Host specialization in the fungal plant pathogen *Zymoseptoria tritici*

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Declaration

I hereby declare that the dissertation entitled "Host specialization in the fungal plant pathogen *Zymoseptoria tritici*" submitted to the Department of Biology, Philipps-University Marburg, is the original and independent work carried out by me under the guidance of the PhD supervisors, and the dissertation is not formed previously on the basis of any award of Degree, Diploma or other similar titles.

Marburg, den 10.12.2014

Stephan Poppe

Krise ist ein produktiver Zustand. Man muss ihr nur den Beigeschmack der Katastrophe nehmen.
(Max Frisch)

Summary

The three closely related plant pathogenic species *Zymoseptoria tritici* (synonym: *Mycosphaerella graminicola*), *Z. pseudotritici* and *Z. ardabiliae* are hemi-biotrophic, ascomycete fungi with different host ranges. *Z. tritici* emerged at the onset of agriculture and is specialized to its host *Triticum aestivum* (bread wheat). In contrast *Z. pseudotritici* and *Z. ardabiliae* infect different wild grass species at the center of origin and diversification of the *Zymoseptoria* grass pathogens and are unable to infect wheat.

Accelerated evolution of single genes is a key feature of pathogen adaptation following a host shift. Genes under positive selection exhibit an elevated ratio of non-synonymous (replacement) mutations to synonymous (silent) mutations. A ratio above 1 is indicative of positive selection and these positively selected genes can be identified by comparative genome analyses of closely related species that infect different hosts. From a comparative genome study of *Zymoseptoria* species the four positively selected genes *Zt80707*, *Zt89160*, *Zt103264*, and *Zt110804* were chosen without *a priori* information about gene function or structure.

In this study it was shown that the four selected genes are up-regulated *in planta.* The genes are not involved in axenic growth of *Z. tritici* as shown by an *in vitro* assay, supporting a pathogenicity related role of the four gene products. The virulence of *Z. tritici* on wheat was significantly reduced for the two deletion mutants (Δ*Zt80707* and Δ*Zt103264*) and both showed an impaired development of the asexual fruiting bodies (pycnidia) on wheat.

The deletion of *Zt89160* caused a hypervirulent reaction in wheat while the fourth mutant (Δ*Zt110804*) showed no detectable change in virulence-phenotype. The protein Zt80707 was moreover shown to be exclusively secreted in *Z. tritici* indicating that positive selection in this protein most likely relates to a novel extracellular function. Adaptation to the host plant wheat was shown for the proteins Zt80707 and Zt89160 as both deletion phenotypes could not or only partially be restored when the genes in *Z. tritici* were replaced with their orthologs of *Z. pseudotritici*.

The presented results demonstrate that evolutionary predictions provide a strong tool for the identification of genes involved in host adaptation and pathogen development. In addition it was shown that adaptive evolution during host specialization also strongly affects non-secreted proteins without effector function (Zt89160, Zt103264, and Zt110804).

Zusammenfassung

Die drei nah verwandten Ascomyceten *Zymoseptoria tritici* (Synonym: *Mycosphaerella graminicola*), *Z. pseudotritici* und *Z. ardabiliae* sind hemibiotrophe Pflanzenpathogene mit verschieden Wirtsspektren. *Z. tritici* tritt seit den Anfängen der Landwirtschaft auf und ist auf die Wirtspflanze Weizen (*Triticum aestivum*) spezialisiert. Hingegen können *Z. pseudotritici* und *Z. ardabiliae* Weizen nicht infizieren und befallen verschiedene Wildgräser.

Beschleunigte Evolution von einzelnen Genen ist ein Hauptmerkmal der Anpassung von pathogenen nach einem Wirtswechsel. Positiv selektionierte Gene zeigen eine erhöhte Rate von nicht-synonymen (sinnverändernden) Mutationen zu synonymen (stillen) Mutationen. Eine Rate über 1 deutet auf positive Selektion hin und diese positiv selektionierten Gene können durch Genomvergleiche von nah verwandten Arten mit verschiedenen Wirtsspektren identifiziert werden. Durch einen Genomvergleich der beschriebenen *Zymoseptoria* Arten wurden die vier positiv selektionierten Gene *Zt80707*, *Zt89160*, *Zt103264*, und *Zt110804* ausgesucht.

In dieser Arbeit wurde gezeigt das die vier identifizierten Gene *in planta* hochreguliert sind und durch ein *in vitro* Stress-Assay konnte belegt werden, dass diese Gene keinen Einfluss auf das axenische Wachstum von *Z. tritici* haben. Beide Ergebnisse unterstützen eine mögliche Rolle der Gene in der Pathogenität von *Z. tritici*. Die Deletion von *Zt80707* und *Zt103264* führte zu einer signifikanten Reduktion der Virulenz von *Z. tritici* auf Weizen und beide Deletionsmutanten zeigten eine beeinträchtigte Entwicklung der asexuellen Fruchtkörper (Pyknidien). Die Deletion von Zt89160 führte zu einer Hypervirulenz auf Weizen während der vierte Deletionsstamm (Δ*Zt110804*) keine Veränderung der Virulenz zeigte. Ferner wurde gezeigt, dass das Protein Zt80707 ausschließlich in *Z. tritici* sekretiert ist, was darauf hindeutet, dass die positive Selektion dieses Proteins mit der neuen extrazellulären Funktion in Verbindung steht. Eine Anpassung an die Wirtspflanze Weizen konnte für die Proteine Zt80707 und Zt89160 demonstriert werden, da beide Deletions-Phänotypen nicht oder nur teilweise wiederhergestellt werden konnten, wenn die korrespondierenden Gene durch deren Orthologe von *Z. pseudotritici* ersetzt werden.

Die präsentierten Ergebnisse verdeutlichen, dass evolutionäre Voraussagen ein hervorragendes Mittel sind um Gene zu identifizieren, die in der Anpassung an den Wirt und die Entwicklung von Pathogenen involviert sind. Zusätzlich wurde gezeigt, dass adaptive Evolution durch die Anpassung an einen Wirt auch nicht sekretierte Proteine beeinflusst.

Glossary

Δ	deletion
AA	amino acid(s)
ad	fill up to end volume
Amp	Ampicillin
ATMT	*Agrobacterium tumefaciens* mediated transformation
bp	basepair(s)
BSA	bovine serum albumin
cDNA	complementary DNA
C-terminal	carboxy terminal
DIC	differential interference contrast
DMSO	dimethyl sulfoxide
DNA	Deoxyribonucleic acid
dpi	days post infection
EDTA	Ethylendiamintetraacetic acid
f.c.	final concentration
fwd	forward
G418	Geneticin
GAPDH	glyceraldehyde 3-phosphate dehydrogenase
gDNA	genomic DNA
GFP	green fluorescent protein
h	hour(s)
$H_20_{bid.}$	twice distillated water
Hyg	Hygromycin B
in planta	inside plant tissue
kb	kilo base pairs
kDa	kilo Dalton
Km	Kanamycin
LysM	lysine motif
min	minute(s)
M	molar (g/L)
ncr	non coding region
N-terminal	amino terminal
OD_{600}	optical density at 600 nm
ORF	open reading frame
PAGE	polyacrylamide gel electrophoresis
PAMP	pathogen-associated molecular pattern
PCR	polymerase chain reaction
PI	propidium iodide
qRT-PCR	quantitative real-time PCR
rev	reverse
RNA	ribonucleic acid
rpm	rounds per minute
RT	room temperature
s	second(s)
SDS	sodium dodecyl sulfate
STB	septoria tritici blotch
Tris	tris (hydroxymethyl) aminomethane
U	unit (enzyme activity)
WGA	wheat germ agglutinin
WT	wild type
YMS	yeast maltose sucrose

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1 Introduction

1.1 The wheat pathogen *Zymoseptoria tritici*

Zymoseptoria tritici (synonym *Mycosphaerella graminicola*) is a plant pathogenic hemi-biotrophic ascomycete fungus that belongs to the genus Mycosphaerella. With more than 10.000 species, this is the largest genus of plant pathogenic fungi including species like *Mycosphaerella fijiensis*, the causal agent of Black sigatoka on banana and many other severe pathogens of agricultural crops. *Z. tritici* is globally distributed and is the causal agent of septoria tritici blotch (STB) which is one of the most important foliar diseases of wheat worldwide (Goodwin et al. 2011) and is the most important foliar disease of winter wheat in western European countries (Mullins et al. 2011). The yield loss caused by *Z. tritici* can be up to 20 - 50% and globally more than 100 Mio Dollar are spent on fungicides to fight STB (Ponomarenko et al. 2011). Under natural conditions *Z. tritici* has a sexual cycle resulting in high genotypic diversity in field populations. Furthermore, airborne ascospores can be dispersed over 100 of kilometers. Because of this high genetic variability and long distance dispersal potential, this pathogen is difficult to control (Wittenberg et al. 2009; Zhan et al. 2003).

Z. tritici describes the sexual form (teleomorph) of the pathogen that forms perithecia or pseudothecia on infected plant leaves which are giving rise to airborne ascospores. In 1842 the asexual form (anamorph) of the pathogen *Septoria tritici* was first described to be the causal agent of STB (Desmazières 1842). The anamorph forms pycnidia on infected leaves that are giving rise to the asexual spores (pycnidiospores). In 1972 both forms were identified to be the same species (Sanderson 1972).

The infection cycle of *Z. tritici* starts with the initial infection of wheat by airborne ascospores, which can overwinter on previous seasons' crops (Fig. 1A). When the wheat seedlings are growing in spring, germ tubes emerging from the ascospores are infecting the seedlings by directed growth towards the stomata, the natural openings of plant leaves. As *Z. tritici* is not penetrating the host tissue by appressoria or haustoria (Kema et al. 1996), this is the only way for the fungus to attack the plant. In humid conditions the spore germination starts within 12 h after leaf contact (Ponomarenko et al. 2011).

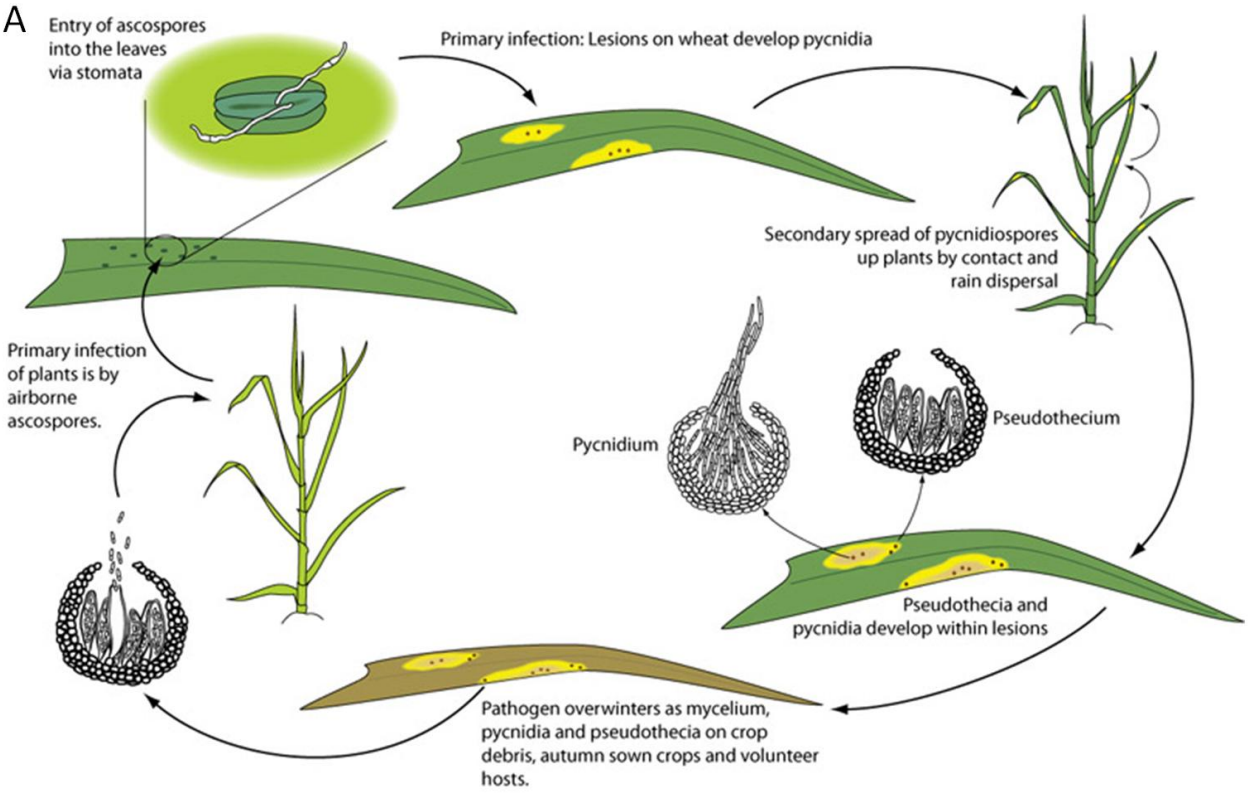

Figure 1: Scheme of the *Z. tritici* lifecycle on wheat and symptoms of STB A) Disease cycle of *Z. tritici* on wheat. The primary infection starts with infection hyphae germinating from ascospores that are entering the leaves via stomata. After up to two weeks of biotrophic growth the fungus switches to necrotrophic growth and lesions on the leaves can be detected. They are expanding into necrotic blotches. Within these asexual fruiting bodies (pycnidia) pycnidiospores are formed. They are spread by contact or rain splash and are the cause of the secondary spread within an infected field. In the end of the growing season the fungus produces more sexual fructifications (perithecia). The ascospores enable the fungus to overwinter and to survive crop-free periods. **B)** Symptoms of *Z. tritici* on bread wheat **C)** Magnification of a *Z. tritici* infected wheat leaf. The pycnidia are spaced regularly within the stomata of the plant. Figure modified after (Ponomarenko et al. 2011).

After the initial infection a latent period begins by the establishment of an intercellular hyphal network in the mesophyll tissue. This hyphal growth resembles endophytic rather than intimate biotrophic growth and involves little increase in biomass (Brunner et al. 2013; Mehrabi and Kema 2006). The latent period can be up to two weeks before visible symptoms can be detected on the infected leaf. Then *Z. tritici* switches from biotrophic to necrotrophic growth coinciding with a massive collapse of plant cells and the chlorotic lesions visible in the latent phase are turning necrotic. An induced toxin production is discussed as the responsible trigger for the switch but it has not been proven (Ponomarenko et al. 2011).

The blotches are expanding in the direction of the vascular bundles forming long necrotic lesions (Fig. 1B). Within these the fructifications (pycnidia or perithecia) are formed inside the sub stomatal cavities resulting in a regular pattern (Fig. 1C). Splash dispersed pycnidiospores are spread within a wheat field by leaf-to-leaf contact or rain dispersal. They are responsible for the secondary spread within a field and are also the primary inoculum for the infection of winter wheat that is sowed in autumn. *Z. tritici* survives host free periods or winters primarily in the form of ascospores which are the primary inoculum for the next growing season.

The genome of the reference isolate IPO323 was sequenced from telomere to telomere revealing 13 core chromosomes (CCs) and eight accessory chromosomes (ACs) what is an unusually high number compared to other fungal species (Goodwin et al. 2011; Wittenberg et al. 2009). The ACs are repeat rich and similar to dispensable, supernumerary or B-chromosomes described for several thousand eukaryotes to distinguish them from the core chromosomes that are shared between all individuals of a species (Houben et al. 2014). In several plant pathogenic fungi these ACs are present and well characterized (Covert 1998). In *Nectria haematococca* for example a family of cytochrome P-450 (Pda) genes necessary for detoxification of an antimicrobial compound produced by garden pea (*Pisum sativum L.*) is located on a small meiotically unstable chromosome dispensable for normal growth (Miao et al. 1991). In *Z. tritici* however these chromosomes are discussed to be not truly dispensable as many of these meiotically unstable chromosomes might play an adaptive role in pathogen evolution and are considered as accessory (Croll and McDonald 2012). In comparison to the core chromosomes they seem to evolve under less selective constraint (Stukenbrock et al. 2011) and they show an elevated rate of translocations, intrachromosomal recombination and nondisjunction during meiosis in *Z. tritici* (Wittenberg et al. 2009; Croll et al. 2013). They carry many coding genes which might be

necessary for survival in some environments and recent studies show that some genes located on the ACs are also up-regulated *in planta* suggesting a role in pathogenicity (Kellner et al. 2014). However their functional relevance especially during infection is still poorly understood.

1.2 The *Zymoseptoria* species complex

The history of *Z. tritici* can be dated back to the Fertile Crescent ~11.000 years ago. There it emerged as a pathogen of wheat coinciding with the domestication of this crop plant (Stukenbrock et al. 2007; Gopher et al. 2002; Zohary et al. 2000; Moore et al. 2000). The speciation of *Z. tritici* occurred with strong host specialization and adaptation to the agro-ecosystem because the host plant wheat has been under a high selection pressure to increase yield production. This selection pressure was accompanied by drastic changes in genetic diversity of the crop species.

To better understand the evolution of *Z. tritici*, closely related species have been collected from a variety of wild grasses in the Middle East and their genomes were sequenced. Thereby it was discovered that the domestication of wheat and the separation of *Z. tritici* and its closest relative *Z. pseudotritici* simultaneously happened ~11.000 years ago (Stukenbrock et al. 2007). Since then *Z. tritici* co-evolved with its host plant and adapted to the strong directional selection pressure applied by farmers with resistant wheat cultivars and fungicides aimed to control STB. The long co-evolution with wheat has resulted in a highly specialized pathogen of wheat that is difficult to control. The two closest related species *Z. pseudotritici* and *Z. ardabiliae* show similar biology in terms of infection, disease cycle and the caused symptoms on their host plants. However they are still specialized to the conditions in the natural grasslands in the Middle East and they can infect a mixture of wild grass species like the grass species from which they were isolated: couch grass (*Elymus repens*), orchard grass (*Dactylis glomerata*) and annual ryegrass (*Lolium multiflorum*) (Stukenbrock et al. 2007). In contrast to the agro-ecosystem, a pathogen adapted to natural grasslands will benefit from the ability to infect a variety of host plants. However, the wider host range of *Z. pseudotritici* and *Z. ardabiliae* putatively reduces the virulence of these pathogens as they have to maintain the ability to cope with a bigger variety of specialized plant defense mechanisms (Muthamilarasan and Prasad 2013). During the biotrophic phase for example an effector that is advantageous on one host plant can be detrimental on another plant species (Collmer et al. 2000; de Jonge et al. 2011). The evolution of those two wild

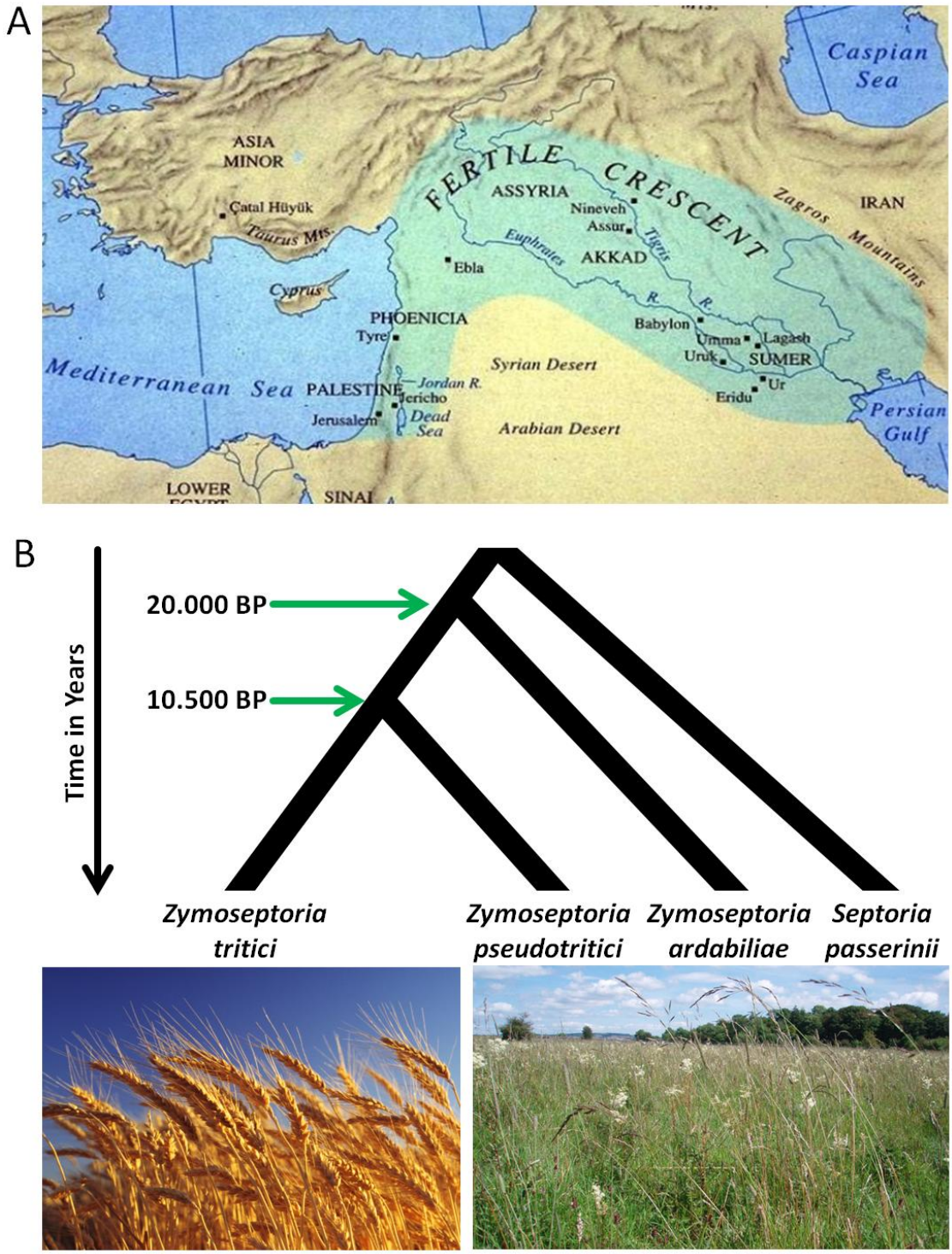

Figure 2: Scheme of the evolutionary history of the *Zymoseptoria* species complex. A) The domestication of bread wheat began ~11.000 years ago in the Fertile Crescent. **B)** The speciation of *Z. tritici* and *Z. pseudotritici* has taken place ~11.000 years ago (Stukenbrock et al. 2007). The ancestral species *Z. pseudotritici* and *Z. ardabiliae* are still adapted to the natural grasslands in die Middle East and remained generalistic plant pathogens with a wide host range. *Z. tritici* in contrast specialized to the wheat agro-ecosystem during this time and had to adapt to the high selection pressure that farmers applied on wheat to increase yield production.

grass pathogens stays in contrast to *Z. tritici*, which is highly specialized to wheat and also more virulent than its related species (Stukenbrock et al. 2011). Thereby the *Zymoseptoria* species complex provides a good model system to compare host specialization and adaptation like the evolution of effector genes and virulence related genes in specialist and generalist pathogens.

1.3 Host specificity

Host specificity describes the level of adaptation of a parasite to a specific host species. For plant pathogenic fungi the host specificity differs a lot. For example necrotrophic fungi like *Sclerotinia sclerotiorum* or *Botrytis cinerea*, the causal agent of grey rot, that can infect over 200 eudicot hosts have a wide host range (MacFarlane H.H. 1968). In contrast to that *Z. tritici* has a very narrow host range that is restricted to bread wheat *Triticum aestivum* and durum wheat (*T. durum*) but it occasionally infects other grass species like barley or some species of grasses (Stukenbrock et al. 2011; Ponomarenko et al. 2011; Ginkel and Scharen 1987). On non-host plants like couch grass (*E. repens*) or annual ryegrass (*L. multiflorum*) *Z. tritici* is able to penetrate the leaf surface via the stomata but the infection hyphae is stopped in the sub-stomatal cavity and massive autofluorescence can be observed likely due to a hypersensitive response (HR) of the plant (Master thesis, S Poppe). This suggests an early recognition by the host plant and that *Z. tritici* can suppress early defense mechanisms in susceptible wheat lines.

The host range is determined by genes that allow growth of the pathogen on a host or block it. Thus, a particular gene product can allow propagation of the fungus on and inside the host tissue while the gene product can be disadvantageous on another host plant and lead to an induction of defenses of the host. In many filamentous plant pathogens this interaction with the host is often facilitated by small secreted proteins (effectors) but also enzymes that are responsible for the synthesis of toxins (Manning et al. 2010; Doehlemann et al. 2009; de Jonge et al. 2011). Successful pathogens are able to subvert their host plants and interfere with the different layers of the host immune system which was illustrated by the zigzag-model (Jones and Dangl 2006). The attacking pathogen is initially recognized by pathogen-associated molecular patterns (PAMPs) that can be fungal chitin, which is part of the fungal cell wall, or bacterial flagellin. Successful pathogens are able to suppress this broad range PAMP-triggered immunity (PTI) by secretion of effectors. However, also these effectors can be recognized by the plant resistance

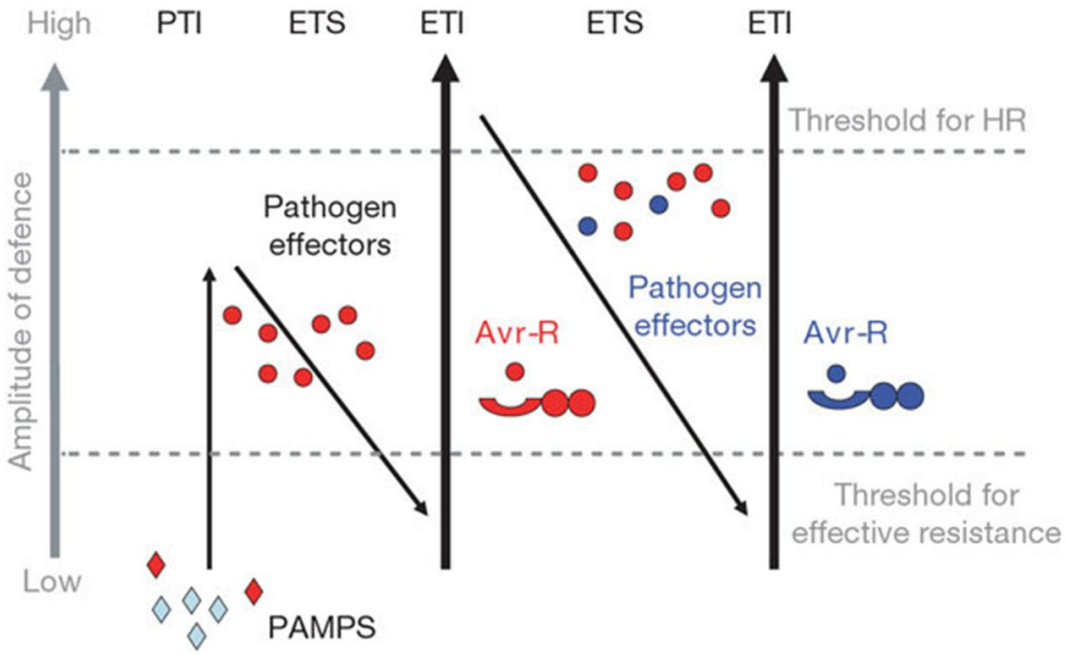

Figure 3: The zigzag model of Jones and Dangl illustrates the quantitative output of the plant immune system. In the beginning of an infection plants detect microbial/pathogen-associated molecular patterns, the so called MAMPs/PAMPs (red diamonds). They trigger the PAMP-triggered immunity (PTI). Only successful pathogens deliver effectors that can interfere with PTI leading to effector-triggered susceptibility (ETS). When one of these effectors (indicated in red) is recognized by the plant resistance effector-triggered immunity (ETI) is activated that is an amplified version of PTI that often passes the threshold for induction of hypersensitive cell death (HR). Finally those pathogen isolates that have lost this specific effector or perhaps gained new ones that can help this isolate to suppress ETI are selected. Also new plant isolates are selected that can recognize one of the newly acquired effectors, resulting again in ETI

leading to effector-triggered immunity (ETI), an amplified version of PTI that often passes a threshold for induction of hypersensitive cell death (HR).

Toxins as virulence factors are widespread among necrotrophic fungi like *Botrytis cinerea* or *Sclerotinia sclerotiorum* (Li et al. 2004; Choquer et al. 2007). They cause death of the affected tissue allowing the fungus to feed saprophytically on the dead plant tissue. As this type of pathogen attack mostly is unspecific, this is a reason for wider host spectra of necrotrophic fungi compared to biotrophic ones that are dependent on living host plant tissue. However, also host-selective toxins have been described already for the necrotrophic fungus *Pyrenophora tritici-repentis*, the causal agent of tan spot of wheat (Pandelova et al. 2012) indicating that toxins might be more specific than expected.

Biotrophic pathogens do not use toxins as virulence factors but secreted effector proteins, which are generally species-specific or even isolate-specific (de Jonge et al. 2011). They secrete these

effectors mainly at hyphal tips or via haustoria which were originally considered to be feeding structures only (Panstruga and Dodds 2009). These highly specialized haustoria penetrate the plant cell wall and establish an increased interaction surface between the pathogen and its host that is used for uptake of nutrients and release of effector proteins, consequently leading to a narrow host range of biotrophic fungi.

Specialization of fungal plant pathogens to their hosts has been shown to be a strong driver of pathogen diversification and speciation. For some species it has been shown that the ability to infect particular host genotypes depends on the presence and sequence composition of single avirulence or effector genes. In other species virulence may be quantitative and determined by multiple quantitative traits (Schulze-Lefert and Panstruga 2011). The genes encoding effectors are exposed to an increased mutational pressure that is driven by an arms race between the pathogen and its host. These genes therefore often show a higher rate of DNA mutations altering the amino acid sequence (non-synonymous mutations) in contrast to synonymous DNA mutations which do not alter the amino acid sequence. New mutations are beneficial for pathogens to overcome host defenses that adapted to the changes of the host the fastest. It results in an accelerated evolution of pathogen effectors and their targets in the host plant (van der Does and Rep 2007).

In addition to necrotrophic and biotrophic plant pathogens there is a third type of pathogen life style that combines these; the so called hemi-biotrophic fungi like *Z. tritici*. The infection process is divided into two phases. The initial infection is an often symptomless biotrophic phase in which the fungus enters and spreads in the host plant. Like biotrophic fungi the hemi-biotrophic species do not kill the host plant during the first phase of infection and are able to avoid the plant defense mechanisms. After being established in the host the fungus induces host cell death and changes to a necrotrophic life style (Ponomarenko et al. 2011). The production of spores takes place in the necrotrophic phase (Li et al. 2004; Mehrabi and Kema 2006; Choquer et al. 2007).

However recent studies with the model necrotrophic fungi *Cochliobolus heterostrophus*, *Botrytis cinerea* and *Sclerotinia sclerotiorum* suggest that also these species do not immediately kill the host plant but instead affect plant programmed cell death (PCD) pathways for their own benefit (Williams et al. 2011). This demonstrates that also necrotrophic fungi seem to interact with the host plant defenses and may be more specific than previously considered.

Fungal plant pathogens must be sophisticated manipulators of plant defenses and host cell metabolism as successful infection and reproduction requires a fine-tuned adaptation at different stages of the pathogen development and host interaction. This involves genetic traits related to virulence as well as *in planta* nutrient uptake and reproduction. The defeat of host resistance is achieved by the secretion of effector proteins, which directly or indirectly interact with specific targets in the host (Kamoun 2007; Van Der Hoorn and Kamoun 2008). The successful suppression of host defenses allows the further infection and colonization of host tissues. The *in planta* growth and reproduction of fungal pathogen involves the uptake and metabolism of plant derived carbohydrates. This is a crucial process as it has been shown that the extracellular release of monosaccharides triggers plant defense responses (Herbers et al. 1996). The uptake of nutrients differs among plant pathogens. Some biotrophic pathogens have evolved particular feeding structures like haustoria to explore a larger surface for the uptake of accessible sugars. In *Ustilago maydis* e.g. the carbohydrate uptake during the biotrophic phase is facilitated by the plasma membrane-localized saccharose transporter (Srt1) that has been shown to be necessary for full virulence (Wahl et al. 2010). This transporter can compete efficiently with plant saccharose transporters and takes up the disaccharide saccharose without producing apoplastic signals that trigger plant defenses (Djamei and Kahmann 2012). Necrotrophic and hemi-biotrophic pathogens rely on an arsenal of toxins and plant cell wall degrading enzymes to kill the host plant cells and metabolize more complex carbohydrates of the plant cell wall (Solomon et al. 2003). Aside the specific interaction between host and pathogen, fungal pathogens co-occur with a community of microbial endophytes. So far, very little is known about the influence and diversity of co-existing endophytic species, however the endophytic microbial community may include both mutualistic and antagonistic species.

One of the central questions of today's ecological genetics is how pathogens become specialized to their respective hosts and become able to interfere with host defenses, how they interact with co-existing microbial communities, how they adapt to particular host-derived nutrients and how they successfully reproduce in host tissues. In plant pathogens only few studies have focused on positively selected genes involved in this specialization process (Jonge et al. 2012; Hacquard et al. 2013; Rouxel et al. 2011). In *Phytophthora infestans* and *P. mirabilis* for example specialization of a secreted protease to protease targets unique to their respective host plants has

been shown (Dong et al. 2014). However for the majority of plant pathogens the genomic and molecular basis of adaptation is still unknown.

Host driven speciation implies mutations of genes involved in the interaction between pathogen and host from the initial infection and defeat of host defenses to the uptake of nutrients, multiplication and reproduction in distinct host tissues. Specialization to distinct host species will thus be mediated by divergent selection of key genes involved in all host depended processes. The evolutionary footprint of such divergent selection is an accumulation and excess of adaptive mutations in these genes. Closely related species, which have diverged recently, provide ideal model systems to identify and study genetic traits underlying host specialization (Stukenbrock 2013).

The *Zymoseptoria* species complex is a powerful model system to study host specialization. The genomes of the two species *Z. pseudotritici* and *Z. ardabiliae* have been sequenced with the aim of characterizing genome evolution in the *Zymoseptoria* species complex. Due to the fact that the genomes have been sequenced completely it is possible to identify differences that have evolved during the speciation of *Z. tritici* (Stukenbrock et al. 2010; Stukenbrock 2013). A comparative population genomic study using genome data from 13 isolates allowed the comparison of more than 9000 homologous genes of *Z. tritici*, *Z. pseudotritici*, *Z. ardabiliae* and an out-group species *Septoria passerinii* (Stukenbrock et al. 2011). Analyses of branch specific d_N/d_S ratio and within species pN/pS ratios revealed that divergence and evolution of the wheat pathogen *Z. tritici* have been associated with an efficient genome-wide fixation of adaptive mutations. Further evolutionary analyses of homologous genes identified a small number of genes, which in particular have been subjected to strong positive selection during divergence of the three *Zymoseptoria* species. All these genes showed an increased ratio of non synonymous to synonymous substitutions (d_N/d_S ratio) between the three *Zymoseptoria* species (Stukenbrock et al. 2011). For the majority of fast evolving genes in *Zymoseptoria* we have no information in protein function. These genes are mainly unclassified genes, in several cases with orthologs in other plant pathogenic species. Four of those genes (*Zt80707*, *Zt89160*, *Zt103264* and *Zt110804*) were chosen for functional characterization solely because of their increased d_N/d_S ratio without further *a priori* information about gene function or structure. A functional characterization of these candidate genes is necessary to link predicted importance with functional relevance.

Molecular studies of *Z. tritici* have been sparse so far as this plant pathogenic fungus is not a classical model organism many molecular tools still have to be established. Commonly used techniques like mutations by targeted recombination have just been established for *Z. tritici* (Bowler et al. 2010). For *Z. pseudotritici* and *Z. ardabiliae* this has never been tested.
In addition the *Z. tritici* wheat pathosystem also lacks a large-scale plant assay to assess even small virulence differences of *Z. tritici* on wheat. Possible *Z. tritici* caused symptoms for quantification of virulence are the amount of necrosis and fruiting bodies as well as the time until symptoms can be detected and the biomass development of the pathogen. Other studies have applied an *in vitro* detached leaf assay to evaluate difference between *Z. tritici* strains (Arraiano et al. 2001). However natural senescence of detached leaves challenges the recognition and quantification of fungal caused necrosis. Hence the infection of living plants is the most appropriate infection method to quantify disease of deletion strains as this method has been used already to estimate virulence of effector gene deletion strains and apathogenic mutants (Marshall et al. 2011; Lee et al. 2013).

1.4 Aims of this thesis

Accelerated evolution of single secreted genes has been remarkably shown to be a key feature of pathogen adaptation following a host jump (Dong et al. 2014). However, this study was solely focused on secreted proteins and this pathogen adaptation has never been demonstrated for fungal plant pathogens. Here I want to test our hypothesis that adaptive non-synonymous mutations fixed in the four identified genes *Zt80707*, *Zt89160*, *Zt103264* and *Zt110804* reflect adaptation to distinct host niches during host specialization and speciation of the closely related plant pathogens *Z. tritici*, *Z. pseudotritici* and *Z. ardabiliae*. These genes were chosen from a genome scan in *Zymoseptoria* as outliers showing signatures of positive selection and remarkably only *Zt80707* encodes a putative signal peptide. So far it has been shown that the deletion of one of the four genes (*Zt80707*) has a strong impact on virulence and reproduction of *Z. tritici* in wheat (Master thesis, S Poppe). The function of this gene and the other three identified genes is so far not known.

- The first aim of this study is to elucidate the underlying role of the four selected genes showing strong signatures of positive selection in *Z. tritici* by a characterization of the

obtained mutant lineages. In addition the specialization of the candidate gene products will be shown by replacing the candidate genes in *Z. tritici* with their homologous genes of the wild grass pathogens *Z. pseudotritici* and *Z. ardabiliae*. Therefore a basic understanding of the infection process of *Z. tritici* on wheat and the two wild grass pathogens *Z. pseudotritici* and *Z. ardabiliae* on couch grass (*E. repens*), orchard grass (*D. glomerata*) and annual ryegrass (*L. multiflorum*) was initially necessary. I aimed to obtain this knowledge by microscopic analysis and use this data for the characterization of possible differences in the infection process of mutant lineages.

- A second aim is to establish a virulence assay for the *Z. tritici* wheat pathosystem as the ability to resolve small virulence differences between strains is essential to evaluate the replacement strains introduced above.
- The third aim of this thesis was to establish the purple false brome (*Brachypodium distachyon*) as a host plant for the wild grass pathogens as this is a model grass species. The genome of *B. distachyon* is completely sequenced (Draper et al. 2001) and inbred lines are available allowing reproducible infection experiments. In addition many molecular tools for this wild grass are established facilitating future experiments on *Z. pseudotritici* and *Z. ardabiliae*.

2 Results

2.1 The four candidate genes *Zt80707*, *Zt89160*, *Zt103264* & *Zt110804*

Prior to the molecular work the open reading frames (ORFs) of the candidate genes had to be confirmed as 14.2% of the annotated *Z. tritici* genes are missing a start and/or stop codon (A Bhattacharyya, personal communication). Also the annotation of the candidate gene *Zt103264* (http://genome.jgi-psf.org/cgi-bin/dispGeneModel?db=Mycgr3&id=103264) was lacking start and stop codons and the annotated intron started with the nucleotides TA and ended with TG. Because the majority of introns starts with GT and ends with AG (Chen and Moore 2014) and the missing start- and stop codon, the annotation of *Zt103264* seems unlikely and had to be confirmed. This was also necessary for *Zt80707* that shows only little homology to genes of other plant pathogenic fungi in an NCBI-BLAST (Altschul et al. 1990, 1997) search. The ORF confirmation was not conducted for the two genes *Zt89160* and *Zt110804* as they showed high homology to genes of other fungal species.

2.1.1 Gene structure of *Zt80707* and *Zt103264* and their homologs differs

To identify the CDS of the two genes *Zt80707* and *Zt103264* of *Z. tritici* and their homologs of *Z. pseudotritici* and *Z. ardabiliae* (Table 1) the 5’ and 3’ RACE Systems for Rapid Amplification of cDNA Ends were used. Thereby it is possible to identify the 5'- and the 3' ends of the candidate genes mRNA. According to the scanning model of translation (Hinnebusch 2011), the first AUG of the mRNA is the translation start site and those were assigned as the start codons for the genes *Zt80707* and *Zt103264* (Fig. 4). Consequently, the sequence upstream of the AUG is the 5’ UTR of the respective transcript. With the results of the 3' RACE PCR it was possible to identify the stop codons of both genes. Thereby the complete ORFs of both genes in all the three species were determined (Fig. 4). The initial ORF for *Zt80707* predicted by JGI encoding a 125 AA protein was confirmed by this experiment. The homologous genes in *Z. pseudotritici* and *Z. ardabiliae* however, have a later transcription start site and thereby a later start codon resulting in a shorter protein of 101 amino acids (AAs) in *Z. pseudotritici* and 78 AAs in *Z. ardabiliae* (Fig. 5A). In addition the homolog of *Z. ardabiliae* has an earlier stop codon than *Z. tritici* leading to a final protein of 78 AAs in the related wild grass pathogen. A NCBI-BLAST search using the

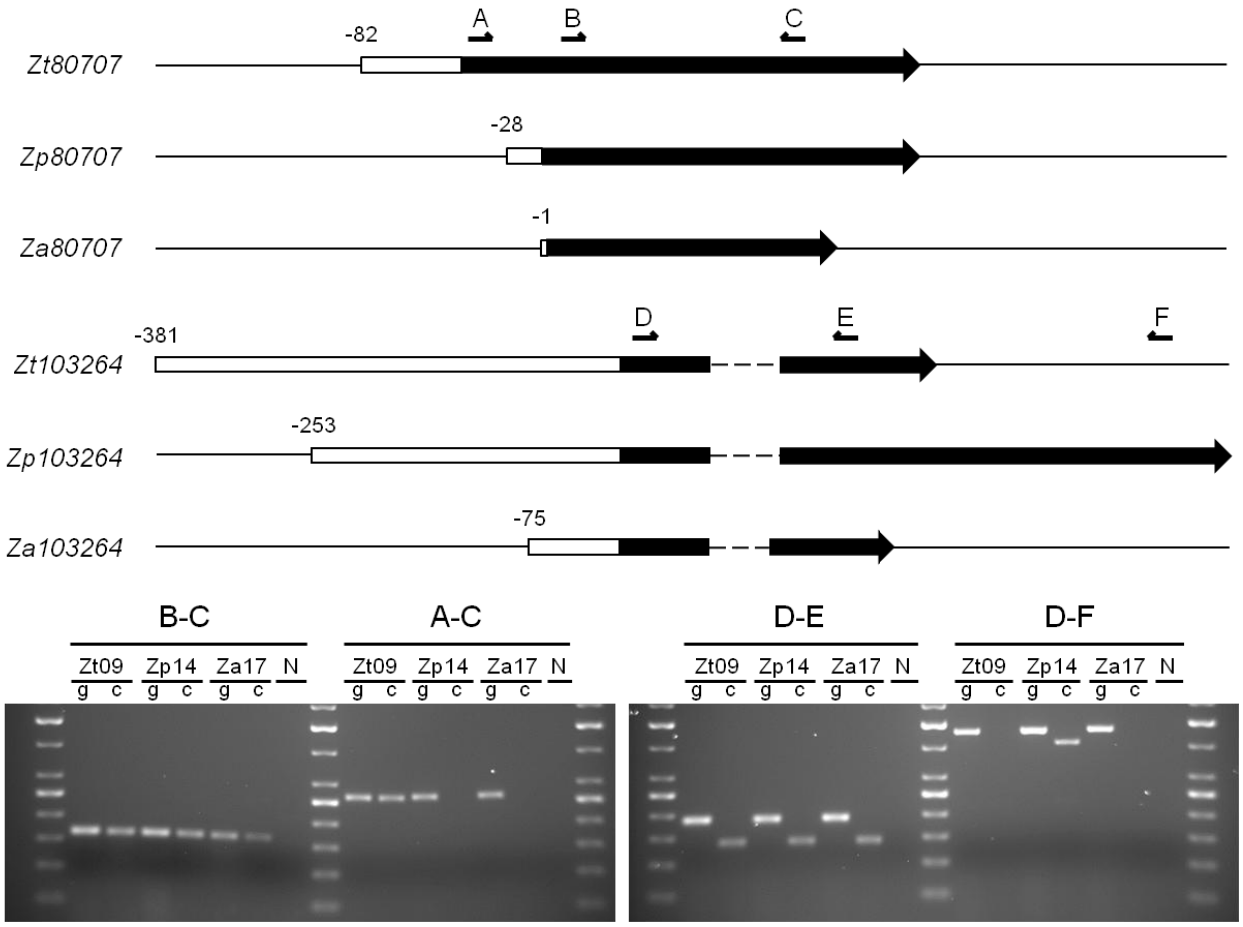

Figure 4: ORFs of *Zt80707* and *Zt103264* and their homologs in *Z. pseudotritici* and *Z. ardabiliae*. A) Results of the identification of the *Zt80707* and *Zt103264* transcripts in *Z. tritici*, *Z. pseudotritici* and *Z. ardabiliae* by 5' and 3' RACE PCR. ORFs are depicted in black and the 5' UTRs in white boxes. The transcription start site at the beginning of the 5' UTR is given relative to the start codon of each transcript. **B)** Confirmation of the identified ORFs by PCRs on genomic DNA (left lanes) and cDNA (right lanes) of *Z. tritici* (Zt09), *Z. pseudotritici* (Zp14) and *Z. ardabiliae* (Za17). The location of the primers (A-F) used for the PCRs are indicated above.

ORF of *Zt80707* was conducted resulting in no homology to other known genes. The gene *Zt103264* was identified to be located on the opposite DNA strand as was predicted by JGI. Also for this gene structural difference between the three species could be found as they have the same start codon but different stop codon positions resulting in proteins of 65 AAs in *Z. tritici*, 146 AAs in *Z. pseudotritici* and 54 AAs in *Z. ardabiliae* (Fig. 5D). The *Z. pseudotritici* isolates Zp12 and Zp13 however, also harbor premature stop codons (nonsense mutations) leading to the complete 147 AAs protein only in the isolates Zp14 – Zp16 (Fig. 5D). Also this gene shows no homology to other known genes according to a NCBI-BLAST search conducted using the ORF of *Zt103264*.

Both ORFs identified with the RACE PCR experiments and the ORFs of their homologs were confirmed by an independent RT-PCR experiment with two primer combinations each (Fig. 4). For *Zt80707* and its homologs the primer combination B-C was leading to a PCR product on genomic and cDNA for all three species confirming presence of the transcript and specificity of the primers. The primer combination A-C however, was just leading to a PCR product on cDNA of *Z. tritici* confirming the 5' extension of *Zt80707*. Specificity of the primers in *Z. pseudotritici* and *Z. ardabiliae* has been shown using the respective gDNA (Fig. 4). In case of *Zt103264* and its homologs specificity of the used primers and presence of the transcript has been shown using the primer combination D-E. This PCR was leading to a product on genomic and cDNA in all three species (Fig. 4). The longer transcript in *Z. pseudotritici* has been confirmed using the primer combination D-F. Amplification of this product was only possible with cDNA of Zp14 as template.

Following the identification of the *Zt80707* and *Zt103264* ORFs including their homologs in the related wild grass pathogens, the comparative analysis to identify positive selection was repeated for those two genes and the present information about the four candidate genes is summarized in Table 1.

Table 1

List of genes chosen for this study

Gene ID	***Zt80707***	***Zt89160***	***Zt103264***	***Zt110804***
Location	Chromosome 5	Chromosome 1	Chromosome 2	Chromosome 9
Zt09 coordinates	0657996-0658613	2089079-2090359	1992714-1993521	1317146-1318829
Length in *Z. tritici*_Zt09 [AAs]	125	393	65	316
Length in *Z. pseudotritici*_Zp14 [AAs]	101	393	146	316
Length in *Z. ardabiliae*_Za17 [AAs]	78	393	54	316
d_N/d_S *Z. tritici* vs. *Z. pseudotritici*	5.825	5.392	2.303	3.533
Predicted function	Unknown/secreted	RCC1 domain	Unknown	Unknown

2.1.2 *Zt80707* encodes a functional signal peptide only in *Z. tritici*

The signal peptide in the *Zt80707* transcript (Fig. 5A red line) of *Z. tritici* was computationally predicted using SignalP (Bendtsen et al. 2004). As shown above this signal peptide is not transcribed in *Z. pseudotritici* and *Z. ardabiliae* (Fig. 4). To confirm that this signal peptide is translated in the Zt80707 protein and targets the translated protein for secretion, an *in vitro* secretion assay was designed. Because of the weak expression of *Zt80707* and its homologs in *Z. pseudotritici* and *Z. ardabiliae* in axenic culture (See Chapter 2.2) the constitutive gpdA

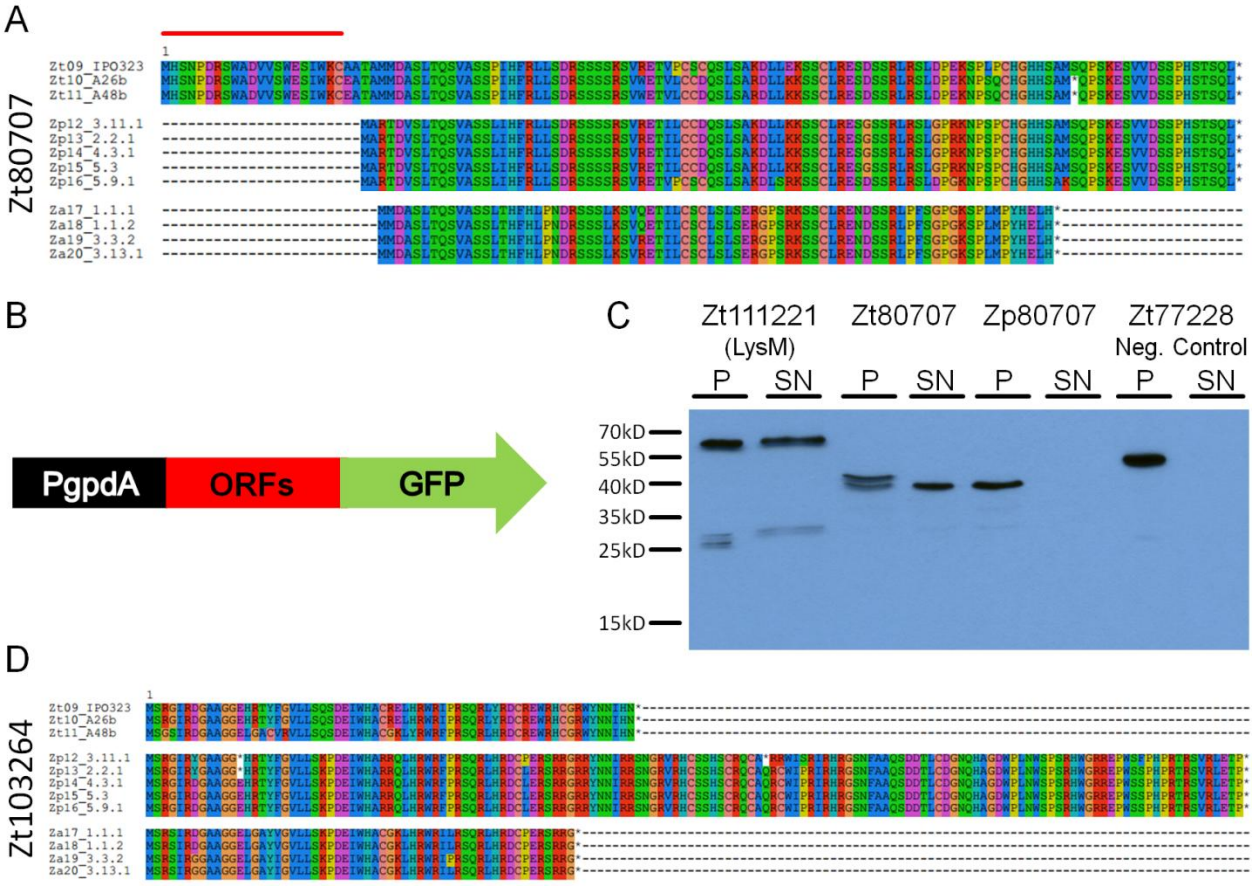

Figure 5: Influence of selection pressure on the proteins Zt80707 and Zt103264. A) Protein alignment of Zt80707 of various *Z. tritici* (Zt), *Z. pseudotritici* (Zp) and *Z. ardabiliae* (Za) isolates showing the acquired predicted signal peptide of Zt80707 in *Z. tritici* (red line). **B)** Scheme of the expression construct for the secretion assay. Genes are under control of the constitutive gpdA promoter and c-terminally tagged with GFP. This construct was cloned into a non coding region on chromosome 1 (chr1ncr). **C)** Western-Blot of Zt111221-GFP (LysM effector), Zt80707-GFP, Zp80707-GFP (isolate Zp13) and Zt77228-GFP (lysis control) proteins detected in the pellet fraction (P) and the culture supernatant (SN) with an anti-GFP antibody. Presence of the GFP tagged gene products in the supernatant confirms secretion. **D)** Protein alignment of Zt103264 of various *Z. tritici* (Zt), *Z. pseudotritici* (Zp) and *Z. ardabiliae* (Za) isolates showing different protein structures between the species and nonsense mutations in the isolates Zp12 and Zp13.

promoter from *Aspergillus nidulans* (Mikkelsen et al. 2003) was used to express *Zt80707* and *Zp80707* (isolate Zp13) *in vitro* from *Z. tritici* cells. Furthermore, both genes were fused with a C-terminal green fluorescent protein (GFP) tag (Fig. 5B). As positive control for protein secretion the well-characterized LysM (Lysine Motifs) effector protein Zt111221 (Marshall et al. 2011; de Jonge et al. 2010) was used. As a negative control the non-secreted protein Zt77228 (a predicted member of the intramitochondrial sorting protein family) was used. The two genes encoding Zt111221 and Zt77228 were expressed as *Zt80707* and *Zp80707* with a C-terminal GFP tag under the control of the gpdA promoter. Western blot analyses confirmed that Zt80707 is present in both the pellet and supernatant fraction of axenically grown *Z. tritici* cells as also shown for the LysM positive control (Fig. 5C). On the other hand, the homologous protein from *Z. pseudotritici* was only detectable in the pellet fraction. These results support the presence of a functional signal peptide in Zt80707 of *Z. tritici*.

2.1.3 *Zt89160* putatively encodes a functional RCC1 domain

The gene *Zt89160* is, in terms of gene structure, highly conserved between *Z. tritici*, *Z. pseudotritici* and *Z. ardabiliae* and encodes a 394 AA protein in each of the three species (Fig. 6A). An NCBI-BLAST search revealed high homology of this protein also to other dothideomycetes like *Aureobasidium pullulans* and *Dothistroma septosporum*, the causal agent of red band needle blight on pines. However, a putative function in pathogenicity has never been shown.

The C-terminal region of this protein contains an RCC1 (regulator of chromosome condensation) domain showing high homology to well studied model organisms as the brown rat (*Rattus norvegicus*), the house mouse (*Mus musculus*) and several *Drosophila* species. The homologous protein of *Drosophila melanogaster* has been crystallized showing a ring like RCC1 β-propeller domain and it was shown that loops within this RCC1 β-propeller domain interact with the histone component and also with the DNA component of the nucleosome core particle (Makde et al. 2010).

To evaluate if Zt89160 has similar functions in *Z. tritici*, the protein structure has been predicted using the I-TASSER server (Roy et al. 2010). This server is based on a composite approach of many threading (fold recognition) programs for sequence to structure alignments. The quality of the predicted protein structure is evaluated using the C-score and the TM-score. The C-score is a

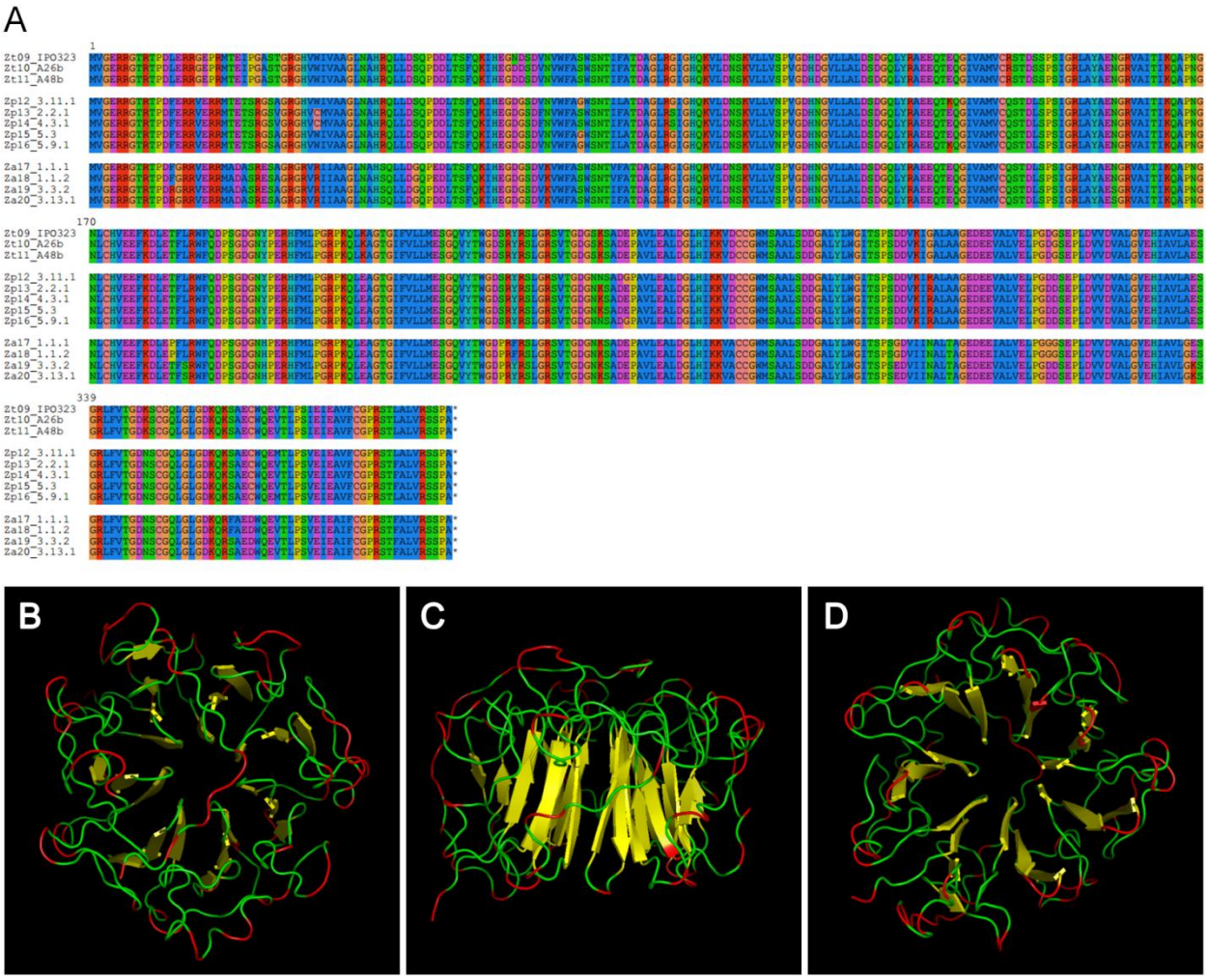

Figure 6: Influence of selection pressure on the protein Zt89160. A) Protein alignment of Zt89160 of various *Z. tritici* (Zt), *Z. pseudotritici* (Zp) and *Z. ardabiliae* (Za) isolates. **B)** Top view, side view **(C)** and bottom view **(D)** of the Zt89160 protein structure predicted with I-TASSER. Beta sheets are depicted in yellow and the accessible amino acids on the surface of the protein are shown in red.

confidence score for estimating the quality of models predicted by I-TASSER. It is calculated based on the significance of threading template alignments and the convergence parameters of the structure assembly simulations. It typically ranges from -5 to 2 with higher values indicating a better quality of the model. The TM-score is a measure of global structural similarity between query and template protein and values > 0.5 are generally indicating a correct topology.

The predicted model for Zt89160 also resembles a ring like RCC1 β-propeller structure containing multiple lateral loops (Fig. 6B-D). This high structural similarity to other well-described RCC1 domain containing proteins indicates a similar DNA and protein binding function of this protein in *Z. tritici*. The C-score of this predicted model is -0.4 and the TM-score

is 0.66 indicating a good quality prediction. However, for the final confirmation of the predicted protein structure a crystallization of Zt89160 is necessary.

2.1.4 Majority of adaptive mutations is located on the protein surface of Zt89160

To investigate whether the adaptive mutations of Zt89160 are evenly distributed over the protein or located in distinct regions of the protein, all non synonymous mutations between Zt89160 (isolate Zt09) and its *Z. pseudotritici* homolog Zp89160 (isolate Zp13) were analyzed. Therefore these mutations were categorized into two groups of mutations located in the center of the protein or on its surface (Table 2) where they are accessible as the loops on the protein surface have been shown to be involved in the interaction with other proteins and DNA in *D. melanogaster*.

Thereby it was possible to show that 83 out of 393 amino acids (21 %) of Zt89160 are accessible on the protein surface. In contrast to that the majority of adaptive mutations (61 %) can be found within these amino acids on the protein surface. This significant accumulation of adaptive mutations of amino acids on the protein surface indicates that the mutational pressure detected for this protein may be driven by the interaction with other proteins or DNA.

Table 2

Distribution of adaptive mutations within Zt89160

	AAs in total	**AAs central / percentage**	**AAs surface / percentage**
Complete protein	393	310 / 78.9 %	83 / 21.1 %
Adaptive mutations	18	7 / 38.9 %	11 / 61.1 %

2.1.5 *Zt110804* encodes a proline-rich hypothetical protein of unknown function

Also for the gene *Zt110804* it was not necessary to confirm its ORF by RACE-PCR as an NCBI-BLAST analysis identified many other homologous genes of unknown function mainly in other dothideomycete species like *M. fijiensis* or *D. septosporum*. Except for the adaptive mutations between *Z. tritici*, *Z. pseudotritici* and *Z. ardabiliae* it is highly conserved and shows no structural differences between the three species (Fig. 7). It encodes a 316 AAs protein of unknown function. The only identifiable domain is a proline-rich region (AAs 122 – 287).

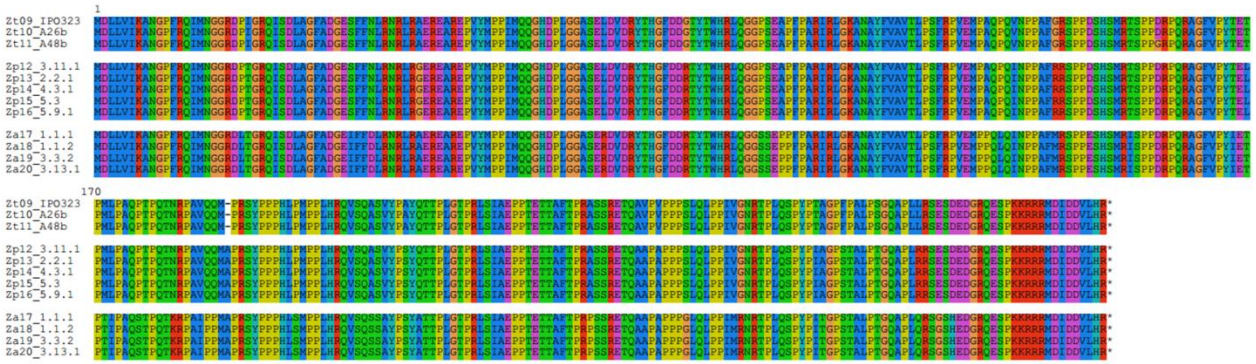

Figure 7: Influence of selection pressure on the protein Zt110804. Protein alignment of Zt110804 of various *Z. tritici* (Zt), *Z. pseudotritici* (Zp) and *Z. ardabiliae* (Za) species. A proline-rich region containing 43 prolines was identified between AAs 122 and 287.

Within this stretch of 165 residues the protein contains 43 prolines. Proline rich regions (PRRs) have been shown to be involved in binding as they can act as "sticky arms" leading to rapid and non-specific binding to a range of proteins (Williamson 1994).

However, the prediction of a reliable protein structure was not possible for this protein. That is why it cannot be shown that the PRR is located on the protein surface and involved in binding of other proteins as here hypothesized. A crystallization of this protein would help to shed some light onto the function of this protein.

2.1.6 *Zt80707* and *Zt103264* are located in repeat rich regions

To investigate if the different gene structures observed for *Zt80707*, *Zt103264* and their homologs in *Z. pseudotritici* and *Z. ardabiliae* were caused by frequent non-homologous recombination events at their loci, the synteny of the four candidate gene loci was analyzed. Therefore the four candidate genes and their neighboring genes were blasted in the genomes of *Z. pseudotritici* (isolate Zp13) and *Z. ardabiliae* (isolate Za17). The presence and orientation of all neighboring genes located on the same scaffold as the candidate genes homologs in the two wild grass pathogens are summarized in Figure 8. This analysis showed that the sequence surrounding *Zt89160* and *Zt110804* is highly syntenic except for two insertion events in *Z. tritici* that occurred distantly to the candidate genes. For the two genes *Zt80707* and *Zt103264* however, gene deletions and insertions as well as gene inversions could be detected in close proximity to the candidate genes. Those findings correlate with the presence of transposable and repetitive elements within the loci of *Zt80707* and *Zt103264* that putatively caused these synteny breaks.

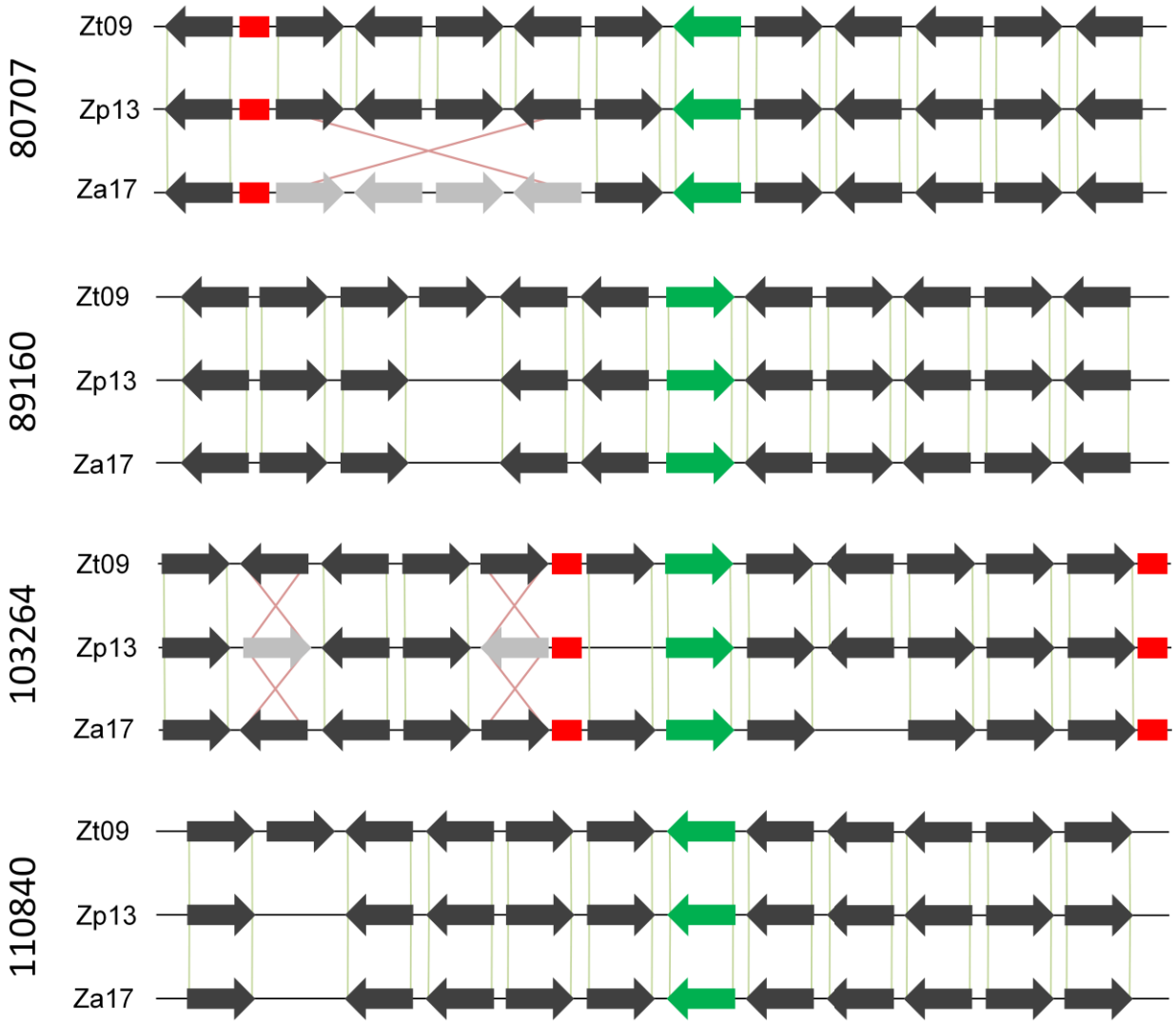

Figure 8: Synteny analysis of positively selected candidate genes. Loci of *Zt80707*, *Zt89160*, *Zt103264* and *Zt110804* compared to their homologous loci in *Z. pseudotritici* (Zp13) and *Z. ardabiliae* (Za17). The four candidate genes are depicted in green, their neighboring genes in dark grey and inverted genes in light grey. Missing genes are shown with a gap. Repetitive DNA is depicted with red boxes.

2.2 Analysis of candidate-gene expression

2.2.1 Synthesis of cDNA from extracted total RNA of *Z. tritici*

Total RNA was extracted from *Z. tritici* axenic culture and from several time points during plant infection (4 dpi, 7 dpi, 14 dpi and 28 dpi) to compare in planta expression levels with the expression of the candidate genes in axenic culture. The 4 dpi and 8 dpi time points were chosen to analyze candidate gene expression during the biotrophic phase of infection. Two weeks after infection the pathogen induces necrosis and switches from biotrophic to necrotrophic growth. This phase is represented by the 14 dpi stage. At 28 dpi the infected area of the leaf is dead and the necrotic tissue was used to analyze *Z. tritici* gene expression during necrotrophic growth. Following DNaseI digestion to remove any residual genomic DNA, cDNA synthesis was conducted using reverse transcriptase. Successful removal of gDNA was verified with a PCR (Fig. 9) using intron spanning primers oES1026 & 1027 (*Z. tritici* GAPDH) as pure cDNA without any gDNA contamination is absolutely crucial for qRT-PCR and RACE-PCR. This test PCR was conducted for all samples.

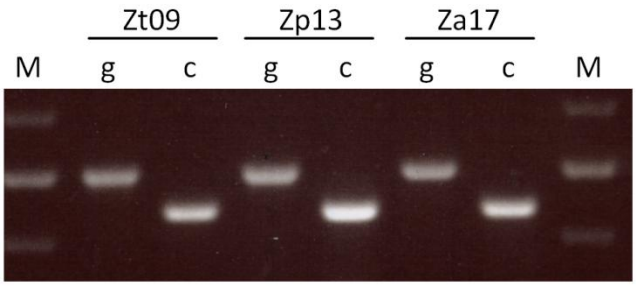

Figure 9: cDNA confirmation. Test PCR using intron spanning primers for fungal GAPDH on genomic (g) and cDNA (c) of *Z. tritici* (Zt09), *Z. pseudotritici* (Zp13) and *Z. ardabiliae* to verify complete removal of genomic DNA prior to expression analyses.

2.2.2 Candidate genes are up-regulated *in planta*

For the analysis of *Z. tritici gene* expression the fluorescent dye SYBR-Green was used that similar to Ethidium bromide intercalates into double stranded DNA. This allows measuring the amount of double stranded DNA in a PCR. The primers for this reaction have to fulfill many criteria to ensure specificity of the method. As side products of the PCR would also be measured, the primers have to be absolutely specific for the desired PCR product and should not lead to primer dimers as those additionally would distort the measurement. The length of the PCR products should not exceed 80 - 120 bp to allow comparison of the different PCRs as longer products lead to the intercalation of more SYBR-Green and a stronger signal without an increased amount of PCR products. These restrictions also ensure comparable PCR efficiency

among all reactions. The efficiency of the PCR reaction is also influenced by the binding ability of the primers that can be modified by the annealing temperature and the primer length. Another important factor is the binding site of the primers. For the cDNA synthesis oligo-dT primers that bind the 3' poly-A tail of the mRNA have been used. Because the synthesis is carried out in 3' to 5' direction, the qRT-PCR primers should be located in the 3' region of the gene to ensure that the efficiency of the reverse transcriptase, which is decreasing with ongoing synthesis, does not influence the measurement.

The gene expression values have been normalized to the constitutively expressed *Z. tritici* housekeeping gene GAPDH. The four candidate genes *Zt80707*, *Zt89160*, *Zt103264* and *Zt110804* are almost not expressed in axenic culture (< 0.01 % of GAPDH expression) but are up-regulated *in planta* supporting a function during pathogenicity. The genes however, show different expression patterns on wheat. While the expression of *Zt89160*, *Zt103264* and *Zt110804* is highest during the biotrophic phase and is decreasing during the advancing infection (Fig. 10), *Zt80707* expression is highest during the switch from biotrophic to necrotrophic growth.

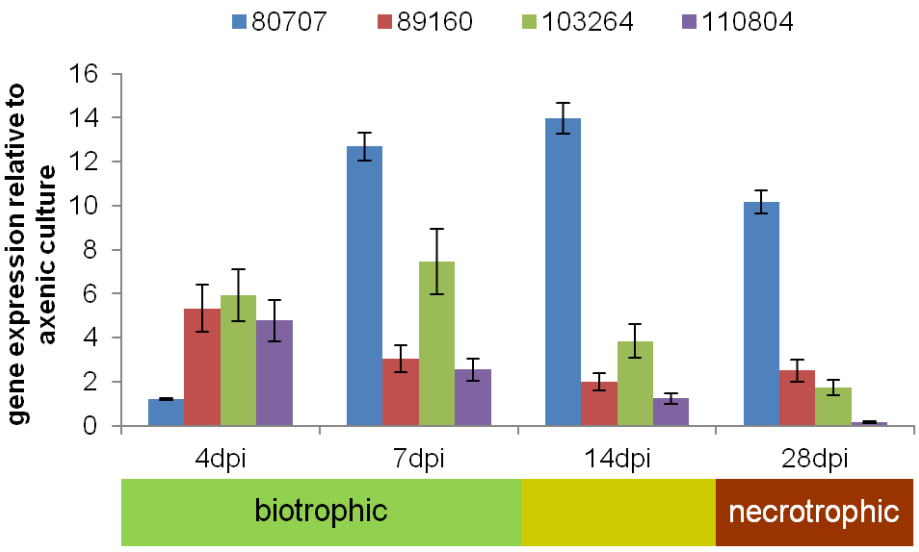

Figure 10: Expression-data for the selected candidate genes. In planta expression data for the four positively selected candidate genes *Zt80707*, *Zt89160*, *Zt103264* and *Zt110804* of *Z. tritici*, relative to axenic culture expression. Values are normalized to GAPDH, a constitutively expressed housekeeping control.

2.3 Generation and identification of *Z. tritici* mutant strains

2.3.1 Generation of a plasmid backbone for Gibson assembly

Targeted gene deletion for *Z. tritici* using *Agrobacterium tumefaciens* mediated transformation (ATMT) was established prior to this work (Bowler et al. 2010; Master thesis S. Poppe, 2011). This approach is based on a modified *A. tumefaciens* TI (tumor inducing) plasmid containing two endonuclease recognition sites (ENRS) flanking the construct that eventually is transferred to the fungal cell. As the transformation constructs have to be generated by an overlap-PCR and ligated with the vector backbone, the approach is very laborious.

One possible way of improving and accelerating this procedure is the Gibson assembly (Gibson et al. 2009). Thereby it is possible to generate the transformation construct and ligate it with the vector backbone in one reaction. An essential prerequisite is a transformation vector backbone with a multiple cloning site (MCS) between the ENRSs containing restriction sites for blunt-cutting restriction-endonucleases. This was generated by modifying the standard transformation already established (Master thesis S. Poppe, 2011) and adding a MCS to it (Fig. 11).

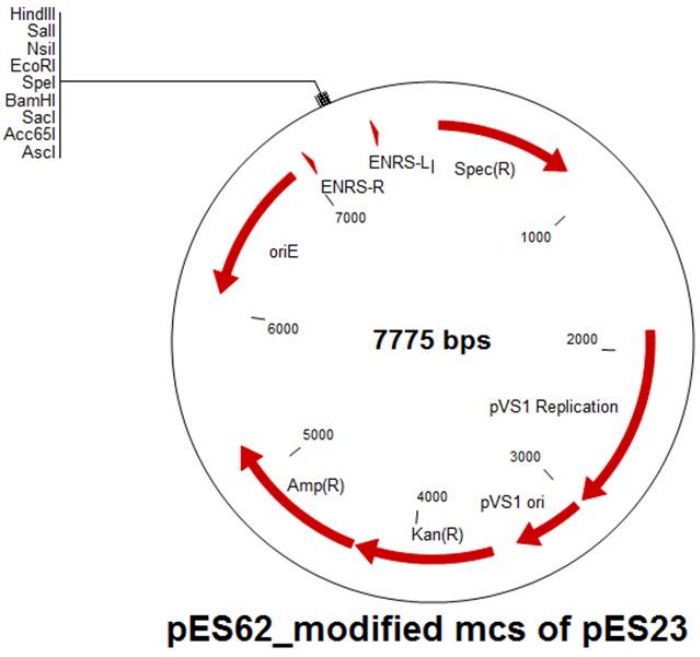

Figure 11: ATMT plasmid pES62. A multiple cloning site was added to the plasmid pES23 resulting in a transformation plasmid that is compatible with Gibson assembly approach. The MCS is located between the two endonuclease recognition sites (ENRS) necessary for cleavage of the transformation construct.

2.3.2 Construct generation for *A. tumefaciens* mediated transformation

Transformation of *Z. tritici* was conducted using the Gibson assembly mentioned above. Therefore each required fragment was amplified by PCR using primers containing 5' overhangs overlapping with the neighboring fragments (Fig. 12). Thereby it was possible to generate the

desired plasmids containing the complete transformation constructs each in one "Gibson reaction". This was achieved by the concerted action of a 5’ exonuclease, a DNA polymerase and a DNA ligase. The generated transformation plasmid was verified by sequencing analysis and could be directly used for ATMT. After successful transformation, all generated mutant strains were verified using Southern blot analysis.

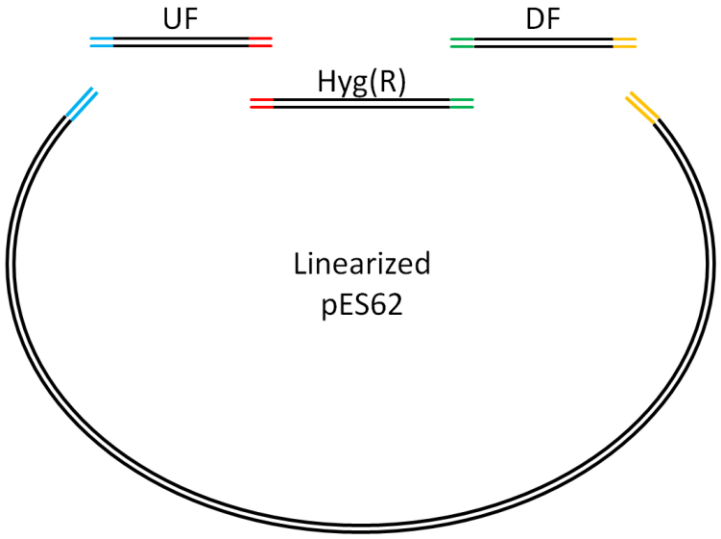

Figure 12: Scheme for the generation of a transformation plasmid by Gibson assembly. Because of the matching overlaps between the single fragments the Gibson assembly will result in one single plasmid containing the complete transformation construct.

2.4 Phenotypic analysis of *Z. tritici* mutant strains

First, single cells of all generated mutant strains were investigated with a light microscope to address if the mutant phenotype was similar to the wild type strain. The independent deletion of all candidate genes did not influence the morphology or axenic growth.

2.4.1 The reaction of *Z. tritici* mutants to abiotic stresses was not influenced by deletion of the candidate genes

To evaluate whether any observed phenotype of the deletion mutants relates to the host-pathogen interaction or to the basic growth performance of the strains, an *in vitro* stress assay was conducted. Therefore fungal cells were grown on YMS plates containing NaCl (1.5 M), H_2O_2 (2 mM), Congored (500 µg/ml) and Calcofluor (200 µg/ml) allowing us to compare the sensitivity of the mutant strains to osmotic and oxidative cell wall stresses (Fig. 13). In addition the strains

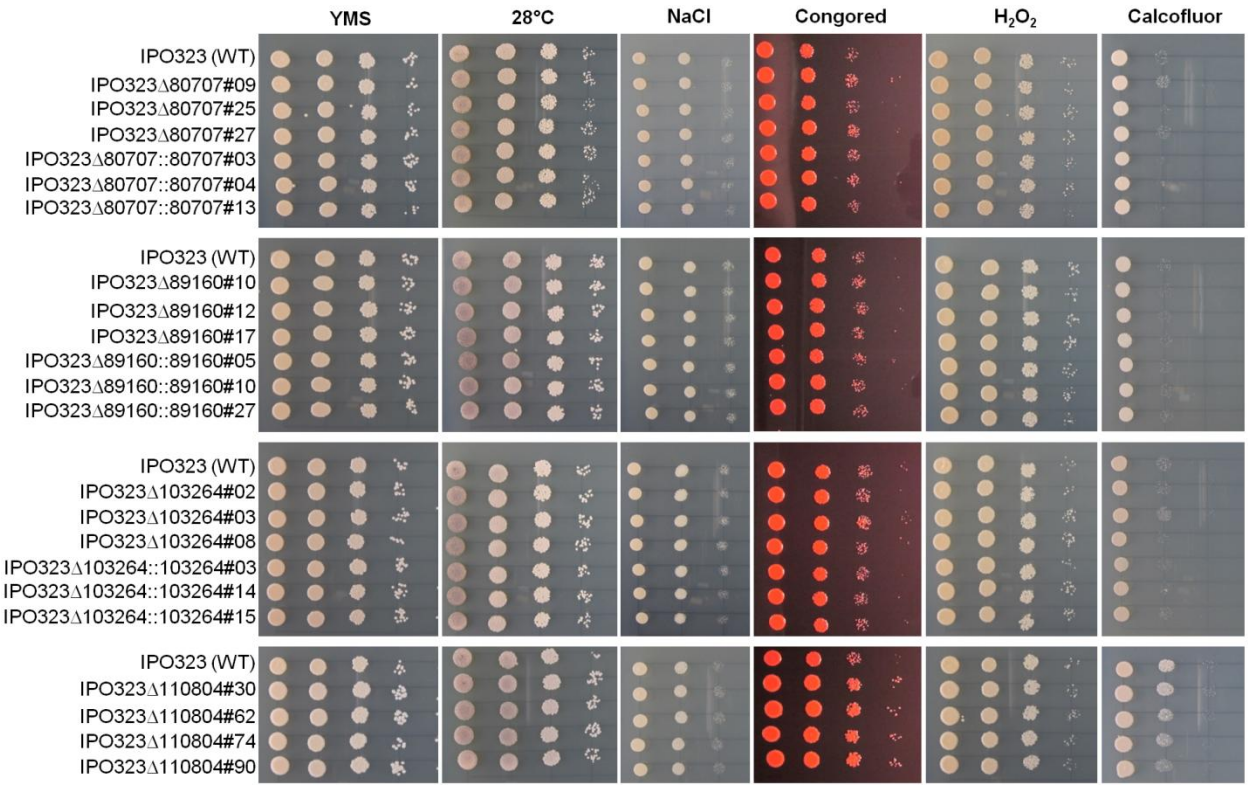

Figure 13: Stress assay using independent transformants of the generated deletion- and complementation strains. Three independent transformants of each generated deletion- and complementation strain for the genes *Zt80707*, *Zt89160* and *Zt103264* were tested under multiple abiotic stress conditions. The deletion of *Zt110804* was not complemented. The following conditions were applied: Heat stress (28°C), NaCl (1.5M), Congored (500µg/ml), H_2O_2 (2mM) and Calcofluor (200µg/ml).

were incubated at 28°C on standard YMS medium to assess temperature sensitivity. Three independent transformants were tested for each mutant strain and in none of the applied conditions a difference to the wild type strain IPO323 was observed (Fig. 13) supporting a pathogenicity related role of the genes.

2.4.2 Deletion of candidate genes influences pathogenicity of *Z. tritici* on wheat

Plant experiments were conducted to determine virulence levels of *Z. tritici* mutant strains on the susceptible wheat variety Obelisk in comparison to the wild type strain. Every plant experiment was conducted twice using two biological replicates for each infection. Evaluation of the symptoms was done 28 days post infection (dpi) by assessing the percentage of necrotic leaf area and the percentage of leaf area covered with pycnidia caused by the *Z. tritici* strains (Fig. 14). The symptoms were evaluated using 6 different categories ranging from 0 (without any visible symptoms), 1 (1-20%), 2 (21-40%), 3 (41-60%), 4 (61-80) to 5 (81-100%). The amount of necrosis was unaffected for plants infected with IPO323Δ*Zt80707*, IPO323Δ*Zt89160* and IPO323Δ*Zt110804* in comparison to the wild type IPO323. However, leaves infected with IPO323Δ*Zt103264* showed significantly lower levels of necrosis. Production of pycnidia was significantly reduced on leaves infected with the deletion mutants IPO323Δ*Zt80707* and IPO323Δ*Zt103264*. The deletion mutant IPO323Δ*Zt89160* caused a significantly higher amount of pycnidia resembling a hypervirulent phenotype. The pycnidia levels of the deletion mutant IPO323Δ*Zt110804* were, as for the necrosis levels, similar to wild type infected leaves. To confirm that the phenotypes observed for the different deletion mutants were solely caused by the deletion of the candidate genes the ORFs of the genes were reintroduced into the endogenous loci in the respective deletion strains. Plant infections with these complementation strains showed that wild type virulence levels could be restored for all three candidate genes that caused a phenotype *in planta* (Fig. 14). Thereby a pathogenicity related role of *Zt80707*, *Zt89160* and *Zt103264* was shown for the *Z. tritici* wheat interaction.

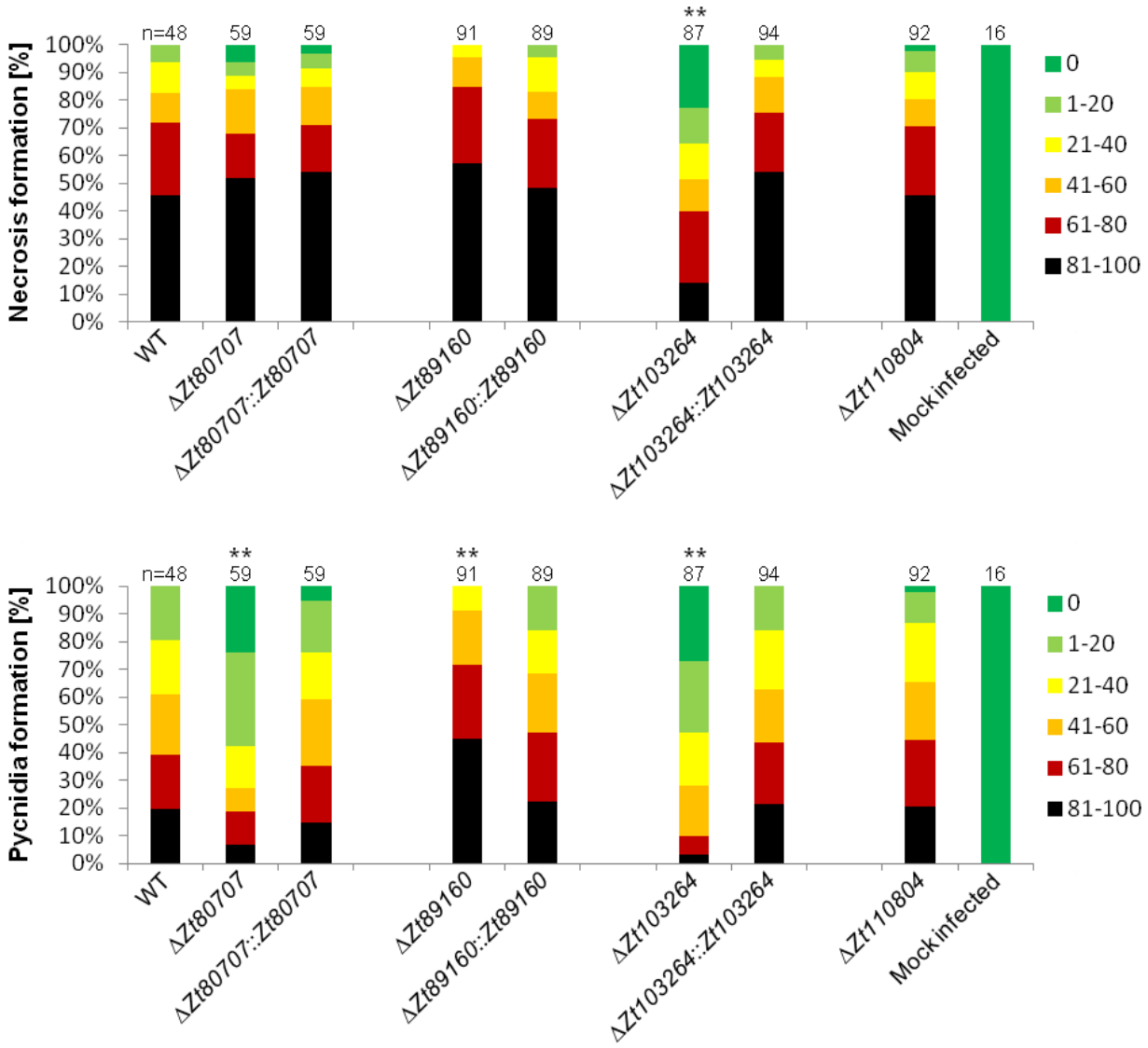

Figure 14: Virulence evaluation of the deletion- , complementation strains. Virulence levels of the *Z. tritici* wild type strain (IPO323), the four deletion strains (IPO323Δ*Zt80707*, IPO323Δ*Zt89160*, IPO323Δ*Zt103264* and IPO323Δ*Zt110804*) and their respective complementation strains on the wheat variety Obelisk. The percentaged leaf area covered with necrosis and the leaf area covered with pycnidia were evaluated 28dpi using 6 categories ranging from 0 (without any visible symptoms), 1 (1-20%), 2 (21-40%), 3 (41-60%), 4 (61-80) to 5 (81-100%). Category 5 indicates a fully necrotic leaf area completely covered with pycnidia. ** $p<0.01$. *p*-values were calculated by Student's *t*-test.

2.4.3 Deletion of *Zt80707* and *Zt103264* impairs development of pycnidia

As shown above (Fig. 14) the IPO323Δ*Zt80707* and IPO323Δ*Zt103264* deletion mutants have reduced levels of pycnidia on wheat leaves 28 dpi. They furthermore show an impaired development of pycnidia since pycnidiospores were exuded only from pycnidia on wild type

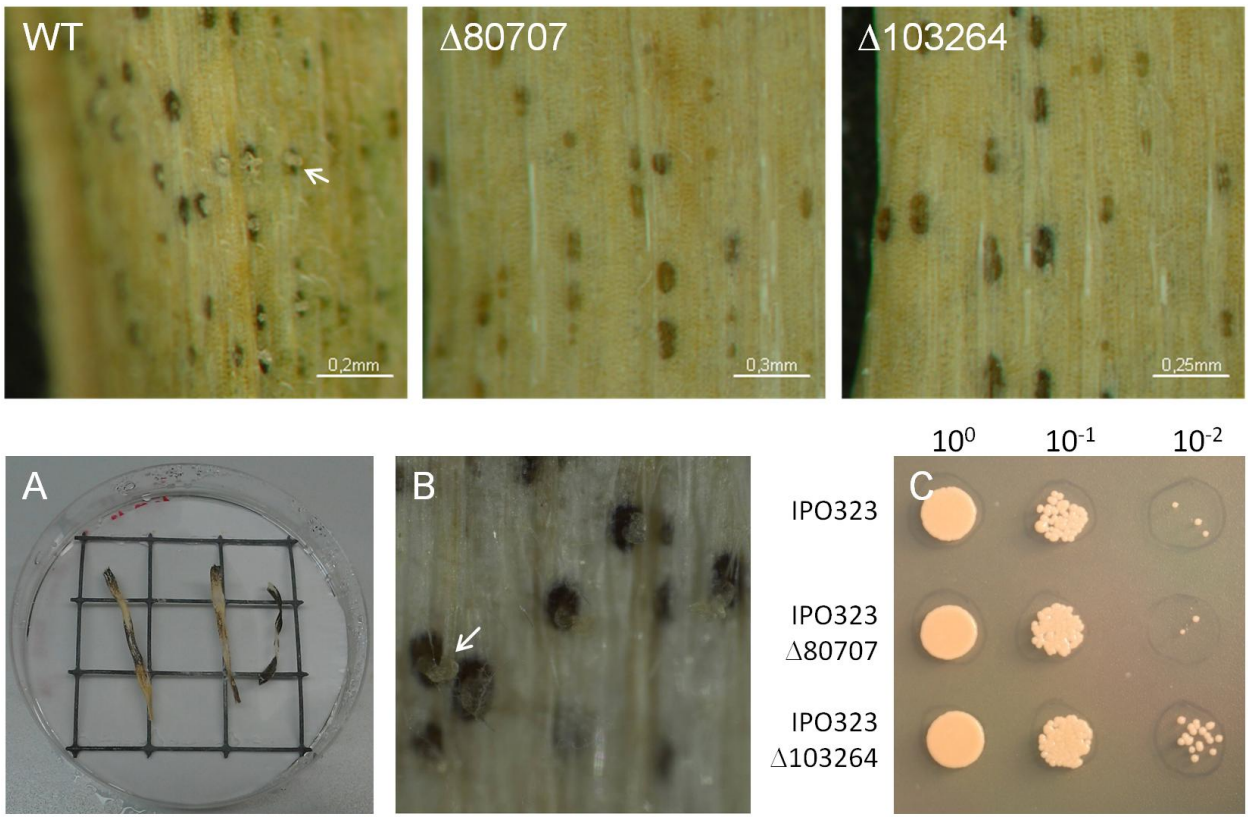

Figure 15: Phenotypic analysis of the deletion strains. Close-ups of necrotic wheat leafs 28days after infection with the wild type IPO323, IPO323Δ*Zt80707* and IPO323Δ*Zt103264*. Bars are given. Pycnidiospores are visible atop the wild type pycnidia (WT – white arrow). **A)** Leaf samples were surface sterilized and incubated under high humidity conditions. Thereby the oozing was induzed. **B)** Following this incubation the spores were also exuded from the pycnidia of the two deletion strains (shown for IPO323Δ*Zt80707* – white arrow). **C)** Isolated spores were solved in water and a dilution series was loaded on a YMS plate.

infected leaves 28 dpi but not from the two mutants (Fig. 15). Extending the infection in the phytochamber by two more weeks did still not lead to visible pycnidiospores on the pycnidia of the deletion mutant infected leaves. To evaluate the viability of the pycnidiospores a qualitative assay was conducted comparing the pycnidia from leaf samples infected with the wild type and the two deletion mutants IPO323Δ*Zt80707* and IPO323Δ*Zt103264*. Therefore leaves were harvested to induce oozing (release of spores) from pycnidia *in vitro* under high humidity conditions (Fig. 15A). Pycnidiospores were released from pycnidia of both wild type and deletion mutant after fourteen days (Fig. 15B). To assess and compare their viability, spores were isolated from each leaf sample and a dilution series was prepared to determine the proportions of germinating spores (Fig. 15C). No significant difference was observed in the

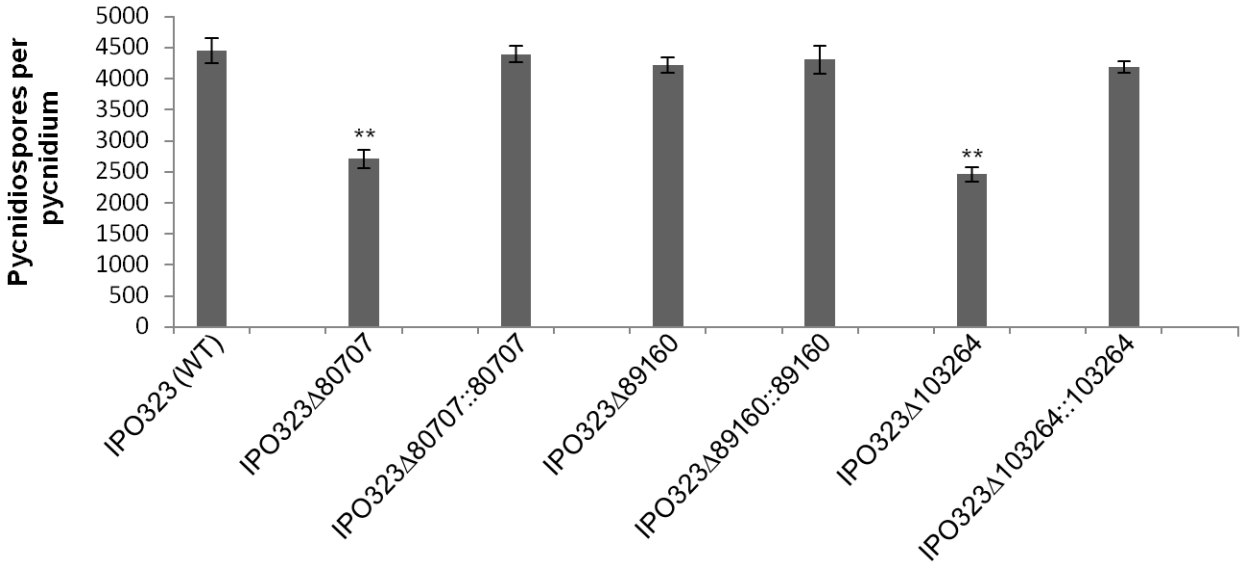

Figure 16: Quantification of pycnidiospores per pycnidium. Following the induced oozing, the pycnidiospores were solved in sterile water and the amount of pycnidia was counted. Thereby it was possible to estimate the ratio of the counted amount of pycnidiospores isolated from a known number of pycnidia. The deletion of *Zt80707* and *Zt103264* was significantly reducing the amount of pycnidiospores per pycnidium compared to the wild type IPO323.** $p<0.01$. *p*-values were calculated by Student's *t*-test.

viability of spores from wild type and deletion mutants signifying that the deletions of *Zt80707* and *Zt103264* have no qualitative effect on the pycnidiospores.

To further investigate the macroscopically observed quantitative differences in pycnidiospore production between deletion mutants of *Zt80707* and *Zt103264* and the wild type, the amount of pycnidiospores per pycnidium was assessed. Therefore oozing of pycnidia on harvested leaves was induced as before. This time the number of pycnidia per leaf (three technical replicates) was also counted and a "Neubauer-improved" counting chamber was used to count the amount of pycnidiospores isolated from the oozing pycnidia. The number of pycnidiospores in the spore suspensions was divided by the number of pycnidia on each leaf from which the spores were isolated from. Thereby it was possible to calculate the amount of pycnidiospores per pycnidium and show that the pycnidia of the two deletion strains IPO323Δ*Zt80707* and IPO323Δ*Zt103264* contained significantly less pycnidiospores in comparison to the wild type pycnidia (Fig. 16). For both deletion strains this effect on pycnidia could be rescued by reintroducing the respective gene into the deletion strain (Fig. 16).

The developmental defect of the pycnidia formation in IPO323Δ*Zt80707* and IPO323Δ*Zt103264* was furthermore visualized using confocal microscopy. For this, infected leaf samples of 14 and 28 dpi were stained using a WGA-FITC / Propidium iodide double staining to stain both the fungus and plant cells. The width of the pycnidia (n=50 for each strain and each time point) was measured on wheat leaves infected by wild type and the two deletion mutants. Hereby, it was first of all shown that the size of the pycnidia produced by wild type *Z. tritici* is almost unchanged from 14 dpi to 28 dpi at 50-60µm (Fig. 17). Furthermore, it was shown that the mean pycnidia

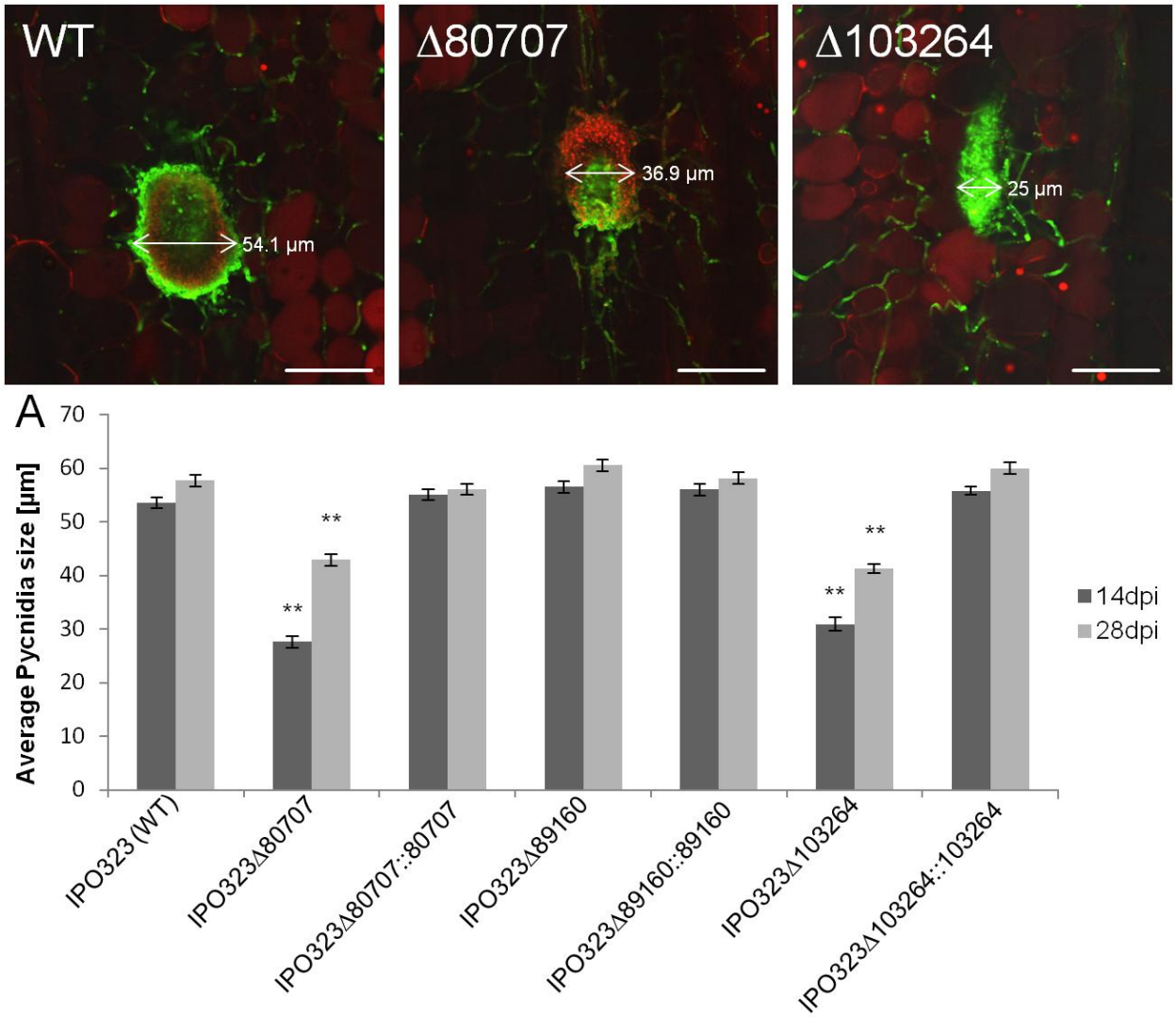

Figure 17: Microscopic analysis of the deletion strains. Images of WGA-FITC / Propidium iodide double stained pycnidia on necrotic wheat leafs 28days after infection with the wild type IPO323, IPO323Δ*Zt80707* and IPO323Δ*Zt103264*. Fungal cell walls are depicted in green and plant cells as well as fungal and plant nuclei are stained in red. Scale bar: 50µm **A)** Average size of pycnidia measured 14 and 28dpi. For each strain and time point 50 pycnidia were measured. Significant differences to the average size of the wild type pycnidia are indicated by two stars and were calculated using the Student's *t*-test.

sizes of IPO323Δ*Zt80707* and IPO323Δ*Zt103264* were significantly smaller than the wild type pycnidia at both time points of infection (Fig. 17A). To confirm that this effect is due to the deletion of the two genes the respective ORFs were reintroduced into the endogenous loci. For both genes this could restore wild type pycnidia development.

2.4.4 Gene products of *Zt80707* and *Zt89160* are specialized to wheat

According to our hypothesis that positively selected amino acid changes in *Z. tritici* have played a role during speciation and adaptation to the host plant wheat, the importance of species-specific substitutions was tested. Therefore the genes *Zt80707*, *Zt89160* and *Zt103264* were replaced with the respective homologous genes from *Z. pseudotritici* and *Z. ardabiliae*.

For the replacement of *Zt80707* the full-length homologs from *Z. pseudotritici* (isolate Zp13) and *Z. ardabiliae* (isolate Za17) were used to replace the gene in *Z. tritici*. Furthermore a fusion construct with the *Zt80707* signal peptide (until the cleavage site at position 25) and the ORF of the Zp13 homolog was generated (Δ*Zt80707*::sp + *Zp80707*, Zp13). This was to assess whether the homolog of *Z. pseudotritici* can complement the gene deletion of *Zt80707* in *Z. tritici* if secreted as the *Z. tritici* homolog. Thereby it was shown that wild type virulence could not be restored in any of the replacement strains lacking the signal peptide of *Z. tritici* (Fig. 18). However, with the fusion construct of the *Zt80707* signal peptide and the Zp13 ORF it was possible to partially restore wild type virulence levels suggesting that the protein encoded by *Zt80707* plays an essential role in the extracellular space of *Z. tritici*. The experiment suggests that substitutions in Zp80707*,* acquired since the divergence of *Z. tritici* and *Z. pseudotritici,* still allow the protein to fulfill the same function as Zt80707 but with a reduced efficiency as the virulence of this replacement strain still significantly differs from wild type virulence.

Also the replacement of *Zt89160* with the homologous gene from *Z. pseudotritici* (isolate Zp13) could not restore wild type virulence in *Z. tritici*. The replacement strain showed the same hyper-virulent phenotype as the mutant suggesting that adaptive substitutions in *Zt89160* indeed have been important for the divergent specialization of *Z. tritici*.

The deletion of *Zt103264* was replaced with the orthologs of *Z. pseudotritici* (isolate Zp14) and *Z. ardabiliae* (isolate Za17). The introduction of both homologs restored virulence levels of the wild type *Z. tritici* isolate, suggesting that adaptive substitutions in this gene do not directly relate to host specialization in *Z. tritici.*

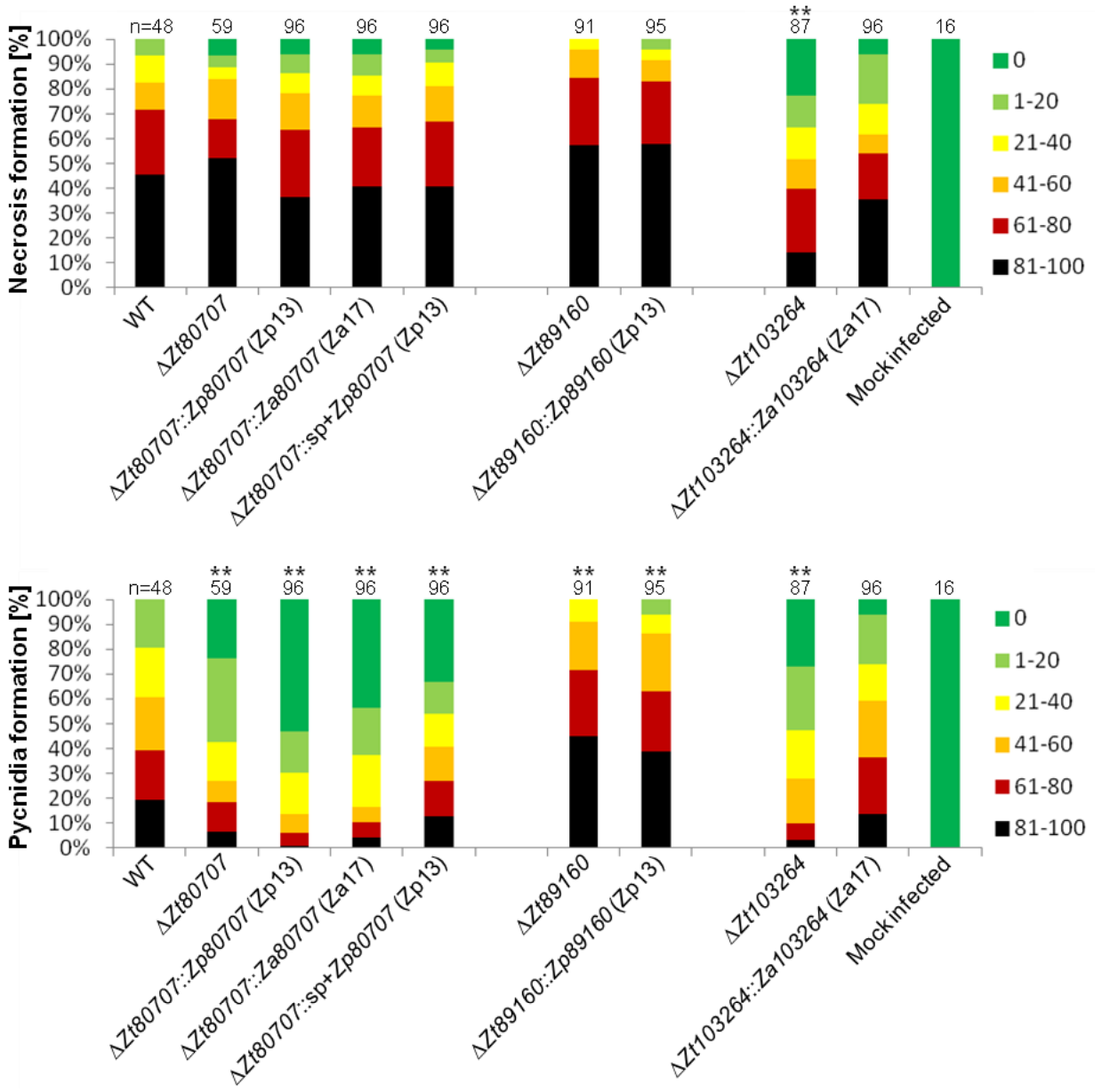

Figure 18: Virulence evaluation of the deletion- , complementation- and replacement strains. Virulence levels of the *Z. tritici* wild type strain (IPO323), the four deletion strains (IPO323Δ*Zt80707*, IPO323Δ*Zt89160*, IPO323Δ*Zt103264* and IPO323Δ*Zt110804*) and their respective replacement strains on the wheat variety Obelisk. The percentaged leaf area covered with necrosis and the leaf area covered with pycnidia were evaluated 28dpi using 6 categories ranging from 0 (without any visible symptoms), 1 (1-20%), 2 (21-40%), 3 (41-60%), 4 (61-80) to 5 (81-100%). Category 5 indicates a fully necrotic leaf area completely covered with pycnidia. ** $p<0.01$. *p*-values were calculated by Student's *t*-test.

2.5 Microscopic analysis of *Z. tritici*, *Z. pseudotritici* & *Z. ardabiliae*

To follow and investigate the infection of *Z. tritici*, *Z. pseudotritici* and *Z. ardabiliae in planta* confocal laser scanning microscopy was used. A wheat germ agglutinin (WGA) based staining was chosen as one abundant part of fungal cell walls is chitin and the best characterized chitin-binding protein is WGA (Lee et al. 1993). To stain the plant cells, propidium iodide that also accumulates in fungal and plant nuclei was used.

2.5.1 Infection cycle of *Z. tritici*

The infection of *Z. tritici* is initiated with an infection hyphae emerging from germinating spores 2dpi. On monocotyledons these hyphae grow directed towards the stomata, the natural openings of the plants, and enter the plant through these (Fig. 19 - 2dpi). Following the penetration the fungus locally proliferates beyond the infected stomata in the mesophyll tissue without any signs of a plant resistance response in the susceptible cultivar Obelisk (Fig. 19 - 4dpi) and it only grows in the apoplast, the space between the plant cells. Because of the slow increase of fungal biomass, this phase is also considered as a latent phase and not as a classical biotrophic phase. However, *Z. tritici* also needs to actively suppress the plant innate immune system for successful proliferation inside the plant leaves. This was visualized by infecting the nonhost plant *Brachypodium distachyon* with *Z. tritici*. Following the entry of the stomatal openings, *Z. tritici* was recognized and a defense reaction was induced in *B. distachyon*. This was observed as the penetrating hyphae caused massive autofluorescence corresponding to a hypersensitive response (HR) of the plant (Fig. 19 - Bd 4dpi).

In case of the compatible *Z. tritici* wheat interaction however, the fungus grows within the infected leaves and two weeks after the initial infection the fungus switches from biotrophic to necrotrophic growth coinciding with the formation of macroscopically recognizable lesions on the infected leaves. Those lesions then develop into the necrotic blotches characteristic for STB. Within these blotches the asexual fruiting bodies (pycnidia) are formed in the sub stomatal cavities (Fig. 19 - 28dpi). When the maturation of the pycnidia is completed, pycnidiospores are released under humid conditions from open stomata. Released spores are subsequently spread by water droplets.

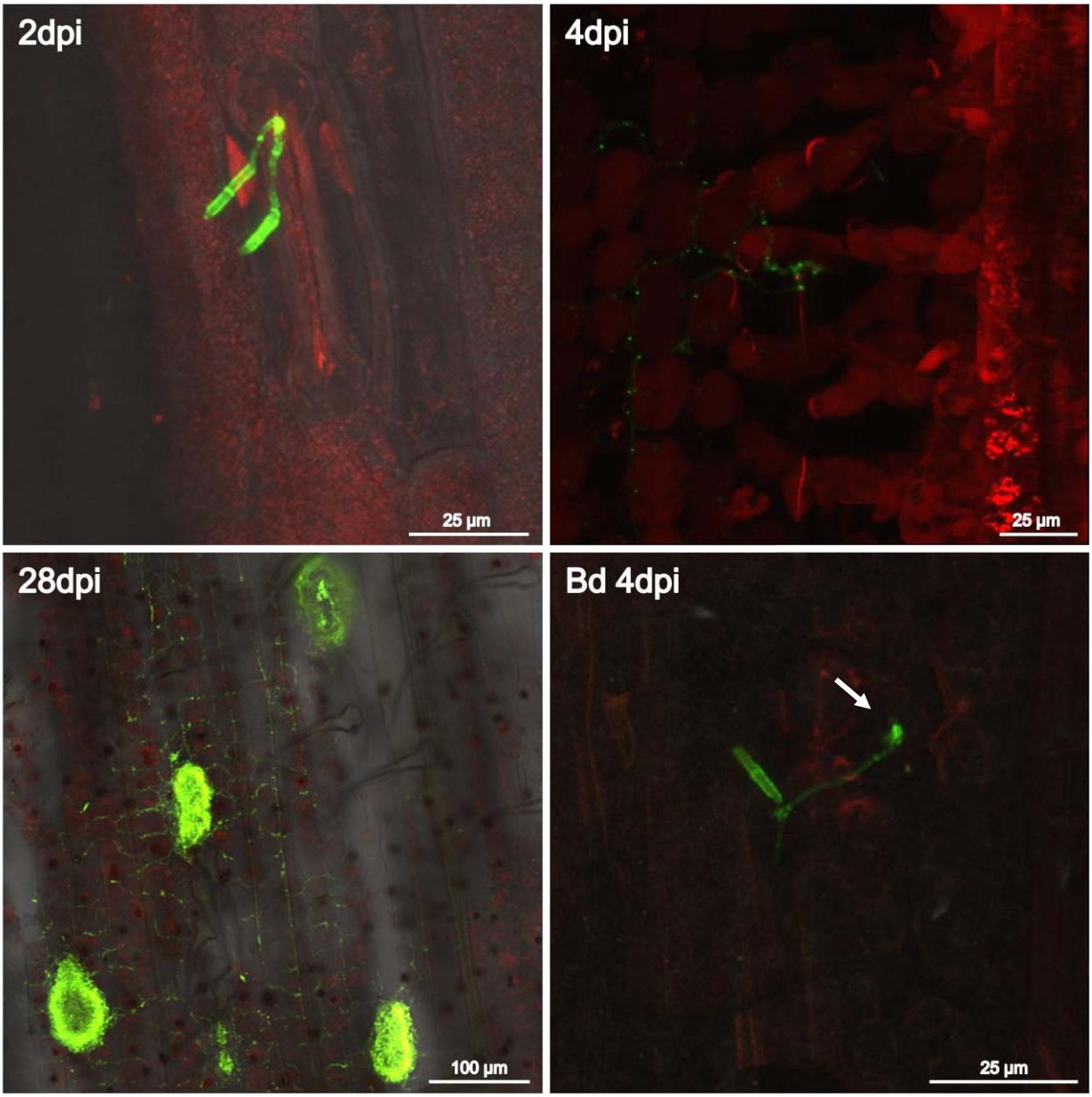

Figure 19: Confocal microscopy images of *Z. tritici* on wheat. Fungal cells are depicted in green and plant cells in red. *Z. tritici* penetrates the stomata 2dpi and afterwards grows intercellularly inside the mesophyll tissue at 4dpi. Four weeks after the initial infection pycnidia formation in the sub stomatal cavities is completed. In the incompatible interaction of *Z. tritici* and *Brachypodium distachyon* the penetrating hyphae (Bd 4dpi - arrow) is causing a hypersensitive response of the plant and an arrest of the infection.

2.5.2 *Z. pseudotritici* and *Z. ardabiliae* cannot infect *B. distachyon*

The ability of *Z. pseudotritici* and *Z. ardabiliae* to infect the wild grass species *L. multiflorum* and *E. repens* was previously demonstrated (Master thesis S Poppe, 2011). However, for both of these grass species no inbred lines or molecular tools are established. Furthermore, these grasses grow very slow. As an alternative host, it was here aimed to establish the model grass species *B. distachyon* as host plant for *Z. pseudotritici* and *Z. ardabiliae*. Therefore the isolates Bd21, Bd21-3 and Bd3-1 (kindly provided by T. Marcel) were tested since these have a high germination rate (<50 %) without the need of a vernalization, and are highly susceptible to different plant pathogenic fungi (Barbieri et al. 2012).

Both *Z. pseudotritici* (Zp) and *Z. ardabiliae* (Za) are able to germinate on the leaves of *B. distachyon* and the hyphae emerging from the germinating spores show directed growth towards the stomata. However, the infection of both wild grass pathogenic fungi was stopped in the sub stomatal cavity of all three *B. distachyon* varieties as the penetrating hyphae induced HR at 7 dpi (Fig. 20). Thereby it was shown that the three tested *B. distachyon* isolates are resistant to *Z. pseudotritici* and *Z. ardabiliae*, and that *B. distachyon* unfortunately could not be used as a model host for *Zymoseptoria* pathogens.

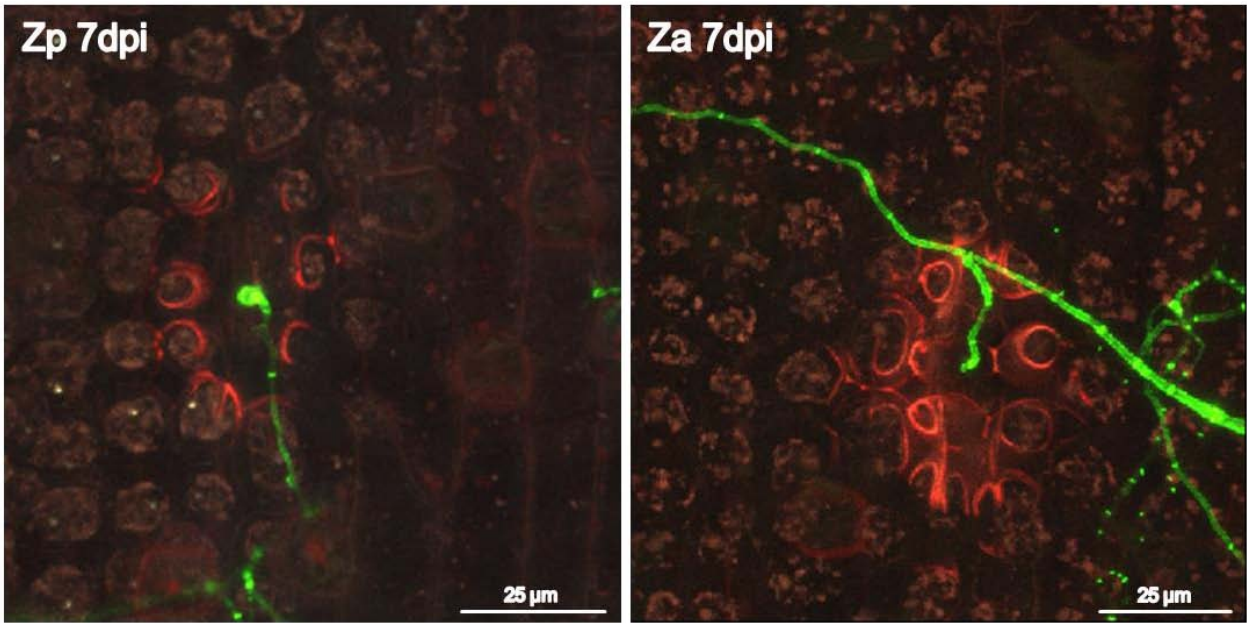

Figure 20: Confocal microscopy images of *Z. pseudotritici* and *Z. ardabiliae* on *B. distachyon*. Z-stacks of penetrated stomata have been made. Fungal cells are depicted in green and plant cells in red. Penetration hyphae of *Z. pseudotritici* and *Z. ardabiliae* are causing HR on *B. distachyon* and cannot infect the tested varieties Bd21, Bd21-3 and Bd3-1.

3 Discussion

In this thesis the functional role of four positively selected genes *Zt90707*, *Zt89160*, *Zt103264* and *Zt110804* in *Zymoseptoria tritici* was characterized. A focus was on the influence of these genes on pathogenicity and adaptation to the host *Triticum aestivum*, bread wheat. Expression analyses revealed a putative function during pathogenicity, as the four genes are not expressed *in vitro* but are up-regulated during pathogenicity. Furthermore, an *in vitro* stress assay of deletion mutants showed no phenotypic difference to the wild type during axenic growth. Interestingly, three of the four single gene deletion mutants showed a significantly altered virulence-phenotype during infection of wheat. To address the role of positively selected amino acid substitutions, the genes *Zt80707*, *Zt89160* and *Zt103264* were replaced with the homologous genes of *Z. pseudotritici* and *Z. ardabiliae*. With the replacement of *Zt80707* and *Zt89160* it was possible to show that adaptive evolution of these two genes relate to virulence in wheat since the *Z. pseudotritici* homologs could not restore wild type virulence of *Z. tritici*. The adaptive mutation of *Zt103264* could not be related to host specialization as the replacement of this gene with ortholog of *Z. ardabiliae* (isolate Za17) restored wild type virulence levels on wheat. In addition RACE-PCR analyses revealed that *Zt80707* and *Zt103264* have not only accumulated amino acid changes but also show different gene structures between *Z. tritici*, *Z. pseudotritici* and *Z. ardabiliae*. Notably, the gene *Zt80707* encodes a signal peptide which is not transcribed in the homologous genes of *Z. pseudotritici* and *Z. ardabiliae* indicating a novel extracellular function of the encoded protein in *Z. tritici*.

Below, results of the studies presented here will be discussed including the here established virulence assay for *Z. tritici*, phenotypes of the deletion strains as well as possible functions of the three genes influencing virulence of *Z. tritici*. Furthermore the structural changes of *Zt80707* and *Zt103264* that have accompanied species divergence are contemplated. The method of comparative genomics and evolutionary predictions of positively selected genes to identify key-players in the specialization of fungal pathogens to their host will also be evaluated. Finally future objectives and perspectives are considered.

3.1 Establishment of a pathogenicity assay for *Z. tritici* on wheat

Pathogenicity is often facilitated by multiple factors and deletion of single genes in pathogenic species is often not abolishing pathogenicity completely (Santhanam et al. 2012; Wichmann and Bergelson 2004; Brefort et al. 2014). Virulence assays that allow the detection of small quantitative differences between mutants and wild type strains are essential when multiple genes determine virulence. Prior to this work, two techniques were used to evaluate *Z. tritici* virulence on wheat. One used detached wheat leaves infected with *Z. tritici* on square Petri dishes (Arraiano et al. 2001). Here the ends of the leaves are covered in water agar to avoid early senescence. This approach appears promising as it allows high throughput screening of mutant strains. However, natural senescence of detached leaves challenges the recognition and quantification of fungal caused necrosis. It was hereby concluded that the infection of living plants is the most appropriate infection method for *Z. tritici* to quantify and compare disease of different strains. For *Z. tritici* it is namely absolutely crucial for the virulence assay to distinguish between senescence and fungal caused necrosis. Another approach developed for *Z. tritici* uses living 17 days old wheat seedlings. Hereby, a marked area of the second leaf of seedlings is infected by evenly applying a spore solution with a brush. This technique was successfully used to estimate virulence of LysM-effector gene deletion strains that were almost apathogenic (Marshall et al. 2011; Lee et al. 2013). In addition the authors measured the biomass development of the wild type and mutant strains *in planta* which also appears a promising approach to quantify small virulence alterations of mutant strains. A modified version of the latter *in planta* approach was developed for the studies described here. For this the amount of fungal caused necrosis and pycnidia were used to determine the virulence of *Z. tritici* on bread wheat. These two parameters were evaluated 28 dpi using six categories ranging from 0 (without any visible symptoms), 1 (1-20%), 2 (21-40%), 3 (41-60%), 4 (61-80) to 5 (81-100%). Category 5 reflects a fully necrotic leaf area completely covered with pycnidia. With this novel and fine scale plant assay it was possible to detect even small differences in virulence of deletion mutants of the *Z. tritici*-wheat-pathosystem.

A challenge of this *in planta* assay is the high variability between *Z. tritici* infected leaves ranging from leaves completely covered with pycnidia to leaves which only show few symptoms (Fig. 14). To achieve representative and reproducible results the number of infected plants per strain has to be high (50 plants per strain). Every experiment was done with three independent

biological replicates and conducted using two independent transformants for each mutant strain. Thereby it was possible to detect statistically significant and reproducible changes of virulence for mutant strains of *Z. tritici* on bread wheat as required to resolve small virulence differences between strains.

3.2 The structural differences of *Zt80707* and *Zt103264*

A RACE-PCR approach was used to verify the previous computational predictions of gene structure of the candidate gene of *Z. tritici* (Goodwin et al. 2011) and their homologs in the related wild grass pathogenic species *Z. pseudotritici* and *Z. ardabiliae*. It was shown that the two genes *Zt80707* and *Zt103264* have dramatic alterations in transcript length between the three species. The transcription start of *Zt80707* is located 23 AAs upstream of the start codon in *Z. pseudotritici* and 25 AAs upstream of the start codon in *Z. ardabiliae*. Interestingly the extra translated peptide of *Z. tritici* is a signal peptide that targets Zt80707 for secretion. This was confirmed experimentally by a Western-Blot analysis documenting a secretion function of the signal peptide. The Western blot analysis was design to discriminate between the presence of the protein Zt80707 in the pellet and supernatant fraction of *Z. tritici* cell cultures. Detection of Zt80707 but not the homologous protein of *Z. pseudotritici* in the supernatant proved the relevance of the signal peptide in Z. tritici. The functionality of the signal peptide was also shown by the infection of wheat with the replacement strains. Replacement of the *Zt80707* ORF with the ORF of *Zp80707* was not influencing the virulence compared to the *Zt80707* deletion strain. However, the fusion of the *Zt80707* signal peptide with the ORF of *Zp80707* led to a partial recovery of virulence in infected wheat seedlings. This indicates that despite the acquired adaptive mutations of Zt80707, the orthologous protein of *Z. pseudotritici* still can fulfill the extracellular function of Zt80707 but in a less efficient manner.

Figure 21: Influence of selection pressure on the protein Zt80707. Protein alignment of the candidate gene *Zt80707* of *Z. tritici* (Zt), *Z. pseudotritici* (Zp) and *Z. ardabiliae* (Za). The start codons are highlighted (red stars) and the alignment demonstrates the length difference of the homologous proteins. The 23 amino acid stretch at the N-terminal end of the *Z. tritici* homolog is predicted to encode a signal peptide (red line).

The extracellular localization of Zt80707 suggests that the adaptive substitutions in this gene could relate to a new function of the protein in the apoplastic space. Nevertheless the secretion of Zt80707 to the extracellular space appears to be imperfect as two putative isoforms of the protein can be detected in the pellet fraction in similar abundance. Those two possible isoforms could correlate to the protein with a cleaved signal peptide (38.5kDa) and the protein with the remaining signal peptide (40.9kDa). The computationally predicted "secretion probability" of Zt80707 is low compared to the LysM positive control protein furthermore suggesting that this new secretion signal has a lower efficiency. The LysM positive control protein itself was expected at a size of 51.5kDa but was detected at 60-70kDa what can be explained by *N*-glycosylation. This was already shown for a LysM effector in *M. oryzae* (Chen et al. 2014) and for other effector proteins like Pep1 and Pit1 in *Ustilago maydis* (Doehlemann et al. 2009, 2011; Hemetsberger et al. 2012) and Ecp6 *in Cladosporium fulvum* (de Jonge et al. 2010). The *N*-glycosylation pathway is conserved among filamentous fungi, and this post translational modification has been discussed as a possible common mechanism to regulate the function of effectors during host infection (Chen et al. 2014). The lacking *N*-glycosylation and the imperfect secretion of Zt80707 may show that the transcription of the signal peptide was not lost in *Z. pseudotritici* and *Z. ardabiliae* after divergence of the *Z. tritici* lineage but rather recently gained in *Z. tritici*. However, further investigation of the protein in *Z. pseudotritici* and *Z. ardabiliae* is necessary to further characterize the functional evolution of this protein.

RACE-PCR showed that the *Zt103264* ORF differs from the JGI prediction. It was here shown that the gene is transcribed in the opposite direction and in a different reading frame. Also this gene shows structural differences between the three species as it is eight amino acids shorter in *Z. tritici* as in *Z. ardabiliae*. Interestingly, in a population sample of *Z. pseudotritici* two distinct alleles can be found; one with an open reading frame of 146 amino acids, 81 amino acids longer than the allele of *Z. tritici* and another with a premature stop-codons leading to a nonsense mutation. The importance of the structural differences remains unclear but supports the strong divergent selection pressure, which the gene has evolved under.

3.3 Influence of *Zt80707*, *Zt89160* and *Zt103264* on the pathogenicity of *Z. tritici*

Quantitative real time PCR analyses showed that the four positively selected candidate genes *Zt80707*, *Zt89160*, *Zt103264* and *Zt110804* are almost not expressed in axenic culture however all four genes are up-regulated during the infection of wheat suggesting an influence of the genes on pathogenicity. Additionally, *in vitro* phenotyping of single gene deletion mutants was conducted. Thereby it was possible to show that the mutants' sensitivity to abiotic stresses was not significantly different compared to the wild type *Z. tritici* strain IPO323. The expression of all four genes was confined to biotrophic and necrotrophic growth in wheat.

The virulence of three (*Zt80707*, *Zt89160* and *Zt103264*) of the four deletion mutants on wheat was significantly altered. While the deletion of these three candidate genes in *Z. tritici* had an impact on virulence and pathogen development, the mutants showed distinct phenotypes. The deletion of *Zt80707* and *Zt103264* led to a reduced amount of pycnidia. After 28 dpi, pycnidia were smaller and did not exude spores as observed on wheat leaves infected by the wild type strain. Extending the infection for two weeks did also not lead to mature pycnidia. This severe phenotype suggested that *Zt80707* and *Zt103264* deletion mutants lost their ability to asexually reproduce. To further investigate this, surface sterilized 28 dpi leaves were incubated under high humid conditions. Thereby it was possible to induce excretion of pycnidiospores for both deletion strains showing that pycnidiospores are produced in the mutants. These findings indicate that the IPO323Δ*Zt80707* and IPO323Δ*Zt103264* mutants can produce viable pycnidiospores but are mainly affected in the maturation of pycnidia.

The induction of pycnidiospore excretion was also applied to determine the amount of released pycnidiospores per pycnidium. Therefore the amount of released pycnidiospores was counted and divided by the number of pycnidia on a given leaf area. This revealed a significantly reduced amount of pycnidiospores per pycnidia for the IPO323Δ*Zt80707* and IPO323Δ*Zt103264* mutants supporting that the development of the pycnidia is impaired in the mutants. The developmental defect of the two deletion mutants was confirmed using confocal microscopy. This showed that the pycnidia size of both deletion strains is significantly smaller in comparison to the wild type. As both *Zt80707* and *Zt103264* are upregulated during early host colonization, they might be involved in the establishment of biotrophic growth and defeat of host defense. The impaired maturation of pycnidia would thereby be a side effect caused by the impaired pathogen

development. However, formation of pycnidia starts already at 8-10 dpi indicating that both gene products might be involved in the early pycnidia formation.

Despite the fact that the deletion of both genes has a similar impact on virulence, it remains unclear if the two gene products also have similar functions. For none of the genes it has been possible to assign a protein function or known motifs. The putatively different localization of both proteins makes a similar function unlikely as *Zt80707* encodes a functional signal peptide and *Zt103264* not. However, also non-conventional secretion of pathogenicity related proteins has been described (Rodrigues and Djordjevic 2012). Localization studies of both proteins using fluorescent tags or immunolocalization could shed light on a possible co-localization and thereby a similar function. A double deletion mutant would be a good way to determine whether there is an additive effect of the gene products (Wichmann and Bergelson 2004). In this context it would be worthwhile to investigate whether the production of one protein is dependent on the presence of the other by qRT-PCR in the two deletion strains.

The deletion of *Zt89160* encoding a protein with an RCC1 (Regulator of Chromosome Condensation) domain, causes a hypervirulent phenotype of *Z. tritici* on wheat. In mammals and *Drosophila* species RCC1 domain containing proteins were described to be involved in critical eukaryotic cellular functions including nuclear transport, nuclear envelop assembly and mitosis (Makde et al. 2010; Hetzer et al. 2002). Also in soybean (*Glycine max*) two putative RCC1 domain-encoding genes have been described (Narayanan et al. 2009). Those two genes are specifically down-regulated in soybean in an incompatible interaction following *Phytophthora sojae* infection. The authors speculate that nucleocytoplasmic trafficking may be suppressed as a resistance response during early infection of *Phytophthora sojae*. Nevertheless, it remains unclear if the RCC1 domain is functional in the two soybean proteins as they are highly diverged compared to the well described human homolog (Meier 2007) and a similar function of RCC1 domain containing proteins has never been shown in plants. For fungal plant pathogens a function of RCC1 domain containing proteins still needs to be demonstrated. Nevertheless, high sequence similarity of Zt89160 to its homolog of *S. cerevisiae*, in particular of the RCC1 domain, indicates comparable functions of the two proteins (Kalashnikova et al. 2013).

Mutant hypervirulence has only been described in a few examples from fungal plant pathogens (Baldwin et al. 2006; Degrassi et al. 2010; Kamper et al. 2006; Meir et al. 2009). Considering the predicted function of the RCC1 domain in Zt89160, the protein could be involved in the

regulation of virulence related genes of *Z. tritici*. The hemi-biotrophic life-style of *Z. tritici* requires a fine tuned regulation of transcription during host infection. The switch from biotrophic to necrotrophic feeding and regulation involved in this shift may be affected in the deletion mutant. Full transcriptome sequencing of the IPO323Δ*Zt89160* mutant would allow further investigation of a putative role of Zt89160 in gene regulation. A significant excess of adaptive mutations identified in Zt89160 are located on the protein surface possibly reflecting changes of the Zt89160 binding sites as compared to putative binding sites of 89160 homologs of *Z. pseudotritici* and *Z. ardabiliae*. However, it remains unclear if species specific substitutions are involved in binding of DNA or other proteins. Therefore a Co-Immunoprecipitation would be helpful to first show the ability of Zt89160 to bind other proteins or DNA and then the involvement of those mutated sites in binding of DNA or proteins can be investigated.

3.4 Zt80707 and Zt89160 are host specific pathogenicity factors

We hypothesized that genes evolving under positive selection likely have played a role during divergent adaptation and host specialization. Here it was indeed shown that three out of four genes investigated play a role during host infection of *Z. tritici*. To further investigate if species-specific substitutions in *Z. tritici* are important for the infection of wheat, the *Z. tritici* genes were replaced with homologous genes from *Z. pseudotritici* and *Z. ardabiliae* and the functional implications were evaluated. The replacement of *Zt80707* with its orthologs of *Z. pseudotritici* and *Z. ardabiliae* lacking a signal peptide did not show any complementation in terms of virulence on wheat. The fusion of the *Z. tritici* signal peptide and the *Z. pseudotritici* ORF (isolate Zp13) could partially restore wild type virulence levels as it produced significantly more pycnidia than the deletion strain but also significantly less pycnidia than the wild type. Together these results show that the acquisition of the signal peptide and the accumulation of adaptive substitutions in *Zt80707* have been important during specialization of *Z. tritici* to wheat.

The molecular function of the protein Zt80707 remains unclear as it shows no homology to any known proteins. Nevertheless it is a small secreted protein, which may play a role in pathogenicity as an effector protein (Jones and Dangl 2006; Donofrio and Raman 2012). Proteins secreted by infecting hyphae could have a function in the host apoplast to inhibit proteases secreted by the host plant as defensive response to the infection (Khang et al. 2010; Doehlemann et al. 2009), or they could be transported to the plant cell with a function in the cytoplasm or an

organelle of the plant cell (Kale 2012). It is possible that the protein leads to a change of the fungal cell wall making it possible to evade plant resistance mechanisms (Tian et al. 2007). Another way of evading the plant resistance is to inhibit it directly. Hereby the protein could be transported to the nucleus of the plant cell and function as transcription factor that inhibits expression plant resistance genes. It has also been shown that secreted effectors inhibit host proteins that are part of the plant resistance mechanism (Catanzariti et al. 2007). However, the gene *Zt80707* is not just expressed during the initial infection as other well characterized effector proteins (Jonge et al. 2012; Doehlemann et al. 2009) but also during necrotrophic growth, as an interaction between a hemi-biotrophic fungus and its host plant by secreted effectors should only be possible during the biotrophic phase when the host plant is still alive. Thereby, the expression pattern of *Zt80707* makes a putative function as effector unlikely but supports a possible role of the encoded protein in fruiting body formation as the fruiting body maturation takes place in both, the late biotrophic and the necrotrophic phase of infection. However, it remains unclear how a secreted protein might be involved in fruiting body formation as a secreted protein has never been described to be involved in this process (Teichert et al. 2014).

The same specialization has been shown for *Zt89160* as the ortholog of *Z. pseudotritici* was could not complement the hypervirulence phenotype of the deletion mutant on wheat. Similar protein specialization was shown for the oomycete protease inhibitor EPIC1 of *P. infestans* and *P. mirabilis* targeting host specific extracellular defense proteases (Dong et al. 2014). We here show a similar importance of species specific substitutions. Another novel finding is the putative process of secreting and modifying a primarily cytoplasmatic protein to adapt to a new host as it was here shown for Zt80707. The understanding of the molecular function of *Zt80707* and *Zt89160* will likely shed light on the mechanisms of specialization and adaptation to new hosts.

3.5 Perspectives

It was possible to show a strong correlation between evolutionary predictions and virulence function in a plant pathogen. Previous studies in prokaryote and eukaryote pathogenic species have likewise demonstrated accelerated evolution of virulence related genes (Dong et al. 2014; Baltrus et al. 2011; Raffaele et al. 2010). A focus in these previous studies has been the evolution of effector encoding genes typically recognized as small, secreted proteins. The here selected candidate genes however were selected only according to evolutionary predictions and no *a priori* information about gene function or structure. Another possible unbiased approach would be to investigate the adaptation of expression. Transcriptional analysis of *Z. tritici* on the compatible host wheat and the incompatible host *B. distachyon* revealed distinct regulatory programs during infection of wheat (Kellner et al. 2014). However, a similar RNA-seq dataset for the closely related wild grass pathogens on their respective host plants is still missing as a compatible host plant has not been established in the laboratory yet. The availability of such a dataset would allow to identify differentially regulated genes between *Z. tritici* and the wild grass pathogens on the respective host plant and together with the here used genome data it is possible to focus on mutations in promoter regions to identify the genes that are under mutational pressure for altered expression as this can be a feature of adaptation to a new host plant, too.

In any case it would be necessary to gain more insight into the molecular function of genes involved specialization and adaptation to new hosts to better understand the molecular mechanism behind host adaptation of pathogens. Therefore it would be worthwhile to investigate the localization of the identified proteins to better understand their mode of action. Especially the localization of Zt80707 is intriguing as it has been shown that a fraction of the mature protein remains inside the cell with an uncleaved signal peptide. Hence, this protein could also have an intracellular function.

Additionally, interaction partners of the candidate proteins could be identified either by Yeast Two-Hybrid screening or a Co-Immunoprecipitation. Thereby it would be possible to draw conclusions about pathways or mechanisms that the candidate proteins are involved in. As soon as soluble protein can be purified, it is also possible to resolve the crystal structure of the candidate proteins and also confirm the homology based structure prediction for Zt89160. Thereby, it can be verified that the observed adaptive mutations are indeed located on the protein surface of Zt89160. For the other identified proteins a reliable structure prediction was not

possible because of the lack of homology to other proteins. Thus crystallization of those proteins could shed light on their function. Co-Immunoprecipitation would also enable us to investigate if Zt89160 possesses DNA binding ability and if this putative DNA binding ability is sequence specific.

Lastly, the specialization of Zt80707 and Zt89160 and their respective homologs of *Z. pseudotritici* and *Z. ardabiliae* to their respective host plants could also be demonstrated using the two wild grass pathogenic species. However, establishment of *B. distachyon* as host plant for *Z. pseudotritici* and *Z. ardabiliae* failed. Therefore it would be worthwhile to establish a plant pathosystem for both species and generate inbred lines of host grasses permitting reproducible infection results. Nevertheless, B. distachyon can also be used as non-host plant for *Z. pseudotritici* and *Z. ardabiliae* as it was done for *Z. tritici* (Kellner et al. 2014) to identify host specific expression patterns of genes putatively involved in pathogenicity.

4 Material and Methods

4.1 Material and ordering sources

4.1.1 Chemicals

The chemicals and enzymes that were used for this project were mainly supplied by Difco (Augsburg), Invitrogen (Karlsruhe), Merck (Darmstadt), Roche (Mannheim), Roth (Karlsruhe), and Sigma-Aldrich (Deisenhofen).

4.1.2 Buffers and solutions

Standard buffers and solutions were made according to (Ausubel et al. 1987) and (Sambrook et al. 2001). Special buffers and solutions are mentioned in the according methods. All media, solutions and buffers were if necessary autoclaved for 5 min at 121°C. Heat sensitive solutions were sterilized using a filter (Pore size 0.2 µm, Merck) and a vacuum pump.

4.1.3 Enzymes and antibodies

Restriction endonucleases were obtained from New England Biolabs (Frankfurt) and ligations were conducted using T4-Ligase of Roche. For standard PCR reactions NEB Taq-Polymerase was used. The other PCR reactions were conducted using PHUSION-Polymerase (Finnzymes/NEB). Antibodies were obtained from Roche and NEB. Enzymatic digestion of DNA was conducted with DNase I (Ambion/Invitrogen).

4.1.4 Used kits

To elute DNA out of agarose gels the Wizard SC Gel and PCR Clean-Up System (Promega, Mannheim) was used. This kit was also used to purify DNA of PCR reactions. The GeneRuler 1 kb DNA Ladder of New England Biolabs was used as a size marker. For the extraction of plasmid-DNA of *E. coli* the QIAprep Spin Miniprep Kit of QIAgen was used. The TOPO TA Cloning Kit was used for cloning of PCR products. Special kits are mentioned and described in the respective methods.

4.2 Cell cultures

4.2.1 Cultivation of *Escherichia coli* and *Agrobacterium tumefaciens*

E. coli strains were either cultivated as shaking cultures in dYT-medium at 37°C and 200 rpm or on solid dYT-medium (2% (w/v) Bactoagar) under aerobic conditions at 37°C. The antibiotics Ampicillin (100 µg/ml) or Kanamycin (50 µg/ml) were added. *A. tumefaciens* was incubated on the same media but at 28°C.

dYT medium	16 g Bacto Tryptone 10 g Yeast extract 5 g NaCl (20 g Bactoagar) *ad* 1 l with H_2O_{bid}

4.2.2 Cultivation of *Z. tritici, Z. pseudotritici and Z. ardabiliae*

Z. tritici, Z. pseudotritici and Z. ardabiliae strains were either cultivated as shaking cultures in YMS medium at 140 rpm or on solid YMS medium under aerobic conditions at 18°C. Shaking cultures were inoculated using cultures that were kept on plates for maximal one month at 4°C. The glycerol stock cultures were made by adding 50% YMS-Glycerol to a densely grown culture and they were stored at -80°C and always streaked out on solid media plates before further working steps were done.

YMS medium	4 g Yeast extract 4 g Malt extract 4 g Sucrose *ad* 1 l with H_2O_{bid}
YMS-Glycerol	2 g Yeast extract 2 g Malt extract 2 g Sucrose 447.43 g Glycerol *ad* 500 ml with H_2O_{bid}

4.2.3 Identification of the cell density

The cell density of shaking cultures was estimated in two ways. The first way was to count the cells using a "Neubauer-improved" counting chamber. The second way was the measurement of the optical density of the *Z. tritici* shaking culture at 600 nm (OD_{600}). Therefore a Novospec II photometer (Pharmacia Biotech, Munich) was used. To guarantee a linear correlation the measured cultures were diluted (mostly 1:10) to get an OD_{600} value below 0.8. The reference was the OD_{600} of the respective medium. For *Z. tritici* an OD_{600} of 0.6 correlates with 1 x 10^7 cells/ml.

4.3 Strains, oligonucleotides and plasmids

4.3.1 *E. coli* strains

The TOP10 strain (Invitrogen) was used for all experiments. It is a derivate of *E. coli* K12 and has the genotype: F-, mcrA, Δ(mrr-hsdRMS-mcrBC), Φ80lacZΔM15, ΔlacX74, deoR, recA1, araD139, Δ(ara-leu)7697, galU, galK, rpsL(StrR), endA1, nupG.

4.3.2 *A. tumefaciens* strains

The AGL1 strain was used for all experiments (Lazo et al. 1991). It has the genotype: AGL0 (C58 pTiBo542) recA::bla. The marker genes are Rifampicin and Carbenicillin.

4.3.3 *Z. tritici*, *Z. pseudotritici* and *Z. ardabiliae* strains

In this chapter all used strains (Table 3) and strains generated during this project (Table 4) are summarized. For the generation of deletion strains, the genes were replaced with resistance cassettes (Bowler et al. 2010). All strains have been confirmed by Southern analyses to verify correct integration events. The plasmids for the generation of the strains are summarized in chapter 4.3.5.

Table 3: Strains used in this project

Strain	Genotype	Resistance[1]	Reference
Zt09 (IPO323)	mat 1-1	-	(Goodwin et al. 2011)
Zp13	mat 1-1	-	(Stukenbrock 2013)
Zp14	mat 1-2	-	(Stukenbrock 2013)
Za17	mat 1-1	-	(Stukenbrock 2013)
Zt28	mat 1-1 Δ*Zt80707*	Hyg	Master thesis S Poppe

[1] Hygromycin (Hyg)

Table 4: Strains generated in this project

Strain	Genotype	Resistance[1]
Zt107	mat 1-1 Δ*Zt80707*::*Zt80707*	G418
Zt114	mat 1-1 Δ*Zt89160*	Hyg
Zt119	mat 1-1 Δ*Zt110804*	Hyg
Zt126	mat 1-1 Δ*Zt103264*	Hyg
Zt142	mat 1-1 Δ*Zt103264*::*Zt103264*	G418
Zt143	mat 1-1 Δ*Zt89160*::*Zt89160*	G418
Zt145	mat 1-1 Δ*Zt80707*::*Zp13_80707*	G418
Zt146	mat 1-1 Δ*Zt80707*::*Za17_80707*	G418
Zt172	mat 1-1 Δ*Zt89160*::*Zp13_89160*	G418
Zt187	mat 1-1 Δ*Zt103264*::*Za17_103264*	G418
Zt192	mat 1-1 Δ*Zt80707*::*Zp13_80707*-SP	G418
Zt209	mat 1-1 chr1ncr_PgpdA *Zt111221*-GFP	G418
Zt170	mat 1-1 Δ*Zt80707*, chr1ncr_PgpdA *Zt80707*-GFP	G418
Zt212	mat 1-1 chr1ncr_PgpdA *Zp80707*-GFP	G418
Zt165	mat 1-1 chr1ncr_PgpdA *Zt77228*-GFP	G418

[1] Hygromycin (Hyg), Geneticin (G418)

4.3.4 Oligonucleotides

In table 5 all oligonucleotides used for this project are summarized. They were taken for the generation of plasmids, qRT-PCR experiments and 5'-RACE-PCR experiments.

Table 5: used oligonucleotides

Name	Sequence 5' -> 3'[1]	Application[2]
oES174	CGATCGGCCTAGACGGCCTGATATTGAAGGAGCATTTT	*Zt80707* deletion – Hyg-R [F]
oES175	CGATCGGCCGCATCGGCCTAGCAGATCTCTATTCCTTT	*Zt80707* deletion – Hyg-R [R]
oES176	CGATCAAGCTTGATTCGACACGCATTGCGCAAAGGC	*Zt80707* deletion – UF [F]
oES177	CGATCGGCCGTCTAGGCCATTGGAGTGCATTATTAGCG	*Zt80707* deletion – UF [R]
oES178	CGATCGGCCGATGCGGCCATTCTGGGTATCCGTATAGC GTCT	*Zt80707* deletion – DF [F]
oES179	CGATCGAATTCGCCATACCGACTCCTACCCTTCCTC	*Zt80707* deletion – DF [R]
oES1021	GTACCATCGACCACAATGAC	*Zt103264* qRT-PCR [F]
oES1022	ATGCCTGTAGGGAACTTCAC	*Zt103264* qRT-PCR [R]
oES199	CGAGCTTGACGCAGAGTGTG	*Zt80707* qRT-PCR [F]
oES200	ACAGGCTCTGGCAGGAAC	*Zt80707* qRT-PCR [R]
oES786	TGTCGGCAAGGTCATTCCAG	*Zt99044*(GAPDH) qRT-PCR [F]
oES787	ATGCGGCAGGTCAAGTCAAC	*Zt99044*(GAPDH) qRT-PCR [R]
oES883	GCGGAGATGGCAACTATCCC	*Zt89160* qRT-PCR [F]
oES884	ACTTGGCCGCTTTCCATCAG	*Zt89160* qRT-PCR [R]
oES885	ATCATATCCGCCGCCACATC	*Zt110804* qRT-PCR [F]
oES886	TCTAGGCGTGCCTAAAGGAG	*Zt110804* qRT-PCR [R]
oES865	GATCGGCGCGCCCACTTTCAGGCTGCCATAAC	*Zt89160* deletion – UF [F]
oES866	TTCAATATCAGGCCGTCTTGCATGTCTCCGTCGATTTC	*Zt89160* deletion – UF [R]
oES867	TCAGATCATGCTGGGCTCATCGGCCTAGCAGATCTCTA	*Zt89160* deletion – Hyg-R [F]
oES868	AATCGACGGAGACATGCAAGACGGCCTGATATTGAAG G	*Zt89160* deletion – Hyg-R [R]
oES869	GAGATCTGCTAGGCCGATGAGCCCAGCATGATCTGAAC	*Zt89160* deletion – DF [F]
oES870	GATCCCTGCAGGCTGCTGACGCATCAAAGTCC	*Zt89160* deletion – DF [R]
oES871	GATCGAATTCTGGAAGGTGTTTCGGATGTC	*Zt110804* deletion – UF [F]
oES872	TTCAATATCAGGCCGTCTGAGCTTGTGCTGCTAATTGC	*Zt110804* deletion – UF [R]
oES873	TAGCTCAACAGTCATGCGATCGGCCTAGCAGATCTCTA	*Zt110804* deletion – Hyg-R [F]
oES874	AATTAGCAGCACAAGCTCAGACGGCCTGATATTGAAGG	*Zt110804* deletion – Hyg-R [R]

oES875	GAGATCTGCTAGGCCGATCGCATGACTGTTGAGCTAGG	*Zt110804* deletion – DF [F]
oES876	GATCCCTGCAGGGCGTGCCGGTTCTGATTAAG	*Zt110804* deletion – DF [R]
oES973	AGCTACGAATTCGTGCTGTCGCGAAAGTAG	*Zt103264* deletion – UF [F]
oES974	CTCCTTCAATATCAAAGCTGACTGCACCAGCCATAG	*Zt103264* deletion – UF [R]
oES975	AATAGAGATCTGCTAGCCTGTGCGGTGGTCTCTTTG	*Zt103264* deletion – DF [F]
oES976	AAGCTTAGCTTAAGGAGGCGTTGACGATAG	*Zt103264* deletion – DF [R]
oES978	ATGGCTGGTGCAGTCAGCTTTGATATTGAAGGAGC	*Zt103264* deletion – Hyg-R [F]
oES979	AAGAGACCACCGCACAGGCTAGCAGATCTCTATTC	*Zt103264* deletion – Hyg-R [R]
oES705	GATCTCCTGCAGGCATCCGAATCGTCCCAATCC	*Zt80707* complementation – UF [F]
oES706	GATCTGGCGCGCCGCTAGATGGCGCCCTATCAC	*Zt80707* complementation – UF [R]
oES654	GATCGGATCCAGATTCGCCCTTCGTATCGG	*Zt80707* complementation – DF [F]
oES655	GCTAGATGGCGCCCTATCAC	*Zt80707* complementation – DF [R]
oES656	GATCGAATTCTCAGACCGGCGACTGCAACC	*Zt80707* compl. – G418-R [F]
oES657	GATCGGATCCACACGCGACCTCAGAAGAAC	*Zt80707* compl. – G418-R [R]
oES1044	GATCGAATTCACTGCTACCATATGAGTCGC	*Zt103264* compl. – UF [F]
oES1045	CAGTCGCCGGTCTGACTATGGCTGGTGCAGTCACG	*Zt103264* compl. – UF [R]
oES1046	CTGCACCAGCCATAGTCAGACCGGCGACTGCAACC	*Zt103264* compl.– G418-R [F]
oES1047	GCTGGTGCAGTCACGACACGCGACCTCAGAAGAAC	*Zt103264* compl. – G418-R [R]
oES1048	TCTGAGGTCGCGTGTCGTGACTGCACCAGCCATAG	*Zt103264* compl. – DF [F]
oES1049	GATCGTCGACTAGCTTCTGTGACAAGGTGG	*Zt103264* compl. – DF [R]
oES1099	TTTACCTGCAGGTCGAAGCCCGAAGCTGAATG	*Zt89160* compl. – UF [F]
oES1100	CAGTCGCCGGTCTGAATGCGAGAGGAGGGAGTATG	*Zt89160* compl. – UF [R]
oES1101	TCCCTCCTCTCGCATTCAGACCGGCGACTGCAACC	*Zt89160* compl. – G418-R [F]
oES1053	GATCATGCTGGGCTCACACGCGACCTCAGAAGAAC	*Zt89160* compl. – G418-R [R]
oES1054	TCTGAGGTCGCGTGTGAGCCCAGCATGATCTGAAC	*Zt89160* complementation – DF [F]
oES1055	GATCCCTGCAGGCTGCTGACGCATCAAAGTCC	*Zt89160* complementation – DF [R]
oES1131	GATCGAATTCCAAACTGCTTACCCAGACC	*Zp80707* replacement – UF [F]
oES1132	ATTGGAGTGCGTTGTTAGCGGCGAATGCTCTGC	*Zp80707* replacement – UF [R]

oES1133	GAGCATTCGCCGCTAACAACGCACTCCAATCCAG	*Zp80707* replacement – ORF [F]
oES1134	AGTCGCCGGTCTGAAGATACCCAGAATACGTTGCC	*Zp80707* replacement – ORF [R]
oES1135	CGTATTCTGGGTATCTTCAGACCGGCGACTGCAAC	*Zp80707* replacement – G418-R [F]
oES1136	GCGAGACGCTATACGACGCGACCTCAGAAGAACTC	*Zp80707* replacement – G418-R [R]
oES1137	CTTCTGAGGTCGCGTCGTATAGCGTCTCGCATCC	*Zp80707* replacement – DF [F]
oES1138	GATCGAATTCCGCCATACCGACTCCTACC	*Zp80707* replacement – DF [R]
oES1449	CAGACGTATTCGCCAGGTGTATGTCCAGAGGCATACGATA	*Zp13_103264* repl. – ORF [F]
oES1450	GGTTGCAGTCGCCGGTCTGAGATCGTCTAATGTTGTTGTACCGTCG	*Zp13_103264* repl. – ORF [R]
oES1451	TACAACAACATTAGACGATCTCAGACCGGCGACTGCAACC	*Zp13_103264* repl. – G418-R [F]
oES1452	TCACGTAGGCGACCATCTGAACACGCGACCTCAGAAGAAC	*Zp13_103264* repl. – G418-R [R]
oES1453	ATTGGCCGCAGCGGCCATTTCGAATCCTCTTGCCCAGACG	*Zp13_103264* replacement – UF [F]
oES1454	TATCGTATGCCTCTGGACATACACCTGGCGAATACGTCTG	*Zp13_103264* replacement – UF [R]
oES1455	GTTCTTCTGAGGTCGCGTGTTCAGATGGTCGCCTACGTGAC	*Zp13_103264* replacement – DF [F]
oES1456	CCGGCGCGCCCAATTGATTTCGCTGTAGCCAGATACTGTG	*Zp13_103264* replacement – DF [R]
oES1457	GCACCTGGCGCGTTGGGAAGATGGTGGGGGAGAGGAG	*Zp89160* replacement – ORF [F]
oES1458	GGTTGCAGTCGCCGGTCTGATCATGCTGGGCTCGAACGG	*Zp89160* replacement – ORF [R]
oES1459	GTTCTTCTGAGGTCGCGTGTTCTGAACGATTCAATTCATGACCC	*Zp89160* replacement – DF [F]
oES1460	CCGGCGCGCCCAATTGATTTCTGCTGACGCATCAAAGTCC	*Zp89160* replacement – DF [R]
oES1461	CCCCTCCTCTCCCCCACCATCTTCCCAACGCGCCAGG	*Zp89160* replacement – UF [F]
oES1462	ATTGGCCGCAGCGGCCATTTACTGCGCACAGCCAAGATGC	*Zp89160* replacement – UF [R]
oES1463	TCCGTTCGAGCCCAGCATGATCAGACCGGCGACTGCAACC	*Zp89160* replacement – G418-R [F]

oES1464	CATGAATTGAATCGTTCAGAACACGCGACCTCAGAAGAAC	*Zp89160* replacement – G418-R [R]
oES1494	TCTCGTATGCCTCGGGACATACACCTGGCGAATACGTCTG	*Zp14_103264* replacement – UF [R]
oES1495	CAGACGTATTCGCCAGGTGTATGTCCCGAGGCATACGAG	*Zp14_103264* repl. – ORF [F]
oES1496	GGTTGCAGTCGCCGGTCTGACTACGGCGTCTCCAGGC	*Zp14_103264* repl. – ORF [R]
oES1497	TTCGCCTGGAGACGCCGTAGTCAGACCGGCGACTGCAACC	*Zp14_103264* repl. – G418-R [F]
oES1596	ATTGGCCGCAGCGGCCATTTCCCAAACTGCTTACCCAGACC	*Za80707* replacement – UF [F]
oES1597	AGATCTGGATTGGAGTGCATTATTAGCGGCGAATGCTCTGC	*Za80707* replacement – UF [R]
oES1598	CAGAGCATTCGCCGCTAATAATGCACTCCAATCCAGATCTG	*Za80707* replacement – ORF [F]
oES1599	GGTTGCAGTCGCCGGTCTGATCAGTGAAGCTCATGATATGGC	*Za80707* replacement – ORF [R]
oES1600	CATATCATGAGCTTCACTGATCAGACCGGCGACTGCAACC	*Za80707* replacement – G418-R [F]
oES1601	AGACGCTATACGGATACCCAACACGCGACCTCAGAAGAAC	*Za80707* replacement – G418-R [R]
oES1602	GTTCTTCTGAGGTCGCGTGTTGGGTATCCGTATAGCGTCTC	*Za80707* replacement – DF [F]
oES1603	CCGGCGCGCCCAATTGATTTTACCGACTCCTACCCTTCCTC	*Za80707* replacement – DF [R]
oES1604	CTCACGTCCGTCCTCGCCATCGCCGCGCACTTCCAAATAC	*Zp80707*-SP replacement – UF [R]
oES1605	GTATTTGGAAGTGCGCGGCGATGGCGAGGACGGACGTGAG	*Zp80707*-SP replacement – ORF [F]
oES1606	GGTTGCAGTCGCCGGTCTGACTACAGCTGGGAGGTTGAATG	*Zp80707*-SP repl. – ORF [R]
oES1607	ATTCAACCTCCCAGCTGTAGTCAGACCGGCGACTGCAACC	*Zp80707*-SP repl. – G418-R [F]
oES1331	CTGACTACTAGTGGTACCCGGGGATCTTTC	Secretion assay *Zt80707* - PgpdA [F]
oES1367	CTGGATTGGAGTGCATGAATATACTGAAGATGGG	Secretion assay *Zt80707* - PgpdA [R]
oES1368	ATCTTCAGTATATTCATGCACTCCAATCCAGATC	Secretion assay *Zt80707* - ORF [F]

oES690	GGCCGTGGCGATGCTCAGCTGGGAGGTTGAATGTG	Secretion assay *Zt80707* - ORF [R]
oES691	AGCATCGCCACGGCCATGGTGAGCAAGGGCGAGGA	Secretion assay *Zt80707* - bb [F]
oES1130	TACTAGACTAGTACCTGATATTGAAGGAGC	Secretion assay *Zt80707* - bb [R]
oES8	TGGAAAGCGGGCAGTGAG	Secretion assay *Zt77228* - bb [F]
oES1726	CCATCTTCAGTATATTCGATATGCAGAACATCTTCCTCGC	Secretion assay *Zt111221* (LysM) [F]
oES1727	ATGGCCGTGGCGATGCTGATGGCAGACGCAGTGATGTTGTG	Secretion assay *Zt111221* (LysM) [R]
oES1731	CCATCTTCAGTATATTCGATATGGCGAGGACGGACGTG	Secretion assay *Zp80707* [F]
oES1732	ATGGCCGTGGCGATGCTGATCAGCTGGGAGGTTGAATGTG	Secretion assay *Zp80707* [R]
oES202	GACGACCGACTCCTTTGATG	5'RACE-PCR *Zt80707* – synthesis [R]
oES43	GGCAATGGGCTCTTCTCTGG	5'RACE-PCR *Zt80707* – GSP [R]
oES121	ATCGTTCTCGCGAAGGCAGC	5'RACE-PCR *Zt80707* – NGSP [R]
oES859	CGACCACAATGACGCCATTC	5'RACE-PCR *Zt103264*–synthesis [R]
oES46	GACGCCATTCTCGACAGTCC	5'RACE-PCR *Zt103264* – GSP [R]
oES124	CTATGCAAGCGCTGGCTCCG	5'RACE-PCR *Zt103264* – NGSP [R]
oES998	CTGAGTGATGCCCATGAC	5'RACE-PCR *Zp80707* – GSP [R]
oES996	CATGATATGGCATCAATGG	5'RACE-PCR *Za80707*–synthesis [R]
oES997	TCCCTGGACCCGAGAATG	5'RACE-PCR *Za80707* – GSP [R]
oES999	CGCGACCATTTGATCGTC	5'RACE-PCR*Zp103264*–synthesis [R]
oES1000	ACCTGTGAAGTTGCCTAC	5'RACE-PCR *Zp103264* – NGSP [R]
oES1001	GTTGTTGTACCGTCAGCC	5'RACE-PCR*Za103264*–synthesis [R]
oES1002	GTGAAGTTTCCCACAGGC	5'RACE-PCR *Za103264* – NGSP [R]

[1] Sequences are shown in 5' to 3' direction
[2] Oligonucleotides hybridize with the sense-strand [R] or with the complementary strand [F] of the corresponding gene.

4.3.5 Plasmids

In table 6 all plasmids generated during this project are summarized. They were all verified by restriction analyses and when parts of the plasmids were amplified by PCR, DNA sequences were verified by sequencing analyses (Eurofins MWG, Ebersberg).

Table 6: Plasmids used in this project

Name	Application	Resistance[1]	Reference
pES23	*Zt80707* deletion	Amp, Km, Hyg	Master thesis S Poppe
pES46	*Zt80707* complementation	Amp, Km, G418	This study
pES64	*Zt89160* deletion	Amp, Km, Hyg	This study
pES65	*Zt110804* deletion	Amp, Km, Hyg	This study
pES74	*Zt103264* deletion	Amp, Km, Hyg	This study
pES86	*Zt103264* complementation	Amp, Km, G418	This study
pES87	*Zt89160* complementation	Amp, Km, G418	This study
pES98	*Zp80707* (Zp13) replacement	Amp, Km, G418	This study
pES99	*Za80707* (Za17) replacement	Amp, Km, G418	This study
pES126	*Zp89160* (Zp13) replacement	Amp, Km, G418	This study
pES127	*Za103264* (Za17) replacement	Amp, Km, G418	This study
pES143	*Zp80707*-SP (Zp13) replacement	Amp, Km, G418	This study
pES150	*Z. tritici* secretion assay	Km, G418	This study

[1] Hygromycin (Hyg), Geneticin (G418), Ampicillin (Amp), Kanamycin (Km)

4.4 Microbiological Methods

4.4.1 Infection of *Triticum aestivum* and wild grasses with *Z. tritici*

This protocol is a modified version of (Rudd et al. 2007).

Seeds of the wheat cultivar Obelisk (Wiersum Plantbreeding, Winschoten, Netherlands) and wild grasses were pregerminated in sterile conditions in plastic boxes containing water for one week (for wild grasses two weeks). Afterwards the seedlings were planted (four plants per pot) and grown for one week.

A seven day old plate culture of IPO323 or the respective transformant that was used for the infection was used to generate a 50ml YMS liquid culture that was incubated shaking at 18°C for 3 days. This pre-culture was used to adjust the spore concentration of the infection solution to 1 x

10^8 cells/ml. This was either done by counting with a "Neubauer improved" counting chamber or by measuring the OD_{600} of the pre-culture and adjusting it to OD_{600} of 6 which corresponds to the latter spore concentration. The respective volume of spore solution was centrifuged (3.200 rpm, 10 min, 4°C, Heraeus Biofuge Stratos) and resuspended in 50 ml water.

The infection was done by brushing the spore solution on a marked area of the second leaf. After 30min of drying time the seedlings were placed into autoclaving bags together with 500 ml of water. After an initial 48-h incubation period at 100% humidity, the infected plants were incubated at 22°C with a 16-h light period at 75% humidity for another 26 days.

To investigate the effect of gene deletion *in planta*, disease development of wild type and mutant infected plant leaves 28 days post infection (dpi) was compared. Symptoms recognized as fungal caused necrosis and pycnidia were evaluated. To quantify disease levels, a scoring scheme of six categories (0, 1-20, 21-40, 41-60, 61-80 and 81-100) representing the percentage leaf area covered with necrosis and with pycnidia was used.

4.4.2 Phenotypic assays

To test whether any observed phenotype relates to the host-pathogen interaction or to basic growth performance of the mutants, an *in vitro* assay of wild type and deletion mutants was done. At first, single cells of the wild type and the deletion mutants were investigated microscopically using a light microscope (Leica DM750). Next, a stress assay to investigate whether deletion mutants were affected in their response to cell stress reagents was conducted. 4µl of a spore suspension containing 1 x 10^7 spores/ml and 6 1:10 dilutions of a dilution series were pipette on stress-plates and incubated for six days at 18°C. Fungal cells were grown on plates containing NaCl (1.5 M), H_2O_2 (2 mM), Congored (500 µg/ml) and Calcofluor (200 µg/ml) to compare the sensitivity of strains to osmotic and oxidative cell wall stresses. They were also incubated at 28°C to assess temperature sensitivity.

Pycnidiospore viability from wild type and the *Zt80707* and *Zt103264* deletion mutants infected leaves was compared. Therefore infected leaves were harvested four weeks after infection and surface sterilized using 5% Sodium-hypochlorite and 70% ethanol. They were incubated under high-humidity conditions for seven days on a metal grid in a sealed Petri dish in the phytochamber with the same light settings as for the infection experiment (see above). The high humidity conditions in the Petri dish induce the oozing of pycnidia and the release of spores. The

whole leaf samples were vortexed gently in 500µl sterile H_2O and 1:10 dilution series were made with 3 steps. 3 µl of every dilution was pipetted on YMS and YMS-Hygromycin plates. The proportions of germinating spores were compared between the wild type and the deletion strains. To investigate the quantitative difference between deletion mutants of *Zt80707* and *Zt103264* and wild type, the amount of pycnidiospores per pycnidium was investigated. Therefore, oozing of pycnidia on harvested leaves was induced and then a "Neubauer-improved" counting chamber was used to count the amount of pycnidiospores isolated from the oozing pycnidia. Then the pycnidia on those leaves were counted. The number of pycnidiospores in the spore suspensions was divided by the number of pycnidia on each leaf from which the spores where isolated from to estimate the amount of pycnidiospores per pycnidium.

4.4.3 Transformation

4.4.3.1 Rubidium chloride mediated transformation of *E. coli*

This protocol is a modified version of (Cohen et al. 1972). For the generation of competent *E. coli* cells 100 ml dYT-medium including 10 mM $MgCl_2$ and 10 mM $MgSO_4$ was made. This medium was inoculated with 1 ml of a fresh TOP10 overnight culture and incubated at 37°C and 200 rpm until it reached OD_{600} of 0.5. The cells were harvested (3000 rpm, 15 min, 4°C; Heraeus Biofuge Stratos) and resuspended in 33 ml of ice cold RF1-solution. After 30-60 min incubation on ice, cells were spun down (3.000 rpm, 15 min, 4°C; Heraeus Biofuge Stratos), resuspended in 5 ml ice cold RF2-solution and incubated on ice for 15 min. The suspension was aliquoted (50 µl per tube), frozen in liquid nitrogen and stored at -80°C.

RF1-solution	100 mM RbCl 50 mM $MnCl_2$ x $4H_2O$ 30 mM K-Acetate 10 mM $CaCl_2$ x $2H_2O$ 15% (v/v) Glycerol in $H_2O_{bid.}$ pH 5.8 (with Acetate); sterile filtrated
RF2-solution	10 mM MOPS 10 mM RbCl 75 mM $CaCl_2$ x $2H_2O$ 15% (v/v) Glycerol in $H_2O_{bid.}$ pH 5.8 (with NaOH); sterile filtrated

For transformation chemical competent cells were thawed on ice for 10 minutes and also the Eppendorf tubes for the reaction were kept on ice before transformation.
For each transformation the following amounts were mixed.

	Re-Transformation	TOPO-cloning	Ligation
DNA amount	1 µl (0,5µg)	5 µl	10 µl
E. coli	15 µl	80 µl	100 µl

After a 30min incubation on ice the cells were heat shocked at 42°C for 45sec. 5 volumes of dYT-medium were added and the cells were incubated shaking at 37°C for 30min. Cells were streaked out on YT-Amp plates (or the respective resistance) and incubated over night at 37 °C.

4.4.3.2 Transformation of *Z. tritici*

This protocol is a modified version of (Bowler et al. 2010). One week before transformation the according *Z. tritici*, *Z. pseudotritici* or *Z. ardabiliae* strain was streaked out from long term storage at -80°C on Yeast Malt Sucrose plate and incubated at 18°C for 4-5 days.
Two days before transformation four single colonies of the respective transformed *A. tumefaciens* strains were inoculated in 2.5ml of dYT medium in a test tube with Rifampicin (100 µg/ml), Carbenicillin (50 µg/ml) and Kanamycin (40 µg/ml) and incubated shaking (200 rpm) at 28°C for 36-40h.
At the day of transformation a 1:10 dilution of the *A. tumefaciens* pre-cultures with water was prepared. The OD_{600} was measured and adjusted to an OD_{600} of 0.15 in induction medium in a total volume of 5ml in a plastic tube. Incubate 750 µl of the culture at 28°C for 6-8h to a final OD_{600} of 0.3.
Plates of IM-Agar were poured. The fungal cells grown before were scraped off from the plates and resuspended in 1ml sterile water. The cell concentration was determined by counting with a "Neubauer improved" counting chamber and adjusted to 10.000 cells/ml. After that 100 µl of the latter *Zymoseptoria* solution was mixed with 100 µl *A. tumefaciens* culture with an OD_{600} of 0.25 – 0.35. This mixture was streaked out on a nitrocellulose membrane that lied on an Induction medium Agar plate and incubated at 18°C for 2-6 days. After the primary incubation this filter was transferred to an YMS plate containing Hygromycin (150 µg/ml) and Cefotaxime (200

µg/ml). Two weeks later single colonies were picked and patched in order to verify their resistance to Hygromycin that was used as resistance marker. The next step was to singularize those colonies twice on YMS-Hyg plates.
Potential candidates were verified using a Southern-Blot analysis.

Induction medium	dYT medium Carbenicillin (100µg/ml) Rifampicin (50µg/ml) Kanamycin (40µg/ml) Acetosyringone (200µM) 0.4% Glucose (50%)
Induction medium Agar	dYT solid medium Carbenicillin (100µg/ml) Rifampicin (50µg/ml) Kanamycin (40µg/ml) Acetosyringone (200µM) 0.4% Glucose (50%)

4.5 Biomolecular techniques

Standard techniques like purification and precipitation of DNA or cloning techniques are described in detail in (Ausubel et al. 1987) and (Sambrook et al. 2001). The concentration of nucleic acids was estimated using Nanodrop (Nanodrop Technologies, Delaware, USA). The purity of deoxyribonucleic acids was estimated with the ratio of OD_{260} to OD_{280}. Pure DNA has a ratio of 1.8 to 1.9. For pure RNA the value ranges from 2.0 to 2.2. Lower values mean a protein contamination and higher values stand for a contamination with salts or sugars.

4.5.1 In vitro modification of nucleic acids

4.5.1.1 Restriction of DNA

For the restriction of double stranded DNA Type II restriction-endonucleases were used according to the supplier's reaction-conditions of each enzyme. The incubation was conducted for at least 1-3 hours at the recommended temperature (usually 37°C – 50°C). An Aliquot was loaded on an Agarose-gel, separated and analyzed. A typical restriction setup was:

X µl DNA (0.5 - 10 µg)
2 µl enzyme specific NEB buffer (10x)
2 µl 10 x Bovine Serum Albumin (if needed)
0.5 U endonuclease
ad 20 µl $H_2O_{bid.}$

4.5.1.2 Ligation of DNA

T4-DNA-Ligase was used for linking two double stranded DNA-molecules to each other covalently. For the ligation of vectors and inserts a molar vector : insert ratio of 1:3 – 1:5 was adjusted. For blunt-end reactions the amount of insert was usually increased (Vector : Insert ratio of at least 1:5). A typical ligation approach:

x mol Vector
3x - 10x mol Insert
1.5 µl T4-DNA-Ligase buffer (10x)
1 µl T4-DNA-Ligase (400 U/µl)
ad 15 µl with $H_2O_{bid.}$

The incubation was usually conducted over night (ca. 16 h) at 16°C in a water bath. For small inserts (<2 kb) with sticky ends an incubation for 15 min at room temperature was sufficient.

4.5.1.3 Polymerase chain reaction

For the amplification of DNA-fragments the polymerase chain reaction (PCR) was used. For fragments smaller than 1 kb the Taq polymerase (NEB) was used. Fragments for plasmids were created using the Phusion polymerase which possesses proof reading and has a reduced mutation rate. A PCR reaction consists of: Initial denaturing – (denaturing – annealing – elongation) x 35 – final elongation. The annealing temperature of oligonucleotides was estimated using the program Clone Manager and the elongation time was adjusted to the synthesis speed of the polymerase. A typical approach looked like the following:

NEB Taq polymerase:

~50 µg template DNA
250 µM dNTPs
0.2 µM Fwd. primer
0.2 µM Rev. primer
1 x concentrated polymerase buffer
1 U Taq polymerase
ad 50 µl with $H_2O_{bid.}$

Phusion polymerase:

~50 µg template DNA
250 µM dNTPs
0.2 µM Fwd. primer
0.2 µM Rev. primer
1 x concentrated HF-buffer
0.5 U Phusion polymerase
ad 50 µl with $H_2O_{bid.}$

PCR reactions were conducted in either the T-personal 48 or the T-Professional Standard Gradient Thermocycler of Biometra with the following program:

NEB Taq polymerase:

Step 1) 95°C – 2 min
Step 2) 95°C – 30 sec
Step 3) 55-65°C – 30 sec } 30 – 35 x
Step 4) 68°C – 60 sec/kb
Step 5) 68°C – 5 min

Phusion polymerase:

Step 1) 98°C – 30 sec
Step 2) 98°C – 10 sec
Step 3) 55-65°C – 20 sec } 30 – 35 x
Step 4) 72°C – 15 sec/kb
Step 5) 72°C – 5 min

4.5.2 Isolation of nucleic acids

4.5.2.1 "Quick & Dirty" DNA-Isolation of *Z. tritici*

This method was used for preliminary screenings of transformants in order to save time and avoid unnecessary working with Phenol/Chloroform. For this isolation 200µl of an overnight culture in YMS liquid medium was centrifuged (13.000 rpm, 30 sec, RT, Heraeus Fresco 17) and the supernatant was discarded. The pellet was boiled in a microwave for 1min at 900W and a 1.5 ml Eppendorf tube (13.000 rpm, 30 sec, RT, Heraeus Fresco 17) afterwards solved in 50µl TE buffer. After a repetition of the boiling step the tube was centrifuged for 3 min at 13.000 rpm and the supernatant was used for PCR reactions.

4.5.2.2 DNA-Isolation of *Z. tritici* with Phenol/Chloroform

This method is a modified version of (Hoffman and Winston 1987). For this isolation 1.5 ml of an overnight culture in YMS liquid medium were pelleted together with 0.2 g glass beats in a 2 ml Eppendorf tube (13.000 rpm, 30 sec, RT, Heraeus Fresco 17). The supernatant was decanted and the pellet was dissolved in 400µl *Zymoseptoria* lysis buffer and 400µl TE-

Phenol/Chloroform. The samples were incubated shaking for 10 min using a Vibrax-VXR shaker (IKA) and after the phase separation (13.000 rpm, 5 min, RT, Heraeus Fresco 17) 400µl of the supernatant were transferred to a new Eppendorf tube and precipitated with 1 ml ethanol. After the centrifugation (13.000 rpm, 30 sec, RT, Heraeus Fresco 17) the dried pellet was solved in 50µl TE with 20µg/ml RNase A, resuspended at 50°C for 10 min and then stored at -20°C.

Zymoseptoria lysis buffer:	10 mM Tris-HCl, pH 8.0 10 mM EDTA 1% (w/v) SDS 10 % Triton X 100 100 mM NaCl in $H_2O_{bid.}$
TE-Phenol/Chloroform:	Mixture of equal volumes of Phenol (equilibrated with TE buffer) and Chloroform

4.5.2.3 RNA isolation of *Z. tritici*

Filter-tips, RNase free tubes and gloves were used for all steps of the protocol. Samples were mortared in liquid nitrogen and filled into Eppendorf tubes to an approximate volume of 0.5 ml. Then 1 ml TRIZOL-reagent was added to the mortared tissue and immediately solved to prevent the TRIZOL from freezing. The samples were centrifuged (12000 g, 10 min, 4°C, Heraeus Fresco 17) and the supernatant was transferred to a new tube, 200µl Chloroform was added and the tube was inverted multiple times. Following centrifugation (12000 g, 15 min) the upper aqueous phase was transferred to a new tube and 500 µl Isopropanol was added and samples were incubated at RT for 10 min. Following centrifugation (12000 g, 10 min), the supernatant was discarded and the pellet was washed and vortexed in 1ml 75% ethanol. After centrifugation (7500 g, 5 min), the supernatant was discarded and the pellet was dried but not completely. It was solved in 20-50µl DEPC-H_2O and incubated at 55-60°C for 10 min.

4.5.3 Separation and analysis of nucleic acids

4.5.3.1 Agarose gel electrophoresis of DNA

Gel electrophoresis enables specific separation of DNA according to its size in a small electric field. The agarose concentration of the gels varied between 0.8% and 2%. For the generation of

the gels the respective amount of agarose was solved in 1x TAE or 0.5X TBE by boiling it and after cooling down (approximately 60°C) Ethidium bromide was added (final concentration: 0.5µg/ml). The agarose solution was poured into a gel form and for solidification at room temperature. After that it was transferred into a gel chamber and covered with the respective buffer. The DNA was now loaded into the gel on the side of the cathode (therefore the DNA was solved in a non-denaturing loading buffer) and was exposed to an electric field of 5 -10 V/cm. Under these conditions the DNA migrates according to its size as a polyanion to the anode. The DNA was visualized by radiating it at UV light (254 nm) which lead to an excitation of the Ethidium bromide that intercalates in the DNA or RNA and a following emission of low-energy photons of the more long-wave red part of the spectrum.

5 x TBE buffer	440 mM Tris base 440 mM Boric acid 10 mM Na_2-EDTA In $H_2O_{bid.}$
50 x TAE buffer	2 M Tris base 2 M Acetic acid 50 mM Na2-EDTA In $H_2O_{bid.}$
6 x non-denaturing loading buffer	50% (w/v) Sucrose 0.01% (w/v) Bromophenol blue In TE buffer

4.5.3.2 Transfer and detection of DNA on membranes [modified from (Southern 1975)]

10-25µg of isolated genomic DNA of the transformants was cut with an endonuclease in a total volume of 20µl according to the recommended conditions. After the separation of the DNA by gel electrophoresis the nucleic acids were transferred to a nylon membrane by a capillary blot. First of all the gels were shaken in 0.25 M HCl for 20 minutes to depurinate the containing DNA fragments. After that the gel was shaken in 0.4 M NaOH for 20 min to solve the Hydrogen bonds. These preliminary steps were conducted to facilitate the transfer of the DNA to the nylon membrane by a capillary blot. In this process the transfer solution (0.4 M NaOH) was sucked by capillary forces from a buffer reservoir through the gel into a stack of paper towels that was placed on top of the blot. By this buffer stream the DNA fragments were eluted from the gel

transferred to the positively charged nylon membrane (Roche) that lied between the gel and the paper towels. The capillary blot was normally conducted over night but for at least 3 h. For the detection of the wanted bands the membranes were prehybridized in Southern hybridization buffer for 30 min at 65°C in a hybridization oven. After that the probe was denatured in hybridization buffer at 100°C for 10 min und exchanged with the prehybridization buffer. The hybridization was conducted over night for approximately 16 h. The detection of luminescence is separated into multiple steps. All of them are done in the hybridization tube at room temperature:

- 2x 20 min washing with Southern wash buffer (the first time still at 65°C)
- 5 min washing with 30 ml DIG wash buffer
- 30 min incubation in 30 ml DIG II buffer (blocking reaction)
- 30 min incubation in 10 ml antibody solution
- 2x 15 min washing in 30 ml DIG wash buffer
- 5 min equilibration in 30 ml DIG III
- 5 min incubation in 10 ml CDP-Star-solution
- shrink-wrap membrane in plastic foil and incubate for 15 min at 37°C
- apply Rontgen film in the darkroom and develop it. Adjust developing times to the signal

Southern hybridization buffer	0.5 M Na-Phosphate buffer, pH 7,0 7 % SDS in $H_2O_{bid.}$
Southern wash buffer	0.1 M Na-Phosphate buffer, pH 7,0 1 % SDS
Antibody solution	1 µl Anti-DIG-AP (Roche) / 10ml DIG2 buffer AB centrifuged for 5min at 10.000 rpm before use
CDP-Star-solution	100 µl CDP-Star (Roche) in 10ml DIG3 buffer
DIG I	0.1 M Maleic acid 0.15 M NaCl Adjust pH 7.5 with 5M NaOH
DIG II	Blocking solution 1:10 in DIG I
Blocking solution	10% Blocking reagent (Roche) in DIG I

DIG III	0.1 M NaCl 0.05 M $MgCl_2x6H_2O$ Adjust pH 9.5 with 1 M Tris-HCl
DIG-Wash	0.3 % (v/v) Tween20 in DIG I

4.5.3.3 Quantitative RT-PCR

For expression analysis 1 µg was used for a DNase I digestion (Ambion/Invitrogen) to remove residual genomic DNA. Then the RNA was reverse transcribed into cDNA using the First Strand cDNA Synthesis Kit (Thermo Scientific). The cDNA samples were used in a qRT-PCR experiment using the iQ SYBR Green Supermix Kit (Bio-Rad, Munich, Germany), gene specific primers (Table 5) and an annealing temperature of 59°C. The PCR was conducted in a CFX96 Real-Time PCR Detection System (Bio-Rad) with the constitutively expressed control gene *Zt99044*, a Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) using the following protocol:

Step 1) 95°C – 3 min
Step 2) 95°C – 10 sec
Step 3) 59°C – 10 sec } 40 x
Step 4) 72°C – 30 sec/kb

The specificity of the amplification was verified using the melting curve of the CFX96 Real-Time PCR Detection System. The calculation of the ct-value (threshold cycle), which describes the cycle at which the fluorescence is higher than the background fluorescence, was done using the Bio-Rad CFX Manager Software. The expression of *Z. tritici* genes was depicted relative to the constitutively expressed control gene *Zt99044* (GAPDH). Three independent experiments were conducted and the mean of the values was used for the analysis.

4.5.3.4 5'- and 3'- RACE-PCR (Rapid Amplification of cDNA Ends)

Rapid Amplification of cDNA Ends (RACE) is a procedure for amplification of nucleic acid sequences from a messenger RNA template between a defined internal site and unknown sequences at either the 3' or the 5' -end of the mRNA.

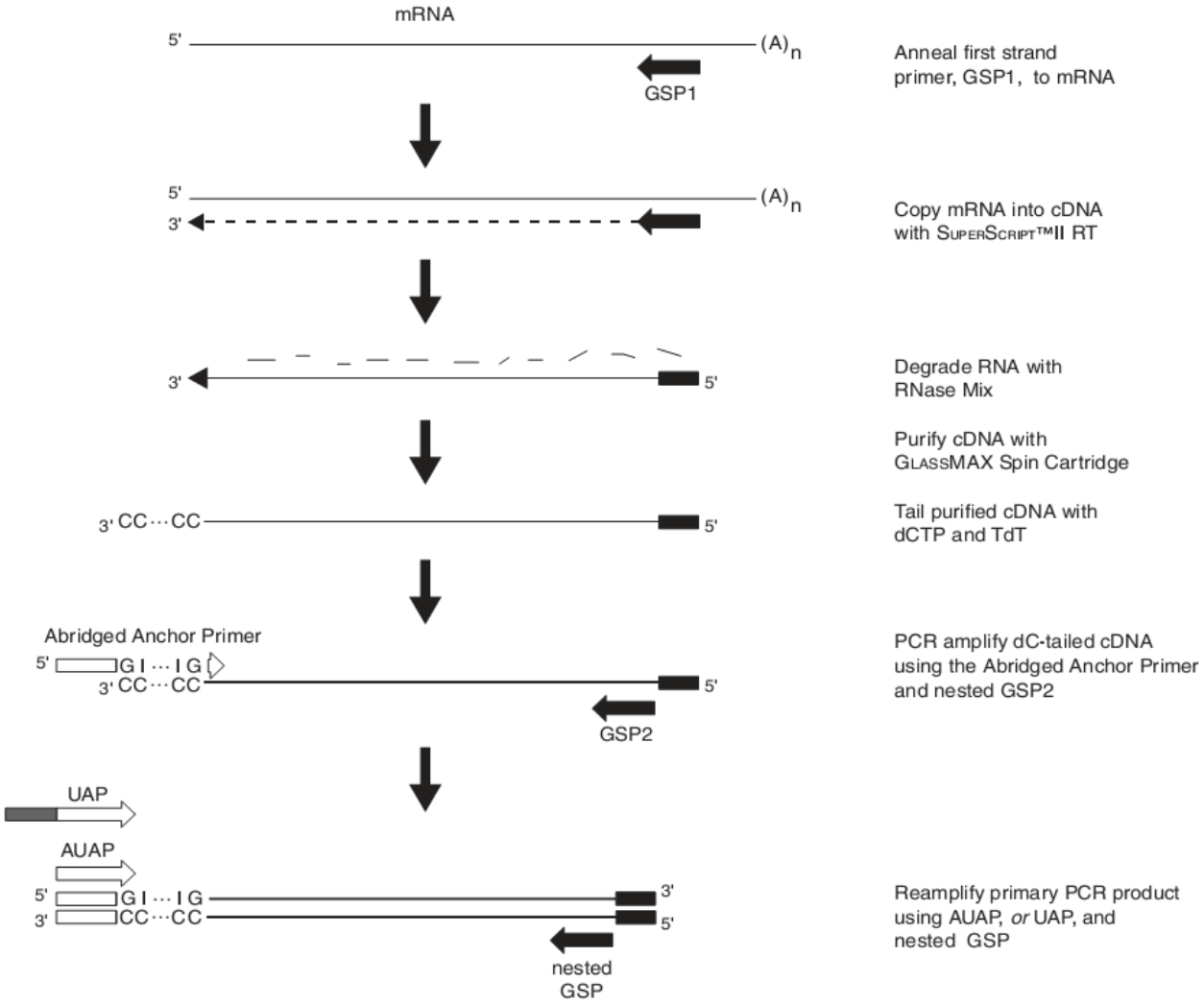

Figure 22: Overview of the 5' RACE procedure

3' RACE takes advantage of the natural poly(A) tail in mRNA as a generic priming site for PCR amplification. In this procedure, mRNAs are converted into cDNA using reverse transcriptase (RT) and an oligo-dT adapter primer. Specific cDNA is then directly amplified by PCR using a gene-specific primer (GSP) that anneals to a region of known exon sequences and an adapter primer that targets the poly (A) tail region. This permits the capture of unknown 3'-mRNA sequences that lie between the exon and the poly (A) tail and the identification of the direction of transcription.

5' RACE, or "anchored" PCR, is a technique that facilitates the isolation and characterization of 5' ends from low-copy messages (Frohman 1993). First strand cDNA synthesis is primed using a gene-specific antisense oligonucleotide (GSP1) permitting cDNA conversion of specific mRNA, or related families of mRNAs, and maximizes the potential for complete extension to the 5' end

of the message (Fig. 22). Following cDNA synthesis, the first strand product is purified from unincorporated dNTPs and GSP1. TdT (Terminal deoxynucleotidyl transferase) is used to add homopolymeric tails to the 3' ends of the cDNA. Tailed cDNA is then amplified by PCR using a mixture of three primers: a nested gene-specific primer (GSP2), which anneals 3' to GSP1; and a combination of a complementary homopolymer containing anchor primer and corresponding adapter primer which permit amplification from the homopolymeric tail. This allows amplification of unknown sequences between the GSP2 and the 5'-end of the mRNA and also identifies the direction of transcription.

Both techniques were conducted for *Zt80707*, *Zt103264* and their homologs in *Z. pseudotritici* and *Z. ardabiliae* according to the supplier's protocol.

4.6 Biochemical methods

4.6.1 Protein extraction

4.6.1.1 Protein extraction of *Z. tritici* axenic culture

Z. tritici was grown in 50ml YMS medium at 200 rpm and 18°C for 72 h until an OD_{600} of 1. For protein extraction, cultures were centrifuged (10.000 g, 15 min, 4°C; Heraeus Biofuge Stratos) and the cells were mortared in liquid nitrogen. The resulting powder was transferred into an Eppendorf tube to an approximate volume of 0.5 ml and the extraction of proteins was conducted using the peqGOLD TriFast™-kit (Peqlab, Erlangen, Germany) according to the supplier's protocol.

4.6.1.2 Protein extraction of *Z. tritici* culture supernatants

Z. tritici was grown in 50ml YMS medium at 200 rpm and 18°C for 72 h until an OD_{600} of 1. For protein extraction of the culture supernatant, cultures were centrifuged (10.000 g, 15 min, 4°C; Heraeus Biofuge Stratos) and 40 ml of the supernatant was lyophilized in an Alpha 1-4 freeze drier (Christ, Osterode, Germany). The freeze dried culture was dissolved in 1 ml $H_2O_{bid.}$ and a TCA precipitation (Sambrook et al. 2001) was conducted. The resulting protein pellet was solved in 50 µl 1% SDS and 5 – 15 µl were used for a SDS polyacrylamide gel electrophoresis (SDS-PAGE).

100% TCA solution	220 g Trichloroacetic acid in 100 ml $H_2O_{bid.}$

4.6.2 Transfer and detection of proteins on membranes

4.6.3.1 SDS polyacrylamide gel electrophoresis

For the separation of proteins a discontinuous sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) was conducted (Laemmli 1970). With this method proteins get a constant negative charge by binding to SDS, what makes the separation in an electrical field possible. The gels consist of a stacking and a running gel. The stacking gel has the function to concentrate the proteins in one layer before they enter the running gel. Inside the running gel the proteins were separated according to their size in the polyacrylamide grid. The molecular weight

of the separated proteins was estimated with a comparison to an applied pre-stained protein ladder (10 – 170 kDa; PageRuler Prestained Protein Ladder; Thermo Scientific). The gels were loaded in gel chambers (Mini Protean Tetra System; Bio-Rad, Munich), filled with SDS running buffer and the separation was carried out at 15 mA/gel in the stacking gel and 20 mA/gel in the running gel.

2 x Laemmli sample buffer	4 ml 10% SDS 1.2 ml 1 M Tris-HCl, pH 6.8 200 µl 1% Bromophenol blue 2 ml 1 M Dithiothreitol (DTT) *ad* 10 ml $H_2O_{bid.}$
10 x SDS running buffer	72.05 g Glycine 25 ml SDS (20%) 15.15 g Tris-Base *ad* 500 ml $H_2O_{bid.}$
Stacking gel	5% (v/v) Acrylamide 0.1% (w/v) SDS 125 mM Tris-HCl, pH 6.8 To start the polymerization: 0.1% (w/v) Ammonium persulfate 0.05% (v/v) TEMED
Running gel	15% (v/v) Acrylamide 0.1% (w/v) SDS 375 mM Tris-HCl, pH 8.8 To start the polymerization: 0.1% (w/v) Ammonium persulfate 0.05% (v/v) TEMED

The proteins separated in the polyacrylamide gel were transferred to a nitrocellulose membrane (Amersham Hybond ECL Nitrocellulose Membrane; GE Healthcare) with an electrophoretic transfer (Trans-Blot SD Transfer Cell; Bio-Rad). One layer of Whatman paper (3 mm) was soaked with transfer buffer and covered with the membrane. The gel was placed on the membrane, air bubbles were removed and it was covered with another soaked Whatman paper. The transfer was conducted with 15 V and 500 mA for 90 min.

Western transfer buffer	12.5 ml 1 M Tris-HCl, pH 10.4 7.20 g Glycine 75 ml Ethanol *ad* 500 ml $H_2O_{bid.}$

The membrane was incubated with blocking solution at RT for 1 h, shortly washed with TBST buffer and then the antibody solution containing the primary α-GFP antibody (1 : 5000; Anti-GFP Mouse IgG, monoclonal antibody; Roche) was added and incubated at RT for 1 h. Then the membrane was washed three times with TBST for 10 min and afterwards incubated in the antibody solution containing the secondary HRP (Horseradish peroxidase) linked α-Mouse antibody (1 : 2000; Anti-mouse IgG; NEB) at RT for 1 h. Following three more washing steps in TBST, the chemiluminescence reaction was conducted using the Amersham ECL Prime Western Blotting Detection Reagent (GE Healthcare, Freiburg, Germany) according to the supplier's protocol. Then the membrane was sealed in plastic foil and developed in a dark room. The film (Medical X-Ray Screen; CEA, Assamstadt) was exposed for 60 s – 6 h depending on the signal strength and developed in a developing machine (QX-60; Konica, Munich).

TBST	150 mM NaCl 0.05% (v/v) Tween20 in 50 mM Tris-HCl, pH 7.5
Blocking solution	5% (w/v) Skimmed milk powder in TBST
Antibody solution	Antibodies diluted in 2.5% (w/v) Skimmed milk powder in TBST

4.7 Staining and microscopy

4.7.1 Staining WGA-FITC / Propidium iodide

Harvested leaf samples were de-stained over night (or longer) in 2 ml Eppendorf tubes in 100% ethanol. Ethanol was exchanged if necessary. After that the leaves were incubated in 10% KOH at 85°C for 30 min. Then the samples were washed 3-4 times with PBS (pH 7.4) and staining solution was added. Inside the staining solution the samples were vacuum infiltrated at 100 mbar using a vacuum pump (cvc3000; vacuubrand/VWR). The staining solution was collected for reuse and the samples were de-stained in PBS and stored in the dark at 4°C until microscopy.

Staining solution	20 µg/ml Propidium iodide 10 µg/ml WGA-FITC 0.02% Tween 20 In 1 x PBS (pH 7.4)	
Stock solutions	Propidium iodide:	10 mg/ml in PBS, stored at 4°C
	WGA-FITC:	1 mg/ml in H_2O, stored at 4°C in the dark
	10% KOH:	20 g 85% KOH pellets in 100 ml H_2O

4.7.2 Light microscopy

Light microscopic pictures where either taken using a microscope (DM750; Leica, Wetzlar) or a binocular (S8APO; Leica) and a camera (DFC450; Leica) that could be mounted on the camera and the microscope. Pictures were made and edited using the Leica Application Suite.

4.7.3 Confocal microscopy

The confocal microscopic pictures were made using a Leica TCS SP5 II Confocal Laser Scanning Microscope (Leica, Wetzlar). The filter wavelengths that were used for the excitation were 488nm (for FITC) and 561 nm (for Propidium iodide). The fluorophors were excited by using an Argon and DPSS Laser. Pictures were made and edited using the Leica Application Suite.

4.8 Bioinformatic analyses

Nucleotide sequences were taken from the database GenBank of the National Center of Biotechnology Information (NCBI; www.ncbi.nlm.nih.gov/) and the *Z. tritici* database of the Joint Genome Institute (JGI; http://genome.jgi-psf.org/Mycgr3/Mycgr3.home.html). They were translated into amino acid sequences using the translate tool of the Expert Protein Analysis System (ExPASy; http://web.expasy.org/translate/). The ExPASy portal also provides tools to estimate the molecular weight of proteins (http://web.expasy.org/compute_pi/) and to search for conserved domains or motifs inside amino acid sequences (http://prosite.expasy.org/). For the identification of N-terminal signal peptides, SignalP was used (Bendtsen et al. 2004), that also could predict the putative cleavage site of the signal peptidase. Alignments of nucleotide- and amino acid sequences were done using the program Seaview (Gouy et al. 2010) and positive

selection analyses were conducted using DnaSP (Librado and Rozas 2009). The prediction of protein 3D structures was done using the I-TASSER server (Roy et al. 2010). Visualization and modification of the modeled protein structures was conducted using the PyMOL Viewer (Schrödinger, LLC 2010).

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