FUNCTIONAL ANALYSIS OF FUNGAL EFFECTOR CANDIDATE SSP (SMALL SECRETED PROTEIN) GENES OF SEPTORIA LEAF BLOTCH PATHOGEN (ZYMOSEPTORIA TRITICI) IN WHEAT

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ABSTRACT

FUNCTIONAL ANALYSIS OF FUNGAL EFFECTOR CANDIDATE SSP (SMALL SECRETED PROTEIN) GENES OF SEPTORIA LEAF BLOTCH PATHOGEN (ZYMOSEPTORIA TRITICI) IN WHEAT

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Wheat (Triticum aestivum L.) is one of the main cereals sown world-wide. It constitutes a significant portion of daily calorie intake in many parts of the world. Despite lots of studies conducted over many years to increase the yield, there are significant losses in wheat production due to biotic and abiotic factors. Septoria tritici blotch is one of the most challenging disease among biotic stress factors.

Septoria Leaf Blotch is one of the most important fungal disease in Turkey and the world, causing many concerns in wheat fields. Upon adequate conditions *Zymoseptoria tritici* causes direct yield loss proportionally to chlorophyll loss on leaves. Primarily, the mechanism of disease formation of the fungus needs to be understood for development of effective strategies against this pathogen. It is known that when the fungus and host plant come across following the infection, pathogens secrete small proteins called effectors. Some of these effectors affect the functioning of plant proteins, suppressing its resistance and facilitating disease formation.

In this project, some of the Small Secreted Proteins (SSPs) genes on *Z. tritici* genome are knocked out via *Agrobacterium* mediated DNA fragment transfer and utilizing pathogen's homologous recombination mechanism.

The effect of the knocked-out genes in fungal virulence were investigated using classical virulence tests on these gene knock out mutant isolates. Also, development process of mutant isolates on synthetic media were monitored to investigate effects of the deleted gene on phenotypic features of the fungi.

Keywords: Zymoseptoria tritici, Septoria leaf blotch, wheat fungal pathogen, gene knock out, homologous recombination, small secreted protein (SSP), effector proteins

ÖZ

BUĞDAYDA SORUN OLAN SEPTORYA YAPRAK LEKESİ (ZYMOSEPTORİA TRİTİCİ) PATOJENİ'NDE FUNGAL EFEKTÖR ADAYI SSP (SMALL SECRETED PROTEİN) GENLERİNİN FONKSİYONEL ANALİZİ

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Buğday (Triticum aestivum L.) dünyada ekilen başlıca tahıllardan birisidir. Dünyanın birçok yerinde günlük kalori alımının önemli bir kısmını kapsamaktadır. Uzun yıllar boyunca, buğday verimini arttırmaya yönelik yapılan birçok çalışmaya rağmen, meydana gelen biyotik ve abiyotik etkenler sebebiyle buğday ürünlerinde belirgin kayıplar gerçekleşmektedir. Septorya yaprak leke hastalığı, biyotik faktörler içinde mücadele edilmesi en zor olan hastalıklardan biridir.

Septorya yaprak leke hastalığı Türkiye'de ve dünyada buğday alanlarında sorun olan önemli fungal hastalıklardan birisidir. Bu hastalığa neden olan *Zymoseptoria tritici* uygun şartlar oluştuğunda, yaprakta klorofil alanının azalması ile doğru orantılı olarak ürün kayıplarına neden olmaktadır. Bu fungusa karşı etkin bir strateji geliştirmek için, öncelikle fungusun hastalık oluşturma mekanizmasının anlaşılması gerekmektedir.

Patojen ile konukçusu olan bitki karşılaştığında, patojen tarafından bazı küçük proteinlerin (efektörlerin) salgılandığı bilinmektedir. Bu efektörlerin bazıları

bitkideki proteinlerin işleyişine etki ederek, dayanıklılığı baskılar ve hastalık oluşumunu kolaylaştırır.

Bu projede, belirlenmiş bazı Small Secreted Proteins (SSPs) efektör aday genlerinin *Z. tritici* genomundan *Agrobacterium* aracılığıyla gönderilen DNA fragmentleri ile patojen hücresinin homolog rekombinasyon mekanizmasından yararlanılarak silinmiştir. Elde edilen bu mutant izolatlarla buğday üzerinde klasik reaksiyon denemeleri yapılarak, fungusun virülensliği üzerinde silinen genin bir etkisi olup olmadığı ve fungusun diğer metabolik fonksiyonları incelenmiştir. Mutant izolatların ayrıca yapay ortamda gelişim süreci takip edilerek, silinen genin fungusun fenotipik özelliklerine olan etkileri gözlemlenmiştir.

Anahtar Kelimeler: Zymoseptoria tritici, septorya yaprak çürüklüğü, buğday fungal patojeni, gen silme, homolog rekombinasyon, küçük salgılanan proteinler, efektör proteinleri

Dedicated to my family

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ABBREVIATIONS

As	Acetosyringone
Avr	Avirulence factor
Carb	Carbenicillin
CDB	Czapek-Dox broth
CDD	Conserved Domain Database
CEBiP	Chitin Elicitor Binding Protein
CERK	Chitin Elicitor Receptor Kinase
cm	Centimeter
DC	Direct Current
ddH_2O	Double Distilled (laboratory graded) H_2O
dH_2O	Distilled H_2O
DNA	Deoxyribonucleic acid
dpi	Days Post Inoculation
ETD	Effector Triggered Defense
ETS	Effector-Triggered Susceptibility
FPLC	Fast Protein Liquid Chromatography
g	Gram
GFG	Gene-for-Gene
GOI	Gene of Interest
GWAS	Genome-Wide Association Study
h	Hour
hph	Hygromycin-B-Phosphotransferase
HR	Hypersensitive Response
Hyg	Hygromycin
iGFG	Inverse Gene-for-Gene
IM	Induction Media
Kan	Kanamycin
kb	Kilobase
KO	Knock Out
KOG	EuKaryotic Orthologous Groups
L	Liter
LB	Lysogeny Broth
LC-MS	Liquid Chromatography-Mass Spectrometry

LysM	Lysine Motif
M	Molar
max.	Maximum
Mb	Mega Base
MCS	Multiple Cloning Site
mg	Milligram
min	Minute
min.	Minimum
mL	Milliliter
mM	Millimolar
mm	Millimeter
µg	Microgram
µL	Microliter
µM	Micromolar
µm	Micrometer
NB-LRR	Nuclear Binding - Leucine Rich Repeat
NEP	Necrosis and Ethylene-inducing Peptide
NHEJ	Non-Homologous End Joining
NIP	Necrosis-Inducing Protein
NLP	NEP Like Protein
nM	Nanomolar
PAMP	Pathogen Associated Molecular Patterns
PCR	Polymerization Chain Reaction
PDA	Potato Dextrose Agar
PDB	Potato Dextrose Broth
PFAM database	Protein Families Database
pM	Picomolar
PRR	Pattern Recognition Receptors
PTI	PAMP-Triggered Immunity
QTL	Quantitative Trait Loci
R gene	Resistance gene
R protein	Resistance Protein
RH	Relative Humidity
Rif	Rifampicin
RLP	Receptor-Like Proteins
RNA	Ribonucleic Acid
ROS	Reactive Oxygen Species
rpm	Rotation per Minute
RT	Room Temperature

s	Second
S gene	Susceptibility gene
S protein	Susceptibility protein
S.D.	Standard Deviation
SDHI	Succinate Dehydrogenase Inhibitor
STB	Septoria Tritici Blotch
T4SS	Type IV Secretion System
TBE	Tris/Borate/EDTA
T-DNA	Transfer DNA
Ti plasmid	Tumor Inducing Plasmid
Tim	Timentin
Tm	Melting temperature
UV	Ultra Violet
V	Volt
Vir	Virulence factor
WA	Water Agar
x "g"	Times standard g-force (9.81 m/s^2)
YPD	Yeast Extract–Peptone–Dextrose

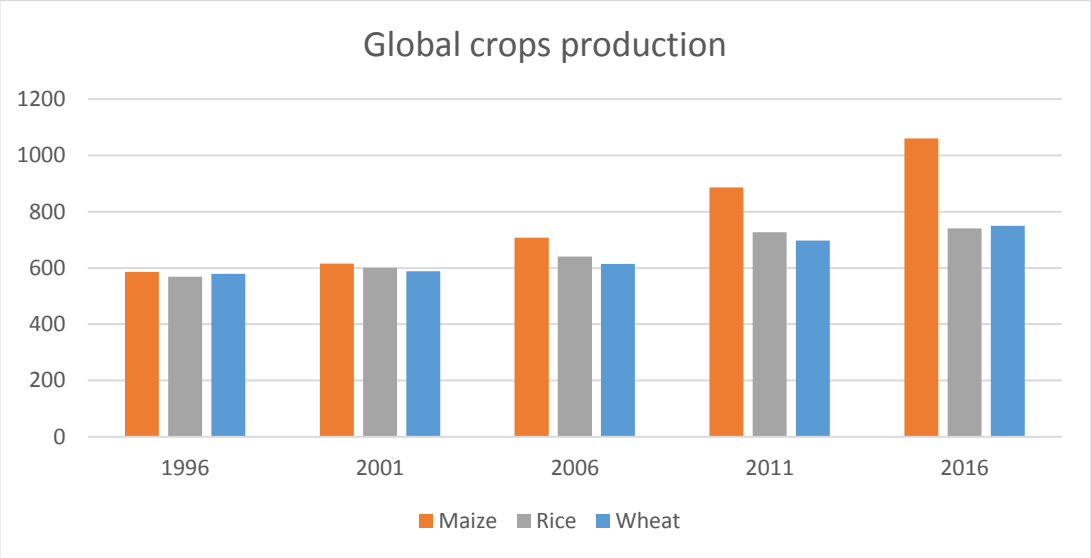
CHAPTER 1

INTRODUCTION

1.1 Wheat (*Triticum* spp.) and its importance

Wheat species were domesticated in the region of fertile crescent as early as 8500 B.C. as staple source of food (Weiss *et al*. 2012). Bread wheat or common wheat (Triticum aestivum L.) and durum wheat or pasta wheat (Triticum turgidum L. ssp. durum), are the most cultivated wheat species, with total of 749.5 million metric tonnes produced in 2016 (Table1.1) comprising around 20% of daily calorie and protein intake worldwide. Turkey produces around 2.7% of the total world wheat, which constitute a major part of the population daily diet (FAOSTAT 2018). Based on current trends it is expected around 38% growth in wheat production globally by 2050 (Ray, Mueller, West, & Foley, 2013).

Table 1.1 Last twenty years of production of the top three crops globally in million metric tonnes (FAOSTAT 2018)



Major abiotic stress factors in wheat causing yield loss can be listed as drought, flood, extreme temperatures, nutrient deprivation, high soil salinity and osmotic stress (Halford, Curtis, Chen, & Huang, 2015). On the other hand, major biotic stress in wheat are caused by bacteria, funguses, viruses, insects etc. Diseases like powdery mildew, fusarium head blight, leaf spots (septoria leaf blotch, stagonospora leaf blotch, and tan spot), rusts (leaf, stem and stripe rusts) are among most devastating fungal diseases (Everts, Leath, & Finney, 2001; D. Marshall, 2009).

1.2 *Zymoseptoria tritici*

Zymoseptoria tritici (previously known as *Mycosphaerella graminicola*) is a filamentous fungus belonging to ascomycete phylum. It consists both biotrophic and necrotrophic phases of infection (hemibiotrophic) (Kema *et al*., 1996). *Z. tritici* can grow in asexual (anamorph) and sexual (teleomorph) forms generating fruiting bodies of pycnidia and pseudothecia respectively, to spread the spores (Figure 1.1).

During asexual propagation, starting at the transition stage and continuing throughout the late stage of infection, on the necrotophic areas of leaves, *Z. tritici* forms sub-epidermal dark asexual fruiting bodies called pycnidia (60 – 200 μm), where infectious spores can be extruded (Figure 1.2, A and B). Pycnidia contain many mature pycnidiospores, ready to spread the disease to other leaves by water splash. *Z. tritici* is a dimorphic fungus which can grow in yeast-like sporulating form and mycelial form (Orton *et al*., 2011). While hyphal growth is essential for penetration through stomata, growth and colonization, yeast-like growth is necessary for spore formation. The fungus can form two types of pycnidiospores: micropycnidiospores and macropycnidiospores. Micropycnidiospores are single celled (8 – 10.5 x 0.8 – 1 μm) whereas macropycnidiospores are 4 – 6 celled elongated structures (35 – 98 μm x 1 – 3 μm) (Figure 1.2, C and D). Both types of spore inoculum can cause the disease (Shearer & Wilcoxson, 1978; Shipton *et al*., 1971; Yemelin *et al*., 2017).

In the case of sexual reproduction, the pathogen produces sexual fruiting bodies, during the late saprotrophic phase of growth (4 – 6 weeks after the initial infection), called pseudothecia (65 – 117 μm) which harbors many asci composed of 8 ascospores (10 – 15 x 2 – 3 μm) which are made up of two asymmetric cells (Figure 1.2, F). Ascospores can propagate thorough air and can spread the disease over hundreds of kilometers (Harrat *et al*., 2017; Sanderson, 1972; Wittenberg *et al*., 2009).

In vitro cultivation of the pathogen on common culture media such as potato dextrose agar (PDA) results in pinkish, mucous mass producing predominantly micropycnidiospores.

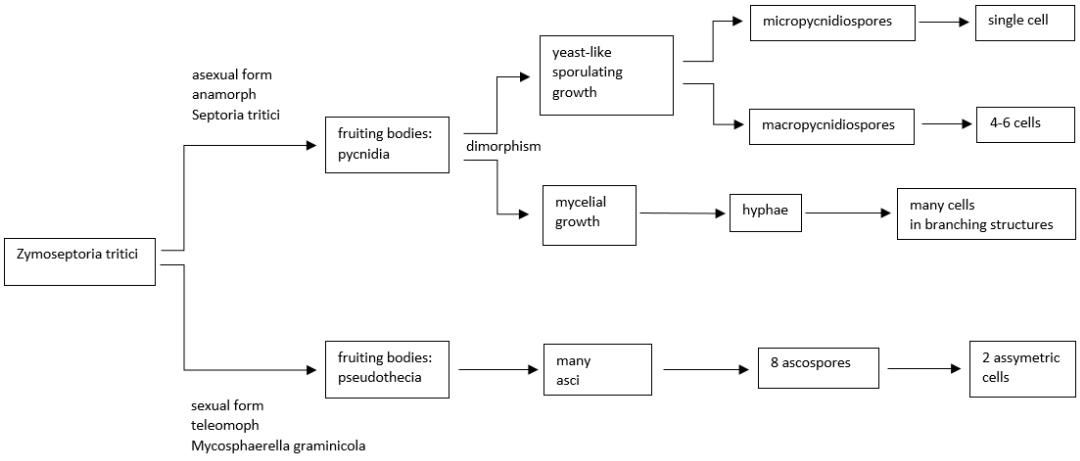


Figure 1.1 Scheme of different growth forms of *Zymoseptoria tritici.*

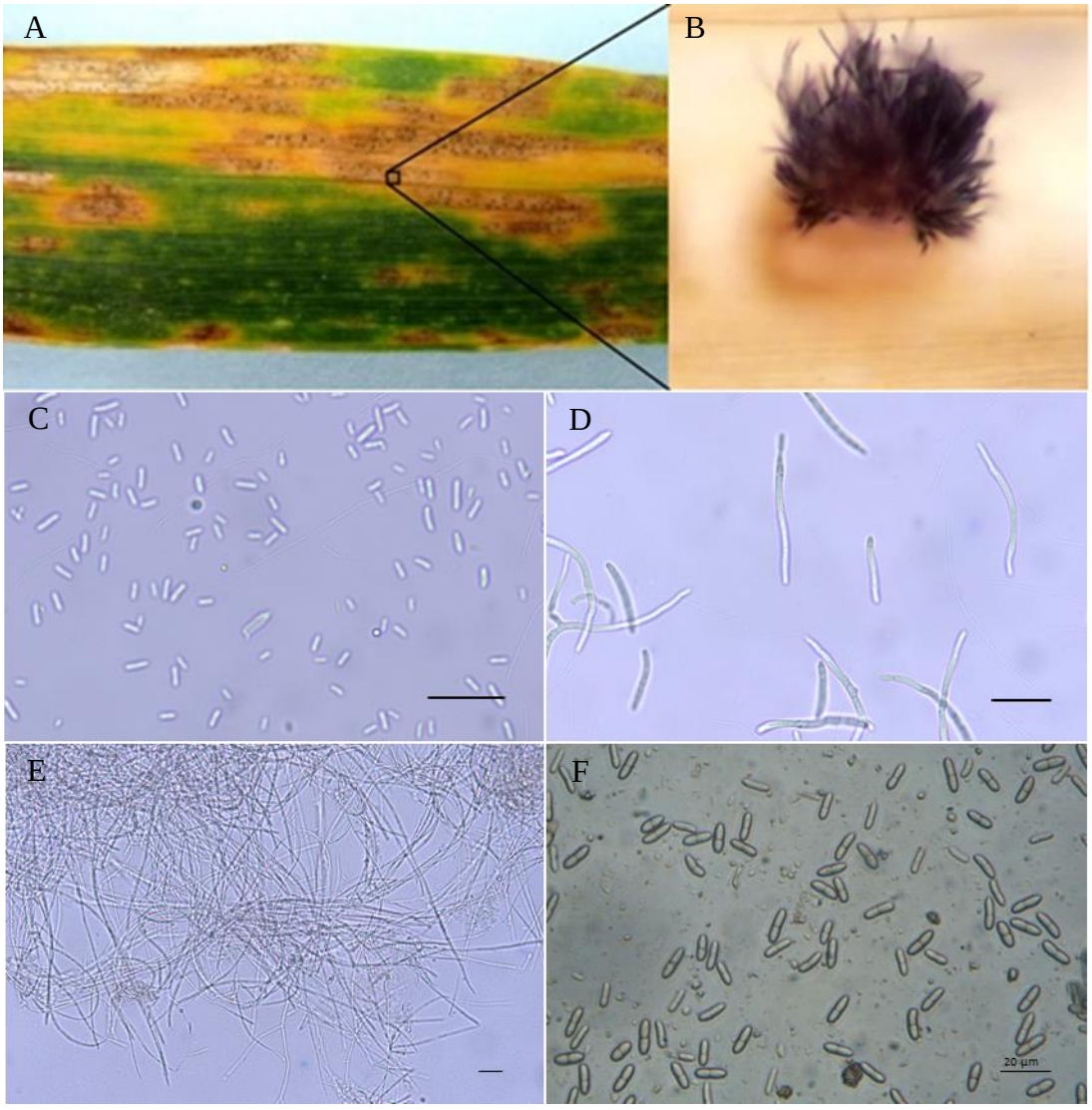


Figure 1.2 Growth forms of *Z. tritici*. A) Necrotic regions of wheat leaf and pycnidia formations, B) Pycnidium (Somai-Jemmali *et al*., 2017), C) Micropycnidiospores, D) Macropycnidiospores, E) Hyphal growth, F) Sexual ascospores (Mary Burrows, Montana State University, bugwood.org retrieved on 11.12.2018). Scale bar 100 µm.

1.2.1 Genetics of *Zymoseptoria tritici*

Full genome sequence of *Z. tritici* Dutch field isolate IPO323 strain, regarded as reference strain, has been sequenced in 2011, revealing 39.7-Mb genome length and 21 chromosomes and 10,933 predicted genes (Goodwin *et al*., 2011). The smallest eight chromosomes are not vital for survival and can be lost whitout affecting the pathogenicity. These accesory chromosomes seems to have been incorporated in the genome through horizontal gene transfer providing the pathogen a great genomic plasticity. Translocations and inversions of chromosome chunks, chromosome length and copy number polymorphisms are also observed augmenting the plasticity of the genome. This genomic polimorphism provide convenience for pathogen population to overcome unfavorable biotic and abiotic conditions (Orton *et al*., 2011; Wittenberg *et al*., 2009). Distinctive features of *Z. tritici* pathogen is low number of cellulose degradation enzymes and increased number of protein degrading enzymes, comparing to other fungal pathogens, suggesting different strategy of pathogenicity and resource acquiring (Goodwin *et al*. 2011). There are 492 predicted secreted proteins with 50 candidate effector proteins (do Amaral *et al*., 2012).

1.3 Septoria Tritici Blotch (STB) disease

Septoria Tritici Blotch (STB) is a foliar disease caused by *Zymoseptoria tritici*, an ascomycete phytopathogen which can infect bread and durum wheat (Triticum aestivum L. and T. turgidum ssp. durum L.) (Figure 1.3). It is a major threat to wheat production globally and it is the most damaging pathogen of wheat in Europe (O'Driscoll *et al*., 2014). Due to presence of high variety of pathogen strains, resistant to multiple fungicides, the control methods of the disease are generally difficult and inefficient, causing yield loss as high as 30 – 50%. Diseased cops produce grains with reduced weight and fewer seeds per spike (Ziv & Eyal, 1977).

The control of STB disease in wheat crops relies on the intensive usage of fungicides such as mixtures of azole and succinate dehydrogenase inhibitor (SDHI) (Torriani *et al*., 2015). Around 70% of fungicides used in EU are sprayed to control the STB and the economic losses are of twofold: i) the direct loss of the wheat harvest due to disease, and ii) the cost of fungicide application, adding around 1.72 billion euros to wheat production expenses in Europe (Fones & Gurr, 2015).

STB development is highly favored by moderate temperatures (19 – 24^0C) and high rainfall. At least 15 hours of leaf wetness following two days of high humidity is necessary for spores to germinate and establish the infection (Pedersen & Hughes, 1993; Mehta, 2014).

Figure 1.3 Appearance of wheat leaves infected by STB on the field. Brown nercrotic areas surrounded by yellow sections are characteristic features of the disease which marks the chlorophyll loss and the outstrech of the disease (Orton *et al*., 2011).

In suitable conditions, after a spore landed on the leaf it germinates after 12 – 48 hours stretching out the hyphae toward stomata. The pathogen hyphae enter through stomata openings initiating the biotrophic phase. This latent phase is manifested by hyphal growth exclusively in intracellular space (apoplast), while remaining undetected. Energy source of the pathogen during this stage is not known with possible candidates being apoplastic sugars, proteins or starch (Eyal *et al*., 1987; Goodwin *et al*., 2011). Following the latent stage after around two weeks (varies between 8 – 20 days) of inoculation, it suddenly changes to necrotrophy resulting in chlorotic lesions, further developing to larger necrotic grey-brown necrotic areas on leaves linked to death of mesophyll cells. Pycnidia, formation is observed within these necrotic regions (Figure 1.4 and 1.5) (Mehrabi & Kema, 2006). It is still debated whether the STB pathogen is a true hemibiotroph or a latent necrotroph fungi (Sánchez-Vallet *et al*., 2015).

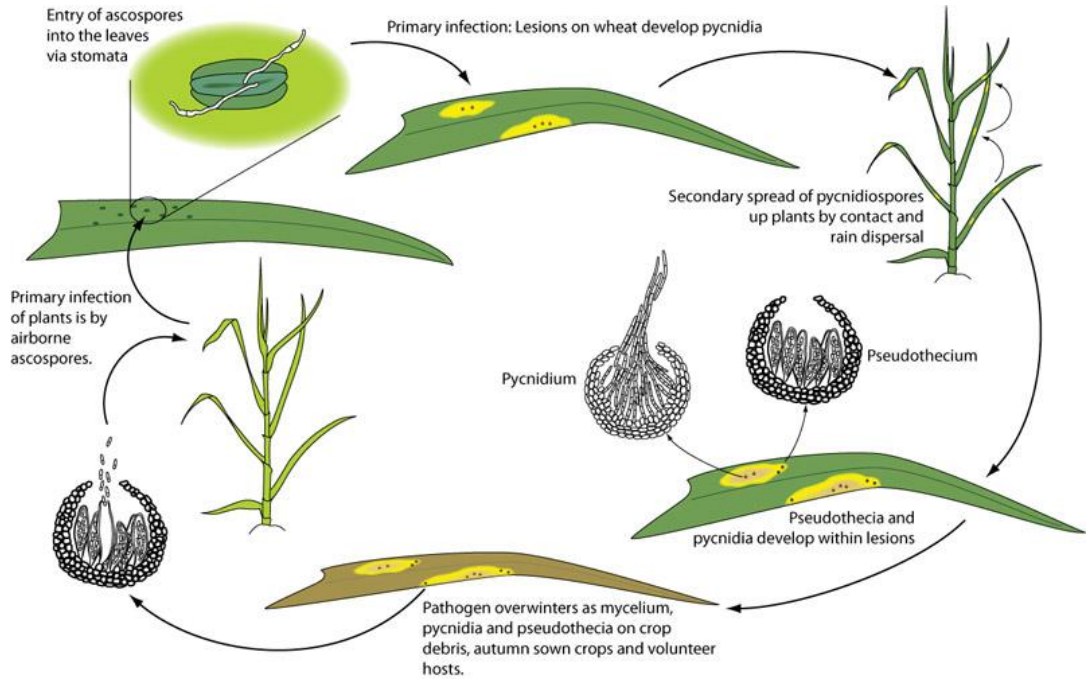


Figure 1.4 The life cycle of *Zymoseptoria tritici* (Ponomarenko *et al*., 2011).

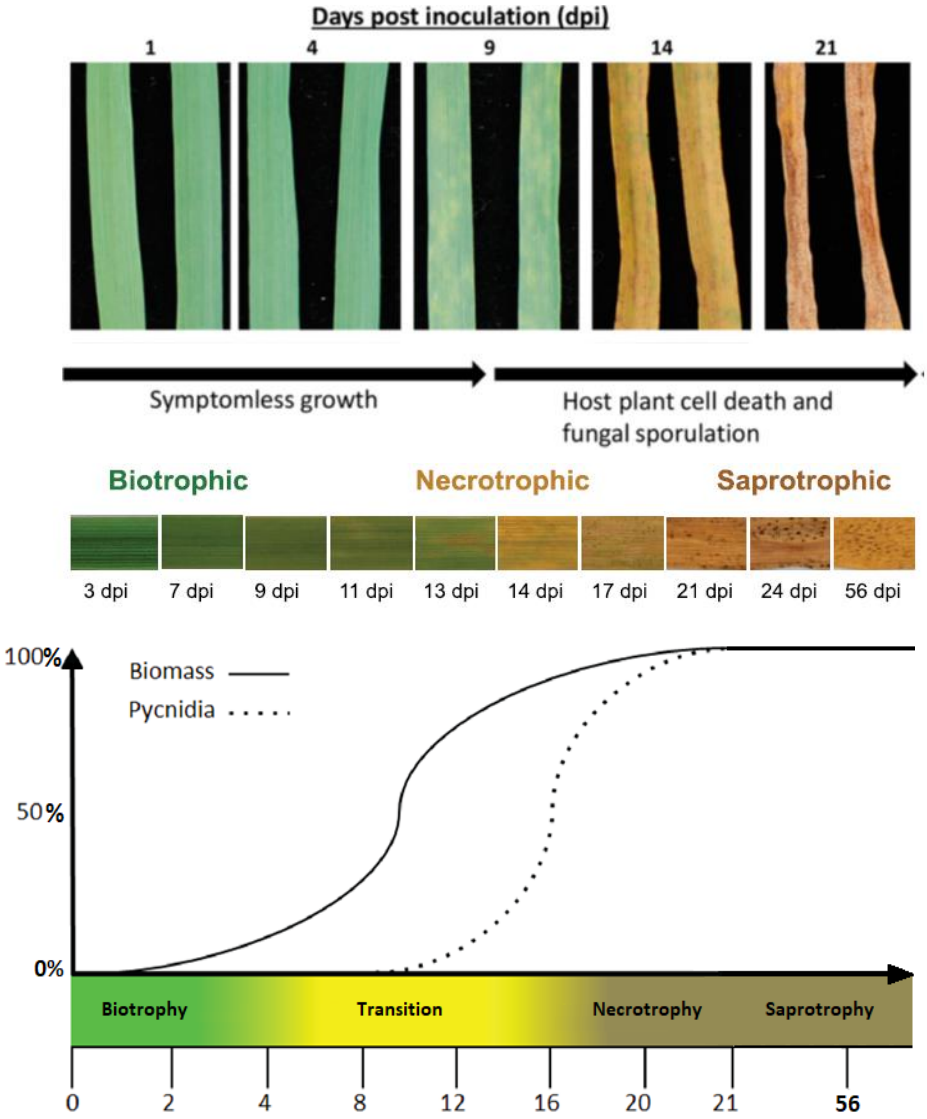


Figure 1.5 Hemibiotrophic lifestyle of *Z. tritici* during infection on wheat leaves. The exponential growth of pathogen and first pycnidia formation occur during transition phase 8 – 14 days post inoculation (dpi). During saprotrophic stage all plant biomass is converted to pathogenic biomass (Palma *et al*., 2015).

It is thought the pathogen possess early latent phase effectors, enabling to reside and grow in apoplast undetected, and the transition stage effectors as necrotrophy initiators, acting upon plant mesophyll cells causing them to collapse and enable the pathogen to utilize plant material for its own growth.

1.4 Plant innate immunity

Plants are continuously in interaction with their surroundings being in contact with many harmful organisms such as viruses, bacteria, fungi, oomycetes, nematodes, and insects (Anderson *et al*., 2006). Plants defend themselves against different invaders by recognizing pathogen associated molecular patterns (PAMPs) by transmembrane pattern recognition receptors (PRRs) activating PAMP-triggered immunity (PTI) as first layer of defense (Figure 1.6). PTI recognize common pathogen molecules such as flagelin, lipopolysaccharides and chitin and it is not selective. Upon recognition, the defense system is activated causing physiological changes such as closing stomatal openings, express antimicrobial agents and initiating hypersensitive response (HR). During HR plants activate apoptotic pathways secreting reactive oxygen species (ROS) from chloroplasts or mitochondria, causing localized cell death, halting further development of the disease (O'Brien *et al*., 2012). On the other hand, pathogens have evolved to secrete effector proteins to disable recognition system of plants and establish the infection. Effector proteins (based on interaction type: virulence factors "Vir" or avirulence factors "Avr") are highly diverse generally small secreted proteins, excreted directly into plant cells or in intracellular space. During the period of plant-pathogen co-evolution, plants also have developed the second layer of defense expressing resistance (R) proteins which can recognize the presence of effector proteins either directly or indirectly and activating effector triggered immunity (ETI) (Boller & He, 2009).

R genes consist of generally intracellular, nuclear binding, and leucine rich repeat proteins (NB-LRR) (Figure 1.6). LRR part of the R proteins is responsible for detection of the specific molecules (effectors) by binding to them, whereas NB part is needed for signal transduction to the corresponding genes in genome, activating the second layer of defense mechanism. Some pathogen effectors can also suppress R genes or escape their detection causing effector-triggered susceptibility (ETS) in plants (Jones & Dangl, 2006; Win *et al*., 2012).

Since some pathogens remain exclusively in apoplast, plants have developed an effector triggered defense (ETD) system which in contrast to ETI does not eliminate the pathogen, but its activity ensures retardation of growth and repress symptoms of the disease. ETD is activated by R genes encoding cell surface receptor-like proteins (RLPs) (Stotz *et al*., 2014).

While HR is particularly effective against biotrophic pathogens, some necrotrophic fungi hijack HR mechanism to cause massive cell death by targeting susceptibility genes (S) for nutrition acquisition. It is called inverse gene-for-gene (iGFG) interaction (Friesen *et al*., 2008).

This hide and seek struggle marks the basis of the plant-pathogen interaction shaping ever changing strategies of virulence and resistance, a coevolution based on gene-for-gene (GFG) hypothesis (Flor, 1971; Ma & Guttman, 2008).

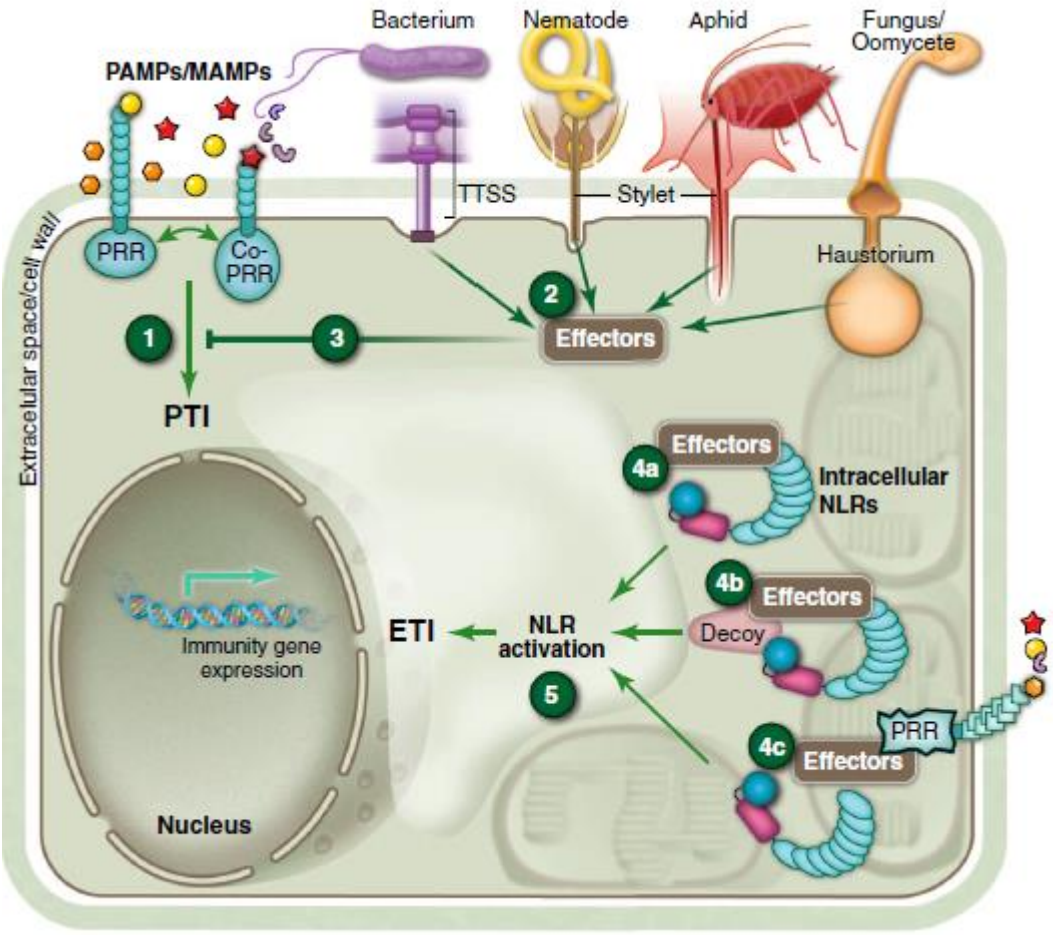


Figure 1.6 Schematic view of the plant immune system (Dangl *et al*., 2013).

1.4.1 Effector proteins and candidate effector investigations

Many organisms that cause pathogenicity on plants secrete effectors to suppress plant immunity activation towards them and facilitate their infection. While bacterial and oomycete effector secretion pathways and their conserved sequences are somewhat known, to this day consensus sequence of motifs of fungal pathogen effectors have not been found and their delivery patterns varies among different fungal species (Kamoun, 2006; Sperschneider *et al*., 2016).

Most of fungal effectors genes code for small secreted proteins and possess N-terminal signal peptide needed for secretion. Most of them are often further

reprocessed N- and sometimes C-terminally by plant and/or fungal proteases. Their length seldom exceeds 200 amino acids and contain multiple cysteine residues that might form cysteine bridges for stability and appropriate folding in rough extracellular conditions. After secretion into apoplast some effectors are translocated into host cells by a yet undiscovered mechanism. (Stergiopoulos & de Wit, 2009). Effector proteins are generally subjected to high rates of mutation due to selective pressure from hosts (Ma & Guttman, 2008) making it difficult to find conserved domains and any homology among them. Diversification of effectors can arise from natural mutations, transposable elements and genomic rearrangement and they can be found in unstable genomic regions such as chromosome ends which are subjected to frequent changes. Despite many years of research, the biology of effectors is still poorly understood (Kamoun, 2006).

Comparative genomics and different bioinformatics tools assist the quest for the new candidate effectors by narrowing down the possible effector gene number and pinpoint high probability effector genes.

SignalP is an online tool which serve for cleavage site and a signal peptide prediction based on algorithms utilizing combination of several artificial neural networks (Petersen *et al*., 2011). It can be used for secretome prediction from total predicted genes.

EffectorP is a web based classifier that applies machine learning to learn from available data and use that knowledge to screen secretome for effector protein prediction (Sperschneider *et al*., 2016).

To further narrow down candidate effector gene number, protein function prediction tools such as TargetP, ProtComp, WoLF PSORT and databases such as PFAM, KOG, CDD can be utilized to exclude in silico annotated genes with predicted functions and proteins with transmembrane domains from the secretome pool.

The remaining unclassified secreted genes with effector like characteristics, can be treated as candidate effectors. However, every candidate effector protein must be cloned and further verified for its function before assuming the effector status.

1.4.2 Studies on *Z. tritici* effector repertoire

The struggle to prevent STB is tough since the pathogen quickly evolves and adapts against resistant wheat cultivars and gain resistance to applied fungicides (Cowger *et al*., 2000; Cools & Fraaije, 2008).

The first effector gene to be discovered in *Z. tritici* is a LysM domain containing gene. It was discovered by homology analysis of CfECP6 effector protein of *Cladosporium fulvum* a biotrophic tomato leaf mold fungus. *Z. tritici* has three LysM effector homologs (Mg3LysM, Mg1LysM, and MgxLysM) which operates by binding to chitin fragments. Only Mg3LysM is able to prevent induction of plant defense through PTI, preventing recognition by plant chitin binding receptors. Chitin, a major component of fungal cell wall, is recognized as PAMP by many organisms activating PAMP triggered immunity. Mg3LysM similar to CfECP6 has three LysM domains but differently it have also protective function for hyphae against hydrolytic plant enzymes named chitinases (Figure 1.7, A) (Marshall *et al*., 2011; de Jonge *et al*., 2010).

Necrosis and ethylene-inducing peptide 1 (NEP1) – like protein (NLP) MgNLP is an effector protein in *Z. tritici* which is upregulated toward end of symptomless stage, but it is not vital to pathogenicity. While MgNLP causes HR response in dicotyledon plant *Arabidopsis thaliana* leaves (Motteram *et al*., 2009), it does not cause cell death in wheat leaves (Kettles & Kanyuka, 2016). MgNLP was found by homology analysis of NLPs which are known to cause cell death in plant leaves. It is possible that MgNLP has a redundant functionality in *Z. tritici* (Figure 1.7, D).

ZtNIP1 and ZtNIP2 (necrosis-inducing protein) are necrosis factors which was found by injecting FPLC (fast protein liquid chromatography) fractions of *Z. tritici* culture filtrates to the wheat leaves. Purification and characterization of culture filtrate by LC-MS (liquid chromatography-mass spectrometry) and testing the candidates lead to discovery of ZtNIP1/2 (M'Barek *et al*., 2015). The mechanism by which ZtNIP1/2 effectors cause cell death in wheat is still unknown (Figure 1.7, B).

Other trials to find effector proteins have applied prediction of secretome in *Z. tritici* revealing 171 candidate effectors (do Amaral *et al*., 2012) and by gene expression analysis of infected tissues during different stages of infection revealing identifying 115 secreted proteins with peak expression at 9dpi (Rudd *et al*., 2015).

The first effector of wheat pathogen *Zymoseptoria tritici* associated in a gene-for-gene interaction is *AvrStb6* identified by Zhong *et al*. (2017) *AvrStb6* is a small, secreted cysteine-rich protein which is an avirulence factor recognized by *Stb6* resistance gene in wheat. *AvrStb6* was discovered using combination of quantitative trait loci (QTL) mapping approach of the progeny of two *Z. tritici* isolates ST99CH_1E4 and ST99CH_1A5 which are virulent and avirulent respectively toward *Stb6* wheat line and genome-wide association study (GWAS) of natural variation of a field population (103 isolates) of *Z. tritici* against *Stb6* containing wheat cultivars. Both QTL and GWAS indicated a gene location at *1A5.g5534* locus is correlated with avirulance to *Stb6* wheat lines. It resides in a highly polymorphic sub-telomeric region of chromosome flanked by transposable elements, which explains the presence of high variability in the *AvrStb6* gene. The gene was functionally validated after a virulent strain of *Z. tritici* lacking *AvrStb6* rendered avirulent against *Stb6* wheat line by ectopic transformation of the gene (Figure 1.7, C) (Solomon, 2017; Zhong *et al*., 2017).

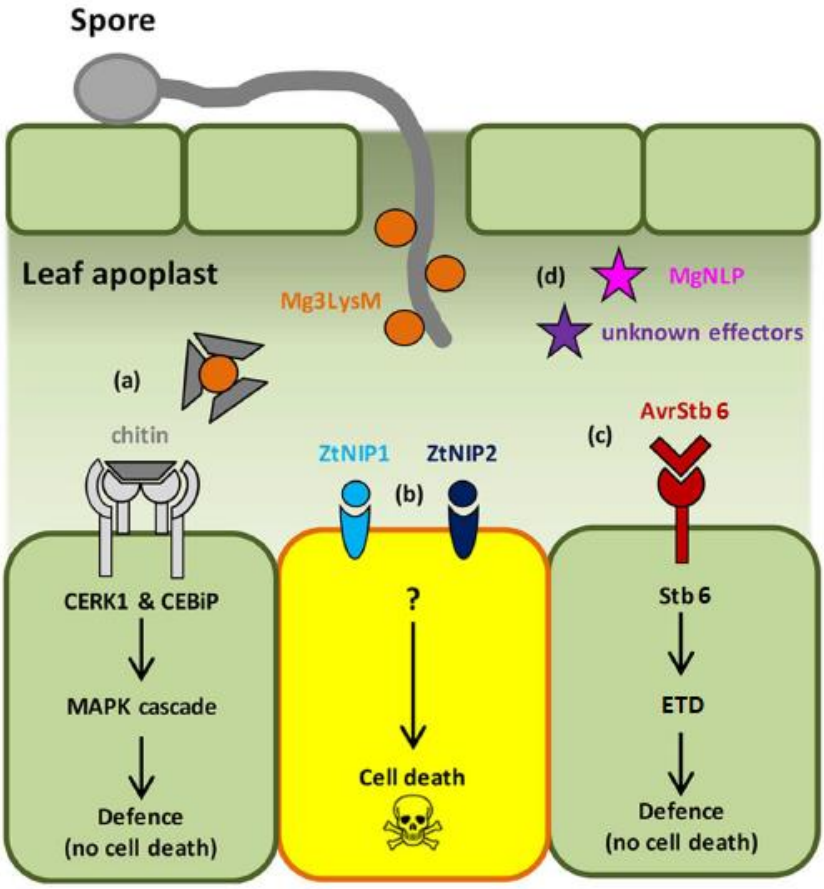


Figure 1.7 Effector molecule interactions between *Zymoseptoria tritici* and wheat cells A) CERK1 and CEBiP recognize chitin fragments whereas Mg3LysM binds to chitin fragments to avoid recognition, B) ZtNIP 1/2 induce cell death by unknown mechanism, C) Stb6 interact with AvrStb6 suppress the pathogenicity; D) MgNLP and yet unknown effectors are secreted aiding and enabling the pathogenicity (adapted from Kettles & Kanyuka, 2016).

1.4.3 Studies on wheat resistance

Chitin binding receptors of the wheat CEBiP (Chitin Elicitor Binding Protein) and CERK1 (Chitin Elicitor Receptor Kinase 1) are responsible for detection of pathogenic chitin oligosaccharides in wheat and are present in many plants. They trigger PTI as a first layer of immunity response.

Hitherto, 21 major wheat *Stb* genes providing qualitative resistance against STB have been discovered (Table 1.2). Qualitative resistance genes recognize specific avirulent pathogen cultivars and ensures strong resistance by gene-for-gene interaction. However, their resistance can be overcome by evolution of corresponding *Avr* gene. The quantitative trait loci resistance, providing quantitative resistance on the other hand, includes typically more than one gene. They offer weak to moderate resistance but have much broader effectiveness (Brown *et al*., 2015).

Stb6 is known to provide gene-for gene resistance and has been recently cloned and identified as a wall-associated receptor kinase needed for detection of a matching apoplastic effector. The *Stb6* protein seems to trigger ETD rather than ETI, slowing down growth of the pathogen but not causing apoptotic response in plant cells (Saintenac *et al*., 2018).

Finding new resistance loci in wheat genome is crucial to develop new resistant cultivars by integrating them in new bred cultivars. Stacking of multiple *Stb* resistance genes against *Z. tritici* by marker assisted breeding or through cysgenics will presumably provide more effective means to fight STB (Kettles & Kanyuka, 2016).

Table 1.2 List of R genes providing qualitative resistance against *Z. tritici* in wheat, their chromosome location and associated markers, *Z. tritici* inoculum interactions by which they were found, plant growth stage at inoculation point and the reference of corresponding research (Brown *et al*., 2015)

Gene	Chromo-some	Associated markers (distance to gene)	Avirulent inoculum	Stage	Resistance source	Reference
Stb1	5BL	Xbarc74 (2.8cM), Xgwm335 (7.4cM)	IN95-Lafayette-1196-WW 1-4 & Purdue local (USA)	S, A	Bulgaria 88	Adhikari et al. (2004a)
Stb2	1BS	Xwmc406 (6cM), Xwmc230 (5cM)	Paskeville local (Australia) (and IPO92034)	A	Veranopolis	Liu et al. (2013)
Stb3	7AS	Xwmc83	Paskeville local isolate (Australia)	A	Israel 493	Goodwin and Thompson (2011)
Stb4	7DS	Xgwm111 (0.7cM)	IN95-Lafayette-1196-WW-1-4, I-89, IPBr1	S, A	Tadinia	Adhikari et al. (2004c)
Stb5	7DS	Xgwm44 (7.2cM)	IPO94269	S, A	Synthetic 6x	Arraiano et al. (2001b)
Stb6	3AS	Xgwm369 (2cM)	IPO323	S, A	Flame, Hereward	Brading et al. (2002)
Stb7	4AL	Xwmc313 (0.3 to 0.5cM), Xwmc219 (1cM)	MG2 (Canada) (and IPO87019)	S	ST6	McCartney et al. (2003)
Stb8	7BL	Xgwm146 (3.5cM), Xgwm577 (5.3cM)	IN95-Lafayette-1196-WW 1-4	A	Synthetic W7984	Adhikari et al. (2003)
Stb9	2BL	Xfbb226 (3.6cM), Xwmc317, Xbarc0129	IPO89011	S	Courtot, Tonic	Chartrain et al. (2009)
Stb10	1Dc	Xgwm848	IPO94269 and ISR8036	S	Kavkaz-K4500	Chartrain et al. (2005c)
Stb11	1BS	Xbarc008 (1cM)	IPO90012	S	TE9111	Chartrain et al. (2005a)
Stb12	4AL	Xwmc219	ISR398 and ISR8036	S	Kavkaz-K4500	Chartrain et al. (2005c)
Stb13	7BL	Xwmc396 (7-9cM)	MG96-36, MG2 (Canada)	S	Salamouni	Cowling (2006)
Stb14	3BS	Xwmc500 (2cM), wmc632 (5cM)	MG2 (Canada)	S	Salamouni	Cowling (2006)
Stb15	6AS	Xpsr904 (14cM)	IPO88004	S	Arina, Riband	Arraiano et al. (2007b)
StbSm3	3AS	barc321 (1.9cM)	MG96-36, MG2 (Canada)	S	Salamouni	Cuthbert (2011)
Stb16q	3DL	Xgwm494 (4.3cM), Xbarc128 (9.9cM)	IPO88018 and IPO94218	S, A	SH M3	Tabib Ghaffary et al. (2012)
Stb17	5AL	Xhbg247 (3.1cM), Xgwm617 (38.3cM)	IPO88018	A	SH M3	Tabib Ghaffary et al. (2012)
Stb18	6DS	Xgpw5176, Xgpw3087	IPO323, IPO98022, IPO89011, IPO98046	S, A	Balance	Tabib Ghaffary et al. (2011)
StbWW	1BS	Xbarc119b (0.9–4.1cM)	79, 2, 1A	S	WW1842, WW2449, WW2451	Raman et al. (2009)
TmStb1	$7A^{m}S$	Xbarc174 (23.5cM)	IPO323	S	MDR043 (T. monococcum)	Jing et al. (2008)

1.5 *Agrobacterium* mediated DNA transfer

Agrobacterium tumefaciens is a rod shaped gram negative soil bacterium which causes tumor formation (crown gall disease) on a wide range of dicotyledonous and some monocotyledonous plants (Cleene & Ley, 1976). The mechanism of pathogenicity of *A. tumefaciens* utilizes transformation of plant cells by inserting the transfer DNA (T-DNA) form its tumor-inducing (Ti) plasmid to plant cells genome. The T-DNA fragment is transferred by type IV secretion system (T4SS) and assisted and protected by virulence (vir) proteins encoded from Ti plasmid as well. After integration of the T-DNA, it hijacks the cell expression mechanism to produce specific plant hormones causing tumor growth and opines which serves as energy source for *A. tumefaciens* itself (Pitzschke & Hirt, 2010).

Scientist harnessed this ability of *A. tumefaciens* to transform cells by engineering the Ti plasmid. For convenience, a system containing two plasmids were designed. Oncogenic and opine genes were removed while genes of virulence remained in helper plasmid (disarmed). The artificial T-DNA binary vector containing left and right border together with multiple cloning sites (MCS) ready for the gene of interest (GOI) insertion and containing the marker gene has been built to work along with vir helper plasmid (Figure 1.8). Moreover, the T-DNA binary vector is designed to be a shuttle vector so that it can be amplified in greater amount in *Escherichia coli* before transformation to *Agrobacterium* and target cell sequentially (Lee & Gelvin, 2007). This system of two plasmids is called binary system and it is a highly effective tool for transformation of plant, bacterial, fungal and even some mammalian cells (Lacroix *et al*., 2006).

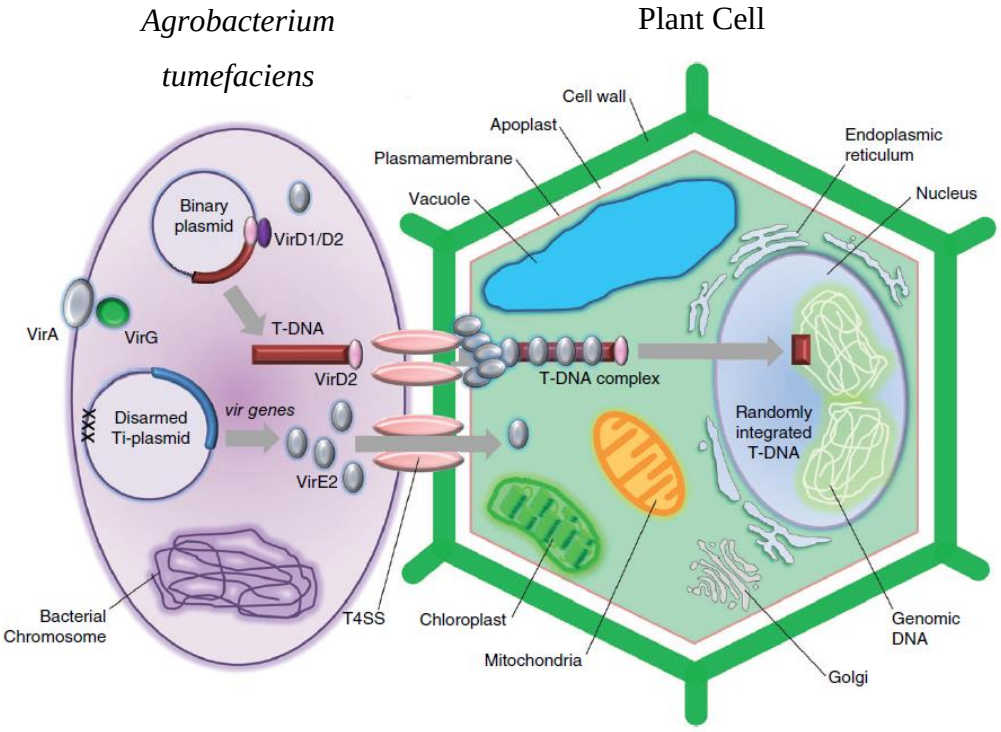


Figure 1.8 Schematic representation of the *Agrobacterium* mediated transformation of the plant cell with engineered binary plasmid system. The T-DNA is transferred and integrated into target cell's genome with help of vir genes (Edgue *et al*., 2017).

1.6 Aim of the research

Septoria tritici blotch is one of the major destructive disease, causing large-scale yield loss in wheat production. Depending on the region and the severity of the disease, different amounts of fungicide applications are performed during the production seasons.

Employment of fungicides, bring about high economic burden to producers, as expenses for fungicides are high, which in turn are reflected in wheat prices. Fungicides are also generally ecologically unfriendly having long lasting harmful impacts on the ecosystem as whole.

The most convenient way of controlling the disease other than fungicide utilization depend primarily on generating of cultivars with multiple resistance genes.

Poor understanding of the genetic and biochemical basis of the pathogenicity, and lack of detailed information about mechanisms of infection and of resistance mechanism had hinder the fighting against the disease (Goodwin *et al*., 2011). The cellular organization and intracellular dynamics of the *Z. tritici* fungus remain also largely unexplored to date (Steinberg, 2015). Development of new durable cultivars require finding of new qualitative and quantitative resistance traits and stacking these genes to discourage resistance-breaking variabilities in the pathogen populations (Marshall, 2009). Thus, generation of new knowledge about this important pathosystem will give the opportunity to establish new means to minimize the yield losses and fight the disease formation on the crops.

Effectors are proteins secreted by pathogen to suppress or activate plant immune mechanism and facilitate the infection process. These effector proteins are targeted by plant R genes to recognize the pathogen and activate the defense mechanism. Depending on the recognition and successful defense response, effectors can be categorized as virulence (disease-inducing) or avirulence (resistance-inducing) factors.

In this study small secreted proteins, as candidate effectors of *Zymoseptoria tritici*, and their roles in virulence and biology of the pathogen are investigated by loss of function analysis.

After full genome sequence of this important wheat pathogen was introduced (Goodwin *et al*., 2011), the possible regions encoding the functional genes in the genome of the fungus were determined by bioinformatics analysis. The analysis also showed up the proteins having secretion signal peptides and candidate effector genes (do Amaral *et al*., 2012). Combining these analyses with transcriptomic data (Rudd *et al*., 2015) eight genes with high probability of effector activity were chosen. Of these eight genes three were knocked out successfully.

Exploiting the ability of *Agrobacterium tumefaciens* of inter-kingdom gene transfer and available T-DNA binary vector system, the designed DNA fragments can be delivered directly to the fungal cells. By applying this versatile transformation system, the desired genes can be targeted precisely and knocked out.

To investigate the role of the chosen candidate effectors, pCHYG binary vector was used for replacement of these genes. pCHYG vector contains Hygromycin-B-phosphotransferase (hph) gene which encode for hygromycin B resistance. Upstream and downstream regions of the targeted genes were cloned to existing hph gene's upstream and downstream regions respectively. This two-side homology around the gene causes innate homologous recombination mechanism of the pathogen occasionally to recombine and interchange the fragments. While the removed gene-bearing fragment is disintegrated by cell's own enzymes, the genome-integrated hph gene will act as a native genomic part expressing Hygromycin-B-phosphotransferase protein. The hygromycin resistance is a great feature as it gives the opportunity to select only the recombined cells, whose efficiency is quite low (around 1 transformation per 100 000 cells). Since there is a possibility of unintended ectopic integration of transferred fragment there is a possibility of occurrence of hygromycin

resistance without loss of the targeted gene. For this reason, colonies were always checked by polymerization chain reaction (PCR) for absence of the gene.

Mutant isolates were grown on liquid and solid media and applied to second leaves of wheat plants. Knowing the phases of infection (1 – 9 dpi latent phase, 9 – 14 transition to phase) changes in disease development, necrotic to green foliar area ratios and pycnidia forming abilities and their percentage in necrotic areas were compared to wild type standard *Z. tritici* isolate IPO323.

Moreover, colony morphology, melanization, growth rates, transition to hyphal growth and their extension length were measured and observed microscopically by using solid Yeast Extract–Peptone–Dextrose (YPD) media in controlled temperatures and known initial cell quantities.

CHAPTER 2

MATERIALS AND METHODS

2.1 *Zymoseptoria tritici* growth and maintenance

Zymoseptoria tritici IPO323 genome is used as reference genome and is available in online genome databases (do Amaral *et al*., 2012). *Z. tritici* was grown on mediums such as PDA (potato dextrose agar), YPD (yeast extract peptone dextrose) agar and YMA (yeast malt agar).

Table 2.1 PDA medium (1000 mL)

N**ame**	**Amount**
Potatoes (sliced washed unpeeled)	20 g
dH2O	1000 mL
Boil and filter out the infusion	
Agar*	20 g
Dextrose (D-Glucose)	20 g
dH2O	fill to 1000 mL
Autoclave	

Table 2.2 YPD medium (1000 mL)

N**ame**	**Amount**
Bacto peptone	20 g
Yeast extract	10 g
Agar*	15 g
Dextrose (D-Glucose)	20 g
dH2O	fill to 1000 mL
Autoclave	

Table 2.3 YMA medium (1000 mL)

N**ame**	**Amount**
Mannitol	10 g
Yeast extract	1 g
Dipotassium phosphate, K_2HPO_4	0.5 g
Magnesium sulfate, $MgSO_4$	0.2 g
Sodium chloride, NaCl	0.1 g
Calcium carbonate, $CaCO_3$	1 g
Agar*	15 g
dH2O	fill to 1000 mL
Autoclave	

* Agar was added to obtain solid media, in liquid media preparation the agar step was omitted.

Growth was done at 17^0C, or at room temperature (RT; 20 – 22^0C) for 4 – 10 days. Rotary shaker at was used at 140 rotation per minute (rpm) in case of liquid medium for 3 – 5 days.

For mycelial simulating condition the fungus was grown at 25^0C. Water agar (WA) was also used to mimic conditions on wheat leaves and stimulate mycelial growth.

Table 2.4 Preparation of 1000 mL water agar

N**ame**	**Amount**
Agar	20 g
dH2O	fill to 1000 mL
Autoclave	

2.2 *E. coli* growth and maintenance

E. coli strain TOP10 (Invitrogen) was used to select the correct ligated vectors and for amplification of the plasmids. *E. coli* was grown in Lysogeny broth (LB) (Table 2.5).

Table 2.5 LB agar medium (1000 mL)

N**ame**	**Amount**
Tryptone	10 g
Yeast extract	5 g
NaCl	10 g
Agar*	15 g
dH2O	fill to 1000 mL
Autoclave	

* Agar was added to obtain solid media, in liquid media preparation the agar step was omitted.

Culture was grown at 37^0C for 18 – 24 hours. Rotary shaker at was used at 220 rpm in case of liquid medium.

For long-term storage 600 µL of liquid *E. coli* culture was mixed with 400 µL of 50% glycerol and stored at -80^0C.

Antibiotics were added to the media after autoclave step when the temperature of the medium was approximately 50°C or less and easy to handle by hand.

2.2.1 Competent *E. coli* preparation

Streak plate of *E. coli* TOP10 was grown on agar LB at 37^0C for 18 – 24 hours. A single colony from agar LB *E. coli* was put on 5 – 10 mL LB in 50 mL falcon flask and was grown for 18 hours at 220 rpm in rotary shaker. Next day 1 mL of grown culture was transferred to 100 mL LB in 250 mL Erlenmeyer flask and let grow for 2 – 3 hours until the density reached around 4.8 x 10^8 cells/mL measured by spectrophotometer absorbance OD_{600} = 0.6 (Optical density measured at wavelength of 600nm). OD_{600} value increases proportionally with cell density (OD_{600} of 1.0 = 8 x 10^8 cells/mL; measured via spectrophotometer, Shimadzu, UV-1601). The 100 mL of culture was poured into two 50 mL falcons and put on ice for 10 minutes. Then

centrifugated for 5 minutes at 5000 rpm at 4^0C and the supernatant was discarded. Pellet is solubilized in cold 10 mL of 0.1 M $CaCl_2$ and was put in ice for 10 minutes. The centrifugation step is repeated for 5 minutes at 5000 rpm at 4^0C and the supernatant was discarded and the pellet was re-solubilized in 10 mL of cold 0.1 M $CaCl_2$ and put on ice for 20 minutes. The tubes were then centrifugated for 5 minutes at 5000 rpm at 4^0C and supernatant was discarded. Cells in pellet were solubilized in 2 mL of cold 0.1M $CaCl_2$ for the last time. The culture was aliquoted to 1.5 mL Eppendorf tubes filling with 100 µL of competent cells ready for transformation. The fresh prepared competent cells were stored at 4^0C for 1 week or mixed with 67 µL of 50% glycerol and stored at -80^0C for longer storage.

2.2.2 *E. coli* transformation

For transformation of *E. coli*, the competent cells in the tubes were incubated with around 100 ng of desired plasmid on ice for 10 minutes. Then heat shock was applied by placing tubes in water bath at 42^0C for 45 seconds and placed on ice again for 5 minutes. Then 200 – 500 µL of LB was added and let grow on rotary shaker at 37^0C for 1 – 2 hours. To reduce the volume, culture was centrifugated at 5000 rpm of 3 minutes. The supernatant was carefully removed by pipette leaving 50 – 100 µL of supernatant over the cells which was used for re-solubilization by carefully pipetting. The cells were spread over agar LB containing the selective antibiotic and let to grow at 37^0C. Colonies were visible 1 – 2 days after inoculation, and were tested by colony PCR. In case of pCHYG plasmid transformation, kanamycin resistance was acquired in colonies. Stock kanamycin solution (50 mg/mL) was added in 1/1000 ratio to media to form final concentration of 50 µg/mL.

2.2.3 Plasmid isolation from *E. coli*

For plasmid isolation Machenerey Nagel Plasmid DNA purification kit (ref: 740588.50) and the procedure in the user manual were applied with slight changes. *E. coli* cells were grown overnight in LB. 1 – 5 mL of the culture was centrifuged at 11000 x g for 30 seconds to collect cells while the supernatant was discarded. Cells in pellet were suspended in 250 µL buffer A1 by pipetting and vortexing until no cell clumps remained. Then, 250 µL buffer A2 was added and mixed by inverting the tubes 6 – 8 times. The mixture was kept at room temperature for 5 min. Afterwards, 300 µL buffer A3 was added and the tube was inverted 6 – 8 times. After mixing thoroughly the lysate was centrifugated at 11000 x g for 5 minutes. The supernatant was transferred carefully with pipette to NucleoSpin® Plasmid Column and centrifugated at 11000 x g for 30 sec. The flow through was discarded and 500 µL of buffer AW preheated to 50^0C was added and centrifugated at 11000 x g for 1 min and flow-through was discard. Then 600 µL of buffer A4 was added and centrifugated at 11000 x g for 1 min. The flow-through was discarded and the empty tube was centrifuged at 11000 x g for 2 min to dry the membrane. The dried column was put on new tube and 50 – 100 µL of ddH_2O of was put carefully on membrane, incubated for 1 min and centrifuged for 1 min at 11000 x g. The flow-through contained purified plasmids. Concentration of collected plasmids were deduced with NanoDrop (Thermo Scientific™ NanoDrop™, One Microvolume UV-Vis Spectrophotometer) absorbance measurements reported in ng/µL. Calculations are based on modified Beer's Law equation. Ratio of absorbance at 260 nm / 280 nm (A_{260} / A_{280}) is used to deduce DNA purity with acceptable interval of 1.8 – 2.0. Generally, plasmid concentration of 100 – 700 ng/µL was obtained.

2.3 *Agrobacterium* growth and maintenance

Agrobacterium tumefaciens strain AGL1 was used for transformation of *Z. tritici*. *Agrobacterium* was grown in LB at 28^0C for 2 days. In case of liquid media rotary shaker was adjusted to 220 rpm. Suitable antibiotic was added to select the cells with desired antibiotic resistance provided by harboring plasmid. *Agrobacterium* AGL1 strain has innate Rifampicin (50 μg/mL) and Carbenicillin (50 μg/mL) resistance which sometimes were used to fight possible contaminations.

For long-term storage 700 μL of liquid *Agrobacterium* culture was mixed with 300 μL of 50% glycerol and stored at -80^0C.

2.3.1 Competent *Agrobacterium* preparation

Streak plate of *Agrobacterium* AGL1 on agar LB containing carbenicillin was grown for 2 days. Single colony was put in 5 mL LB in 50 mL falcon flask and was grown overnight (18 hours) at 28^0C at 180 rpm in rotary shaker. One mL of the grown culture was transferred into 100 mL LB in 250 mL Erlenmeyer flask and let grow at the same conditions for another 18 hours. The concentration of cells was measured with spectrophotometer at OD_{600}, with acceptable absorbance being between 0.5 – 0.7. Culture was divided into two 50 mL falcons and put on ice for 30 minutes. Then centrifugated for 10 minutes at 4000 rpm at 4^0C and the supernatant was discarded. Pellet was solubilized in 5 mL of 20 mM ice cold $CaCl_2$. Centrifugation was done again for 5 minutes at 4000 rpm at 4^0C. Afterwards, the supernatant is discarded and the pellet is re-solubilized in 1 mL of 20 mM ice cold $CaCl_2$ again. The competent cells were aliquoted to 1.5 mL tubes filling 200 μL of competent *Agrobacterium* culture. The fresh competent cells were used directly for transformation or were put in -80^0C for longer storage.

2.3.2 *Agrobacterium* transformation

Transformation of *Agrobacterium* were performed according to Holsters *et al*. (1978) protocol with slight changes. For transformation the competent cells in the tubes were incubated with 500 ng of desired plasmid on ice for 30 minutes in 1.5 ml tubes (5 minutes if freshly prepared cells were used). Then the tubes were put in liquid nitrogen for 5 minutes. The heat shock is applied by placing tubes in water bath at 37^0C for 5 minutes and placed on ice again immediately for 5 minutes. Then 1 mL LB was added and let grow on rotary shaker at 28^0C for 3 – 4 hours. To reduce the volume culture was centrifugated at 5000 rpm of 3 minutes. The supernatant was carefully removed by pipette leaving 50 – 100 µL of supernatant for re-solubilization by carefully pipetting. The cells were spread over agar LB containing the selective antibiotic and let grow at 28^0C for 2 days. After colonies were visible, they were tested by colony PCR (Holsters *et al*., 1978). In case of pCHYG plasmid transformation, kanamycin resistance was acquired in colonies. Stock kanamycin solution (50 mg/mL) was added in 1/1000 ratio to media to form final concentration of 50 µg/mL.

2.4 Knock out cassette construction

Z. tritici IPO323 genome is available in the online database of JGI (https://genome.jgi.doe.gov/Mycgr3/Mycgr3.home.html) and Ensembl-Fungi (http://fungi.ensembl.org/Zymoseptoria_tritici) where the gene sequences as well as genomic regions can be downloaded in standardized FASTA format with existing annotations. To generate gene knock-out cassettes, first of all, the genomic sequence of the target gene with 1 kb upstream and 1 kb downstream sequence was downloaded. Visualizing the sequences, picking up primers, *in silico* enzymatic cut and cloning were performed in Geneious 10.2.5 bioinformatics genetic analysis software (Figure 2.1 and 2.2).

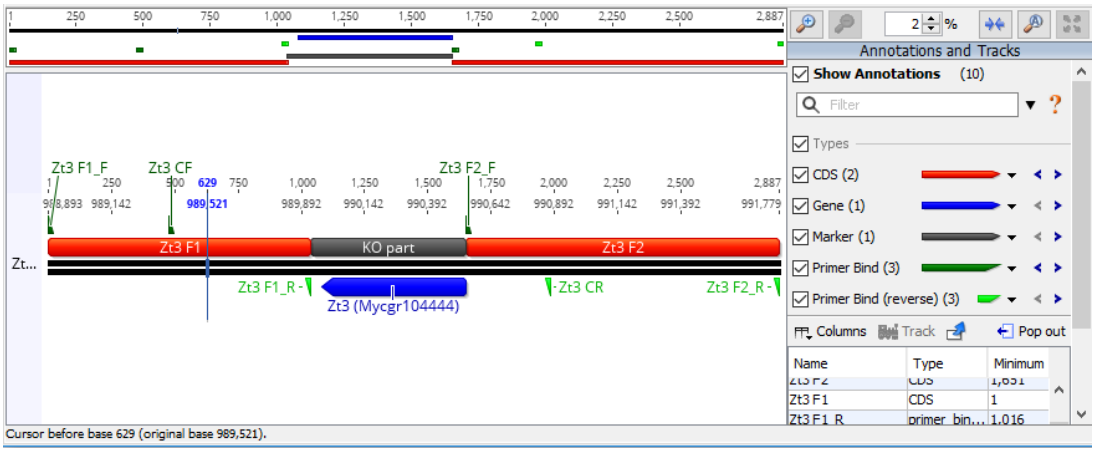


Figure 2.1 *In silico* representation of upstream and downstream genomic sequences of Mycgr104444 (Zt3) gene, the primers and their binding sites and the KO part of DNA (black) which will be replaced by hph gene (Geneious).

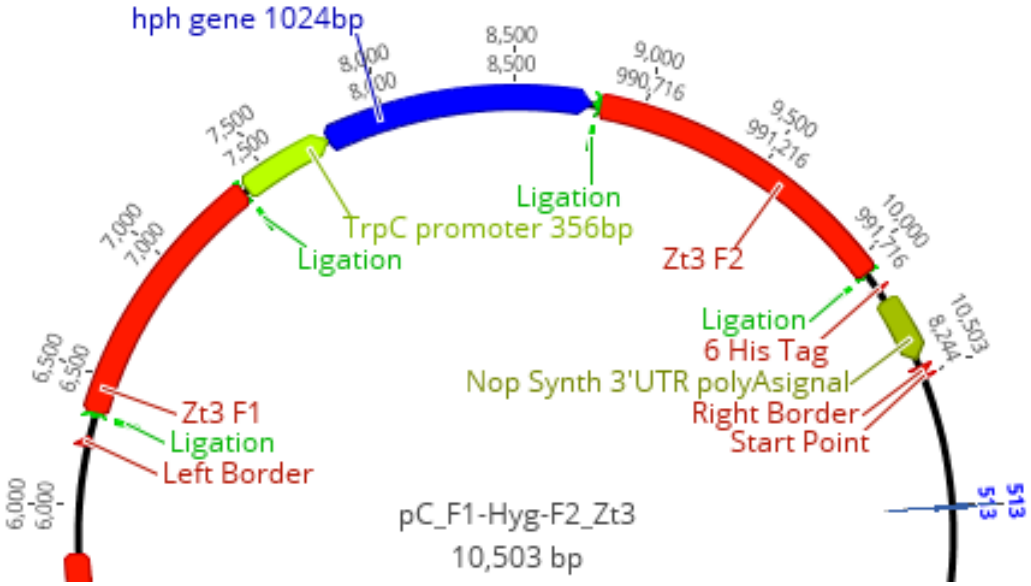


Figure 2.2 Representation of *in silico* cloning of upstream and downstream genomic regions of Mycgr104444 (Zt3) gene in pCHYG vector (pC_F1-Hyg-F2_Zt3) (Geneious).

2.4.1 pCHYG-JK vector

The pCHYG-JK vector is a binary shuttle vector used for transformation of *Z. tritici*. It has kanamycin resistance gene in the backbone and hygromycin resistance (hph) gene in T-DNA region. The hph gene is flanked on both sites by MCS. On the upstream region it contains ApaI, EcoRI, SacI, KpnI single cut sites and on the downstream it contains BamHI, XbaI, SalI, HindIII, NcoI, SpeI, HpaI single cut sites. The whole sequence between left and right borders, marked by specific 25 nucleotides, is recognized as T-DNA and is transferred to the host.

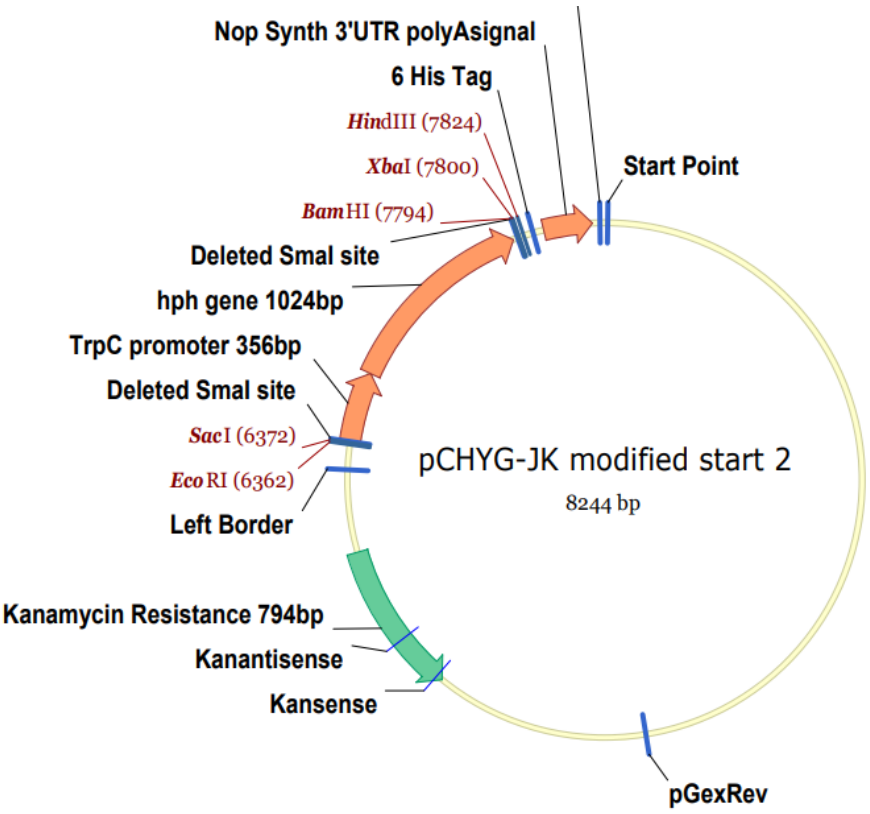


Figure 2.3 Representation of pCHYG-JK binary vector used for *Agrobacterium*-mediated transformation of *Zymoseptoria tritici* (Motteram *et al*., 2009).

2.4.2 Construction of primers

Upstream region of the knock out (KO) targeted gene was designated as F1 (flanking 1) and downstream region as F2 (flanking 2). The flanking region lengths were adjusted to be around 1 kb. The forward and reverse primers for generation of flanking fragments were chosen by picking around 20 bp from desired regions, adding suitable 6 bp for enzymatic cut and add 6 more random nucleotides for enzyme to bind easier on cutting site. The primers were analyzed using Geneious software for GC content (optimally 40 – 60%), close Tm temperatures and checked for primer-dimer and self-dimerization. Forward and reverse primers of F1 and F2 were named as F1F, F1R, F2F and F2R respectively. To check the proper ligation a forward control primer from within the F1 (F1CF) region and a reverse primer from within left side of hph gene (hphLR) was designed. Similarly, a control reverse primer from within F2 (F2CR) and a forward primer from within right side of hph gene (hphRF) was designed (Figure 2.4). Control primers were also chosen by picking 20 bp from desired regions or by automatic generation option in the Geneious software and analyzing them afterwards. To confirm the deletion of the gene in *Z. tritici* a primer in F1 region (Knock-out control forward, KOC-F) and one primer inside gene-of-interest region (Knock-out control reverse KOC-R) was designed. The absence of PCR product using KOC primers indicated the successful gene knock-out. The primers used in this project are shown in Table 2.6.

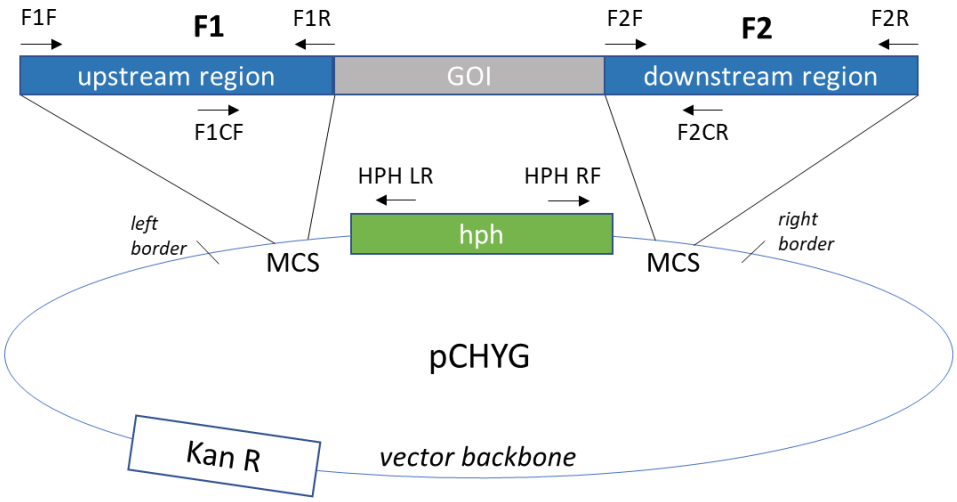


Figure 2.4 Generation of F1 and F2 fragments and ligation to the left and right side MCS of hph gene inside pCHYG vector.

When designed T-DNA enters the cell, because of long flanking regions, the homologous recombination mechanism of *Z. tritici* can engage in exchange of fragments incorporating hph gene in the genome while removing the gene of interest (Figure 2.5).

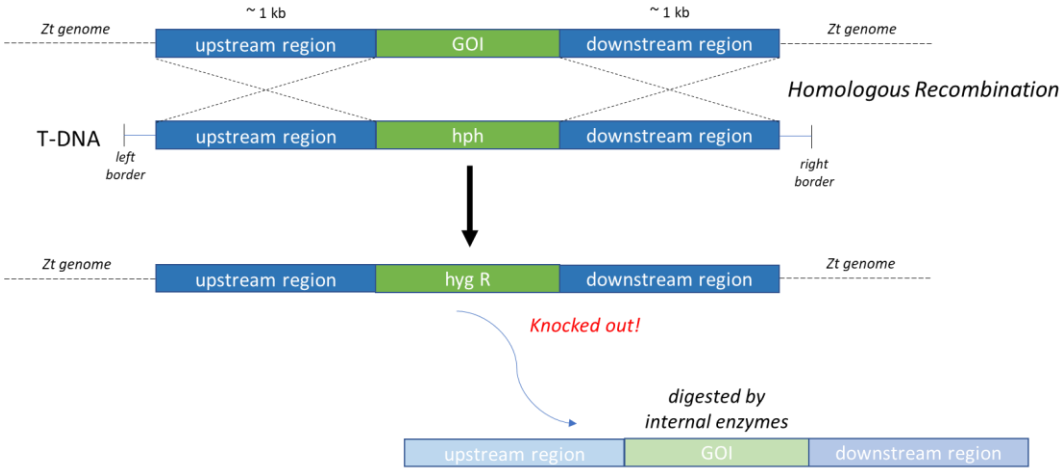


Figure 2.5 Homologous recombination occurring in the *Z. tritici* genome which lead to generation of knock out transformants.

Table 2.6 Table of primers. Six nucleotides in red color are added for recognition by associated enzyme. In blue are six extra random nucleotides added for enzyme binding

Gene No	Gene ID	Primer name	Nucleotide sequence 5' to 3'	Associated enzyme
3	104444	Zt3-F1_F	TAAGCAGGGCCCCGTAACGACGCTAGACTACCC	ApaI
		Zt3-F1_R	TGCTTAGAATTCCACACCAAGTCGTTGCACATT	EcoRI
		Zt3-F1_CF	TCCTTATACGTAGACGCTCCT	
		Zt3-F2_F	TAAGCAGGATCCTCTGCTGGACTGGTGAAGTAC	BamHI
		Zt3-F2_R	TGCTTAGTCGACAGCTCAACTGTACTTCGTGGG	SalI
		Zt3-F2_CR	CAGACCACATGTTGCCACAAG	
		Zt3-KOC-F	TCGAACCAAGTCTTCCCGAAG	
		Zt3-KOC-R	CCTCCAAACTGTCCTCCTTCC	
17	104383	Zt17-F1_F	TAAGCAGGGCCCGCTTACATTGGTCGGGAAAGC	ApaI
		Zt17-F1_R	TGCTTAGAATTCACGAAAATGCGCTATTGCCTC	EcoRI
		Zt17-F1_CF	CTCCGGAGCAGGATTCAAAGT	
		Zt17-F2_F	TAAGCAGGATCCCAAAGTGTCTCAGCTTTGCGG	BamHI
		Zt17-F2_R	TGCTTAGTCGACATTTGCTCGATAGGTCTGGCG	SalI
		Zt17-F2_CR	CCGTCCGATCAGCCATTGATA	
		Zt17-KOC-F	TCGCATCTCGAAGAACCACAA	
		Zt17-KOC-R	AAAAATGTCAGCGAGGTGTGC	
22	105826	Zt22-F1_F	TAAGCAGGGCCCTGTTAGTTAGACGGAAGCTCTGT	ApaI
		Zt22-F1_R	TGCTTAGAATTCAGCAAAACCTCGACTCTCCAT	EcoRI
		Zt22-F1_CF	TGCTCTCAGTCGTCGATGATG	
		Zt22-F2_F	TAAGCAGGATCCGCAACGAACAGCAGGGTTGTC	BamHI
		Zt22-F2_R	TGCTTATCTAGAGTTGCCATGGGGGATTGAAAT	XbaI
		Zt22-F2_CR	CACGGAGAGAAAACTGGTCGA	
		Zt22-KOC-F	TCGTAACTTTGCGCAGAGACT	
		Zt22-KOC-R	TACTCGACAACCCTGCTGTTC	
HPH gene		HPH-LR	TGAACTCCCCAATGTCAAGCA	
		HPH-RF	GACGGCAATTTCGATGATGCA	

2.4.3 Polymerization chain reaction

For fragment generation PCR MyTaq™ HS Mix (Bioline Cat. No. BIO-25045) was used, while for colony PCR MyTaq™ Red Mix (Bioline Cat. No. BIO-21107) was used. PCR was conducted according to manufacturer's protocol with slight changes (Table 2.7).

Table 2.7 Constituents and their amounts used in PCR

Constituents in 200 µL PCR tube	Amount
5x MyTaq Reaction Buffer	5 µL
Forward Primer (10 µM)	1 µL
Reverse Primer (10 µM)	1 µL
MyTaq (HS) DNA Polymerase	0.25 µL
Template DNA	20 - 50 ng
ddH_2O	fill to 25 µL

In case of colony PCR, instead of template DNA, 1 µL of single colony culture grown in LB was added. In case of solid media single colony from plate was picked with a sterile pipette tip and was dipped into PCR mix.

Annealing step in thermocycler varied between 50 – 70^0C and was adjusted according to primer length, GC content and PCR results; increasing in case of multiple bands and lowering if no products were visible. For fragments longer than 1 kb extension time was increased from 10 s up to 30 s (Table 2.8).

Table 2.8 Thermocycler conditions

Step	Temp.	Time	Cycles
Initial denaturation	95^0C	3 min	1
Denaturation	95^0C	15 s	35
Annealing	50 - 70^0C	15 s	
Extension	72^0C	10 – 30 s	
Cooling and keeping	4^0C	∞	1

2.4.4 PCR clean-up and gel extraction

For PCR clean-up and gel extraction Macherey-Nagel NucleoSpin® Gel and PCR Clean-up kit (Ref: 740609.250) was used. Manufacturer's protocol form user manual was used with slight changes. PCR products were adjusted to volume of 50 µL by addition of ddH_2O if necessary. Two volumes of buffer NTI (100 µL) was added and mixed with the PCR product in 200 µL PCR tube. The mix was then transferred to NucleoSpin® Gel and PCR clean-up column and centrifugated for 30 seconds at 11000 x g. The flow-through was discarded and 700 µL of buffer NT3 was added to the column and centrifugated again for 30 seconds at 11000 x g and the flow-through was discarded. NT3 washing step was repeated for better results. The column was centrifugated for 1 min at 11000 x g to remove any remaining of buffer NT3 and to dry the membrane. Column was placed in new 1.5 mL tube, and 30 µL of ddH_2O was added carefully on membrane. After waiting for 1 minute, by centrifugation for 1 minute at 11000 x g the cleaned-up PCR product was obtained as flow-through. Concentration of the solution was measured in NanoDrop.

Gel extraction procedure was the same as PCR clean-up procedure except first steps. The DNA band of interest was visualized under UV using protective mask and was excised carefully using scalpel. The excised gel slice was put into 1.5 mL tube and its weight was measured. For 100 mg gel slice 200mL NTI buffer was added and incubated at 50^0C for 10 minutes. If gel was not completely dissolved the tube was inversed several times and incubated at 50^0C for another 10 minutes. After gel was dissolved completely 700 µL of the mixture was put in NucleoSpin® Gel and PCR clean-up column and centrifugated for 30 seconds at 11000 x g. The flow-through was discarded and any remaining dissolved mixture was re-filled to the column (max. 700 µL at a time) and centrifugated. After all the mix had pass through membrane 700 µL of buffer NT3 was added to the column and centrifugated for 30 seconds at 11000 x g and the flow-through was discarded. NT3 washing step was performed twice for better results. The column was centrifugated for 1 min at 11000 x g to remove any remaining of NT3 and to dry the membrane. Column was placed

in new 1.5 mL tube, and 30 µL of ddH_2O was added carefully on membrane. After waiting for 1 minute, by centrifugation for 1 minute at 11000 x g the cleaned-up gel extracted product was obtained as flow-through. Concentration of the solution was measured in NanoDrop.

2.4.5 Gel electrophoresis

For gel electrophoresis 1% agarose was added to Tris/Borate/EDTA (TBE, Table 2.9) buffer and microwaved until all agarose was solubilized. After cooling to approximately 50°C or below, 1 µL of ethidium bromide (Invitrogen™ UltraPure™ 10 mg/mL Cat. No. 15585011) was added to 100mL liquid agarose solution for visualizing DNA under UV light (Quantum-ST4-110/26MX), and manufacturer's "Quantum ST4" program was used for DNA visualization. Agarose gel was poured in the tank and the comb with 24 wells was dipped for wells to form. After gel was solidified 1 – 5 µL of DNA samples were mixed with 1 µL of 6X Orange DNA loading dye (Thermo scientific #R0631) and loaded to the agarose gel. ddH2O was used if necessary, to complete the sample volume to 6 µL. Electrophoresis ran at 100 – 150 V on DC using power supply (Thermo scientific Owl™ EC3000XL Programmable Power Supply), until bands of different sizes were separated and observed as such in visualizing program. Generally, 30 – 60 minutes were enough for >100bp band separation. O'GeneRuler 100bp Plus DNA ladder (Thermo scientific #SM1153) was used for comparison and band size deduction.

Table 2.9 Preparation of 2L of 5X TBE

Name	**Amount**
Tris	108 g
H_3BO_3 (Boric acid)	55 g
0.5 M EDTA (pH 8)	40 mL
dH_2O	fill to 2000 mL
Mix and adjust pH to 8 by addition of HCl	

2.4.6 Enzymatic cut

NEB enzymes were used for enzymatic cuts which were performed as double digestions in suitable NEB buffers. The commonly used enzymes were ApaI, EcoRI HF, KpnI, BamHI, SalI, XbaI (Cat. No. R0114S, R3101S, R0142S, R0136S, R0138T, R0145S respectively). Double digestion protocols were performed according to manufacturer's protocol with slight changes (Table 2.10, 2.11, 2.12, 2.13). NEB CutSmart, NEB1.1, NEB2.1 and NEB3.1 buffers were included in the enzyme kit.

Table 2.10 Double digestion protocol for ApaI/EcoRI HF

Constituent	Amount	Activity in buffer
10X NEB CutSmart buffer	2.5 µL	
ApaI	0,5 µL	100%
EcoRI HF	0,5 µL	100%
DNA (cleaned up)	500 ng	
dd H2O	to 25 µL	
Incubate at 25^0C for 9 hours		
Incubate at 37^0C for 9 hours		
Inactivation at 65^0C for 20 min		

Table 2.11 Double digestion protocol for ApaI/KpnI

Constituent	Amount	Activity in buffer
10X NEB 1.1 buffer	2.5 µL	
ApaI	0,5 µL	25%
KpnI	0,5 µL	100%
DNA (cleaned up)	500 ng	
dd H2O	to 25 µL	
Incubate at 25^0C for 9 hours		
Incubate at 37^0C for 9 hours		
Inactivation at 65^0C for 20 min		

Table 2.12 Double digestion protocol for BamHI/SalI

Constituent	Amount	Activity in buffer
10X NEB 3.1 buffer	2.5 µL	
BamHI	0,5 µL	100%
SalI	0,5 µL	100%
DNA (cleaned up)	500 ng	
dd H2O	to 25 µL	
Incubate at 37^0C overnight		
Inactivation at 65^0C for 20 min		

Table 2.13 Double digestion protocol for BamHI/XbaI

Constituent	Amount	Activity in buffer
10X NEB 3.1 buffer	2.5 µL	
BamHI	0,5 µL	100%
XbaI	0,5 µL	75%
DNA (cleaned up)	500 ng	
dd H2O	to 25 µL	
Incubate at 37^0C overnight		
Inactivation at 65^0C for 20 min		

The products of enzymatic cut were cleaned with PCR-clean up kit and measured by NanoDrop before usage for ligation reaction.

2.4.7 DNA ligation

For ligation of double digested and cleaned up vector and DNA fragment of known concentration T4 ligase were used. T4 DNA Ligase Buffer (10X) were obtained from NEB (Cat. No. M0202S). T4 ligation procedure was performed according to manufacturer's protocol with slight changes (Table 2.14).

Table 2.14 T4 ligation protocol

Name	Amount	Description
10X T4 buffer	2 µL	
Insert	37.5 ng	double digested and PCR purified
Vector	50 ng	double digested and gel purified
T4 ligase	1 µL	
dH2O	to 20 µL	
Incubate at 16^0C overnight		
Inactivation at 65^0C for 10 min		

2.6 *Zymoseptoria tritici* transformation

For generation of knock outs Z. *tritici* native IPO323 cultivar was used. In some studies Ku70 and Ku80 mutant strains are used, which have these gene deleted providing lower rate ectopic recombination and efficiency increasement up to 85% (Sidhu *et al*., 2015). Ku70 and Ku80 genes are DNA repair mechanism genes which utilize non-homologous end joining (NHEJ) mechanism. Due to functional NHEJ genes in IPO323, off-target mutations can be a problem causing misleading results. Since Ku70 or Ku80 mutant could not be obtained, IPO323 was the choice to work with.

For *Zymoseptoria tritici* transformation Zwiers & Waard (2001) protocol with slight alterations was used.

Six days before transformation two YPD plates were inoculated with *Z. tritici* IPO323 strain from -80^0C stock, sealed with parafilm completely and let grow at room temperature.

Four days before transformation *Agrobacterium* AGL1 strain from -80^0C stock containing the desired cassette for knock-out were inoculated on kanamycin containing agar LB and let grow at 28^0C.

One day before transformation for each *Agrobacterium* plate 40 mL of LB Mannitol (Table 2.15) were poured into 250 mL Erlenmeyer flask and was

inoculated with a loopful of that *Agrobacterium* culture. It was incubated for around 20 hours at 28^0C at 220 rpm in rotary shaker.

For each *Agrobacterium* culture 100 mL of induction media (IM, Table 2.16) was prepared.

On the transformation day 5 IM plates per *Agrobacterium* culture was prepared. After cooling to approximately 50°C or below, acetosyringone from stock solution (at -20^0C; 40 mg/mL) was added at 1/1000 ratio (final concentration 40 µg/mL).

For every 100 mL liquid IM 100 µL of stock acetosyringone (As) was added.

From overnight grown *Agrobacterium* culture 2 mL were pipetted into 2 mL tubes and centrifugated at 16100 x g for 2 minutes. The supernatant was discarded and cells in the pellet were solubilized in 400 µL of IM (As). Tubes were centrifugated again at 16100 x g for 2 minutes and the supernatant was discarded.

Kanamycin (Kan, 50 mg/mL) was added to liquid IM (As) at 1/1000 ratio to obtain IM (As, Kan).

The pellet from 2 mL tubes were re-solubilized in 1 mL IM (As, Kan) then mixed with 9 mL of IM (As, Kan) in 15 mL falcon tube. The absorbance was measured with spectrophotometer at 660 nm. It was diluted to 0.15 in 250 mL flask with IM (As, Kan) to final volume of 9 – 11 mL (OD_{660} / 0.15 = dilution factor). The dilution was confirmed by readings of OD_{660} in spectrophotometer. The flask was put in rotary shaker at 220 rpm at 28^0C for around 3 hours until OD660 had increased to 0.25 (0.19 – 0.26 was acceptable) indicating exponential phase of growth.

Z. tritici spores were scraped with sterile loop from 6-day old YPD culture and mixed into 30 mL IM (As, Kan) in 50 mL falcon tube. The concentration was measured using hemocytometer under microscope and set to $1 – 2 \times 10^7$ spores / mL. Around 5 loopful *Z. tritici* spore culture was enough for achieving this concentration. The tube containing *Z. tritici* suspension was put on ice or in refrigerator (4^0C).

Over the IM (As) plates 200 µL of IM (As, Kan) was spread and cellulose disks (A.A. Packaging limited, 325P Cellulose 80 mm DIA. Disc) were carefully put on top using sterile forceps. The cellulose disks were made sure to be flat and in contact with agar using spreader. Plates were let for around 20 minutes with lids off to dry.

In 1.5 mL tubes 600 µL of *Z. tritici* culture, 600 µL of *Agrobacterium* culture and 6 µL of acetosyringone were mixed. For each isolate, five plates were prepared and 200 µL of the mix was pipetted onto cellulose disk covered IM (As) plates, and spread to the whole surface letting to dry for few minutes with lids off. For negative control one plate was spread only with *Z. tritici*.

Plates were incubated at room temperature for two days. Cellulose disks allow nutrient and water transfer through solid media while restrain cells on the top.

Two days after transformation *Aspergillus nidulans* minimal medium agar (Table 2.20) was prepared and after cooling to approximately 50°C or below, 1 mL timentin (50 mg/mL) and 1 mL hygromycin (50 mg/mL) was added to 500 mL media. The media containing antibiotics poured in the same number of plates as transformation plates. After solidification 100 µL of dH_2O were spread over the plates. Cellulose disks were transferred from 2 dpi agar IM (AS) plates to *A. nidulans* minimal media (Tim, Hyg) inside sterile cabin and sterile forceps and incubated at room temperature without sealing for 14 – 21 days.

Hygromycin was used as transformant selective marker while timentin is needed to kill *Agrobacterium*.

After for 14 – 21 days transformants begin to appear as white spots radiating outwards (Figure 2.6). These colonies were transferred on YPD (Tim, Hyg) plates with sterile loop and let grow for 5 – 7 days at room temperature.

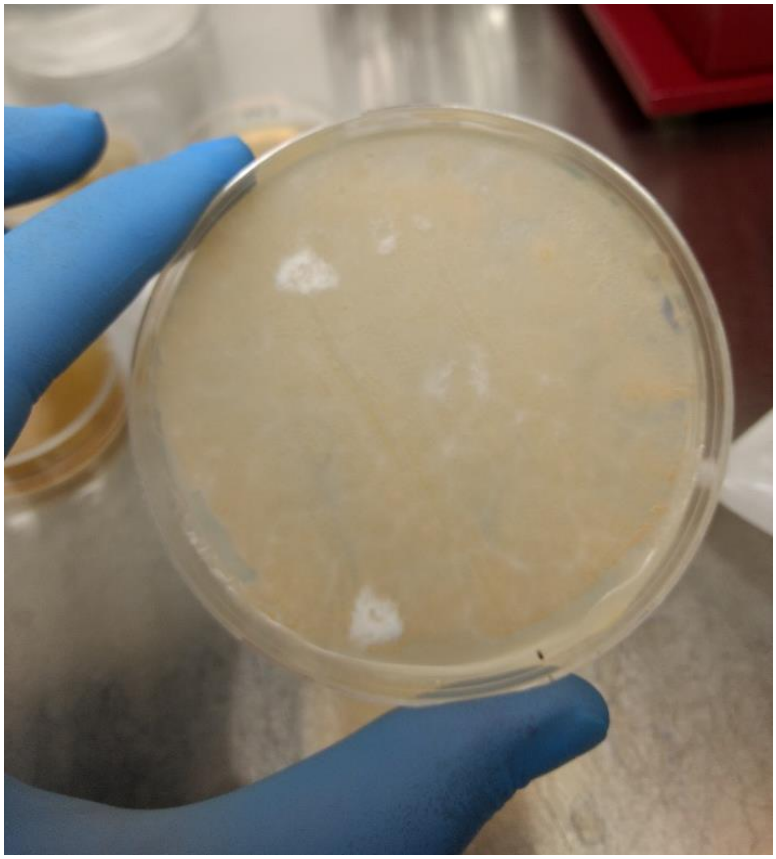


Figure 2.6 Transformed *Z. tritici* colonies on cellulose disks. Colonies appear as white spots radiating outwards.

DNA isolation of transformant *Z. tritici* were performed and checked with PCR for absence of target gene and controlled against foreign organism contamination.

The confirmed knock outs were stored at -80^{0}C inside 2 mL tubes containing 100 µL of 10% glycerol (20 µL of %50 glycerol and 80 µL ddH_2O) by taking 1 – 2 loopful culture and dip in the solution.

Table 2.15 Preparation of 1000 mL of LB Mannitol (low salt)

Constituent	Amount
Bacto Tryptone	10 g
Yeast extract	5 g
NaCl	2.5 g
Mannitol	10 g
dH2O	fill to 1000 mL
Autoclave	

Table 2.16 Preparation of 1000 mL of induction media (IM)

Constituent	Amount	Final concentration
20% glucose	9 mL	10 mM
1M MES (pH 5.3)*	40 mL	40 mM
50% glycerol	10 mL	0.5%
Agar no.1 **	13 g	1.3 %
Complete medium***	fill to 1000 mL	
Autoclave		

* Table 2.17, ** agar was added for solid media, *** Table 2.18

Table 2.17 Preparation of 200 mL of 1M MES buffer

Constituent	Amount
MES	39 g
dH_2O	200 mL
5M KOH	around 3 mL
Adjust pH to 5.3 with 5M KOH	
Store at 4^0C	

Table 2.18 Preparation of 1000 mL complete medium

Constituent	**Amount**
Stock A*	50 mL
Stock B*	50 mL
Stock C*	50 mL
9 mM $FeSO_4$	1 mL
dH2O	fill to 1000 mL
Store at 4^0C for 1 week	

* Table 2.19

Table 2.19 Preparation of stock A, B and C

Stock A		**Stock B**		**Stock C**	
Constituent	**Amount**	**Constituent**	**Amount**	**Constituent**	**Amount**
MgSO4	10 g	K2HPO4	40.5 g	CaCl2	2g
KH2PO4	29 g	(NH4)2SO4	10 g		
NaCl	3 g				
dH2O	fill to 1000 mL	dH2O	fill to 1000 mL	dH2O	fill to 1000 mL
Store at room temperature		Store at room temperature		Store at room temperature	

Table 2.20 Preparation of 1000 mL *Aspergillus nidulans* minimal medium agar plates

Constituent	**Amount**
20X Salts*	50 mL
Trace elements**	1 mL
Glucose	10 g
Agar	10 g
dH_2O	fill to 1000 mL
Adjust pH to 6.5 with 5M KOH (~1mL)	

* Table 2.21, **Table 2.22

Table 2.21 Preparation of 20X salt solution

Constituent	**Amount**
$NaNO_3$	120 g
KCl	10.4 g
$MgSO_4*7H_2O$	10.4 g
KH_2PO_4	30.4 g
dH_2O	fill to 1000 mL
	Autoclave
	Store at 4^0C

Table 2.22 Preparation of trace element solution (add in order)

Constituent	**Amount**
$ZnSO_4*7H_2O$	2.2 g
H_3BO_3	1.1 g
$MnCl_2*4H_2O$	0.5 g
$FeSO_4*7H_2O$	0.5 g
$CoCl_2*5H_2O$	0.16 g
$CuSO_4*5H_2O$	0.16 g
$(NH_4)_6Mo_7O_{24}*4H_2O$	0.11 g
Na4EDTA	5 g
dH_2O	fill to 100 mL
Boil and mix thoroughly	
Cool to 60^0C and adjust pH to 6.5 with 5M KOH	
Store at 4^0C in the dark	

2.6.1 DNA isolation from *Zymoseptoria tritici*

To isolate DNA form *Z. tritici* culture Ron's Fungal DNA Mini Kit was used (Cat. No. 803490). *Z. tritici* was grown on YPD at room temperature for 5 – 7 days (until all the petri surface is filled with the culture). Two – three loop-full of *Z. tritici* culture was soaked in 250 µL of ddH_2O in 2 mL tubes. Few ceramic beads of size 1.4 mm and 2 mm were filled into tube and the fungus is grinded in benchtop oscillator (Roche MagNA Lyser Instrument) at 7000 rpm for 1 minute. The mix was transferred into new 1.5 mL tube leaving the ball-bearings at the bottom. The CH buffer is added immediately to the tube and vortexed at maximum speed for 15 s to mix well the solution. The tube was placed into 70^0C water bath for 10 minutes and inversed occasionally. The tube was centrifuged at 12100 x g for 5 minutes. The supernatant was transferred carefully to new 1.5 mL tube while the pellet was discarded. To the mix, 250 µL of buffer AB was added and mixed well. The mixture was transferred to spin column and centrifugated at 12100 x g for 30 sec. After flow-through was discarded, 200 µL of buffer RB (including RNase A) was added to spin column and centrifugated at 12100 x g for 30 sec. Flow-through was discarded and 400 µL of WB buffer was added and centrifugated at 12100 x g for 30 sec and the flow-through was discarded again. To wash the membrane, 400 µL of 70% ethanol was added and centrifugated at 12100 x g for 30 sec and the flow-through was discarded. The spin column was added into new 1.5 mL tube and 100 µL of ddH_2O was added carefully on the membrane and incubated for 1 minute at RT. Then the centrifugation at 12100 x g was performed for 1 minute and the isolated DNA was collected as flow-through. The concentration of DNA was measured using NanoDrop.

2.7 Preparation and utilization of antibiotics

The antibiotics were solubilized in the suitable media and filtered through 0.20 μm syringe filter (Millex, Cat. No. SLGP033RS) using a sterile syringe. Preparation and utilization of antibiotics were done according to the Table 2.20. All antibiotics were stored in -20^{0}C. Rifampicin tubes were covered with aluminum folio because of light sensitivity.

Table 2.23 Preparation of antibiotics and their usage

Name of antibiotic	Medium	Stock conc.	Dil. factor	Working conc.
Kanamycin	ddH_2O	50 mg/ mL	1/1000	50 μg/mL
Carbenicillin	ddH_2O	50 mg/ mL	1/1000	50 μg/mL
Rifampicin	methanol	50 mg/ mL	1/500	100 μg/mL
Hygromycin	ddH_2O	50 mg/ mL	1/500	100 μg/mL
Timentin	ddH_2O	50 mg/ mL	1/500	100 μg/mL

2.8 Wheat maintenance and disease assay

The Turkish wheat cultivar of winter bread wheat, Pehlivan was chosen for disease assay as it was bred in Turkey (Trakya Agricultural Research Institute, 1998) and it is known to be susceptible of STB disease (Turgay *et al*., 2017).

Ten to fifteen plants were planted on two pots (17 x 24 x 5.5 cm) arranged in linear fashion near the container's edge for each *Z. tritici* culture.

Wheat plants were grown in growth chamber in 16-hour day / 8-hour night cycle. During day period temperature was held at 22^{0}C and during night at 20^{0}C at relative humidity (RH) of 70% for 20 days.

After 20 days of incubation wheat second leaves were stuck on aluminum plate (18.5 x 9.5 x 3.5 cm) placed on soil with double-sided tape to prepare for inoculation (Figure 2.6).

Z. tritici cultures were grown on YPD agar for 7 days at room temperature. The grown spores were picked up using a sterile loop and put on 5 mL of dH_2O. The spore concentration was adjusted to 10^7 spores / mL using hemocytometer counting method. In the concentration adjusted mixture 0.1% Tween 20 (Scharlau, Cat. No. TW00220100) was added as surfactant. The inoculum was spread on the stuck leaves with sterile cotton swabs on the area between two tapes (Figure 2.7). Following application of the disease inoculum, plants were incubated at 95 – 100% RH for 72 hours and at 85% RH for the following incubation period. The temperature was fixed to 22^0C while day/night cycle remained the same. The incubations were done for at least 21 days after inoculation, and constant monitoring of disease progression was performed.

Figure 2.7 Application of fungus to wheat leaves. A) Wheat leaves stuck on aluminum plate with double-sided tape, B) Applying *Z. tritici* culture (10^7 spores/mL) to the wheat leaves with sterile cotton swabs.

2.8.1 STB disease evaluation

For Septroria tritici blotch evaluation, Rosielle's Septoria tritici blotch scale was used (Table 2.20).

Table 2.20 Septria tritici blotch disease 0 – 5 scale (Rosielle, 1972)

Scale	Symptoms in the plant
0 (Imm)	No pycnidia formation, no or very rare necrotic lesions (% 0 - 1)
1 (HR)	No or very rare pycnidia formation, rare yellowish necrotic lesions (% 1 - 10)
2(R)	Little pycnidia formation, visible small necrotic lesions (% 10 - 25)
3 (I)	Visible pycnidia formation, wide necrotic lesions on any part of the leave (% 25 - 50)
4 (S)	Moderate pycnidia formation, remarkably wide necrotic lesions (% 50 - 75)
5 (VS)	Large number of pycnidia, necrotic lesions are seen all over the leaf surface (% 75 - 100)

Imm - Immune, HR – Highly resistant, R – Resistant, I – Intermediate, S – Susceptible, VS – Very susceptible

After disease evaluation the severity of the disease was measured using Tawsend-Heuberger formula.

$$\text{Disease severity (\%)} = [\ \Sigma\ (V \times n) / Z \times N\ ] / 100$$

where V is given Rosielle's disease scale after evaluation, n is number of plants of the particular V scale, Z is the maximum value of the scale (5) and N represent the total number of evaluated plants.

The measurements of the chlorosis, necrotic lesion and pycnidia formation were done using ImageJ (Rasband, W.S., ImageJ 1.52a; U.S. National Institutes of Health, Bethesda, MD; https://imagej.nih.gov/ij/) software.

2.9 Fungal growth assays

Fungal growth assays can be done by using known or unknown concentration of spore mixture spotted on medium. From unknown concentration mixture appearance and switch from spore-forming to mycelial growth can be deduced. With known start concentration growth rate can be deduced as well. To deduce appearance visual examination was performed, whereas for spore-forming to mycelial growth transition visual or microscopic examinations were performed. Growth rate was deduced by measuring the area covered by the fungus. The area was measured with help of ImageJ scaling to the diameter of the plate (5.5 cm).

YPD was used as standard growth medium, while WA was used as mycelial growth inducing medium. PDA and YMA was used to examine differences in melanization and mycelial growth transition.

The spore formation was observed with light microscope (Leica, DFC 280) and visualized on computer using manufacturer's software.

CHAPTER 3

RESULTS AND DISCUSSION

3.1 Candidate effector gene selection

Twenty two proteins from the publication of do Amaral *et al*. (2012) (Table 3.1) with no transmembrane motif, no predicted function and no homology, containing more than 6 cysteines and having more than 10% of cysteine content and smaller than 200 amino acids in length were chosen to work with. The protein pool had been further narrowed down to 8 proteins based on secretome analysis (Rudd *et al*., 2015) (Appendix C). All of the presumed candidate effectors reside on core chromosomes 1 – 13 of *Zymoseptoria tritici*. Of these 8 proteins three were successfully knocked out: Mycgr3G104444 (Zt3), Mycgr3G104383 (Zt17) and Mycgr3G105826 (Zt22). The genes were chosen based on characteristics of expression profiles with possible effector activity (Figure 3.1). Zt3 is activated at 4 dpi reaching its maximum at 14 dpi at the stage of transition from biotrophy to necrotrophy, resembling a necrotrophy activation effector or a host specific toxin. Zt17 is active from the beginning reaching its maximum at 9 dpi, and dropping its expression at saprotrophic stage (21 dpi). It may function as evader against recognition or as activator of necrotrophy. Zt22 is a quite active gene throughout the disease development dropping its expression towards the saprotrophic stage. It may be important for the growth and expansion of the disease. The reason of high expression of Zt22 in PDB relative to CDB may be that PDB is not a defined medium having rich sources of carbon and nitrogen and the fungus grows much faster in PDB compared to defined CDB where the carbon and nitrogen sources are defined.

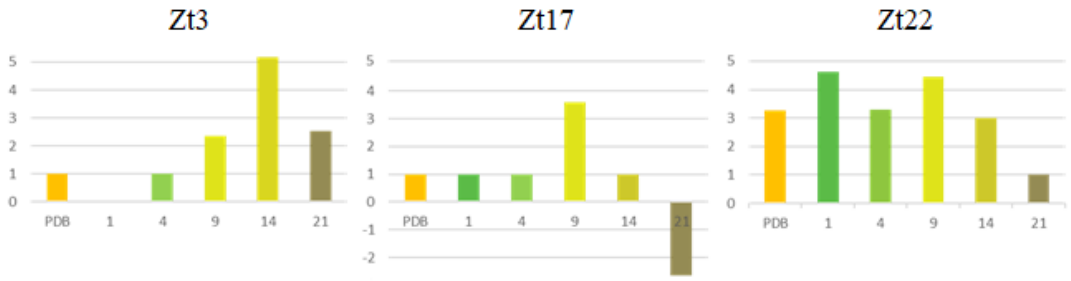


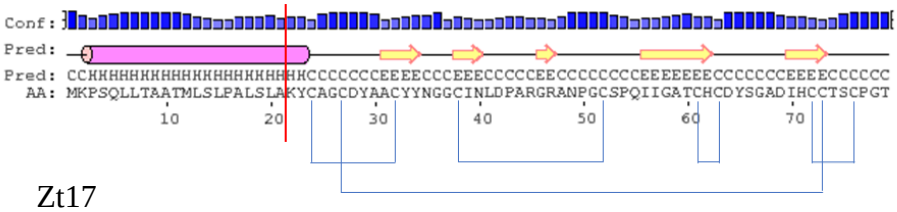
Figure 3.1 Expression profile of Zt3 (104444), Zt17(104383) and Zt22 (105826) on wheat leaves measured at 1, 4, 9, 14 and 21 days post inoculation and expression in PDB (Potato Dextrose Broth). All expression data are given as expression ratios compared to expressions in CDB (Czapek-Dox Broth).

Table 3.1 Twenty-two candidate effectors, the given gene number and JGI-Id, signal peptide length, pre- and post- processing length, cysteine count and percentage, *Z. tritici* specificity, worked on and successful knock outs (in gray) (modified table from do Amaral *et al*., 2012)

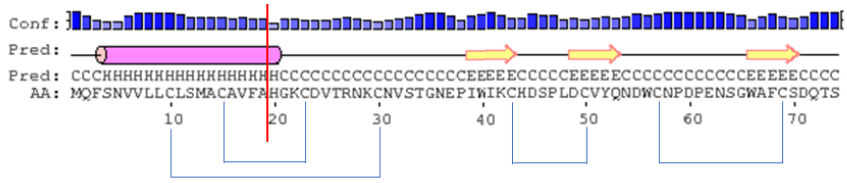
Gene no.	**#id-JGI**	**Pre len**	**Sig len**	**Mature length**	**Cys. count**	**%Cys**	***Z.tritici* specific (<e-5)**	**worked on**	**KO**
Zt1	87205	55	22	33	8	24.24	Y	Y	N
Zt2	83081	53	20	33	6	18.18	Y	Y	N
Zt3	104444	79	21	58	10	17.24	Y	Y	Y
Zt4	82925	57	19	38	6	15.79	Y	N	-
Zt5	81208	59	18	41	6	14.63	Y	N	-
Zt6	82029	66	18	48	7	14.58	Y	N	-
Zt7	101652	77	21	56	8	14.29	Y	N	-
Zt8	79286	63	20	43	6	13.95	Y	Y	N
Zt9	100649	75	17	58	8	13.79	N	Y	N
Zt10	41491	81	18	63	8	12.7	N	N	-
Zt11	79161	68	19	49	6	12.24	Y	N	-
Zt12	99161	164	15	149	18	12.08	N	N	-
Zt13	106125	70	17	53	6	11.32	Y	N	-
Zt14	106502	89	18	71	8	11.27	Y	N	-
Zt15	108482	108	19	89	10	11.24	N	N	-
Zt16	80332	76	21	55	6	10.91	Y	N	-
Zt17	104383	74	19	55	6	10.91	Y	Y	Y
Zt18	83064	75	18	57	6	10.53	Y	N	-
Zt19	104758	118	22	96	10	10.42	Y	Y	N
Zt20	97031	118	22	96	10	10.42	Y	N	-
Zt21	106445	119	22	97	10	10.31	Y	N	-
Zt22	105826	98	20	78	8	10.26	Y	Y	Y

Prediction of secondary structures were done using PSIPRED v3.3 Protein Sequence Analysis Workbench (http://bioinf.cs.ucl.ac.uk/psipred/). The predicted structure i.e. helix or strand is given in second line as drawing and the confidence is given in first line with bars, long bars meaning high confidence whereas smaller bars indicating lower confidence. For cysteine bridge DiANNA 1.1 web server (http://clavius.bc.edu/~clotelab/DiANNA) was used. The cysteine bridge prediction may not reflect the actual disulfide bridges but can us an give an overall idea of possible bonds and 3D conformation. Signal peptide and cleavage sites are predicted by SignalP 4.1 server (http://www.cbs.dtu.dk/services/SignalP) (Figure 3.2). Zt3 is predicted to have a helix structure in N-terminus most of which resides in is the signal peptide region. Five shorter strands, and 5 cysteine bridges are predicted in the processed peptide (C24 – C32, C27 – 73, C38 – C52, C61 – C63 and C72 – C76). Zt17 is predicted to have a helix structure in N-terminus most of which is formed in the signal peptide region and three shorter strands and 4 cysteine bridges (C10 – C30, C15 – C23, C43 – C50 and C57 – C69). Zt22 is predicted to have 3 helices and three strands. Of these, one helix and one strand reside in signal peptide region and there are four cysteine bridges predicted (C26 – C70, C28 – C91, C43 – C96 and C65 – C80).

Zt3



Zt17



Zt22

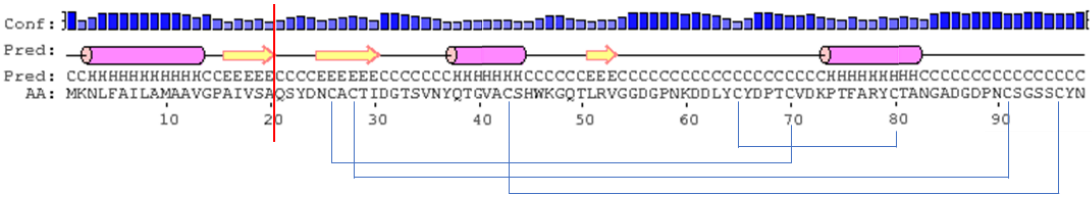


Figure 3.2 Prediction of secondary structures of Zt3, Zt17 and Zt22 by PSIPRED v3.3, cysteine bridges by DiANNA 1.1 web server and signal peptide and site of cleavage by SignalP 4.1 server. Cylinders in pink represent predicted helices, arrows in yellow represent predicted strand structures. Cysteine bridges are shown by blue lines below, connecting predicted cysteine residues taking part in disulfide bridge formation.

For 3D structure prediction I-TASSER (Iterative Threading ASSEmbly Refinement) was used (Figure 3.3) (https://zhanglab.ccmb.med.umich.edu/I-TASSER). For each predicted protein model, it assigns a C-score which shows the confidence of estimation quality with values between -5 to +2, with higher values corresponding to greater confidence of predicted structure and vice versa. The predicted models were viewed in in iC3D Web-based 3D structure viewer (https://www.ncbi.nlm.nih.gov/Structure/icn3d/full.html) (Figure 3.3). Based on prediction, Zt3 has a helix structure in N-terminus followed by coil and two short antiparallel beta strands and short strand at the C-terminus, Zt17 has a helix structure at the N-terminus followed by a long coil and a short strand at the C-terminus, Zt22

has a short helix structure at N-terminus followed by two short anti parallel strands, one long and one shorter helix, and the coil structure at the C-terminus.

Figure 3.3 Prediction of 3D structures by I-TASSER, viewed in viewed in iCn3D. C-scores of predicted models for Zt3, Zt17 and Zt22 are -2.88, -3.32 and -2.52 respectively. Only models with highest C-scores are shown in the figure.

3.2 Generation of cassettes

For generation of cassettes, fragments F1 and F2 of the target gene were generated by PCR, using primers F1F and F1R for F1 and primers F2F and F2R for F2, using IPO323 genomic DNA as template. After checking for correct length in the agarose gel the fragments were purified using PCR purification kit and quantified by NanoDrop. First step was to perform double digestion of F1 fragment and pCHYG plasmid by the same enzymes. After sequent clean-up double digested DNAs, F1 fragment was ligated to pCHYG to generate pC_F1-HYG stable plasmid. The plasmid was transformed to *E. coli* for long term storage and amplification. pC_F1-HYG plasmid was isolated from *E. coli* using plasmid isolation kit, quantified by NanoDrop and double digested with proper enzymes likewise cleaned-up F2 fragment. After sequent clean-up, F2 fragment was ligated to pC_F1-HYG to generate pC_F1-HYG-F2 stable plasmid. The plasmid was transformed to *E. coli* for long term storage and amplification. To check for the correct ligation each fragment was tested with two pairs of primers (Figure 3.2). Expected length were 1060 ,1191,

1261 and 522 bp for Zt3F1, Zt3CF, Zt3F2 and Zt3CR respectively, 1032, 682, 716 and 621bp for Zt17F1, Zt17CF, Zt17F2 and Zt17CR respectively and 1260, 897, 934 and 518 bp for Zt22F1, Zt22CF, Zt22F2 and Zt22CR respectively. After verification of the generated PCR fragment lengths, pC_F1-HYG-F2 plasmid was isolated from corresponding *E. coli* culture and quantified by NanoDrop. The plasmid then was transformed to competent *Agrobacterium*. *Agrobacterium* colonies containing correct plasmid were selected, stored at -80^{0}C, and used for *Z. tritici* transformation.

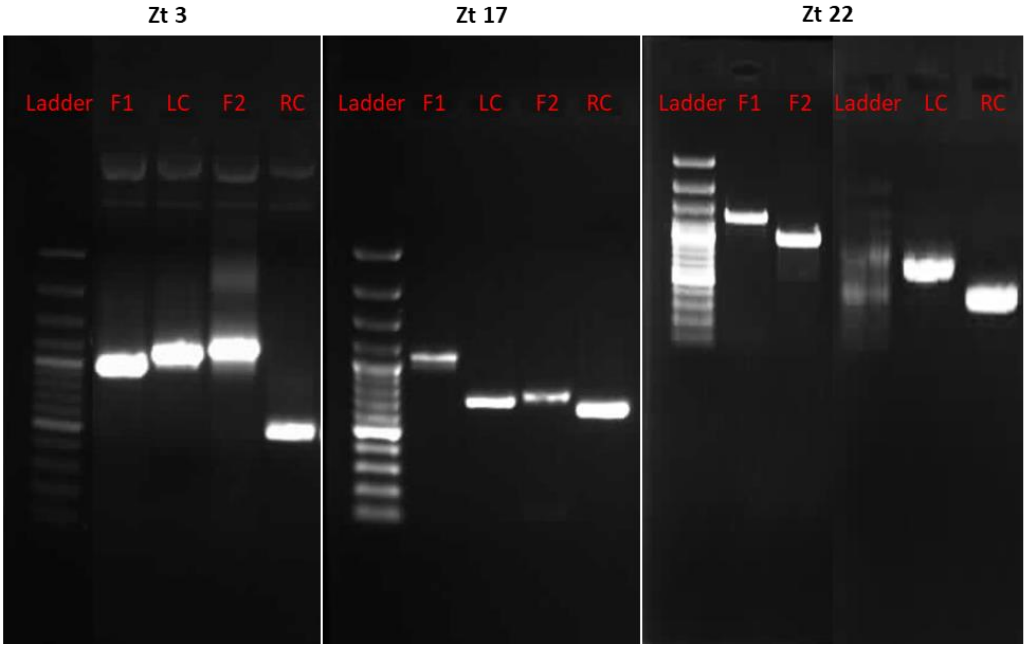


Figure 3.4 Colony PCR confirmation of generation of F1-HYG-F2 cassettes in the pCHYG plasmid.

3.3 Generation of knock outs

To generate *Z. tritici* gene knock-out strains, *Agrobacterium* colonies containing the correct cassette in the pCHYG plasmid were incubated along with *Z. tritici* IPO323 strain. Acetosyringone and induction media were used for induction of *Agrobacterium* and activate its transfer of T-DNA mechanism. After two days of incubation, hygromycin selective media was used to kill non-transformant *Z. tritici*,

and timentin was used to kill remaining *Agrobacterium* colonies. After 2 – 3 weeks of incubation on *Aspergillus nidulans* minimal medium, colonies of transformant *Z. tritici* began to grow in mycelial form, radiating as white spots (Figure 2.6). These colonies were inoculated in fresh YPD solid media with the selective marker (hygromycin). Their DNA was isolated using Ron's kit. Isolated DNA was used as template for PCR and KOC-F and KOC-R primers were used to verify the correct gene knock out practice. Control primers were used also using IPO323 DNA as template as positive control (Figure 3.3). Expected length was 555, 324 and 298 bp for Zt3KOC, Zt17KOC and Zt22KOC respectively.

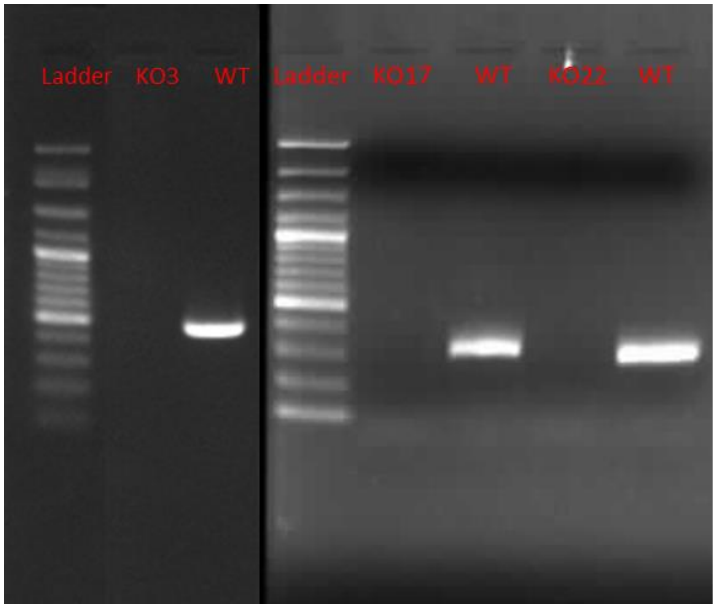


Figure 3.5 Confirmation of gene knock outs by PCR (WT – wild type IPO323).

3.4 Fungal assays

To analyze *Z. tritici* appearance, spore-forming to mycelial growth transition and growth rate the spore concentration was adjusted to 10^7 spores / mL and 1 µL was spotted on YPD growth medium (Figure 3.6). On morphology analysis, it was observed that at 25^0C the growth area of the fungi was greater than in 17^0C which is

an expected result as higher temperatures stimulate hyphal growth radiating faster from the center. While there were not major differences in growth morphology between IPO323 and ΔZt17 and ΔZt22 mutants, the ΔZt3 mutant colony growth was much slower and it was unable to generate mycelial growth at 17^0C and 25^0C throughout first 12 days of incubation. There was slight melanization of the ΔZt3 colony in YPD with greater melanization observed at 25^0C. The melanization process occurs when fungal cells are in stress conditions (Butler & Day, 1998). Even though, the conditions for growing were optimal, melanization, slower growth rate and lack of micellization reflect that cells were in stressful condition due to internal metabolic changes after Zt3 gene knock out.

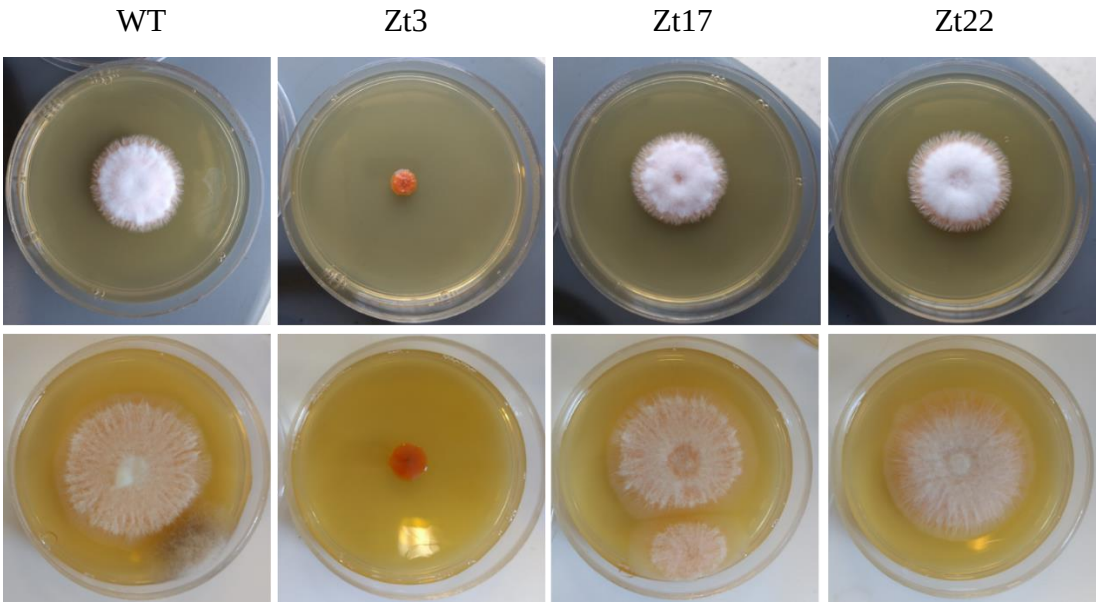


Figure 3.6 Colony morphology of *Zymoseptoria tritici* on PDA medium. IPO323 (WT), and three different mutant strains, ΔZt3 (*ΔMchygr104444)*, ΔZt17 (*ΔMchygr104383*) and ΔZt22 (*ΔMchygr105826*) at 17^0C (upper row), and 25^0C (lower row) at 12 dpi. Inoculation of 10^4 spores (1 µL) were spotted on the middle of the plates.

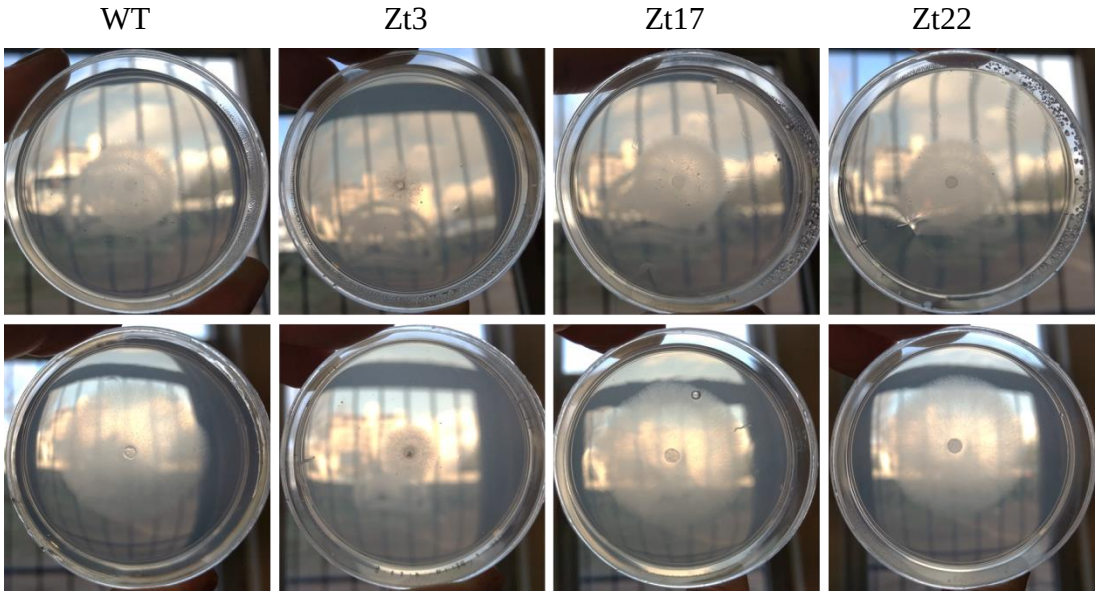


Figure 3.7 Colony morphology of *Zymoseptoria tritici* on WA medium. IPO323 (WT), and three different mutant strains, ΔZt3 (*ΔMchygr104444)*, ΔZt17 (*ΔMchygr104383*) and ΔZt22 (*ΔMchygr105826*) at 17^0C, RT (upper row), and 25^0C (lower row) at 12 dpi. Inoculation of 10^4 spores (1 µL) were spotted on the middle of the plate.

For mycelial growth stimulation water agar was used (Figure 3.7), as it mimics the conditions on wheat leaves where spores germinate stretch their hyphae to enter through stomata. In the WA media only mycelial growth was observed, while spore formation was absent. At 25^0C the growth area of the fungi was greater than at 17^0C which is an expected result, as higher temperatures stimulate hyphal outstretching. There was not a significant difference in growth morphology and rate between IPO323 and ΔZt17 and ΔZt22 mutants, while the ΔZt3 mutant colony growth was severely retarded. It seems that ΔZt3 has not lost completely the mycelial growth capability, but the size of radiation was much lower.

There are studies having been conducted to pinpoint the genes responsible for transition between yeast-like and hyphal growth transition and two genes were identified, absence of which interrupt yeast-like growth. The genes were identified by reverse genetics approach. The identified genes have predicted functions of

oxidative and osmotic stress response and purine biosynthesis (Yemelin *et al*., 2017). On the other hand, Zt3 has no predicted function and is a novel gene affecting the fungus switch between growth forms.

To measure growth rate of fungus under different conditions pictures were taken at specific time intervals. Growth was measured in comparison to plates total area using ImageJ software (Figure 3.7). It can be clearly seen the retardation of growth rate of ΔZt3 from the beginning under all circumstances. The growth rate was higher at higher temperatures. The growth rate at RT and 25^0C on WA was similar while on YPD growth rate at 25^0C was much greater.

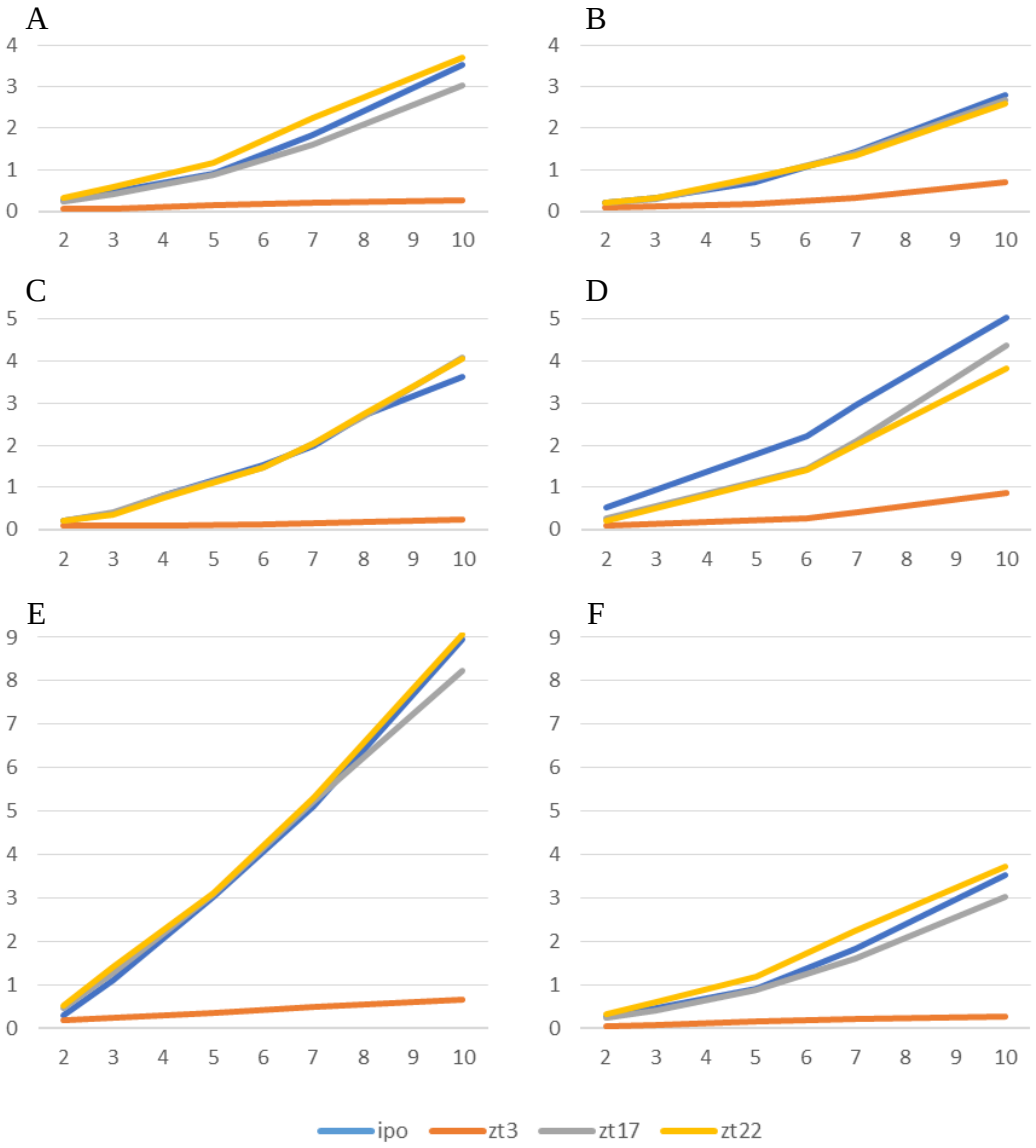


Figure 3.8 Growth curve of IPO323 (WT) ΔZt3, ΔZt17, Δ Zt22 and on A) YPD and B) WA at 17^0C; C) YPD and D) WA at RT; E) YPD and F) WA at 25^0C; throughout 0-10 dpi. X-axis represents days post inoculation and y-axis represents area in cm^2 covered by the fungus. Total area of the plate is 23.75 cm^2.

3.4.1 Growth on different mediums

To investigate melanization, spore formation and mycelial growth differences between IPO323 and ΔZt3 mutant they were grown on PDA, YMA and YPD mediums at 17^0C and 25^0C (Figure 3.7). IPO323 color was pinkish in every medium with white mycelial growth. On the other hand, ΔZt3 mutant were generally darker with blackish appearance on PDA medium. Higher temperature caused increased melanization rate. At 17^0C no mycelial growth was observed in ΔZt3 mutants at any of the tested mediums. The melanization of Zt3 also decreased at 17^0C when hygromycin was present, and at 25^0C temperature combining with hygromycin presence effected negatively the growth of ΔZt3. Small clumps radiating mycelial growth with small propagation regions were developed. The spore formation was observed at all instances and no changes were observed in different mediums and at the different temperatures in terms of spore type generation. In Figure 3.9 spore formations of the wild type and mutants are shown under 40X microscope magnification.

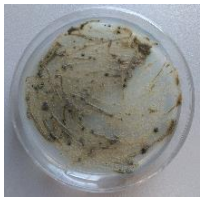
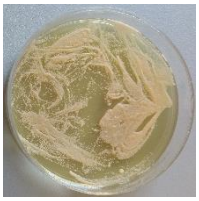
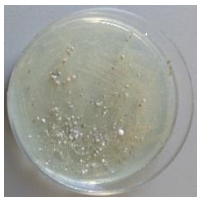
		PDA	YMA	YPD
17^{0}C	**IPO323**			
	ΔZt3			
	+ hyg			
25^{0}C	**IPO323**			
	ΔZt3			
	+ hyg			

Figure 3.9 IPO323 and ΔZt3 at 17^{0}C and 25^{0}C on PDA and YMA at 10 dpi.

3.5 Fungal growth under microscope

To observe spore formation IPO323, ΔZt3, ΔZt17 and ΔZt22 were grown on YPD at 17^0C for three days. The fungal mass was scarped with sterile loop and dipped in sterile water and mixed. 10 µL were spread over microscope slide and covered with cover slip. Observation were done with light microscope at 40X magnification (Figure 3.9). IPO323, ΔZt17 and ΔZt22 generated single celled micropycnidiospores, while ΔZt3 generated solely 4 - 6 celled, elongated macropycnidospores. There was no micropycnidiospore formation in ΔZt3 colonies. The fungus forms macropycnidiospores normally only in pycnidia on the wheat leaves, while micropycnidiospores are observed only on artificial media. According to carried literature reviews the macropycnidiospore formation of *Z. tritici* on artificial media have not been reported to date. It can be concluded that Zt3 must have an important role in mechanism of type of spore formation causing substantial changes.

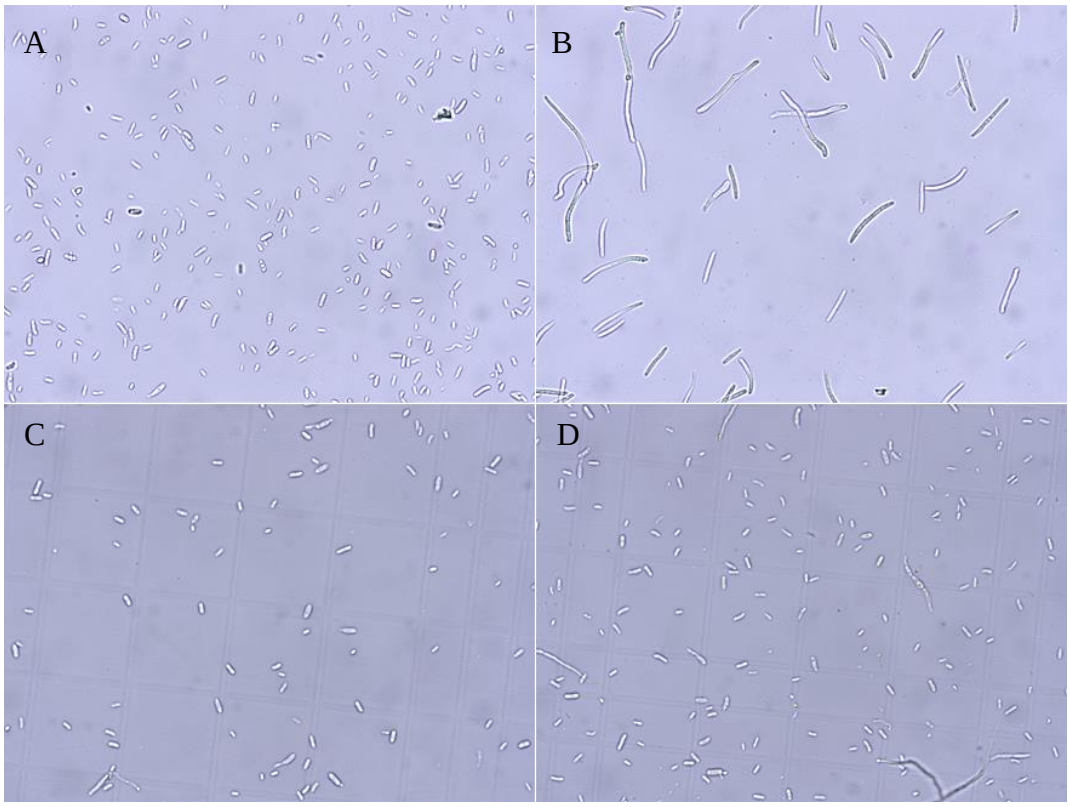


Figure 3.10 Types of spore formation of A) IPO323, B) ΔZt3, C) ΔZt17, D) ΔZt22 under microscope at 40X magnification grown on YPD at 17^0C for three days.

3.6 Wheat disease assays

To examine the pathogenicity of the generated mutants compared to IPO323 (WT) wheat assays were performed. Two pots containing 10 - 15 fourteen days old wheat seedlings were inoculated with wild type, mutant cultivars and the mock for comparison. In Table 3.2 disease severity based on necrotrophic area were designated visually or with help of ImageJ software on 10 infected leaves. Mean, standard deviation and disease scale were measured by statistical means (Table 3.2). IPO323, ΔZt17 and ΔZt22 had similar disease severity, meaning that the gene knock-out has not affect the ability of disease establishment. ΔZt3 had slightly reduced disease severity within the same disease scale as other strains meaning it may have slowed down the disease formation, but it can germinate, colonize the

mesophyll and perform the biotrophic to necrotrophic stage transition. Also, the observed higher S.D. of the disease in ΔZt3 inoculated wheat leaves makes it difficult to reach a definite conclusion about the scale of decline of the disease severity.

Table 3.2 Disease severity on the bread wheat cultivar Pehlivan appointed by Rosielle's Septoria tritici blotch scale and calculated by Townsend-Heuberger formula, disease scale and standard deviation

Culture	Disease severity (%)	Disease scale	S. D.
Control	12	1	4.19
WT (IPO323)	87	5	7.24
ΔZt3 (*ΔMycgr3G104444*)	77	5	4.58
ΔZt17 (*ΔMycgr3G104383*)	85	5	4.00
ΔZt22 (*ΔMycgr3G105826*)	87	5	11.73

Unfortunately, only a few pycnidia were seen on necrotrophic parts of the leaves, despite the knowledge for IPO323 to cause high number of pycnidia on Pehlivan cultivar (Turgay *et al*., 2017). The reason may be due to assay being conducted inside growth chamber rather than in greenhouse for the sake of controlled environment. Natural versus artificial illumination, and its intensity may have had an impact in preventing pycnidia formation. Also, since the conidia formation needs high humidity conditions and our growth chamber had the humidity controller out of order, humidity was kept high manually. The fluctuations in humidity may had resulted in interruption of pycnidia formation. Few pycnidia formation can be seen on leaves inoculated with ΔZt22 and ΔZt17 (Figure 3.10) but they were not enough

to make statistical comparison and since the count is quite below the expected number, making assumptions could be misleading.

The 12% necrotic area on control leaves can be indication of effect of sticking the leaves on sticking tape and the tissue damage during sticking procedure. This can have some effects on disease severity of actual infected leaves and the absolute disease severity may be lower than the observed values. For absolute values field experiments can be more reliable.

Figure 3.11 Appearance of wheat leaves at 21 dpi with different *Z. tritici* cultivars.

CHAPTER 4

CONCLUSION

Zymoseptoria tritici is an ascomycete that causes septoria tritici blotch disease, one of the most devastating foliar disease of bread and durum wheat in temperate climates, causing major economic and staple food loss globally (Fones & Gurr, 2015).

It is known that plant pathogens excrete small proteins to manipulate host defense system by repressing or activating plant cell death pathways. Discovering these effector proteins is a major challenge for researchers as they generally possess no homology and known motifs with each-other and other proteins (Jonge & Thomma, 2009).

In combat against this disease emphasis must be placed on methods of identifying and incorporating durable host-plant resistance that does not encourage resistance-breaking variability in the pathogen populations. As effectors are generally counterparts and targets of resistance genes, understanding their biology has a great potential to generate stable plant protection strategies by stacking multiple qualitative and quantitative resistance genes within the cultivar genome, removing susceptibility genes, and deployment of effector mechanism targeting pesticides (Molinari, 2011).

In the framework of the project, three SSP genes were successfully deleted from the genome of the fungus by using the homologous recombination gene knock-out technique. Some morphological and pathogenic properties of the deleted genes were investigated.

Deletion of Zt3 gene which is one the SSP genes caused substantial changes in yeast-like growth forms, generating solely longer multicellular spores called

macropycnidiospores. These types of spores were observed only on wheat leaves so far, but not in artificial media. Deletion of Zt17 and Zt22 had not caused notable changes in spore formation compared to wild type IPO323.

The Zt3 gene knock out also delayed the transition into mycelial growth form and slowed down the rate of growth and expansion of hyphae in solid media. Melanization of colony was also observed in different mediums, with almost black appearance on PDA medium. Growth rate, transition to mycelial form and extend of growth of Zt17 and Zt22 knocked out *Z. tritici* was similar to wild type IPO323

On the wheat assay Zt3 knock out caused slightly lesser disease symptoms, but it was within the same disease scale of 5, meaning the gene may have a slight influence on the disease mechanism, but the mutant was still able to infect the leaves. The absence of pycnidia formation hindered the analysis of spore formation and effect of knock out genes on count of asexual body production. Also, regeneration and restarting of infectious cycle could not be accomplished.

As future the goal to this project I would recommend confirmation the Zt3 protein localization by adding GFP to its C-terminus, and inserting it to the ΔZt3 strain, investigating whether it is truly secreted outside the cell as predicted by SignalP 4.0. It would tell if it causes metabolic changes inside the cell, or it provides an external signal for the fungus to switch between two sporulation forms. Elucidating down-stream signaling pathway of the gene would help to better understand the fungus' biology and molecular switches.

Restoring the micropycnidospore formation in artificial media and the growth rate and mycelial growth transition period by insertion of native Zt3 gene to ΔZt3 strain would prove the function of the gene, eliminating the possibility that the observed changes are due to off-target gene disruption.

Pycnidia formation ability and the number of the pycnidia formation of the mutant strains compared to wild type IPO323 can be assessed using stable humid condition.

Obtaining or generating IPO323 ΔKu70 or ΔKu80 can be used instead of WT IPO323 to increase the chances of positive results and to reduce off-target fragment integrations. Also using multiple markers would allow more than one gene to be knocked out in the same strain.

Incorporating Cas9 gene in the *Z. tritici* genome, preferably in one of dispensable chromosomes of IPO323, would give the opportunity for easier and multiple gene targeting.

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APPENDICES

A. pCHYG-JK modified start sequence

>pCHYG-JK; 8244 bp

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accatggtagatctgactagtgttaacgctagccaccaccaccaccaccacgtgtg
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gaattacgttaagcatgtaataattaacatgtaatgcatgacgttatttatgagat
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tcgggaattaaactatcagtgtttgacaggatatattggcgggtaaacctaagaga
aaagagcgttta

B. Genomic sequence of Mycgr3G104444 including 1000 bp flanking 5’ and 3’ ends

> Mycgr3G104444 5’ and 3’ flanking 1000 bp; 2570 bp

cggcatctcacggcacacggacgaccactactcccctgcgttcaatcgctgcgggg
tacggtcacagccggtgaatgcatcggagtgctagcgtctttaggccacgaacctc
agacggcatctcacggcacacggacgaccactactcccctgcgttcaatcgctgcg
gggtacggtcacagccggtgaatgcatcggagtgctagcgtctttagcccttcagg
ggtaacacgggcctccattagttcaactcccttgacttcaggtcctccggtatatc
aatcggacggcaccccgatgcgtggcgttattcgccccacgttcgacaggcatcgg
gatcagtgccccttcatggttatcccagagtttgcacgaggcgctgtgaccgatac
ggaggttgtcgccccgtcctgacagtttgcacccatgtcgcggcatcggatcttgc
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gtcgatcgtggtgcactggtcggcaattgaaaacataggcggaacacgaggtgttg
tggaacgacagggagaccaaatgccgaacagtacattgggcaacgaaagacctcgg
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caatattgtggactgcgagctcgataatgtgttgcgccaatgaatttccttgcggc
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aagcaatctttatatccgagagaaaggtagtaaggtatttccggagttccttatct
tctactatttctagtagagattatgcttatcgtagcggtagtagcctaaa

C. Genomic sequence of Mycgr3G104383 including 1000 bp flanking 5’ and 3’ ends

> Mycgr3G104383 5’ and 3’ flanking 1000 bp; 2491 bp

ggggctatgcactacttcttgcttttggtcaaggtgtcgtaccacttagcgatagt
cgtcttgccatggggtaagcgcaggcttattcgacgttgatgtggaacatccaggg
gcaaattcctacatagtatgcagcattttcgaatataggacggaggagctggcagc
agtagttctacaataagcagaacaagaggtgagcaaatacaagacaacagaaatgc
gaagagaagtaagggcaaagtggtgtggcacacgctggatacacttccttacaccc
tcaaggatgtaaatttgagagctcgcagcgacgaaattcgctctccaagggtctct
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D. Genomic sequence of Mycgr3G105826 including 1000 bp flanking 5’ and 3’ ends

> Mycgr3G105826 5’ and 3’ flanking 1000 bp; 2851 bp

agtgtgagaggaacgcgccccagcctgcggtagtgccgtcgctgcagaggtgcagg
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gagcgcggagaggttacctacgaaccgtcgagcaaatgtatctacctcctcgatag
cgaacaagcttcggtcgcatcgaatgcctagcgaaacaccttcgtcgtcgc

E. Twenty-two candidate effectors and their expression profile

Gene order	#id-JGI	Chr	Pre lenth	Signal length	Mature length	Cysteine count	% Cys	Mg specific (<e-5)	working	KO	PDB / CDB	Day1 / CDB	Day4 CDB	Day9 / CDB	Day14 / CDB	Day21 / CDB	genome coord
Zt1	87205	8	55	22	33	8	24.24	Y	Y	N	1	-1.79769e+308	1	2.35941	5.17278	2.52893	8:1342011-1342250
Zt2	83081	13	53	20	33	6	18.18	Y	Y	N	1	1	1	4.03983	2.16656	1	13:1086308-1086905
Zt3	104444	5	79	21	58	10	17.24	Y	Y	Y	1	5.26673	8.77967	11.4141	8.03881	2.50491	5:989971-990541
Zt4	82925	12	57	19	38	6	15.79	Y	N								
Zt5	81208	6	59	18	41	6	14.63	Y	N		3.51305	1	1	1	1	1	6:1302604-1304493
Zt6	82029	9	66	18	48	7	14.58	Y	N								
Zt7	101652	11	77	21	56	8	14.29	Y	N								
Zt8	79286	2	63	20	43	6	13.95	Y	Y	N	1	3.3887	2.75026	4.87453	3.07397	1	2:1007274-1007899
Zt9	100649	7	75	17	58	8	13.79	Y	Y	N	1	4.71524	6.46048	9.28485	6.70039	3.89708	7:263826-264330
Zt10	41491	5	81	18	63	8	12.7	N	N		1	-4.56322	1	-6.18549	1	1	5:454296-454611
Zt11	79161	1	68	19	49	6	12.24	Y	N		1	1	1	1	1	-2.8709	1:6005953-6006585
Zt12	99161	2	164	15	149	18	12.08	N	N		1	-2.66246	1	-3.8161	-3.6679	-3.50482	2:2101446-2102282
Zt13	106125	11	70	17	53	6	11.32	Y	N		1	-2.99166	1	1	1	-2.30588	11:812963-814004
Zt14	106502	13	89	18	71	8	11.27	Y	N		-4.1291	-3.60866	1	-3.12271	1	1	13:656106-656761
Zt15	108482	3	108	19	89	10	11.24	N	N		1	5.95777	4.34629	11.9692	10.2306	3.56385	3:780397-781083
Zt16	80332	4	76	21	55	6	10.91	Y	N		-3.6054	-2.16773	-3.37715	1	1	-4.13202	4:637335-638008
Zt17	104383	5	74	19	55	6	10.91	Y	Y	Y	1	1	1	3.58325	1	-2.65782	5:513930-514421
Zt18	83064	13	75	18	57	6	10.53	Y	N		2.83357	1	-2.04081	1	1	-2.95917	13:920891-921712
Zt19	104758	6	118	22	96	10	10.42	Y	Y	N	1	1	1	3.13046	1	1	6:660758-661490
Zt20	97031	12	118	22	96	10	10.42	Y	N		1	1	1	1	1	-7.19	12:686096-686453
Zt21	106445	13	119	22	97	10	10.31	Y	N		1	1	1	1	-4.15031	-3.52632	13:136801-137670
Zt22	105826	10	98	20	78	8	10.26	Y	Y	Y	3.25904	4.61061	3.29174	4.44508	3.00763	1	10:107364-108215

CURRICULUM VITAE

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EDUCATION

Degree	Institution	Year of Graduation
MS	METU Biochemistry	2015
BS	METU Molecular Biology and Genetics	2012
High School	Gjon Buzuku High School, Prizren	2005

WORK EXPERIENCE

Year	Place	Enrollment
2014-Present	Field Crops Central Research Institute Biotechnology Research Center, Ankara	-Project assistant

FOREIGN LANGUAGES

Advanced English, Advanced Albanian, Advanced Turkish, Intermediate German

PUBLICATIONS

1. 1. Optimization of gene deletion technique on *Zymoseptoria tritici*, for gene function studies, in our country.
Abstract in "Turkey 6th Plant Protection Congress with International Participation", 5 – 8 September 2016.

2. Identification of mutations in *Zymoseptoria tritici* population causing resistance to azole in Turkey. Abstract in "Turkey 6th Plant Protection Congress with International Participation", 5 – 8 September 2016.