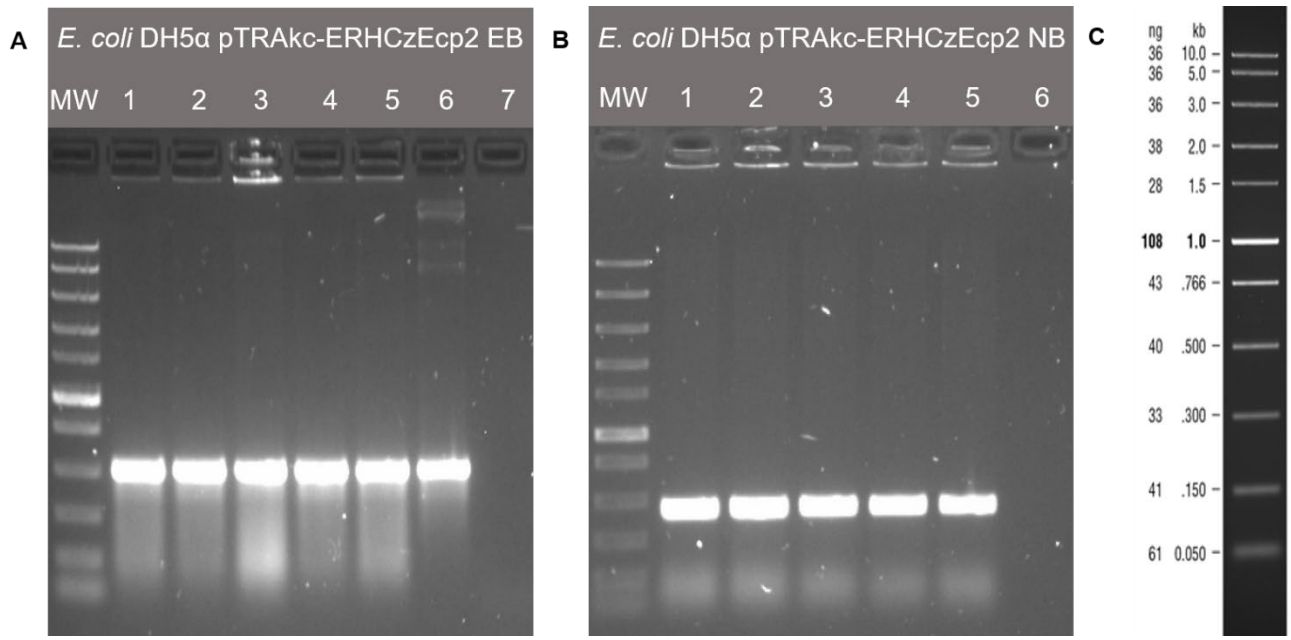

Figure 2.7: Colony PCR of E. coli DH5α pTRAkc-ERHCzEcp2 EB and E. coli DH5α pTRAkc-ERHCzEcp2 NB*. A) E. coli DH5α pTRAkc-ERHCzEcp2 EB (signal peptide) colonies were screened by PCR with a pA35S forward and CzEcp2-BamHI reverse primer (524 bp, lanes 1-5). A positive control was included (524 bp, lane 6) and a negative water control (lane 7). B) E. coli pTRAkc-ERHCzEcp2 NB (LPH signal peptide) colonies were screened by PCR with a pA35S forward and CzEcp2BamHI reverse primer (458 bp, lanes 1-4). Positive (458 bp, lane 5) and negative controls (lane 6) were included. C)The NEB Fast DNA ladder (New England Biolabs) (lanes MW) was used to infer product sizes. Amplicons were separated in a 1.2% agarose gels stained with EtBr.*

Table 2.10: Isolated pTRAkcERHCzEcp2 EB and pTRAkc-ERHCzEcp2 NB quantification using the NanoDrop 2000 Spectrophotometer.

Sample	Concentration	$A_{260/280}$	$A_{260/230}$
pTRAkc-ERHCzEcp2 EB (fungal signal peptide)	134.3 ng/µl	2.09	2.11
pTRAkc-ERHCzEcp2 NB (LPH signal peptide)	121.5 ng/µl	1.93	1.83

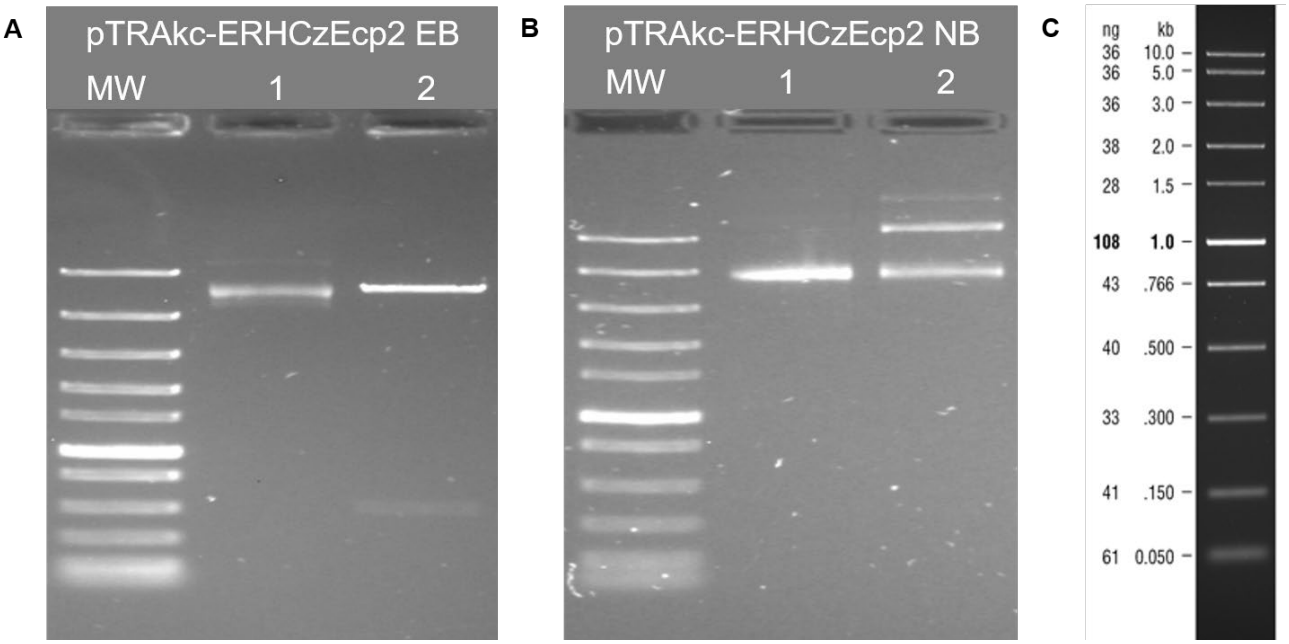

Figure 2.8: Restriction enzyme digestion of pTRAck-ERHCzEcp2 EB and pTRAck-ERHCzEcp2 NB. *A) Uncut pTRAkc-ERHCzEcp2 EB (signal peptide) (lane 1) had three native conformations (open circular, linear and supercoiled respectively). Digested plasmid (lane 2) produced two bands, vector DNA (7 509 bp) and CzEcp2 EB (511 bp). B) Digestion of pTRAkc-ERHCzEcp2 NB (LPH signal peptide) (lane 1) produced a single band (6 219 bp). The uncut plasmid had three native conformations (open circular, linear and supercoiled respectively). C) NEB Fast DNA ladder was used to infer band sizes of each product (lanes MW in both gels). Products on both gels were separated in a 0.8% agarose gel stained with EtBr.*

2.3.5 Agrobacterium transformation with the recombinant binary vectors

Agrobacterium tumefaciens GV3101 (pSOUP+pMP90) was transformed with pTRAkc-ERH, pTRAkc-ERHCzEcp2 EB (fungal signal peptide), pTRAkc-ERHCzEcp2 NB (LPH signal peptide) and pCambia2300 (CAMBIA) respectively. The pSOUP vector already present in the bacterial strain was not a requirement for agroinfiltration. It was retained in the bacteria for consistency with the positive control bacteria carrying the INF1 gene which required pSOUP for T-DNA transfer into the plant cells (*Sophie Mantelin, personal communication*). Transformed colonies were selected on YEB agar (containing carbenicillin, tetracycline and gentamicin). As expected, the positive control bacterial transformation with pCambia2300 grew abundantly. The negative control plate (untransformed *Agrobacterium*) did not have colonies as expected. Colonies were observed for *Agrobacterium*

transformed with the constructs of interest (pTRAkc-ERHCzEcp2 EB and pTRAkc-ERHCzEcp2 NB). Table 2.11 shows the number of colonies obtained for each transformation.

Table 2.11: Transformed A. tumefaciens GV3101 (pSOUP+pMP90) colony growth on selection media.

Plasmid constructs	***Number of single colonies (CFU)***	***Transformation efficiency (CFU/µg)***
Negative control (bacteria only)	0	-
pCambia2300 (positive control)	350	$2.58x10^4$
pTRAkc-ERH	330	$3.31x10^4$
pTRAkc-ERHCzEcp2 EB (*Ecp2* SP)	300	-
pTRAkc-ERHCzEcp2 NB (LPH SP)	235	-

The presence of pTRAkc-ERH, pTRAkc-ERHCzEcp2 EB and pTRAkc-ERHCzEcp2 NB respectively in the transformed *Agrobacterium* was confirmed with colony PCR. The six colonies screened for pTRAkc-ERH (Agro pTRAkc-ERH (no gene)) each had a single band (lanes 1-6, 338 bp). A single band was obtained for the positive control (lane 7, 338 bp) and no band for the negative control (lane 8) as expected (Figure 2.9A). Single bands were obtained for the five colonies (Agro pTRAkc-ERHCzEcp2 EB (complete *CzEcp2*)) screened for pTRAkc-ERHCzEcp2 EB (lanes 1-5, 612 bp). The positive control (lane 6) produced a single band (612 bp) and the negative control (lane 7) did not have bands as expected (Figure 2.9B).

No bands were observed for the colonies (Agro pTRAkc-ERHCzEcp2 (mature *CzEcp2*)) screened for pTRAkc-ERHCzEcp2 NB (lanes 1-4) (Figure 2.10A) which was unexpected. The positive control produced a band as expected (lane 5, 656 bp). No bands were observed for the negative control (lane 6) as expected. Colony PCR (Figure 2.10B) of the same colonies was done using *Agrobacterium* specific primers (Grayburn and Vick 1995). This was done to validate that the lack of amplification was not due to the PCR reactions or *Agrobacterium*. Multiple bands were obtained for each colony (lanes 1-4) including the two positive controls (*Agrobacterium* (pSOUP+pMP90) and *Agrobacterium* pMP90 respectively, lanes 5-6). This was because the primers hybridised to multiple genes and likely that the annealing temperature was not stringent enough. No bands were observed for the negative control (lane 7) as expected.

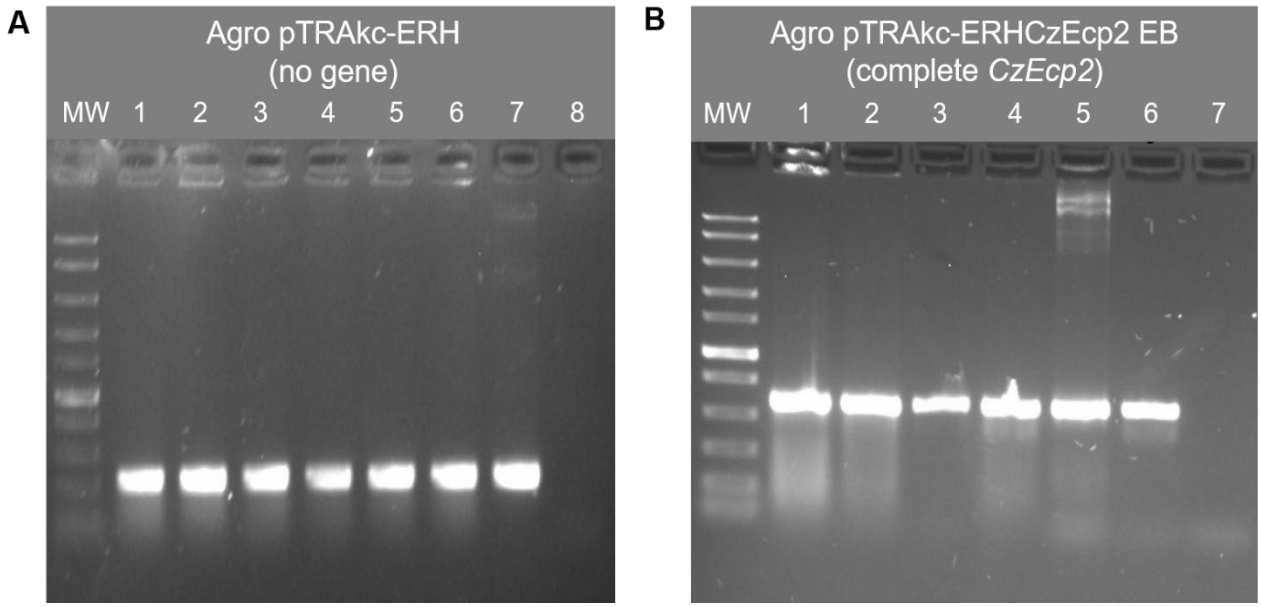

Figure 2.9: Colony PCR of A. tumefaciens GV3101 (pSOUP+pMP90) transformants. *Lane MW showed the Fast DNA ladder (NEB, Ipswich, USA) used to infer band sizes in both gels. A) Amplification of Agrobacterium transformed with empty pTRAkc-ERH (Agro pTRAkc-ERH) with pA35S primers produced a single band (338 bp, lanes 1-6). A positive (338 bp) and negative control were included (lanes 7-8). B) A single band was observed for Agro pTRAkc-ERHCzEcp2 EB (signal peptide) (lanes 1-5, 612 bp) amplified with pA35S forward and CzEcp2-BamHI reverse primers. The two extra bands in lane 5 show plasmid DNA conformations. The positive control (lane 6, 612 bp) produced a band. No band was seen for the negative water control (lane 7). Both gels were 1.2% agarose stained with EtBr.*

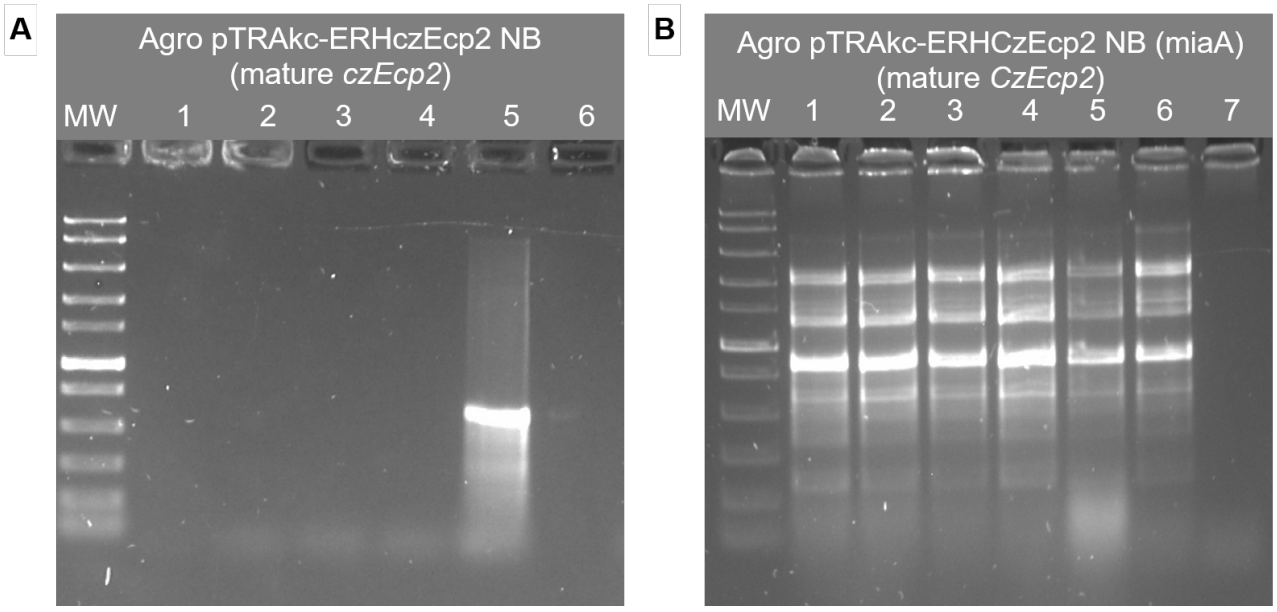

Figure 2.10: Colony PCR of A. tumefaciens GV3101 (pSOUP+pMP90) pTRAkc-ERHCzEcp2 NB transformants. *Lane MW shows the Fast DNA Ladder (NEB, Ipswich, USA) used to infer band sizes in both gels. A) No bands were observed where Agro pTRAkc-ERHCzEcp2 NB (mature CzEcp2) colonies were screened (lanes 1-4) with pA35S forward and CzEcp2-BamHI reverse primers. The positive control (lane 5, 656 bp) produced a single band. No band was obtained for the negative water control (lane 6). B) Multiple bands were observed for the same colonies (lanes 1-4) screened with miaA Agrobacterium specific primers (Grayburn and Vick 1995). This validated that the lack of amplification in A) was not due to the bacteria or the colony PCR. The same number of bands were observed for the non-transformed Agrobacterium positive controls (lanes 5-6) and no bands for lane 7 (negative water control). Both gels were 1.2% agarose stained with EtBr.*

2.3.6 Nicotiana spp. transient agroinfiltration

N. benthamiana, *N. tabacum* cv. Petit Havana and *N. tabacum* cv. LA Burley plants were transiently agroinfiltrated with *A. tumefaciens* GV3101 (pSOUP+pMP90) carrying various vectors. The bacteria carrying pTRAkc-ERH was referred to as Agro pTRAkc-ERH (black boxes). The *Agrobacterium* carrying pTRAkc-ERHCzEcp2 EB (signal peptide) was referred to as Agro pTRAkc-ERHCzEcp2 EB (purple boxes) (Figure 2.11 to Figure 2.13). The *Agrobacterium* for the positive control infiltration carried the *P. infestans* INF1 effector gene (yellow boxes) (Kamoun et al. 1998). Infiltrations with untransformed *A. tumefaciens* GV3101 (pSOUP+pMP90) were also performed on the same leaves (red boxes). The colours of each box represented the same agroinfiltration sample throughout Figure 2.11 to Figure 2.13. Three leaves on each of three plants of the three *Nicotiana* cultivars were agroinfiltrated with the respective transformants and monitored for 10 days.

Necrosis due to expression of the INF1 protein (yellow boxes) was observed on all three *Nicotiana* spp. The most severe INF1 hypersensitive response (HR) lesions were observed on *N. benthamiana* (Figure 2.11A and B). *N. tabacum* cv. Petit Havana (Figure 2.12C) and *N. tabacum* cv. LA Burley (Figure 2.13C) had necrotic spots on some of the INF1 infiltrated leaves. On the three *N. tabacum* cv. Petit Havana leaves (Figure 2.12) and one of *N. tabacum* cv. LA Burley (Figure 2.13C), chlorosis was observed on some leaves where Agro pTRAkc-ERHCzEcp2 EB (signal peptide) (purple boxes) was infiltrated. In *N. benthamiana* (Figure 2.11C), chlorosis was observed where INF1 and pTRAkc-ERHCzEcp2 EB (signal peptide) were infiltrated. Slight yellowing was observed where pTRAkc-ERH was infiltrated into *N. benthamiana* Figure 2.11A (black box).

For the rest of the leaves, where the negative controls (Agro pTRAkc-ERH and *Agrobacterium* only) were infiltrated, no changes to the leaves were observed. Table 2.12 summarises the symptoms observed in all the infiltrated leaves 10 dpi. Figure 2.11A of *N. benthamiana* had a more yellow appearance compared to the other two replicates. This observation creates the impression that all infiltrations resulted in chlorosis. It is suggested however that the yellowing seen where infiltrations were made were due to senescence instead of chlorosis.

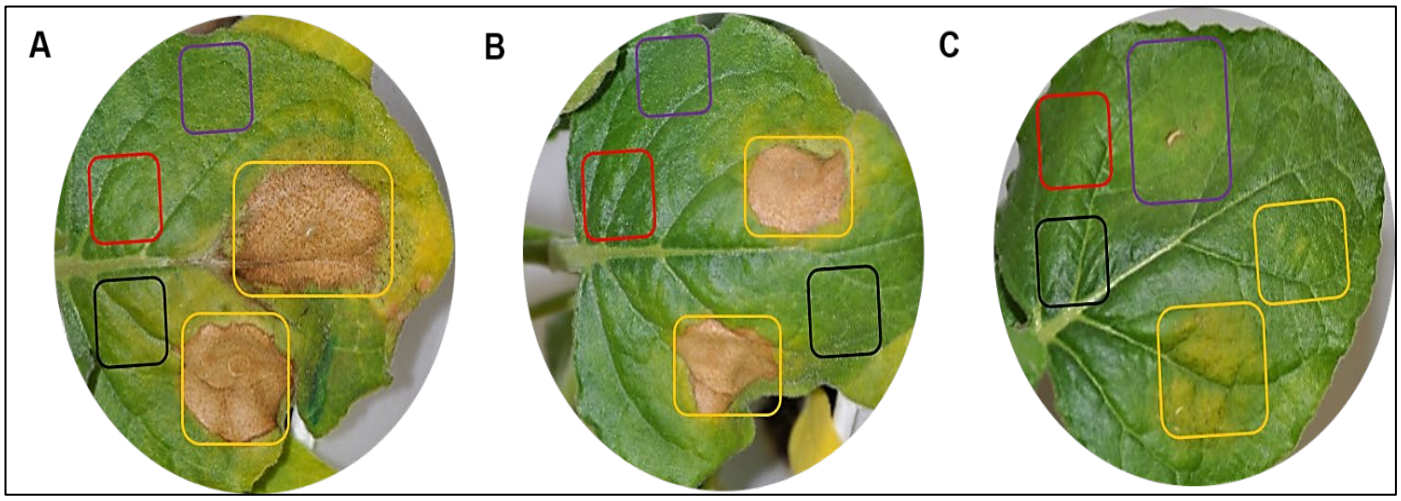

Figure 2.11: Transient agroinfiltration of N. benthamiana leaves. *N. benthamiana leaves were transiently infiltrated with A. tumefaciens GV3101 (pSOUP+pMP90) carrying pTRAkc-ERHCzEcp2 EB (signal peptide) (purple boxes) and pTRAkc-ERH (black boxes) respectively. The positive control infiltration was Agrobacterium carrying a vector with the P. infestans INF1 effector gene (yellow boxes). The red boxes were infiltrated with the Agrobacterium strain only. A) and B) INF1 expression produced severe HR 10 dpi. Infiltration with the other samples did not result in any visible changes to the leaves except slight yellowing for pTRAkc-ERH infiltration. C) Infiltration with INF1 and pTRAkc-ERHCzEcp2 EB resulted in chlorosis. No other visible changes were noted for infiltration with the vector and bacteria only controls.*

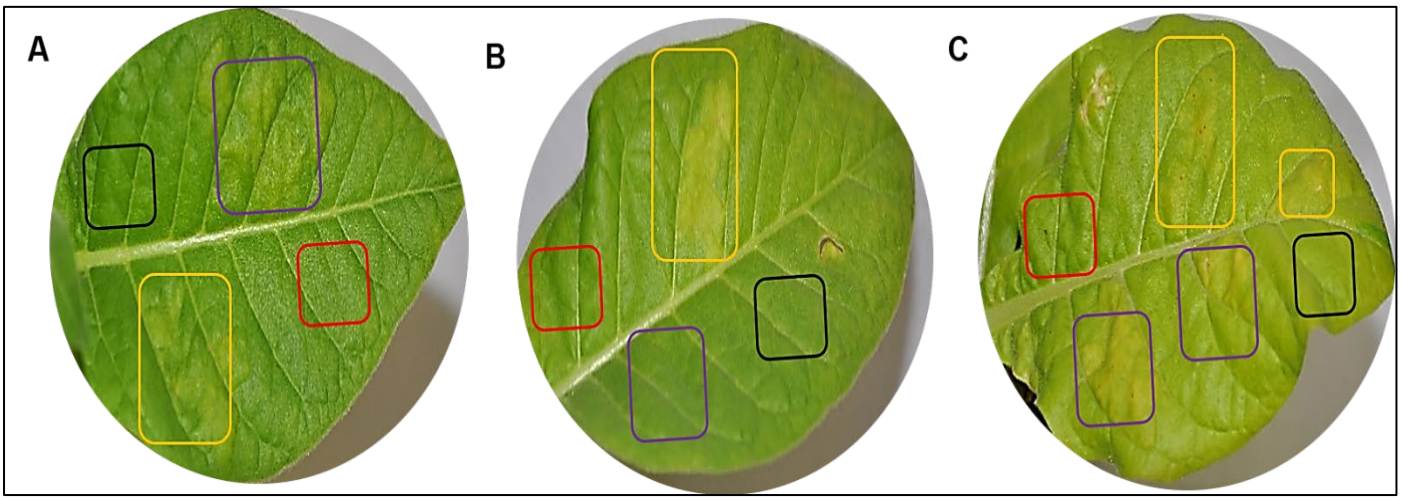

Figure 2.12: Transient agroinfiltration of N. tabacum cv. Petit Havana leaves. *The leaves were infiltrated with A. tumefaciens GV3101 (pSOUP+pMP90) (red boxes) only and the same bacteria carrying pTRAkc-ERH (black boxes). Infiltration was also done with the same Agrobacterium strain carrying the INF1 gene (yellow boxes) and pTRAkc-ERHCzEcp2 EB (signal peptide) (purple boxes) respectively. A) Mild chlorosis was observed 10 dpi where INF1 and pTRAkc-CzEcp2 EB were infiltrated. No changes were observed for the other infiltrations. B) Mild chlorosis was observed for INF1 infiltration. The other infiltrations did not visibly change leaf morphology. C) Infiltration with INF1 resulted in chlorosis and necrotic spots. Chlorosis was also seen for pTRAkc-ERHCzEcp2 EB infiltration. No changes were observed for the negative controls.*

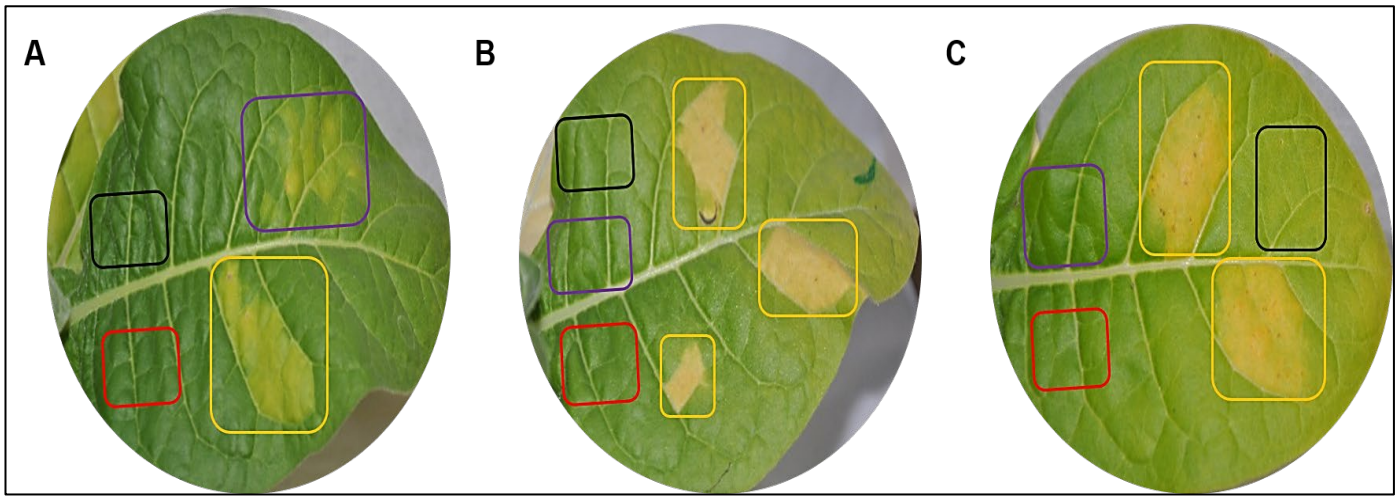

Figure 2.13: Transient agroinfiltration of N. tabacum cv. LA Burley leaves. *The leaves were infiltrated with A. tumefaciens GV3101 (pSOUP+pMP90) (red boxes) only and same the bacteria carrying pTRAkc-ERH (black boxes). Infiltration was also done with the same Agrobacterium strain carrying the INF1 gene (yellow boxes) and pTRAkc-ERHCzEcp2 EB (signal peptide) (purple boxes) respectively. A) Chlorosis was observed 10 dpi where INF1 and pTRAkc-CzEcp2 EB were infiltrated. No changes were observed for the other infiltrations. B) Chlorosis and necrotic spots were observed for INF1. The other infiltrations did not visibly change leaf morphology. C) Chlorosis and necrotic spots were observed 10 dpi for INF1. No changes were observed for the negative controls and pTRAkc-ERHCzEcp2 EB infiltrations.*

Table 2.12: Summary of symptoms observed 10 days post infiltration of Nicotiana spp. with A. tumefaciens GV3101 (pSOUP+pMP90) transformants.

Infiltration	N. benthamiana			N. tabacum cv Petit Havana			N. tabacum cv. LA Burley		
	R1 [1]	R2 [2]	R3 [3]	R1	R2	R3	R1	R2	R3
pTRAkc-ERHCzEcp2 EB	-	-	•	••	-	•••	•••	-	-
pTRAkc-ERH only	•	-	-	-	-	-	-	-	-
INF1	••••	••••	•••	••	••	•••	•••	••••	••••
Agrobacterium only	-	-	-	-	-	-	-	-	-

1, 2 and 3 Biological replicate 1, 2 and 3 respectively for each Nicotiana spp.
•••• Severe hypersensitive response (HR)/necrosis
••• Chlorosis
•• Mild chlorosis
• Yellowing of the leaf
- No visible change in leaf morphology

2.4 Discussion

This study aimed to confirm the presence of *Ecp2* in the *Cercospora zeina* CMW25467 genome and clone it into the pTRAkc-ERH binary vector with and without its signal peptide. The recombinant vector was then agroinfiltrated into *Nicotiana* spp. and the leaves monitored for 10 days for a hypersensitive response. Cloning the complete and mature *CzEcp2* genes was successful. *A. tumefaciens* GV3101 (pSOUP+pMP90) transformation with pTRAkc-ERHCzEcp2 EB (signal peptide) was successful while transformations with pTRAkc-ERHCzEcp2 NB (LPH signal peptide) was not. The plants were therefore only infiltrated with the recombinant vector carrying the complete *CzEcp2* gene (pTRAkc-ERHCzEcp2 EB). A hypersensitive response was not observed for expression of complete *CzEcp2* compared to the positive control, *Phytophthora infestans* INF1. However, in some of the replicates, chlorosis was observed for the construct carrying the complete *CzEcp2* gene. The lack of clearly defined necrosis was not expected but the observation of chlorosis could have indicated a possible development of necrosis if the leaves were monitored for longer than 10 days.

The *Cercospora zeina* genome was searched for a homolog of the extracellular protein 2 (*Ecp2*) effector gene using the NCBI database and RNA sequence data from a previous study (Swart et al. 2017). The *Ecp2* gene from another Dothideomycete, *Cladosporium fulvum* (*Passalora fulva*), was used as a reference to search for the gene within *C. zeina* because the accession was previously characterised (Van den Ackerveken et al. 1993). The *C. fulvum* (*P. fulva*) *Ecp2* nucleotide sequence (Z14024.1) was used in BLASTx analysis against GenBank to search for a putative CzECP2 protein (PKR94769.1) within the *C. zeina* genome. The *C. fulvum* protein accession (CAA78401.1) could also have been used as a starting point in BLASTp analysis because it would have hit the same hypothetical protein within the *C. zeina* genome. However, the use of the protein sequence was avoided because many accessions have been logged under the same name for *C. fulvum* (*P. fulva*) ECP2 therefore increasing the likelihood of choosing the incorrect query accession.

BLASTx analysis against GenBank was used because it allowed the search for a protein that was similar to the *CfEcp2* translated nucleotide query sequence. It broadened the scope of results found because no significant hits were found when BLASTn was done to search for potential *Ecp2* nucleotide sequences within the *C. zeina* genome. Reciprocal BLAST analysis of PKR94769.1 against the *C. zeina* genome assembly (GCA_002844615.1) hit a 14 893 bp contig (MVDW01000239.1) on which the sequences (gDNA, mRNA and protein) for *CzEcp2* were located. This proved the presence of a putative *Ecp2* gene within the *C. zeina* genome. tBLASTn was done because it allowed the use of the putative *C. zeina* ECP2 protein accession (PKR94769.1), to find a putative translated nucleotide sequence within the *C. zeina* genome assembly. The identified nucleotide sequences could then be used for downstream applications including primer design for isolation of the gene from the genome for use in downstream experiments.

The CzECP2 protein accession (PKR94769.1) was used in a BLASTp against GenBank to search for similarity to other protein accessions. The most significant ECP2 hits were against the following Dothideomycetes (de Wit et al. 2012; Crous and Braun 2003; Hyde et al. 2013; Ohm et al. 2012) in order of the highest to the lowest similarity: *Cercospora beticola* (XP_023457559.1); *Cercospora beteroae* (PPJ52722.1); *Pseudocercospora (Mycosphaerella) fijiensis* (XP_007922577.1); *Dothistroma septosporum* (EME39817.1) and *C. fulvum* (*P. fulva*) (CAA78401.1/QDX18258.1). The significant similarity of putative CzECP2 to these ECP2 accessions indicates the possibility that the *C. zeina* accession is also ECP2. It was however important to note that some of the protein sequences that were hit were not characterised as ECP2 but were rather predicted to be hypothetical ECP2 proteins.

Pairwise alignments were done to compare each of the above-mentioned accessions to the putative CzECP2 accession. The complete and mature peptides were aligned against that of CzECP2. The highest similarity was observed for the alignments with *C. beticola* and *C. berteroae*. This result was expected because these fungi are classified in the same genus as *C. zeina* (Crous and Braun 2003). The results obtained in this study validated those obtained in a previous study done by Lombard (2015) in her MSc thesis to prove the presence of *Ecp2* within the *C. zeina* genome using *Ecp2* accessions from other Dothideomycete fungi as a reference.

RNA sequence data from a previous study (Swart et al. 2017) was searched for the presence of RNA transcripts that were hypothesised to be *CzEcp2*. The czeina239g000040 gene model was annotated as *CzEcp2* and assigned the accession PKR94769.1 and therefore hypothesised to be CzECP2. These conclusions matched the results found on NCBI GenBank in the current study. The study done by Swart et al. (2017) was based on growing *C. zeina in vitro* on different media to determine the expression level of putative genes in the genome. This data was compared to the expression of putative genes *in planta*. The highest *Ecp2* transcript counts were obtained in complete medium and cornmeal agar. These findings were used as motivation to select cornmeal agar in the present study to culture *C. zeina* with the expectation that *CzEcp2* expression will be stimulated. It has been hypothesised that cornmeal agar simulates conditions within the maize leaf, therefore encouraging the fungus to express the same genes it would if it were causing infection (GLS) within its host, hence its use to study the expression of apoplastic genes (Swart et al. 2017).

Studies have suggested that high *in planta* expression of effectors is indicative that the genes play a direct role in virulence of the pathogen (Stergiopoulos et al. 2010; De Wit et al. 1985; De Wit and Roseboom 1980; De Wit 2016). Van den Ackerveken et al. (1993) identified *CfEcp2* in the host tomato apoplastic fluids which led the researchers to conclude that it is likely required for pathogenicity. Where mutants of the effector were created, fungal colonisation of tomato was drastically reduced (De Kock et al. 2004; Laugé et al. 1997). The *in planta* reads of *CzEcp2* were higher compared to YPD, V8, PDA broth and PDA agar *in vitro* reads (Swart et al. 2017). This may be an indication of the

importance of the gene for *C. zeina* virulence during host invasion. However, it is essential to note that the *CzEcp2 in vitro* expression in cornmeal agar, which is hypothesised to closely simulate conditions *in planta*, had a much higher read count than *in planta*. This may therefore suggest a nonspecific function for *CzEcp2*. This hypothesis does however require further investigation (De Wit 2016; De Wit and Roseboom 1980; De Wit et al. 1985; De Wit and Spikman 1982).

RNA sequencing is a tool used to determine the transcriptomes of organisms. This technique has been used to elucidate which genes are expressed into proteins and devise gene models that contribute towards genome annotation (Schnable 2019; Wang et al. 2009; Ozsolak and Milos 2011). Genome and RNA sequencing was used in a study (de Jonge et al. 2012) to determine which *Verticillium dahliae* effectors were responsible for eliciting defence against vascular wilt in tomato plants. Previous studies identified Ve-1 as the immune receptor responsible for resistance but had not identified the effector. de Jonge et al. (2012) therefore discovered that Ave1 is the effector recognised by Ve-1 to protect the plant against wilting. This study amongst others showed the relevance of using RNA sequence data to find effectors as was done in the current study.

C. zeina was cultured on V8 agar for 7 days in constant darkness to stimulate conidiation (Meisel et al. 2009). The conidia were then transferred to cornmeal agar to stimulate *CzEcp2* expression under constant light for 7 days (Swart et al. 2017). Cornmeal agar was chosen in this study due to findings from Swart et al. (2017) that a high transcript read of PKR94769.1 (putative CzECP2) was obtained when the fungus was cultured on this media. The orange/red colour surrounding the greenish-grey conidia (Figure 2.3) was the V8 agar and not a potential toxin or different conidia colour. V8 agar was used because previous studies of *C. zeina* showed that conidiation was best stimulated on this media due to its high nutrient content, therefore allowing the slow-growing fungus to survive for longer than 7 days (Muller et al. 2016; Meisel et al. 2009).

C. zeina conidia on the cornmeal agar were the same colour as on the V8 agar as expected. The growth was less dense which could likely be due to the fungus being stressed under constant light conditions or because it was not growing directly on the agar but on cellophane. It may also be due to cornmeal agar being a low nutrient media used to simulate the harsh conditions of the maize leaf (Swart et al. 2017). Cellophane disks were layered on the surface of the cornmeal agar to facilitate easy removal of the conidia prior to RNA extraction (Swart et al. 2017).

RNA was extracted from the *C. zeina* conidia grown on cornmeal agar. The agarose gel image showed three distinct bands for each replicate (Figure 2.4). Each band represented rRNA with 28S being larger and more intense than 18S and 5S being the smallest and faintest band. The amount of tissue used for RNA isolation determined the total amount of RNA observed on the agarose gel. No smears or other bands were observed on the gel which indicated good quality RNA and a lack of gDNA contamination (Loening 1968; Ouyang et al. 2014; Chomczynski and Sacchi 2006).

cDNA was synthesised from the *C. zeina* RNA and amplified in RT-PCR reactions using *CzEcp2* gene specific primers, producing a single band for each replicate as expected. The primers were designed to amplify a cDNA sequence slightly larger than the coding sequence of *CzEcp2* to clone the full sequence into pJET1.2/blunt. The gene of interest was isolated from cDNA instead of gDNA due to the presence of an intron within the gDNA sequence. If cloning was done using the gDNA sequence of *CzEcp2*, the reading frame of the amino acid sequence would have shifted after intron excision. This would have resulted in expression of a different protein instead of CzECP2 and therefore false positive results in downstream experiments.

The full *CzEcp2* sequence was cloned into pJET1.2/blunt to ensure that the start and stop codons were included when the gene was isolated from the recombinant plasmid. The recombinant vector, pJETCzEcp2 was transformed into *E. coli* DH5α cells which were selected on LB-ampicillin agar. Successful ligation of the gene into the vector was indicated by the growth of bacterial colonies. This indicated disruption of the vector lethal gene (*eco47IR*) which was designed to kill bacteria that lacked a recombinant vector (Thermo Fisher Scientific). The transformants were selected on ampicillin because of the ampicillin resistance gene present on the vector backbone.

The colonies that grew were screened in colony PCR reactions using pJET specific primers. The amplicon was 623 bp as expected which indicated the presence of *CzEcp2* within pJETCzEcp2. The amplicon obtained was longer than the initial 575 bp because it included nucleotides from the pJET1.2/blunt vector sequence. Colony PCR was used as a screening technique because it distinguishes false positives from true positives. It is also sensitive and accurate because it takes advantage of the principles of PCR. It also mitigates the waste of reagents and time if an attempt was made to isolate a recombinant plasmid that may not be present in the bacteria (Bergkessel and Guthrie 2013).

The recombinant vector was Sanger sequenced using pJET primers to determine that the sequence was unmuted and to verify the presence of *CzEcp2* within the vector. This also served to prove that *Ecp2* exists within the *C. zeina* genome because it was isolated from cDNA synthesised using RNA extracted from the fungus. The sequenced result was aligned against a reference sequence of in silico cloned pJETCzEcp2. Although the full length of 623 bp was not obtained from sequencing, a large enough fragment was obtained to confidently identify *CzEcp2* within the recombinant vector and therefore conclude that it exists in the genome. The shortened sequence was not due to deletions, but rather due to the sequence coverage. It is likely that the samples were contaminated with salts, PCR reagents or primers hence the sequencer could not pick up all the nucleotide bases (Shetty et al. 2019; Pisapia et al. 2019; Sanger et al. 1977).

An alternative to Sanger sequencing is Next Generation Sequencing (NGS). The principles of this technique are like those of Sanger sequencing except that it is massively parallel. This implies that it

allows for thousands of genes to be sequenced at a time compared to Sanger which allows sequencing of one gene at a time (Kozińska et al. 2019; Shetty et al. 2019; Schuster 2008). NGS is therefore suitable for elucidation of whole genome sequences. For this study Sanger sequencing was better suited because a single gene was being studied.

CzEcp2 was isolated from pJETCzEcp2 with and without its signal peptide. Cloning primers were used to add restriction sites to the gene. EcoRI and BamHI were added to the 5' and 3' ends respectively of the complete sequence with the signal peptide. NcoI and BamHI were added to the 5' and 3' ends respectively of the mature sequence without the signal peptide. The restriction sites were added to create sticky ends on each sequence prior to ligation into the pTRAkc-ERH binary vector. The vector already had these sites in its sequence and was therefore also digested with the same enzymes for insertion of the respective gene sequences. pTRAkc-ERH was cut with the enzymes that corresponded to those added to *CzEcp2* to ensure that the sticky ends matched and therefore re-ligated to form the restriction sites again.

Cutting the vector and gene with EcoRI and BamHI allowed insertion of the complete *CzEcp2* sequence into the vector and the disruption of the vector SEKDEL sequence. The SEKDEL sequence was added for endoplasmic reticulum retention of the protein expressed from a gene of interest (Mortimer et al. 2012; Maclean et al. 2007). This would allow the study of the expression of cytoplasmic proteins. Given the hypothesis that *CzEcp2* is an apoplastic effector gene, the expressed protein needed to be transported to the apoplast through cleavage of the signal peptide (von Heijne 1990), hence the gene was cloned with its native fungal signal peptide and the SEKDEL was disrupted.

Digestion of *CzEcp2* and the vector with NcoI and BamHI allowed ligation of the gene into the vector without its native signal peptide sequence. The gene was fused to an upstream LPH sequence already present on the backbone. The LPH region on the pTRAkc-ERH vector is a plant signal peptide that can be fused to genes of interest to determine if they can be transported to the apoplast after cleavage of the signal peptide (Maclean et al. 2007). Use of the LPH may be more beneficial than the fungal signal peptide because it is a plant signal and may therefore be better recognised by plant machinery. It may be likely that the fungal signal peptide will not be recognised and cleaved and therefore the protein not transported to the appropriate location for function.

CzEcp2 EB (signal peptide) was ligated into pTRAkc-ERH EB, and *CzEcp2* NB was ligated into pTRAkc-ERH NB and fused to the LPH signal peptide. The recombinant vectors were pTRAkc-ERHCzEcp2 EB (fungal signal peptide) and pTRAkc-ERHCzEcp2 NB (LPH signal peptide). They were transformed into chemically competent *E. coli* DH5α cells to obtain a high copy number of each vector. The transformants were selected on LB-ampicillin agar because the binary vector carried an ampicillin resistance gene, therefore only the transformants carrying the recombinant vectors grew

on the selection media. Controls for the ligation reactions included pTRAkc-ERH with no insert DNA and no ligase, and pTRAkc-ERH with ligase and no insert. These reactions were not expected to produce a high number of colonies, especially where ligase was excluded. Theoretically, in the absence of ligase enzymes, a cut sequence should not be able to re-circularise. Insert DNA was excluded to determine the number of transformants obtained by pTRAkc-ERH ligation compared to the recombinant vectors. Given that the number of colonies obtained for the recombinant vector transformations were significantly higher than those of the controls, it was concluded that the ligation and transformation reactions were successful.

The transformed *E. coli* cells were screened for the presence of the recombinant vectors using colony PCR. A pA35S forward primer and *CzEcp2*-BamHI reverse primer were used. Where cells were screened for pTRAkc-ERHCzEcp2 EB and pTRAkc-ERHCzEcp2 NB, a single band was obtained for each amplicon (542 bp and 458 bp respectively) as expected. Where no bands were observed for the negative water control lanes, no amplification was obtained because of the lack of DNA template. This was expected as it indicated a lack of contamination in the reagents used for the colony PCR reactions. Colony PCR was used to determine if the observation of colonies was due to true positives that contained the recombinant vectors (Bergkessel and Guthrie 2013). Three extra bands were observed at the top of the positive control lane and were hypothesised to be the native conformations of the plasmid DNA (top=open circular, middle=linear, bottom=supercoiled) (Chancham and Hughes 2001; Higgins and Vologodskii 2015). The recombinant vectors were extracted from the transformed colonies previously screened. Restriction enzyme digestion of pTRAkc-ERHCzEcp2 EB produced two distinct bands as expected to indicate the presence of the insert within the vector (Smith 1993).

Digestion of pTRAkc-ERHCzEcp2 NB produced a single band. This result was unexpected as the size of the vector band was smaller than expected and the band for *CzEcp2* NB was not visible. This may be due to rearrangement of the vector DNA and potential loss of *CzEcp2* NB along with some other parts of the vector sequence (Nakano et al. 2005; Hiei et al. 1997). It could also be due to the enzymes not cutting adequately because the reaction was not long enough or because the DNA was too concentrated. It may also be due to pTRAkc-ERH re-circularising prior to *CzEcp2* NB insertion during the ligation reactions. Given that the colony PCR screening of the transformants carrying pTRAck-ERHCzEcp2 NB produced bands as expected, the restriction digestion was expected to produce two bands because of the cut sites being recreated. It is likely that a dead colony carrying the vector was picked from the selection plate for the PCR. It is also likely that a colony not carrying the recombinant vector but re-circularised pTRAkc-ERH was unfortunately chosen for plasmid isolation (Bergkessel and Guthrie 2013).

Sanger sequencing was done using the pA35S forward primer and the *CzEcp2*-BamHI reverse primer to verify that the EcoRI, NcoI and BamHI sites were recreated in the recombinant vectors. It also confirmed the presence of unmutated *CzEcp2* with and without the signal peptide in pTRAkc-

ERHCzEcp2 EB and pTRAkc-ERHCzEcp2 NB respectively (Sanger et al. 1977; Schuster 2008). The pTRAkc-ERHCzEcp2 EB sequenced product was 456 bp, which was shorter than the expected 524 bp product. The pTRAkc-ERHCzEcp2 NB sequenced product was 394 bp which was also shorter than the expected 458 bp product. This was hypothesised to be due to poor sequence coverage instead of deletions. The quality of sequencing is dependent on factors such as salt content, and contaminants such as cycle sequencing PCR reagents and primers. If the PCR products were not adequately purified prior to sequencing, it would have reduced the quality of the sequences produced (Sanger et al. 1977; Schuster 2008). Despite the shorter products, enough of the sequence matched the in-silico reference to confirm the presence of unmutated *CzEcp2* within the recombinant vectors. The restriction enzyme sites were not observed due to the shortened sequences; however, restriction enzyme digestion of the recombinant vectors confirmed their presence.

Agrobacterium tumefaciens GV3101 (pSOUP+pMP90) obtained from the JHI Institute (Dundee, United Kingdom) was transformed with pTRAkc-ERHCzEcp2 EB, pTRAkc-ERHCzEcp2 NB and pTRAkc-ERH respectively. Selection for positive transformants was done on YEB agar with carbenicillin, tetracycline and gentamicin antibiotics. The tetracycline was included for maintenance of the pSOUP vector which was not a requirement for transfer of the T-DNA into the plants. It was retained because it was already present within the bacteria when the strains were received. Gentamicin was used to maintain the pMP90 vector which carried all the *vir* genes required for transfer of the T-DNA into the plant cells. Carbenicillin was used to maintain the pTRAkc-ERH recombinant vectors. *Agrobacterium* is intrinsically resistant to ampicillin because it carries a β-lactamase gene, therefore the stronger version, carbenicillin, was used for positive selection (Hellens et al. 2000a; Lee and Gelvin 2008). Despite the bacterium expressing resistance to the ampicillin antibiotic, higher concentrations of carbenicillin can kill it (Lee and Gelvin 2008). The use of ampicillin as a selection marker in *Agrobacterium* transformation is a shortcoming that can be avoided by using antibiotic markers on the binary vectors that the bacteria are susceptible to, for example kanamycin.

Many *Agrobacterium* strains exist for agroinfiltration of plants. Strains that have commonly been used are *A. tumefaciencs* LBA4404 (Ooms et al. 1982) and *A. tumefaciens* AGL1 (Lazo et al. 1991). The GV3101 (pSOUP+pMP90) strain was chosen in this study because it was made available by the JHI Institute and because the *P. infestans* INF1 positive control gene was cloned into the same strain (JHI Institute). An *A. tumefaciens* GV3101::pMP90 strain (Koncz and Schell 1986) was modified by adding the pSOUP vector (Hellens et al. 2000b) to obtain *A. tumefaciens* GV3101 (pSOUP+pMP90) (*Sophie Mantelin, personal communication).* Therefore, the use of the same bacterial background was essential to rule out the possibility that any of the changes seen in the infiltrated areas was due to the bacteria. If the vectors carrying *CzEcp2* were cloned into a different strain, there would have been inconsistencies with maintaining all conditions of infiltration the same except for the presence of gene within the vector and the positive control gene.

Colony PCR was used to screen for positive *Agrobacterium* transformants carrying pTRAkc-ERHCzEcp2 EB, pTRAkc-ERHCzEcp2 NB and pTRAkc-ERH. Observation of bands for pTRAkc-ERHCzEcp2 EB and pTRAkc-ERH indicated that they were present within the picked colonies. This result was expected given the bacterial growth on the selection medium which confirmed that they were not false positives. Colony PCR was used to screen the *Agrobacterium* transformants because extraction of plasmid DNA does not yield high quantities for downstream reactions (Bergkessel and Guthrie 2013). Therefore, poor quality plasmid DNA would easily degrade, not yield good bands in PCR and likely not be digested well with restriction enzymes. Screening bacterial transformants using the colonies as template saves time and circumvents downstream errors.

No bands were observed on the *Agrobacterium* pTRAkc-ERHCzECP2 NB agarose gel. This may be due to the absence of the *CzEcp2* NB (LPH signal peptide) gene within the vector. It may also be caused by primer degradation or human error where one of the PCR reagents was omitted. It may also be likely that the recombinant vector rearranged during *Agrobacterium* transformation and therefore lost the gene of interest or the primer binding sites. The restriction enzyme digest products of pTRAkc-ERHCzEcp2 NB also alluded to the absence of *CzEcp2* NB (LPH signal peptide) or the restriction sites within the recombinant vector. It is likely that the colonies initially screened for *E. coli* transformation lost the vector in subsequent experiments or that colonies lacking the recombinant vector were picked for isolation. This construct was therefore not used for agroinfiltration of *Nicotiana* spp. because of the lack of bands in the colony PCR and the inconsistency of the results. In future it will be necessary to screen a lot more colonies and ensure that the screened colonies are same ones used for plasmid extraction. It is also suggested that the recombinant plasmid gets sequenced again when results look unexpected and that plasmid DNA from more than one colony be extracted should one sample produce unexpected results.

A separate colony PCR was done to verify that the lack of bands observed for *Agrobacterium* pTRAkc-ERHCzEcp2 NB was not due to an error with the colony PCR reaction or that the bacteria were inviable. *Agrobacterium*-specific miaA primers (Grayburn and Vick 1995) were used for the same colonies previously screened and multiple bands were obtained for each sample. This may have been an indication that the PCR was not stringent enough and that the annealing temperature should have been increased. It may also be an indication that the primers were designed to hybridise to multiple genes in the *Agrobacterium* genome. The presence of bands indicates a successful colony PCR reaction with viable *Agrobacterium*. This suggests that the bacteria were likely not carrying pTRAkc-ERHCzEcp2 NB or that the vector lost the gene of interest through rearrangement during *Agrobacterium* transformation.

Leaves of *N. benthamiana, N. tabacum* cv. Petit Havana and *N. tabacum* cv. LA Burley were transiently infiltrated with *Agrobacterium tumefaciens* GV3101 (pSOUP+pMP90) carrying pTRAkc-ERH and pTRAkc-ERHCzEp2 EB. Transient infiltration was done because of the need to study the

expression of *CzEcp2* in short term. The goal of the study was to determine if the gene will be expressed, and if so what kind of a plant defence response would be observed. Stable transformation is the long-term alternative that results in incorporation of a gene of interest into the plant genome. This trait is then inherited by generations of progeny after transformation of the parent plants. Therefore, the trait is stable and maintained within that population (Wroblewski et al. 2005; Leuzinger et al. 2013; Gelvin 2003; Krenek et al. 2015).

The positive control for infiltration was the same bacterial strain carrying a *P. infestans* INF1 gene fused to a PVX vector (JHI Institute, United Kingdom) (Kamoun et al. 1998). This control was included in all the leaves for the three *Nicotiana* spp. to compare to the expression of *CzEcp2*. This effector gene was chosen as a positive control because previous studies have proven that it causes an HR in *Nicotiana* spp. without a cognate R-protein (Kamoun et al. 1998). An *Agrobacterium* only control was infiltrated into all the leaves to rule out defence responses caused by the bacteria instead of the gene.

Where pTRAkc-ERHCzEcp2 EB was infiltrated into the replicates of *N. benthamiana, N. tabacum* cv. Petit Havana and *N. tabacum* cv. LA Burley, necrosis was not observed. Instead, some of the replicates produced chlorotic responses. This result was unexpected due to *CzEcp2* being a homolog of *CfEcp2* which has been shown to cause HR in *Nicotiana* spp. (Laugé et al. 2000b; De Kock et al. 2004; Takken et al. 2000b). The following hypotheses were drawn from these findings; CzECP2 expression could have been hindered by the native fungal signal peptide not being cleaved by the plant machinery. Given that CzECP2 is an apoplastic protein, transport of the mature peptide would be essential for it to perform its function in the correct location. It is also likely that the T-DNA region carrying the gene of interest was not excised from the binary vector and transported into the plant cells (An 1987; Krenek et al. 2015).

Homologs of *CfEcp2* have been identified in other Dothideomycete fungi. In *Mycoshpaerella (Pseudocercospora) fijiensis*, three copies of the gene were identified, *MfEcp2, MfEcp2-2* and *MfEcp2-3,* with the highest identity to *MfEcp2* (57%). These genes were shown to be recognised by Cf-Ecp2 (tomato R-protein) in tomato due to the high conservation of the CfECP2 and MfECP2 amino acid sequences (Stergiopoulos et al. 2010). Three homologs were identified in *Dothistroma septosporum* (*DsEcp2-1, DsEcp2-2* and *DsEcp2-3*) (Stergiopoulos et al. 2010; Bradshaw et al. 2016; de Wit et al. 2012). Studies showed that the gene is highly expressed *in planta* and that it causes HR in nonhost *N. tabacum* plants and tomato plants carrying Cf-Ecp2 (tomato R-protein) (Guo 2015; Bradshaw et al. 2016). This suggests that the R-protein responsible for CfECP2 recognition in tomato recognises DsEcp2-1 due to high amino acid similarity between the effectors. Therefore, an R-protein in the nonhost *Nicotiana* spp. recognised the effector and caused defence responses (Guo 2015).

The CzECP2 mature sequence lacking the signal peptide was aligned against the mature peptides of CfECP2, DsECP2-1 and MfECP2 (*P. fijiensis* ECP2) (Appendix, Figure S3). The alignment shows

that similarity is highest between *C. zeina* and *C. berteroae* compared to *C. fulvum* or *D. septosporum*. *M. fijiensis* (*P. fijiensis*) is also more similar to *C. fulvum* than it is to *C. zeina*. This may explain why *MfEcp2* and *DsEcp2-1* were recognised by Cf-Ecp2 (tomato R-protein) within the *C. fulvum* host, tomato (Stergiopoulos et al. 2010; Guo 2015). Therefore, it is hypothesised that the lower similarity of mature CzECP2 to CfECP2 is the reason for a lack of HR within the tobacco plants compared to what was seen previously for CfECP2 and DsECP2-1 in nonhost plants (Laugé et al. 2000b; De Kock et al. 2004; Laugé et al. 1997). The dominant R-protein within *Nicotiana* spp. that was able to recognise CfECP and DsECP2 may therefore not have recognised CzECP2 due to differences between the amino acid sequences (De Kock et al. 2004).

The observed chlorosis on the *Nicotiana* spp. replicates could have developed into HR if the plants were monitored for longer than 10 days. It has been suggested that fungal proteins expressed in plants can be perceived as PAMPs (pathogen associated molecular patterns) by the plants (Laugé et al. 2000b; Laugé et al. 1997). Therefore, the chlorosis is more likely to be PTI rather than a precursor for ETI where an HR is expected due to recognition of an effector. De Kock et al. (2004) found that *C. fulvum Ecp2* fused to a PR1a signal peptide in a PVX vector caused necrosis in *Nicotiana paniculata, N. tabacum* cv. Petit Havana, *N. tabacum* cv. White Burley and other *Nicotiana* spp. but not in *N. benthamiana*. The researchers proposed that the necrosis was due to recognition of *CfEcp2* by dominant R genes within the plants. Their findings were significant because they suggested that host specific pathogenic effectors are recognised by non-host plants. Their findings were therefore used as motivation to select the *Nicotiana* spp. in this study with an expectation of similar results.

Infiltrations of the three plant types with the pTRAkc-ERH vector control and the wildtype *Agrobacterium* control did not cause any notable changes in leaf morphology. These controls were included to rule out the possibility that any defence responses seen in the leaves were due to background caused by the vector or the bacteria. This allowed for validation that the *CzEcp2* gene expression was responsible for potential plant responses (Laugé et al. 2000b). Expression of INF1 in *N. benthamiana* resulted in severe necrosis in the regions of infiltration. This indicated that the pathogenic protein was recognised in the plant and a hypersensitive response was elicited by the plant to protect itself. It is however likely that the necrosis was not due to direct recognition of INF1 but rather due to its perception by the plant as a PAMP (Kanneganti et al. 2006; Kamoun et al. 1998).

INF1 infiltration of *N. tabacum* cv. Petit Havana and *N. tabacum* cv. LA Burley resulted in chlorosis in all the replicates. A clear HR was expected as observed in *N. benthamiana*, however only necrotic spots were noted on some of the replicates (Kamoun et al. 1998). These necrotic spots are potential indicators of the recognition of INF1 by the tobacco resistance proteins. They could likely have coalesced into larger spots or lesions if the leaves were monitored for longer than 10 days. An alternative positive control in this study could have been the *P. infestans Crn1* or *Crn2* effector genes which have been shown to cause necrosis non-specifically in *N. benthamiana* plants. These genes

were postulated to be similar to INF1 in function but structurally different (Torto et al. 2003). In cases where necrosis was not observed, GFP fluorescence could have been used to determine if the gene of interest was expressed. Therefore, in future an effector gene can be cloned into a binary vector by fusing it upstream to a GFP sequence. If the gene is expressed, GFP will fluoresce green under UV light (Wydro et al. 2006).

One of the three infiltrated *N. benthamiana* leaves had a more yellow appearance across its entire surface compared to the other two replicates. This created the impression that all the infiltrations resulted in chlorosis and necrosis for INF1. It has been hypothesised however that the yellow colour of the leaf was due to senescence rather than chlorosis because of the uniformity and spread of the colour (Niewiadomska et al. 2009). A noticeable point is that the plants were infiltrated at 9 weeks and monitored for 10 days, therefore the older leaves which were chosen for infiltration were starting to senesce. If chlorosis occurred as a result of the infiltrations only, it would have been localised to the demarcated areas.

The findings from this study have led to a potential hypothesis that *CzEcp2* is not a pathogenic effector. Given the absence of clearly defined plant defences within 10 days, the plant likely did not recognise the protein as a threat. This could lead to the assumption that *CzEcp2* does not contribute towards *C. zeina* pathogenicity. With that being said, the evidence from this study is not enough to definitively conclude this claim. Future work involving functional characterisation of *CzEcp2* is required. It is important to note that most of the studies done to elucidate the presence and function of *CfEcp2* were done in the host plant, tomato (Laugé et al. 1997; Wubben et al. 1994; Laugé et al. 2000b; Van den Ackerveken et al. 1993). In some cases, such as the study done by Wubben et al. (1994), proteins expressed in *C. fulvum* infected leaves were studied instead of the gene being cloned for infiltration studies in non-host plants. Given the need for effectors to be recognised by cognate R-proteins, this approach is more likely to yield intense HR.

In future, transformation studies in maize will be necessary to determine if the responses noted in *Nicotiana* spp. are the same in the host plant. This will give definitive proof of the role of effectors in the pathogenicity and virulence of *C. zeina* and facilitate discovery of the cognate maize R-proteins. Although the function of *Ecp2* is still elusive, studies have shown that it is important for the ability of *C. fulvum* to cause infection and produce other important effectors responsible for virulence. It has been suggested that *CfEcp2* is essential for growth of the fungus during host colonisation (Laugé et al. 1998). It has also been suggested that it plays a role in stomatal collapse during invasion, therefore the lack of *CfEcp2* hinders efficient entry into the host cells (Laugé et al. 1997).

In future, the inconsistency in observation of plant defence responses across all replicates of *Nicotiana spp*. in this study could be circumvented through screening a much larger number of plants and infiltrating more leaves on each plant. Even though macroscopic changes were not visible for all

the replicates, it is likely that some type of response occurred microscopically. Given that the infiltrated leaves did not die after 10 days of observation, the plants may have been protecting themselves against the effector. Such responses can be viewed through staining techniques (Trypan blue, DAB) and microscopic analysis of stained tissues (Jambunathan 2010).

The lack of HR within 10 days of observation may be explained by the hypothesis of delayed HR. Apoplastic effectors have been shown to take longer than usual to be recognised by the plant and for defence responses to result. This is explained by effector triggered defence (ETD) instead of effector triggered immunity (ETI) (Stotz et al. 2014). Therefore, in this study it might have been necessary to monitor the plants for longer than 10 days to see a potential HR. The delay may also be explained by the hypothesis that *C. zeina* is a hemibiotrophic pathogen therefore it takes up to 14 days before the first necrotic lesions are observed at which point the fungus has switched to necrotrophy (Nsibo et al. 2019; Ward et al. 1999; Ward and Nowell 1998). Therefore, apoplastic *C. zeina* effectors may take longer to cause an HR when agroinfiltrated into plants if they contribute towards pathogenicity and virulence. In this study the leaves were observed for 10 days due to time constraints, therefore in future longer observation times (> 14 days post infiltration) are recommended.

2.5 Conclusion

This study provided a stepping stone to begin elucidating the effector biology of *C. zeina*. Expression analysis of the putative *CzEcp2* effector gene was conducted by agroinfiltration of *Nicotiana* spp. Given that the molecular mechanisms underlying the pathogenicity and virulence of *C. zeina* are still unknown, it was postulated that the effector biology may play a role in those functions. This study confirmed findings from previous studies that *CzEcp2* is expressed in the fungal genome (Lombard, MSc thesis 2015). It also confirmed that the fungus grows on cornmeal agar and that *CzEcp2* expression is stimulated as was found in Swart et al. (2017). The findings that an *Ecp2* homolog from other Dothideomycete fungi exists within the *C. zeina* genome assembly was also confirmed as previously suggested in another study (Lombard, MSc thesis 2015). *CzEcp2* was successfully cloned with its signal peptide into the pTRAkc-ERH binary vector and transferred into *Nicotiana* spp. Some leaves showed chlorosis indicating potential expression of *CzEcp2* and a precursor response to HR. This finding led to the hypothesis that CzECP2 is potentially pathogenic and that it may be an apoplastic effector given the delayed HR. It was also hypothesised that based on the observation of chlorosis where complete CzECP2 EB may have been expressed, that the fungal signal peptide was cleaved within the plant cells. Functional analysis of this effector and others is required where potential defence responses are studied for longer than 10 days and quantified. This may give insight into how *C. zeina* interacts with its host plant, maize, during GLS infestation.

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Chapter 3

Concluding Remarks and Future Prospects

One of the biggest threats to the survival of the human population is food insecurity (Rukuni 2002; Barrett and Lentz 2010). Climate change has caused a domino effect of negative impacts to the systems responsible for food production (Battisti and Naylor 2009). In some areas of the world rainfall has increased to a point where crops get waterlogged (Luck et al. 2011; Rosenzweig et al. 2002). In other places rainfall has been reduced to the point of droughts (Benhin 2008; Chakraborty et al. 2000). Whether there is too much or too little rain, whether it is too hot or too cold, these imbalances in nature all contribute towards the development of detrimental diseases (Luck et al. 2011). Pathogens have evolved resistance to currently used control measures and new pathogens have emerged that lack control measures (Brent and Hollomon 1995; Mobambo et al. 1993; Delp 1980). Whatever the problems are, in whichever part of the world, it has become essential to solve them with solutions that are not harmful and that function rapidly.

Africa has been postulated to be the most impacted by food insecurity due to climate change. As it stands, the FAO estimated that approximately 21% of the African population is undernourished. Reports from 2018 estimated that there were 821 million people undernourished by the end of 2017 (World Health Organization 2018). As the world population continues to grow, these dire statistics may also grow if food supply is not increased and made accessible to the most poverty-stricken parts of the world. Biotechnology innovations that include water-use efficient cropping systems, and drought tolerant and disease resistant crops are essential for adaptation to the current climate. It may be impossible to create a “super crop” with all these traits, but it is possible to devise other creative ways to mitigate issues relating to the decline in agricultural productivity.

Microorganisms, as minute and sometimes mundane as they may seem, are one of the biggest threats to food security. Some of the most detrimental diseases are those caused to the leaves of agricultural crops. Examples of maize leaf pathogens include but are not limited to *Cochliobolus heterostrophus Cercospora zeina* and *Exserohilum turcicum*. These fungal pathogens cause southern corn leaf blight, grey leaf spot and northern corn leaf blight respectively (Tatum 1971; Perkins and Pedersen 1987; Ward et al. 1999). At present, there is no single control measure that eradicates these pathogens. Most farmers rely on the use of fungicides, intercropping and tillage practices, and planting resistant varieties. (Welz and Geiger 2000; Berger et al. 2014; Korsman et al. 2012; Huang et al. 2010). Smallholder farmers who rely on the use of rainwater for irrigation are the most affected by drought and late rainfall caused by climate change They often practice conservation tillage to preserve soil integrity and use little to no chemicals for protection of their crops against pathogens (Thierfelder and Wall 2012; Gouse et al. 2006; Nsibo et al. 2019).

Grey leaf spot has been shown to reduce maize productivity in *C. zeina* infested farm lands (Ward and Nowell 1998; Nsibo et al. 2019; Ward et al. 1999). The pathogen infection strategy is still unknown (Berger et al. 2014; Swart et al. 2017). The present research aimed to understand the effector biology of the fungus to determine if the *Ecp2* effector exists in the genome and if it can be cloned for transient

infiltration studies. Effector proteins have been shown to influence how a pathogen causes disease and the severity of infection. Plants defend themselves against pathogens through elicitation of HR (hypersensitive response) when R-proteins recognise the effectors (De Wit and Spikman 1982; Joosten and de Wit 1999; de Jonge et al. 2010; Laugé et al. 1997).

This study confirmed the presence of a *Cladosporium fulvum Ecp2* homolog within the *C. zeina* genome, verifying the findings from Lombard (2015) in her MSc thesis. It also confirmed that *CzEcp2* is expressed by the fungus when it is cultured on cornmeal agar (Swart et al. 2017). Evidence was obtained from results generated with the following *in silico* analyses; searching for the gene by BLAST analysis against GenBank, searching RNA sequence data from Swart et al. (2017) for confirmation of the accession, reciprocal BLAST to confirm the gene is in the *C. zeina* genome assembly, BLASTp against GenBank to search for protein homologs in other Dothideomycete fungi; and protein and pairwise alignments of the identified homologs against putative CzECP2.

Other *Cercospora* species (*Cercospora beticola* and *Cercospora berteroae*) also had homologs of *C. fulvum* ECP2. This may be an indication that the *Ecp2* effector is conserved across the *Cercospora* genus. If this hypothesis were tested, it could provide a different aspect to figuring out how some *Cercospora* fungi cause diseases in their hosts. Like *C. zeina*, the causal agent of soybean frogeye leafspot, *Cercospora sojina* Hara, has an unknown infection strategy (Luo et al. 2018). Despite having a fully intact cercosporin toxin biosynthesis (CTB) cluster, Luo et al. (2018) showed that *C. sojina* does not produce cercosporin. This toxin is commonly used by other *Cercospora* species to cause diseases in their hosts. The researchers sequenced the genome of the fungus and found putative effector genes. This therefore may be used as a basis to determine how effector biology influences the pathogenicity of *C. sojina* and other *Cercospora* species.

C. fulvum effector biology has been well studied over the years. Some effectors that have been identified include *Ecp2, Avr4, Avr9* and *Ecp6*. They've been shown to function in various roles regarding how *C. fulvum* causes leaf mould in tomato (Van der Hoorn et al. 2000; Bolton et al. 2008; Van den Ackerveken et al. 1993; van Esse et al. 2007). Gene knock-out studies have proven the necessity of some effectors for the fungus to cause disease in its host (Laugé et al. 1997; Bolton et al. 2008). In future, gene knock out studies using RNA silencing can be conducted where *czecp2* mutants of *C. zeina* are created and used to infect the host plant, maize (Nakayashiki et al. 2005; De Wit 2016; Stergiopoulos and Wit 2009). If GLS severity is reduced, it can be hypothesised that *CzEcp2* is a requirement for fungal proliferation during infection and for virulence of the fungus. CRISPR-Cas9 may be used to study the functions of effector genes through targeted knockouts. This method was proposed to be more efficient and produce less off-target effects for breeding purposes (De Wit 2016; Schuster et al. 2016).

Homologs of other *C. fulvum* effector genes, *Ecp6* and *Avr4,* were searched for in the *C. zeina* genome in a previous study (Lombard, 2015 MSc Thesis). According to the results, putative homologs of these genes exist in the genome as was shown by mapping the predicted sequences using the draft genome at the time. Further analysis is needed to confirm the presence of those genes and their expression in the fungus before it can be concluded definitively that they exist. It is essential to note however that the studies done to identify *Ecp6* in the above-mentioned study resulted in the identification of an incorrect accession (data not shown). Therefore, future work should be dedicated towards identifying the correct effector gene prior to expression analysis studies (Lombard, 2015 MSc Thesis). The scope of candidate effectors searched for in the *C. zeina* genome can be increased using tools such as EffectorP and ApoplastP (Sperschneider et al. 2018; Sperschneider et al. 2016).

The present study proved that *CzEcp2* can be ligated into the pTRAkc-ERH binary vector for *Agrobacterium* infiltration studies. The gene was ligated into the vector with and without its native signal peptide. Cloning with the fungal signal peptide (pTRAkc-ERHCzEcp2 EB) was done to determine if it could be cleaved within non-host *Nicotiana* plants. The observation of chlorosis in some of the infiltrated replicates led to the assumption that the protein was expressed after signal peptide cleavage. Therefore, the *C. zeina* fungal signal peptide can be cleaved within *Nicotiana* species. The cloning strategy developed in the present study for *CzEcp2* expression analysis can be applied to study other *C. zeina* effector genes.

Unfortunately, the construct with the mature peptide (pTRAkc-ERHCzEcp2 NB) was not used for infiltrations due to inconsistency with screening for the presence of mature *CzEcp2* within the construct. This construct served as an alternative to study if *CzEcp2* could result in HR when fused to a plant signal peptide (LPH) on pTRAkc-ERH (Maclean et al. 2007). It was hypothesised that this *Ecp2* construct could have expressed a stronger plant defence response than pTRAkc-ERHCzEcp2 EB with the fungal signal peptide. This stemmed from the idea that the plant machinery would cleave the plant signal peptide more accurately resulting in an increased number of correctly cleaved molecules to perform their function. It is likely that the fungal signal peptide was incorrectly cleaved within plants due to challenges with cross-kingdom recognition of sequences (von Heijne 1990; von Heijne and Abrahmsèn 1989). Therefore, in future studies such a construct should be included when expressing effectors via *Agrobacterium* transient infiltration.

Some studies have used the PVX vector to express candidate effectors within host and nonhost plants using *Agrobacterium* infiltration (Van der Hoorn et al. 2000; Takken et al. 2000b). Takken et al. (2000) developed a cloning strategy using the PVX vector and agroinfiltration. They did this to study *C. fulvum Avr4* expression in tomato and *Ecp2* expression in *Nicotiana* species and found that HR results due to expression of AVR4 and CfECP2. Therefore, in future the PVX vector can be used as an alternative for *C. zeina* effector gene expression studies in host and plants that are not hosts (Chapman et al. 1992).

Nicotiana benthamiana, Nicotiana tabacum cv. Petit Havana and *Nicotiana tabacum* cv. LA Burley were transiently infiltrated with pTRAkc-ERHCzEcp2 EB which resulted in chlorosis for some replicates. A hypersensitive response (HR) was expected on the premise that the effector will be expressed and recognised by R-proteins within the plants (De Kock et al. 2004). Observation of chlorosis was a positive result in that it could have developed into HR if the plants were observed for longer than 10 days, and it was an indication of a defence response. Studies done to characterise *CfEcp2* showed HR in some *Nicotiana* species. These results suggested that an individual gene within the plants recognised CfECP2 and resulted in defence responses because it is a pathogenic protein (Laugé et al. 1998; Laugé et al. 2000b; Laugé et al. 1997; De Kock et al. 2004). In future, when *C. zeina* effectors are infiltrated into nonhost plants, longer observation times may lead to observation of HR. *C. zeina* is a fungus that functions apoplastically, therefore defence against its pathogenic proteins may take longer than 14 days to occur, possibly resulting in effector triggered defence (ETD) (Stotz et al. 2014; Ward et al. 1999; Ward and Nowell 1998).

Given the confirmation that *CzEcp2* is present within *C. zeina* and its similarity to *CfEcp2*, studies can be undertaken to co-infiltrate it into tobacco plants with the tomato cognate R-protein, Cf-ECP2 (Westerink et al. 2004). This may assist in understanding how *CzEcp2* interacts with R-proteins, if at all it does. *CzEcp2* can also be infiltrated into tomato plants to determine if a defence response occurs. Should plant defence responses occur, homologs of Cf-ECP2 can be searched for in the maize genome. Protein immunoprecipitation may then be done to determine which R-proteins *CzEcp2* interacted with (Stergiopoulos et al. 2010; Stergiopoulos et al. 2009).

Recent literature has suggested that effectors (avirulence factors) isolated from fungi grown *in vitro* are non-specific in their function (De Wit 2016). In his review, De Wit (2016) discussed studies that were done to search for apoplastic effectors in fungi. He suggested that the effectors responsible for pathogenic activity within hosts are not expressed *in vitro* but rather *in planta*. He hypothesised that this was due to specific defence responses only occurring in host plants that stimulate specific gene expression within the pathogen. Their studies done to discover *C. fulvum* effectors proved that those expressed *in planta* are either minimally expressed *in vitro* or not expressed at all. Their use of apoplast fluids from infected tomato plants to infiltrate uninfected lines proved that certain effectors (*Avr9* and *Avr4*) are expressed *in planta* and can cause HR in susceptible plants (Van den Ackerveken et al. 1994; De Wit et al. 1985; De Wit 1977). Where effectors were isolated from *in vitro* cultured *C. fulvum*, the responses from the avirulent and virulent strains were identical, implying a non-specific function for those proteins (De Wit and Roseboom 1980; De Wit and Spikman 1982).

Based on suggestions from De Wit (2016), future studies involving effector identification in fungal pathogens should be done *in vitro* and *in planta*. Where apoplastic pathogens are being studied, the apoplast fluids of infected hosts can be isolated and studied for increased expression of pathogenic genes (Joosten 2012; De Wit et al. 1985; De Wit 1977). Given the lack of HR seen when *CzEcp2* was

infiltrated into *Nicotiana* species, it may be suggested that it is not a pathogenic effector. However, this hypothesis would have to be tested by isolating effectors from avirulent *C. zeina* to compare to the virulent strain (De Wit 1977, 2016). Given that the infection mechanism of *C. zeina* is unknown, it may be difficult to create gene knock-outs of the fungus to create avirulent strains because the virulent gene targets are unknown. However, in a previous study, a gene-knockout protocol of *C. zeina* genes was developed (Swart et al. 2017) and can be adapted for studying effector gene mutants of the fungus.

A suggestion for future work is that susceptible maize lines should be infected with virulent *C. zeina*, the apoplast fluids isolated and then infiltrated into uninfected maize to determine the types of defence responses elicited. The apoplast fluid can then be studied for the presence of specific effector proteins (De Wit et al. 1985; De Wit and Spikman 1982). Isolating the cognate R-proteins will help in understanding which plant machinery plays an essential function in maize defence against this pathogen. It will also facilitate the study of effector gene expression in non-host plants through co-infiltration with the R-proteins (Westerink et al. 2004). These studies can also be extended to determine how *C. zeae-maydis* infection of maize influences defence responses and which effectors it secretes during infection.

In Swart et al. (2017), gene expression within *C. zeina* was compared *in planta* and *in vitro*. The results were significant because they showed that there are differences in expression when the fungus is growing within the host and on synthetic media. For *CzEcp2*, expression was higher *in planta* compared to *in vitro* for some media, but lower *in planta* compared to cornmeal agar and complete medium (Swart et al. 2017). The higher *in planta* reads may suggest that it is a pathogenic effector required for *C. zeina* to perform its infectious functions. However, the reads *in planta* are considerably lower than some *in vitro*, which may also suggest a nonspecific function for *CzEcp2* (De Wit 2016; De Wit and Roseboom 1980). In future, it would have to be determined if *CzEcp2* is only important for growth of the fungus *in planta* or if it directly targets defence proteins within maize during infection.

Studying the expression of effectors in non-host plants may be valuable for the identification of alternative R-proteins (De Kock et al. 2004; Laugé et al. 2000b; Mccann 2016). Alternative R genes may be bred into host plants with native R genes. They may either prime the plant for stronger defences or assist in compounding defence against pathogens (Zhu et al. 2012). It may also be possible to use R genes from one plant in a different species provided the pathogens in question are closely related and carry homologs of the same effectors. The presence of more than one R gene within the hosts may also alleviate pathogen resistance caused by the pathogen losing or changing the effector gene or evading recognition through adaptor proteins (Joosten et al. 1994; Dodds and Rathjen 2010). Screening for putative maize R-proteins should be carried out in numerous maize varieties, including susceptible and resistant lines. This may broaden the scope of responses seen and therefore the proteins expressed.

The principles applied in the present study may be extended to other pathogens of maize such as *E. turcicum* that causes NCLB (Perkins and Pedersen 1987). Where effector proteins and cognate host R-proteins of this pathogen are identified, the R genes may be stacked with *C. zeina* R genes in a maize hybrid (Zhu et al. 2012; Condon et al. 2013; Van de Wouw and Idnurm 2019). This may assist in alleviating the combined effects of NCLB and GLS when both pathogens exist on the maize. Breeding crops with the ability to fight off pathogens inherently can potentially reduce the amount of harmful chemicals currently used to control diseases. This will reduce off-target effects in the environment against other insects and contamination of water from run off. It may also reduce the danger of exposing farm workers to harmful chemicals. Various aspects of breeding for resistance using effector and R genes are discussed in Van de Wouw and Idnurm (2019).

In future studies, instead of only looking for HR, it may be valuable to look for early PTI responses such as ROS (reactive oxygen species) when *C. zeina* effectors are being studied (Murshed et al. 2008; Jambunathan 2010; Kohen et al. 2000). Techniques should be devised to determine whether the host and nonhost cell walls are strengthened through callose deposition as a form of early defence (Luna et al. 2011; Ellinger et al. 2013; Yun et al. 2006). The use of staining techniques such as DAB may be used for detection of early plant defence responses (Thordal-Christensen et al. 1997; Alvarez et al. 1998). The use of GFP (green fluorescent protein) tags may be valuable in future to quantify effector gene expression and plant defence responses. This can be achieved by cloning a GFP sequence into a binary vector downstream of the gene of interest to create a translation fusion of the two genes. Therefore, when the gene of interest is expressed, so will the GFP gene. Under UV light, effector gene expression will be perceived as green fluorescence which can be quantified (Richards et al. 2003; Leffel et al. 1997; Chiu et al. 1996).

In conclusion, this study provided insight into the effector biology of *C. zeina* and confirmed the presence of *Ecp2* in the genome. Evidence was provided that a *C. zeina* effector (*CzEcp2*) can be expressed in non-host *Nicotiana* species and that the effector is expressed when the fungus is grown on cornmeal agar. These findings provide a step closer towards understanding the molecular mechanism underlying *C. zeina* infection of maize. A platform has been created for further cloning and expression of candidate effectors from the *C. zeina* genome. The hypothesis of the study which stated that *CzEcp2* will cause HR in *Nicotiana* species was not proven. It led to the following conclusions: that CzECP2 was not transported to the apoplast due to incorrect signal peptide cleavage or the lack of cleavage, that *CzEcp2* is not essential for *C. zeina* pathogenicity hence the lack of HR or that the plants recognised the protein as a PAMP instead of an effector, hence the observation of chlorosis and the lack of HR. The following alternative hypotheses were suggested for future studies: *CzEcp2* elicits early plant defence responses in nonhost *Nicotiana* species.; *CzEcp2* is an apoplast effector and therefore results in delayed HR; *CzEcp2* is not a pathogenic effector hence there is a lack of HR in nonhost *Nicotiana* species; *CzEcp2* fused to a plant signal peptide will result

in transport of the protein to the apoplast in nonhost *Nicotiana* species. Despite the significance of the findings in this study, more research must be invested into understanding the function of *CzEcp2* and deciphering the broader spectrum of *C. zeina* effectors and their cognate R-proteins in maize plants.

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Appendix

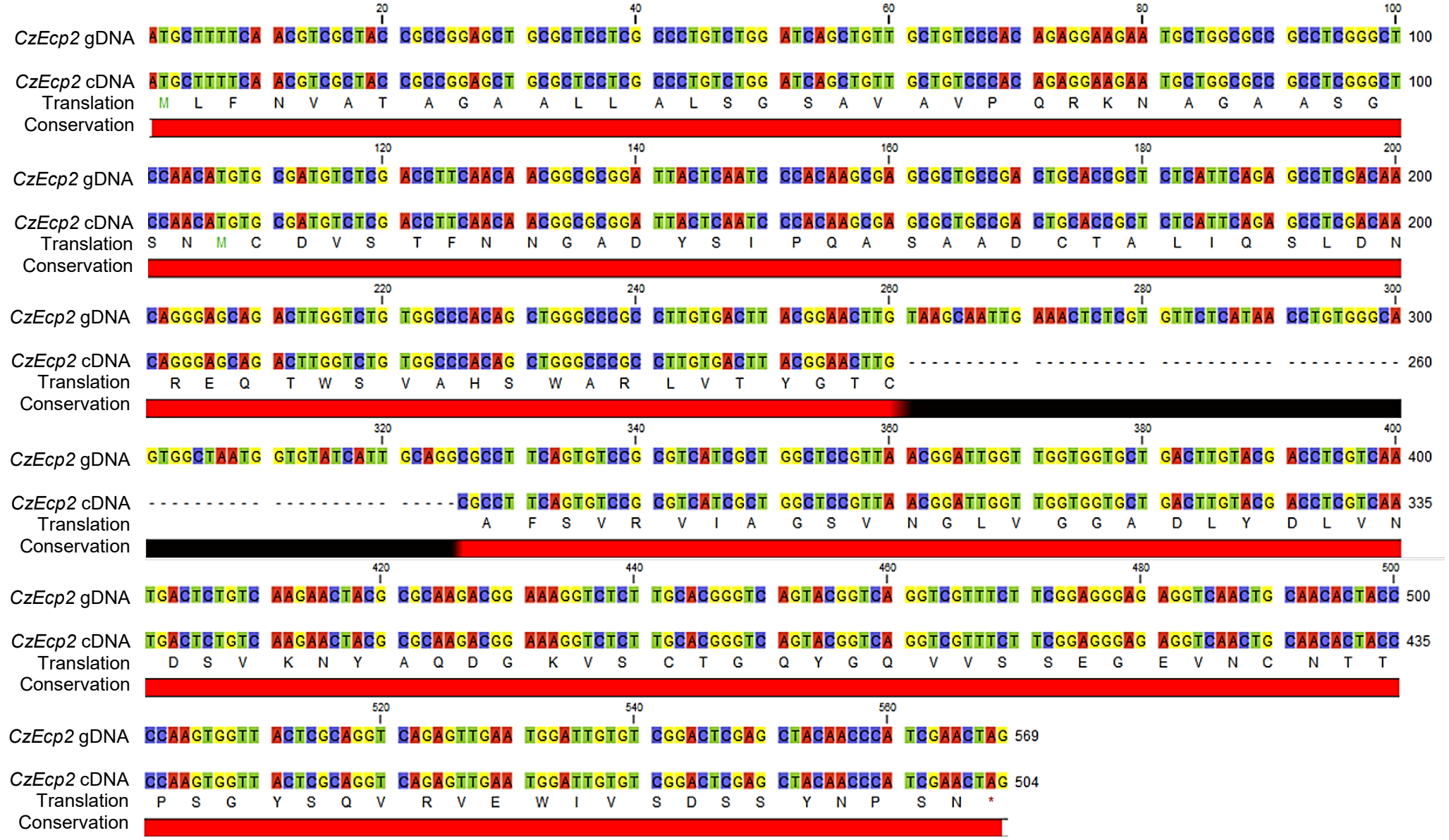


Figure S1: Putative C. zeina Ecp2 alignments with CLC Bio Main Workbench. *Putative CzEcp2 gDNA (569 bp) was aligned to cDNA (504 bp, 65 bp intron highlighted in black). The red bar shows 100% conservation between nucleotides. The colours on each nucleotide were adenine (red), thymine (green), cytosine (blue) or guanine (yellow). The single letter amino acid (Translation) sequence was included to infer the reading frame of CzEcp2 after intron excision.*

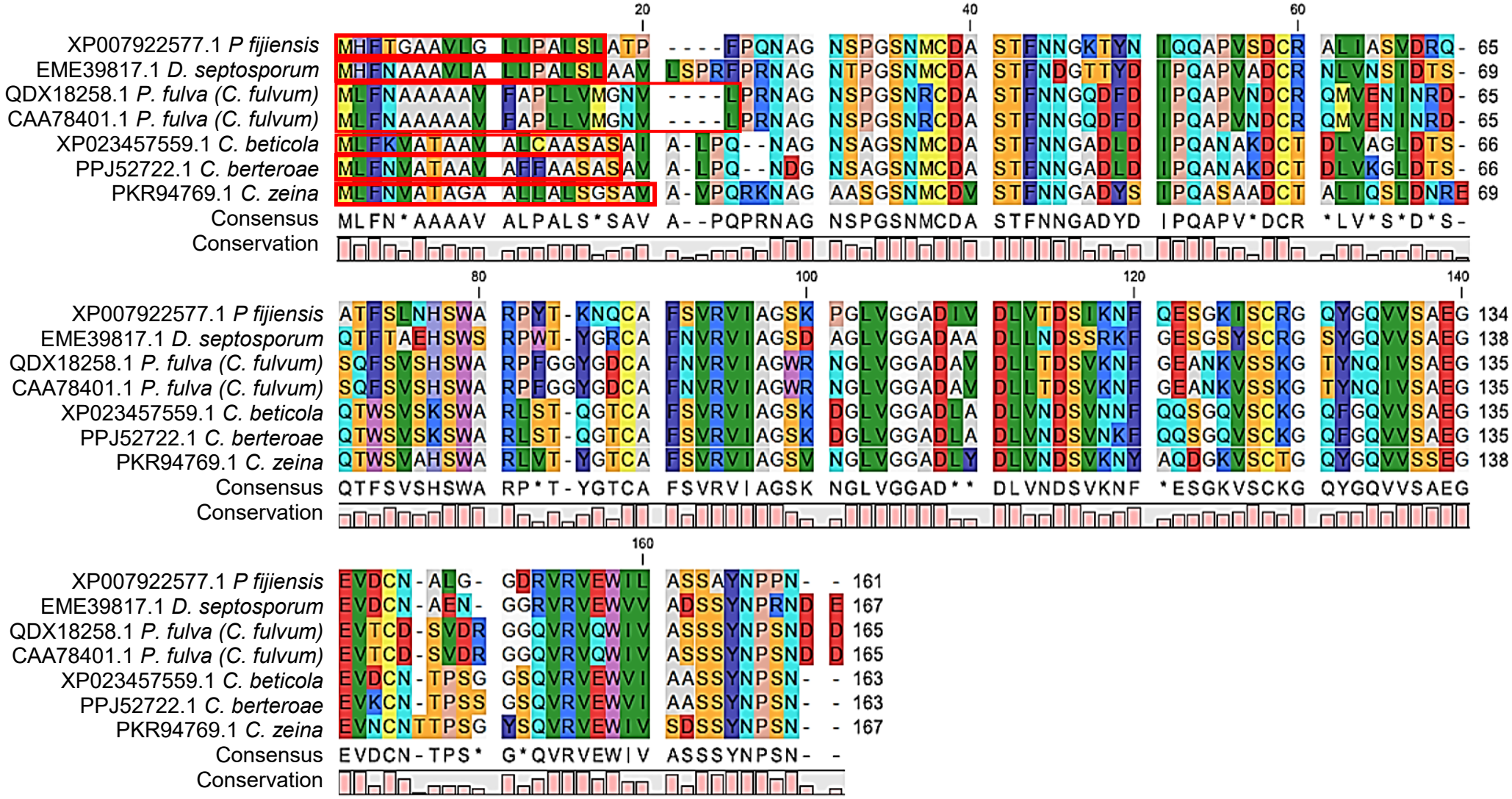


Figure S2: Alignment of putative ECP2 proteins from selected Dothideomycete fungi against putative C. zeina ECP2 using ClustalW. *Complete ECP2 protein accessions from NCBI were aligned against putative C. zeina ECP2 using ClustalW. Alignments were based on the BLOSUM62 matrix. Where the conservation bars were full, complete conservation between amino acid residues was observed. The consensus sequence shows the residues that were most common between the all accessions (full bars). All * symbols represented ambiguous bases (midi bars). All – symbols represented the lack of a residue in some peptides at a given position (extremely flat bars). The sequences highlighted with the red box demarcate the signal peptide (SignalP 5.0) for each accession. Each protein sequence was between 161 to 167 amino acids long.*

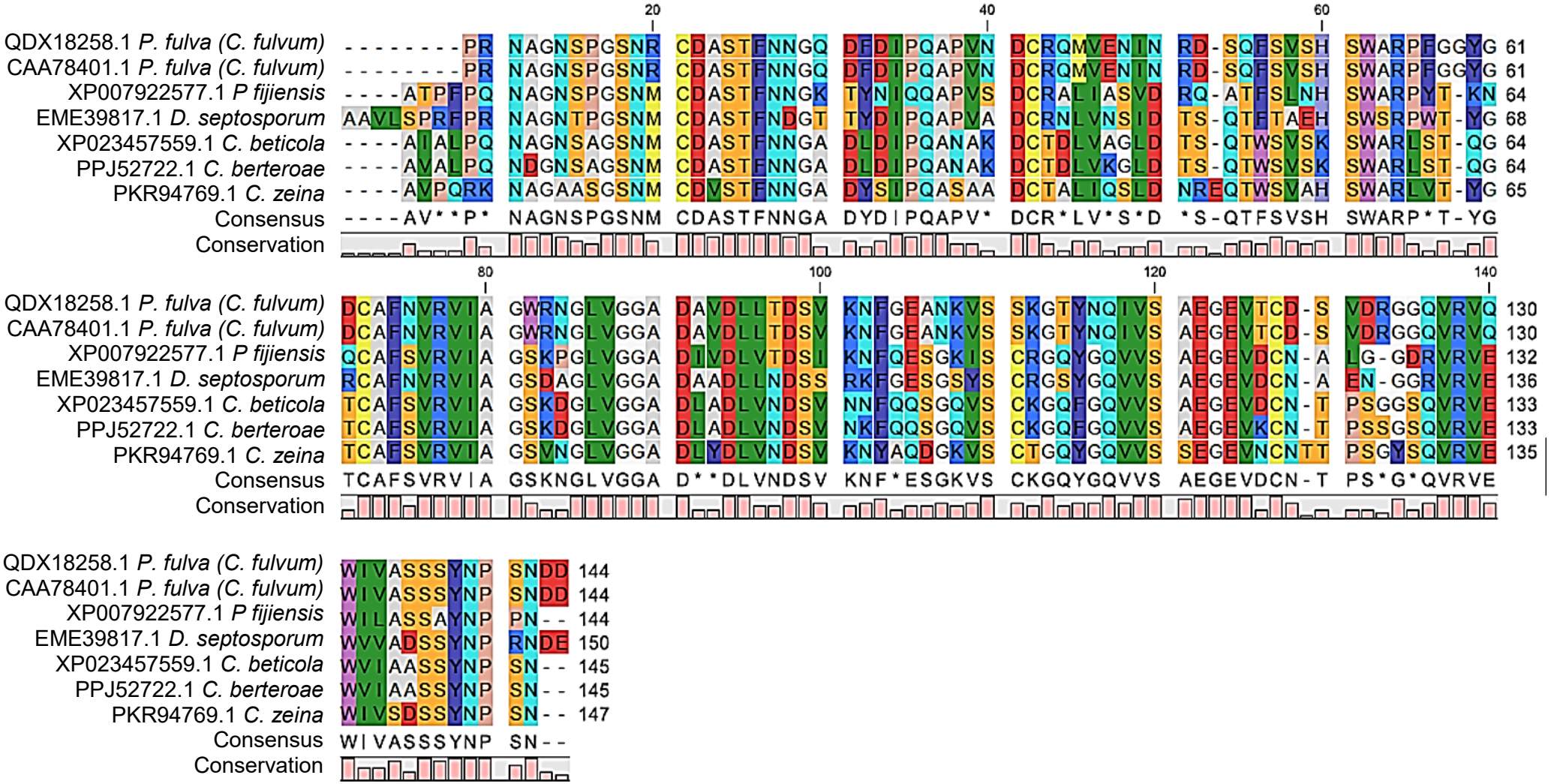
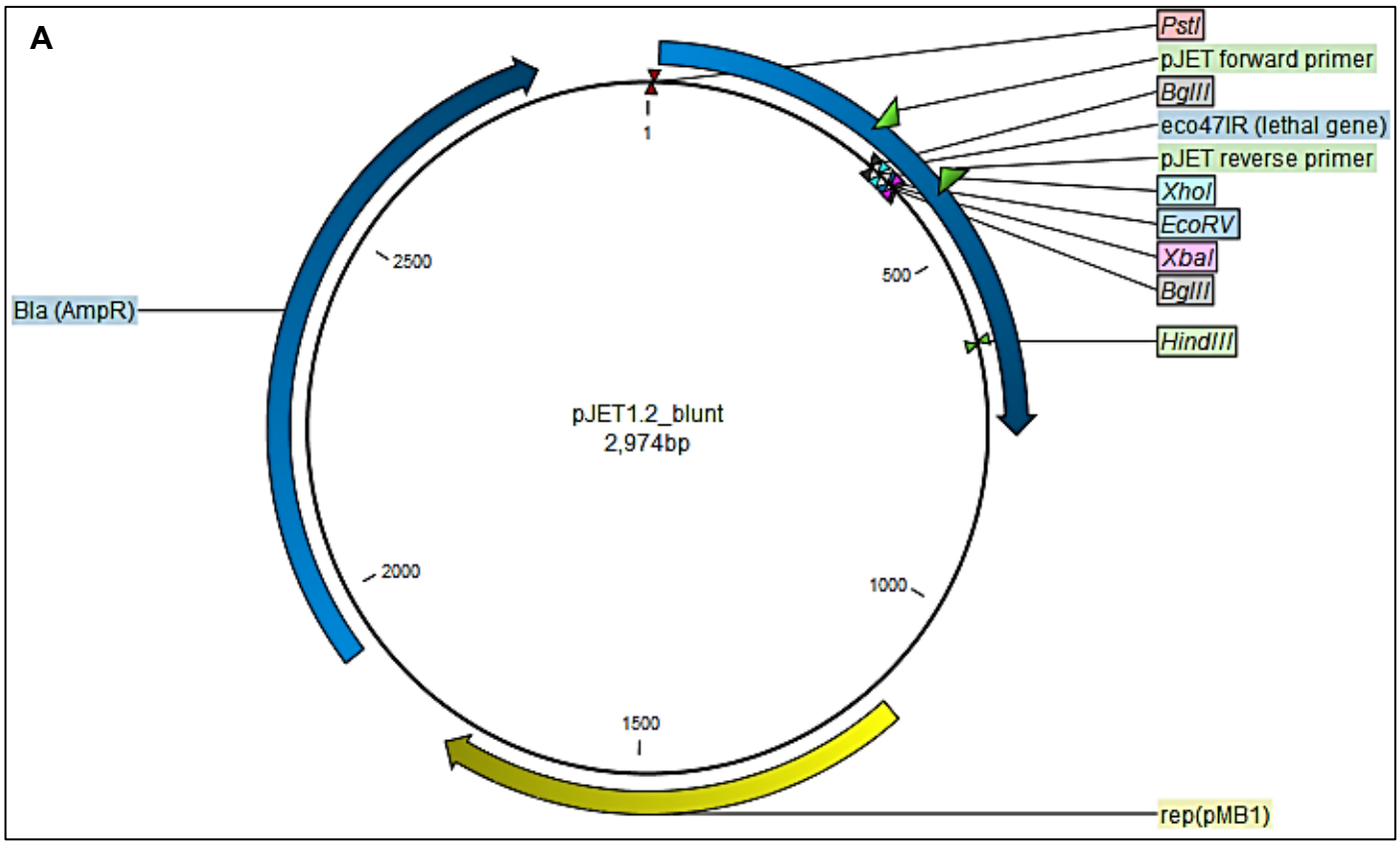


Figure S3: Alignment of putative ECP2 mature peptides from selected Dothideomycete fungi against putative C. zeina ECP2 using ClustalW. *The mature peptides (no signal peptides) of Dothideomycete fungal accessions from NCBI were obtained using SignalP. They were aligned against putative C. zeina ECP2 using ClustalW. They represent the part of the amino acid sequence that is translated into ECP2 and transported to the apoplast. The peptides ranged from 144 to 150 amino acids in length. Alignments were based on the BLOSUM62 matrix. Conservation was observed where amino acid residues matched 100% between all accessions (full bars). The consensus sequence showed the most common residues. All * symbols represented ambiguous bases (midi bars). All – symbols represented the lack of a residue in some peptides at a given position (extremely flat bars).*

Table S1: RNA sequence data analysis of C. zeina gene expression under different in vitro and in planta conditions. Expression of putative CzEcp2 was compared to that of a housekeeping gene (GAPDH) and C. zeina CTB1.

Annotation & Accession [1]	Gene model (NCBI) [2]	Read Counts							
		In planta	In vitro						
		Maize	YPD	V8 agar	PDA broth (pH3)	PDA agar (pH8)	PDA-AP (with nitrogen)	Cornmeal agar	Complete media
CzECP2 PKR94769.1	*Czeina239g000040*	2 740	23	207	87	418	7 805	10 451	23 596
CTB1 PKR98448.1	*Czeina53g000880*	98	1 104	835	639	1 828	1 645	1 834	1 930
GAPDH PKR98223.1	*Czeina49g000150*	3 856	33 204	94 341	126 936	71 793	27 310	51 861	71 193
Median [3]		63	544	511	479	594	610	672	722
Read count for selected gene/Median read count for all C. zeina genes [4]	*CzECP2*	43.3	0.04	0.4	0.2	0.7	12.8	15.6	32.7
	CTB1	1.5	2	1.6	1.3	3.1	44.8	2.7	2.7
	GAPDH	60.9	61	184.6	265	122	2.7	77.2	98.6

1 The annotated gene name and the NCBI protein accession matched by each transcript read from the RNA sequence data.
2 The gene model name in the C. zeina genome sequence matched by the RNA sequence transcripts. Gene models represent a hypothesis that a gene is present on a contig in a genome.
3 The median gives the middle value of a dataset. It was calculated as the median value for all C. zeina genes expressed for each specific treatment.
4 The number of read counts for specific genes (CzEcp2, CTB1 or GAPDH) for each treatment was divided by the median values of all genes obtained for that treatment.



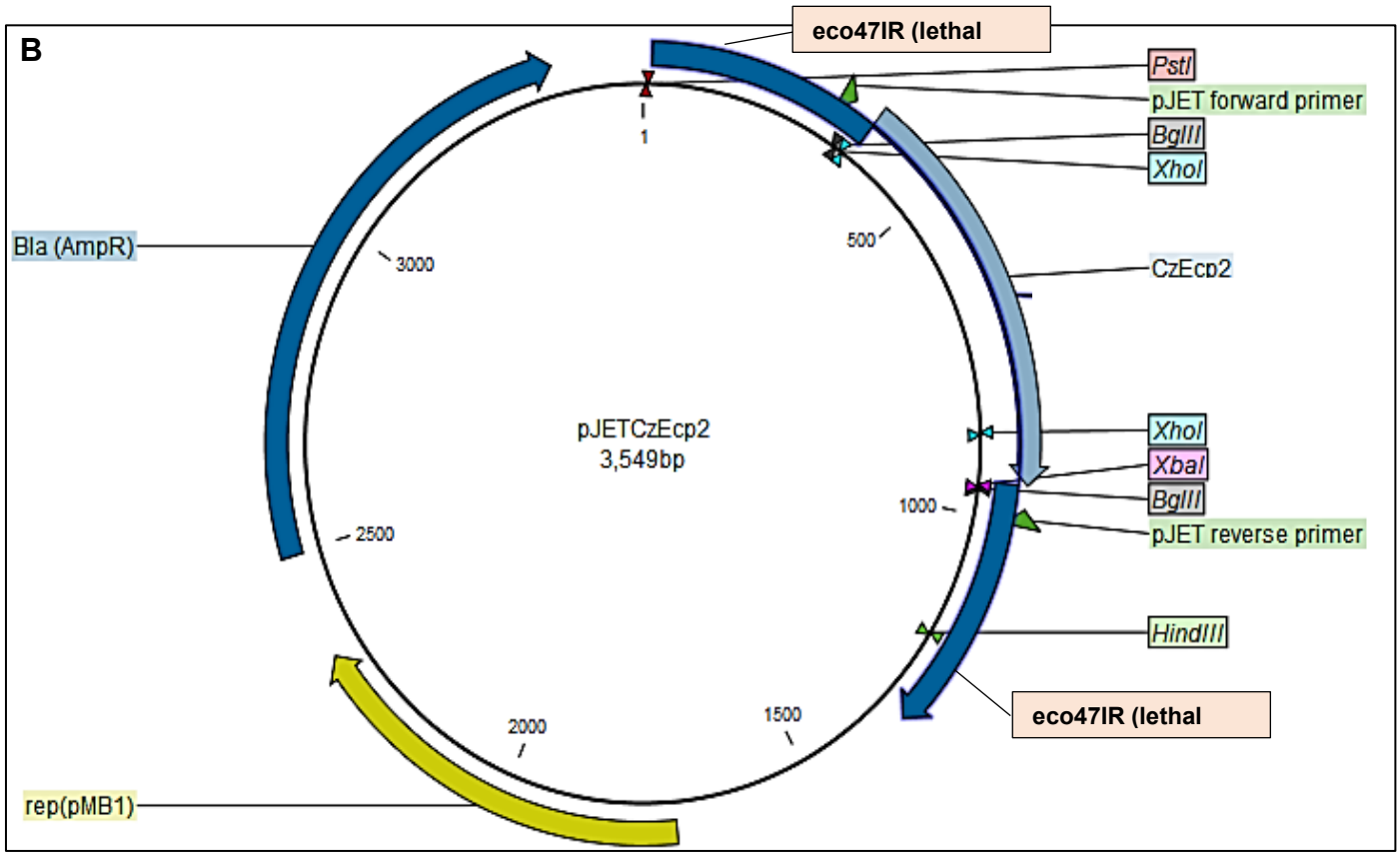


Figure S4: Maps of the PCR cloning vector pJET1.2/blunt with and without C. zeina Ecp2 (pJETCzEcp2). *A) The pJET1.2/blunt PCR vector (Thermo Fisher Scientific) has an eco47RI lethal gene for positive selection of bacterial clones carrying the recombinant vector. Successful cloning into the multiple cloning site disrupts the lethal gene resulting in bacterial growth on ampicillin selection. B) CzEcp2 was cloned into pJET1.2/blunt to obtain the full sequence and replicate the gene to a high copy number in E. coli DH5α cells.*

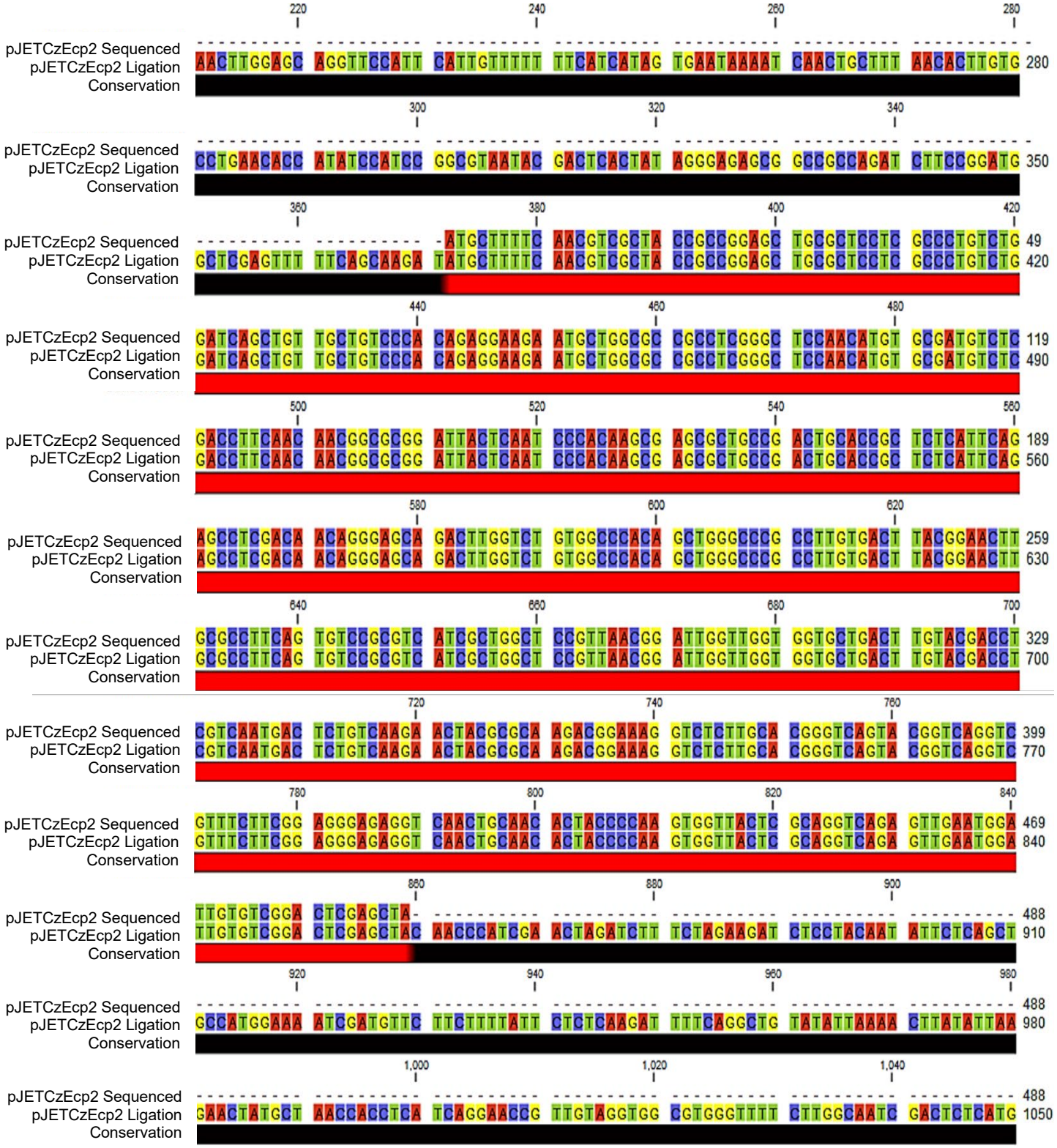


Figure S5: Alignment of sequenced pJETCzEcp2 against the in-silico product (pJETCzEcp2 ligation) using CLC Bio Main Workbench. *pJETCzEcp2 was Sanger sequenced using backbone specific primers to determine the presence of CzEcp2. The product (pJETCzEcp2 Sequenced) was aligned against the in silico recombinant vector (pJETCzEcp2 Ligation, 3 478 bp product). The red bars indicated part of CzEcp2 (488 bp) and 100% conservation of nucleotides. They show where CzEcp2 (originally 575 bp) ligated into the pJET1.2/blunt vector (Thermo Fisher Scientific). The shorter CzEcp2 sequence length was obtained due to poor sequencing coverage. The black bars show the remainder of the in silico recombinant vector sequence (no nucleotide matched to sequenced product, therefore no conservation). The colours on each sequence represent a respective nucleotide: cytosine (blue); thymine (green); guanine (yellow) and adenine (red).*

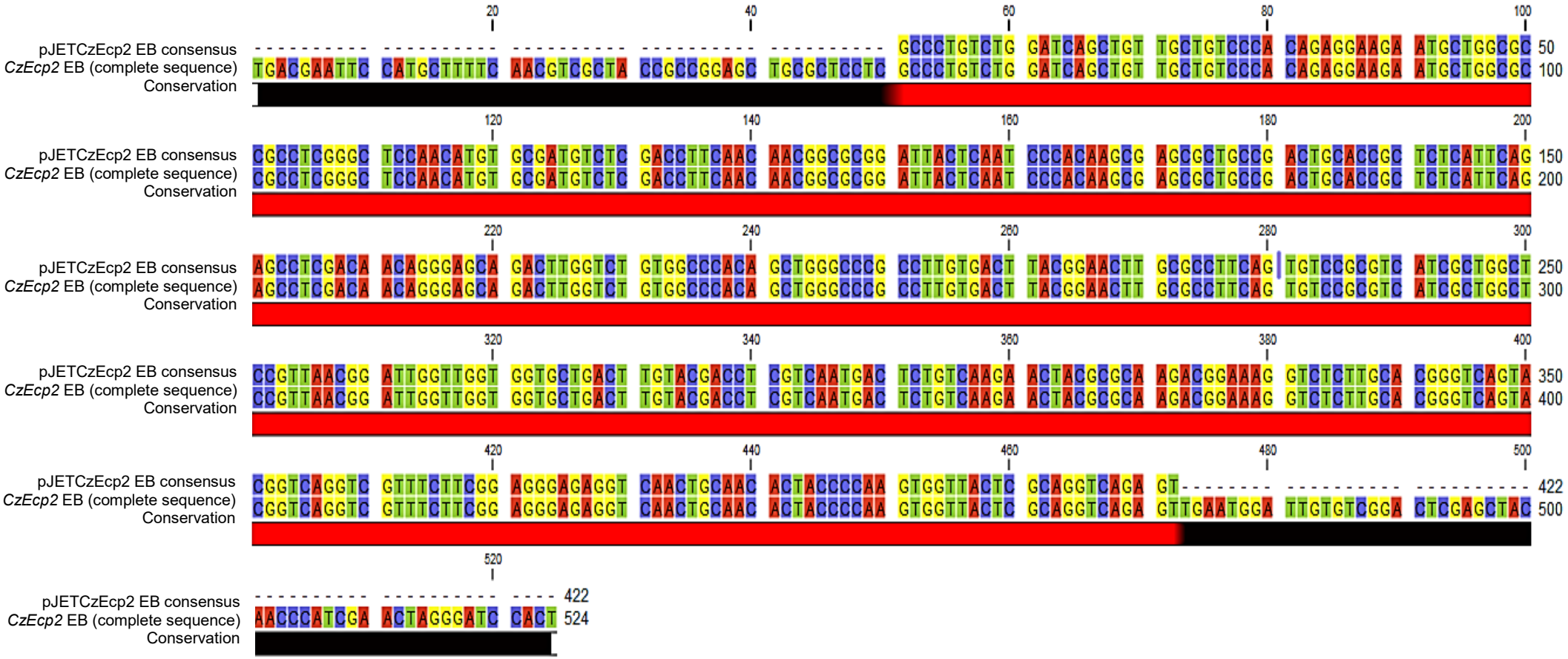


Figure S6: Alignment of Sanger sequenced CzEcp2 EB (fungal peptide) against an in-silico reference CzEcp2 EB (full sequence) using CLC Bio Main Workbench. *pJETCzEcp2 was Sanger sequenced using cloning primers to determine if EcoRI and BamHI sites were added to the 5' and 3' ends of CzEcp2 EB (signal peptide). The alignment of pJETCzEcp2 EB consensus (sequenced product) against the reference CzEcp2 EB (full sequence) showed conservation (red bars) for most of the CzEcp2 EB length. The black bars meant no match between the nucleotides. Sequenced CzEcp2 EB (422 bp) was shorter than the expected 524 bp due to sequence coverage. The different colours on each sequence represented a respective nucleotide: cytosine (blue); thymine (green); guanine (yellow) and adenine (red). Alignment was done with ClustalW and imported to CLC Bio Main Workbench for editing.*

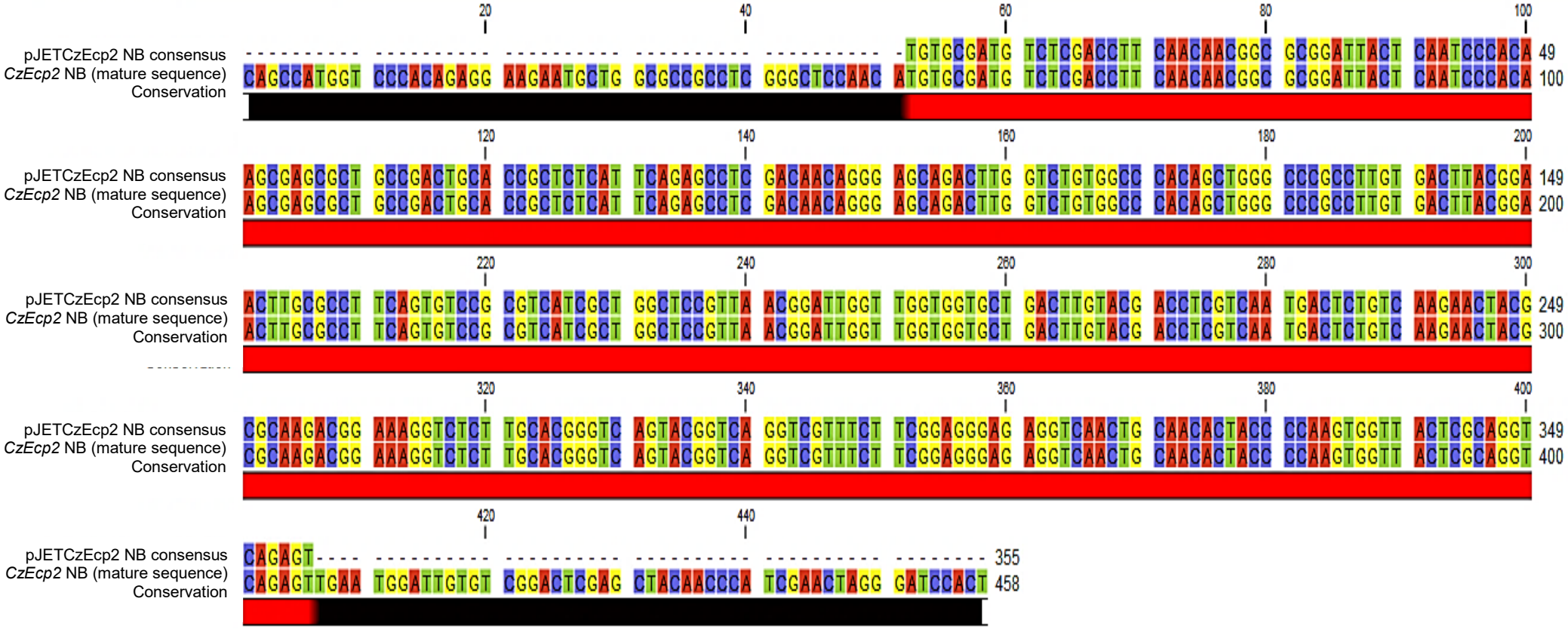


Figure S7: Alignment of Sanger sequenced CzEcp2 NB (LPH signal peptide) against an in-silico reference CzEcp2 NB (mature sequence) using CLC Bio Main Workbench. *pJETCzEcp2 was Sanger sequenced using cloning primers to determine if NcoI and BamHI sites were added to the 5' and 3' ends of CzEcp2 NB (LPH signal peptide). Alignment of pJETCzEcp2 NB consensus (sequenced product) against the reference CzEcp2 NB (mature sequence) showed conservation (red bars) for most of the CzEcp2 NB length (355 bp). The black bars meant no match between the nucleotides. Sequenced CzEcp2 NB was shorter than the expected 458 bp due to sequence coverage. The different colours on each sequence represented a respective nucleotide: cytosine (blue); thymine (green); guanine (yellow) and adenine (red). Alignment was done with ClustalW and imported to CLC Bio Main Workbench for editing*

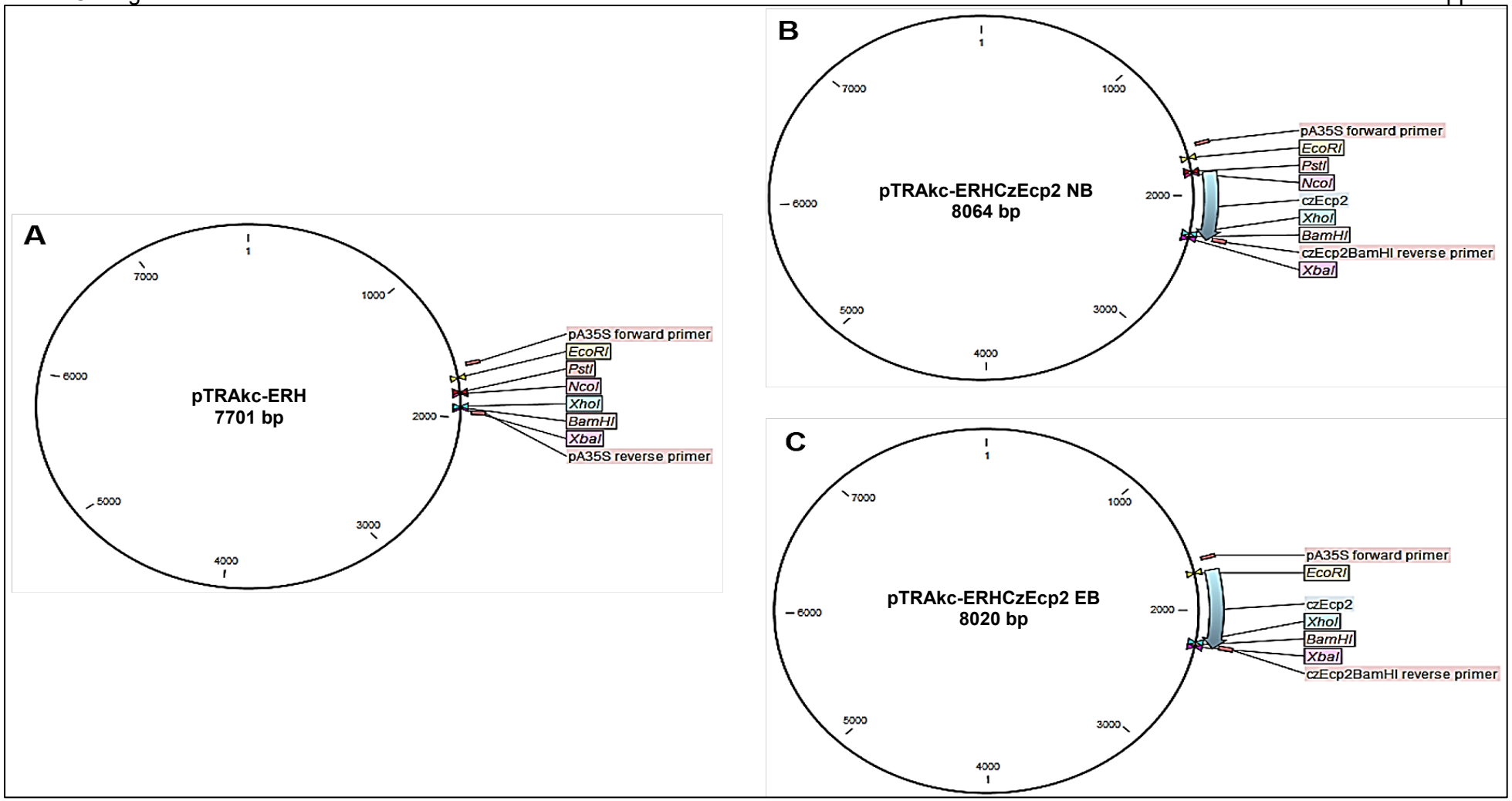


Figure S8: Plasmid maps of the binary vector pTRAkc-ERH and the recombinant binary vectors carrying C. zeina Ecp2 (CzEcp2) with and without its signal peptide. *pTRAkc-ERH was the binary vector used for Agrobacterium-mediated transformation of Nicotiana spp. (Maclean et al. 2007). For expression of CzEcp2 EB (524 bp, with signal peptide, 5' EcoRI and 3' BamHI) and CzEcp2 NB (458 bp, LPH signal peptide, 5' NcoI and 3' BamHI), the respective genes were cloned into pTRAkc-ERH. pTRAkc-ERHCzEcp2 EB and pTRAkc-ERHCzEcp2 NB were respectively transformed into Agrobacterium tumefaciens GV3101 pSOUP+PMP90 (obtained from JHI, Dundee, Scotland). The XhoI site visible before BamHI in A) and B) is present in CzEcp2, not the vector.*

Maclean J, Koekemoer M, Olivier A, Stewart D, Hitzeroth I, Rademacher T, Fischer R, Williamson A-L, Rybicki E (2007) Optimization of human papillomavirus type 16 (HPV-16) L1 expression in plants: comparison of the suitability of different HPV-16 L1 gene variants and different cell-compartment localization. Journal of General Virology 88 (5):1460-1469.

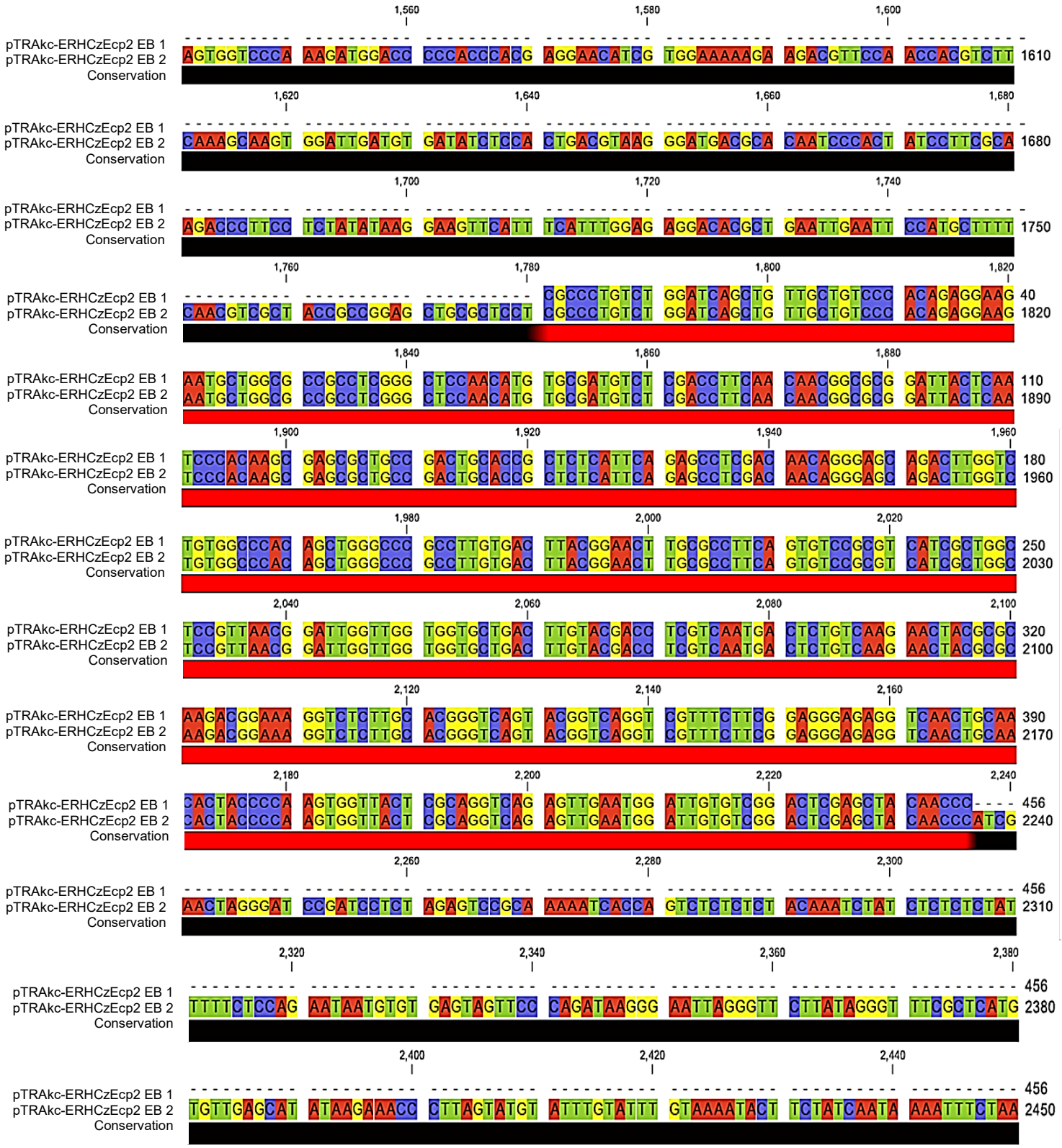


Figure S9: Sanger sequenced pTRAkc-ERHCzEcp2 EB (signal peptide) alignment against in-silico pTRAkc-ERHCzEcp2 EB. *pTRAkc-ERHCzEcp2 EB was Sanger sequenced using a vector-specific forward and insert-specific reverse primer. The product obtained (pTRAkc-ERHCzEcp2 EB 1) was aligned against an in-silico recombinant vector (pTRAkc-ERHCzEcp2 EB 2). The red bars represent 100% conservation between nucleotides including CzEcp2 EB. Where the sequences did not match, the bars were black. The different colours on each sequence represent a respective nucleotide: cytosine (blue); thymine (green); guanine (yellow) and adenine (red). The shorter sequence product (pTRAkc-ERHczEcp2_EB_1) obtained (456 bp instead of 612 bp) was due to the extent of sequencing coverage.*

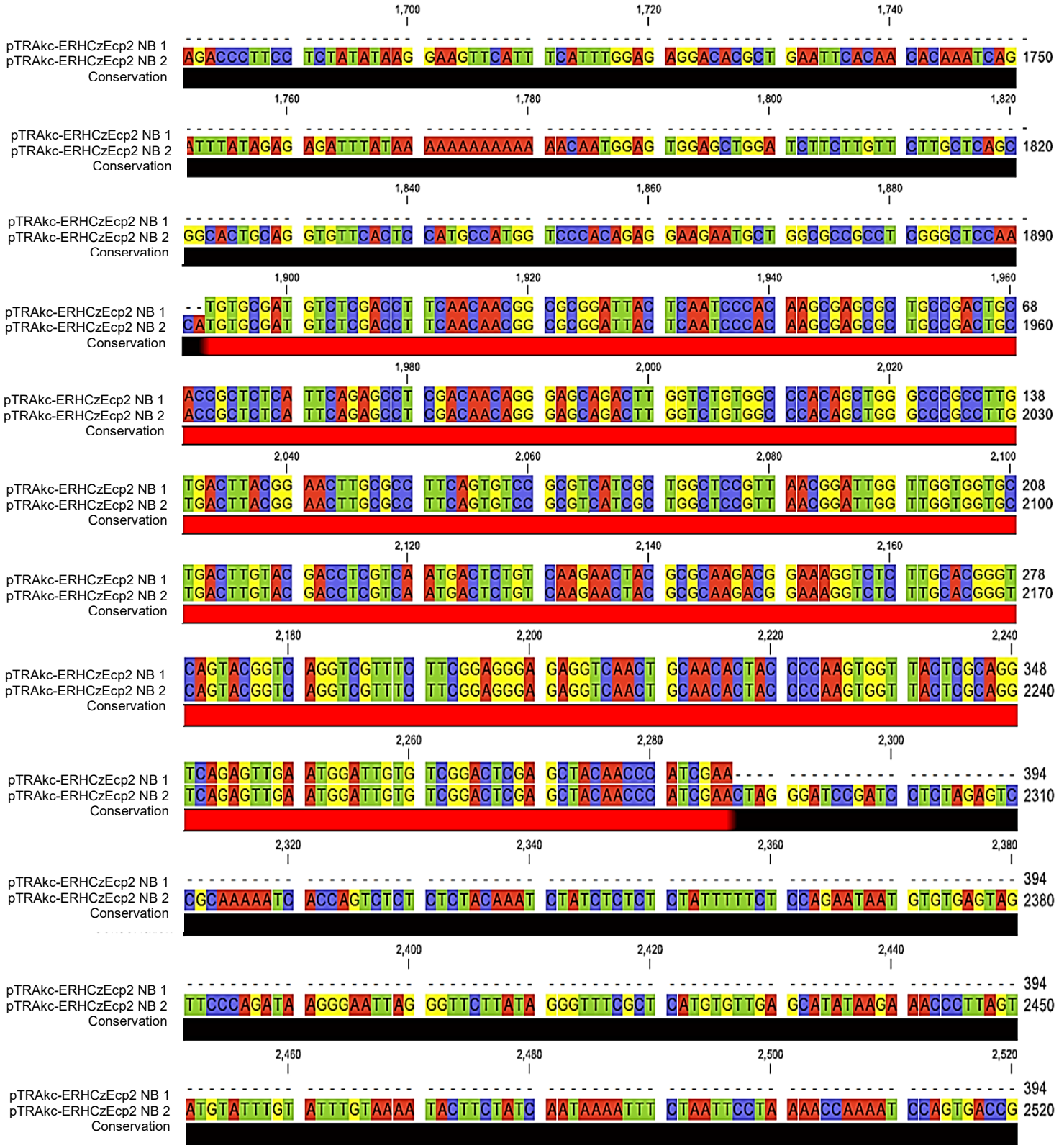


Figure S10: Sanger sequenced pTRAkc-ERHCzEcp2 NB (LPH signal peptide) alignment against in-silico pTRAkc-ERHCzEcp2 NB. *pTRAkc-ERHCzEcp2 NB was Sanger sequenced using a vector-specific forward and insert-specific reverse primer. The product obtained (pTRAkc-ERHCzEcp2 NB 1) was aligned against an in-silico recombinant vector (pTRAkc-ERHCzEcp2 NB 2). The red bars represent 100% conservation between nucleotides including CzEcp2 NB. Where the sequences did not match, the bars were black. The different colours on each sequence represent a respective nucleotide: cytosine (blue); thymine (green); guanine (yellow) and adenine (red). The shorter sequence product (pTRAkc-ERHczEcp2_NB_1) obtained (394 bp instead of 656 bp) was due to the extent of sequencing coverage.*

Summary

Cloning the *Cercospora zeina Ecp2* effector gene for *Agrobacterium*-mediated transient transformation

by

Carol-Ann Crystal Segal

Grey leaf spot (GLS) is a devastating foliar disease of maize. In South Africa, Zimbabwe, China and Brazil the only known causal agent of the disease is *Cercospora zeina*. The infection strategy of *C. zeina* is unknown and at present the best control measures include resistant hybrids and fungicide application. *C. zeina* has been shown to not produce cercosporin, the toxin that its sister species *Cercospora zeae-maydis* uses to produce the exact disease symptoms in maize. It is well known that *C. zeina* functions in the apoplastic space of leaves, however, the molecular mechanisms underlying its disease-causing abilities are still unknown. As studies involving molecular plant-pathogen interactions increase, more research must be invested into understanding how *C. zeina* interacts with its host and which proteins it secretes during the process.

Many pathogens have been shown to use effectors for virulence during host invasion. Effectors are proteins that have specific effects on host and nonhost plants. Some well-studied effectors such as *Avr4, Ecp2* and *Ecp6* are cysteine rich and are regarded as small secreted proteins. They have been shown to play distinct roles that are essential for pathogen virulence. The first aim of this study was to confirm the presence of the extracellular protein 2 (*Ecp2*) effector gene in the *C. zeina* genome. The second aim was to clone the effector into a binary vector for *Agrobacterium*-mediated transient infiltration of *Nicotiana* spp. The hypothesis of this study was that *CzEcp2* will cause a hypersensitive response in nonhost *Nicotiana* spp. *CzEcp2* was found within the *C. zeina* genome and transcriptome and cloned into the pTRAkc-ERH binary vector with and without its signal peptide sequence. The recombinant vectors, pTRAkc-ERHCzEcp2 EB (fungal signal peptide) and pTRAkc-ERHCzEcp2 NB (LPH signal peptide) were transformed into *Agrobacterium tumefaciens* GV3101 pSOUP+pMP90.

The constructs were screened for the presence of *CzEcp2* with and without the signal peptide using colony PCR and Sanger sequencing. The agarose gel showing the amplicons for pTRAkc-ERHCzEcp2 EB amplified with a vector-specific forward and insert-specific reverse primers showed

the products of interest (612 bp). This result confirmed that *CzEcp2* was within the construct and carrying the signal peptide. The colony PCR results for pTRAkc-ERHCzEcp2 NB amplified with the same primers did not yield any bands on the agarose gel. This was likely due to the absence of the gene, a lack of primer binding sites, rearrangement of the sequences in the backbone or human error. This construct was therefore not used for agroinfiltration of the plants in this study. The *CzEcp2* gene was cloned into the binary vector without its signal peptide to determine if it could be expressed when fused to a plant signal peptide. Therefore, in future this construct will have to be recreated to prove this hypothesis.

Transient agroinfiltrations were carried out on *Nicotiana benthamiana, Nicotiana tabacum* cv. Petit Havana and *Nicotiana tabacum* cv. LA Burley. The infiltration controls included the wildtype *Agrobacterium* strain and the *Agrobacterium* strain transformed with the empty vector, pTRAkc-ERH. The positive control to which *CzEcp2* expression was compared was *Phytophthora infestans* INF1. This gene was also in a vector in the same bacterial strain. The pSOUP vector was retained for consistency throughout the study and was not required for *CzEcp2* expression. Therefore, to ensure that the reactions seen in the plants were not due to the bacteria or the vector, all conditions were kept consistent except for the presence of the *CzEcp2* gene.

In *N. benthamiana*, all the replicates expressed a hypersensitive response (HR) where INF1 was infiltrated. This was an indication that the INF1 protein was recognised by the plant resistance (R) proteins. The plant was therefore protecting itself against the pathogenic protein. It is likely however that the plant perceived INF1 as a pathogen-associated molecular pattern and therefore caused a basal defence response. In all the *N. tabacum* replicates, INF1 expression resulted in chlorosis and necrotic spots in some. This is an indication that the plant may not have perceived INF1 as pathogenic and therefore avoided causing severe HR. In all the plant replicates, *CzEcp2* did not cause a HR which was unexpected due to its homology with *Cladosporium fulvum Ecp2* known to cause HR in nonhost plants. In some of the replicates, chlorosis was instead observed. This may have indicated gene expression and therefore protein recognition by the plants. The HR may also have been delayed given that CzECP2 is an apoplastic effector, therefore in future plants should be monitored for longer than 10 days. Where the vector and bacteria controls were infiltrated, no changes were seen as expected.

In conclusion, *Ecp2* is present in the *C. zeina* genome and can be cloned into a binary vector for agroinfiltration studies. This effector gene may have a role in pathogenicity of the fungus, but more studies are required to prove this definitively. Given that this effector was studied, more effectors can be discovered and functionally characterised to determine what role they play in *C. zeina* pathogenicity. This study therefore created a platform towards elucidation of the fungus infection strategy.