Identification and Characterisation of Effector Proteins from *Zymoseptoria tritici*

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STATEMENT OF ORIGINAL AUTHORSHIP

I hereby certify that the submitted work is my own work, was completed while registered as a candidate for the degree stated on the Title Page, and I have not obtained a degree elsewhere on the basis of the research presented in this submitted work.

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Summary

Plant pathogens are known to secrete a large number of secreted proteins termed as effectors into the host plant. These secreted effector proteins play role in the infection and manipulation of plant host defenses for aiding successful colonisation. The filamentous fungal pathogen *Zymoseptoria tritici:* an important pathogen of wheat, also secretes effectors that play key role during host colonisation.

In this thesis, 50 *Zymoseptoria tritici* small secreted proteins (*ZtSSPs*) were identified which fulfilled all the effector characteristics including; (i) small size, (ii) presence of signal peptide, (iii) presence of cysteine residues, (iv) lack of transmembrane domain and (v) lack of functionally annotated domains. Out of these 50, 30 were cloned and characterised initially based on their ability to induce cell death *in planta* using a non-host plant. Five novel candidates that induce cell death in a non-host plant were selected and this cell death was shown to be independent of presence a signal peptide. All five *ZtSSPs* were also involved in activation of diverse defense marker genes and were found to be differentially upregulated during infection suggesting their diverse roles.

One particular *ZtSSP*, *ZtSSP2* is a well conserved effector across isolates and interacts with a wheat host ubiquitin protein. This wheat ubiquitin possess a RING finger E3 ligase domain and plays a key role in ubiquitin mediated cellular processes. The expression of wheat ubiquitin ligase showed that its expression is downregulated at early and late stages of *Z. tritici* infection, suggesting involvement of this ubiquitin in host defense responses. To explore different system for effector characterisation the grass *B. distachyon* was used as a non-host model to study non-host defense and the potential for *Z. tritici* effector screening.

In conclusion secreted effectors of *Z. tritici* play a key role in host defense manipulation. This study on *Z. tritici* candidate effectors has provided the identification of a wheat host effector target and further insights into the plant-pathogen interaction between *Z. tritici*, host plant wheat as well as with the non-hosts *N. benthamiana* and *B. distachyon*.

Abbreviation

ANOVA	Analysis of Variance
BiFc	Bimolecular Fluorescence Complementation
BLAST	Basic Local Alignment Search Tool
cDNA	Coding DNA
cv	Cultivar
CWDEs	Cell wall degrading enzymes
DAB	3,3'- diaminobenzadine
DAMPs	Damage Associated Molecular patterns
DNA	Deoxyribonucleic acid
DPI	Days post inoculation/infection
EST	Expressed sequence Tag
ETI	Effector Triggered Immunity
HR	Hypersensitive Response
IDR	Intrinsic Disordered Regions
JA	Jasmonic Acid
JGI	Joint Genome Institute
LB	Luria Bertani
MAMP	Microbe Associated Molecular Pattern
MAPK	Mitogen Activated Protein Kinase
Mb	Mega base
MEGA	Molecular Evolutionary Genetics Analysis
NB-LRR	Nucleotide Binding Domain and Leucine rich repeats
NCBI	National Centre for Biotechnology Information
OD	Optical Density
PAL	Phenylalanine ammonia lyase
PAMP	Pathogen Associated Molecular Pattern
PCD	Programmed Cell Death
PCR	Polymerase Chain Reaction
PDA	Potato Dextrose Agar
pEG101	pEarlyGate 101
PR	Pathogenesis Related
PRR	Pattern Recognition Receptor
PTI	PAMP triggered Immunity
qRT-PCR	Quantitative Real Time PCR
RNA	Ribonucleic acid

RNAseq	Next generation sequencing of RNA
ROS	Reactive Oxygen Species
RT-PCR	Reverse transcriptase PCR
SA	Salicylic Acid
SNP	Single Nucleotide Polymorphism
SOD	Superoxide Dismutase
YFP	Yellow Fluorescent Protein

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Chapter 1

Zymoseptoria tritici, an Economically Important Fungal Pathogen of Wheat

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Chapter 1
Introduction

World population and food security is one of the biggest concerns worldwide. The world population is predicted to reach almost 10 billion by 2050 (United Nations, Department of Economic and Social Affairs) and this will likely impose a significant pressure on world food production globally. In addition to that, finite resources including fertile land, water and an ever-changing climate also play a part in this battle for food production and accessibility worldwide.

Wheat is one of the most intensely produced cereals worldwide and a key player in the global cereal market. It accounts for about 21% of the food calories and 20% of the protein intake for 4.5 billion people (Braun et al., 2010). With the unprecedented increase in yields achieved through green revolution, wheat has contributed to feed millions because of which it is of high importance to human's worldwide (Shiferaw et al. 2013).

Crop losses due to pathogens, accounts for up to 16% globally (Oerke 2006). Globally, the most damaging fungal diseases of wheat include Fusarium head blight (caused by *F. graminearum*), powdery mildew (caused by *Blumeria graminis*), STB (caused by *Z. tritici*) and stem rust (caused by *Puccinia graminis*) (Dean et al. 2012).Therefore, in order to ensure future food security and increase wheat yields, it is necessary to have reduced wheat yield losses to fungal diseases.

1.1 *Zymoseptoria tritici*: an economically important pathogen of wheat

Zymoseptoria tritici is the causal agent of Septoria tritici blotch (STB), an ascomycete fungus that belongs to the Mycosphaerella genus within the Dothideomycetes family. It was previously known as *Mycosphaerella graminicola* (Fuckel) Schröter in Cohn. *Z. tritici* is the teleomorph of *Septoria tritici* Roberge in Desmaz (Orton et al. 2011). The genome of *Z. tritici*, IPO323 is 39.7Mb in size, of which contains approximately 11000 predicted genes dispersed over 21 chromosomes (Goodwin et al. 2011). Chromosome 1 to 13 are known to be core chromosomes, while the remaining smallest 8 chromosomes are referred to as accessory chromosomes and harbour 646 genes most of whom have relatively low levels of expression during infection (Kellner et al. 2014; Rudd et al. 2015).

STB is characterized by the presence of necrotic lesions on the surface of wheat leaves (Fig 6). These lesion bearing leaves have reduced net photosynthesis, which impacts on growth and

ultimately lowers yield. STB is highly prevalent in wheat growing areas, especially in areas with cool, wet weather such as Ireland. STB outbreaks can reduce wheat yields by up to 50% (Eyal et al. 1987, Fones & Gurr 2015). In Europe, €700 million worth of wheat yield is lost annually to STB (Fones & Gurr 2015; Orton et al. 2011). In addition, around €500 million is spent on fungicides to control STB outbreaks in Germany, France and the United Kingdom alone (Fones & Gurr 2015). The rise in fungicide resistant isolates of *Z. tritici*, (Hayes et al. 2016) it's rapidly evolving and diverse nature, presents a serious threat to global wheat production and compromises food security.

1.2 STB disease Cycle

Z. tritici reproduces both sexually and asexually. The sexual reproduction occurs between two mating types, *MAT1-1* and *MAT1-2* (Kema et al. 1996) and leads to the formation of ascospores. While asexual reproduction leads to pycnidiospores. Sexual reproduction facilitates the spread of new resistance traits over large geographical areas rapidly while the asexual reproductive cycle allows *Z. tritici* to propagate acquired resistant traits (Orton et al. 2011). This ability of *Z. tritici* for rapid gene flow and high genetic diversity makes it a successful pathogen of wheat and it is highly challenging to acquire durable disease control against it (Talbot 2015).

1.2.1 Disease initiation:

Wind-blown ascospores produced in pseudothecia arise from infected crop debris which act as a primary source of infection. Local secondary infections primarily originate from the rain splashed pycnidiospores during the growing season, which are disseminated predominantly by rain splash. The infection occurs mainly in cool and wet weather with optimum temperatures being 16 to 21°C. (Eyal et al. 1987) (Fig. 1).

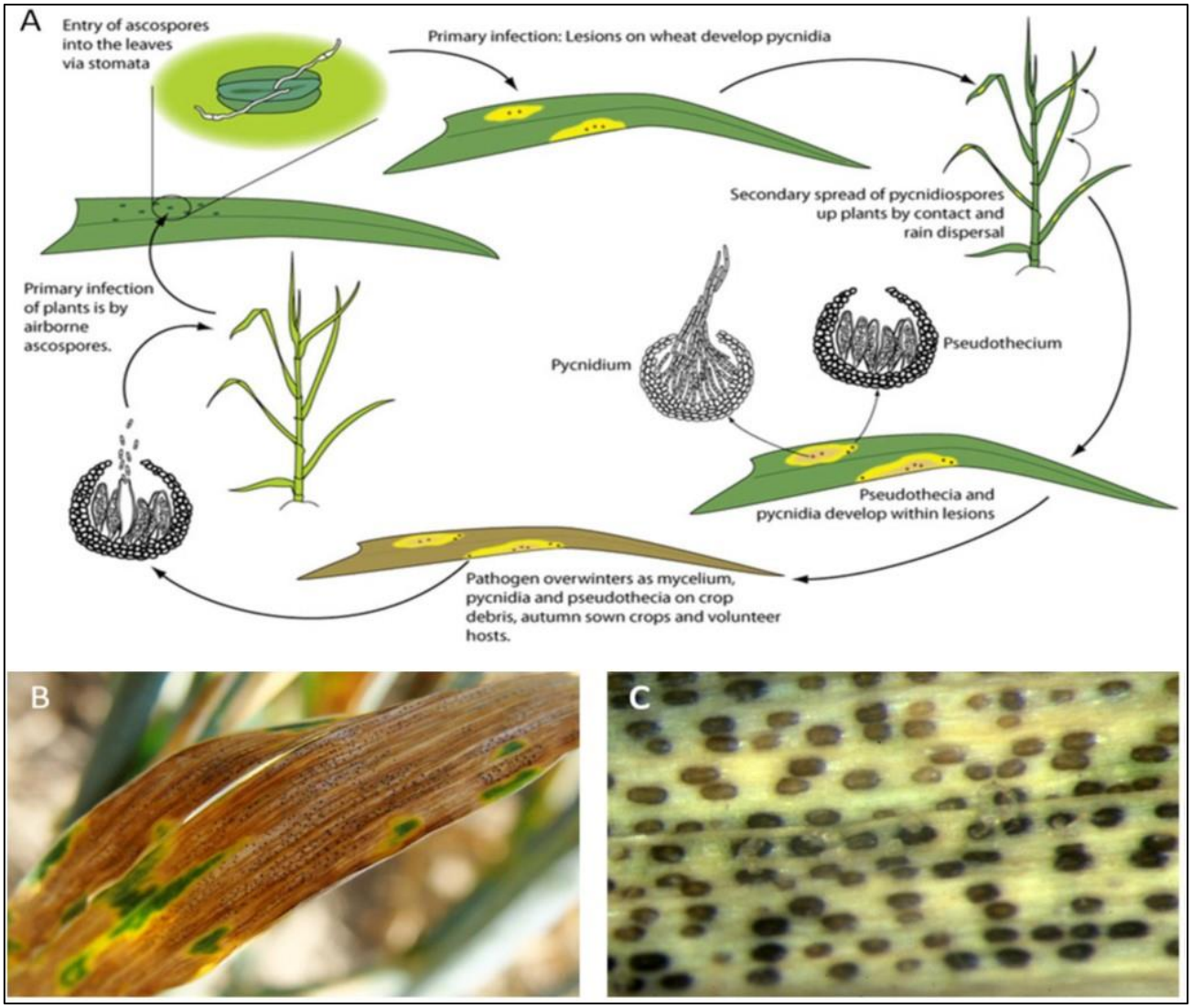

Figure 1. *Z. tritici* disease cycle and symptoms of STB in wheat leaves. (A) Life cycle of the fungal wheat pathogen *Zymoseptoria tritici.* The primary infection starts with rain splashed pycnidiospores or airborne ascospores that enters the leaves via stomata followed by an asymptomatic phase of very slow hyphal growth in the apoplast. After approx. 14 days the fungus switches to necrotrophic growth which results in lesions. Asexual fruiting bodies (pycnidia) develop within these lesions and form asexual pycnidiospores. In the end of the growing season sexual fruiting bodies known as pseudothecia are produced within the lesions which produce the sexual ascospores. B) Symptoms of *Z. tritici* on bread wheat C) Magnification of a *Z. tritici* infected wheat leaf. The pycnidia are spaced regularly within the stomata of the plant.

Adapted from Ponomarenko et al. 2011

The molecular mechanism behind the adherence of *Z. tritici* on to wheat leaves still remains elusive however genetic and transcriptional data suggests that the *Z. tritici* genome encodes class II hydrophobins coding genes, namely *Mycgr3G48129, Mycgr3G40724 Mycgr3G96543* and *Mycgr3G95491* and their level of expression is upregulated at the initial stage of infection (Rudd et al. 2015). Hydrophobins are known to play a role in attachment of fungal hyphae to hydrophobic surfaces (Wosten et al.1994; Linder 2005). The function of hydrophobins is based on its ability to self-assemble at the interface between the hydrophilic cell wall and hydrophobic surface of the host forming an amphipathic membrane (Wosten 2001). Similarly, the hydrophobin gene *Mpg1* in *Magnaporthe grisae* was found to be essential for infection related development and pathogenicity (Talbot et al. 1996). It is possible that the hydrophobins also plays a key role in *Z. tritici* adherence to leaf surfaces.

1.2.2 Host Penetration:

Primary infection starts with infection hyphae germinating from ascospores (12-24hrs after contact) that enter the leaves via the stomata. To date, there is no record of host penetration through the use of specialized structures like haustoria or appressoria. Infection is then followed by a long latent asymptomatic phase (biotrophic phase) which could last up to two weeks before any visual symptoms occur. Recent reports on *Z. tritici* having a long epiphytic growth on leaves, rather than penetration for as long as 10 days was also reported adding to the uncertainty regarding the latent phase (Fones et al. 2017).

It is evident from various plant-pathogen interactions that host penetration by the pathogen involves secretion of plant cell wall degrading enzymes (PCWDEs). Higher expression of PCWDEs in host cells allows cell destruction and also acts as a nutritious supply for the pathogen. Comparative genome analyses of *Z. tritici* with *Stagonospora nodorum* and *Magnaporthe oryzae* showed 28 genes that encode for cell wall degrading enzymes (CWDEs) which tends to be smaller compared to other plant pathogens (Goodwin et al. 2011). Expression profiling of these genes revealed that they are differentially expressed throughout the *Z. tritici* lifestyle. Low levels of expression of these genes are observed in the asymptomatic phase while the majority are expressed in the transition between asymptomatic phase to symptomatic phase or later (Brunner et al. 2013; Rudd et al. 2015). It is believed that this delicate balance of CWDEs expression is achieved by *Z. tritici* in order to avoid the recognition and activation of the wheat PTI response (Rudd et al. 2015).

1.2.3 Host colonisation:

After penetration through stomata, the fungal hyphae remain strictly apoplastic and continue extracellular growth with a relatively small increase in fungal biomass. This latent phase is usually 8 to 14 days where there are no disease symptoms. It is hypothesized that during this latent period the fungus feeds on its stored lipids and fatty acids which provides energy for growth. A study by Rudd et al. 2015 demonstrated that *Z. tritici* genes encoding for enzymes involved in β-oxidation of fatty acids and lipids are upregulated. This results in the use of lipids as an initial source of energy to feed the glyoxylate cycle and supports for slow colonization of wheat leaves. Studies have shown the importance of the β-oxidation of fatty acids and the glyoxylate cycle in other pathogenic fungi and therefore it is believed that stored lipid reserves act as an important source of energy during the early stage of host colonisation (Solomon et al. 2004, Wang et al. 2007).

1.3 Effector paradigm in *Z. tritici*-wheat interaction

The asymptomatic phase is followed by the more aggressive symptomatic or necrotrophic phase. The molecular mechanism behind this sudden change in the lifestyle still remains elusive. This phase is accompanied by the appearance of necrotic lesions on the leaf surface (8 to 14 days post infection (dpi)) which is followed by the formation of black pycnidia within the lesions at around 21 to 28 days post infection. The transition to disease symptoms features a very high level of expression of transcripts encoding secreted proteins with 35% of these proteins having no functional annotations (Rudd et al. 2015).

Z. tritici has been predicted to produce a high number of small secreted proteins throughout its interaction with wheat (Morais do Amaral et al. 2012), however only a handful of them have been functionally characterised. The functionally characterised effector proteins, *Mg3LysM* and *Mg1LysM* were also found to be upregulated at this transition phase which suggests that *Z. tritici* are able to suppress the chitin mediated plant defence response at the necrotrophic phase along with an increase in fungal chitin or biomass (Yang et al. 2013).

Another *Z. tritici* effector belongs to the Necrosis and Ethylene-Inducing Peptide 1 (NEP1)-like (NLP) family of proteins termed as MgNLP. It has been shown to induce cell death in *Arabidopsis*, but not in wheat (Motteram et al. 2009). In addition, Z. *tritici* secretes two necrosis inducing protein effectors (ZtNIP1 and ZtNIP2), which induce cell death and chlorosis but only in some wheat cultivars (Barek et al. 2015). However, little is known about the mechanism of cell death and how much this might contribute to virulence. The *Z. tritici* effector *AvrStb6* has

been shown to be involved in a gene-for-gene interaction with the *septoria tritici blotch* resistance wheat gene *Stb6*. Map based cloning of *Stb6* revelaed that this resistance gene encodes a wall associated receptor kinase (WAK) (Saintenac et al. 2018). Similar to other effector proteins, AvrStb6 is small, cysteine-rich and has a high degree of sequence polymorphism, which could potentially be an adaptation to avoid recognition by the host (Zhong et al. 2017). Interestingly, direct interaction of *Avrstb6* and *Stb6* has not been confirmed and further molecular understanding on this interaction could provide insights on novel disease resistance biology. Recently, another avirulence effector *Avr3D1* has been identified that is specifically recognised by wheat cultivars harbouring the *Stb7* resistance gene (Meile et al. 2018).

A number of the *Z. tritici* candidate effectors were found to induce a cell death phenotype when expressed in the non-host plant *Nicotina benthamaina* (Kettles et al. 2017). It was demonstrated that two receptor-like kinases, Brassinosteriod Insensitive 1 (BRI1)-Associated Receptor Kinase 1 *(*BAK1*)* and the Suppressor of BIR1-1 (SOBIR1), play a key role in the recognition of these effector candidates. One of the candidate effectors was subsequently identified as a novel fungal PAMP, termed Cell Death-Inducing 1 (*ZtCDI1*) (Franco-Orozco et al. 2017). Similarly, another candidate (*Zt-6*) was characterized as a secreted ribonuclease which possess a cytotoxic activity against microbes as well as plants (Kettles et al. 2018).

As the necrotrophic phase continues, the disease symptoms become more visible with an increase in fungal biomass and plants cell death at multiple sites of infected tissues. The increased fungal biomass causes the infected tissues to collapse with the fungi sporulating asexually. These asexual spores within the defeated host tissue acts as a secondary inoculum which upon rain splashed or wind dispersal initiates another STB disease cycle (Kema et al. 1996; Suffert et al. 2016).

1.4 Plant Immunity

Plants are under constant exposure to pathogens such as fungi, bacteria, oomycetes, viruses and nematodes. Unlike vertebrates that possess an adaptive immune system, plants rely solely on an innate immune system to recognize and respond to pathogen attacks (Ausubel, 2005).

The plant immune system is able to provide resistance against the majority of pathogens. The term non-host resistance involves all genotypes of a plant species that are resistant to all strains of a pathogen. Alternately, if a pathogen is virulent on some plant genotypes while some cultivars are resistant to certain strains of pathogen then this interaction is known as compatible interaction. This type of interaction leads to plants being susceptible to pathogens and the development of disease whereas, in an incompatible interaction pathogen growth in the plant is limited and the plant is resistant to the pathogen.

1.4.1 Molecular Plant-Pathogen interactions.

Our current understanding of a plant- pathogen interactions is centred on the Zig-Zag model (Jones & Dangl 2006) (Fig.2). This model includes extracellular PAMP-recognition receptors (PRRs) that detect chemical elicitors; broadly conserved pathogen molecules (pathogen/microbe-associated molecular patterns, PAMP/MAMPs), and/or damage-associated molecular patterns (DAMPs) (Mazzotta & Kemmerling 2011). Upon recognition of these foreign molecules by PRRs, the plant innate immune system responds by initiating defence responses termed as PAMP-triggered immunity (PTI) (Jones & Dangl 2006) (Fig. 2). Successful pathogens are able to counteract the initial extracellular layer of immunity by producing effector proteins. These effectors are small secreted proteins and have evolved to bypass the initial defence response (effector triggered susceptibility (ETS)) (Fig.2). However, resistant plants possess Resistance (R) genes, These R proteins recognise some of the pathogen effectors either through direct binding or by sensing the perturbing activity of an effector on host components (Cui H et al. 2015). Upon recognition, the plant activates the second layer of defence response termed as effector triggered immunity (ETI) (Fig.2). ETI often leads to a hypersensitive resistance response (HR) mediated programmed cell death (Jones & Dangl 2006; Deslandes & Rivas 2012). However, microbes may evade recognition by the loss/mutation of effectors or by secreting novel effector proteins (Pendleton et al. 2014).

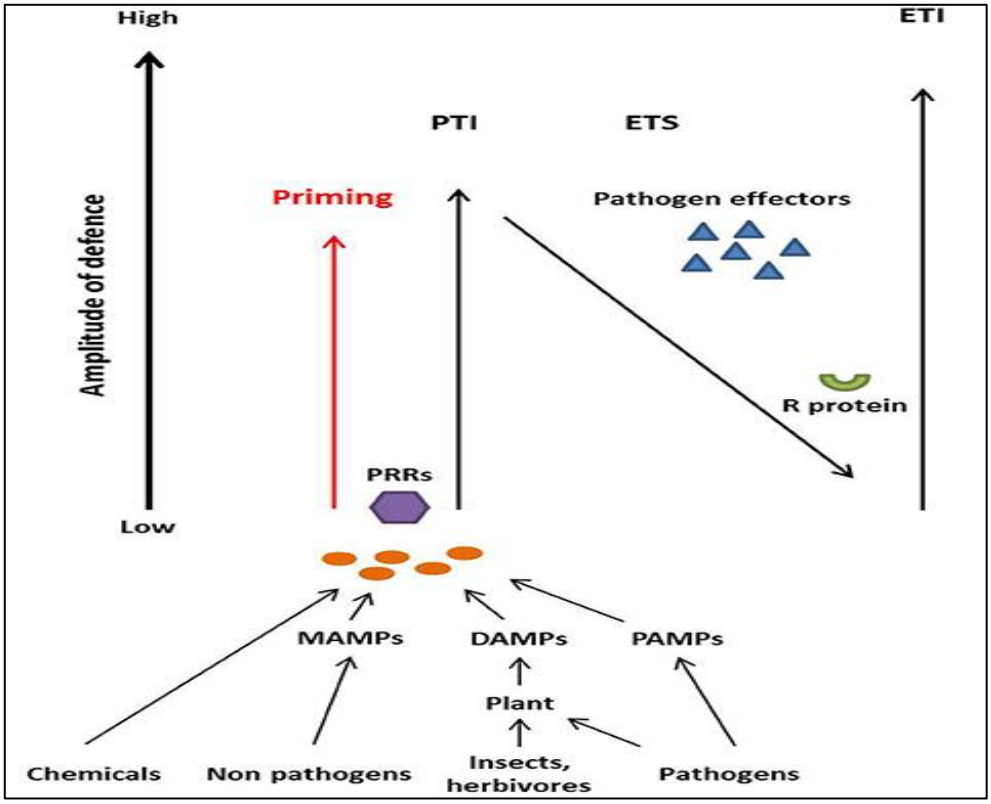

Fig 2. Zig-Zag model of plant immunity. Plant recognition of chemical elicitors, microbe associated molecular patterns (MAMPs) from non-pathogenic microbes, pathogen associated molecular patterns (PAMPs) and damage associated molecular patterns (DAMPs) occurs through pattern recognition receptors (PRRs) leading to induction of pattern triggered immunity (PTI). Pathogen secrete effectors to overcome PTI resulting in effector triggered susceptibility (ETS). In parallel plants evolve to recognise these effectors by R protein leading to Effector triggered immunity ETI).

Adapted from Wiesel et al. 2014

1.4.2 Pattern Recognition Receptor (PRR) provides basal immunity against pathogen invasion

PRR proteins are usually plasma membrane bound receptor kinases and broadly fall into two class: receptor like kinase proteins (RLKs) and receptor like proteins (RLPs) (Fig. 3). Both of these receptors have an ectodomain for ligand binding (ECD) and a single transmembrane domain. RLKs contain an extra cytoplasmic kinase domain responsible for activation of downstream signalling (Wang et al. 2014).

PRRs can be further subdivided on the basis of their ligand binding domains (ECD). PRRs with leucine-rich repeat (LRR) domain bind PAMPs including bacterial flagellin, elongation factor Tu (EF-Tu) or endogenous AtPep peptides (Zipfel C 2014). While, PRRs containing Lysine motifs (LysM) binds to carbohydrate containing ligands such as fungal chitin and bacterial peptidoglycan. In addition to that, lectin-type PRRs bind bacterial lipopolysaccharides (LPS) or extracellular ATP (adenosine triphosphate). PRRs with epidermal growth factor

(EGF)-like domains recognize plant cell-wall derived oligogalacturonides (Gust A.A & Felix G 2014; Ranf et al. 2015).

One of the best studied bacterial PAMP is *Flg22*, a subunit from bacterial flagellin which is recognised by the PRR receptor FLAGELLIN SENSITIVE2 (FLS2). Similarly, bacterial elongation factor EF-Tu is recognised by the EF-Tu receptor (EFR) in the *Brassicaceae* (Zipfel et al. 2006). FLS2 and EFR associates with another RLK Brassinosteroid insensitive 1-assciated receptor kinase 1 (BAK1) upon PAMP perception and initiates the immune response. The immune response typically includes production of reactive oxygen species (ROS), the induction of MAP (mitogen-activated protein) kinase (MAPK) signalling, callose deposition at the site of infection and transcriptional activation of defence-related genes.

1.4.3 Wall associated Kinases (WAK's) a new player in plant immunity

Studies have associated PRR mainly RLKs like wall-associated receptor kinases (WAK) with resistance against plant pathogens (He et al. 1999, Verica & He 2002). These WAKs are unique receptor-like kinase genes encoding an extracytoplasmic WAK domain, a cytoplasmic serine/Threonine protein kinase (STK) domain and an extracellular domain similar to the epidermal growth factor like calcium binding (EGF) domain. Arabidopsis WAK1 was shown to be induced in response to infection with *Pseudomonas syringae* and overexpression of WAK1 in *A. thaliana* resulted in higher resistant to *Botrytis cinerea* (Verica & He 2002; Brutus et al. 2010). Map-based cloning of a major head smut quantitative resistance locus (qHSR1) identified *Zea mays* Wall associated Kinase (*ZmWAK*) gene providing quantitative resistance to head smut (Zuo et al. 2015). While, map-based cloning of wheat *P. nodorum* resistance gene *Snn1* also revealed an active role for a wall-associated kinase in host triggered effector mediated susceptibility in wheat (Shi et al. 2016). Recent map-based cloning of the *Z. tritici* resistance wheat gene *Stb6* demonstrated that *Stb6* encodes a WAK like receptor kinase (Fig. 3) (Saintenac et al. 2018). These studies highlight gene-for gene disease resistance apart from that mediated by classical NB-LRR resistance genes.

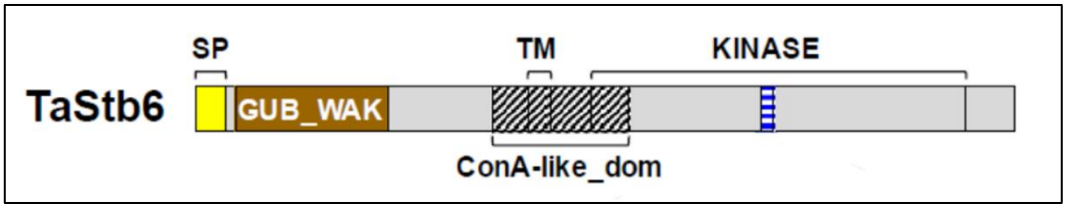

Fig 3. Gene Structure of wheat *Stb6* with WAK like domains. A signal sequence (SP) in yellow followed by a galacturonan-binding domain (GUB_WAK), a transmembrane region (TM), a concanavalin A-like domain and a Ser/Thr kinase domain. Interestingly, Ta*Stb6* lacks an epidermal growth factor (EGF)-like calcium binding domain that are commonly found in WAKs.

Adapted from Saintenac et al. 2018

1.4.4 The second layer of the plant defence response is mediated by resistance (R) genes.

The majority of plant resistance proteins encode for immune receptors containing a central nucleotide-binding (NB) domain and a C-terminal leucine-rich repeat (LRR) domain. Plant resistance proteins can be divided into two subclasses depending on their N-terminal domain (Meyers et al. 2003) (Fig. 4). One of the largest classes being the Toll/interleukin-1 (TIR)-NB-LRRs receptors, which shares a homology to the Drosophila Toll immune receptors and mammals Interleukin-1 receptor intracellular signalling domains. The second most common class includes a CC-NB-LRRs receptors which possesses a predicted coiled-coil (CC) domain at the N-terminus. These NB-LRR proteins together with CC and TIR domains play a key role in pathogen effector recognition and subsequent defence activation.

Following activation of PTI or ETI, plant hormones tend to act as a key players in triggering plant immune signalling networks (Bari & Jones 2009; Pieterse et al., 2009). Salicylic acid (SA), Jasmonic Acid (JA) and Ethylene (ET) are major defense associated hormones. SA is known to be predominantly involved in resistance to biotrophic pathogens while JA & ET regulate resistance against necrotrophic pathogens (Thomma et al. 1998; Glazebrook 2005). However, there are several studies that highlight the role of cross talk between SA, JA and other hormones during pathogen challenge (Derksen et al. 2013).

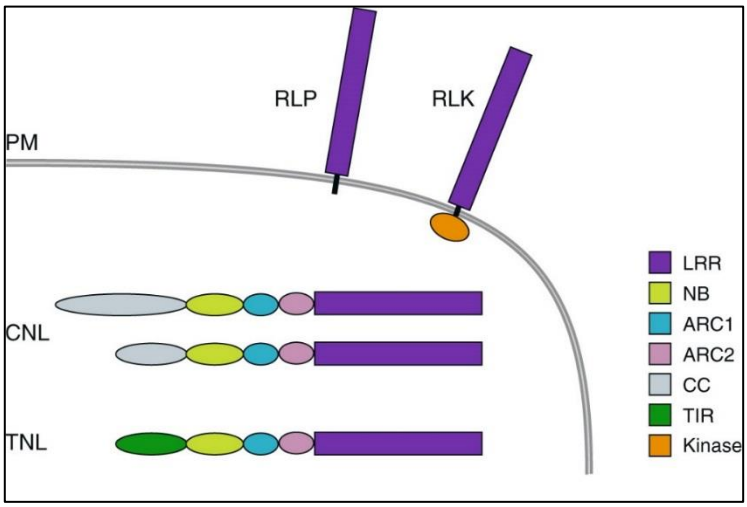

Fig 4. Schematic representation of different R protein classes. The receptor like protein (RLP) and receptor like kinase (RLK) contains an extracellular Leucine Rich Repeats (LRR) domain and are present on the plasma membrane (PM) whereas, CNL [N-terminal Coiled coil (CC) domain] and TNL (N-terminal [Toll/interleukin 1-like receptor (TIR domain) represents another classe of R proteins that can be present in the cytoplasm, nuclear, membrane bound with a central NB-ARC domain fused with LRR domain. NB refers to nucleotide binding domain, ARC refers to Nucleotide-Binding adaptor shared by Apaf1, R genes and CED4.

Adapted from Ooijen et al. 2007

1.4.5 Zig-Zag model is not comprehensive

The Zigzag model provides a conceptual framework for understanding plant-pathogen interactions and also incorporates effector dynamics into the model. However, the model is not complete. The interaction that takes place during plant symbiosis and necrotrophic interactions are not described within the scope of this model. In the case of a necrotrophic pathogen, plant cell death is a representation of the plant's susceptibility towards a pathogen whereas, in the zigzag model cell death results in effector triggered immunity (ETI), typical for biotrophic pathogens *(*Dickman & de Figueiredo 2013*)*.

Similarly, the zigzag model includes various ordered response events within a plant immune system such as; detection by PRRs followed by the action of effectors to bypass the PTI response. However, there exists accumulating evidence that shows blurred lines between PAMPs and effectors and PRRs and R proteins in relation to the zigzag model. One example is *Ecp6* from the tomato pathogen *Cladosporium fulvum*. The fungal effector *Ecp6* is highly conserved (orthologs termed LysM) among the fungal kingdom and competes with the plant LysM-containing CEBiP receptor for the binding of chitin oligomers that are released during host colonization *(*de Jonge et al. 2010*)*. Similarly, Nep1-like proteins (NLPs) that are present within bacteria, fungi and oomycetes acts as a MAMPs (Oome et. al 2014). Interestingly, NLPs have conserved domains of 20-24 amino acids that act as an inducers of the immune response (Bohm et al. 2014) showing MAMP like epitopes within an effector molecule. In addition to the NB-LRRs, cell wall-associated kinases (WAKs) have been shown to be involved in avirulence effector recognition for example *Stb6* which recognises the *Z. tritici* effector *AvrStb6* (Saintenac et al. 2018). This recognition does not induce hypersensitive mediated cell death.

1.5 Molecular models of Effector recognition

1.5.1 Direct Recognition

The presence of R proteins and their interaction with pathogen effectors was first described by the gene for gene model (Flor, 1971). The model suggests that the interaction of plant and pathogen results in either a compatible interaction with either the absence of the resistance (R) gene or complementary pathogen avirulence (*Avr*) gene or incompatible interaction with the R gene and *Avr* gene present. This effector recognition can occur by physical binding with an NLR protein and subsequent signalling similar to a receptor-ligand model.

1.5.2 Guard Model

Since the pathogen repertoire of effectors is huge and diverse, plants must be able to recognise multiple effectors through indirect R protein mediated effector recognition. This concept led to an extension of the gene for gene model to the Guard hypothesis by Van der Biezen & Jones (1998). The guard model (Fig. 5.1) predicts that R proteins are under surveillance by key effector targets (guardees) in plants and that any effector induced modification of these key proteins results in activation of host ETI. The guard model allows a single R protein to recognise multiple effectors that interact with the same guardee (de Witt, 2007; Jones & Dangl 2006). A good illustration of this model is the *Arabidopsis* RPM1 (Resistance to *P. syringae* pv. Maculicola 1) interacting protein 4 (RIN4). RIN4 is guarded by two CC-NB-LRR proteins RPM1 and (Resistance to *P. syringae* 2) RPS2. RIN4 is targeted for phosphorylation or cleavage by at least four independent bacterial effectors (*AvrRpm1*, *AvrB*, *AvrRpt2* and *HopF2*). Targeted phosphorylation and cleavage of RIN4 leads to activation of RPM1 and subsequent plant defence signalling (Mackey et al. 2002; Wilton M et al. 2010; Axtell & Staskawicz 2003). Another well characterised example of the guard model involves tomato C. *fulvum* resistance gene (*Cf-2*), a transmembrane receptor providing dual resistance by recognizing the perturbation of tomato apoplastic cysteine protease namely; Rcr3 and PIP1 by *C. fulvum* effector *Avr2* and effector from parasitic nematode *Globodera rostochiensis* venom allergen-like protein *(*Gr-VAP1) (Torres et al. 2012).

1.5.3 Decoy model

There is growing evidence of indirect effector recognition that is inconsistent with the guard hypothesis resulting in the so called a Decoy model (Fig. 5.2) (Van der Hoorn & Kamoun 2008). Considering an evolutionary point of view with respect to the guard model; if a plant possesses an R protein then the guardee is under selection pressure to favour its interaction with the effector protein and thus an increase in pathogen perception.

However, lack of an R protein in plants would again result in selection pressure to avoid interaction of the guardee with effectors and thus limit detection and modification by pathogen effectors. This opposing selection force creates an unstable situation where R genes are polymorphic (presence/absence). Therefore, to relax the evolutionary constraint on the guardee protein, plants can produce decoy proteins that are structurally similar to the guardee protein and are also monitored by R proteins.

Unlike guardees, these decoys specialize in perception by mimicking effector target proteins and thus incorporates the idea of multiple effector host targets which the guard model lacks. For example, the P. syringae effectors *AvrPto* and *AvrPtoB* target the kinase domains of PAMP-detecting RLKs, FLS2 and EFR. However, the tomato serine/threonine (Ser/Thr) kinase *Pto* can also interact with these effectors and activate the NLR pseudomonas resistance and fenthion sensitivity (*Prf*) that triggers immune signalling. Thus, *Pto* acts as a decoy and activates Prf mediated defence responses (Mucyn et al 2006; Oh et al. 2011).

Indirect recognition likely contributes to broad spectrum recognition of pathogen effectors and this mode of recognition can occur broadly considering a limited number of PRRs and NLRs present in plants. In addition to that interspecific and intraspecific pathogen effectors are known to target broadly conserved host proteins that are interconnected to various processes and are so called as 'immune hubs' for promoting infection (Mukhtar et al. 2011; Weßling et al. 2014).

1.5.4 Integrated decoy model

The integrated decoy model (Fig. 5.3) was conceptualized on the basis of reports regarding dual NB-LRR genes which encode unusual integrated domains that confer pathogen resistance. Sinapidou et al. 2004 first reported a pair of R proteins; recognition of *Peronospora parasitica* 2 (RPP 2A, 2B) required for disease resistance to an oomycete. A pair of *Arabidopsis* NB-LRRs, Resistance to *Pseudomonas syringae* 4 (RPS4) with Resistance to *Ralstonia solanacearum* 1 (RRS1-R) acts cooperatively to detect two effectors PopP2 from *Ralstonia solanacearum*, and *AvrRps4*, from *Pseudomonas syringae* bacteria (Le Roux et al. 2015;

Sarris *et al.* 2015). Lack of recognition (via the absence of the R protein pair) of these effectors dislodges the defence-promoting WRKY transcription factors from their binding sites resulting in suppression of host defence. In the presence of the R protein pair, the WRKY domain in RRS1-R acts as an effector target 'decoy' which upon binding by PopP2 and AvrRps4 results in downstream defence signalling. Similarly, in rice, the NLR *Pyricularia oryzae* resistance K (*Pik-1, Pik-2*) and R-gene analog 5 (RGA5) possess an integrated heavy metal-associated (HMA) domain that interacts directly with the effector proteins from *M. oryzae* activating disease resistance (Cesari et al. 2013; Maqbool et al. 2015).

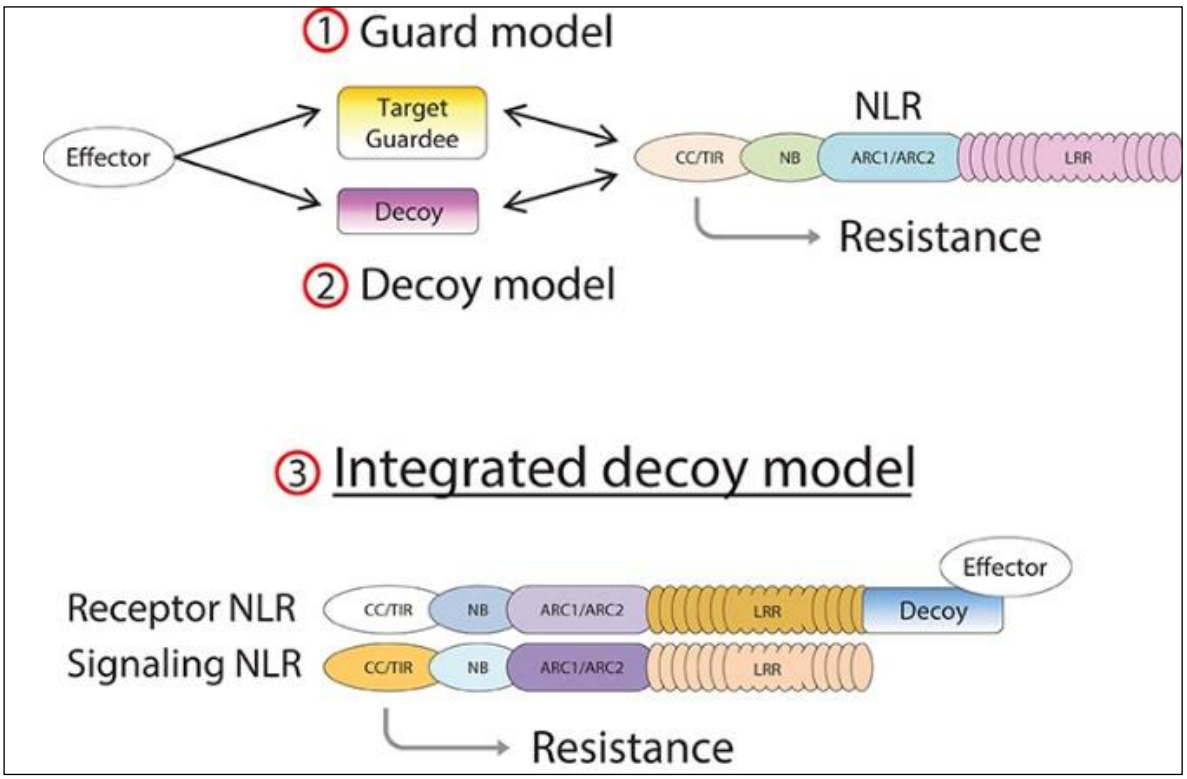

Fig 5. The guard model (1) suggests an indirect interaction between R protein and effectors. The effector interacts with the guardee protein and this interaction results in R-protein mediated immunity. The decoy model (2) refers to a decoy protein mimicking effector targets resulting in perception and activation of defence response. The integrated decoy model (3) represents a pair of R protein acting together in which binding of effector to a decoy protein results in triggering of downstream defence response.

Adapted from Cesari et al. 2014

1.6 Decoy engineering: Alternative strategy to resistance breeding

The ability of plant immune system to respond against pathogen invasion heavily relies on its ability to detect the invaders through its receptors and co-receptor kinases. Engineering of these receptor variants recognised by the effector could help evade effector mediated manipulation and engineer disease resistance. One successful proof of concept included; a modification of a decoy RLK protein PBS1 (protein kinase superfamily protein 1) with a protease cleavage site for the tobacco etch virus (TEV) protease that allowed activation of *RPS5* (Resistance to *pseudomonas syringae* 5) leading to resistance against *P. syringae* and TEV (Kim et al. 2016). Similarly, recent crystal structures of the NLR Pik Heavy metal associated (HMA) domain complexed with effector *Avr-PikD* and an unrecognised effector *Avr-PikE* showed that the C-terminus of the HMA domain played a key role in the interaction with effectors. The HMA domain represents the polymorphic regions of this NLR allowing recognition of multiple effectors (Concepcion et al. 2018). Therefore, these studies highlight the possibility of engineering decoys/guardees to provide broader spectrum resistance against pathogens. However, before proceeding it is central to establish whether such alteration within a decoys residues affects its structure and function. On the contrary, such modulation within the decoy protein might also allow further selection pressure in the pathogen effector repertoire and thus ultimately result in gain of virulence.

1.7 Effector Biology

The discovery that plant pathogenic gram negative bacteria deliver secreted protein into hosts through a type III secretion system (T3SS) (Abramovitch et al. 2006, Zhou & Chai 2008) was the first use of the term "effectors". As well as contributing to virulence, these proteins are involved in recognition by corresponding plant receptors thus, effectors overcomes the limitation of the term 'avirulence'. "Effectors" have been defined by Kamoun (2006) as pathogen secreted molecules that manipulate host cell structure and function thereby facilitating infection or triggering defence responses. While Birch and colleagues (2014) referred to effectors as "any protein synthesized by a pathogen that is exported to a potential host, which has the effect of making the host environment beneficial to the pathogen". However, it has been reported that biotrophic pathogens also use nonproteinaceous effectors (e.g secondary metabolites, low molecular weight compunds for example, coronatine and syringolin) to manipulate their host system (Panstruga & Dodds, 2009; Bohnert et al. 2004). Studies have shown that filamentous plant pathogens also secretes photodynamic toxins e.g.

rubellins by *R. collo-cygni* (Heiser et al. 2003). However, through out this study, effectors are considered as a small secreted proteins (SSPs) containing cysteine residues and an N-terminal signal peptide for extracellular secretion, low sequence homology with known proteins and the ability to manipulate host physiological and morphological processes.

Pathogen effectors are classified as either apoplastic or cytosolic on the basis of its site of function (Kamoun 2006, 2007). Apoplastic effectors are secreted into the plant extracellular space; the apoplast, where they interact with extracellular targets and surface receptors, whereas cytosolic effectors are translocated inside the plant cell by specialized delivery structures which may include infection vesicles, appressoria or haustoria (Djamei et al. 2011; Park et al. 2012). Efficient delivery of these effectors is required for successful infection and host colonisation.

1.7.1 Apoplastic Effectors

Apoplastic effectors are often small and cysteine rich which possibly form disulfide bridges to stabilize the protein after secretion from the pathogen and remain in the extracellular spaces (Kamoun 2006).

1.7.1.1 Host protease inhibitors

Effectors play a central role in protecting pathogens from various host proteases and chitinases and also manipulate plant responses in various ways. A major group of secreted proteins is cell wall degrading enzymes (CWDEs) that hydrolyse plant cell wall polymers such as cellulose and pectins. However, lack of evidence of the contribution of these enzymes to virulence raises a question mark and they may not fall into the effector category (Doehlemann & Hemetsberger 2013). Papain-like cysteine proteases (PLCPs) are a key component of the plant immune response (Doehlemann & Hemetsberger 2013). Some of the pathogen effectors that target plant proteases are the *Phytophthora infestans* effector *Avrblb2*, cystatin-like effector proteins (EPICs) EPIC1 and EPIC2B that target protease C14 (Kaschani et al. 2010). Similarly, the apoplastic effector *Avr2* from *Cladosporium fulvum* inhibits the tomato cysteine protease Rcr3 that is required for activation of basal defence responses (Esse et al. 2008). The apoplastic effector *Pit2* is also known to inhibit a cysteine protease in maize (Mueller et al. 2013). Similarly, serine protease inhibitors *Epi1* and *Epi10* inhibit the Pathogenesis related subtilisin-like serine protease P69B of tomato (Tian et al. 2004).

A class of effector glucanase inhibitor proteins (GIPs) from *Phytophthora sojae; GIP1* interacts with the soybean endoglucanase enzyme EgaseA and prevents release of oligosaccharides and subsequent PTI (Rose et al. 2002). Additionally, the *P sojae* Xyloglucan-specific EndoGlucanase (PsXEG) effector PsXLP1 (known to be a paralogous decoy molecule of PsXEG1), binds to the soyabean Glucanase inhibitor protein 1 (GmGIP1) with higher affinity than PsXEG1 thereby preventing inhibition of PsXEG1 by the plant host endoglucanase (Ma et al. 2017).

1.7.1.2 Host chitin-binders

A group of apoplastic effectors that protects the pathogen from host chitinases and chitin induced immunity contain LysM domains. The effectors Mg1LysM and Mg3LysM from *Zymoseptoria tritici* binds to chitin at the fungal cell wall to protect it from hydrolytic enzymes (Marshall et al. 2011). Similarly, the *Cladosporium fulvum* effector *Ecp6*, contains three LysM domains that sequesters chitin oligomers originating from the fungal cell wall before they are detected by plant PRRs, preventing PTI (de Jonge et al. 2010; Sanchez-Vallet et al. 2013). The LysM effector *Slp1* from *M. oryzae* also binds chitin to suppress chitin-induced plant immunity (Mentlak et al. 2012). These effectors compete with plant PRR proteins namely chitin-elicitor binding protein (CEBiP) and receptor chitin elicitor receptor kinase 1 (CERK1) that mediate PTI through chitin mediated recognition of the pathogen.

1.7.1.3 Antioxidant Suppressors

Similarly, pathogen apoplastic effectors also interact with host reactive oxygen species (ROS) generating systems. ROS are key molecules in the plant defence system. Plant NADPH oxidase (NOX) and apoplastic/cell-wall associated peroxidases (POX) are involved in ROS generation. The accumulation and release of ROS occurs in response to pathogen attack and these molecules have direct antimicrobial action and simultaneously act as a key signal transduction molecules. The maize pathogen *Ustilago maydis* effector *Pep1* (protein essential during penetration-1) plays a crucial role in host penetration and interacts with the maize peroxidase POX12 thereby inhibiting ROS production (Hemetsberger et al. 2012).

1.7.2 Cytoplasmic Effectors

Cytoplasmic or intracellular effectors are translocated into the host cytoplasm where they interact with numerous plant components and manipulate host mechanisms. In some oomycete species translocation usually involves a characteristic conserved motif namely RxLR (Arginine, any amino acid, Leucine, Arginine) or LFLAK (Leucine, Glutamine, Leucine, Phenylalanine, Leucine, Alanine, Lysine) domain. Proteins belonging to the Crinkler (CRN) family of proteins consist of an N-terminus LFLAK domain. These oomycete effectors carrying such motifs that allow effectors to translocate into host cells. In contrast the exact role of N-terminal signal peptides in cell entry is not clearly defined in fungal pathogens. Barley powdery mildew fungus *B. garmanis* f.sp. *hordei* possess a conserved N-terminal [YFW]xC motif (Godfrey et al. 2010). However, fungal effectors appear to share very limited sequence conservation apart from a few effectors that contains a LysM domain. This could be attributed to the rapid evolution and genome plasticity within fungal genomes (Croll et al. 2012)

1.7.2.1 Targeting host defence pathways

The maize pathogen *Ustilago maydis* effector proteins, *Tin2* and chorismate mutase (*Cmu1*) function in the cytoplasm of maize cells. *Cmu1* is translocated into plant cells and was identified as a key for pathogen virulence (Djamei et al. 2011). The fungal secreted enzyme Cmu1 is able to bind to maize chorismate mutase; *ZmCm2* which is involved in salicylic acid production (a plant hormone that activates plant immunity and induces cell death at high concentrations). This ultimately shifts the plant metabolism towards biosynthesis aromatic amino acids rather than salicylic acid. Similarly, the effector *Tin2* has been shown to promote disease by diverting plant metabolism to anthocyanin biosynthesis rather than lignin biosynthesis though interaction with the host protein kinase *ZmTTK1* (a positive regulator of anthocyanin biosynthesis) (Tanaka et al. 2014).

The cytoplasmic effector of *Ustilago maydis,* Seedling efficient effector 1 (See1) interacts with the maize homolog of suppressor of G2 allele of *skp1* (SGT1). SGT1 acts in cell cycle progression in yeast (Dubacq et al. 2002) and is an essential component of innate immunity in plant and animals (Shirasu, 2009; Zhang et al. 2010). This interaction of See1 with SGT1 interferes with MAPK- induced phosphorylation of SGT1 resulting in the potential modulation of immune response and reactivation of plant DNA synthesis in maize leaves which supports tumor formation (Redkar et al. 2015). *Magnaporthe oryzae* effectors IUG6, IUG9 (isolate unique genes) which are delivered into the rice cytoplasm suppress Bax-mediated programmed

cell death in tobacco leaves (Dong et al. 2015). In addition, *M. oryzae* also secretes antibiotic biosynthesis monooxygenase (Abm) that acts on the plant hormone JA and converts it to hydroxylated JA thus attenuating JA accumulation and plant defense (Patkar et al. 2015).

1.7.2.2 Targeting host ubiquitin system

The covalent attachment of ubiquitin molecules to a protein is termed as ubiquitination. Subsequently, after attachment of ubiquitin molecules to a protein, it undergoes degradation by the 26S proteasome. This system often termed as ubiquitin-proteasome system (UPS) is crucial to the plant in order to maintain its proteome plasticity. Ubiquitin mediated degradation of stress responsive proteins until a stress signal is perceived prevents over accumulation or degradation of negative response regulators. Some pathogen effectors are known to target this system in order to facilitate infection. *Magnaporthe oryzae* effector *AvrPiz-t* delivered into rice sheath epidermal cells during infection targets proteasome activity by interacting with the RING E3 ubiquitin ligase AvrPiz-t Interacting Protein 6 (APIP6). This interaction leads to degradation and suppression of ROS production during PTI in rice *(*Park et al. 2012*)*. Similarly, several cytoplasmic effectors are known to inhibit host programmed cell death (PCD) that restricts biotrophic pathogen growth. *Phytophthora infestans* effector AVR3a strongly suppresses infestin 1 (INF1)-triggered cell death (ICD) by binding and stabilizing a potato U-box E3 ubiquitin ligase CMPG1 (Bos et al. 2010).

1.7.3 Necrotrophic Effectors

Necrotrophic fungal pathogens obtain nutrients from dead host cells after pathogen invasion which is then followed by the colonisation of the pathogen. In the case of necrotrophs, induction of programmed cell death (PCD) would provide the pathogen optimum conditions to grow. Necrotrophic pathogen effectors are also termed as host specific toxins (HSTs) critical for pathogen virulence.

The best studied HST is ToxA which is produced by two fungal pathogens of wheat *P. nodorum* and *P. tritici-repentis* (Liu et al. 2006; Ciuffetti et al. 1997). The exact function of ToxA is still unknown, but it has been shown that ToxA interacts with a chloroplast protein, ToxA binding protein 1 (ToxABP1) (Manning et al. 2007). Similarly, it was found that ToxA targets wheat plastocyanin which is an important component of the electron transport chain of photosynthesis (Tai et al. 2007) and it is likely that disruption of the electron transport chain

generates ROS inducing cell death. In addition, ToxA interacts with wheat pathogenicity related protein PR-1-5 protein (Lu et al. 2014). Similarly, a second effector secreted by *P. nodorum*, SnTox3 also plays a key role in fungal virulence. It has been recently identified that this SnTox3 interacts with a broad range of wheat PR-1 protein families, suggesting wheat PR-1 proteins are key targets for host defence manipulation (Breen et al. 2016).

There is an emerging concept of utilizing necrotrophic effector's ability to induce cell death/ necrosis as a tool for screening varieties that are sensitive to a fungal pathogen. The idea is based on rapid screening of wheat varieties on the basis of disease symptoms developed after application of specific purified fungal toxins. All major wheat breeders have adopted this as a selection strategy for breeding (Fig.6).

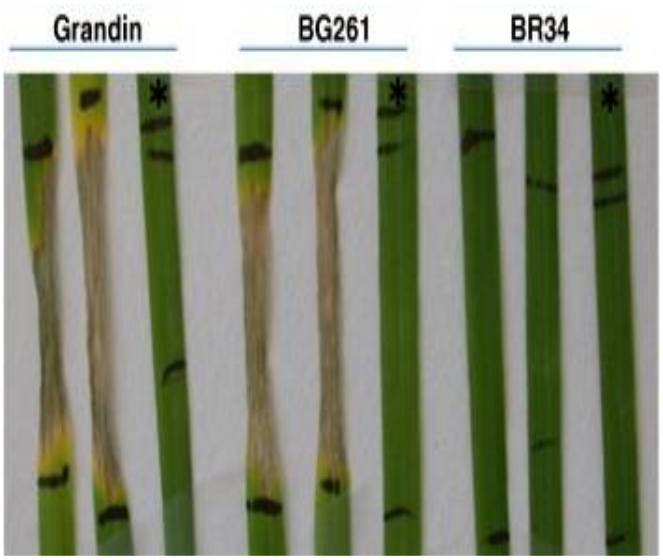

Fig 6. An illustration of how effector assisted resistance breeding works. Wheat leaves are infiltrated with *S. nodorum* ToxA protein. Cultivar Grandin and BG261 are susceptible to ToxA as they carry the dominant Tsn1 allele that confers ToxA sensitivity while BR34 carries the recessive Tsn1 allele and are insensitive to ToxA. The breeders can select the ToxA insensitive cultivar to increase resistance to corresponding pathogen.

Adapted from Oliver RP & Solomon PS. 2010

1.7.4 Translocation of fungal effectors inside host plant

Unlike the bacterial Type III secretion systems (T3SSs) utilized by many bacteria to deliver effector proteins into host cells, fungal and oomycete pathogens have diverse effector delivery systems upon infection. Biotrophic and hemibiotrophic fungal pathogens secrete effectors that are translocated to the host cytoplasm or apoplast using specialized infection structures such as haustoria or appressoria (Koeck et al. 2011; Petre & Kamoun 2014). These feeding structures play an important role in trafficking effectors into host cells and in gaining nutrients from the host (Voegele & Mendgen 2011).

The rice blast fungus *Magnaporthe oryzae* shows two distinct secretion systems for the delivery of apoplastic and cytoplasmic effectors (Giraldo et al. 2013). Cytoplasmic effectors tend to accumulate in a membrane rich structure termed as a biotrophic interface complex (BIC), and then are ultimately translocated across the extra invasive hyphal membrane (EIHM) to the host cytoplasm (Khang et al. 2010). While the apoplastic effectors aren't associated with the BIC and are dispersed in the extracellular space between fungal cell wall and the extra-invasive–hyphal membrane (Zhang & Xu 2014). The delivery of cytosolic effectors from the filamentous plant pathogens into the host cells has infrequently been visualised directly and still remains elusive (Wang et al. 2017).

Studies on some obligate (powdery mildew) and facultative biotrophs (smut fungi) have shown the presence of exosomes and extracellular vesicle (EV) bodies between the interface of fungi and the host membrane cells. These EVs could be used by fungi to secrete effectors into the host (An Q et al. 2006; Rutter & Innes, 2016). The *P tritici-repentis* secreted ToxA was found to be internalised into plant cell through its Arginyl-Glycyl-Aspartic (RGD) motif (Meinhardt et al. 2002). Similarly, domains in the N-terminus of the flax rust fungus *Melampsora lini* effectors *AvrM* and *AvrL567* are shown to be involved the uptake into plant cells (Rafiqi et al. 2010; Thomas et al. 2013). A growing number of studies with tagged fungal effectors may provide better insights into fungal effector trafficking from pathogen to host cell.

1.8 Scope of Thesis

Plant pathogen secrete proteins that facilitate successful colonisation of the plant host or to establish a compatible interaction with the host. These small secreted proteins or effector proteins are able to suppress or counteract plant defence responses. Much of the recent research in the field of pathogen effectors has gained knowledge from bacterial effectors, while the contribution of pathogenic fungal effectors during plant disease are still far from comprehensive. This is particularly true for effectors of *Z. tritici.*

The completion of the *Z. tritici* genome sequence by Goodwin et al. 2011 and the detailed protein secretome analysis by do Amaral et al. 2012 provided insights into the candidate effector repertoire of *Z. tritici.* To date only a small number of effectors contributing to *Z. tritici* pathogenesis in wheat have been functionally characterized. With this in mind the aim of this study was to identify *Z. tritici* effector candidates that play a key role in host manipulation.

Chapter 1 includes general introduction to lifestyle features of *Z. tritici* (biotrophic and necrotrophic phase) and host response, plant immune system and effector biology.

Chapter 2 descibes identification of cell death inducing *Z. tritici* effector candidates using a screen in the non-host model plant *N. benthamiana*. The activation of plant defense pathways by expression of *Z. tritici* small secreted proteins *(ZtSSP's)* was studied.

Chapter 3 describes effector candidate's polymorphism and identification of host interacting partners of the effector candidate (*ZtSSP2*).

Chapter 4 includes investigation of *B. distachyon* as a model for *Z. tritici* and in comparison to the host wheat (*T. aestivum)*.

Chapter 5 explores general discussion from effector identification to effector mediated resistance bredding. The chapter will also summarise the importance of the findings in this thesis and future direction in *Z. tritici* research.

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Chapter 2

Zymoseptoria tritici small secreted proteins induce defence responses in the non-host plant *Nicotiana benthamiana*

A manuscript is in preparation with work from Chapter 2 and 3:

"Screening of small secreted proteins of *Z. tritici* reveals a candidate protein that targets host ubiquitin system".

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2.1 ABSTRACT

Septoria Tritici Blotch, caused by the ascomycete fungus *Zymoseptoria tritici*, is a major threat to wheat production worldwide. The *Z. tritici* genome encodes for many small secreted proteins (*ZtSSP*) that likely play a key role in the successful colonisation of host tissues. In this study, thirty of these *ZtSSP* were screened for the induction of cell death in the non-host model plant *Nicotiana benthamiana*. Five novel candidates were identified that resulted in the rapid accumulation of H_2O_2 and cell death. Transient overexpression of these five candidates also resulted in the upregulation of defense marker genes in *N. benthamiana*. Lastly, cell death inducing candidates were differentially regulated through out *Z. tritici* infection in wheat.

2.2 INTRODUCTION

The plant immune system provides resistance against most pathogens. The term non-host resistance (NHR) is defined as resistance shown by all genotypes of a plant species to all strains of a given pathogen. This form of plant defence has received attention as a source of durable resistance (Nuernberger and Lipka 2005; Heath 2000).

Plant innate immunity includes the recognition of broadly conserved pathogen associated molecular patterns (PAMPs) for example fungal chitin, by plant pattern recognition receptors (PRRs). This recognition initiates PAMP-triggered immunity (PTI) to mount a primary defense (Jones & Dangl 2006). The PTI response typically includes the production of reactive oxygen species (ROS), MAP (mitogen-activated protein) kinase (MAPK) signalling, callose deposition at the site of infection and transcriptional activation of defence-related genes (Jones & Dangl 2006; Zipfel 2009, 2014; Zhang et al. 2007). Successful pathogens can counteract this immunity by producing effector proteins. These proteins are typically secreted proteins, small, cysteine rich and are known to manipulate host physiology and interfere with plant immunity (Dou & Zhou 2012). Effector proteins have evolved to bypass the initial defence response (PTI) resulting in a scenario termed Effector triggered susceptibility (ETS). In return, plants also possess Resistance (R) genes which upon recognition of pathogen effectors, activates Effector triggered immunity (ETI) (Jones & Dangl 2006; Deslandes & Rivas 2012).

Septoria tritici blotch (STB), caused by *Zymoseptoria tritici* is one of the most prevalent and economically devastating disease in wheat growing areas worldwide (Saintenac et al. 2018). Twenty-one major resistance genes to *Z. tritici* have been identified, but till date few have been characterised (Brown et al. 2015). Recently, a major STB resistance gene *Stb6* was successfully cloned and characterised as encoding a cell wall receptor (WAK) kinase (Saintenac et al. 2018). *Stb6* provides resistance through gene-for-gene interaction with recognition of the corresponding pathogen effector *Avrstb6* (Zhong et al. 2017).

Z. tritici is known to produce a series of small secreted proteins (SSPs) throughout its interaction with wheat during colonisation (Morais do Amaral et al. 2012; Mirzadi Gohari et al. 2015; Rudd et al. 2015). Apart from *Avrstb6* only handful of *Z. tritici* effectors have been characterised for their role in pathogenesis. For example, two *Z. tritici* effectors contain a lysin motif (LysM) namely; *Mg3LysM* and *Mg1LysM* which play an important role during the initial

symptomless period of *Z tritici* infection (Marshall et al. 2011). *Mg3LysM* competes with host chitin receptors by binding fungal chitin fragments (Lee et al. 2014). Another *Z. tritici* effector belongs to the Necrosis and Ethylene-Inducing Peptide 1 (NEP1)-like (NLP) family of proteins termed as MgNLP. It has been shown to induce cell death in *Arabidopsis*, but not in wheat (Motteram et al. 2009). In addition, Z. *tritici* secretes two necrosis inducing protein effectors (ZtNIP1 and ZtNIP2), which induce cell death and chlorosis in some wheat cultivars (M. Barek et al. 2015). However, little is known about the mechanism of cell death and how much this might contribute to virulence.

Recently, several *Z. tritici* candidate effectors were found to induce a cell death phenotype when expressed in the non-host plant *Nicotina benthamiana* (Kettles et al. 2017). It was demonstrated that two receptor-like kinases, Brassinosteriod Insensitive 1 (BRI1)-Associated Receptor Kinase 1 *(*BAK1*)* and the Suppressor of *BIR1-1* (SOBIR1), play a key role in the recognition of these effector candidates. One of these candidate effectors was subsequently identified as a novel fungal PAMP, termed Cell Death-Inducing 1 (ZtCDI1) (Franco-Orozco et al. 2017). Similarly, another candidate (Zt-6) was characterized as a secreted ribonuclease which possess a cytotoxic activity against microbes as well as plants (Kettles et al. 2018).

Host cell death and necrosis are observed during the necrotrophic phase of *Z. tritici* infection in wheat. Therefore, this study involved identification and characterization of *Z. tritici* effector candidates that could induce cell death. Thirty *Z. tritici* small secreted proteins (*ZtSSP*) were screened by transient over-expression in *N. benthamiana*. Nine were found to induce varying levels of cell chlorosis and cell death. Four out of these nine were previously reported to induce cell death by Kettles et al. 2017. Expression of the remaining five candidates in *N. benthamiana* leads to ion leakage, induction of responses including, hydrogen peroxide (H_2O_2) and upregulation of several defence marker genes of salicylic acid (SA) and jasmonic acid (JA) pathway.

2.3 MATERIALS AND METHODS

2.3.1 Plant material, fungal strains, and growth conditions.

Nicotiana benthamiana and wheat (*Triticum aestivum*) cv. Remus, cv. Kanzler was used in this study. *N. benthamiana* plants were grown and maintained in the growth chambers at 16-hour light at 22°C and 8-hour dark at 18°C throughout the experiments and 4-6-week *N. benthamaina* plants were used for all the experiment. Wheat seeds (cv. Remus) were surface sterilised and incubated for 3 days at 4 ºC for seed stratification and then incubated for 4 days at room temperature without illumination to allow germination. Germinated seeds were then transferred into plastic pots with John Innes Compost No. 2 (Westland Horticulture, UK) and grown in a controlled chamber at 16 hours of daylight/8-hour night cycle at 19°C/12ºC and watered every two days. For biolistic studies, wheat cv. Kanzler seedlings were grown in pots containing soil. Seven leaves of 7-day-old plants were used for particle bombardment.

The Irish *Z. tritici* isolate 560.11 (Lynch et al. 2016) was used to infect the susceptible wheat cultivar Remus. Prior to use, the isolate was cultured on potato dextrose agar (PDA) and grown at 20ºC under a 12-hour light/12 hour near ultraviolet (NUV) darkness for approximately 7 days. Fourteen-day old wheat seedlings were used for inoculation. Fungal spores from the PDA cultures were harvested and spore concentration was adjusted to $1 \times 10^6 ml^{-1}$ in water containing 0.02% Tween 20. Spore suspensions (15mls) were sprayed using hand-held spray bottles. Control plants were sprayed with 15ml of 0.02% Tween 20 solution. Inoculated plants were then covered with polythene bags to ensure high humidity. After 72 h, the polythene bags were removed.

2.3.2 Effector Selection and Cloning

The publicly available secretome dataset from Morais do Amaral et al. 2012 was mined to identify non-annotated *Z. tritici* small secreted proteins (*ZtSSPs*). 262 candidate genes with EST support were screened based on small size (50 to 300 amino acids) resulting in 102 SSP's. These were sorted based on number of cysteine residues which resulted in 90 SSP's with multiple cysteines (≥1). Candidate proteins were then analysed for the presence of any functional domain using NCBI blast and CDD database (Bauer et al. 2010). Out of 90 candidates 50 had no functional domain (Table 1) and were termed as hypothetical proteins. The amino acid sequence was then used to predict effector properties and any apoplastic

localisation using EffectorP & ApoplasticP (Table 1) (Sperschneider J et al. 2015, 2017). These 50 hypothetical proteins were then selected for screening in *N. benthamiana*.

Candidate genes were amplified from cDNA from RNA isolated from wheat (cv. Remus) infected with *Z tritici* (560.11) without the signal peptide using Phusion High Fidelity Polymerase (New England Biolabs) and primers flanked with gateway adapter sequence (Appendix 1). Similarly, cell death inducing *Z tritici* candidates were further cloned with signal peptide. AttB-flanked PCR products were purified using QIA quick PCR Purification Kit (Qiagen) and cloned into pDONR207 (Invitrogen) using BP clonase II enzyme mix (Thermo Fisher Scientific) and subsequently cloned into the binary vector pEARLYGATE 101 (pEG101) (Earley et al. 2006) using LR clonase II enzyme. All entry clones were sequence verified by sequencing (Macrogen Europe) before LR reaction.

2.3.3 Agrobacterium mediated transient expression of effectors

A tumefaciens strain GV3101 was transformed by electroporation with pEARLYGATE101: effector constructs and grown for 48 hrs at 28°C 220rpm in LB medium with the antibiotic gentamycin (25mg/ml) and kanamycin (50mg/ml). Transformed cells were harvested by centrifugation and suspended in infiltration buffer (10mM $MgCl_2$, 10mM 2-(N-mopholino) ethanesulfonic acid (MES) pH 5.6 and acetosyringone 150µM) at an absorbance 600nm (OD600) of 1-1.5. The bacterial suspension was left at room temperature for 2hr before infiltration. Infiltration was performed on 4-6-week-old *N. benthamiana* leaves using a 1ml needless syringe. Symptom development and images were taken 4-6 days post infiltration.

2.3.4 Trypan Blue and DAB staining

Cell death was stained using Trypan Blue (Koch and Slusarenko 1990) while heating, leaf samples for 5-10 minutes. Leaves were briefly rinsed in water and de-stained in choral hydrate (2.5g/ml H_2O) overnight. For analysis of H_2O_2, leaves were placed in a freshly made 1mg/ml 3,3'- diaminobenzadine (DAB) (Sigma) solution as described by Thordal-Christensen et al. 1997. Leaves were then cleared by boiling in bleaching solution (ethanol: acetic acid: glycerol=3:1:1) with heat (~10 min) until all chlorophyll was removed. To quantify H_2O_2 accumulation from the pictures, total leaf area and stained area was measured using ImageJ (Bethesda, MD, USA), and the ratio (stained area/total leaf area) was calculated.

2.3.5 Electrolyte Leakage Measurement

To quantify the cell death phenotype, electrolyte leakage was measured according to Huang et al. 2013. Four leaf discs were collected from *A. tumefaciens* infiltrated area and immersed in 10 ml sterile milliQ water in 12 well cell culture plates. The plates were incubated in the growth chamber (16-hour light at 22°C and 8-hour night at 18°C) and conductivity measured by Delta Ohm HD 9213R1 conductivity meter.

2.3.6 RNA extraction and RT-PCR

100 mg of infected leaves per sample was collected at different days post infection (dpi), frozen in liquid nitrogen and stored at -80 ºC. Total RNA was extracted from *Z. tritici* infected wheat leaves using the RNeasy Mini Kit (Qiagen) and *N. benthamiana* leaves using Trizol (Invitrogen) following manufacturer's instructions. The RNA was then subjected to on-column DNase treatment (Sigma). Quantification of total RNA was carried out using a Nanodrop ND-1000 spectrophotometer. Reverse transcription of 1-2ug RNA was carried out using Omniscript RT Kit (Qiagen). Semi-Quantitative PCR was conducted to quantify the expression of effector candidates in *N. benthamiana* leaves using gene specific primers (Appendix 1). PCR conditions were as follows: 1 cycle of 5min at 95°C; 35 cycles of 30s at 95°C, 35s at 50-60°C, 35s at 72°C; and a final cycle of 72°C for 5min. PCR products were visualized after electrophoresis in 1-2% agarose gel.

Real-Time quantitative PCR was carried out in 12.5 µl reactions including 1.25 µl of a 1:5 (v/v) dilution of cDNA, 0.2 µM of primers, and 1× SYBR Premix Ex Taq (Tli RNase H plus, RR420A; Takara). PCR conditions were as follows: 1 cycle of 1 min at 95°C; 40 cycles of 5 s at 95°C and 20 s at 60°C; and a final cycle of 1 min at 95°C, 30 s, at 55°C, and 30 s at 95°C for the dissociation curve. The threshold cycle (Ct) values obtained by qRT-PCR were used to calculate the relative gene expression using the Equation $2^{-(\text{Ct target gene} - \text{Ct housekeeping gene})}$ as described previously (Livak and Schmittgen 2001). *Z. tritici tubulin* gene and *N. benthamiana actin* gene were used as housekeeping controls.

2.3.7 Statistical Analysis

Statistical analysis of the data was carried out using the R statistical software (R Core Team, 2016). All the data from the studies were checked for normal distribution and when necessary, variances were stabilized using Box-cox transformation. A generalised linear model was used to test all the data and significant differences were determined using the Tukey test at $p<0.05$.

2.4RESULTS

2.4.1 *Insilico* selection of *Z. tritici* small secreted protein candidates *(ZtSSPs).*

The publicly available secretome dataset from Amaral et al. 2012 was mined to identify non-annotated *Z. tritici* small secreted proteins (*ZtSSPs*). 262 candidate genes with expressed sequence tag (EST) support were screened based on small size (50 to 300 amino acids) resulting in 102 *ZtSSPs*. These proteins were then sorted based on number of cysteine residues (≥1) which resulted in 90 *ZtSSPs*. Candidate proteins were then analysed for the presence of any functional domain using NCBI blast and CDD database (Bauer et al. 2011). This resulted in the identification of 50 proteins without functional annotation (Table 1). These candidates were further confirmed in the RNA-Seq dataset of Longbow infected with IPO323 7dpi (*Mascerello* unpublished), 8 of the selected candidates were not present in this dataset. Further analysis of these candidates showed that 58% of these candidates were predicted to be an effector protein while, 82% of them were predicted to be an apoplastic proteins (Table 1) (Sperschneider et al. 2018).

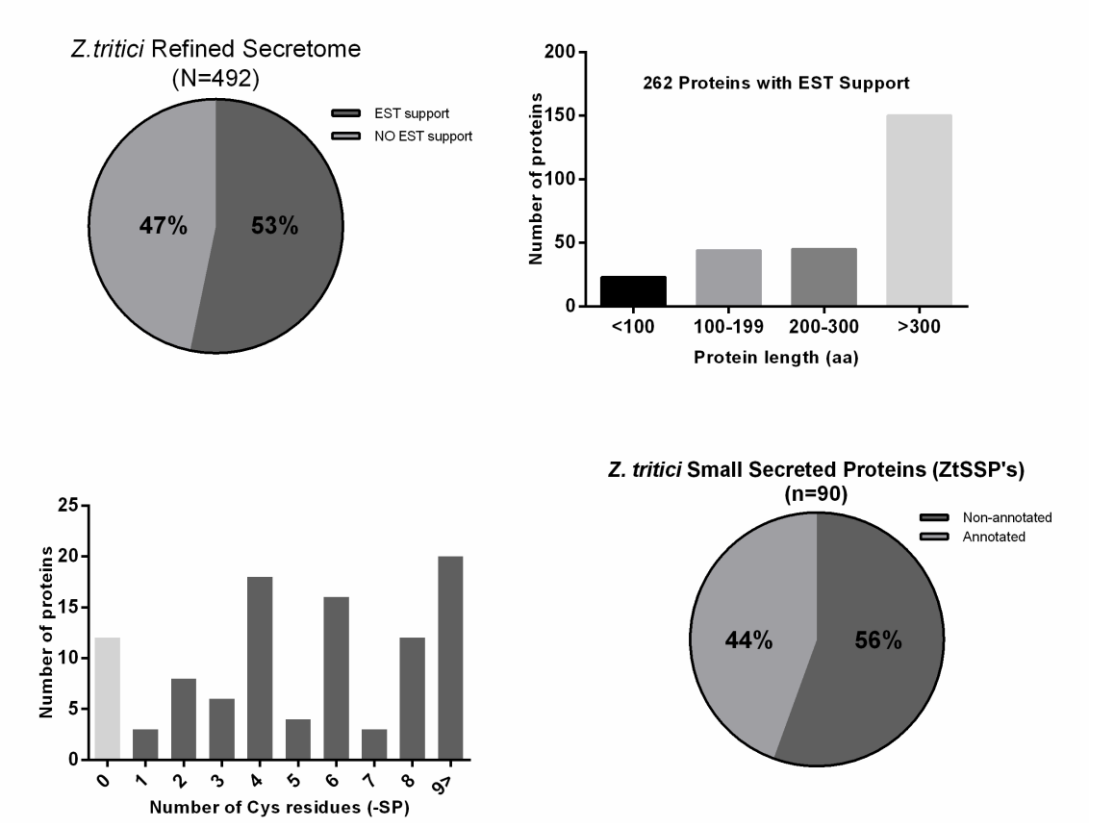


Fig. 1 *In silico* selection of non-annotated small secreted proteins (*ZtSSP*) of *Z. tritici*. (A) Selection of small proteins, 492 proteins encoded by the *Z. tritici* genome with EST support using doAmaral et al. 2012. (B) Proteins were classified based on their size and proteins with 50-300 (-SP) amino acid length were selected. (C) Numbers of cysteine residues in the selected mature proteins after removing signal peptide. (D) Proteins were categorised based on the presence of functional domain by searching NCBI CDD database.

Table 1: **List of putative candidate effector proteins of *Z. tritici*.** Gene ID corresponds to JGI gene ID, Gene annotation were obtained from JGI database and blast search with NCBI CDD database. EffectorP 1.0 and ApoplasticP 1.0 was used to predict whether proteins were predicted effectors and their localisation respectively: '+' and '-'indicates positive and negative prediction.

Codes	Gene ID	Size (aminoacids)	Gene annotation	RNAseq Support (Mascarello unpublished)	EffectorP 1.0 Prediction	ApoplastP 1.0 Prediction	
						Full length	No Signal Peptide
ZtSSP1	Mycgr3G73448	180	Hypothetical	✓	+	-	-
ZtSSP2	Mycgr3G105265	200	Hypothetical	✓	-	+	+
ZtSSP3	Mycgr3G81079	258	Hypothetical	✓	+	-	-
ZtSSP4	Mycgr3G103091	83	Hypothetical	✓	-	+	+
ZtSSP5	Mycgr3G99161	165	Hypothetical	✓	+	+	+
ZtSSP6	Mycgr3G100649	76	Hypothetical	✓	+	+	+
ZtSSP7	Mycgr3G102617	158	Hypothetical	✓	+	+	+
ZtSSP8	Mycgr3G103900	130	Hypothetical	✓	+	+	+
ZtSSP9	Mycgr3G104000	181	Hypothetical	✓	+	-	-
ZtSSP10	Mycgr3G104404	180	Hypothetical	✓	-	+	+
ZtSSP11	Mycgr3G104444	80	Hypothetical	✓	+	+	+
ZtSSP12	Mycgr3G104794	158	Hypothetical	✓	+	-	-
ZtSSP13	Mycgr3G105182	144	Hypothetical	✓	+	+	+
ZtSSP14	Mycgr3G105223	189	Hypothetical	✓	+	+	+
ZtSSP15	Mycgr3G105659	183	Hypothetical	✓	-	-	-
ZtSSP16	Mycgr3G105826	99	Hypothetical	✓	+	+	+
ZtSSP17	Mycgr3G105896	193	Hypothetical	✓	+	-	-

ZtSSP18	Mycgr3G106445	120	Hypothetical	✓	+	+	+
ZtSSP19	Mycgr3G108482	109	Hypothetical	✓	+	+	+
ZtSSP20	Mycgr3G110220	132	Hypothetical	✓	+	+	+
ZtSSP21	Mycgr3G111008	220	Hypothetical	✓	-	+	+
ZtSSP22	Mycgr3G111382	191	Hypothetical	✓	-	+	+
ZtSSP23	Mycgr3G67799	273	Hypothetical	✓	-	+	+
ZtSSP24	Mycgr3G68477	315	Hypothetical	✓	-	+	+
ZtSSP25	Mycgr3G79783	185	Hypothetical	✓	-	+	+
ZtSSP26	Mycgr3G90001	287	Hypothetical	✓	-	+	+
ZtSSP27	Mycgr3G101652	77	Hypothetical	✓	+	+	+
Zt-4	Mycgr3G104697	150	Hypothetical	✓	-	+	+
Zt-5	Mycgr3G108774	304	Hypothetical	✓	-	+	+
Zt-10	Mycgr3G111505	198	Hypothetical	✓	-	+	+
Zt-14	Mycgr3G107286	117	Hypothetical	✓	+	+	+
32	Mycgr3G102996	164	Hypothetical	✓	+	+	_
33	Mycgr3G103254	101	Hypothetical	✓	+	+	+
34	Mycgr3G103792	197	Hypothetical	✓	+	+	+
35	Mycgr3G104758	119	Hypothetical	✓	+	+	+
36	Mycgr3G104867	171	Hypothetical	✓	-	+	+
37	Mycgr3G105677	199	Hypothetical	✓	+	+	+
38	Mycgr3G106106	157	Hypothetical	✓	-	+	+
39	Mycgr3G106345	164	Hypothetical	✓	-	+	+
40	Mycgr3G106502	90	Hypothetical	✓	+	+	+

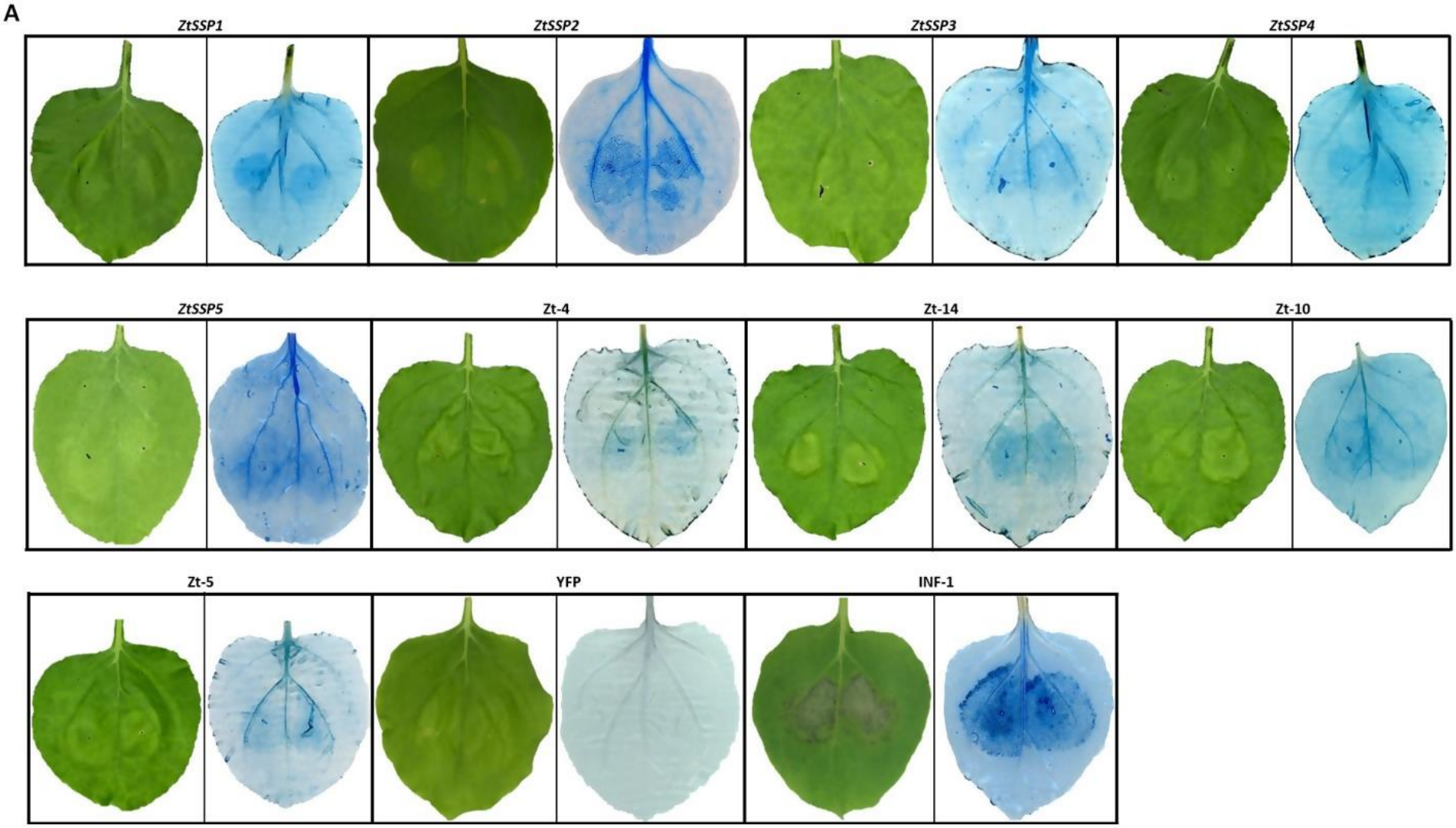
41	Mycgr3G107824	137	Hypothetical	×	-	+	+
42	Mycgr3G108329	193	Hypothetical	×	+	-	-
43	Mycgr3G108877	112	Hypothetical	×	+	+	+
44	Mycgr3G110052	180	Hypothetical	✓	+	-	-
45	Mycgr3G110144	222	Hypothetical	×	-	+	+
46	Mycgr3G41315	196	Hypothetical	✓	-	-	-
47	Mycgr3G69789	308	Hypothetical	✓	-	+	+
48	Mycgr3G95672	142	Hypothetical	×	+	+	+
49	Mycgr3G99124	113	Hypothetical	×	+	+	+
50	Mycgr3G99917	311	Hypothetical	×	-	+	+

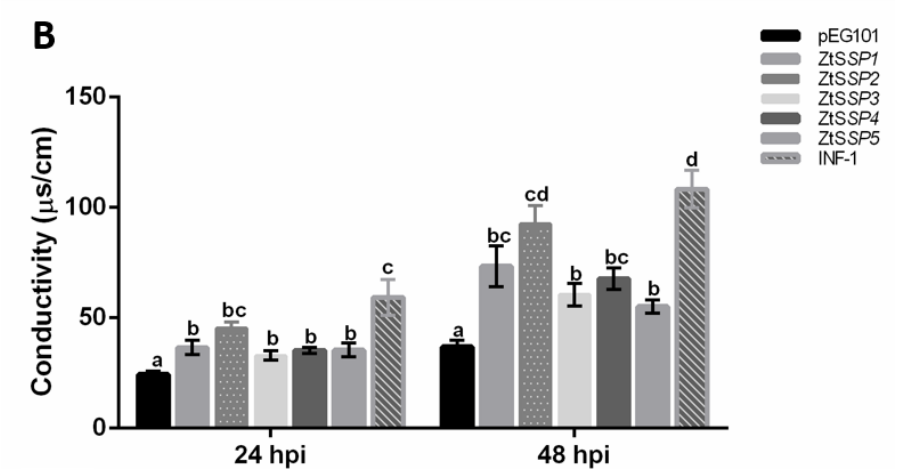
2.4.2 Transient expression of *ZtSSP* triggers prominent cell chlorosis in *N. benthamiana*.

Cell death symptoms in wheat infected with *Z tritici* can be detected with trypan blue staining from 4 days post infiltration (dpi) onwards (Reilly & Karki et al. unpublished). To test whether the *Z. tritici* candidate effector proteins induce cell death, cloned *ZtSSPs* were transiently overexpressed in *N. benthamiana* leaves using *A. tumefaciens*. Agro-infiltration in *N. bethamiana* has been widely used as a rapid assay to identify genes from an array of pathogens such as *M. oryzae, rust and Z. tritici* that regulate plant immunity (Chen et al. 2013; Qi et al. 2016, Kettles et al 2017).The agro-infiltrated leaves were then visualised at 4-5 dpi for appearance of cell death. Of the thirty candidates tested (Fig 2A, 3A) nine were found to induce differing levels of leaf chlorosis and subsequent staining with trypan blue which resulted in the visualisation of dead cells (Fig 2A). The vector alone and PEG: YFP did not induce plant cell death.

The candidates *ZtSSP1, ZtSSP2, ZtSSP3, ZtSSP4, ZtSSP5, Zt-4, Zt-5, Zt-10* and *Zt-14* in this study were found to induce cell death. Of these, five candidates, namely *ZtSSP1, ZtSSP2, ZtSSP3, ZtSSP4 and ZtSSP5* are unique to this study while four candidates, *Zt-4*, *Zt-5*, *Zt-10* and *Zt-14* were previously reported by Kettles et al. 2017. Quantification of the cell death phenotype induced by *ZtSSPs* were performed by measuring ion leakage (Fig 2B). A significant amount of ion leakage was measured at 24 and 48 hours post inoculation (hpi) after infiltration of *A. tumefaciens* harbouring *ZtSSP1-5* compared to the vector control. Full length (+SP) *ZtSSP1-5* also induces a cell death phenotype similar to that induced by candidates lacking the signal peptide (2C).

Expression of these candidate effectors in *N. benthamiana* leaves were confirmed using RT-PCR performed on leaf discs taken from sites infiltrated with infiltration buffer, Vector control (*ccdB* gene), and PEG:*ZtSSP1-27* (Fig 2D, 3A & 3B).


A
ZtSSP1
ZtSSP2
ZtSSP3
ZtSSP4
ZtSSP5
Zt-4
Zt-14
Zt-10
Zt-5
YFP
INF-1


B
pEG101
ZtSSP1
ZtSSP2
ZtSSP3
ZtSSP4
ZtSSP5
INF-1
Conductivity (µs/cm)
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a
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cd
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24 hpi
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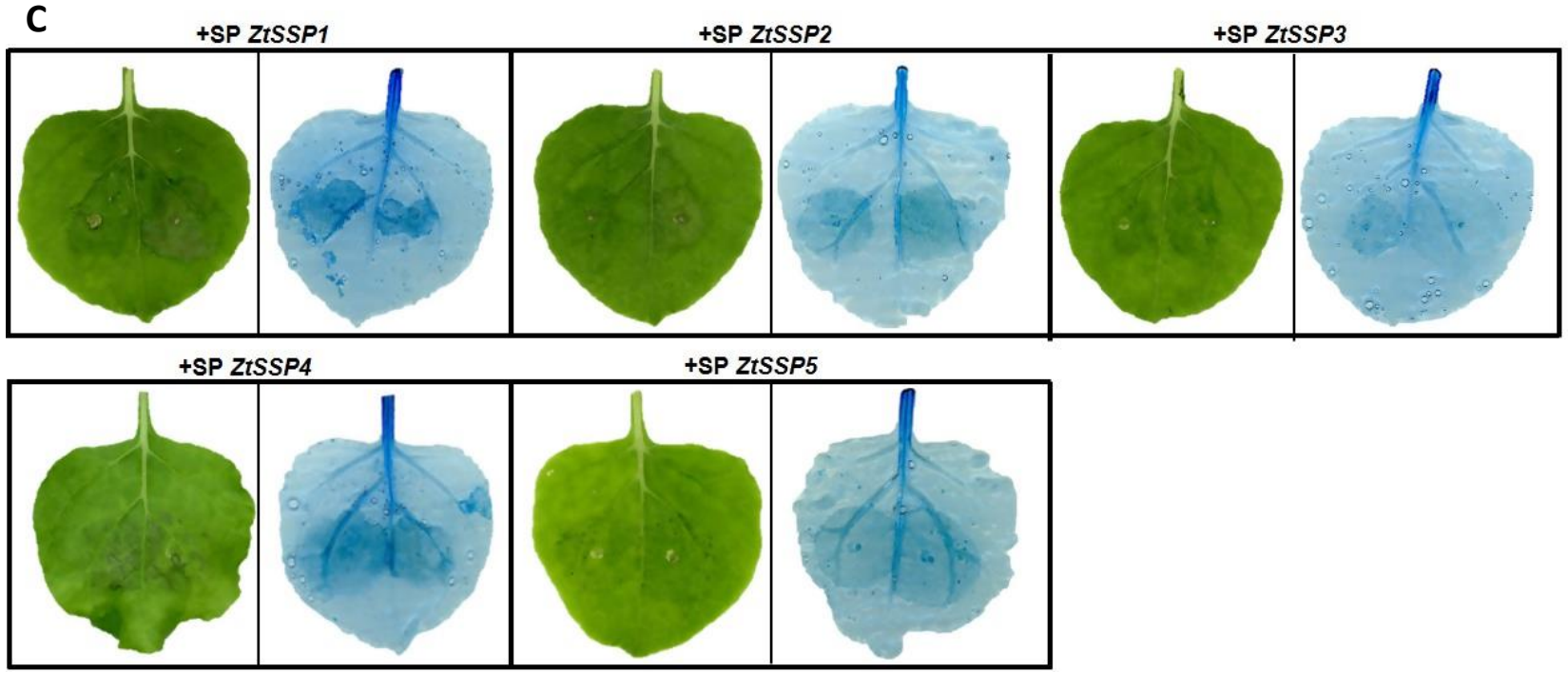
C
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+SP ZtSSP3
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+SP ZtSSP5

D

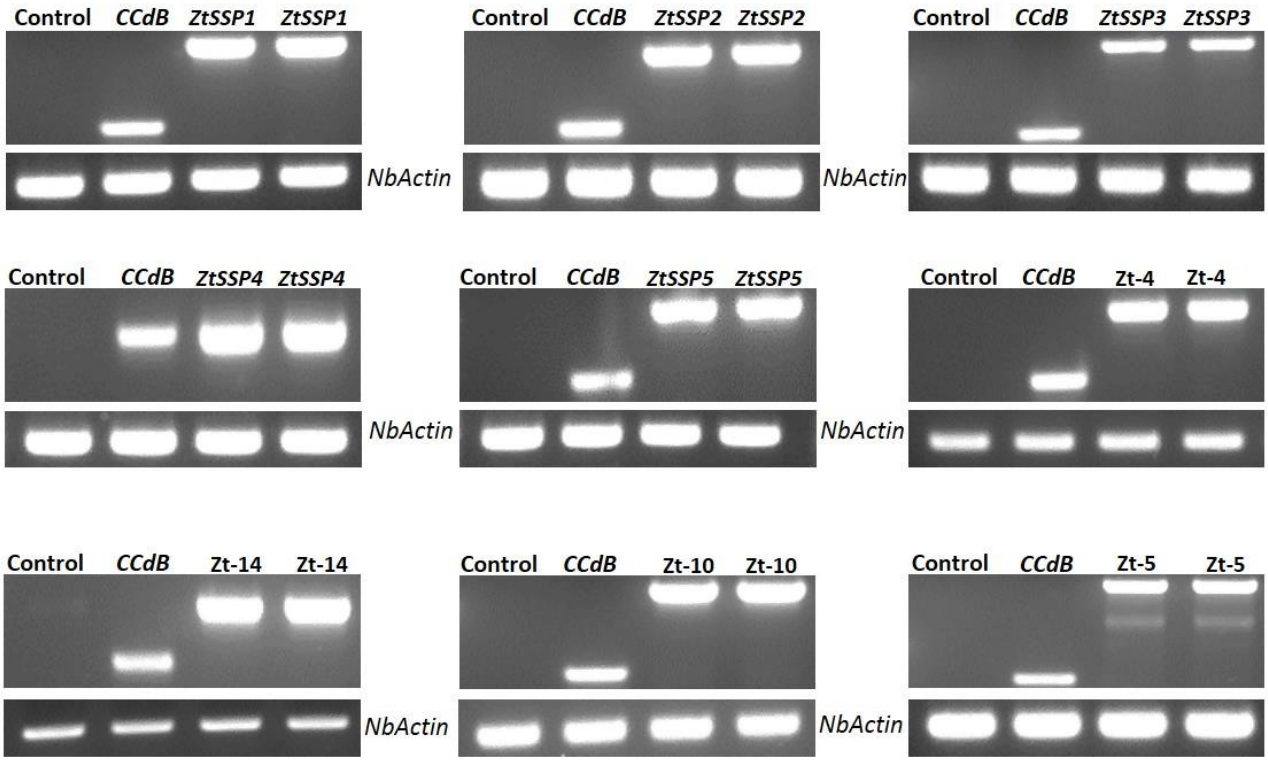
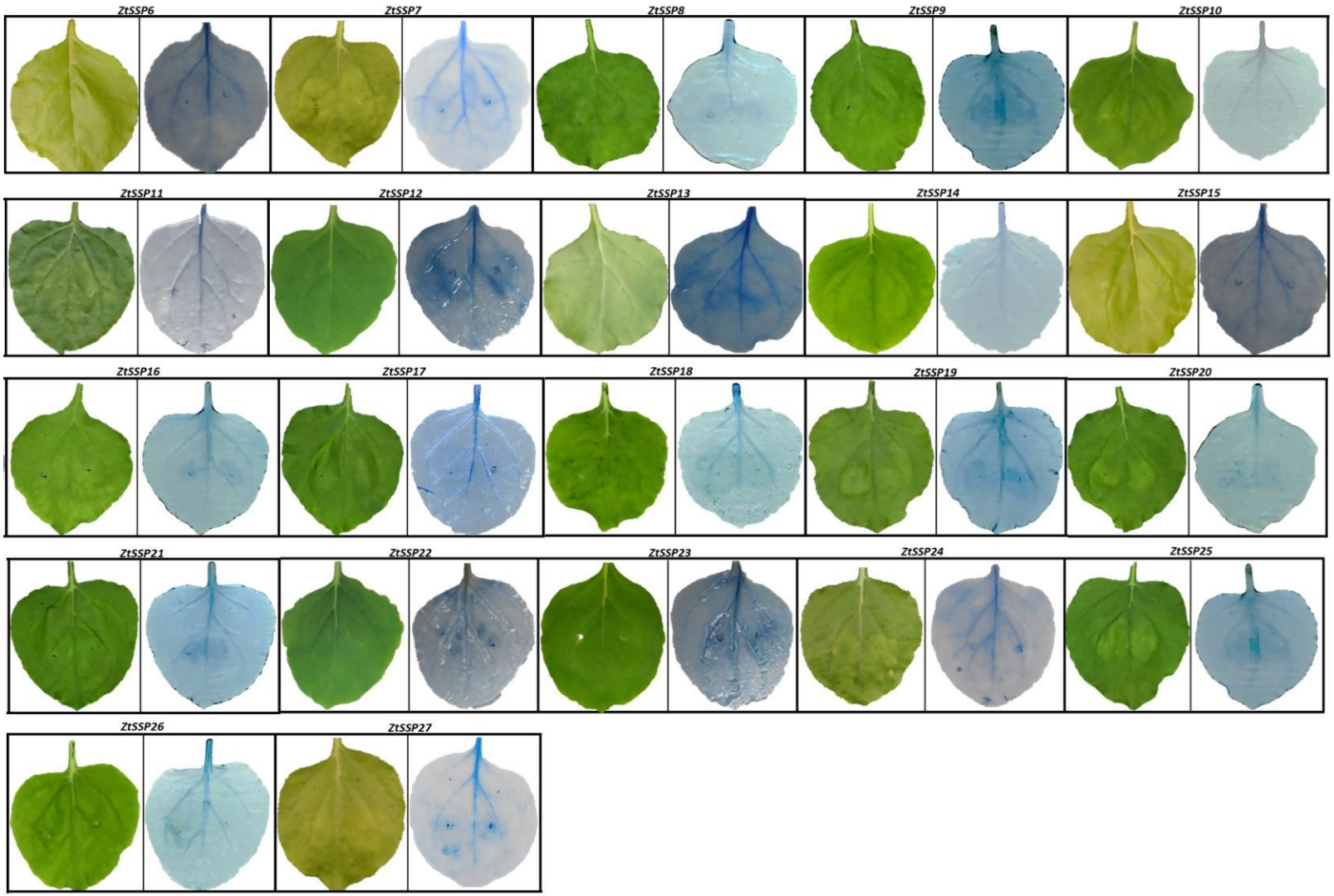


Fig. 2. Transient expression of small secreted proteins from *Z. tritici* induce cell death in non-host *N. benthamiana*. Leaves were stained with trypan blue at 4 dpi. (A) Phenotype obtained after infiltration of cell death inducing candidates. Candidate effectors (*Zt-4, Zt-5, Zt10, Zt-14*) in our list overlaps with study from Kettles et al. 2017 which was identified as cell death inducers. (B) Electrolyte leakage was measured in the infiltrated leaves at the indicated time points after agroinfiltration (hpi: hours post infiltration). (C) Phenotype after infiltration of full length (+SP) of 5 cell death inducing candidates. (D) Expression of *ZtSSPs* were validated using semi-quantitative PCR using gene specific primers. Experiment was repeated three times (n=8) and error bars represent the standard error mean (SEM). Different letters represents significant differences determined Tukey test ($P<0.05$) compared to control.

A


ZtSSP6
ZtSSP7
ZtSSP8
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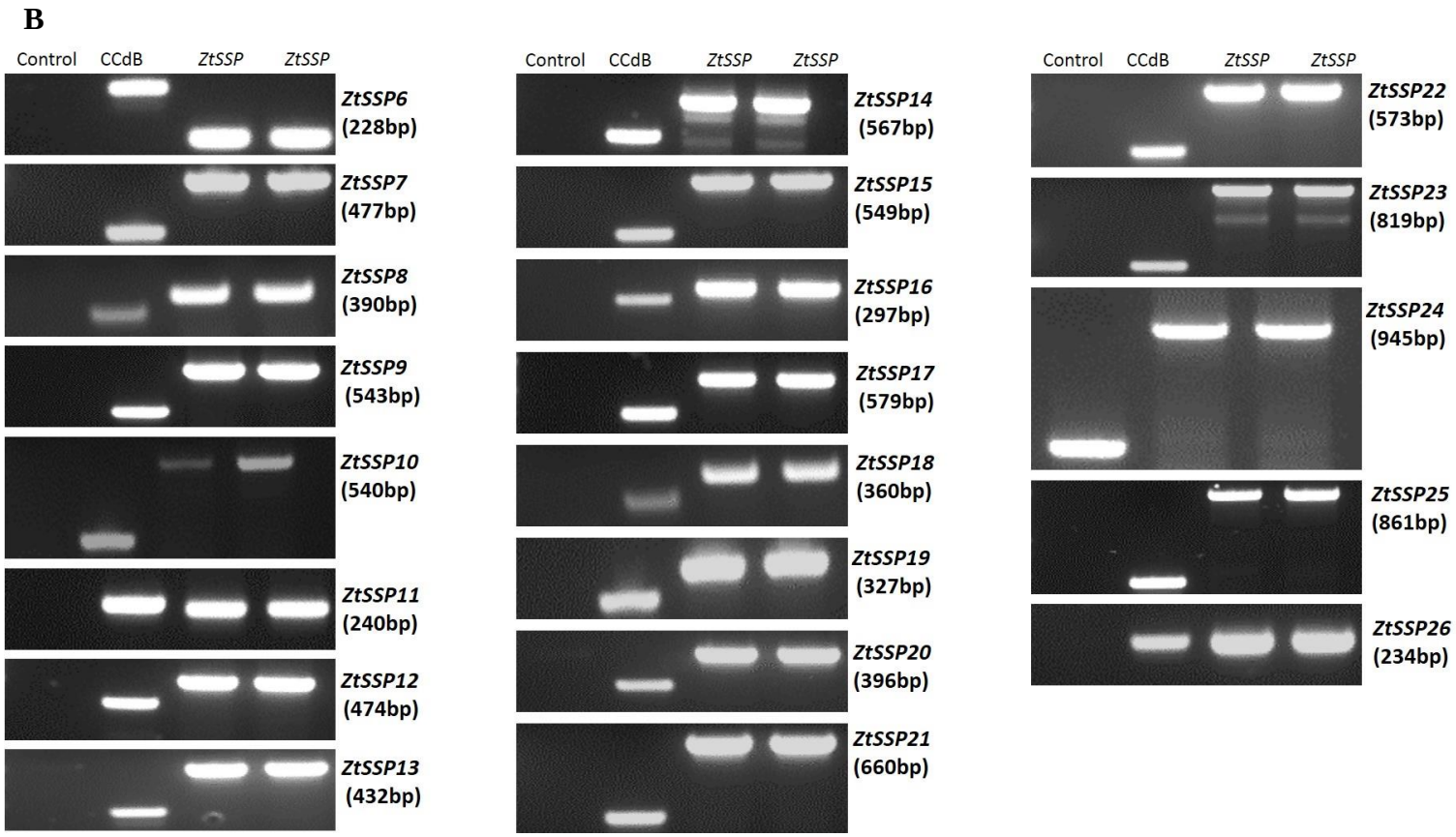

Fig. 3.**Transient expression of small secreted proteins from *Z. tritici* in non-host *N. benthamiana***. **(A)** Agrobacterium harbouring effector plasmids were infilterated into 4-6 week old *N. benthamiana* leaves. Leaves were visualized at 4 dpi (left) and subsequently stained with trypan blue at 4 dpi (right). **(B)** Expression validation of effector in *N. benthamina.* Total RNA was extracted from *N. benthamaina* leaves at 36 h post inoculation. Gene specific primers were used to amplify effector expression. The controls included tissue from infiltration buffer infiltrated sites and *ccdB* gene present in the plasmid pEARLYGATE101 plasmid lacking a cloned insert.

2.4.3 Expression of *ZtSSP* resulted in accumulation of H_2O_2 in *N. benthamiana*

Rapid accumulation of H_2O_2 as a part of oxidative burst is a well-known phenomenon in both host and non-host resistance responses (Huckelhoven et al. 2001; Trujillo et *al.* 2004). To examine if the cell death phenotype obtained by *ZtSSP1-5* in *N. benthamiana* is associated with the rapid accumulation of H_2O_2, leaves expressing *ZtSSP1-5* were stained for H_2O_2 at 48 hpi. The expression of the candidates resulted in rapid accumulation of H_2O_2 (Fig 4A). The relative staining was significantly higher ($P<0.01$) in all the *ZtSSP1-5* infiltrated leaves compared to vector control leaves (Fig 4B).

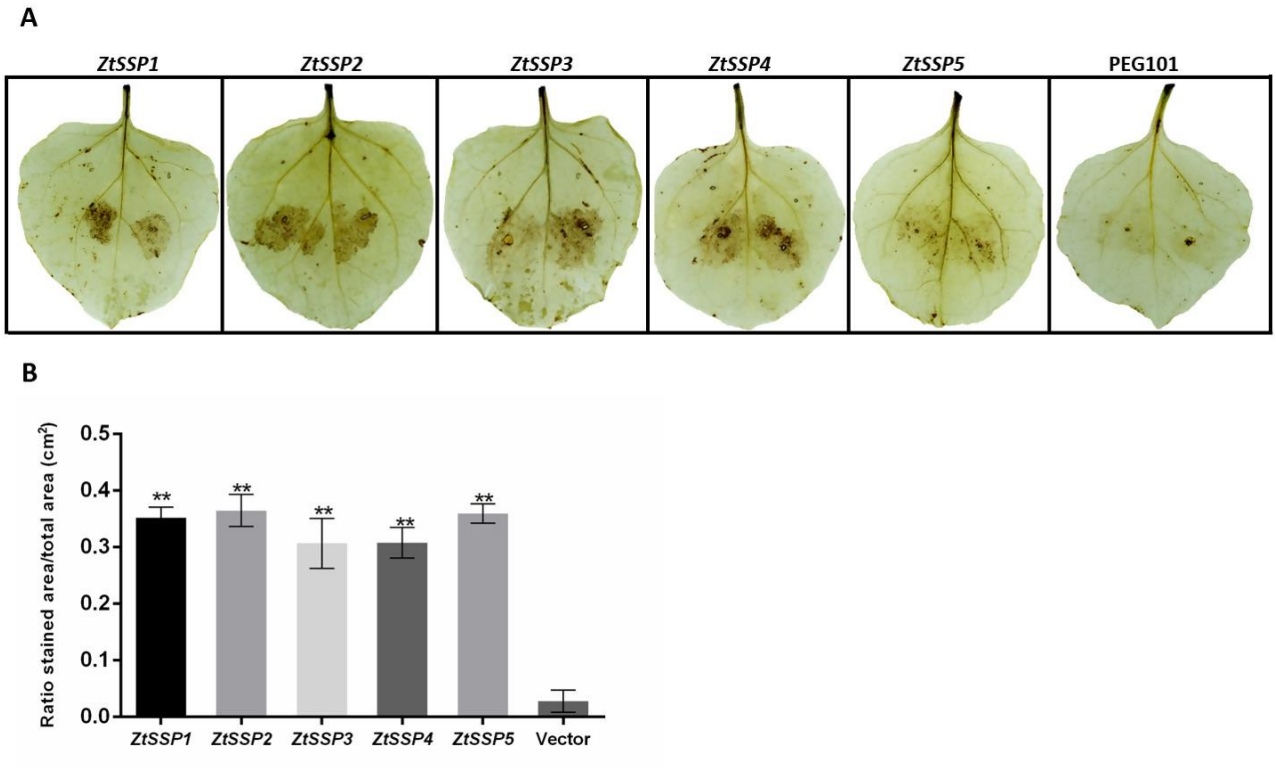

Fig. 4. Accumulation of H_2O_2 in *N. benthamiana* leaves expressing *ZtSSP* candidate proteins at 2 dpi. Representation of leaf image showing H_2O_2 accumulation in the leaves as detected using DAB staining. The quantification of H_2O_2 stained area relative to total leaf area was measured using ImageJ (Bethesda, MD, USA), and the ratio (stained area/total leaf area) was calculated. Experiment was repeated three times (n=5) and error bars represent the standard error mean (SEM). The asterisk represents significant differences determined Tukey test (** $P<0.01$).

2.4.4 Expression of *ZtSSP* candidates in *N. benthamiana* induces expression of plant defense

To investigate the role of *ZtSSP* in activation of *N. benthamiana* defence associated genes, the relative transcript levels of key defence marker genes; Pathogenesis-related (PR) genes *PR1a*, *PR2*, *PR3 (chitinase)*, *Endochitinase B* and transcription regulator *WRKY12* were assessed (Fig 5). *ZtSSP1* and *ZtSSP3* were found to significantly induce expression of *PR1a* at 4dpi compared to the control ($p<0.05$). Interestingly, candidate *ZtSSP4* significantly reduced the expression of *PR1a* and *PR2* at 4dpi ($p<0.05$) compared to control. However, in contrast, *PR2* was not induced by any of the candidates tested. Candidate *ZtSSP2* was found to highly induce expression of *PR3 at* 4dpi ($p<0.01$) while, *ZtSSP3* induced *PR3* at 2dpi and 4dpi ($p<0.05$). There was also significant downregulation ($p<0.01$) of *PR3* expression with *ZtSSP1* at 4dpi. Similarly, the candidate *ZtSSP3* was found to induce expression of *endochitinase B* compared to the control at 2dpi. The transcriptional regulator *WRKY12* is known to play diverse role in biotic and abiotic stress (Li et al. 2016). Candidate *ZtSSP3* and *ZtSSP5* induce expression of *WRKY12* at 2dpi ($p<0.05$), while, it was significantly up-regulated by candidate *ZtSSP2*, *ZtSSP3* and *ZtSSP5* at 4 dpi. The expression of *WRKY12* was significantly downregulated by candidate *ZtSSP4 at* 4dpi ($p<0.0001$). Therefore, each *Z. tritici* candidate triggers the activation or repression of a unique set of defence genes related to different pathways suggesting their diverse role during interaction with the host.

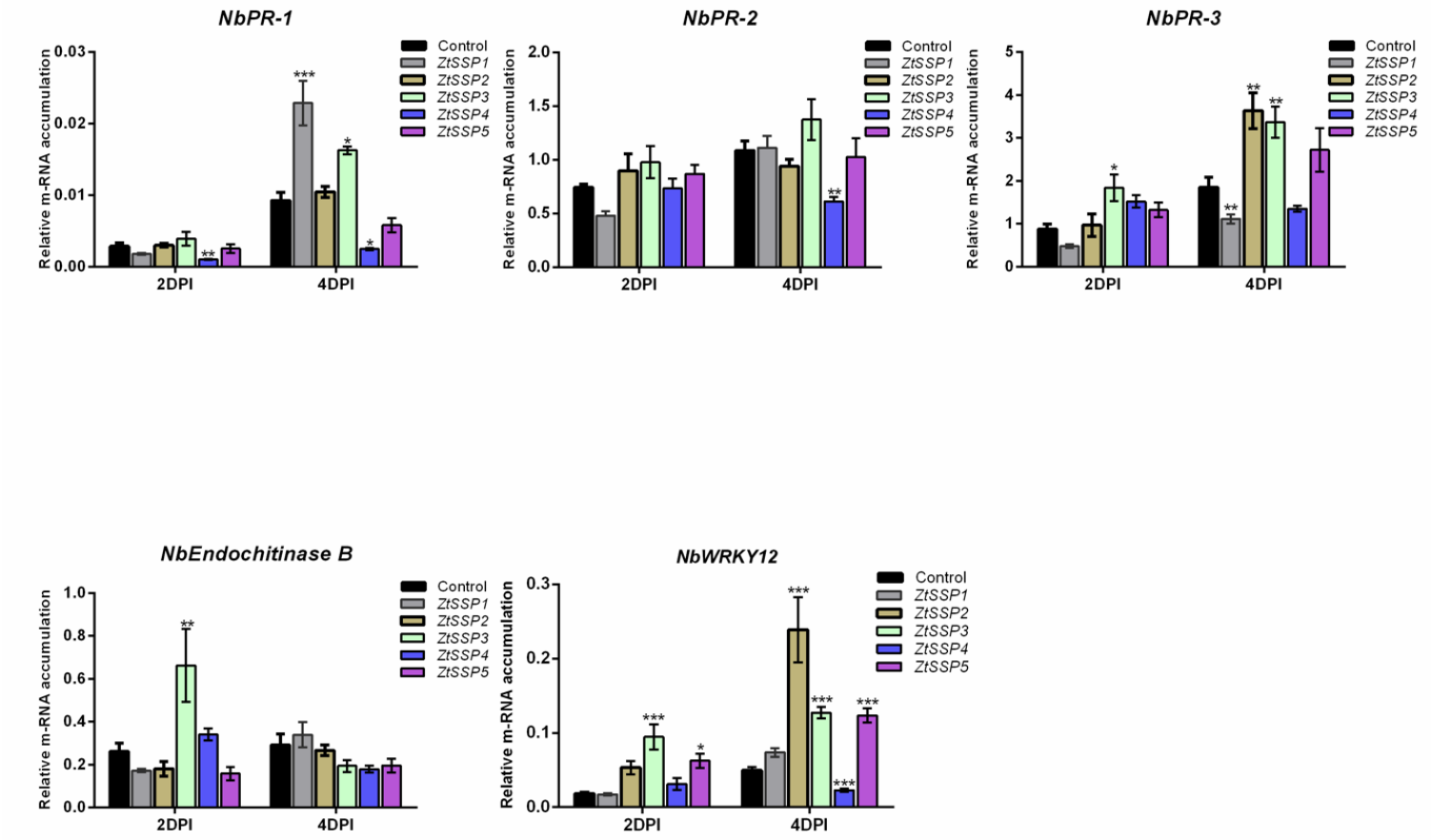

Fig. 5. Gene expression of several defense genes in *N. benthamina* leaves transiently expressing *ZtSSP* candidates. Total RNA was extracted from *N. benthamina* leaves infiltrated with *A. tumefaciens* carrying either PEG101: *ZtSSP* or PEG101-empty (Control) at 2 and 4 dpi. RNA was reverse transcribed into c-DNA. The gene expression was measured relative to the actin house keeping gene. Bars represent means ± standard error mean (SEM) from three biological replicates. The asterisk on top of bar represents significant differences determined by Tukey test against Empty vector control at the same time point (* for P<0.05, **for P<0.01 and *** for P<0.0001).

2.4.5 *ZtSSP* candidate genes expression during infection in wheat

The pathogen effector genes are known to be induced transcriptionally during infection of the host plant (Stergiopoulos & de Wit 2009). Quantitative reverse transcription PCR (qRT-PCR) was carried out to determine regulation of candidate *ZtSSP's* during infection. The *Z. tritici* isolate 560.11 (Lynch et al. 2016) was used to infect wheat (cv. Remus) and the expression of *ZtSSPs* determined at 2, 4, 8, 10, 14, 21 days post inoculation (dpi) (Fig. 6). Based on expression analysis these genes could be divided into three groups. The first group includes *ZtSSP1, ZtSSP3 and ZtSSP5* which was highly expressed at 21 dpi (Appendix 6 for disease symptoms at 21dpi), while the second group of candidates includes *ZtSSP2* only which was expressed at 2 dpi through to 21dpi, while the final group which includes *ZtSSP4* only was highly induced at 14 dpi.

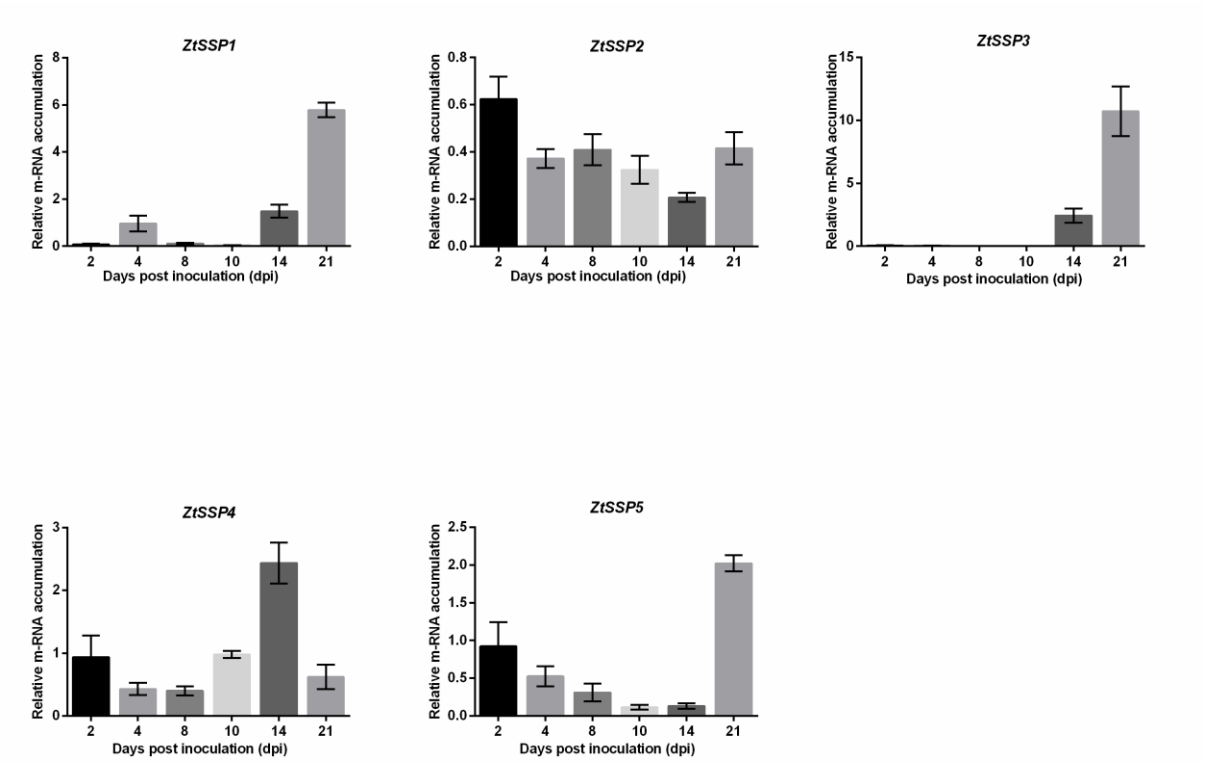

Fig.6. Gene expression analysis of small secreted protein (*ZtSSP1-5*) at 2, 4, 8, 10, 14, 21 days post inoculation of wheat (cv.Remus) with *Z. tritici* isolate 560.11. RNA was extracted from wheat infected leaves followed by reverse transcription into cDNA. The qPCR was performed on cDNA using specific primers for each candidate gene. The expression of each ZtSSPs was measured relative to that of house keeping gene *β-tubulin* (*Z. tritici*). The error bars represent the mean relative expression of three independent experiments ± SEM.

2.5 DISCUSSION

N. benthamiana is used as a model plant for a rapid and efficient screen of diverse pathogen effector candidates. For example, a study by Chen et al. 2013 identified five cell death inducing proteins (MoCDIP) from *M. oryzae* using transient expression in rice protoplasts. Four out of five MoCDIP also induced cell death in *N. benthamaina*. Similarly, an immune suppression assay was carried out for effectors of the soyabean rust fungus (Qi et al. 2016). The candidate effector of soyabean rust pathogen *P. pachyrhizi (*PpEC23) was able to suppress the HR induced by *Pseudomonas syringae* pv. tomato strain DC3000 (*Pst* DC3000) in soyabean leaves as well as in *N. benthamiana*. Another example of effector suppression was reported from wheat rust (Ramachandran et al. 2017) where an effector candidate that supressed PCD in *N. benthamiana* was also found to suppress the HR in wheat. Screening of *Z. tritici* necrosis and ethylene inducing peptide 1 (Nep-1)-like proteins (MgNLP) showed that this protein induced cell death in *Arabidopsis* and tobacco leaves. However, infiltration of the MgNLP failed to induce cell death activity in wheat (Motteram et al. 2009). Recent study by Kettles et al. 2018 identified a potent cell death inducer (Zt6) of *Z. tritici* using *N. benthamiana* that in addition, could induce cell death in wheat.

Candidate effector proteins are known to have less sequence similarity to annotated proteins (Stergiopoulous & de Wit 2009). Therefore, non-annotated proteins were selected for this study. Firstly, no prominent cell death phenotype was observed from any of the candidates tested. It was found that nine candidates could induced leaf chlorosis at 4-5 days post infiltration and subsequent cell death was visualised with trypan blue staining. This observation was similar that made by Kettles et al. 2017 where the phenotype observed was vector dependent. This resulted in the identification of five novel cell death inducing candidates of *Z. tritici* in this study.

In order to test whether the signal peptide had any effect on the phenotype induced by the five candidates, these *ZtSPP*s were further cloned with signal peptide and re-tested for induction of a cell death phenotype (Fig 2c). Interestingly, no major difference in the phenotype induced was observed when expressed with or without a SP for these 5 effector candidates. There are reports of candidate effectors without a SP displaying mild to no phenotype (Chen et al. 2013; Ma et al. 2015; Fang et al. 2016; Kettles et al. 2017). Recently it was reported that the presence of a SP is not the signal that separates apoplastic proteins from non-apoplastic proteins (Sperschneider et al., 2017). The localisation predictor ApoplastP (Sperschneider et al., 2017)

suggests that irrespective of the presence or absence of a SP, three of the cell death inducing proteins were localised in the apoplast (Table 1). A similar observation was reported by Habash et al. 2017 where a putative effector protein from *H. schachtii* without SP was found to be localised in the apoplast. However, one cannot rule out the possibility that these proteins could be recognized in the *N. benthamiana* cytoplasm since *ZtSSP1* and *ZtSSP3* are not predicted to be apoplastic (Table 1). The latter case was true for oomycete RxLR effectors that induced cell death in the absence of a signal peptide (Morgan & Kamoun 2007) suggesting recognition by cytoplasmic receptors. Thus, the exact localisation of these candidate proteins with and without signal peptide remains to be further explored.

One of the common attributes of PTI is the rapid accumulation of ROS at challenged sites (Torres 2010). It was shown that receptors of *N. benthamaina* namely; *NbBAK1* and *NbSOBIR1* are involved in recognition of *Z. tritici* effectors (Kettles et al. 2017). Therefore, the results also suggest that expression of *ZtSSPs* in *N. benthamaina* resulted in a cell death phenotype and ROS accumulation most likely due to early perception and a subsequent PTI response.

Fungal pathogen effectors are known to have diverse functions and are shown to be differentially regulated throughout infection (Chen et al. 2013). Here, in this study the five *ZtSSP* candidates were also differentially expressed and have variations in their expression profile during infection of host plant wheat (Fig 6). Despite all five *ZtSSP's* inducing cell death, there was differences in the activation of defence associated marker genes in *N. benthamaina*. *PR1* and *PR2* are salicylic acid responsive genes and are typically induced by biotrophic pathogens (Uknes et al. 1992). Candidates *ZtSSP1* and *ZtSSP3* were found to significantly upregulate expression of *PR1* while *ZtSSP4* downregulated *PR1a* and *PR-2* expression at 4dpi compared to the control. This suggests that expression of candidates *ZtSSP1* and *ZtSSP3* may target salicylic mediated defence pathways. Additionally, the Jasmonic acid pathway and the *WRKY* transcription factor play a role in resistance against necrotrophic pathogens (Wang et al. 2017; Spoel et al. 2012; Zheng et al. 2006). Study by Raffaello & Asiegbu 2017 found that transient expression of the secreted protein (HaSSP30) from the necrotrophic pathogen *Heterobasidion annosum* led to cell death in *N. benthamiana* and triggered induction of genes such as *PR3*, *PR4*, and *WRKY12*. Similarly, in our study candidate *ZtSSP2 and ZtSSP3* significantly up-regulated expression of *PR3 and WRKY12* at 4dpi. *ZtSSP3* was also found to induce expression of *endochitinase B* and *WRKY12* at 2dpi. This suggests that candidate *ZtSSP2* and *ZtSSP3* might also play a role in the necrotrophic stage of *Z. tritici* infection. Gene

expression of the candidates showed that they are expressed at the early necrotrophic and late necrotrophic stage of *Z. tritici* infection that potentially correlate with their cell death inducing ability.

N. benthamiana is phylogenetically distant to monocot wheat and it is surprising that an un-adapted *Z. tritici* pathogen proteins (*ZtSSP*) induces PCD. This finding and the study of Kettles et al. 2017 raises an interesting scenario where multiple *ZtSSP* candidates were recognised by a non-host plant, although *Z. tritici* is known to be a specialized pathogen of wheat. It could be hypothesized that *N. benthamiana* possesses a number of receptors that recognise specific *Z. tritici* effectors. There could be a few another possible explanations that perception is by an indirect recognition of these *ZtSSP* candidates via a guard or decoy protein (van der Biezen & Jones1998b; Innes 2004). In addition, it has been shown that effector proteins from evolutionary diverse pathogen targets a limited number of host protein hubs (Mukhtar et al. 2011). In this context, un-adapted pathogen effector proteins could be monitored indirectly through any cellular and physiological perturbation caused by the protein or guard or decoy proteins that subsequently activates the non-host immune system. Therefore, the activation of defense and cell death might be as a result of an effector target present in both wheat and *N. benthamiana*.

In conclusion, this study identified five novel *ZtSSP* candidates that induce cell death in the non-host plant *N. benthamiana*. The expression of these candidates in *N. benthamiana* resulted in rapid induction of ROS, cell death and expression of key defense markers. The findings from this study could potentially facilate further investigation into non-host immune receptors or conserved plant hubs which are effector tragets. Once identified these proteins could act as a source for engineering durable resistance in wheat against *Z. tritici*.

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Chapter 3

Z. tritici candidate effector polymorphism and identification of wheat host interacting partners

A manuscript is in preparation with work from Chapter 2 and 3:

"Screening of small secreted proteins of *Z. tritici* reveals a candidate protein that targets host ubiquitin system".

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3.1 ABSTRACT

The wheat infecting fungal pathogen *Zymoseptoria tritici* is known to produces array of small secreted proteins predicted to be play role in its pathogenicity. Previously five ZtSSP candidates were identified that induce cell death in the non-host plant *N. benthamiana*. These cell death inducing candidates were validated to be functionally secreted using a yeast secretion assay. SNP analysis with 54 isolates of *Z. tritici* showed that four out of the five candidates were present in all isolates and showed some degree of polymorphism. A yeast two-hybrid assay using one of the prominent candidate *ZtSSP2* showed that it interacts with wheat E3 ubiquitin ligase in yeast and *in planta*. Expression analysis of wheat ubiquitin ligase suggests that wheat infected *Z. tritici* plants showed significant downregulation of *TaE3UBQ* expression at specific time points (4 dpi and 21 dpi) suggesting its potential role in regulating defence signalling during the wheat-*Z. tritici* interaction.

3.2 INTRODUCTION

Plant possess a multi-tier defense system against pathogen infection. The first layer involves the recognition of pathogen associated molecular patterns (PAMPs) through cell associated pattern recognition receptors (PRR) resulting in PAMP triggered immunity (PTI) and the second layer involves recognition of pathogen secreted effector proteins by plant resistance (R) protein which results in effector triggered immunity (ETI) (Jones & Dangl 2006; Chisholm et al. 2006; Schwessinger & Zipfel 2008).

One of the key regulatory process involved in plant defense signalling involves ubiquitination (Craig et al. 2009; Trujillo & Shirasu et al. 2010). Post translational modification of proteins via ubiquitination plays a key role in maintaining protein abundance, function and localisation, thus, it is vital for various cellular processes (Deshaies & Joazeiro 2009; Komander & Rape 2012). Protein ubiquitination usually involves three main enzymes; ubiquitin-activating enzymes (E1), ubiquitin-conjugating enzymes (E2) and ubiquitin ligases (E3). Protein ubiquitination involves activation of ubiquitin by enzyme E1, which is then transferred to the ubiquitin conjugating enzyme (E2) and finally the E3 ligase recruits the E2-ubiquitin and the target protein and transfers the ubiquitin to the target protein. Attachment of ubiquitin to a substrate protein can result in modification of protein activity, localization or degradation by 26S proteasome (Smalle & Vierstra 2004; Metzger et al. 2014).

There are two major classes of E3 ligase: the HECT (homologous to E6-AP carboxyl terminus) and the RING (really interesting new gene ligases) (Pickart 2001; Buetow & Huang 2016). Recent studies have highlighted the important role of E3 ligases during host pathogen interaction. For example, the rapid induction of *Arabidopsis Toxicos en Levadura* (*ATL*) and *Oryzae sativa* EL5(elicitor responsive RING-H2) protein against fungal elicitors, both of which belongs to RING-H2 type E3 ligase and suggests a role in early defense activation (Hondo et al. 2007; Takai et al. 2002). Study by Dhawan et al. 2009 showed the role of *A. thaliana* RING E3 ligase *HUB1* (histone monoubiquitination 1) in mediating resistance against a necrotrophic fungal pathogen. Additionally, the role of *Erysiphe necator*-induced RING finger protein 1 (EIRP1) in mediating resistance against powdery mildew was reported through ubiquitin mediated degradation of the *Vitis pseudoreticulata* WRKY transcription factor 11 (*VpWRKY11*) (Yu et al. 2013).

In addition to resistance response, studies have highlighted a role of these E3 ligase to enhance susceptibility to pathogen infection. For example: The effector *AVR3a* from *P. infestans* interacts and stabilizes the host U-box E3 ligase CMPG1 (Cys, Met, Pro and Gly protein 1) that is required for INF-1 triggered cell death. The Avr3a interaction with CMPG1 leads to CMPG1 modification and thus prevent host cell death induction during infection (Bos et al. 2010). Similarly, rice blast fungus effector *AvrPiz-t* interacts with the rice RING E3 ubiquitin ligase *AvrPiz-t* Interacting Protein 6 (APIP6). This interaction was shown to reduce the APIP6 E3 ligase activity *in vitro* and thus supressing APIP6 mediated defense activation in plants (Park et al. 2012).

In this study, the five cell death inducing *Z. tritici* effector candidates (*ZtSSPs*) identified previously (Karki et al. unpublished; Chapter 2) were further characterised. The *ZtSSPs* were tested for their *in vitro* secretion assay, disordered propensity and sequence variations. One *ZtSSP* namely, *ZtSSP2* was selected for further characterisation based on its ability to induce cell death and activate JA-mediated defense in a non-host *N. benthamiana*. *ZtSSP2* is a small secreted protein (22KDa) with ten-cysteine residues that appears to be well conserved within *Z. tritici* isolates and other ascomycete fungi. Furthermore, *ZtSSP2* interacts with host E3 ubiquitin ligase (*TaE3UBQ*). This study establishes that *ZtSSP2* likely targets a wheat E3 ubiquitin ligase to regulate host immunity and establish further colonisation.

3.3 MATERIALS AND METHODS

3.3.1 Validation of protein secretion using a yeast secretion system

A gateway compatible vector for yeast secretion assay and *suc2* yeast mutant (strain SEY6210) was constructed as described in Plett et al. 2017. The assay was performed as described in Oh et al. 2009. Briefly, the invertase (*SUC2)* gene with and without signal peptide was amplified from the yeast strain BY4741 with a linker (HA-tag-Kex2 cleavage site) added between the gateway reading frame and *SUC2* gene. This construct was then ligated into pGADT7 vector and verified by sequencing. Candidate *ZtSSPs* were cloned into the yeast secretion vector in frame with N terminus of *SUC* gene and transformed into *suc2* yeast mutant. Transformants were PCR validated and then selected on a synthetic dropout medium (minus Trp and leu) plates. Invertase secretion was assayed by plating positive clones onto YPRAA plates. Invertase activity was determined by reduction of colourless TTC to red coloured triphenylformazan (TPF).

3.3.2 Bioinformatics analysis of *ZtSSPs*

The protein sequence of *Z. tritici* candidate proteins were obtained from JGI (http://genome.jgi.doe.gov/Mycg3/Mycg3.home.html) and were analysed for disordered propensity using programs like PONDR VL-XT, IUPred and PrDOS. BLASTp search was performed using the NCBI (National Centre for Biotechnology Information) BLAST service (http://blast.ncbi.nlm.nih.gov/Blast.cgi) and uniprot blast (http://www.uniprot.org/blast/). Multiple alignments were generated using ClustalW2 (http://www.clustal.org), which was then used to generate the phylogenetic analysis using MEGA 7 (Kumar 2016). Maximum likelihood phylogenetic trees were generated using the Jones-Taylor-Thornton (JTT) models. The bootstrap values were obtained from 1000 bootstrap replicates.

3.3.3 Yeast two-hybrid analysis

A yeast two-hybrid screening was performed by Hybrigenics services, SAS (http://www.hybrigenics-services.com). Briefly, the c-DNA of (-SP) *ZtSSP2* was cloned into pB27 vector as an N-LexA-bait-C fusion to Lex A. The construct was used as a bait to screen c-DNA library of wheat leaves inoculated with *Z. tritici*. A total of 76.4 million clones were screened and positive clones were selected on selective medium lacking Trp, Leu, and His supplemented with 0.5mM 3AT. The prey fragments from positive clones were amplified and

sequenced. The sequences were then used to identify corresponding proteins in the NCBI GenBank database.

For analysis of specific interaction CDS of *TaE3UBQ* and (-SP) *ZtSSP2* were cloned into the vector pDONR207 using Gateway cloning technology (primers listed in Appendix 1). They were then recombined into bait and prey vectors derived from pGADT7 and pGBKT7 plasmids (Clontech, USA). Analysis of protein-protein interactions was performed using the Gal4 two-hybrid assay as described in Perochon et al. 2010. As a negative control to ensure specific interactions with *ZtSSP2*, another effector candidate Zt-10 was cloned to use in this study.

3.3.4 *In-planta* validation of protein-protein interaction.

For the *in planta* analysis of the interaction between *ZtSSP2* and *TaE3UBQ* the coding sequences were cloned in the gateway vector pDONR207 (Invitrogen, USA) and were subsequently cloned into the bimolecular fluorescence complementation (BiFC) vectors pDEST-GW VYCE, pDEST-VYCE GW, pDEST-GW VYNE and pDEST-VYNE GW (Gehl et al. 2009). This resulted in constructs where in proteins were fused at either the N or C terminal to the YFP C-terminal (YFP^C) or N-terminal fragment (YFP^N). Vectors were transformed into *A. tumefaciens* strain GV3101 by electroporation. *Agrobacterium* transformants containing the plasmids were selected on LB agar plates containing 10 μgml^{-1} rifampicin, 20 μgml^{-1} gentamicin and 50 μgml^{-1} kanamycin. A mix of *Agrobacterium* transformants was prepared: OD600 = 0.5 and 0.5 and 0.1 of YFP^C construct, YFPN and P^{19} silencing construct respectively. This mix was syringe-infiltrated into leaf epidermal cells of 3-4 week old *Nicotiana benthamiana*, by making a small injury into the leaf and pressure infiltrating with *Agrobacterium* transformants. Images were analysed using a confocal laser scanning microsope (Olympus fluoview FV1000). The YFP excitation was performed at 515 nm and emission was detected in 525 to 600nm range.

3.3.5 Statistics

Statistical analyses were performed using GraphPad Prism 7.0 software. Description of the various tests are indicated in the figure legends.

3.4 RESULTS

3.4.1 Validation of *Z. tritici* small secreted proteins (*ZtSSPs*) secretion using yeast expression system.

In order to validate that the cell death inducing candidates are secreted, a yeast secretion system was utilised. The full length *ZtSSP*s were cloned upstream of a *SUC2* invertase plasmid lacking its native signal sequence (Fig 1A) (Plett et al. 2017). The plasmid was transformed into *suc2* mutant yeast stains which are unable to grow in media with sucrose as source of energy. All the *ZtSSP: SUC2* constructs and full length *SUC2* construct (positive control) enable the *suc2* mutant yeast stain to grow on synthetic drop out media (-TL) and YPRAA media (Fig 1B). The constructs also catalysed the reduction of 2, 3, 5-triphenyltetrazolium chloride (TTC) to red colored triphenylformazan (TPF) while, yeast stain carrying the vector alone (negative control) could not grow on both SD-TL, YPRAA and TTC added cultures remain colorless. Overall, our result suggests that the 5 candidates are indeed secreted proteins.

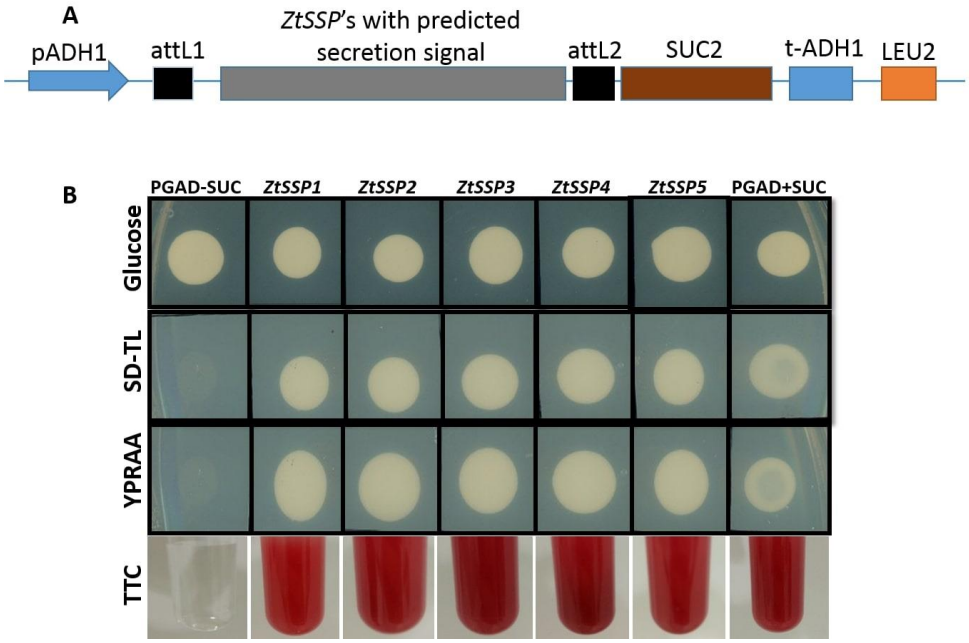


Fig. 1. Use of Yeast Invertase secretion system to validate *Z. tritici* proteins are secreted proteins. (A) Design of the gateway compatible yeast pGADT7-GWY-Suc2 vector and Invertase mutant yeast strain SEY6210 used for the secretion assay (Zhou unpublished). **(B)** Yeast strain carrying the *ZtSSP's* with secretion signal fused in frame with the Invertase gene Suc2 were able to grow in sucrose containing drop out media (SD-TL) and YPRAA media (contains raffinose instead of sucrose therefore cells will grow if Invertase is secreted. Similarly reduction of TTC to red formazan indicating secretion of Invertase. SEY6210 carrying pGADT7-GWY-Suc2 vector was used as negative control while SEY6210 with pGADT7-GWY-Suc2 (Full length) acts as positive control.

3.4.2 *Z. tritici* candidate proteins have intrinsic disordered regions

The function of a protein is centrally dependent upon its well-defined structure however, recent studies on the proteome of eukaryotes suggest that a high number of polypeptides possess regions that lack a defined structure and hence are referred to as intrinsically disordered regions (IDR) (Marin et al. 2013; Vincent & Schnell 2016). Proteins with these IDR regions lack functional domain and thus tend to evolve more rapidly than proteins with defined structural domains (Chen et al. 2006; Brown et al. 2011; Nilsson et al. 2011).

Plant pathogens including bacterial and fungal effectors are enriched with IDR regions and are known to be actively involved in diverse functions such as cellular signalling, post translation modifications and protein-protein interactions (Marin et al. 2013; Dyson 2016). Here, analysis of the cell death inducing candidates of *Z. tritici* for presence of IDR regions using various prediction programs was conduceted (PONDR VL-XT, IUPred and PrDOS) (Romero et al. 2001; Dosztanyi et al. 2005; Ishida et al. 2007). The results suggest that candidates *ZtSSP1-5* at least possess one or more IDR regions within its sequences (Fig 2). The candidate *ZtSSP3* and *ZtSSP2* had regions with higher IDR i.e. 36.5% and 26.5% respectively while *ZtSSP1*, *ZtSSP4* and *ZtSSP5* showed 12.29%, 23.17% and 8.53% IDR regions respectively.

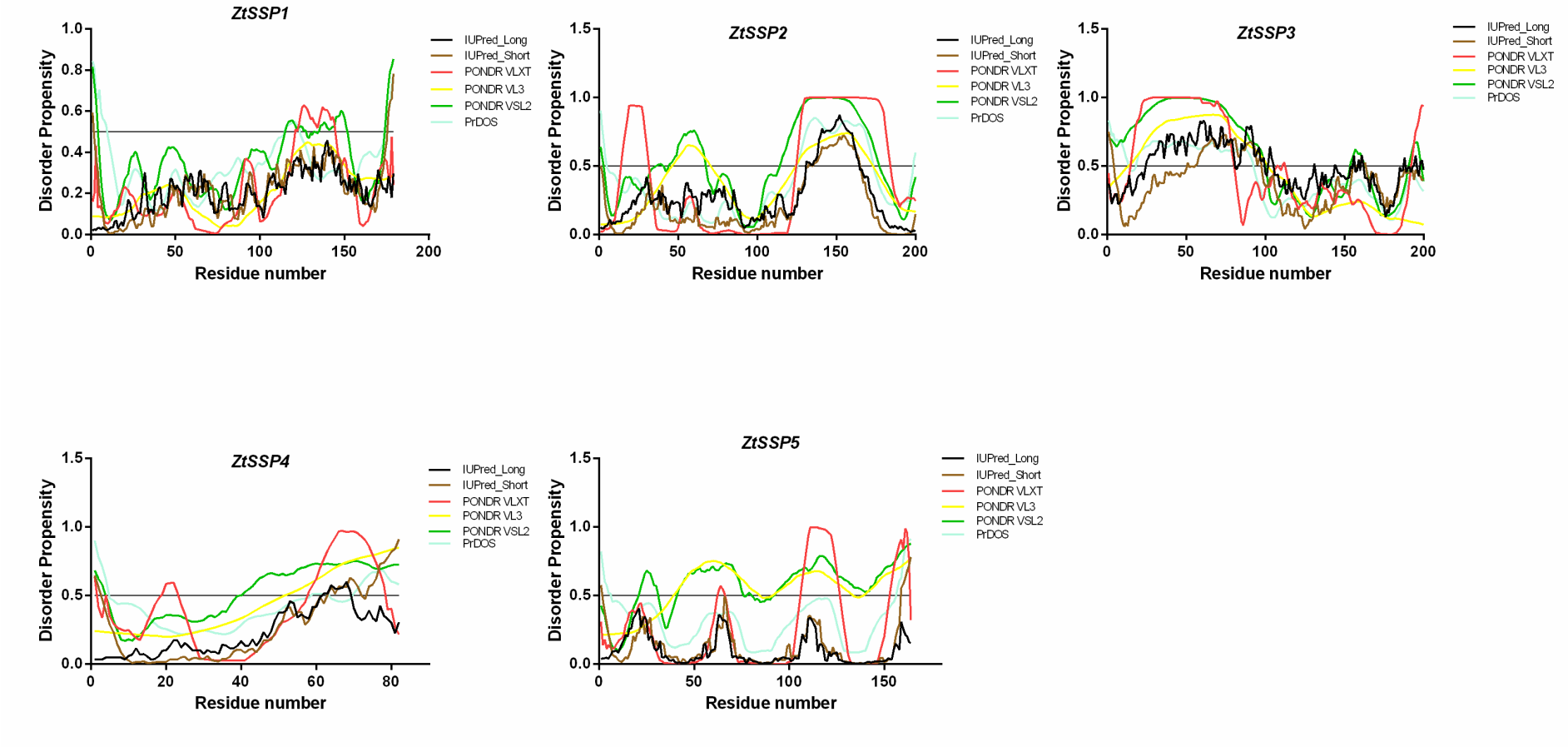


Fig. 2. Evaluating *ZtSSP* candidate proteins disorder propensity. The plot represents intrinsic-disorder predictions results by *IUPred _Long* (Black line); *IUPred_Short* (Brown); *PONDR VLXT* (Red); *PONDR VL3* (Yellow); *PONDR VSL2* (green line) and *PrDOS* (blue line). In this analysis the threshold of disordered propensity score was set at score of 0.5. Any disordered score above 0.5 were considered to correspond to the disordered regions while the score below 0.5 were considered flexible.

3.4.3 Four out of five *ZtSSP* are conserved effector candidates.

In order to determine whether the five effector candidates cloned from the Irish isolate 560.11 (Lynch et al. 2016) possess any allelic variations in other *Z. tritici* isolates, coding sequence from 54 Irish field isolates were examined (Welch et al. 2018). The sequences of these five effector candidates were aligned from the reference isolate IPO323 and Irish *Z. tritici* field isolates where they were present (collected from different wheat cultivars at two location in Ireland i.e. Carlow & Cork (Welch et al. 2018). All five effectors from 560.11 share similar coding sequences with reference isolate IPO323. Four out of five *ZtSSP's* (*ZtSSP1*, *ZtSSP2*, *ZtSSP3* and *ZtSSP5*) were present in all the isolates of *Z. tritici*. Candidate *ZtSSP4* was present only in IPO323 and 560.11 but absent in all of the field isolates of *Z. tritici* compared.

Two isolates (C12c and S19c) have four single nucleotide polymorphisms (SNP's) in the CDS sequence of *ZtSSP1* (Table 2, Appendix 3). Similarly, for *ZtSSP2*; 28 isolates out of 54 had a single SNP at position 131, leading to a change in amino acid from valine to alanine (Table 2, Appendix 3). For *ZtSSP3* three isolates (E15c, I14c and I18c) had SNP's at position 40 and 56. *ZtSSP5* had the highest SNP's among all the candidate tested with seven different SNP's at various position (Table 2, Appendix 3).

Table 2. SNPs of *ZtSSP* candidates among *Z. tritici* isolates

Candidate	SNPs*	Isolates
ZtSSP1	43 E-D 113 I-V 145 S-T 174 D-E	C12c S19c S19c S19c
ZtSSP2	131 V-A	28 isolates
ZtSSP3	40 Y-H 56 P-L	I18c E15c and I14c
ZtSSP5	Insertion T at 74 26 K-N 28 I-M 112 Y-C 128 T-A 148 N-K 156 T-A	22 isolates E18c E18c C17c, I14OP, S15op,E10c 24 isolates E9OP 26 isolates

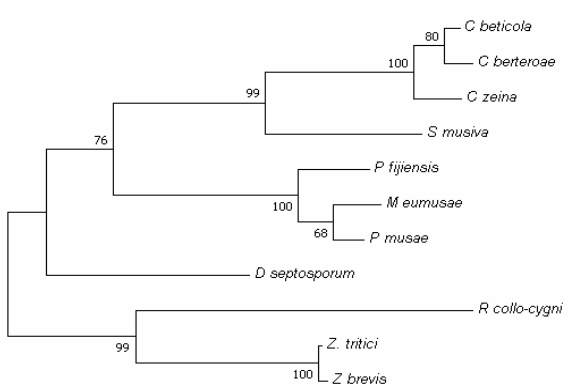
*: The coding sequence of *ZtSSPs* from IPO323 was taken as a reference sequence and only non-synonymous SNP's are shown with position.

3.4.4 Homologues of *ZtSSP2* are conserved across Ascomycete fungi.

In order to determine whether ZtSSP2 homologous proteins are present in other plant pathogenic fungi, the ZtSSP2 protein sequence was queried against the National centre for Biotechnology Information (NCBI) protein database using BLASTP. This resulted in the identification of homologues in other Ascomycetes within the family *Mycosphaerellaceae*, including the barley pathogen *Ramularia collo-cygni* which was 53% identical to *ZtSSP2*, the conifer infecting fungi *Dothistroma septosporum* which share 54% sequence identity and the banana pathogen *Mycosphaerella fijiensis* which shares 51% sequence identity.

All the homologues of ZtSSP2 protein identified were of similar size, possess an N-terminal signal peptide and contained the ten conserved cysteine residues (Fig 3B). This result suggests that the candidate ZtSSP2 protein is a core effector and its functional homologue is present and conserved in other plant pathogenic fungi.

(A)



(B)

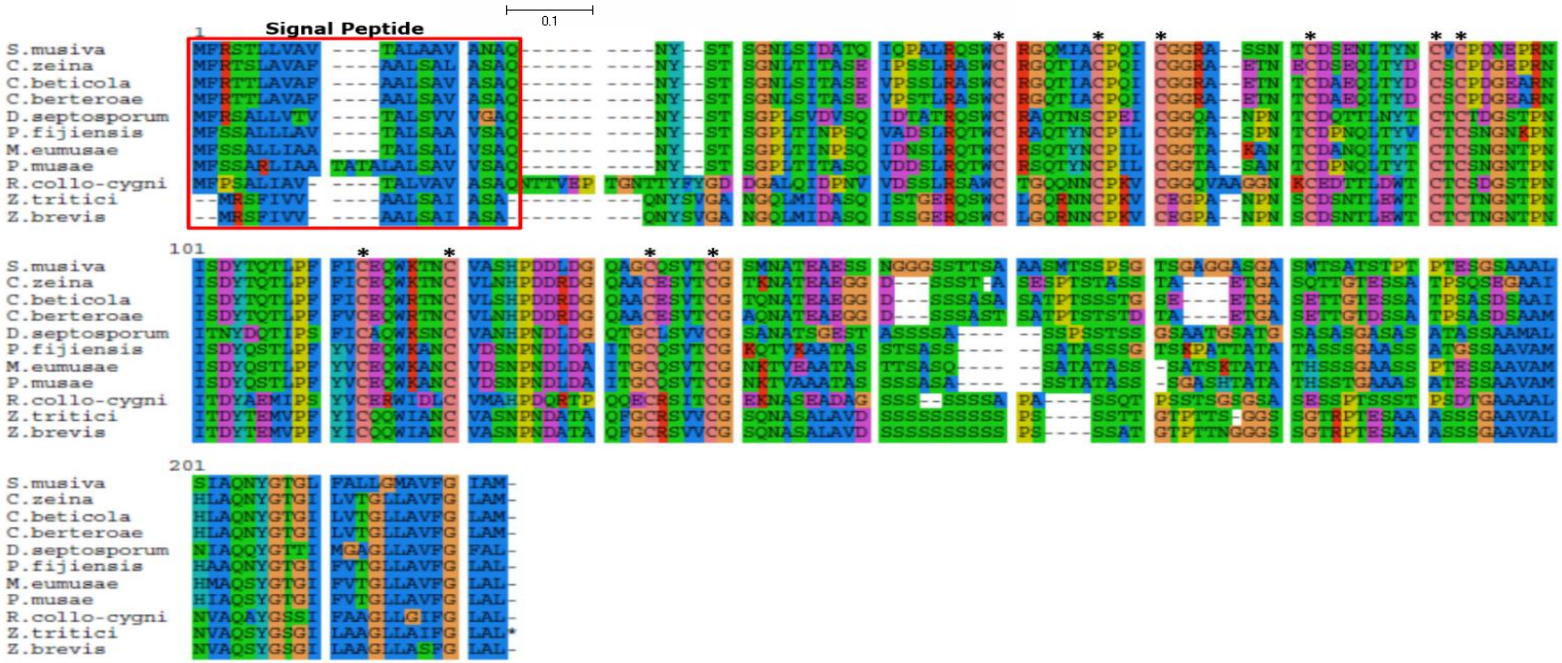


Fig. 3. *ZtSSP2* (*Mycgr3G105265*) homologues are widely present across Ascomycetes. A. The Unrooted maximum likelihood phylogeny of *ZtSSP2* and related homologue protein from the ascomycete fungi *Septoria musiva*, *Cercospora Zeina*, *Cercospora beticola*, *Cercospora berteroae*, *Dothistroma septosporum*, *Pseudocercospora fijiensis*, *Mycosphaerella eumusae*, *Pseudocercospora musae*, *Ramularia collo-cygni*, *Zymoseptoria brevis* generated with MEGA7 (Kumar et al. 2016), Bootstrap values are based on 1000 replications. **B.** Protein alignment of mature protein sequence of *Z. tritici ZtSSP2* with its homologues from other Ascomycete fungi using CLUSTAL OMEGA. Asterisk indicated the conserved cysteine residues and different colours represents identical or similar amino acids.Signal peptide predicted using SignalP 4.1.

3.4.5 Candidate *ZtSSP2* interacts with wheat E3 ubiquitin ligase *in vitro*.

Previous screening study using the non-host plant *N. benthamiana* suggested that the candidate protein ZtSSP2 displayed a prominent cell death phenotype compared to other candidates tested and its expression profiling also showed that the candidate was differentially regulated throughout the *Z. tritici* infection cycle. Therefore a yeast two-hybrid screen was conducted in order to determine if candidate protein ZtSSP2 could interact with any component of the wheat host. Using ZtSSP2 protein as a bait, a cDNA expression library generated from *Z. tritici* infected wheat leaves was screened. Putative interacting wheat proteins were identified (Table 3) including a wheat C3H2C3-type RING E3 ubiquitin ligase protein (TaE3UBQ), wheat heavy metal associated isoprenylated plant protein-39 (TaHMA-39) and endo-1,3;1,4-beta-D-glucanase like protein. Among these interactors, TaE3UBQ protein was selected for further analysis as pathogen effectors ave been reported to target the host ubiquitin system to manipulate host defense (Park et al. 2012). *ZtSSP2* might be hypothesized to interact with E3 ligase and thereby manipulate the wheat host ubiquitin proteasome system. The protein-protein interaction was confirmed in yeast (*Saccharomyces cerevisiae*) using a galactose-responsive transcription factor GAL4-based yeast two-hybrid system (Fig 4).

Table 3. List of wheat protein identified as a potential interactors with *Z. tritici* candidate *ZtSSP2*.

BLAST against *T.aestivum* TGACv1	*PBS® Score	Best BLASTP match	AA Size
TRIAE_CS42_1BL_TGACv1_030593_AA0095040.1	A	Heavymetal-associated isoprenylated plant protein 39-like (LOC109766401)	224
TRIAE_CS42_1AL_TGACv1_000743_AA0018220.1			
TRIAE_CS42_7DL_TGACv1_604456_AA1998280.4	A	Endoplasmin homolog (LOC109732828)	634
TRIAE_CS42_7AL_TGACv1_556087_AA1755000.3			
TRIAE_CS42_7BL_TGACv1_578233_AA1891510.4			
TRIAE_CS42_1DS_TGACv1_082290_AA0264360.1	B	E3 ubiquitin-protein ligase At1g12760-like (LOC109745851)	420
TRIAE_CS42_1BL_TGACv1_030333_AA0087160.1	B	Endo-1,3;1,4-beta-D-glucanase-like (LOC109778325)	240
TRIAE_CS42_1DL_TGACv1_061296_AA0191610.1			
TRIAE_CS42_5DL_TGACv1_435835_AA1455370.1	D	Ankyrin repeat domain-containing protein 2A-like (LOC109746370)	398
TRIAE_CS42_5BL_TGACv1_406247_AA1342930.1			

*The interactors listed above represents candidates that have highest global PBS® score in yeast two hybrid. PBS score is a score that is generated automatically using sets of algorithm that specify the interaction represented in the screen.

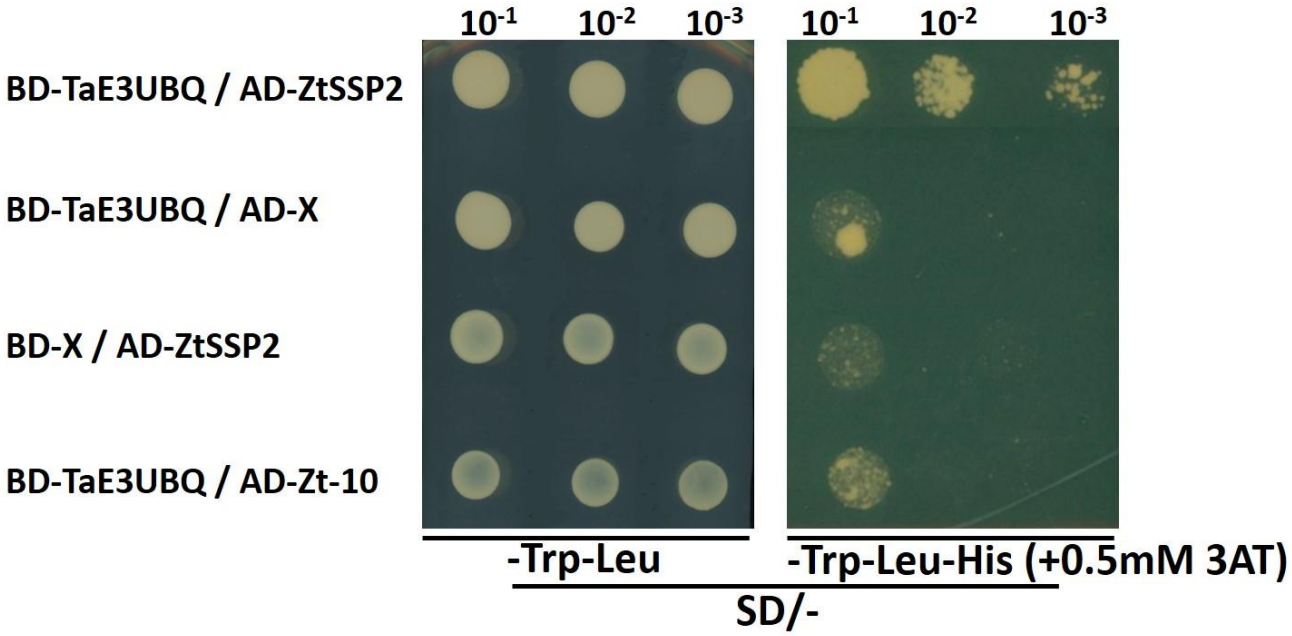


Fig. 4. Interaction of *ZtSSP2* with *TaE3UBQ*. Yeast two-hybrid assay using the yeast transformed with *TaE3UBQ* and *ZtSSP2* cloned in the Gal4 bait and prey vectors. Yeast were grown for 7 days under selective Trp/Leu/His drop out medium in presence of 0.3mM 3-amino-1,2,4-triazole (3-AT) or a non-selective Trp/Leu drop out medium (-TL) conditions. BD represents Binding domain & AD, activating domain. Experiment was repeated three times with similar result.

3.4.6 *TaE3UBQ* represents a C3H2C3 RING type E3 ligase.

The full length cDNA of *TaE3UBQ (*TraesCS1D02G119700) was obtained by comparing the clone sequence with *EnsemblPlants* IWGSC database. BLASTp showed that the *TaE3UBQ* has two additional homeologs namely: TraesCS1A02G118800 and TraesCS1B02G138300 sharing 99.3 and 97% similarity with its 1D variant. The *TaE3UBQ* ORF encodes a RING finger protein of 420 amino acids, with a theoretical *pI* value of 6.06 and a deduced molecular mass of 46.2 kDa (Fig 5a). Protein sequence analysis using NCBI CDD (Bauer et al. 2017) and SMART (http://smart.embl.de) programs showed a C-terminus C3H2C3 Zinc-finger domain and four transmembrane regions at the N-terminus (Fig 5a). The *TaE3UBQ* phylogenetic analysis revealed that the RING finger domain is highly conserved among various plant species (Fig 5b, 5c).

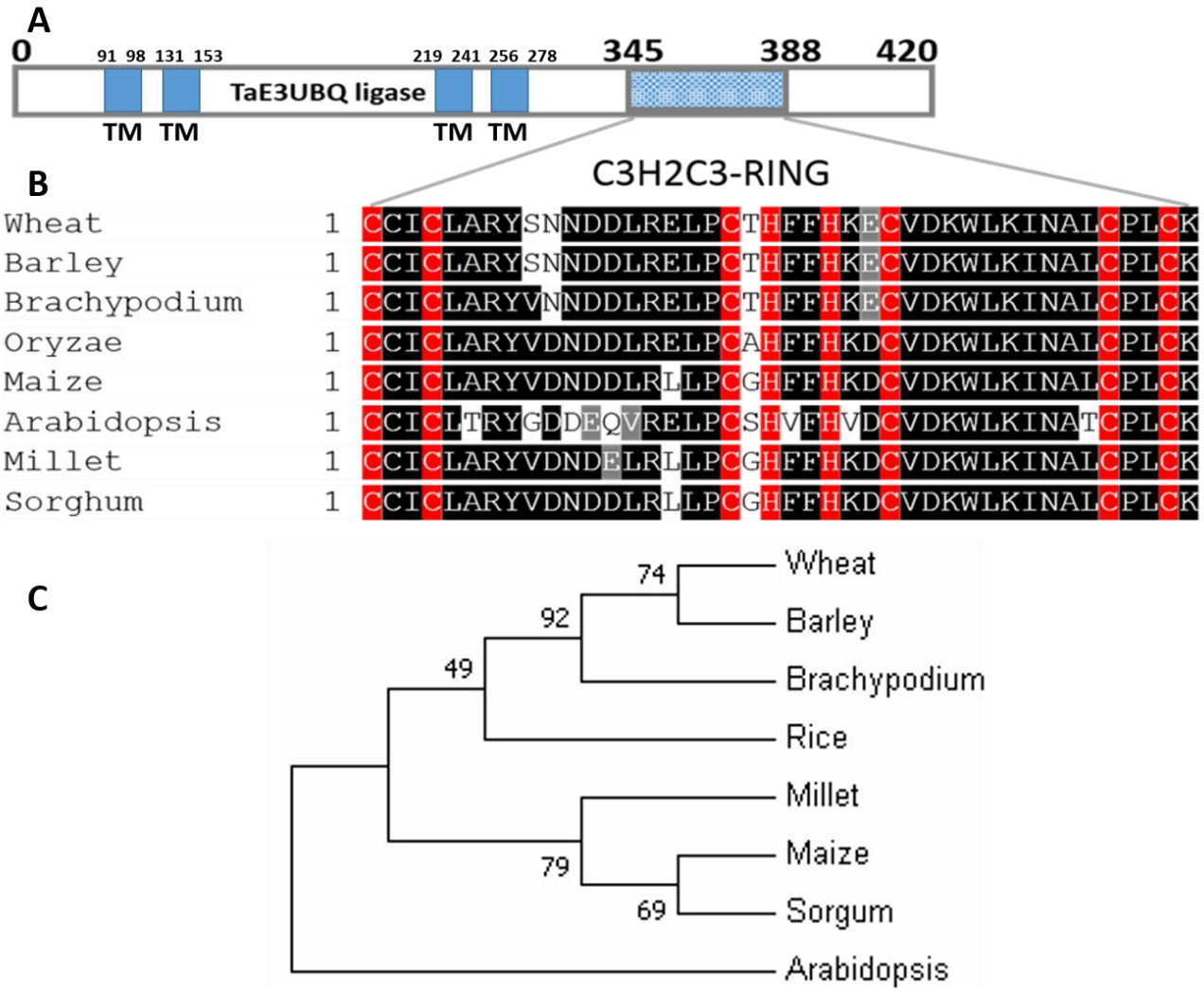


Fig. 5. Sequence analysis of *T. aestivum* E3 UBQ ligase protein (*TaE3UBQ*). (A) Schematic representation of the structure of *TaE3UBQ* and the conserved RING finger motif. (B) Sequence alignment of the C3H2C3-type RING finger conserved motifs from different species. (C) The phylogenetic relationship of TaE3UBQ and its closely related homologs from *H. vulgare* (GenBank accession no. BAJ95361.1), *B. distachyon* (GenBank accession no. XP_003568812), *Oryza sativa* (GenBank accession no. XP_015640584), *P. miliaceum* (GenBank accession no. RLN29883.1), *Zea mays* (GenBank accession no. PWZ17186.1), *Sorghum bicolor* (GenBank accession no. XP_002439378), *A. thaliana* (GenBank accession no. NP_178156.1). The phylogenetic tree was constructed using Mega7 Molecular Phylogenetic analysis by Maximum Likelihood method with 1000 bootstrap replicates.

3.4.7 Candidate *ZtSSP2* interacts with wheat E3 ubiquitin ligase *in planta*

The interaction between *ZtSSP2* and *TaUBQ* was investigated *in planta* using the BiFC system (Gehl et al. 2009). *ZtSSP2* and *TaUBQ* were fused at their N terminus with either the N-terminus or C-terminus part of the YFP. The resulting constructs were then co-expressed using *Agrobacterium* infiltration in *N. benthamiana* leaves.

A strong YFP signal was observed which appears to be membrane localised, when YFP^N–TaE3UBQ was co-infiltrated with YFP^C-ZtSSP2 (Fig. 6). The *ZtSSP2* is predicted to be apoplastic and the *TaE3UBQ* is predicted to be plasma membrane localised (ApoplasticP, BUSCA prediction) (Table 1 and Appendix 4). In order to confirm the interaction of *TaE3UBQ* was specific to *ZtSSP2*, co-infiltration with another effector; YFP^C-Zt-10 and YFP^N–TaE3UBQ was performed. NoYFP fluorescence was obsereved with this construct suggesting no interaction of TaE3UBQ with candidate Zt-10. For positive control YFP^N–NAC and YFP^C-NAC were used and this interaction was observed in the nucleus (Kahla 2016).

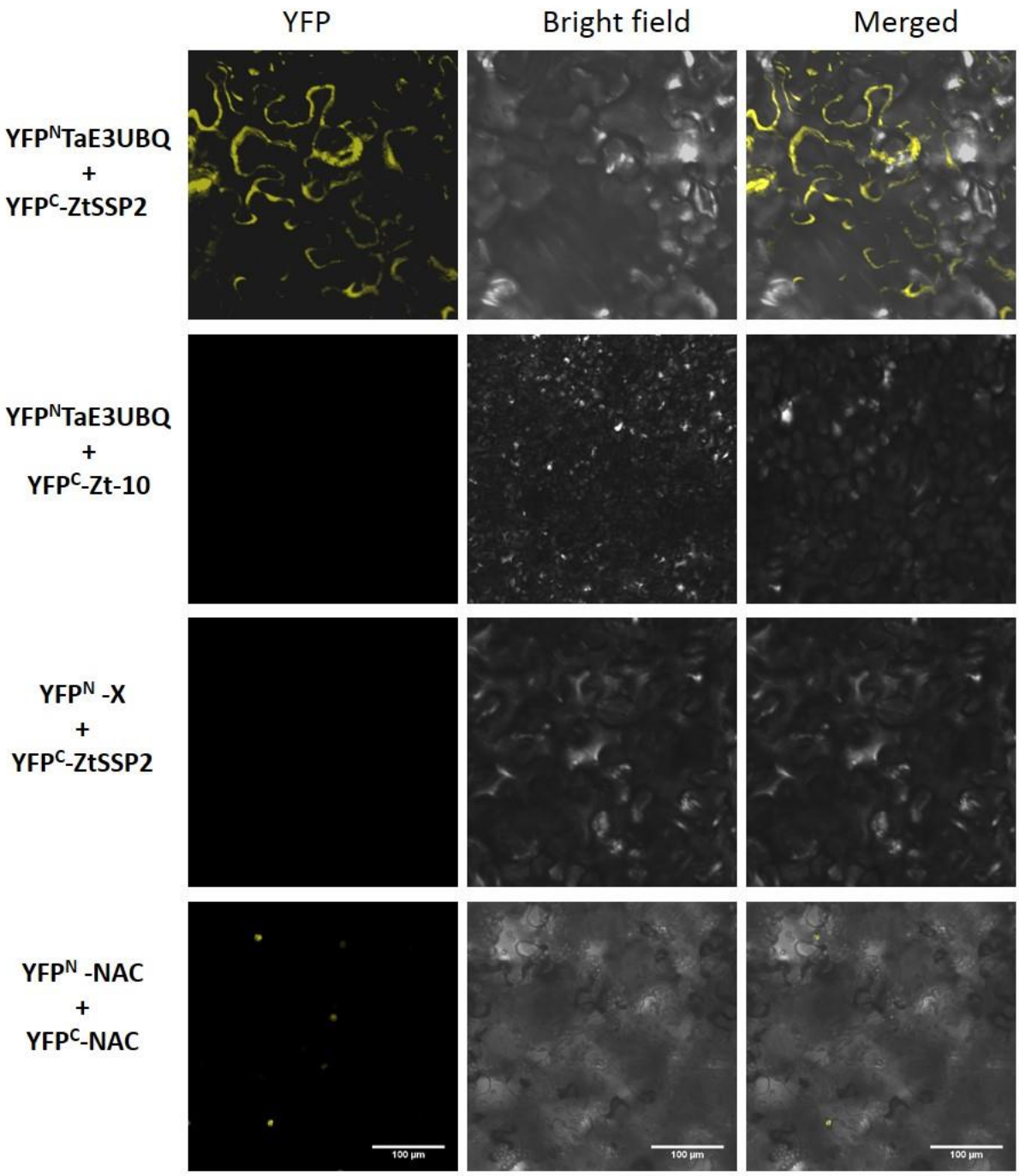


Fig. 6. Validation of *in planta* interaction of *Z. tritici* effector candidate *ZtSSP2 with* wheat Ubiquitin protein. Confocal microscopy images of representative *N. benthamiana* epidermal leaf cells expressing proteins fused to the N- or C-terminal part of the YFP as indicated. YFP, bright field are shown both separately and as an overlay. Scale bar is 100µm. In both experiment B and C, candidate Zt-10 was used as a negative control and NAC was used as positive control (Kahla 2016).

3.4.8 *TaE3UBQ* expression is downregulated by *Z. tritici* infection

The expression of *TaE3UBQ* was further investigated during infection of wheat leaves by *Z. tritici* (isolate 560.11). The relative expression of *TaE3UBQ* was compared with mock (Tween20) inoculated control plants (Fig.7). There was a significant difference in the transcript levels in the *Z. tritici* infected leaves compared to healthy plants at 4 dpi and 21dpi. This significant downregulation of *TaE3UBQ* at 4 dpi coincides with the early biotrophic phase of host colonisation by *Z. tritici.* Similarly, downregulation of *TaE3UBQ* was found at 21dpi coinciding with the late necrotrophic phase.

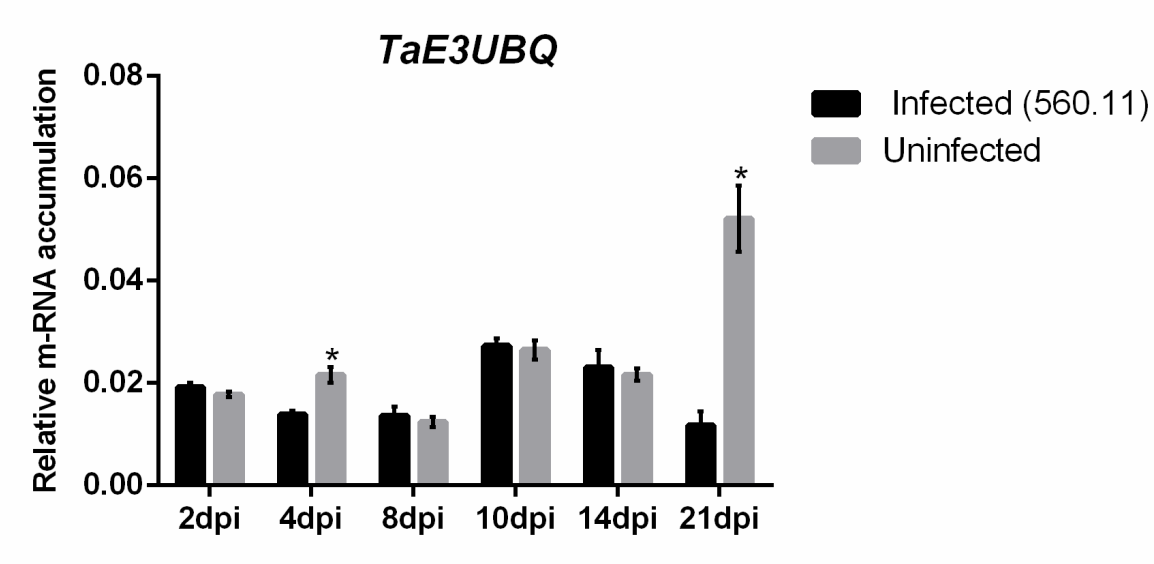


Fig. 7. Real-Time Polymerase chain Reaction (qRT-PCR) of *TaE3UBQ* expression in *Z. tritici* infected wheat leaves and in control leaves till 21 days post infection. *TaTubulin* was used as housekeeping gene reference. Control plants were sprayed with sterile water with 0.02% Tween 20. Data are the mean ± SEM of three independent biological samples with three technical repeitions. Student's t-test ($P<0.05$).

3.5 DISCUSSION

The major component of the filamentous plant pathogens secretome are often small secreted cysteine rich proteins (SSPs) (Stergiopoulos & Pierre 2009). These SSPs are often small (300aa) with high cysteine residues and presence of secretion signal at the N-terminus. SSPs from plant pathogens play key role in subverting host plant immunity and facilitating colonisation. (Hogenhout et al. 2009; Rafiqi et al. 2012). The *Z. tritici* secretome predicts hundreds of these SSP however, till date only handful of these have been characterized for their role in pathogenicity (Gohari et al. 2015; Rudd et al. 2015).

Fungal virulence factors are known to be secreted and cysteine-rich proteins, therefore, these secreted proteins from plant pathogens play a significant roles in pathogenesis (Stergiopoulos & Pierre, 2009). Bioinformatics analysis of *Z. trtici* showed that *ZtSSP1-5* had an N-terminal secretion signal (SignalP 2.0 program, http: //www.cbs.dtu.dk/services/SignalP-2.0/.). Therefore, yeast secretion assay (Jacobs et al. 1997) was used to test whether ZtSSP1-5 are indeed secereted proteins (Fig. 1) and confirm the prediction obtained with the SignalP program.

SNP analyses showed that the protein coding sequence of four out of five *ZtSSP* candidates are maintained in the various field isolates of *Z. tritici*. One of the candidates *ZtSSP4* was not present in any of these field isolates tested suggesting loss of that effector gene variant in field isolates. It can be hypothesize that this loss of effector in field isolates might reflect the natural selection pressure for pathogen fitness. These mechanisms of deletion or loss of effector in fungal pathogen are common and thus may be a result of avoiding recognition by cognate host proteins and ultimately lower fitness costs (Rouxel et al. 2003). Recent analysis on the pangenome of *Z. tritici* also showed that an avirulence effctor *Zt_8_609* displayed a presence-absence polymorphism within species (Hartmann et al. 2017; Plissonneau et al. 2018). Based on the analysis with field isolates of *Z. tritici*, the candidates *ZtSSP1, ZtSSP3 and ZtSSP5* had at least three SNPs in their coding sequences. The high number of non-synonymous SNPs was found in *ZtSSP5* resulting in seven different *ZtSSP5* isoforms. These mutations within the effector protein might play role in avoiding recognition and also maintain the effector function (Levin et al. 2000).

The candidate *ZtSSP2* was found to be highly conserved with only one SNP (131V-A) that was found in 28 isolates. Similarly, ZtSSP2 protein sequence was queried against the NCBI protein database using BLASTP which resulted in identification of homologous proteins in other Ascomycetes, including the pine infecting hemibiotroph *D. septosporum*, banana infecting *P. fijiensis*, and barley pathogen *R. collo-cygni*.The conservation of this protein in hemibiotrophic and necrotrophic plant pathogens might reflect the importance of this candidate and its homologues for these fungal pathogens in targeting a common plant defense. Recent evidence suggests that a range of virulence effectors from plant pathogens concenter into a limited number of host cellular targets "Hubs" inorder to subvert the host defense and enhance virulence (Mukhtar et al. 2011). One of the key regulatory networks in plant defense is an ubiquitin-proteasome system (UPS). This UPS regulates multiple aspects of plant immunity involving recognition, receptor protein accumulation and subsequent defense signalling (Marino et al. 2012; Ustun et al. 2016). Therefore manipulation of host UPS by effectors is central to increasing pathogen virulence.

Here, candidate effector *ZtSSP2* was found to physically interact with a host E3 Ubiquitin ligase (*TaE3UBQ*) both in yeast and *in planta* (Fig. 5 & Fig. 8). Domain analysis of *TaE3UBQ* showed that it possesses a RING-finger domain. There exists accumulating evidence that the E3 ubiquitin ligase acts as a key regulator of plant immunity and signalling (Trujillo & Shirasu 2010; Marino et al. 2012). In rice, the resistance gene *Xa21* (*Xanthomonas oryzae pv. oryzae locus* 21) was shown to require a RING- E3 ubiquitin XA21 binding protein 3 (XB3) which plays a key role in accumulation of XA21 protein and *Xa21* mediated disease resistance (Wang et al. 2006). In *Arabidopsis* the role of Plant U-Box 12 (PUB12); a U-box E3 ligase is involved in the PTI response against bacterial flagellin through Flagellin sensing 2 (FLS2) (Lu et al. 2011). Similarly, *Arabidopsis Tóxicos en Levadura* (ATL) family of RING finger E3 ligase (ATL9) was found to be induced by fungal chitin and is involved in resistance against biotrophic fungal pathogen (Deng et al. 2017).

These E3 ligases are central to plant immune responses and are also known targets for pathogen secreted effector proteins. One such recent example was effector *AvrPiz-t* from the blast fungus *M. oryzae*. *AvrPiz-t* has been shown to interact and inhibit the rice RING-type E3 Ubiquitin ligase (APIP6) *in vitro* resulting in suppression of APIP6 mediated PTI response (Park et al. 2012).

Therefore, the effector candidate *ZtSSP2* interaction with wheat E3 ubiquitin ligase (*TaE3UBQ*) may suppress the immune response mediated by *TaE3UBQ* in wheat. Expression analysis of healthy and *Z. tritici* infected wheat leaves showed that the infected leaves had significantly lower expression of *TaE3UBQ* at early (4dpi) and later stages of infection (21dpi) compared to healthy leaves (Fig.7) suggesting downregulation of *TaE3UBQ* regulation is required for successful host colonisation. Alternatively, it is also possible that the wheat ubiquitin system might be able to recognise the effector candidate *ZtSSP2*, resulting in ubiquitin mediated degradation of *ZtSSP2* as a host defense response.

Therefore, further insights are required to establish the role of *TaE3UBQ* in the *Z. tritici*-wheat interaction. Virus induced gene silencing (VIGS), wheat TILLING (target induced local lesions in genomes) lines for E3 ubiquitin could provide a confirmation of the role of *TaE3UBQ* in disease resistance and similarly, generation of *ZtSSP2* knock out mutants could also establish its role in *Z. tritici* infection.

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Chapter 4

B. distachyon as a non-host model for screening *Z. tritici* effector candidates

A manuscript including some of this work has been submitted to Phytopathology:

Responses of the non-host grass *Brachypodium distachyon* to isolates of the fungal pathogen *Zymoseptoria tritici*, compared to the wheat host.

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4.1 ABSTRACT

Fungal pathogens are known to cause some of the most devastating diseases in plants that has a huge impact on global food security. These fungal pathogens have a repertoire of proteins termed effectors, which are key to their pathogenicity. These pathogen secreted proteins are secreted into host cells and thereby control the host plant physiology. They are also known to subvert the host defense mechanism thus, promoting host susceptibility. With the advent of next generation sequencing and computational bioinformatics, a high number of these effector proteins can be predicted from pathogen genome. With a high number of these proteins predicted, a rapid and efficient method to screen these candidates is critical for understanding their role in disease. In this study first, infection of *B.distachyon* using *Z. tritici* isolates was performed and non-host defense mechanism (NHR) of *B.distachyon* and *T. aestivum* was compared. This suggested that NHR of *B. distachyon* involves rapid upregulation of ROS, JA and the phenylpropanoid pathway compared to susceptible wheat. Secondly, a biolistic based single cell transient assay was performed to screen *Z. tritici* candidate effector proteins in two-member of the Poaceae the non-host *B. distachyon* and host *T. aestivum*.

4.2 INTRODUCTION

With the recent advances in whole genome sequencing and in-silico prediction, large set of effector candidates are identified from fungal pathogens of cereals. *Zymoseptoria tritici* is an ascomycete fungus that causes Septoria tritici blotch (STB) disease on wheat. The genome of *Zymoseptoria tritici* encodes number of putative effector candidate proteins that are thought to play a key role during both phases of its lifecycle and contributing to its virulence (Gohari et al. 2015; Rudd et al. 2015). However, a limited number of effector candidates have been characterised till date in *Z. tritici*. These include the lysin motif (LysM)-containing effectors *Mg3LysM* and *Mg1LysM* which have been identified as playing an important role during the initial symptomless period of infection by suppressing chitin mediated activation of host defenses (Marshall et al. 2011). Similarly, some of the candidate effectors have been shown to induce cell death in non-host *N. benthamiana* (Kettles et al. 2017). In addition, one of the cell death inducing candidate was characterised as a phytotoxic ribonuclease (Kettles et al. 2018). *Z. tritici* effector *AvrStb6* has been shown to be involved in a gene-for-gene interaction with the wheat resistance gene *Stb6* (Zhong et al. 2016). Another avirulence effector *Avr3D1* has been recently identified to be recognised by wheat cultivars harbouring the *Stb7* resistance gene (Meile et al. 2018).

High number of *Z. tritici* small secreted protein candidates have no known functional domain and in addition, lack of high-throughput functional assays that are available to express these candidate proteins in wheat, has halted their functional characterisation. Studies on fungal pathogen effectors have utilised A. *tumefaciens* mediated transient expression system in a non-host plant *N. benthamiana* to overexpress the candidate proteins (Chen et al. 2013; Qi et al. 2016; Petre et al. 2016; Ramachandran et al. 2017; Kettles et al. 2017). However, *A. tumefaciens* mediated transient expression system is inapplicable for cereals. In addition to that overexpression in the non-host system *N. benthamiana* may not reflect the localisation and targeting and function as in natural host infection because of phylogenetic divergence between *N. benthamiana* (dicot) and Wheat (monocot) plants.

Transient overexpression using biolistic methods to investigate effector activity has been successful in monocots. The assay is based on the observation that a typical resistance response involves a HR mediated cell death and this cell death ultimately results in reduced expression of the reporter construct used (Levine et al. 1994; Jia et al. 2000; Bai et al. 2012). Similarly, co-transformation of candidate protein over-expression constructs with *B-peru/C1*-expression

plasmid and β-glucuronidase (GUS) was reported by Pliego et al. 2013 and Eichmann et al. 2010 for screening *B. graminis* effector proteins in Barley. This assay involves utilisation of the additional cell death marker anthocyanin and GUS as a transfection reference marker.

***B. distachyon* as a model to study STB**

Over the past decade, there have been an increasing number of studies which have used *B. distachyon* as a non-host model against different pathogens (Fitzgerald et al. 2015). These include studies on the economically important pathogens *Fusarium graminearium* and *Puccinia graminis* (Figueroa et al. 2013; Peraldi et al. 2011). A single study investigating the non-host response (NHR) of different *B. distachyon* accessions against *Z. tritici* has been reported till date (O'Driscoll et al. 2015). *B. distachyon* has the potential to serve as a model plant system especially for cereal diseases; as it is closely related to the Triticeae family and also has small diploid genome size (~355 Mbp) (O'Driscoll et al. 2015). However, in this study one isolate of *Z. tritici* was used. Therefore, the non-host defense response (NHR) of *B. distachyon* to different two different isolates (IPO323 and 560.11) was compared to wheat in this study (Lynch et al. 2016; Kema & Silfhout 1997).Understanding NHR in *B. distachyon* against diseases of Triticeae (cereals) may therefore provide valuable insights into future pathogen control strategies.

A biolistic based single cell assay was also investigated that could be applicable to screen effector proteins in grasses. A β-glucuronidase based biolistic approach was first utilised in non-host plant *B. distachyon* However, low transformation efficiency and higher variation within experiments in *B. distachyon* leaves were observed. An assay similar to Pliego et al. 2013 was then adopted to screen effector candidates of *Z. tritici* for induction of cell death in wheat.

In this study, the NHR response of *B. distachyon* against two isolates of *Z. tritici* was explored and compared with the host wheat.Secondly, the likelihood of utilizing a transient expression assay for *Z. tritici* effector screening was assessed on a non-host and host plant.

4.3 MATERIAL AND METHODS

4.3.1 Plant and fungal material

Seeds of wild-type *B. distachyon* (*Bd21*) and the spring wheat cultivar Remus were stratified at 4^{o}C for 3 days and then incubated in the dark at room temperature for 4 days to allow germination. Germinated seeds were transferred to pots containing one-part vermiculite and three-part peat soil. The pots were then placed in a growth chamber under a 16 hour daylight/8-hour night cycle at 19^{o}C:12^{o}C and watered every 2 days.

Z. tritici isolates IPO323 (Kema & Silfhout 1997) and 560.11 (Lynch et al. 2016) were used for this study. Prior to use, each isolate was cultured on potato dextrose agar (PDA) and grown at 20^{o}C under white light supplemented with blue/black ultraviolet (UV-A) light under a 12:12 hour light: dark photo cycle for approximately 7 days. Fourteen-day old wheat seedlings were used for inoculation. Fungal spores from the PDA cultures were harvested using deionised water and spore concentration was adjusted to 1 x 10^{6}ml^{-1} in water containing 0.02% Tween20 solution. Spore suspensions (15mls) were sprayed using hand-held spray bottles. Control plants were sprayed with 15ml of 0.02% Tween20 solution. Inoculated plants were then covered with polythene bags for 48 hours to ensure high humidity.

4.3.2 Disease and pycnidia scores

Seedlings of the *Z. tritici* susceptible wheat cv. Remus and wild type *B. distachyon* (*Bd-21*) were inoculated with *Z. tritici* isolates IPO323 or 560.11. Inoculated plants were incubated under high humidity for 48 hours and subsequently at normal humidity at 22°C under a 16:8-hour light: dark photocycle. Diseased leaves were harvested at 10, 14, 17 and 21 days post inoculation. The samples were boiled for 10 minutes in ethanol to remove chlorophyll and were then mounted on glass slides for scoring. Using microscopy, leaves were visually rated for percentage disease coverage and for number of pycnidia.

4.3.3 Single-Cell Transient Gene Expression Assay

Single-cell transient gene expression assays using biolistic delivery of plasmid DNA into *B. distachyon* (*Bd-21*) epidermal cells were carried out as described previously by Bai et al. 2013. Briefly a reporter plasmid pCambia 1305.1 (GUSplus) and plasmids expressing individual *Z. tritici* effectors controlled by the 35s promoter were mixed before coating of gold particles (molar ratio of 1:1; 2 µg of total DNA). Seven *B. distachyon* leaves were placed in petridish

containing water agar with benzimidazol (85µM). The leaves were then transformed with a particle inflow gun of the model PDS-1000/He (Bio-Rad).

Screening of candidates for cell death in wheat was performed as previously described by (Pliego et al. 2013). Briefly, seven leaves of 7-day old wheat cv. Kanzler were co-bombarded (PDS-1000/He System, Bio-Rad) with 7µg of pEG101: *ZtSSP* (over-expression), 7µg pUbiGUS (β-glucuronidase reporter for transformation efficiency) and 7µg of the *B-Peru/C1*-expression plasmid pBC17 (Schweizer et al. 2000) for anthocyanin production. 4 days post bombardment, cells accumulating anthocyanin was counted and the leaves were then stained with 5-bromo-4-chloro-3-indolyl glucuronide (X-gluc) solution overnight at 37°C. Finally leaves were destained with trichloroacetic acid (Duchkov et al. 2005) and number of cells that had visible GUS stain were counted.

4.3.4 RNA extraction and quantitative real time PCR

For plant gene expression studies, wheat and *B. distachyon* leaves were sampled at 0.5, 48, 96 and 144 hours after inoculation (hpi) with either of the two different *Z. tritici* isolates. All samples were immediately frozen in liquid nitrogen and stored at -80°C until use. Total RNA was extracted from 100mg of plant tissue using RNeasy plant Mini Kit (Qiagen, The Netherlands) and subjected to on-column DNase treatment (Sigma). Quantification of total RNA was carried out using Nanodrop ND-1000 spectrophotometer. Reverse transcription of RNA (1µg) was carried out using Omniscript RT Kit (Qiagen) and oligo (dT) primer using manufacturer's instructions.

Six defence marker genes (*PR1*, *PR2*, *PR3*, *SOD*, *CAT*, *PAL* and *OPR3*) and 2 reference genes (*Actin* and *GAPDH*) in both wheat and *B. distachyon* were analysed by qPCR (Appendix 1). Real-Time quantitative PCR was carried out in 12.5 µl reactions including 1.25 µl of a 1:5 (v/v) dilution of cDNA, 0.2 µM of primers, and 1× SYBR Premix Ex Taq (Tli RNase H plus, RR420A; Takara). PCR conditions were as follows: 1 cycle of 1 min at 95°C; 40 cycles of 5 s at 95°C and 20 s at 60°C; and a final cycle of 1 min at 95°C, 30 s, at 55°C, and 30 s at 95°C for the dissociation curve. The threshold cycle (Ct) values obtained by qRT-PCR were used to calculate the Normalised fold change was calculated for each time relative to mock using the $2^{-\Delta\Delta Ct}$ described by Livak and Schmittgen (2001) and Ct Reference = geometrical mean (Ct GAPDH: Ct Tub).

4.3.5 DAB and Trypan Staining

For analysis of H_2O_2, leaf sections were placed in 3, 3' diaminobenzidine (DAB) (1mg/ml) pH 3.8 for 4 hours in the dark. Leaf samples were then placed in 90% ethanol with heat for 10 minutes to remove chlorophyll. Cell death was stained using Trypan Blue (Koch and Slusarenko, 1990) while heating leaf samples for 20 minutes, before rinsing in water and de-staining in choral hydrate (2.5g/ml H_2O) overnight. Leaf samples were mounted onto slides with 80% glycerol and examined under a light microscope (Leica DM5500B). Images were recorded with a Leica DFC310FX digital camera.

4.3.6 Statistical Analysis

All the data from the studies were checked for normal distribution and when necessary, variances were stabilized using Box-cox transformation. A logistic regression model with a binomial distribution and a zero-inflation model with a Poisson distribution were used to analyse disease and pycnidia score data respectively using R. For gene expression studies, values correspond to the average of data from three technical repetitions from two biological samples. Statistical analysis was conducted using ANOVA incorporating Tukey's significant difference test at $P \leq 0.05$ in Graph Pad Prism (version 5.03 for windows; GraphPad software, San Diego, CA, United States).

4.4 RESULTS

4.4.1 NHR of *B. distachyon* to *Z. tritici* isolates

The reponse of *T. aestivum* (cv. Remus) and *B. distachyon* (*Bd-21*) to infection with an Irish *Z. tritici* isolate (560.11) and the reference Dutch isolate (IPO323) was investigated.

Disease symptoms (including necrosis) caused by the *Z. tritici* isolate 560.11 on wheat were more severe compared to the symptoms caused by the Dutch isolate, IPO323 (Fig. 1A). At 14, 17 and 21 days-post inoculation (dpi), disease levels on wheat were significantly ($p<0.05$) higher on plants inoculated with isolate 560.11 than plants inoculated with IPO323 (Fig 1C). Pycnidia coverage on wheat infected with isolate 560.11 was also significantly ($p<0.05$) higher than plants infected with IPO323 at 14, 17 and 21 dpi (Fig 1E).

Macroscopic symptoms were observed in *B. distachyon* as a result of a likely defense response to infection with both of the *Z. tritici* isolates (Fig. 1B). Symptoms were visible earlier in *B. distachyon* (10 dpi) than on the wheat cv. Remus (Fig. 1A). Symptoms on *B. distachyon* plants inoculated with the 560.11 isolate were significantly ($p<0.05$) higher compared to those inoculated with the IPO323 isolate at all time points (Fig. 1D). In addition, the symptoms caused by the *Z. tritici* isolates on *B. distachyon* were predominantly visible as the production of brown pigments rather than significant necrosis (Fig. 1B). No pycnidia were observed in infection of *B.distachyon* with either isolate at 21 dpi.

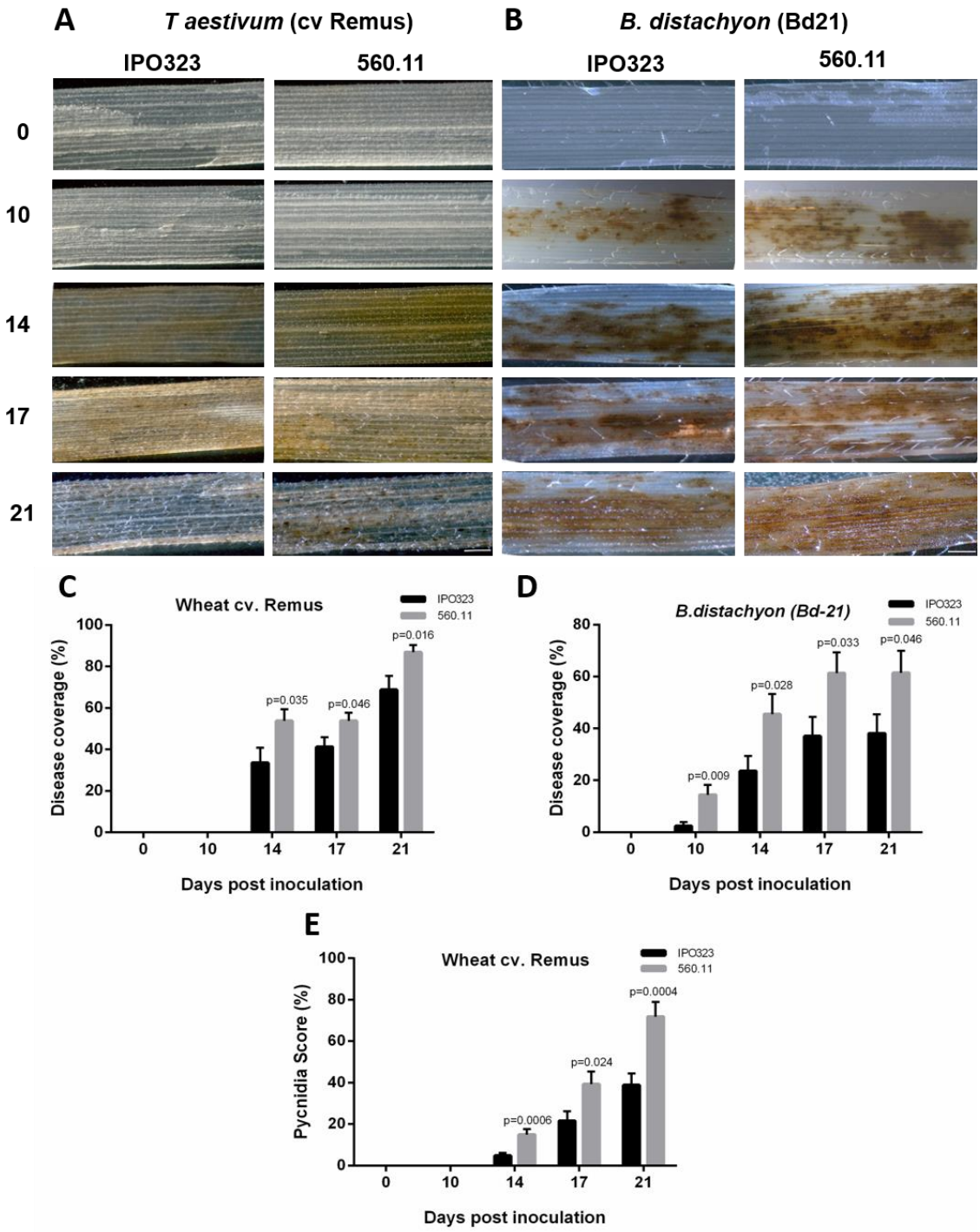

Fig. 1. Progression of symptoms on *T. aestivum* cv. Remus (A) and *B. distachyon* (B) induced by *Z. tritici* isolate IPO323 and 560.11. Infected leaves were cleared of chlorophyll and symptoms were recorded at (A) 10, (B) 14, (C) 17 and (D) 21dpi. Images are representative from three independent experiments (n=3). Symptom coverage at 10, 14, 17 and 21 days post inoculation on *T. aestivum* cv. Remus (C) and *B. distachyon* (D). Pycnidia coverage on Remus (E). Diseased leaves were visually rated for symptoms and pycnidia coverage. Each data point represents ± standard error of the mean of three independent experiments and corresponding p-values shown above bars. Scale bar = 2mm.

4.4.2 Defence gene expression following challenge of wheat and *B. distachyon* with two *Z. tritici* isolates

Defence gene induction in the non-host grass *B. distachyon* compared to the wheat host was investigated following inoculation with the *Z. tritici* isolates, 560.11 and IPO323. Quantitative real-time PCR (qRT-PCR) was performed to assess the relative expression of defence genes involved in Salicylic acid (SA) and Jasmonic acid (JA) mediated pathways, as well as Phenolic and Oxidative stress pathways.

4.4.2.1 SA signalling pathway marker gene expression

To explore a potential role for the SA signalling pathway in the interaction of *Z. tritici* with wheat and *B. distachyon* at early stages of infection (0.5, 48, 96 and 144 hours post inoculation (hpi)), expression of *PR1* (*Pathogenesis-Related*), *PR2, NPR1 (Non-expresser of PR genes 1)*, and *PAL* (*Phenylalanine Ammonia Lyase*) were investigated. The presence of fungal transcripts in the infected leaves was confirmed using semi-quantitative PCR using primer for *Z. tritici tubulin* gene (Appendix 5)

TaPR1 transcripts were significantly induced in wheat plants inoculated with IPO323 compared to mock controls at all time points analysed as were wheat seedlings inoculated with 560.11 at 144 hpi. Wheat seedlings inoculated with IPO323 also showed significantly higher transcript levels of *PR1* compared to those inoculated with the 560.11 isolate between 0.5 and 96 hpi while at the later timepoint of 144 hpi, plants inoculated with 560. 11 had higher *PR1* levels (Fig 2.1A). Similarly, *TaPR2* and *TaNPR1* transcripts were significantly higher in wheat plants infected with IPO323 at 0.5 hpi compared to this inoculated with 560.11 (Fig.2.1C, 2.2A). While, *TaPR2* increased again at 144 hpi to significantly higher levels following IPO323 inoculation than 560.11.

BdPR1 was found to be significantly induced only at 144 hpi with IPO323 (Fig. 2.1B), and was higher in comparison to plants inoculated with the 560.11 isolate While no significant difference in relative transcript levels of SA-mediated *BdPR2 and BdNPR1* genes were found in *B. distachyon* plants inoculated with either of the two *Z. tritici* isolates (Fig. 2.1D, 2.2B).

No significant induction of *TaPAL* was observed compared to mock control with both isolates (2.2C). However, a significant induction of *BdPAL* was observed in *B. distachyon*

relative to the mock control and was significantly different when challenged with the 560.11 isolate compared to IPO323 at 48 hpi, 96 hpi (Fig. 2.2D).

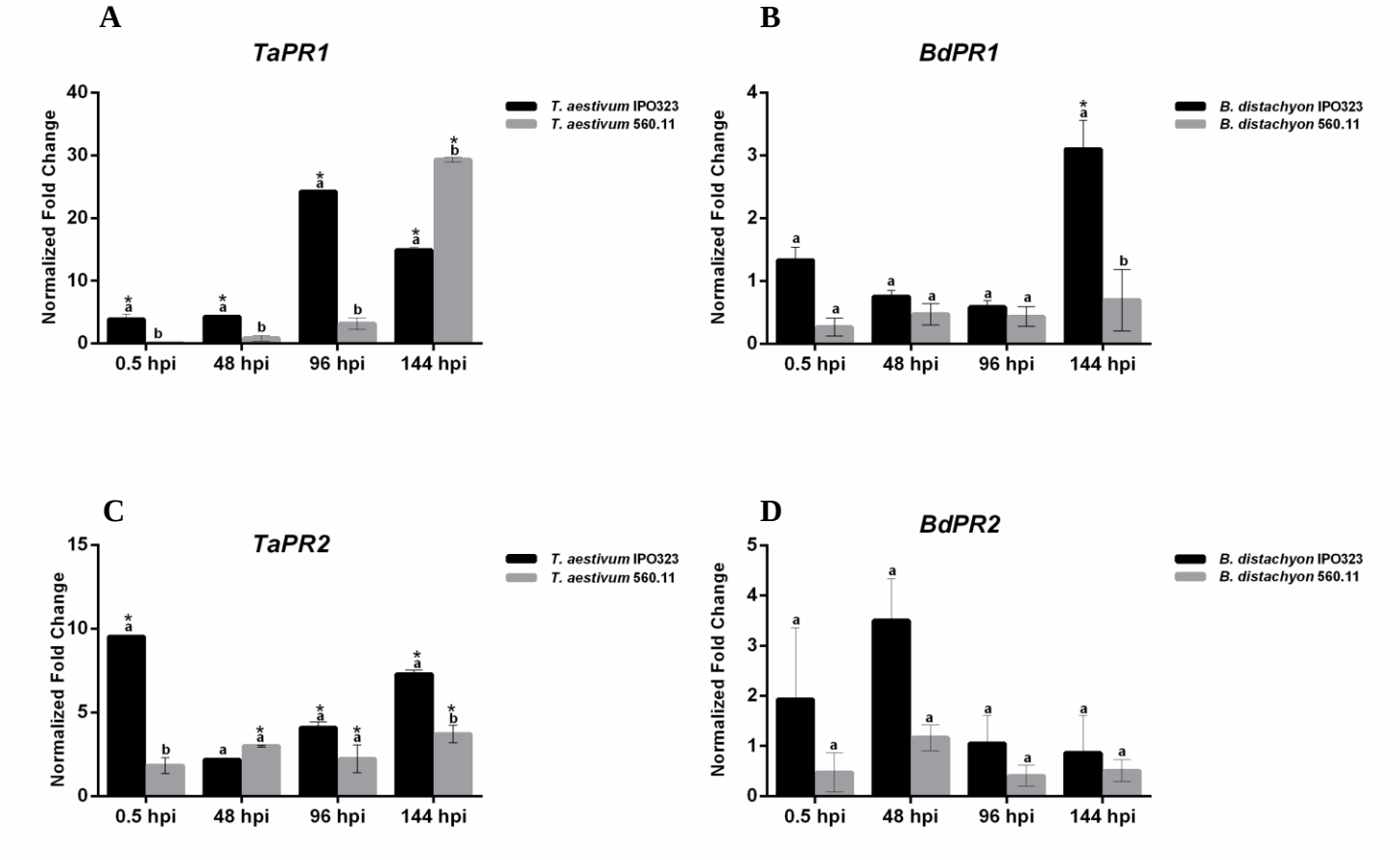

Fig. 2.1. Expression profile of *PR-1* (A), (B) and *PR-2* (C), (D) genes in *T. aestivum* (A), (C) and *B. distachyon* (B), (D) after infection by *Z. tritici*. qRT-PCR was used to assess gene expression (fold change) in leaves infected with *Z. tritici* isolates IPO323 and 560.11 relative to mock inoculations. Normalisation was carried out using *Actin* and *GAPDH* of respective *T. aestivum* and *B. distachyon.* Results represent mean of three biological replicates and two technical replicates (error bars indicates ±SEM). Means with the same letters are not significantly different while means with asterisk are significantly different from mock treated control determined using Tukey test at P≤ 0.05.

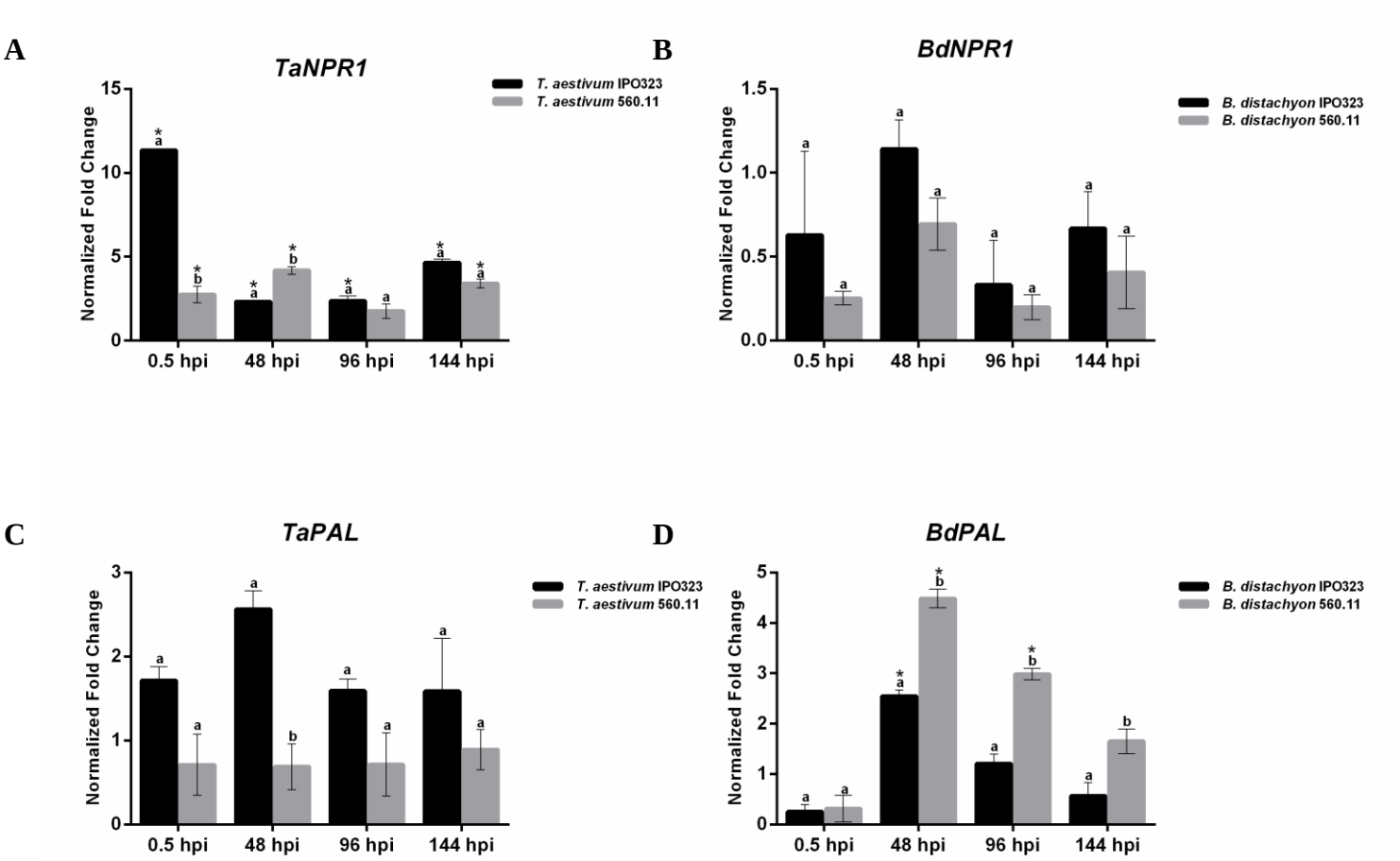

Figure 2.2. Expression profile of *NPR-1* (A), (B) and *PAL* (C), (D) genes in *T. aestivum* (A), (C*)* and *B. distachyon* (B), (D) after infection by *Z. tritici.* qRT-PCR was used to assess gene expression (fold change) in leaves infected with *Z. tritici* isolates IPO323 and 560.11 relative to mock inoculations. Normalisation was carried out using *Actin* and *GAPDH* of respective *T. aestivum* and *B. distachyon.* Results represent mean of three biological replicates and two technical replicates (error bars indicates ±SEM). Means with the same letters are not significantly different while means with asterisk are significantly different from mock treated control determined using Tukey test at P≤ 0.05.

4.4.2.2 JA signalling pathway marker gene expression

To explore the role of the JA signalling pathway to *Z. tritici* infection, the expression of *PR3* and *Oxo phytodienoate reductase 3 (OPR3*) was investigated. *TaPR3* transcript accumulation was induced and observed to be significantly higher ($p<0.05$) at 0.5 hpi in wheat plants infected with IPO323 (Fig. 3A). At 48 and 96 hpi, wheat infected with both isolates showed a significant induction of *TaPR3* accumulation compared to the mock control (Fig 3A). In infected *B. distachyon* plants, *BdPR3* transcripts were induced relative to mock control at 48 and 144 hpi with IPO323 and between 48 to 144hpi for 560.11 isolate. There was a significant difference in transcript accumulation of *BdPR3* at 48 and 96 hpi between the two *Z. tritici* isolates where 560.11 had higher *BdPR3* transcript levels (Fig. 3B). Levels of *TaOPR3* and *BdOPR3* transcripts were not found to be induced following infection with either *Z. tritici* isolate (Fig. 3D).

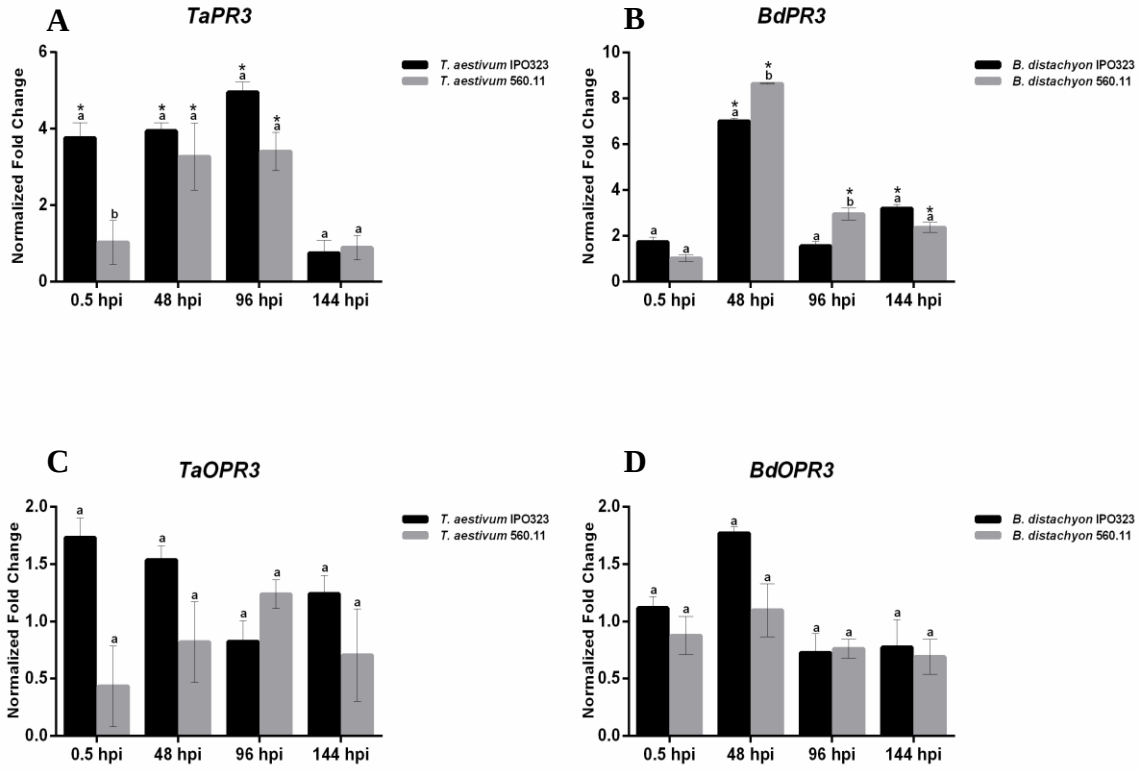

Fig. 3. Expression profile of *PR-3 (*A), (B) *and OPR-3* (C), (D) genes in *T. aestivum (*A), (C) and *B. distachyon* (B), (D) after infection by *Z. tritici.* qRT-PCR was used to assess gene expression (fold change) in leaves infected with *Z. tritici* isolates IPO323 and 560.11 relative to mock inoculations. Normalisation was carried out using *Actin* and *GAPDH* of respective *T. aestivum* and *B. distachyon.* Results represent mean of three biological replicates and two technical replicates (error bars indicates ±SEM). Means with the same letters are not significantly different while means with asterisk are significantly different from mock treated control determined using Tukey test at P≤ 0.05.

4.4.2.3 ROS production and catalase expression

Significant induction of *TaSOD* was observed at 0.5, 48, 96 hpi and 144 hpi in wheat plants infected with IPO323 compared to the mock control and the 560.11 isolate and (Fig. 4A). Similarly, *TaCAT* was significantly induced by IPO323 at 48 hpi compared to mock control and compared to inoculation with the 560.11 isolate (Fig. 4C). In *B. distachyon*, there was no significant difference in induction of *BdSOD* by the two isolates but *BdSOD* was induced relative to the control at 48 hpi and 144 hpi (Fig. 4B). In contrast, *BdCAT* was induced and differentially expressed in *B. distachyon* plants infected with either the IPO323 or 560.11 isolate at 0.5 hpi to 96 hpi (Fig. 4D).

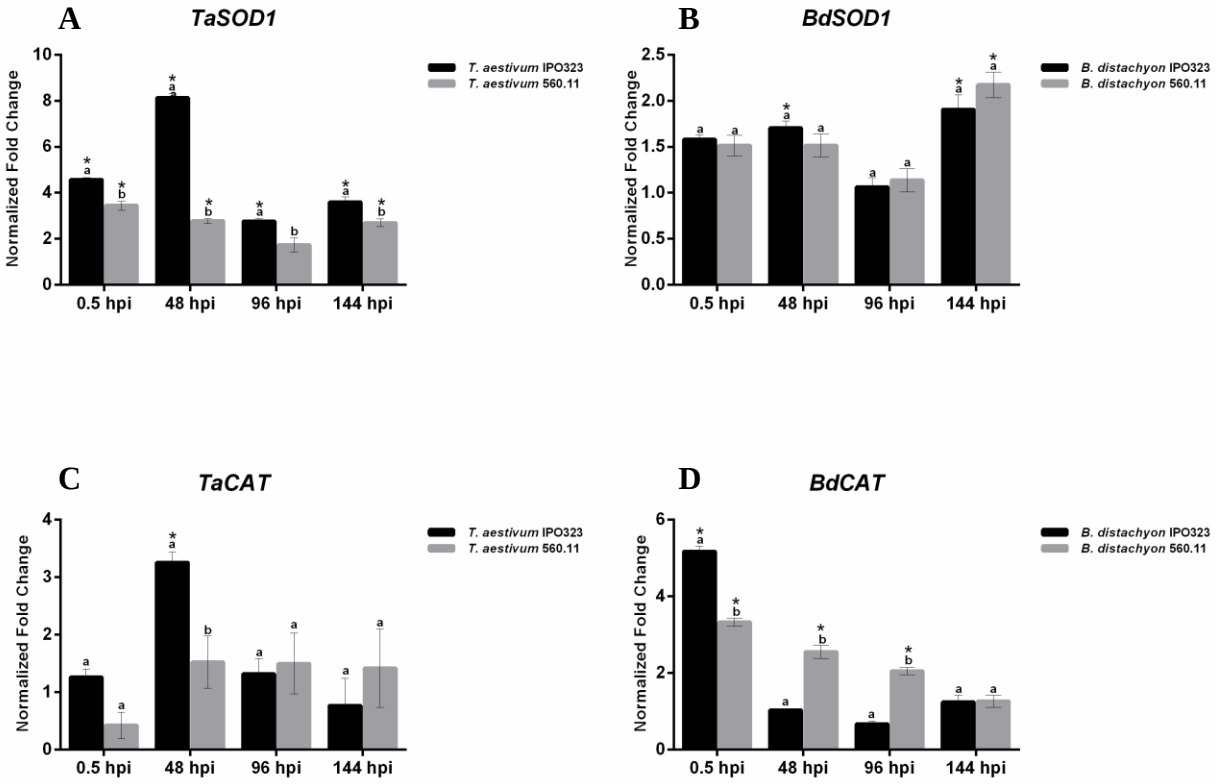

Fig. 4. Expression of *Superoxide dismutase (SOD)* (A), (B) and *Catalase (CAT)* (C), (D) gene in *T. aestivum* (A), (C) and *B. distachyon* (B), (D) after infection by *Z. tritici.* qRT-PCR was used to assess gene expression (fold change) in leaves infected with *Z. tritici* isolates IPO323 and 560.11 relative to mock inoculations. Normalisation was carried out using *Actin* and *GAPDH* of respective *T. aestivum* and *B. distachyon.* Results represent mean of three biological replicates and two technical replicates (error bars indicates ±SEM). Means with the same letters are not significantly different while means with asterisk are significantly different from mock treated control determined using Tukey test at P≤ 0.05.

4.4.3 Screening of *Z. tritici* effectors in *Brachypodium distachyon* using biolistic methods

In order to develop an alternative screening method for *Z. tritici* effectors in the model grass, biolistic methods was used to deliver a plasmid overexpressing *ZtSSP* candidates into *B. distachyon* leaves.The leave were stained for GUS (β-glucuronidase) cells as score for transformation and with trypan blue or 3, 3′-Diaminobenzidine (DAB) to record cell death and H_2O_2 production respectively. The candidate proteins *ZtSSP24* and *ZtSSP47* were screened however, no significant difference in the number of cells showing cell death or hydrogen peroxide accumulation compared to the empty vector control was found for either of the tested effector candidates (Fig 5).

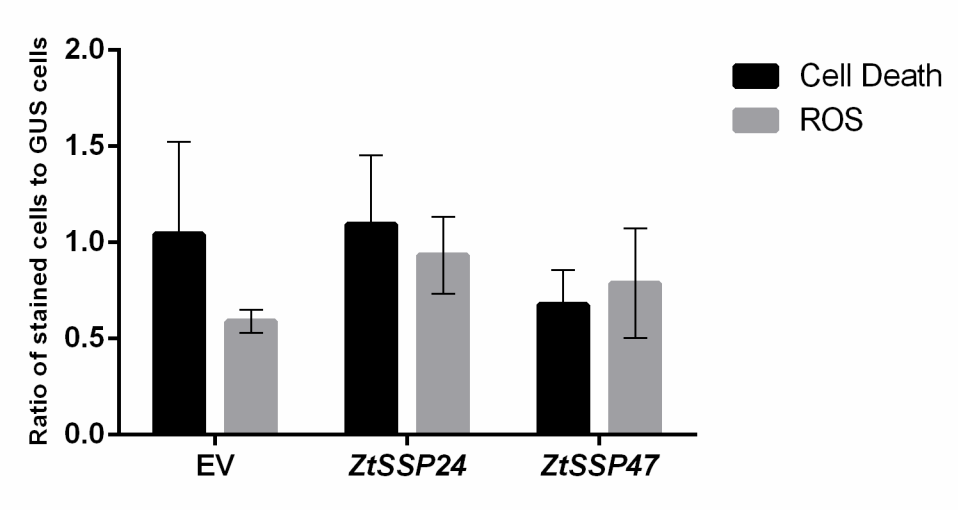

Fig. 5. Screening *Z. tritici* ZtSSP's in *Brachypodium distachyon*. Leaves of *B. distachyon* were co-bombarded with plasmid overexpressing *Z. tritici* effector protein candidates and plasmid pCAMBIA1305.1 for GUS expression. Three shots per construct were carried out and the leaves were then incubated in dark for 2 days. Each construct was then subjected to staining for transformation via GUS as well as for PCD with trypan blue and hydrogen peroxide with DAB. Bars represents the standard deviation with average of three independent experiments (n=21).

4.4.4 *ZtSSP* candidates did not induce cell death in wheat.

In order to assess whether any of the ZtSSPs contribute to cell death and thus the necrotrophic stage of *Z. tritici* infection in the host plant wheat; an assay was used where, the transient expression of the maize transcription factor *B-Peru* and *C1* leads to anthocyanin accumulation. The anthocyanin can be used as a cell death marker as it accumulates in intact vacuoles of viable cells (Pliego et al., 2013)

Thirteen pEG101: *ZtSSPs* over expression constructs were co-bombarded with pUbiGUS (cell death insensitive transformation marker) and anthocyanin expression plasmid pBC17 into wheat leaves. Co-bombardment of pBC17 with pUbiGUS and the vector pEG101 was used as a negative control while Zt-6 (Kettles et al. 2018) previously reported to induce cell death in wheat was used as a positive control. The number of cells accumulating anthocyanin (Fig. 6A) were counted and then the leaves were subsequently stained for GUS activity. The number of cells stained with GUS (Fig. 6B) was further counted and the ratio of anthocyanin to GUS cells was used as an estimate for cell death. The empty vector control (pEG101) resulted in the average ratio of 1.27, while the positive control (pEG101: Zt-6) showed reduced ratio of 0.3 (Fig. 6C). The anthocyanin to GUS ratios obtained from bombardment of thirteen ZtSSP constructs were not significantly different to the control suggesting no cell death activity by these candidates.

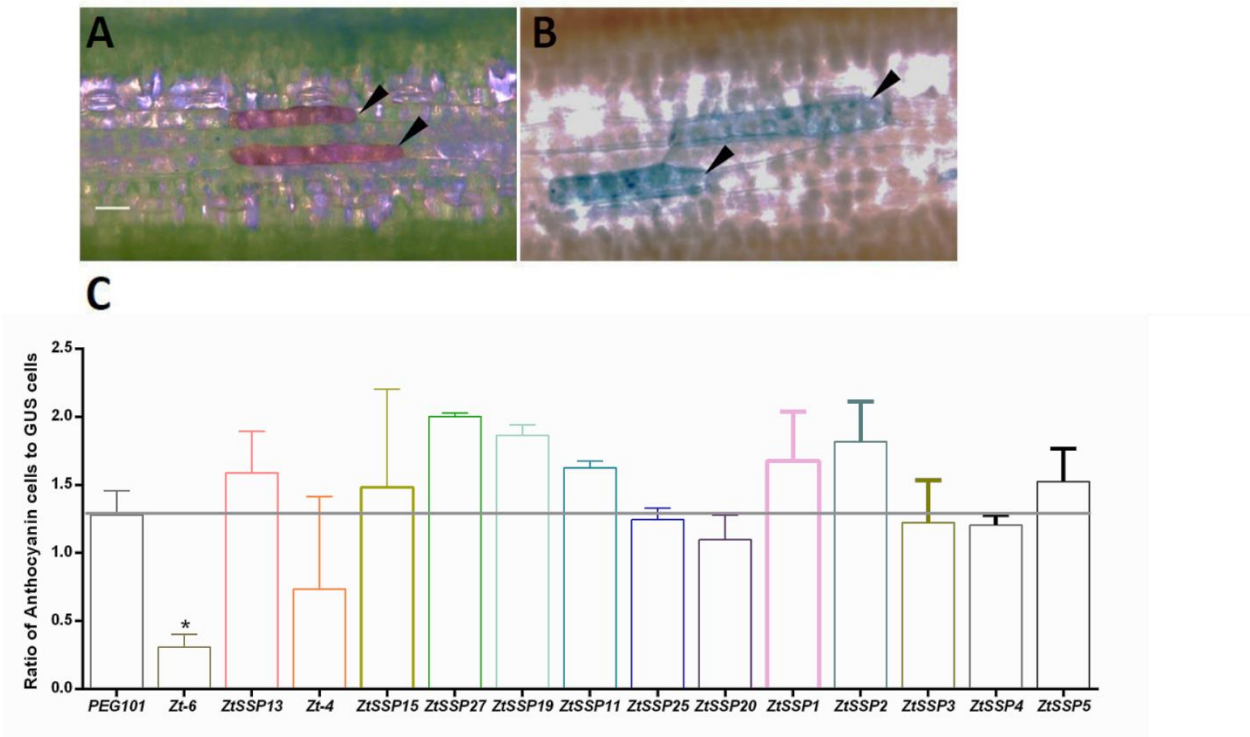

Fig 6. Cell death assay in wheat leaves. Wheat leaves cv. Kanzler were co-bombarded with pUbiGus as a transformation marker, pEG101 for overexpression of *Z. tritici* small secreted protein candidates, and the *B-Peru/C1-expression* plasmid pBC17 that induces anthocyanin accumulation in wheat epidermal cells. (A) Unstained wheat leaf showing epidermal cells that accumulate anthocyanin (arrow; bar=20 μm) 4 days after bombardment and (B) β-glucuronidase (GUS) expressing cells after GUS staining). (C) Quantification of the relative number of anthocyanin producing cells calculated as the ratio of anthocyanin accumulated cells to the number of β-glucuronidase (GUS) expressing cells. Co-transformation of pBC17 with the empty vector pEG101 and pEG101: Zt-6 served as negative and positive control respectively. Values are the means of four independent experiments with n= 28 leaves counted for each construct. (Bars=+SEM). The asterisk on top of bar represents significant differences determined by Tukey test (* for $P<0.05$).

4.5 DISCUSSION

Non-host defense involves up-regulation of ROS, JA and phenylpropanoid pathway in *B. distachyon* compared to *T. aestivum*.

To compare the interactions between the host *T. aestivum* and non-host grass *B. distachyon*, both were infected with two different *Z. tritici* isolates. The *Z. tritici* isolates tested induced rapid disease symptoms on inoculated *B. distachyon* leaves which further developed into dark brown lesions with brown pigment accumulation. Phenylalanine ammonia-lyase (PAL) catalyses the production of cinnamic acid from phenylalanine, which can be converted into phenolic compounds. These compounds are in turn oxidised and polymerized into brown pigments. Role of *PAL* and associated polyphenols have been associated with resistance in tomato against bacterial wilt pathogen *Ralstonia solanacearun* (Vanita et al. 2009), Similarly resistance in rice against *Laodelphax striatellus* involves higher accumulation of *PAL* and other phenolic compounds in resistance variety compared to susceptible one (Duan et al. 2014). Similarly, there was a consistent increased levels of brown pigment observed in *B. distachyon* following challenge with the 560.11 *Z. tritici* isolate (Ngadze et al. 2012). In addition, higher transcript levels of *BdPAL* were found in *B. distachyon* leaves infected with the 560.11 isolate from 48 to 144 hpi than with IPO323. No induction of *TaPAL* by either *Z. tritici* isolate was found in the susceptible wheat cultivar Remus. However, expression of the wheat *TaPAL* gene has been previously reported to be induced by *Z. tritici* and found to be higher in the STB semi-resistant wheat cultivar, Premio (Ors et al. 2017).

The role of reactive oxygen species has been extensively studied in both host and non-host defence responses (Hückelhoven & Kogel, 2003; Nimchuk et al. 2003). Moreover, H_2O_2 has been shown to play a key role in the outcome of the wheat-*Z. tritici* interaction (Shetty et al. 2003; 2007). *Superoxide dismutase* (*SOD*) and *Catalase* are known to break down superoxide radicals (O_2^-) and H_2O_2 respectively, and play key role in antioxidant defence against the pathogen (Zhang et al. 2008; Bednarski et al. 2013). Wheat SOD (*TaSOD*) expression was significantly higher in IPO323 infected samples compared to plants infected with the 560.11 isolate. This supports H_2O_2 as a defence response in wheat and may yexplain the lower disease symptoms and pycnidia counts found following infection of IPO323. A lower accumulation of H_2O_2 during infection with the 560.11 isolate would agrees with susceptible interactions observed by Shetty et al. 2003. Similarly, higher pycnidia production was observed on plants

infected with the 560.11 isolate, suggesting higher virulence of *Z. tritici* 560.11 compared to IPO323 on the wheat cv. Remus.

The interaction between *B. distachyon* and the *Z. tritici* 560.11 and IPO323 isolates resulted in the early onset of H_2O_2 accumulation in epidermal cells associated with *Z. tritici* spores. H_2O_2 accumulation in *B. distachyon* occurs as early as two dpi in epidermal cells following challenge with both isolates (data not shown). The induction of *BdSOD* with both isolates at 48hpi correlates with this observation In addition, significantly lower expression levels of the antioxidant *BdCAT* were observed at 0.5 hpi on plants inoculated with the *Z. tritici* 560.11 isolate compared to IPO323. Together this suggests that H_2O_2 accumulation could also play a key role in NHR of *B. distachyon* against *Z. tritici*.

The role of plant hormones in defence against pathogen infection has been well characterised (Bari and Jones, 2009; Pieterse et al. 2009). Higher induction of SA- related *PR* genes against *Z. tritici* has been observed in resistant cultivars, while little induction of these genes was observed in susceptible cultivars at initial stages of infection (Shetty et al. 2003; Ray et al. 2003; Adhikari et al. 2007). *TaPR1*, *TaPR2* and *TaNPR1* were induced in wheat following *Z. tritici* infection and this was earlier and transcript levels were higher with IPO323. This suggests that SA-mediated signalling is triggered in wheat possibly as a defence response against *Z. tritici.* This result is in agreement with previous research where higher expression of *PR* genes was observed in the susceptible cultivar Alixan compared to resistant cultivar Atigo (Ors et al. 2017).

The JA signalling pathway is known to mediate defence against necrotrophic and hemi-biotrophic pathogens (Li & Yen 2008). No differential expression of *TaPR3* and *TaOPR3* between isolates was observed with wheat, with the exception of *TaPR3* induction by IPO323 at 0.5 hpi. However, *BdPR3* was found to be induced at 48, 96 and 144 hpi in *B. distachyon* following *Z. tritici* inoculation but to higher levels at 48 hpi and 96 hpi with the 560.11 isolate than IPO323 suggesting role of JA against *Z. tritici* in non-host resistance (Fig. 7B). Taken together, the expression profiles of these key defence marker genes suggests that ROS, SA, JA and the phenylpropanoid pathway have roles in limiting disease and pycnidia progression in wheat against *Z. tritici* (Ors et al. 2017; Shetty et al. 2003, 2007). Although, SA may promote disease and PCD later in infection. While ROS, JA and the phenylpropanoid pathway are key for NHR of *B. distachyon* against *Z. tritici* infection at the end of the latent period.

Transient screening of *Z. tritici* effector in *B. distachyon*

Comparision of the infection and resistance response of non-host plant *B. distachyon* against *Z. tritici* isolates showed that NHR of *B. distachyon* exhibits an early symptoms (browning) compared to susceptible wheat. In addition, the response of *B. distachyon* also showed isolate specific responses with 560.11. Therefore, the isolate specific responses observed in a non-host *B. distachyon* prompted the screening of *Z. tritici* candidate effectors in a monocot grass system.

A number of studies have successfully utilised *A. tumefaciens* mediated transient expression in *N. benthamiana* as a substitute screening system (Petre et al. 2016; Liu et al. 2016; Qi et al. 2016; Cheng et al. 2017; Ramachandran et al. 2017; Kettles et al. 2017; Qi et al. 2018). Petre et al. 2016 identified a candidate effector from wheat stripe rust (*Puccinia striiformis f.sp. tritici*) that interacts with plant processing bodies. Similarly, effector candidate 6 *(PEC6)* from same fungus was found to suppress ROS accumulation and callose deposition in *N. bethamiana* as well as interact with wheat adenosine kinases (Liu et al. 2016). Concurrently with this study Kettles et al. 2017 identified number of candidate protein from *Z. tritici* that induce cell death in *N. bethamaina*. Apart from induction of phenotype, this non-host system has allowed subcellular localisation of proteins using a fluorescent protein tag fused with candidate effector proteins, such as the *P. pachyrhizi* effector candidate 23 protein (PpEC23) was reported to be localised in the nucleus and interact with a host transcription factor, similarly an effector from yellow rust (*Puccinia striiformis f s p tritici*) *PST02549* accumulated in the nucleus and cytosol and was found to interact with component of host P bodies (Qi et al. 2016; Petre et al. 2016).

However, *A. tumefaciens* mediated expression has not been successful for monocot grasses. In addition to that dicot plants act as a strictly non-host to pathogen of cereals therefore, any of the induced phenotypes might not always reflect the phenotype in the natural host. Similarly, protein accumulation and localisation in a surrogate plant again may not always reflect their true localisation in the host environment.

Therefore, a GUS-mediated single cell transient assay (Bai et al. 2013) was investigated using *B. distachyon*. Subsequent cell staining using DAB and trypan blue would visualise the phenotype induced. Two candidates *ZtSSP24* and *ZtSSP47* were transiently expressed in *B. distachyon* leaves but no H_2O_2 or cell death phenotype was observed compared to control. This

suggested that these candidates failed to induce a defense response in *B. distachyon*. However, the biolistic transformation in *B. distachyon* also suffered with a very low transformation efficiency (~ 25-50 cells) as indicated by the low number of GUS cells (Fig 5). To overcome this limitation, young leaves from 3 to 5 growth stages were used along with the varying amount of gold particles and DNA concentration however, transformation efficiency still suffered from lower efficiency. This may suggest that *B. distachyon* leaves may not be well suited for biolistic assays. In contrast to biolistic approach other over-expression assays like bacterial mediated or viral overexpression system could be more suitable in *B. distachyon* leaves.

Z. tritici effector candidate did not induce cell death in *T. aestivum*

An assay reported by Pliego et al. 2013 was previously used to screen fifty *Blumeria graminis f. sp. hordei* candidate effectors in barley by using host-induced gene silencing and subsequently identified eight candidates that contribute to infection. The same assay was used here to screen *Z. tritici* candidate effectors in wheat. Thirteen *ZtSSP* candidates tested were not found to induce cell death in wheat leaves. This may be explained by a host specialized function or cultivar specific response of these *Z. tritici* candidate proteins. Since *Z. tritici* is specialised to infect wheat, its effector candidates may be able to avoid recognition by wheat cognate receptors.

Insertions or polymorphism within the regulatory regions of effector genes could also play role in alteration of effector expression during host colonisation.This might ultimately play role in intial subvert of host defense with lower expression and higher expression of cell death inducing effector in later necrotrophic stage resulting in host cell death (Rudd et al. 2015; Toruno et al. 2016). Therefore, transient biolistic assay might not be an apposite approach to capture cell death as this transient system relies on visualisation quite early i.e 2-4 days post bombardment.

Additionally, there is absence of hypersensitive mediated cell death during interaction of wheat resistance gene (R) *Stb6* with corresponding avirulence (Avr) effector *Avrstb6* (Rudd et al. 2008). Therefore, cell death assays may not be an appropriate assay for functional screening of these candidates. Alternatively, *Z. tritici* effectors might play role in suppressing wheat defense mediated cell death responses especially during the early biotrophic phase where cell death is

not favoured. Co-bombardment of effector candidates with a cell death inducer protein such as *Zt6* (Kettles et al. 2017) could help identify any cell death suppressing *Z. tritici* candidates.

An alternative to the biolistic mediated transient expression system, is a bacterial T3SS mediated delivery by *P. fluorescens*. Pf0-1 strain Effector-to-Host Analyzer (EtHAn) was developed to deliver pathogen effectors into wheat (Yin & Hulbert 2010; Upadhyaya et al. 2013). Using this system Upadhyaya et al. 2013 screened wheat stem rust effectors and identified one candidate that could induce HR response in wheat. Similarly, there are few studies utilizing virus-mediated overexpression (VOX) for protein expression in cereals (Clement et al. 2018). These alternative approaches could hold potential to screen for *Z. tritici* effectors in monocot cereals.

In conclusion, screening different *ZtSSP* candidates for induction of cell death in non-host *B.distachyon* and in a host plant wheat did not identify any candidates that induce cell death. In addition the defense response of both *B. distachyon* and wheat against *Z. tritici* isolates is isolate specific. However, active ROS and the phenylpropanoid pathway may have a role in the NHR response of *B.distachyon* to *Z. tritici* compared to host wheat.

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Chapter 5

General Discussion

The journey from effector identification to effector mediated resistance in crops

5.1 ABSTRACT

Filamentous plant pathogens causes diverse diseases and thus impose a serious threat to agriculture system and food production worldwide causing reduction in crop yields. These pathogens are known to secrete effector proteins (small secreted proteins, SSPs) that are key to their pathogenicity. These proteins are involved in the manipulation of host immune system and various other cellular and physiological processes contributing to disease. With the advent of next generation sequencing and various 'omics' tools, rapid identification of these candidate proteins has been possible. However, this identification has not been followed by rapid characterisation of their function. Therefore, effector characterisation and identification of effector interacting host susceptible factors would provide insights on manipulation of the host defense system by effector. Such molecular understanding of effector function and its susceptible host targets could help design future strategies to protect crops against pathogens.

5.2 INTRODUCTION

Crop production faces tremendous challenges whether it be the rapidly changing environmental conditions or damage to yields by plant pathogens. Management of diseases on small gain cereals is heavily relient on the use of chemical fungicides (Morton & Staub 2008). This has led to a scenario where fungal pathogens are acquiring fungicide resistance while there is a political demand for the reduction of fungicide applications. Therefore, there is a need for alternative approaches to control septoria tritici blotch (STB). This could be through new sources of resistance such as durable resistance genes, a better understanding of pathogen biology, and the identification of key virulence effectors and their susceptible host targets.

5.2.1 Effector Discovery in *Zymoseptoria tritici*

With the advent of next generation sequencing, high number of fungal genomic sequences and transcriptomic data are now publically available and providing better insights into fungal pathogen genomes and their effector repertoire. This genetic information has allowed the identification and *in silico* prediction of candidate effector proteins. The prediction is usually through presence of signatures or motifs (conserved amino acid sequence in the protein structure) such as N-terminal putative secretion signals, CRN proteins, RXLR-motifs. These features have allowed to catalogue high numbers of candidate effectors from bacterial and oomycete pathogens to be catalouged (McDermott et al. 2011; Jiang et al. 2008; Hass et al. 2009). However, prediction of candidate effectors from fungal pathogens is more challenging due to lack of such common motifs in their sequences owing to their highly plastic genomes (Hane et al. 2011; Plissonneau et al. 2018).

Here the publicly available secretome dataset from Amaral et al. 2012 was mined to identify non-annotated *Z. tritici* small secreted proteins (*ZtSSPs*). This dataset had used SignalP v3.0 to predict secreted proteins. This was refined here by selecting for those ZtSSPs with expressed sequence tag (EST) support, a small size (50 -300 amino acids) and the presence of cysteine residues.Using this approach I identified 90 candidates that were potential effectors. Further analysis using the NCBI conserved domain database resulted in 50 non-annotated or hypothetical proteins while remaining 40 had a known functional domain. These 50 hypothetical proteins were selected for further functional analysis. The generalised approach used in this study allowed the quick selection of candidate effectors from the dataset however,

this approach of selection holds potential risk of eliminating larger or cysteine poor effector proteins.

5.2.2 Machine learning as a tool to identify *Zymoseptoria tritici* effectors

Computational prediction models utilising general properties of effector proteins has been applied as a starting point to screen candidates for further functional analysis. One of the first machine learning tools for prediction fungal effectors was EffectorP 1.0 (Sperschneider et al. 2016). Sperschneider et al. 2016 reported improvement of prediction from fungal secretome datasets using sequence derived properties like length, molecular weight, protein net charge as well as cysteine, serine and tryptophan content. Recently, an updated version EffectorP 2.0 was released and reported to have improved prediction through training on negative dataset and achieved higher prediction accuracy (Sperschneider et al. 2018a).

In addition to prediction machine learning models, subcellular localisation of candidate effectors can be predicted using LOCALIZER and ApoplastP respectively (Sperschneider et al. 2017, 2018b). This machine learning approach provides faster identification however the performance of such machine learning approaches relies heavily on training datasets and selection pipelines included, owing to the fact that these effector proteins are highly diverse accurate predictions are difficult. In this thesis, EffectorP, ApoplastP was utilised to screen for candidate effector from *Z. tritici*. One of the candidate protein ZtSSP2 was not predicted to be an effector however, using functional assays it was validated that the candidate ZtSSP2 is indeed a well conserved protein with effector characteristics and has a functional secretion signal. This result reflects the inaccuracy of the prediction softwares in identification of potential effector candidate and a heavy reliance on these tools could result in the omission of key candidates. In addition, the lack of functionally validated effectors also limits the datasets need for training such programs. Despite these drawbacks as higher number of pathogen effectors are identified and validated experimentally, these data would potentially improve such predictions.

5.2.3 Comparative genomics for *Zymoseptoria tritici* effector identification

Recent advances in next generation sequencing has allowed genomic comparisons and accurate information on genetic variations within a pathogen population. Genomic insights on plant fungal pathogens have shown that fungal genomes are highly polymorphic in nature described as “two-speed genome” model suggesting fungal genomic regions that are gene rich and

undergo lower evolutionary changes and a fast evolving repeat-rich regions enriched with effectors (Stukenbrock & Croll 2014; Dong et al. 2015). These highly evolving regions in the genomes often includes effector genes which play key roles in pathogenicity. Rapid adaptation including gene deletion, effector gene polymorphisms and transposable element insertions was reported in *Z. tritici* populations (Croll & McDonald, 2012; Hartmann et al. 2016, Plissonneau et al., 2018). Studies utilising association genetic mapping in *Z. tritici* have identified the highly polymorphic effector *AvrStb6* that evades recognition of wheat cultivars carrying the corresponding resistance gene, *Stb6* (Zhong et al. 2017). In addition to that recent study by Meile et al. 2018 also utilised genetic mapping to identify another highly polymorphic effector *Avr3D1* that evades recognition by wheat cultivars carrying *Stb7* resistance gene. This approach has been highly successful and holds potential in identifying potent virulence effector candidates from pathogen populations.

In this thesis, five cell death candidates identified in first chapter were analysed for presence/absence andfor sequence polymorphisms in Irish field isolates (Welch et al. 2017). Recombination events leading to loss of effector gene and gain in virulence occurs frequently in filamentous plants (Croll & McDonald, 2012; Hartmann et al. 2017). Candidate *ZtSSP4* was absent in all the Irish field isolates suggesting a loss of function variation in Irish field isolates compared to reference and 560.11 isolates. Using genome assembly one can predict whether there is any transposable elements rich regions in close proximity to *ZtSSP4* which has led to gene loss in field isolates. The remaining four candidates *ZtSPP1-3* and *ZtSSP5* were present in 57 field isolates with few single nucleotide polymorphism in their protein sequence suggesting these four candidates represents a more conserevedse of *Z. tritici* effector candidates. Transient overexpression of these a candidates in a non-host system showed possible recognition and activation of defense response which suggests presence of recognition receptors in distant dicot non-host plant.

5.2.4 Identification of effectors using random mutagenesis

Random mutagenesis has been widely utilised as a tool to identify genes that are required for virulence in the host plant (Kahmann & Basse 1999; Hamer et al. 2001). *Agrobacterium tumefaciens*-mediated transformation (ATMT) has been extensively used to disrupt fungal genes from pathogens including *F. oxysporum* (Mullins et al. 2001), *C. graminicola* (Muench et al. 2011) and *Z. tritici* (Gohari et al. 2014; Tiley et al. 2018). A random mutagenesis screen

on *V. dahliae* followed by pathogenicity test and sequence analysis resulted in identification of 58 candidate pathogenicity and effector genes (Santhanam et al. 2016).

Such a strategy could be ideal to generate ATMT or EMS mutant library of *Z. tritici*. These mutants can then be subsequently tested for pathogenicity in various wheat cultivar possessing known *Stb* resistant genes. Mutants that displayed reduced virulence or in contrast those that trigger disease resistance and localised host cell death could then be analysed to identify novel effectors and pathogenicity factors of *Z. tritici*.

5.2.5 Effectors as a tools for pathogen resistance breeding

Novel breeding programs involves identification of resistant traits using gene mapping and marker assisted breeding. However, rapid evolution of pathogen genome and limited knowledge on the resistance mechanism still impose a challenge in mordern agriculture. Therefore, if core sets of pathogen effectors that contribute to pathogenicity are known then, these effectors can be used to screen for resistant germplasm that have recognition specificity to such effector candidates. This effector assisted rapid screening of plant germplasm provides a powerful tool for breeding resistant crops (Vleeshouwers & Oliver 2004). *Agrobacterium tumefaciens* proteins or potato virus X (PVX) mediated expression of effector proteins in dicot plants has provided a rapid functional analysis of effectors *in planta*.

Effector screening was utilised for *Z. tritici* candidates in the second chapter of this thesis. The Z. *tritici* candidates were screened using *A. tumefaciens* mediated expression in a non-host plant model *N. benthamiana*. The elicitor activity of five novel candidates was identified based on the cell death phenotype induced. This result showed that non-adapted pathogen effectors are recognised by a non-host plant suggesting that the non-host plant could act as a source of resistance. Once identified these receptors may be a tool for durable resistance viagenetic engineering. One such example involved the expression of *Arabidopsis NPR1* in strawberry which provided resistance to anthracnose (*Colletotrichum* spp.), powdery mildew (*Podosphaera aphanis*), and bacterial angular leaf spot (*Xanthomonas fragariae*) (Silva et al. 2015). Transgenic tomato plants with *Arabidopsis EFR* was shown to provide broad spectrum resistance in tomato against bacterial diseases (Lacombe et al. 2010). With such successful reports of resistance the potential use of gene editing techniques such as clustered regularly interspaced short pallindromic repeats (CRISPR) to transfer of single or even multiple target

genes for NHR to distantly related plant species could be an effective approach to achieve durable resistance.

Necrotrophic effectors of *Pyrenophora tritici-repentis* PtrToxA was utilised to screen for wheat cultivars susceptible to tan spot. This rapid assay was rapidly adopted by breeders as it eliminates the process of phenotyping of wheat cultivars against tan spot in field (Vleeshouwers & Oliver 2004). Such an approach would be an ideal scenario if one can identify key necrotrophic effectors against *Z. tritici*.

Since, *A. tumefaciens* mediated transient expression is not amenable to monocots a biolistic approach of effector screening was utilised. However, lack of recognition of these effector in wheat suggested potential host specificity or a cultivar specific response. Apart from the assay used in this thesis, alternatively, bacterial and viral based vectors for expressing effectors into monocot cereals could provide a valuable tool for screening *Z. tritici* effector candidates in different cultivars of wheat and identification of resistant cultivar.

5.2.6 Susceptibility genes as a source of resistance

The utilisation of susceptibility genes can be an alternative approach to resistance breeding. These so called susceptibility factors are appropriated by pathogen effectors to manipulate host defense mechanism. One of such susceptible factors identified was potato NPH3/RPT2-LIKE1 protein (StNLR1) that interacts with *P. infestans* RXLR effector Pi02860. Interaction of Pi02860 with StNLR1 results in increased interaction with Switch-Associated Protein 70 (SWAP70), thus targeting SWAP70 for proteasomal degradation, thereby suppressing SWAP70 mediated plant immunity (Qin et al. 2018). Similarly, the effector *Avr3a* interacts and stabilizes the host E3 ligase CMPG1 thereby suppressing infestin 1 (INF1) mediated cell death (Bos et al. 2010). In the third chapter of this thesis a wheat E3 ubiquitin ligase was identified that interacts with one of the *Z. tritici* effector (*ZtSSP2*).

Together with the evidence from *P. infestans* effector candidates, it can be hypothesized that *Z. tritici* effector *ZtSSP2* might be interacting with host E3UBQ to manipulate host defense mechanism in a similar fashion. Further, work will shed light on mechanism of host immune modulation by *Z. tritici* effector. With the advent of genome editing techniques like CRISPR/Cas9 (clustered regularly interspaced short palindromic repeats) and transcription activator-like effector nucleases (TALENs) it is possible to target host susceptible genes to

make a subtle change in the sequences that could lead to inactivation of so called susceptible factors. Such genomic editing tools has been successfully used in crops including tomato to silence the downy mildew resistance gene (DMR6) and provide broad-spectrum disease resistance (Thomazella et al. 2016). Similarly, targeted mutation in the rice ethylene responsive factor, *OsERF922* leds to an increased resistance to *M. oryzae* (Liu et al. 2012).

5.3 Conclusion

In summary, this study used a bioinformatic based *in silico* prediction for identification of candidate effectors from *Z. tritici*. These effector candidates are differentially expressed throughout the *Z. tritici* infection and induce a cell death phenotype in a non-host plant. Functional characterisation of one of the candidates (ZtSSP2) showed that it interacts with a wheat ubiquitin ligase which provided a novel insights into the molecular mechanism of host defense manipulation by *Z. tritici*. *B. distachyon* was investigated as a non-host model for studying the *Z. tritici* infection and screening effector candidates. Non-host defense signalling pathways were found to play a key role in limiting *Z. tritici* infection in *B. distachyon*. The result of this research has identified novel effector candidates and their role in pathogencity particularly for ZtSSP2. Further work on these effectors could provide comprehensive understandings on *Z. tritici*-wheat interaction and allow the development of novel methods of control.

5.4 Future Work

This study has identified some of the key effector candidates from *Z. tritici* and shed light on its mechanism to manipulate host defenses. Of special interest in the future is to establish the role of wheat E3 ubiquitin ligase in regulating defense against *Z. tritici*. Potential silencing of wheat E3 ubiquitin ligase and subsequent identification of its downstream targets would provide a conceptual frame work on the pathway involved and targeted by *Z. tritici* effector ZtSSP2. A knowndown *Z. tritici* mutant of *ZtSSP2* would confirm its role in pathogen virulence. Future work should focus on characterising the other candidate effectors identified here. Subcellular localisation and identification of effector targets will also hold key for subsequent effector research in this *Z.tritici*-wheat pathosystem. Furthermore, an alternative approach to that used here would be to identify effectors that could suppress cell death

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SCIENTIFIC CONTRIBUTIONS

Tiley A.M., **Karki S.J.**, and Feechan A. 2018. *Zymoseptoria Tritici*. In: eLS. John Wiley & Sons Ltd, Chichester. http://www.els.net [doi: 10.1002/9780470015902.a0027948].

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Karki S.J., Zhou B.B., Feechan A. Screening of small secreted proteins of *Z. tritici* reveals a candidate protein that targets host ubiquitin system. In preparation.

PATENT

Karki S.J and Feechan A. 2018. Small secreted polypeptides for use in treating or preventing microbial infections in plants.UK patent number GB1814950.0 (Status: Pending).

CONFERENCE PROCEEDINGS

Plant Science Seminar Series, Oral presentation, January 2017 & May 2018.University College Dublin, Ireland.

Irish Plant Scientist's Association Meeting (IPSAM), Oral presentation. Limerick Institute of Technology (June 2017) and University College Dublin (June 2018), Ireland.

CerealPath Annual Symposium, Oral presentation, University College Dublin (December 2016) and University of Natural Resources and Life Sciences, Vienna (BOKU) (December 2018).

Zymoseptoria tritici community meeting, Poster presentation, September 2017. University of Kiel, Germany.

International Congress of Plant pathology (ICPP), Poster presentation. July 2018. Boston, United States

MODULES UNDERTAKEN

Biologicals and Bioactives for Plant Disease Control, ECTS credit 4, March 2017, University of Copenhagen, Denmark.

PSC Summer School 2016: Agriculture in Transformation, ECTS credit 3, September 2016, Zürich-Basel Plant Science Center, University of Zurich, Switzerland.

CerealPath Soft Skill, ECTS credit 2.5, April 2016, John Innes Centre & Limagrain, UK.

Creative Thinking and Innovation Training, ECTS credit 5, April 2016, University College Dublin, Ireland.

CerealPath Intro to Research, ECTS credit 2.5, January 2016, University College Dublin, Ireland.

Working with Biological Data, ECTS credit 5, January 2017, University College Dublin, Ireland.

Non-UCD Accredited Prior Learning, ECTS credit 10, January 2016, University College Dublin, Ireland.

6. APPENDIX

Appendix 1: List of Primers used in this project

Primer Name	Primer Sequence (5' - 3')	Purpose	Source
100649F	GGGGACAAGTTTGTACAAAAAAGCAGGCTTCATGCTTCCAGATGGAGACGAACC	Cloning	This study
100649R	GGGGACCACTTTGTACAAGAAAGCTGGGTGCTAAGCGCAATCCGCCGAAC	Cloning	This study
102617F	GGGGACAAGTTTGTACAAAAAAGCAGGCTTCATGCGACGTGCAAACGTTCAAAG	Cloning	This study
102617R	GGGGACCACTTTGTACAAGAAAGCTGGGTGTCAAGCCTTTAAACAAGGATCCG	Cloning	This study
103091F	GGGGACAAGTTTGTACAAAAAAGCAGGCTTCATGCTTCCCAAACCCATCGACGC	Cloning	This study
103091R	GGGGACCACTTTGTACAAGAAAGCTGGGTGTCAGCAGAAAATGCCATCGGCG	Cloning	This study
103900F	GGGGACAAGTTTGTACAAAAAAGCAGGCTTCATGGACAGCCTCGTTGTCAGGC	Cloning	This study
103900R	GGGGACCACTTTGTACAAGAAAGCTGGGTGTCAAGGAGGTGTACCGTTGTGT	Cloning	This study
104000F	GGGGACAAGTTTGTACAAAAAAGCAGGCTTCATGGGTCTTCTCTCCAAGCGTGG	Cloning	This study
104000R	GGGGACCACTTTGTACAAGAAAGCTGGGTGCTCCTCGTGGAGATGCTAGATG	Cloning	This study
104404F	GGGGACAAGTTTGTACAAAAAAGCAGGCTTCATGGTCCACTACCTCACTCCTG	Cloning	This study
104404R	GGGGACCACTTTGTACAAGAAAGCTGGGTGTTAGTTGTGAGGAGGAAGCGTC	Cloning	This study
104444F	GGGGACAAGTTTGTACAAAAAAGCAGGCTTCATGAAATACTGCGCCGGGTGCG	Cloning	This study
104444R	GGGGACCACTTTGTACAAGAAAGCTGGGTGTCAAGTTCCTGGGCACGATGTG	Cloning	This study
104697F	GGGGACAAGTTTGTACAAAAAAGCAGGCTTCATGGCCCCGGCTCCTGTC	Cloning	This study
104697R	GGGGACCACTTTGTACAAGAAAGCTGGGTGTTAAAGCTGGGCAGGAGTGTAGC	Cloning	This study
104794F	GGGGACAAGTTTGTACAAAAAAGCAGGCTTCATGCACTCCGACACCATCTGGC	Cloning	This study
104794R	GGGGACCACTTTGTACAAGAAAGCTGGGTGCTACTTCGCAGAGCCCTGCTC	Cloning	This study
105182F	GGGGACAAGTTTGTACAAAAAAGCAGGCTTCATGATCAGCGTTCCCGACCTCAAG	Cloning	This study
105182R	GGGGACCACTTTGTACAAGAAAGCTGGGTGCTAGACGGTAGCCCTGTTCTC	Cloning	This study
105223F	GGGGACAAGTTTGTACAAAAAAGCAGGCTTCATGGCACCTCAGCACCAGCG	Cloning	This study
105223R	GGGGACCACTTTGTACAAGAAAGCTGGGTGTCAGTTGTACGTGCAGCTGGG	Cloning	This study
105265F	GGGGACAAGTTTGTACAAAAAAGCAGGCTTCATGCAAAACTACTCCGTCGGC	Cloning	This study
105265R	GGGGACCACTTTGTACAAGAAAGCTGGGTGTTACAGAGCCAAGCCGAAAATG	Cloning	This study
105659F	GGGGACAAGTTTGTACAAAAAAGCAGGCTTCATGGAAGAGAGCATCCCCTACAAG	Cloning	This study
105659R	GGGGACCACTTTGTACAAGAAAGCTGGGTGCTACGAGTTGCCTCTCAATCCC	Cloning	This study
105826F	GGGGACAAGTTTGTACAAAAAAGCAGGCTTCATGCAAAGTTACGACAACTGTGC	Cloning	This study
105826R	GGGGACCACTTTGTACAAGAAAGCTGGGTGTCAATTGTAGCAGCTGGAACC	Cloning	This study
105896F	GGGGACAAGTTTGTACAAAAAAGCAGGCTTCATGATTCCGCCGGGTACAACATTC	Cloning	This study
105896R	GGGGACCACTTTGTACAAGAAAGCTGGGTGCTAGTAGTACGCCCAAGCTTCC	Cloning	This study
106445F	GGGGACAAGTTTGTACAAAAAAGCAGGCTTCATGTCTGACTATCAAGGCGGTGC	Cloning	This study
106445R	GGGGACCACTTTGTACAAGAAAGCTGGGTGCTATTGACATCCGGGAGGGAA	Cloning	This study
107286F	GGGGACAAGTTTGTACAAAAAAGCAGGCTTCATGTGCGTTGACTTTGGCTTCAC	Cloning	This study
107286R	GGGGACCACTTTGTACAAGAAAGCTGGGTGCCTAACAGAACGGCGTGGCG	Cloning	This study

108482F	GGGGACAAGTTTGTACAAAAAAGCAGGCTTCATGAGAACGTCGTTTCACAAAG	Cloning	This study
108482R	GGGGACCACTTTGTACAAGAAAGCTGGGTGCTAACAGTTGTGTCCACAATTC	Cloning	This study
108774F	GGGGACAAGTTTGTACAAAAAAGCAGGCTTCATGGCGCTCACCTGCAAGACCC	Cloning	This study
108774R	GGGGACCACTTTGTACAAGAAAGCTGGGTGTCATTGTGTTTCCGCTCCCGCC	Cloning	This study
110220F	GGGGACAAGTTTGTACAAAAAAGCAGGCTTCATGGGCACCTGTGCTTTGC	Cloning	This study
110220R	GGGGACCACTTTGTACAAGAAAGCTGGGTGCTAACCACAGACCCATACGCC	Cloning	This study
111008R	GGGGACCACTTTGTACAAGAAAGCTGGGTGCTAGAACGCGAGAGCGGCC	Cloning	This study
111382F	GGGGACAAGTTTGTACAAAAAAGCAGGCTTCATGCAGTCCTCCTCCACCTCAG	Cloning	This study
111382R	GGGGACCACTTTGTACAAGAAAGCTGGGTGTCACAACATCGCAGCCAACCC	Cloning	This study
111505F	GGGGACAAGTTTGTACAAAAAAGCAGGCTTCATGCTCAGACGACCCAGTC	Cloning	This study
111505R	GGGGACCACTTTGTACAAGAAAGCTGGGTGCTAATAGGCCGCAGAGTATCT	Cloning	This study
67799F	GGGGACAAGTTTGTACAAAAAAGCAGGCTTCATGCAGCAGACTGATGACTGC	Cloning	This study
67799R	GGGGACCACTTTGTACAAGAAAGCTGGGTGCTAGTGACCACCCTTCCCAC	Cloning	This study
68477F	GGGGACAAGTTTGTACAAAAAAGCAGGCTTCATGCAGGAGGAGCCCTGCAAC	Cloning	This study
68477R	GGGGACCACTTTGTACAAGAAAGCTGGGTGTCAAACCTGCACCGGGAATTGG	Cloning	This study
73448F	GGGGACAAGTTTGTACAAAAAAGCAGGCTTCATGATTGACCTCTATGCCTC	Cloning	This study
73448R	GGGGACCACTTTGTACAAGAAAGCTGGGTGCTACTGCGCAATCTTGTAATC	Cloning	This study
81079F	GGGGACAAGTTTGTACAAAAAAGCAGGCTTCATGTCGCCTGCGCCAACTG	Cloning	This study
81079R	GGGGACCACTTTGTACAAGAAAGCTGGGTGTCAAAGCATGCCGATGTGGTCG	Cloning	This study
90001F	GGGGACAAGTTTGTACAAAAAAGCAGGCTTCATGCTCCCTCAGGCTTCCAC	Cloning	This study
90001R	GGGGACCACTTTGTACAAGAAAGCTGGGTGTTAAAGCATGGCGAGGGCC	Cloning	This study
99161F	GGGGACAAGTTTGTACAAAAAAGCAGGCTTCATGGATCCCGCTCCCAAAC	Cloning	This study
99161R	GGGGACCACTTTGTACAAGAAAGCTGGGTGCTAAGCTTTCTTCGACGGGC	Cloning	This study
101652F	GGGGACAAGTTTGTACAAAAAAGCAGGCTTCATGCAGAAGCCCGGCCCA	Cloning	This study
101652R	GGGGACCACTTTGTACAAGAAAGCTGGGTGCTAGGGACACTTGCCCTTGCA	Cloning	This study
SP 105265F	GGGGACAAGTTTGTACAAAAAAGCAGGCTTCATGCGTTCCTTCATCGTTGT	Cloning	This study
SP 105265R	GGGGACCACTTTGTACAAGAAAGCTGGGTGTCAGAGCCA	Cloning	This study
SP103091F	GGGGACAAGTTTGTACAAAAAAGCAGGCTTCATGAAGACCACTCAGATCTTTCTC	Cloning	This study
SP 103091R	GGGG AC CAC TTT GTA CAA GAA AGC TGG GTGGCAGAAAATGCCATCGG	Cloning	This study
SP 73448F	GGGG ACA AGT TTG TAC AAA AAA GCA GGC TTCATGCATTTCTCCCACATCTTG	Cloning	This study
SP 73448R	GGGGACCACTTTGTACAAGAAAGCTGGGTGACTG CGC AAT CTT GTA ATC GTC	Cloning	This study
SP 81079F	GGGG ACA AGT TTG TAC AAA AAA GCA GGC TTCATGCGTTTCACCACCGCC	Cloning	This study
SP 81079R	GGGGACCACTTTGTACAAGAAAGCTGGGTGA AAG CAT GCC GAT GTG GTC G	Cloning	This study
SP 99161F	GGGGACAAGTTTGTACAAAAAAGCAGGCTTCATGCTCGCCACGGTCCTC	Cloning	This study
SP 99161R	GGGGACCACTTTGTACAAGAAAGCTGGGTGTAGCTTTCTTCGACGGGCAAG	Cloning	This study
NbPR1a F	CGACCAGGTAGCAGCCTATG	qRT-PCR	Qi et al 2016
NbPR1a R	TCTCAACAGCCTTAGCAGCC	qRT-PCR	Qi et al 2016
NbPR2 F	GGGCTGTTAATTTGCAGTATCC	qRT-PCR	Qi et al 2016
NbPR2 R	GGTTTATAACATCTTGGTCTGATGG	qRT-PCR	Qi et al 2016
NbWRKY12 F	CTCATCAGCTAGTTCATTTGATGC	qRT-PCR	Qi et al 2016
NbWRKY12 R	AGCTCGGTCTTTGTTCTAAAAGC	qRT-PCR	Qi et al 2016
NbPR3F	TGCCTTTTTCGGTCAAACTT	qRT-PCR	Raffaello & Asiegbu 2017

NbPR3R	TGTAAATGGTTCTGCACTCAGG	qRT-PCR	Raffaello & Asiegbu 2017
NbERF1F	GTTAACGCCGTCAAGTTGGT	qRT-PCR	Raffaello & Asiegbu 2017
NbERF1R	AGAGGCGGCACCTCAAATA	qRT-PCR	Raffaello & Asiegbu 2017
Endochitinase F	GCCTTTATCAATGCTGCTAGG	qRT-PCR	Raffaello & Asiegbu 2017
EndochitinaseR	ATCCTCGGGCAGTAGTATCG	qRT-PCR	Raffaello & Asiegbu 2017
Nb Actin	TACCACCGGTATTGTGTTGG	qRT-PCR	Raffaello & Asiegbu 2017
Nb Actin	TCATAAATTGGGACGGTGTG	qRT-PCR	Raffaello & Asiegbu 2017
73448F	GGGAGAACATCTGTCTCGGC	qRT-PCR	This study
73448R	CACCCTCGAAGACCATCTCG	qRT-PCR	This study
81079F	CAGCCCAACGAAGCAAACAA	qRT-PCR	This study
81079R	ATCGGTGTGGGTGTGAAGAC	qRT-PCR	This study
99161F	CTGCTTCGAAAACTGCCCAG	qRT-PCR	This study
99161R	ACCGCATTCGTTGATCAGGT	qRT-PCR	This study
103091F	ACTTCCCAAACCCATCGACG	qRT-PCR	This study
103091R	ACTCCACAGCATCACCATCG	qRT-PCR	This study
105265F	TCACCGACTACACCGAGATG	qRT-PCR	This study
105265R	CGTGGAGGAGGAAGGAGAAG	qRT-PCR	This study
Mg Tub F	ATCTACCGCGGAAAGGTGTCCA	qRT-PCR	Rudd et al 2015
Mg Tub R	TGGTCGCCGACACGCTTAAAGAG	qRT-PCR	Rudd et al 2015
35s F (CD3-687)	CCTTCGCAAGACCCTTCCTC GACATGTTGTCGCAAAATTCGCC	Cloning	This study
TaPR1	CAATAACCTCGGCGTCTTCATCAC TTATTTACTCGCTCGGTCCCTCTG	qRT-PCR	Casassola et al 2015
TaGAPDH	GGCCGGGATTGCTCTGAACG TGGTGCTGTGCATGTGACGG	qRT-PCR	Guo et al 2011
Ta-ATub	ATCTCCAACTCCACCAGTGTCG TCATCGCCCTCATCACCGTC	qRT-PCR	Ding et al 2016
TaCAT	CCATGAGATCAAGGCCATCT ATCTTACATGCTCGGCTTGG	qRT-PCR	Sheoran et al 2015
TaMnSOD	CAGAGGGTGCTGCTTTACAA GGTCACAAGAGGGTCCTGAT	qRT-PCR	Sheoran et al 2015
TaPAL	CAAGATGGTCGAGGCTTACC CGAAGTCGATCATGAAGCAA	qRT-PCR	Casassola et al 2015
TaOPR3	TCGCCCTTCATGGACTACATG TAGAGGATGCCGTGGTCGTT	qRT-PCR	This study
TaPR3	CCTCCATTATCTCGCAGTCGCTC CGCCGTAGTTGTAGAACCCCTTG	qRT-PCR	Ding et al 2016
TaPR2	CGCCAACGTGTACCCCTACTT TCTCGGAAATCACCACCTTCAC	qRT-PCR	Ding et al 2016
BdTUA6	ACCAACCTTGTGCCCTATCC GGGCACCAGTCAACAAACTG	qRT-PCR	Hong et al 2008
BdPAL	ATTCAGGCTATCCTTGCTGAGG AGGAGCTTCCTTCCAAGATGTG	qRT-PCR	Gill et al 2015
BdNPR1	AGCTTCAACTCGACCAGCAT CGATCACCACATCATTGAGC	qRT-PCR	Kouzai et al 2016
BdPR2	CATCAACTCCATGCGGATCTAC GGCGATGTACTTGATGTTGACC	qRT-PCR	Gill et al 2015
BdGADPH	TTGCTCTCCAGAGCGATGAC CTCCACGACATAATCGGCAC	qRT-PCR	Hong et al 2008

BdPR1	AGCTCTGGCATCATCAGCATCC CGTTGTGTGGGTCCAGGAAATC	qRT-PCR	Mandadi and Scholthof 2012
BdPR-3	GCTCGGCTGATTGTTCAACACG TTGCCCGAACCACAAATATGCC	qRT-PCR	Mandadi and Scholthof 2012
BdOPR3	ACCCATTTCTTCTCGAATGATCCC ACACGTGCAAGTACGGAAAGAAAG	qRT-PCR	Mandadi and Scholthof 2012
BdCAT	CCCGGAGAGTCTGCATATGT GTGCCTCCAACAGTAACAGC	qRT-PCR	Glover et al 2014
BdMnSOD	GCGCAATCAAGTTCAACGG TCACCACCACCCTCACTG	qRT-PCR	Glover et al 2014

Appendix 2: Table: Blastp analysis of 5 *Z. tritici* small secreted proteins (*ZtSSP1-5*) showing 5 best hits using NCBI Blast.

Candidate	Accession	E-value	Description
ZtSSP1	gi|398396310|ref|XP_003851613.1|;gi|339471493|gb|EGP86589.1|	4.71E-131	hypothetical protein MYCGRDRAFT_73448 [*Zymoseptoria tritici* IPO323]
ZtSSP1	gi|1191700176|emb|SMQ51967.1|;gi|1200896422|emb|SMY25608.1|; gi|1272996252|emb|SMR54529.1|;gi|1273008191|emb|SMR56422.1|	1.64E-110	unnamed protein product [*Zymoseptoria tritici* ST99CH_3D7]
ZtSSP1	gi|796707315|gb|KJX98610.1|	5.22E-106	hypothetical protein TI39_contig402g00025 [*Zymoseptoria brevis*]
ZtSSP1	gi|996596831|gb|KXH37665.1|	1.22E-53	hypothetical protein CNYM01_12331 [*Colletotrichum nymphaeae* SA-01]
ZtSSP1	gi|1248276004|ref|XP_022469368.1|;gi|1088683749|gb|OHE92198.1|	3.92E-52	hypothetical protein CORC01_12492 [*Colletotrichum orchidophilum*]
ZtSSP2	gi|398394613|ref|XP_003850765.1|;gi|339470644|gb|EGP85741.1|	1.80E-138	hypothetical protein MYCGRDRAFT_105265 [*Zymoseptoria tritici* IPO323]
ZtSSP2	gi|1191700841|emb|SMQ52631.1|	4.93E-137	unnamed protein product [*Zymoseptoria tritici* ST99CH_3D7]
ZtSSP2	gi|796695845|gb|KJX94569.1|	1.46E-133	hypothetical protein TI39_contig4175g00002 [*Zymoseptoria brevis*]
ZtSSP2	gi|1002202811|gb|KXS98511.1|	1.99E-52	hypothetical protein AC578_5524 [*Mycosphaerella eumusae*]
ZtSSP2	gi|452837379|gb|EME39321.1|	1.42E-50	hypothetical protein DOTSEDRAFT_83110 [*Dothistroma septosporum NZE10*]
ZtSSP3	gi|398395850|ref|XP_003851383.1|;gi|339471263|gb|EGP86359.1|; gi|1191699722|emb|SMQ51513.1|;gi|1200895969|emb|SMY25155.1|; gi|1272995794|emb|SMR53588.1|;gi|1273007737|emb|SMR55968.1|	0	hypothetical protein MYCGRDRAFT_81079 [*Zymoseptoria tritici* IPO323]
ZtSSP3	gi|796705367|gb|KJX97062.1|	0	hypothetical protein TI39_contig567g00001 [*Zymoseptoria brevis*]
ZtSSP3	gi|1273343885|gb|PIA96405.1|	9.21E-87	hypothetical protein CB0940_10792 [*Cercospora beticola*]
ZtSSP3	gi|1271960261|emb|CZT14242.1|	2.06E-81	uncharacterized protein RCC_00217 [*Ramularia collo-cygni*]
ZtSSP3	gi|1030135095|ref|XP_016760600.1|;gi|453084435|gb|EMF12479.1|	2.95E-81	hypothetical protein SEPMUDRAFT_117066 [*Sphaerulina musiva SO2202*]
ZtSSP4	gi|398408503|ref|XP_003855717.1|;gi|339475601| gb|EGP90693.1|;gi|1191695515|emb|SMQ47310.1|	1.79E-48	hypothetical protein MYCGRDRAFT_103091 [*Zymoseptoria tritici IPO323*]

ZtSSP4	gi|1200891801|emb|SMY20991.1|;gi|1272991564| emb|SMR45839.1|;gi|1273003546|emb|SMR47089.1|	1.35E-11	unnamed protein product [Zymoseptoria tritici ST99CH_1A5]
ZtSSP4	gi|796710390|gb|KJY01360.1|	2.78E-11	hypothetical protein TI39_contig295g00016 [*Zymoseptoria brevis*]
ZtSSP4	gi|1191700896|emb|SMQ52686.1|	0.025	unnamed protein product *[Zymoseptoria tritici* ST99CH_3D7]
ZtSSP4	gi|1247342183|gb|PCI04238.1|	9.3	hypothetical protein COB78_07890 [*Rhizobiales bacterium]*
ZtSSP5	gi|398407033|ref|XP_003854982.1|;gi|339474866| gb|EGP89958.1|;gi|1273003965|emb|SMR47508.1|	1.63E-112	hypothetical protein MYCGRDRAFT_99161 [*Zymoseptoria tritici IPO323*]
ZtSSP5	gi|1191695931|emb|SMQ47726.1|	2.14E-111	unnamed protein product [Zymoseptoria tritici ST99CH_3D7]
ZtSSP5	gi|1272991984|emb|SMR46259.1|	2.93E-111	unnamed protein product [Zymoseptoria tritici ST99CH_1E4]
ZtSSP5	gi|1200892218|emb|SMY21408.1|	1.18E-110	unnamed protein product [Zymoseptoria tritici ST99CH_1A5]
ZtSSP5	gi|913799705|emb|CRK13081.1|;gi|913822740|emb|CRK38593.1|	1.84E-30	hypothetical protein BN1708_002445 [*Verticillium longisporum*]

Appendix 3: Multiple sequence alignement of *ZtSSP's*


ZtSSP2
SP
323 1 MRSFIVVAALSAIASAQNYSVGANGQLMIDASQISTGERQSWCLGQRNNCPKVCEGPANPNSCDSNTLEWTCTCTNGNTPNITDYTEMVPFYICQQWIANCVASNPNDATAQFGCRSVVCGSQNASALAVDSSSSSSSSSPSSSTTGTPTTSGGSSGTRPTESAAASSSGAAVALNVAQSYGSGILAAGLLAIFGLAL
560.11 1 MRSFIVVAALSAIASAQNYSVGANGQLMIDASQISTGERQSWCLGQRNNCPKVCEGPANPNSCDSNTLEWTCTCTNGNTPNITDYTEMVPFYICQQWIANCVASNPNDATAQFGCRSVVCGSQNASALAVDSSSSSSSSSPSSSTTGTPTTSGGSSGTRPTESAAASSSGAAVALNVAQSYGSGILAAGLLAIFGLAL
C7c 1 MRSFIVVAALSAIASAQNYSVGANGQLMIDASQISTGERQSWCLGQRNNCPKVCEGPANPNSCDSNTLEWTCTCTNGNTPNITDYTEMVPFYICQQWIANCVASNPNDATAQFGCRSVVCGSQNASALAVDSSSSSSSSSPSSSTTGTPTTSGGSSGTRPTESAAASSSGAAVALNVAQSYGSGILAAGLLAIFGLAL
C10op 1 MRSFIVVAALSAIASAQNYSVGANGQLMIDASQISTGERQSWCLGQRNNCPKVCEGPANPNSCDSNTLEWTCTCTNGNTPNITDYTEMVPFYICQQWIANCVASNPNDATAQFGCRSVVCGSQNASALAVDSSSSSSSSSPSSSTTGTPTTSGGSSGTRPTESAAASSSGAAVALNVAQSYGSGILAAGLLAIFGLAL
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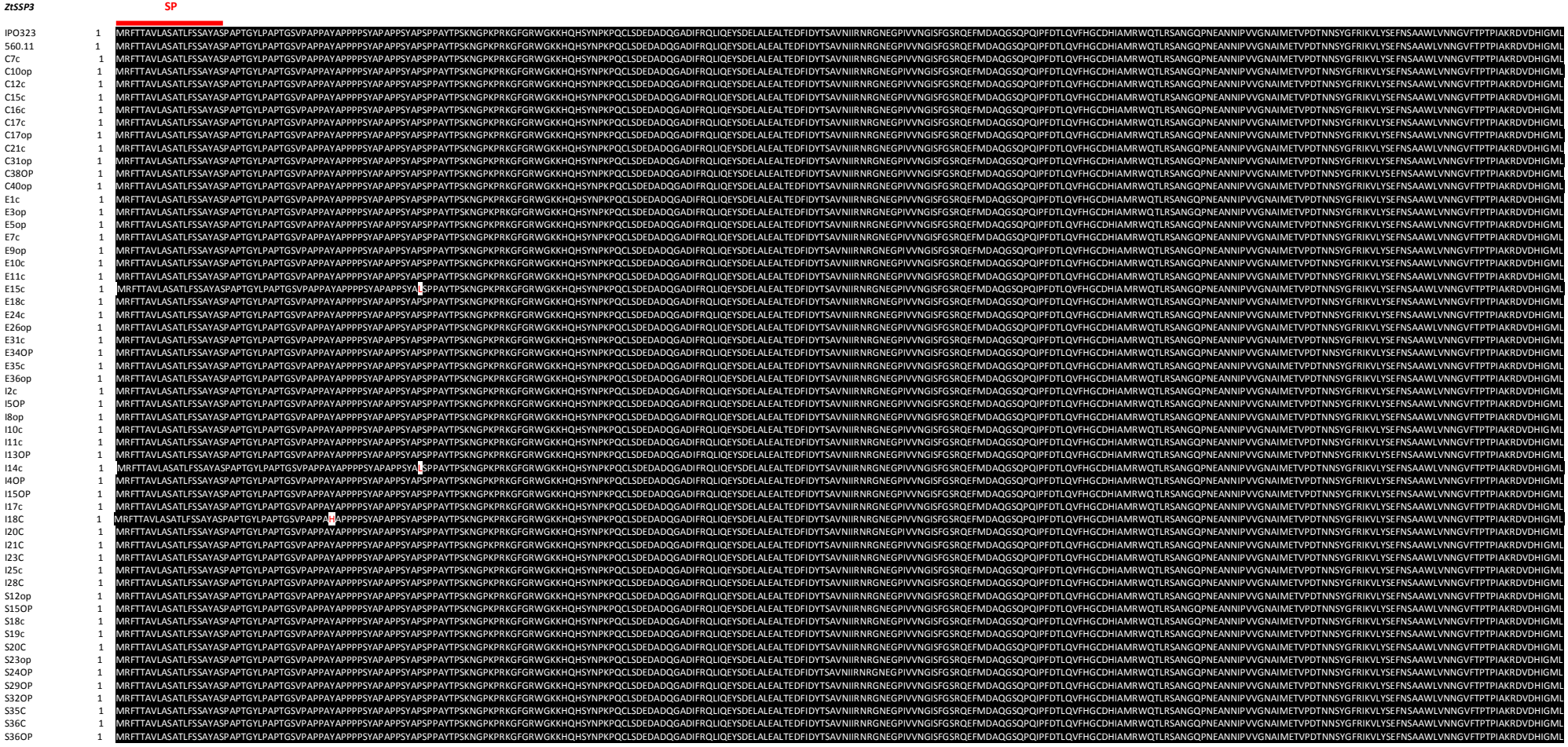
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I15op
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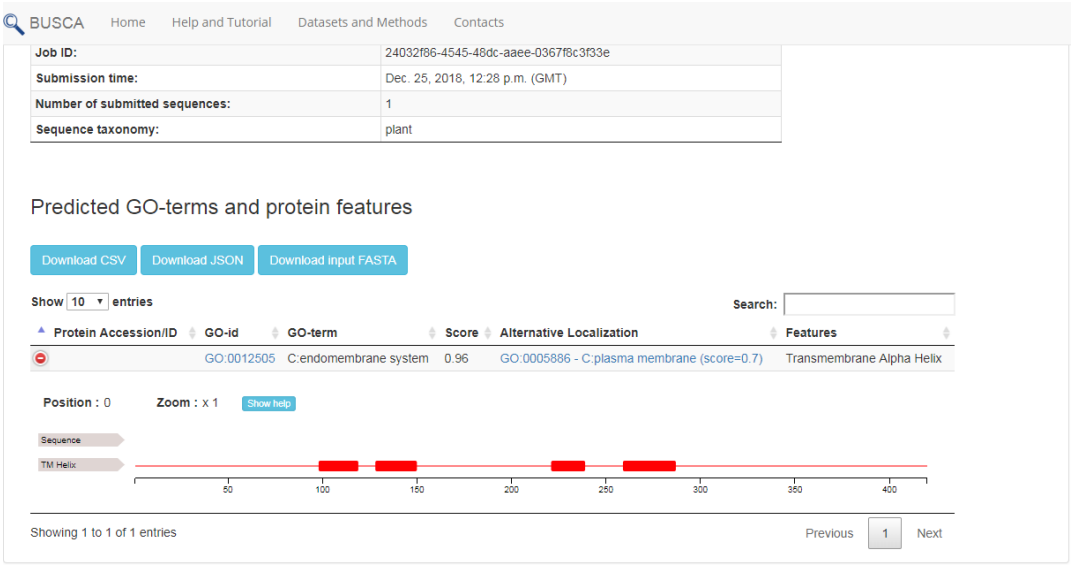
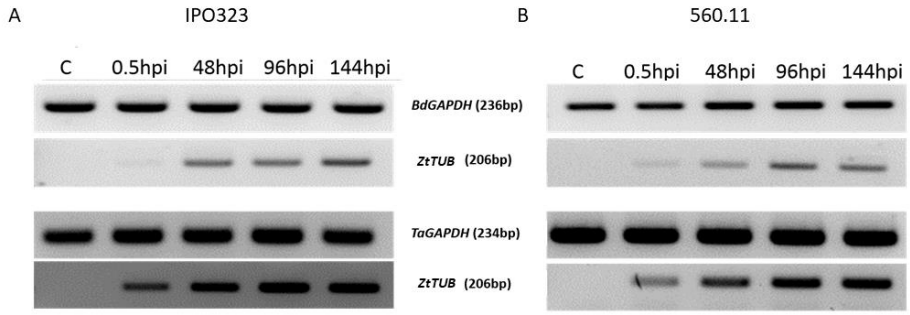
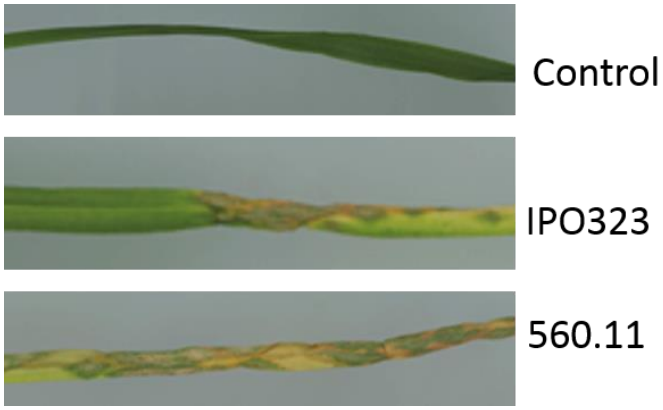
S35c
S36OP

Appendix 4: Prediction of TaUBQ localisation using BUSCA prediction software





Appendix 5: Semi-quantitative RT-PCR in *B. distachyon* (Bd-21) and *T. aestivum* (Cv. Remus). PCR was carried out using primers for *BdGAPDH* and *TaGAPDH* respectively and *Z. tritici Tubulin* (*ZtTUB*) at 0.5, 48, 96 and 144 hpi after inoculation with *Z. tritici* isolates (A) IPO323 and (B) 560.11. Representative image of two independent experiments



Appendix 6: Wheat infected with *Z. tritici* isolates IPO323 and 560.11 at 21dpi. The symptoms represents the necrotrophic stage of *Z. tritici* lifecycle.

Figure adapted from Mascarello et al. unpublished