Epigenetics of the plant pathogenic fungus *Zymoseptoria tritici*

Thesis

Bachelor of Science

Department of Biology

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1 Summary/ Zusammenfassung

1.1 Summary

In this bachelor thesis, two epigenetic mechanisms were analysed in the hemibiotrophic plant pathogenic fungus *Zymoseptoria tritici*.

The first objective was to study the role of the histone methyltransferases KMT1 and KMT6 in virulence of *Z. tritici*. They are important for transcriptional gene regulation, as these proteins methylate lysine residues on the histone H3 and thereby contribute to formation of heterochromatin, which leads to silencing of genes and transposable elements or low gene transcription. To study the effect of KMT1 and KMT6 on virulence a plant infection assay was conducted on wheat, the host plant of *Z. tritici.* The infection symptoms were compared between a *Z. tritici* wild type strain and deletion mutants lacking the histone methytransferases KMT1 or KMT6, as well as a double deletion mutant.

Plants infected with the *kmt1* deletion mutant showed strongly decreased infection symptoms. This could indicate a role of KMT1 for the virulence or for normal growth of the fungus. The histone methyltransferase KMT6 seems to have less influence on the infection process. Infection induced symptoms on the leaves were just slightly decreased compared to leaves infected with the wild type strain of *Z. tritici*. The double deletion mutant on the other hand caused comparable low infection symptoms on the plants as the *kmt1* deletion mutant.

The second objective of this thesis was to study DNA methylation in *Z. tritici*. In most of its strains DNA methylation is absent, because the gene *dim2* encoding for DNA methylation is not functional. However, in the isolate Zt10, collected from Iran DNA methylation is still present because it contains a functional *dim2* gene. During this thesis DNA methylation in the stain Zt09 should be restored by integrating the functional *dim2* gene from Zt10. Also DNA methylation should be inactivated in Zt10 by replacing the functional *dim2* gene with a resistance cassette for the antibiotic hygromycin. Plasmids were created to integrate the respective inserts into the genome of *Z. tritici* by *A. tumefaciens* mediated transformation. It was possible to establish Zt10 deletion mutants for dim2, whereas no positive transformation of Zt09 and thereby integration of a functional *dim2* gene could be confirmed.

1.2 Zusammenfassung

In dieser Bachelorarbeit wurden zwei epigenetische Mechanismen im hemibiotrophen und

pflanzenpathogenen Pilz *Zymoseptoria tritici* untersucht.

Ein Ziel dieser Arbeit war es, die Rolle der Histon-Methyltransferasen KMT1 und KMT6 in der Virulenz von *Z. tritici* zu untersuchen. Diese Proteine sind wichtige Faktoren in der transkriptionellen Genregulation, da sie für die Methylierung von Lysinresten am Histon H3 verantwortlich sind und somit zur Ausbildung von Heterochromatin beitragen, was zu einer geringen Transkriptionsrate oder zur Stilllegung von Genen führt. Um die Auswirkung von KMT1 und KMT6 auf die Virulenz von *Z. tritici* zu untersuchen, wurde die Wirtspflanze Weizen mit unterschiedlichen *Z. tritici* Deletionsstämmen infiziert. Hierbei wurde die Virulenz eines Wildtypstammes verglichen mit der von Deletionsmutanten, denen entweder KMT1, KMT6 oder beide Histon-Methyltransferasen fehlen. Pflanzen, die mit *kmt1* Deletionsmutanten infiziert wurden entwickelten stark reduzierte Infektionssymptome. Daraus lässt sich auf eine mögliche Funktion von KMT1 auf die Virulenz oder das Wachstum des Pilzes schließen. Die Histon-Methyltransferase KMT6 scheint weniger Einfluss auf die Infektion zu haben. Pflanzen die mit einer *kmt6* Deletionsmutanten infiziert wurden zeigten im Vergleich zum Wildtyp leicht reduzierte Symptome. Die Doppeldeletionsmutante hingegen zeigte eine vergleichbar schwache Virulenz wie die *kmt1* Deletionsmutante.

Der zweite Teil dieser Arbeit beschäftigt sich mit der DNA Methylierung in *Z. tritici*. In den meisten *Z. tritici* Stämmen ist DNA Methylierung nicht vorhanden, da das dafür erforderliche Gen *dim2* nicht funktionsfähig ist. Im Stamm Zt10, welcher aus dem Iran stammt, ist dieses Gen hingegen aktiv und somit eine DNA Methylierung vorhanden. Ziel dieser Arbeit war es, das funktionsfähige *dim2* Gen aus Zt10 in das Genom des *Z. tritici* Stammes Zt09 zu integrieren und dadurch die DNA Methylierung wieder herzustellen. Ein weiteres Ziel war das *dim2* Gen in Zt10 durch eine Resistenzkassette für das Antibiotikum Hygromycin zu ersetzen, wodurch die DNA Methylierung inaktiviert würde. Es wurden Plasmide mit den erforderlichen Integrationskonstrukten hergestellt, welche durch *A. tumefaciens* vermittelte Transformation in die *Z. tritici* Stämme integriert werden sollten. Das *dim2* Deletionskonstrukt konnte erfolgreich in das Genom des Stammes Zt10 integriert werden. Jedoch konnte kein positiv transformierter Zt09 Kandidat mit einem funktionsfähigen *dim2* Gen bestätigt werden.

2 Introduction

2.1 The wheat pathogen Zymoseptoria tritici

2.1.1 Lifestyle and origin of *Z. tritici*

Z. tritici (synonym: *Mycospherella graminicola*) is a filamentous ascomycete fungus that belongs to the class of Dothideomyces. It is a plant pathogenic fungus which can cause the disease *Septoria tritici blotch* (STB) on its host plant wheat (*Triticum aestivum*).

It is highly specified to its host and yet there is no record of *Z. tritici* infecting other plants. The lifestyle of *Z. tritici* can be described as hemibiotrophic, as a biotrophic infection is followed by a necrotrophic phase **(**Ponomarenko et al., 2011). The infection takes place through germinating hyphae of (sexual) ascospores or (asexual) pycnidiospores entering open stomata on the leaf surface. After infection the filamentous hyphae proliferates biotrophically into the intercellular spaces. In this phase there are no symptoms caused by the fungus. After a switch of lifestyle STB symptoms appear in the following necrotrophic phase.

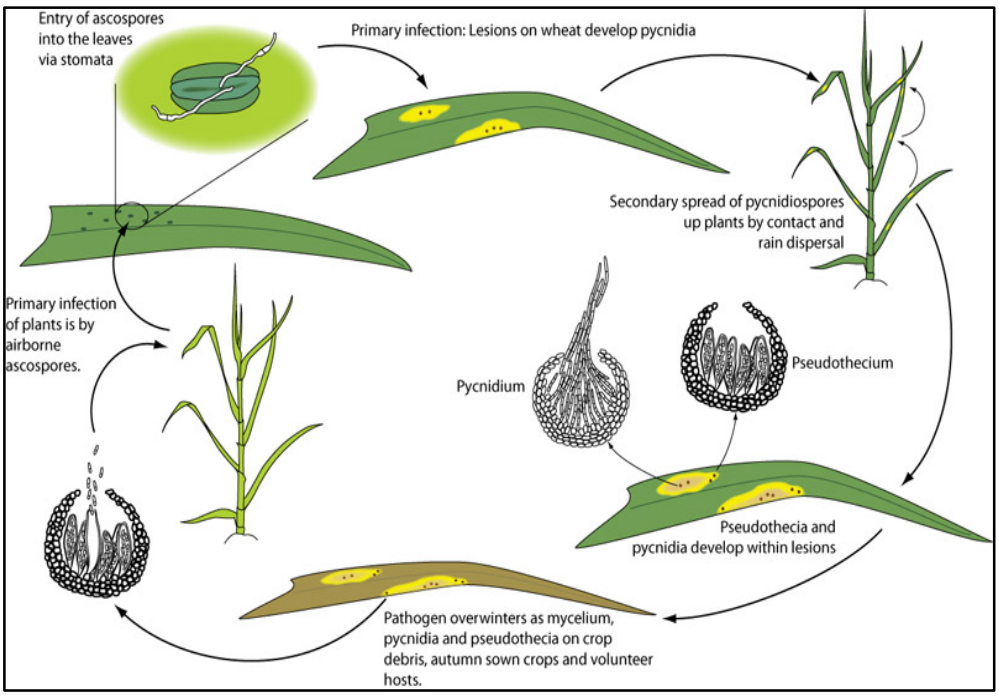


Figure 1: Life and infection cycle of *Z. tritici* on wheat (Ponomarenko et al., 2011). Sexually produced ascospores infecting the leaf though stomata. After a phase of symptomless biotrophic growth, the fungal lifestyle shifts to necrotrophy and first symptoms appear. Asexual pycnidiospores get dispersed by rain and plant contact, thereby infecting other plants. The pathogen can overwinter on the host in form of inactive spores.

The disease typically causes necrotic lesions and pycnidiospores on the leafs (Figure 1). STB is a common wheat disease worldwide. Because it reduces the yield, STB has an impact on food availability and economics. *Z. tritici* co-evolved with its host since the beginning of wheat domestication around 8.000-9.000 BC in the Fertile Crescent, today a region in the middle east (Stukenbrock et al., 2007).

2.1.2 Genome of Z. tritici

The reference strain of *Z. tritici* (IPO323) has a haploid set of 21 chromosomes. 13 of these chromosomes are essential for the fungus, they are called core chromosomes and can be found

in all isolates (Goodwin et al., 2011). The remaining 8 chromosomes are dispensable, meaning they are not present in every isolate and a functional importance of these chromosomes could not be detected yet (Goodwin et al., 2011). In conclusion, different *Z. tritici* isolates can have a different number of chromosomes. The dispensable chromosomes differ from core chromosomes (Schotanus et al. 2015). Characteristic for dispensable chromosomes is a twofold higher proportion of repetitive sequences (Figure 2), as well as low gene transcription a lower gene content. (Kellner et al. 2014) The amount of repetitive DNA can be correlated to the high frequency of transposable elements (TEs). An enrichment of H3K27me3 resulting in heterochromatin formation on the dispensable chromosomes could be an explanation for the low transcription rate (Schotanus et al. 2015).

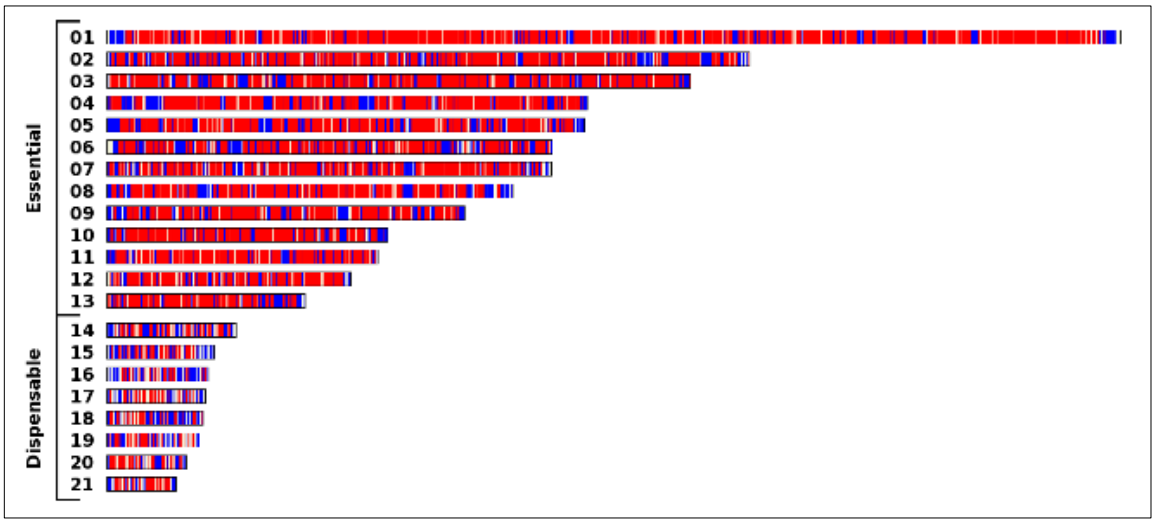


Figure 2. The chromosomes of reference isolate IPO323. The core chromosomes 1-13 and the accessory chromosomes 14-21 (Goodwin et al., 2011). They differ in their size (1,8-6.08 MB for chore chromosomes and 409-774 kb for accessory chromosomes) and content of repetitive sequences (indicated in blue).

2.2 Epigenetic regulation of gene expression

2.2.1 Methylation of DNA

One way how gene expression is regulated in eukaryotes is methylation of DNA. It is an important factor for gene regulation and is therefore involved in numerous cellular processes and the development of illnesses such as cancer (Phillips, 2008). It is a common and important epigenetic mechanism for inactivation of genes. Methylation can occur on the cytosine bases of the DNA. Due to enzymes called DNA-methyltransferases (DNMTs) methyl groups can be added or maintained on the cytosine bases. DNMTs can be differentiated into de novo DNMTs that add an initial methyl group to the DNA, and maintenance DNMTs that copy the methylation to the new DNA strand after replication (Phillips, 2008). Within the eukaryotes,

plants show the highest rate of methylated cytosines in their genome. In fungi, DNA methylation mainly occurs on repetitive sequences or on transposable elements (TEs). Here, the methylation appears to be a mechanism to prevent the TEs from further amplification in the genome. Certain species like *Z. tritici* (IPO323) do not have a functional DNA methylation machinery (Dhillon et al., 2010).

2.2.2 Role of DNA methylation in *Z. tritici*

In the genome of *Z. tritici* DNA methylation is thought to be absent. A genome-wide methylation assay of the reference strain IPO323 showed lacking cytosine methylation, because the gene encoding for the methyltransferase *dim2* is inactivated. Based on sequence data and Southern Blot (M. Möller, unpublished data, Dhillon et al., 2010) DNA methylation is present in the sister species *Z. ardabiliae* and *Z. pseudotritici*. Loss of DNA methylation in *Z. tritici* probably occurred due repeat induced point mutation (RIP). This is a type of genome mutation, that is so far only observed in fungal taxa. It targets duplicated sequences in both DNA strands and induce single nucleotide point mutations (SNPs), thereby the nucleotides adenine and thymine replace guanine and cytosine. This can lead to missense or nonsense mutations in the open reading frame (ORF) (Galagan and Selker, 2004). During the evolution of *Z. tritici,* the single-copy DNMT gene *dim2* has been amplified several times. Thus the *dim2* gene became susceptible to RIP. To this point RIP has not been experimentally verified in *Z. tritici*, but repetitive sequences show strong characteristics of RIP (such as low GC-content and high ratio of transitions to transversions) that would indicate its existence. It is supposed that through these point mutations the *dim2* gene got deactivated and DNA methylation is lacking. The original, no longer functional template for the *dim2* gene can be found on chromosome 6. Due to TE mediated amplification or segmental duplication there are 23 replicates of the inactivated *dim2* gene in the genome of *Z. tritici* (Dhillon et al., 2010).

Contrary to the reference strain IPO323 an isolate with an intact *dim2* copy was found in Iran (Zt10). In this strain DNA methylation is likely to be active (M. Möller, E. Stukenbrock, unpublished). In conclusion, DNA methylation can be seen as a polymorphic trait within the species *Z. tritici*. The Iranian isolate and its difference in DNA methylation compared to the reference strain IPO323 may play a role in understanding how *Z. tritici* became a highly specialized and successful pathogen. It offers a way to examine the consequences of DNA methylation within the taxa *Z. tritici* and the *Zymoseptoria* species complex.

2.2.3 Methylation of histones

A further process which regulates gene expression is methylation of histones. It takes place when methyl groups are transferred to the amino acid tails of histones (Greer and Shi, 2012). Histones are proteins that interact with DNA to form nucleosomes. A nucleosome is a unity of a histone octamere and 147 base pairs (bp) of DNA. The octamere consists of dimers of the core histones H2A, H2B, H3 and H4. Depending on the amino acid of the histone tail that is methylated and how many methyl groups are transferred, the bond between histone and DNA gets weaker or stronger. So due to the strength of this bonds, the nucleosomes form dynamic chromatin structures which are crucial for genome organization and gene expression (Strahl and Allis, 2000).

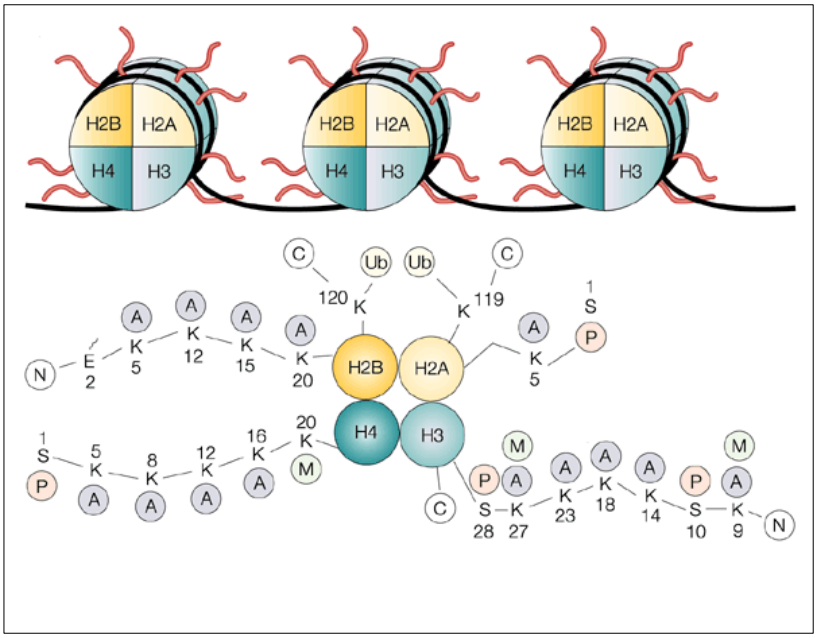


Figure 3. Nucleosome and histone structure. A nucleosome consists of a unit of four histone dimers (H2A, H2B, H3 and H4) and 147bp of DNA which is twisted around the group of histones. They are target of modifications that influences gene expression, which include the histone methylation (Marks et al., 2001).

If the methylation weakens the bond between histones and DNA, the DNA is uncoiled and more accessible for proteins involved in transcription and transcriptional regulation. This loose chromatin structure is called euchromatin. Methylation of histone H3 on lysine 4 (H3K4), histone H3 on lysine 48 (H3K48), and histone H3 on lysine 79 (H3K79) are examples for euchromatin associated histone modifications (Noma et al., 2001). If the methylation causes a stronger bond between DNA and histones it becomes compact heterochromatin. Here the transcription is typically low, also genes can be completely silenced. Methylation of histone H3 lysine 9 (H3K9) and histone H3 lysine 27 (H3K27) is often found in these regions (Nakayama et al., 2001, Cao et al., 2002). Heterochromatin has two appearances, the constitutive heterochromatin remains compact while facultative heterochromatin is more dynamic. The enzymes transferring methyl groups to the amino acids are called histone-methyltransferases.

As histone methylation plays an important role in gene regulation it is involved in various developmental processes, such as the development of illnesses and inheritance.

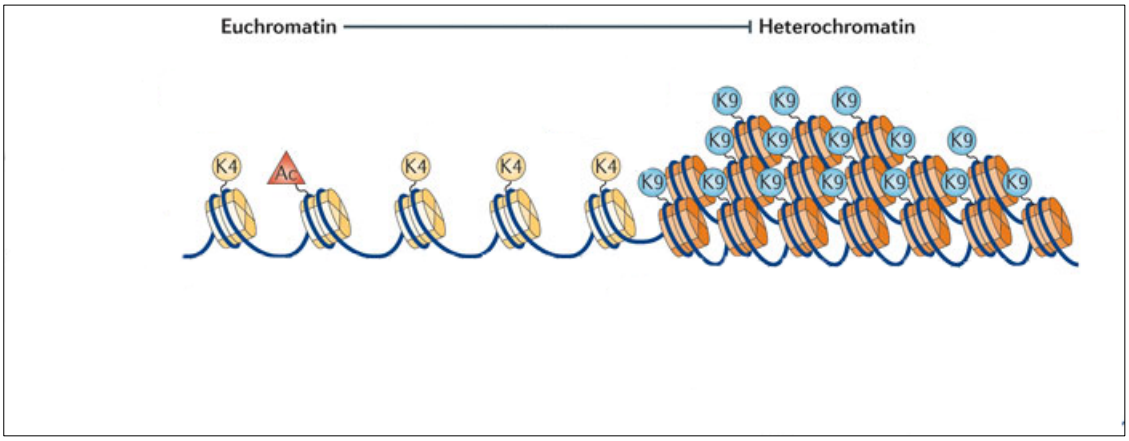


Figure 4. Euchromatin and heterochromation formation based on different nucleosome structures. Regions with methylation of H3K9 are associated with the compact heterochromatin and low gene transcription. Methylation of H3K4 is often found in regions with loosely packed euchromatin (modified from Gaspar-Maia et al., 2011).

2.2.4 Role of histone methylation in virulence of *Z. tritici*

In *Z. tritici* one main difference between core and dispensable chromosomes is the elevated level of tri-methylation of histone H3 lysine 27 (H3K27me3), which is mediated by the histone methyltransferase KMT6. In contrast to the core chromosomes, H3K27me3 is covering the entire length of all dispensable chromosomes (Schotanus et al., 2015). The histone methyltransferase KMT1 is responsible for tri-methylation of histone H3 lysine K9 (H3K9me3), that is linked to transposable elements and repeats both on core and dispensable chromosomes. Both histone methyltransferases are associated with heterochromatin and gene silencing.

The histone methyltransferase KMT6 was studied in the cereal pathogen *Fusarium graminearum* and was found to be involved in the regulation of secondary metabolites, pathogenicity associated and species specific genes (Connolly et al., 2013). *Kmt6* deletion mutants showed growth defects, were sterile and expressed genes for mycotoxins, pigments and

other secondary metabolites. A majority of silenced genes in the wild type showed an enrichment of H3K27me3 and through deletion of *kmt6* additional 14 percent of the genome could be expressed (Connolly et al., 2013).

The histone methyltransferase KMT1 was studied in the context of epigenetic regulation in the plant pathogenic fungus *Leptoshaeria maculans* (Soyer et al., 2014). Here, H3K9me3 has been shown to be involved in the transcriptional regulation of effector genes that are important for the infection process.

2.3 Objectives of this bachelor project

One aim of this bachelor project is to study DNA methylation in *Z. tritici.* We will try to restore the DNA methylation system in the *Z. tritici* strain Zt09. Furthermore we will delete the functional *dim2* gene in the strain Zt10. Therefore, two plasmids will be created and used for transformation of the respective *Z. tritici* strains. We aim to create mutant strains that allow us to study the effect and function of DNA methylation within the species of *Z. tritici*.

The specific goals are:

- create a plasmid to restore DNA in Zt09 by integrating a functional *dim2* gene from Zt10
- create a plasmid to delete *dim2* in the Iranian isolate Zt10
- transform the *Z. tritici* strains with the created plasmids

The second objective is to study the role of histone methylation in virulence of *Z. tritici.* We will use deletion mutants that lack the genes encoding for the histone methyltransferase KMT6 (responsible for tri-methylation of H3K27), KMT1 (responsible for tri-methylation of H3K9) and a double mutant which is lacking both KMT1 and KMT6. Using a plant infection assays of wheat (*T. aestivum*) we will analyse if wild type (WT) and mutants have a different ability to infect its host. Thereby we will be able to see if the removal of certain histone marks has an impact on pathogenicity in *Z. tritici* and then compare our results to the previous studies that indicate that these histone marks have an impact on virulence in *L. maculans* and *F. graminearum*.

The specific goals are:

- use plant infection assay to compare the mutant virulence to WT infection patterns
- using mutant strains which lack the histone methyltransferase KMT1, KMT6 and a double mutant
- evaluate infection patterns through daily manual screening for necrosis and pycnidia

3 Materials and Methods

3.1 Material and ordering sources

Chemicals and enzymes

Chemicals and antibiotics used for this bachelor thesis were provided by Difco, Merck, Roche, Sigma-Aldrich, Ivitrogen and Roth. Enzymes used for restriction digest were provided by New England Biolabs GmbH (NEB). For PCR-reactions PHUSION DNA-Polymerase (NEB) was used.

Enzyme	Application
EcoR V HF	restriction digest of pES61
EcoR I HF	restriction digest of pES188
Sph I HF	restriction digest of pES188
Sal I HF	restriction digest of gDNA Zt10_Δ*dim2*

Buffers and solutions

Buffers and solution were produced according to Ausubel et al. (1987) and Sambrock et al. (2001). If necessary all buffers and solutions were autoclaved for five minutes at 121°C. Further buffers and solutions are mentioned in the respective method.

Kits

The Wizard® SV Gel and PCR Clean-Up System (Promega) was used for purification of plasmid DNA. The plasmid Midi Kit and Mini Kit (QIAgen) were used for extraction of plasmid DNA. Kits are listed in the appendix.

Oligonucleotides

Name/ Local code	Nucleotide sequence (5'→3')	Application
oES879	CCGAGAAGGACCCAGCAAAC	test PCR for GAP DH on Zt10 *dim2* deletion mutants
oES1006	GTCAAGTCAACGACGGAAAC	test PCR for GAP DH on Zt10 *dim2* deletion mutants
oES2347	GAATAGAGATCTGCTAGCCCCGCGAATGGCATGTGTCAGC	amplification of *Zt10 gDNA* for UF *of dim2* deletion construct in ES188
oES2348	TACGAATTCTTAATTAAGATTTCTCACGCGGTATGGCAGG	amplification of *Zt10 gDNA* for UF *of dim2* deletion construct in ES188 amplification of Zt09 gDNA for DF of *dim2* integration construct in ES189
oES2349	GCTCCTTCAATATCAAAGCTCACGCTCTTCGCTAGTTCTC	amplification of *Zt10 gDNA* for DF *of dim2* deletion construct in ES188
oES2350	TCGAGGGTACCGAGCTCGATCAACAAGGCGTTTCAGGAGG	amplification of Zt10 gDNA for DF of *dim2* deletion construct in ES188 amplification of Zt09 gDNA

		for UF of *dim2* integration construct in ES189
oES2351	GCTGACACATGCCATTC GCGGGGCTAGCAGATCT CTATTC	amplification of hygromycin resistance from ES72 for *dim2* deletion construct in ES188
oES2352	GAGAACTAGCGAAGAGC GTGAGCTTTGATATTGA AGGAGC	amplification of hygromycin resistance from ES72 for *dim2* deletion construct in ES188
oES2353	GCTGACACATGCTATTC GCGTTCGCGATGGCGGC CATTAC	amplification of *dim2*-orf from Zt10 gDNA for *dim2* integration construct in ES189
oES2354	GCTCCTTCAATATCAAA GCTACTAGCGAAGAGCG TGTAAG	amplification of *dim2*-orf from Zt10 gDNA for *dim2* integration construct in ES189
oES2355	GAATAGAGATCTGCTAG CCCGCCGCCATTCTAGC ACCAAC	amplification of Zt09 gDNA for UF of *dim2* integration construct in ES189
oES2356	GTAATGGCCGCCATCGC GAACGCGAATAGCATGT GTCAGC	amplification of Zt09 gDNA for DF of *dim2* integration construct in ES189
oES2357	GTTGGTGCTAGAATGGC GGCGGGCTAGCAGATCT CTATTC	amplification of hygromycin resistance from ES72 for *dim2* integration construct in ES189
oES2358	CTTACACGCTCTTCGCTA GTAGCTTTGATATTGAA GGAGC	amplification of hygromycin resistance from ES72 for *dim2* integration construct in ES189
oES2296	GCTCGTACCAATGACCA AAC	test PCR for *dim2* on Zt10 *dim2* deletion mutants
oES2297	ATCGGACCTCGTCACTCT TG	test PCR for *dim2* on Zt10 *dim2* deletion mutants

Plants and fungi strains

For the plant infection assay wheat plants (*Triticum aestivum*) from cultivar Obelisk (Wiersum Plantbreeding) were used. *Z. tritici* strains Zt09, Zt110, Zt219 and Zt125 were used to infect the plants. Fungal gDNA from Zt09 and Zt10 was used for construction of mutant strains with altered DNA methylation systems.

Strain	Isolate name	Characteristics	Provider

Zt09	IPO323_Δchr18	wild type strain missing DNA methylation	Gert Kema
Zt10		wild type strain functional DNA-methylation system	Bruce McDonald
Zt110	IPO323_Δ*kmt6*	IPO323 mutant with *kmt6* deletion construct	Mareike Möller
Zt125	IPO323_Δ*kmt1*	IPO323 mutant with *kmt1* deletion construct	Mareike Möller
Zt219	IPO323_Δ*kmt1*_Δ*kmt6*	IPO323 mutant with *kmt1* and kmt6 deletion construct	Petra Happel

Plasmids

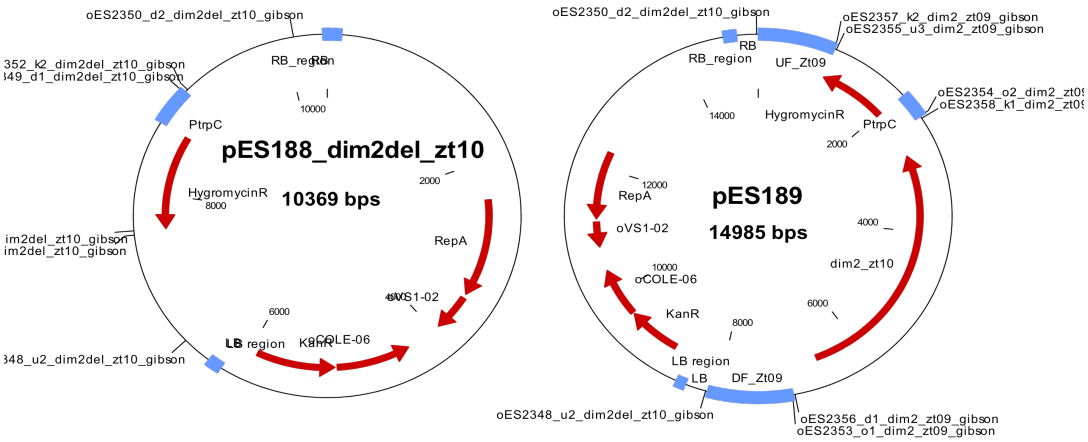


Figure 5. Plasmids constructed for modifications of the DNA methylation of the *Z. tritici* strains Zt09 and Zt10. Both plasmids contain a backbone (pES61) with a kanamycin resistance and origins of replications for *E. coli* and *A. tumefaciens*. The plasmid pES188 is intended for Zt10 transformation. It contains a hygromycin resistance, which shall replace a functional *dim2* gene. For integration into the fungal genome it also contains the Zt10 upstream and downstream flanking regions of the target locus. The plasmid pES189 is constructed to integrate the functional gene *dim2* into the genome of Zt09. This integration construct consists of a hygromycin resistance, the gene *dim2* and respective upstream and downstream flanking region of Zt09.

Plasmid name	Established by/ Reference	Application
ES61	Petra Happel	vector/ backbone construct
ES188	this study	deletion of DNA-methylation system (*dim2* gene) in Zt10
ES189	this study	integration of DNA-methylation system (*dim2* gene) in Zt09

3.2 Molecular standard techniques

Determine nucleic acid concentration

To measure the nucleic acid concentration a photometer (Nanodrop Thermo Fisher Scientific) was used. Therefore, a single drop (1µl) of the sample is applied on the apparatus and respective data is collected.

Determine cell culture concentration

To determine the density of a liquid cell culture, cells can be counted under a microscope using a "Neubauer improved" counting chamber. The chamber has small cells that contain an accurately defined amount of liquid. The number of counted cells in this defined amount of liquid is basis for the calculation of the amount of cells in the whole cell culture. Formula used to compute amount of cells:

$$\frac{countedcells \times dilution}{countedareas(mm^2) \times chamberdepth(mm)} = \frac{cells}{\mu l}$$

Another method is the determination the optical density (OD) of a cell culture. Therefore, a photometer (Nanodrop Thermo Fisher Scientific) was used. To ensure a linear correlation of the samples, the measured cultures were diluted (mostly 1:10) to get an OD600 value below 0.8. The reference was the OD600 of the respective medium.

Agarose gel electrophoresis

The agarose gel electrophoresis was used to separate and identify DNA-sequences based on their size. In order to produce the gel matrix, agarose was dissolved by boiling it in 1xTAE buffer. The agarose gel concentration depended on the different purposes of the gel. After the medium was cooled down (to approximately 60°C) Midori Green (5µl/100ml 1xTAE) was added. The DNA-stain Midori Green is used to visualize the DNA fragments under UV light. Afterwards the solution is poured into a gel-tray and cooled down to room temperature. The solidified gel was transferred into a gel chamber, covered with 1xTAE buffer and then loaded with DNA samples. DNA is negatively charged and therefore migrates towards the anode, if voltage is applied (100-130V for 45 to 60 minutes). To visualize the DNA-fragments the GelDocTM XR+ (Bio-Rad) system was used. One application for the agarose gel electrophoresis was the purification of PCR fragments. Therefore, a whole sample was applied to the gel and the respective bands were cut out under UV-light for further use.

Isolation of DNA

To purify and isolate DNA different methods were applied, depending on the needed amount of DNA and the required quality of purification.

Isolation of genomic DNA with Phenol/Chloroform

This method is a modified version of (Hoffman and Winston, 1987). It was used to isolate genomic DNA (gDNA) from cells of the respective *Z. tritici* strains. A cell culture was grown in liquid YMS medium and pelleted (Heraeus Biofuge 15, 5min 13.000rpm) in 2ml Eppendorf tubes. Afterwards the supernatant was removed. At this point the cell pellets could be frozen and stored for further use. The pellets were resuspended in 500µl *Z. tritici* lysis buffer and 500µl Phenol/Chloroform (proportion 1:1). 0.2g glass beads were added and the Eppendorf tubes were incubated on a Vibrax-VXR shaker (30 minutes) to break the fungal cells, followed by centrifugation (15min, 13.000rpm, Heraeus Biofuge 15). Thereby the solution divided into 2 phases, 400µl from the upper phase (containing the gDNA) was pipetted into a 1,5ml Eppendorf tube, containing 500µl ethanol. Afterwards the samples were frozen (20 minutes, -20°C) and centrifuged (5min, 13.000rpm, Heraeus Biofuge 15). The gDNA precipitated due to the ethanol and the cold. The supernatant was discarded. The gDNA pellet was resuspended in 50µl TE with 20µg/ml RNAse A on a Thermomixer (10min, approximately 50°C).

Z. tritici 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_2Obid.
TE-Phenol/Chloroform (Mixture of equal volumes of Phenol (equilibrated with TE buffer) and Chloroform)

Boiling Preparation

The protocol for boiling preparation was established by Petra Happel. It was used to isolate plasmid DNA after the Gibson Assembly. The isolated DNA was not as efficiently cleared as accomplished by other mentioned protocols, but was an efficient way to scan for positively assembled candidates.

Therefore, a colony of *E. coli* was inoculated over night (37°C) in 2 ml test tubes in dYT medium with kanamycin. The cultures were transferred into 2ml Eppendorf tubes and centrifuged (2 minutes, 13.000rpm) then the supernatant was discarded. STET buffer (200µl) and lysozyme (20µl) were added and then vortexed until the pellet was dissolved. The Eppendorf tubes were transferred into a Thermomixer (Eppendorf) (1 minute at 95°C) and then centrifuged (10 minutes, 13000 rpm), again. The pellet was removed with a toothpick and 20µl NaOAc (3M) and 500µl 2-Propanol were added and mixed by thoroughly inverting the tube. The tubes were centrifuged (10 minutes, 13.000rpm) and the supernatant was discarded. Afterwards the pellet was washed by adding 500µl ethanol (70%) for a few minutes. After discarding the ethanol, the tubes were centrifuged for some seconds to collect the residual ethanol which then was discarded as well. 100µl TE-RNase A was added and the tubes were incubated in a Thermomixer (15 minutes, 37°C).

High quality plasmid DNA purification and extraction

For a high quality purification of plasmid DNA the QIAGEN Plasmid Midi Kit/ Mini Kit was used. The protocols can be found in the appendix.

DNA purification

The Wizard® SV Gel and PCR Clean-Up System was used in combination with gel electrophoresis to purify DNA out of gel slices after PCR. The protocol can be found in the

appendix.

Quick DNA extraction

To extract DNA in a fast way *Z. tritici* colonies were picked into 50µl 25mM NaOH and transferred into a Thermomixer (10 minutes, 98°C). Afterwards 50µl 40mM TrisHCl (pH 5.5) was added. The extracted DNA should be used within a day. This method was used to extract gDNA for test PRCs in which positive candidates (for example from Zt10+pES188) could be confirmed.

Polymerase Chain Reaction (PCR)

PCR is a method for in-vitro DNA amplification. Therefore, the enzyme DNA polymerase is necessary. PCR was used to amplify the inserts for plasmid construction (pES 188 and pES 189) as well as to confirm positively transformed Zt10_ Δ*dim2* candidates. For both PCRs Phusion DNA-Polymerase (NEB) was used. This polymerase has a 3'-5' exonuclease activity (proofreading) which is important for high accuracy. It elongates approximately 1Kb per 30 seconds. PCRs were conducted in a S1000TM Thermal Cycler (Bio Rad). A standard protocol was used:

	Temperature	Time
1.) initial denaturation of template DNA (hotstart at 98°C)	98°C	30 seconds
2.) denaturation of template DNA	98°C	8 seconds
3.) annealing: binding of primers	60°C	20 seconds
4.) elongation: Polymerase amplifies template DNA	72°C	30 seconds/kb
5.) final elongation:	72°C	300 seconds
6.) cooling period to avoid unwanted enzyme reactions	15°C	

Step 2-5 were repeated for 35-36 cycles.

GIBSON ASSEMBLY

The Gibson Assembly was used to assemble the plasmids pES188 and pES189 out of the previously amplified PCR products. The assembly works based on enzyme activity under isothermal conditions. To assemble the plasmids the Gibson Script was followed. 15µl Gibson Master Mix (NEB), the inserts, a vector and H_20 were added to a total amount of 20µl. Used inserts were the respective upstream and downstream flanking regions and a hygromycin resistance. For the integration of a methylation system in pES189 a functional *dim2* gene was inserted as well. The used vector was the previously restricted (with EcoRV) plasmid pES61.

To estimate the needed amount of inserts, their concentration (ng/µl) was measured with the Nanodrop and their length (bp) was identified using the program Clone Manager. The mixture was incubated (60 minutes, 50°C) in a PCR cycler (T-Professional Thermocycler, Biometra). During the incubation the enzymes form the Gibson Master Mix assembled the plasmid. First a exonuclease (5'-3') produced a single stranded 3' overlap. The complementary overlaps from the respective fragments annealed to double stranded DNA. A DNA-polymerase elongated the 3' ends and finally a DNA ligase closed the nicks. Thereupon an *E.coli* transformation was conducted with the assembled plasmids.

DNA restriction digest

The restriction is based on the activity of endonuclease enzymes, which cut into the DNA at specific recognition sequences and thereby introduce breaks with sticky ends or overlaps. This method is an important tool for cloning and transformation, because genetic material can be introduced accurately into a specific location in the DNA. It is also important to analyse the occurrence of specific DNA sequences in combination with gel electrophoresis.

Restriction enzyme	Application
EcoR5 HF	restriction digest of pES61
EcoR1 HF	restriction digest of pES188
Sph1 HF	restriction digest of pES188
Sal1 HF	restriction digest of gDNA Zt10_Δ*dim2*

To restrict the double stranded DNA a protocol according to the supplier's (NEB) instructions was followed. A typical preparation was:

1µg plasmid DNA
2µl enzyme specific buffer
2µl 10X Bovine Serum Albumin (BSA)
0.5U restriction endonuclease
Ad 20µl with H_2Obid.

The DNA was incubated for at least 1-3 hours or better over night at the recommended temperature (mostly at 37°C). Afterwards an aliquot was loaded into a gel electrophoresis gel to check for the amount of DNA and to evaluate, if the circular plasmid DNA was cut at the expected sites.

Southern Blot

This method modified from Southern (1975) is used to verify specific DNA sequences. Restriction enzymes were used to cut the DNA specifically for following identification of the respective sequence. The Southern Blot was used to visualize the deletion of the *dim2* gene in the genome of *Zt10*. Genomic DNA from the transformed *Z. tritici* candidates was restricted specifically. This made it possible to verify the occurrence of the respective DNA sequence. Therefore 10-25µg gDNA was cut with a fitting restriction endonuclease enzyme (in this case SAL1-HF) in a total volume of 25µl (over night, 37°C). A gel electrophoresis (0.8% TAE agarose gel, 2.5 hours, 90 Volt) followed, to separate the DNA according to its size. In order to transfer the DNA to a Hybond N+ membrane it needed to be depurinated and neutralized. For the depurination the gel was incubated in 0.25M HCl (15 minutes) under gentle shaking. To neutralize the gel was incubated in 0.4M NaOH (for 15 minutes) so the Hydrogen bonds split up. Afterwards the DNA was transferred out of the gel and onto a membrane due to capillary force. Therefore a Whatman paper (filter paper) and paper towels were piled up on the membrane. Weight was applied to the top of the paper towels and NaOH was soaked through this pile. Due to the capillary force the molecules were pulled out of the gel and into the positively charged membrane. The blot was conducted over night and then dissembled. To visualize the DNA bands, different treatments in an incubation tube were conducted on the membrane.

First the membrane got pre-hybridized in Southern Hybridisation buffer (30 minutes, 65°C). For the next step DIG labelled probes were added to 15ml hybridization buffer and boiled in a water bath (100°C) for 15 minutes. Then the Pre-Hybridiation buffer was replaced by the probe and the membrane was incubated (over night, 65°C). Afterwards a luminescence detection of the DIG labelled DNA followed. Therefore, the membrane needed to be washed repeatedly in a hybridization tube with different buffers. First it was washed twice in Southern wash buffer (15 minutes, 65°C), followed by DIG wash buffer (5 minutes, 25°C). For a blocking reaction 1g skimmed milk powder was added to into 20ml DIG buffer to wash the membrane (30 minutes, 25°C). Afterwards the membrane was washed in antibody solution (30 minutes, 25°C) and then twice in DIG wash buffer (15 minutes, 25°C). Finally the membrane was equilibrated

in DIG buffer 3 (10 minutes, 25°C). Then it was taken out of the hybridization tube and incubated with CDP Star solution (15 minutes, without light). Afterwords the membrane was analysed ChemiDoc™ MP Integrating system (Bio-Rad).

Following buffers were used:

-Southern hybridization buffer:
0,5M $NaPO_4$ buffer, pH7.0
7% SDS
in H_20_{bid}

-Southern wash buffer:
0.1M $NaPO_4$ buffer, pH7.0
1% SDS
in H_20_{bid}

-DIG1:
0.1M maleic acid
0.15M NaCl
Dissolve maleic acid in H_2O_{bid}
Adjust to pH 7.5 with NaOH

-DIG-wash:
0.3% Tween-20 in DIG1

-DIG2
blocking solution 1:10 in DIG1

-DIG3
0.1M NaCl
0.05 $MgCl_2$ in H_2O_{bid}
Adjust to pH 9.5 with 1M Tris-HCl

-Antibody solution:
1µl Anti-DIGAP /10ml DIG buffer 2

-CDP Star solution:
100µl CDP Star in 10ml DIG buffer 3

3.3 Microbiology

Cultivation of *E. coli*

E. coli cells were cultivated in a liquid shaking culture (200rpm) in the following medium:

dYT medium:
16 g Bacto Tryptone
10 g Yeast extract
5 g NaCl
Ad 1l with H2O

Transformation of *E. coli*

Transformation of *E. coli* was used to amplify pES61 (which was used as a backbone construct for the plasmids ES188 and ES189), as well as to amplify the plasmids ES188 and ES189. Chemical competent *E. coli* (TOP10) cells were thawed on ice for 10 minutes. For each transformation reaction 50µl *E. coli* cells were mixed with 1µl of the respective plasmid DNA and incubated on ice for 30 minutes. Then the cells were treated with a heat shock (45 seconds, 42°C) to enable the entry of the plasmid into the cell. After 2 more minutes on ice, 5 volumes of dYT medium were added, followed by a regeneration time on the Thermomixer (45-60 minutes, 37°C) under gentle shaking. The cells were plated on solid dYT-Kanamycin medium and incubated (over night, 37°C). Due to the kanamycin treatment positively transformed cells were selected, because they were able to express the kanamycin resistance gene from plasmid pES61.

Transformation of electrocompetent *A. tumefaciens*

Transformed *A. tumefaciens* mediated the transformation of *Z. tritici.* To transform the *Agrobacterium* an Eppendorf tube containing 50µl electrocompetent *A. tumefaciens* cells was thawed on ice. 1µl plasmid DNA was added and kept on ice for one minute. The cell suspension was pipetted into a pre-chilled electroporation cuvette and inserted into the electroporation apparatus. Through two shocks (200Ohm, 25µF) the cells got electroporated. 950µl pre-warmed dYT medium (28°C) was added into the electroporation cuvette and then pipetted into a test tube. The cells regenerated while incubated on a shaker (200rpm, 28°C) for two hours (one cell cycle lasts around 58 minutes). 200µl of the regenerated *A. tumefaciens* cells were streaked out on one half of a dYT medium plate, containing carbenicillin (100µg/ml), rifampicin (50µg/ml)

and kanamycin (40µg/ml). They were singularized twice to generate single colonies. The plates were incubated at 28°C for two days.

Cultivation of Z. *tritici*

Z. tritici cultures were cultivated under aerobic conditions at 18°C. To cultivate *Z. tritici* cells on solid medium, cells were streaked out on YMS agar. To generate a liquid cell culture YMS medium was inoculated with *Z. tritici* cells that were grown on solid YMS agar. They were incubated on a shaking table at 200 rpm.

YMS liquid medium:
4g yeast extract
4g malt extract
4g sucrose
(16g agar for solid medium)
Ad 1l H2O

A. tumefaciens* mediated transformation of *Z. tritici

This protocol is a modified version of Bowler et al. (2010). One week before the transformation, the respective *Z. tritici* strains (ES09 and ES10) were streaked out from long term storage (-80°C) on YMS plates and incubated for five days at 18°C. One day prior to the transformation four single colonies of transformed *A. tumefaciens* were inoculated in liquid dYT medium with antibiotics. One test tube contains 2.5ml liquid dYT medium with 10µl carbenicillin (stock 100mg/ml), 10µl rifampicin (stock 50 mg/ml), 40µl kanamycin (stock 10 mg/ml). This preculture was incubated for 24 hours on a shaking table (200rpm, 28°C).

At the day of transformation a 1:10 dilution of the *A. tumefaciens* pre-cultures was prepared. The OD_{600} of the pre-cultures was measured and diluted to an OD_{600} of 0.15 in induction medium (IM) in a total volume of 5ml. A 50 ml plastic test tube was used for the induction. The culture was incubated (3h, 28°C) to a final OD_{600} of approximately 0.3. To prepare the IM Agar plates carbenicillin (stock 100mg/ml), rifampicin (stock 50mg/ml), kanamycin (stock 10mg/ml), acetosyringone (stock 100mM) and glucose were added to the warm IM Agar and plates were poured (approximately 15 to 20 ml were used for each plate). Hybond-N+ membrane was placed on the solidified IM agar plates. Cells from the already grown *Z. tritici* cultures were scraped off the agar plates and resuspended in 1ml distilled water. The amount of cells was determined using the "Neubauer improved" counting chamber. It was adjusted to a cell density of $1x10^7$ cells/ml. 100µl of *Z. tritici* cell suspension and 100µl *A. tumefaciens* culture were mixed and placed on Hybond-N+ membranes. After an incubation (2 to 3 days, 18°C) the membranes were peeled of the plates and transferred to a YMS agar plate with hygromycin (stock 50mg/ml) and cefotaxime (stock 50 mg/ml) and incubated at 18°C. Due to cefotaxime

the *A. tumefaciens* cells were eliminated. Hygromycin selected for the *Z. tritici* cells that integrated a hygromycin resistance. After colonies got visible (after approximately 10 days) they were patched onto a YMS hygromycin plate and incubated (7 days, 18°C) again. To singel the colonies a second time, they were patched to an YMS only plate and incubated again for 7 days at 18°C. To verify positively transformed candidates a test PCR followed.

3.4 Plant infection assay

Infection of wheat plants with Z. tritici

This protocol is a modified version of Poppe et al. (2015). Seeds of wheat (*Triticum aestivum*) cultivar Oblisk (Wiersum Plantbreeding) were pre-germinated in a phytochamber for five days. Therefore they were put into plastic boxes with wet filter paper. Afterwards the seedlings were planted, three seedlings into one plastic pot and grown for seven days.

Afterwards the second leaf (leaf which develops secondly during plant growth) of each plant was infected with *Z. tritici* spore solution. Two biological replicates of the *Z. tritici* strains Zt09, Zt110, Zt125, Zt219 were used for the infection.

An area on the upper side of the leaf was marked and the spore solution was applied with a brush. During the infection procedure the plants were randomized to ensure a double blind experiment. To enable an efficient infection, the plants were put into autoclave bags with water. The bags were sealed for an incubation time of 48 hours to achieve an air humidity of 100 percent. This leads to an opening of the stomata, which facilitates the infection. During the following plant infection assay the plants were located in a phytochamber under following conditions: temperature 20°C, 16 hour light period, 90% air humidity.

To prepare the spore solution a five day old *Z. tritici* culture was used to inoculate a 50ml YMS liquid pre-culture, that was incubated (2 days, 18°C) on a shaking plate (200rpm). 5ml of this pre-culture were used to inoculate a 50ml YMS liquid main-culture (2 days, 18°C). Using a "Neubauer improved" counting chamber the cell density was detected. Then the cell culture was centrifuged (10 minutes, 3200rpm) and resuspended in water to adjust the cell density to 1x 10^8 cells/ml. To lower the hydrophobic properties of the leaf surface 0.1 percent Tween20 was added to the cells.

Observation of symptoms, leaf harvesting and evaluation methods

For 21 days the second leaf of each plant was manually screened for the first occurrence of pycnidia and necrosis. 21 days post infection (dpi) the leafs were harvested for further evaluation. Therefore, the leaves were cut, stuck to a paper and dried (at 4°C). A scoring scheme with six graduations (0, 1-20, 21-40, 41-60, 61-80, 81-100) representing the percentage of necrotic leaf area or pycnidia coverage was used to evaluate the symptoms manually. Afterwards the leaves were scanned (HP Photosmart, HP) and evaluated automatically with the image processing software ImageJ (using the batch processing macro: settings_V15_default) for pycnidia count and necrosis coverage per cm^2. For statistical support of the data the non-parametric Wilcoxon signed-rank test was used. This test can be applied on two paired samples, to prove the statistical significance of the results (here significance is given if p-value < 0.05.

4 Results

4.1 *Z. tritici* strains with modified DNA methylation

In this bachelor thesis we aimed to establish *Z. tritici* strains with modifications in their DNA methylation system. The strain Zt09 is lacking functional DNA methylation. Here the gene

dim2 should be integrated to restore the methylation. In contrast to Zt09 the *Z. tritici* strain Zt10 has a functional *dim2* gene, and thus DNA methylation is present. Here we aimed delete DNA methylation by replacing the gene *dim2* by an encoding sequence for hygromycin resistance.
Two plasmids containing either the *dim2* deletion construct in Zt10 (pES188) or integration of dim2 in Zt09 (pES189) were planned and constructed for transformation in *Z. tritici*. The plasmids consist of a backbone (pES61) containing elements for amplifications in *E. coli* and *A. tumefaciens* with an encoding sequence for resistance to the antibiotic kanamycin, which is used as a selection marker for transformation in this bacteria. The inserts of the plasmids contain the sequences that will be integrated into the genome of *Z. tritici*. For integration into a specific target sequence of the Zt09 and Zt10 genomes, both plasmids do also contain the upstream and downstream flanking regions of their genomic target locus, necessary for DNA integration by homologous recombination, as well as an insert with the encoding sequence for the planned modifications of the DNA methylation system. Both inserts have an encoding sequence for the antibiotic hygromycin, which is a selection marker for successful integration of the plasmid into the fungal strains.
The plasmid pES188 should be transformed into Zt10 to replace the *dim2* encoding sequence with a deletion construct in the form of a hygromycin resistance. For integration via homologous recombination the plasmid needed to contain upstream and downstream flanking regions of the genomic target sequences from Zt10. For Zt09 transformation the plasmid pES189 was constructed. The insert contains a hygromycin resistance, as well as a functional *dim2* open reading frame originating from Zt10. For upstream and downstream flanking regions sequences from Zt09 were used.

To amplify the fragments for the inserts of the plasmids, PCRs were conducted on genomic DNA from Zt09 and Zt10, as well as on the plasmid pES72 to obtain the hygromycin encoding sequence. Gel-electrophoresis allowed us to visualize the PCR results (Figure 6.) Afterwards the PCR products were cut out of the gel and were purified. The backbone was restricted with the endonuclease enzyme EcoRV.

Figure 6. Gel-electrophoresis of fragments needed for the construction of the plasmids pES 188 and pES 189. The plasmid pES61 is shown as well. The amplified fragments appear to have the

expected base pair length (pES188: upstream flanking region (UF) 1110bp, downstream flanking region (DF) 1077bp, encoding region for hygromycin resistance (hyg) 1443bp; pES189: upstream flanking region 1010bp, downstream flanking regions 1110bp, encoding region for hygromycin resistance 1443bp, encoding sequence for the gene *dim2* 4723bp).

Gibson Assembly was conducted to assemble the plasmids out of the different inserts and the backbone (pES61). *E. coli* transformation followed immediately to amplify the plasmids. To confirm correct assembly the plasmids needed to be restricted (restriction digest of pES188 with SphI; restriction digest of pES189 with EcoRI). From each plasmid one correctly assembled sample was chosen for sequencing (pES 188: candidate #4; pES 189: candidate #3).

Figure 7. Gel electrophoresis of pES188 restricted with the endonuclease enzyme SphI. All plasmids except #3 are showing the expected base pair length of 7962bp, 1218bp, 856bp and 333bp. The lane for the base pair length 333bp is only slightly visible.

Figure 8. Gel electrophoresis of pES189 restricted with the endonuclease enzyme EcoRI. The samples #3, #9, #11 and #12 were assembled correctly. They show the bands for the expected base pair length of 9585bp, 4501bp and 899bp. The lane for a base pair length of 899bp is only slightly visible.

A transformation of electrocompetent *A. tumefaciens* followed. Three days later the *A. tumefaciens* mediated *Z. tritici* transformation was conducted. Two days after the transformation, the transformed cells were transferred to YMS plates containing the antibiotics hygromycin and cefotaxime. Hygromycin was used as selection marker for *Z. tritici.* Only the colonies which integrated the insert and thereby a sequence encoding for hygromycin resistance were able to grow. Also the *Z. tritici* cells are resistant to cefotaxime, so it can be used to eliminate only the *A. tumefaciens* cells. Colonies got visible after 13 days. 168 colonies of the transformed Zt10 x pES188 were growing on the transformation plate, but only five colonies of Zt09 x pES189 were growing on the other transformation plate. Because of the small number of viable colonies, the Zt09 x pES189 transformation was repeated twice, but did not lead to candidate transformants that could be further tested for integration of the construct.

Out of the 168 colonies of Zt10 x pES188 PCR screenings confirmed that seven clones were positively transformed (#8, #68, #101, #125, #135, #141, #146). To reassure and visualize the deletion of the gene *dim2* in the fungal genome a Southern Blot was conducted. Genomic DNA of the candidates was cut with the enzyme SalI. This enzyme has two restriction sites in the encoding sequence for *dim2* but not in the hygromycin deletion construct. The detection of bands is enabled due to digoxigenin (DIG) labelled DNA-probes. The upstream and

downstream flanking regions of the *dim2* were chosen as probes. Thereby they form characteristic restriction patterns for the deletion mutant and the Zt10 WT. For Zt10 WT two bands with the lengths of 3568bp and 2023bp were expected. The candidates with a correctly integrated construct should only show one band with a length of 4820bp, which could be confirmed for all seven of them.

Figure 9. Southern blot of Zt10 *dim2* deletion candidates and a WT control. The genomic DNA was cut with the endonuclease enzyme SalI. Expected bands for mutants with a correctly integrated *dim2* deletion construct have a length of 4820bp. The WT control should show two bands with 2023bp and 3568bp.

4.2 Role of KMT1 and KMT6 in virulence of *Z. tritici*

To investigate the role of histone methyltransferases KMT6 and KMT1 in virulence of *Z. tritici*, a plant infection assay was conducted on the host plant *T. aestivum*. The ability to infect the plant with *Z. tritici* was compared between *Z. tritici* mutants lacking *kmt6* or *kmt1*, as well as a double deletion mutant. Two biological replicates (transformants) were used each (Δ*kmt6*: transformants #283 and #365; Δ*kmt1*: transformants #68 and# 80; Δ*kmt6_kmt1*: transformants #23 and #116). The mutants were established prior to this study. Two biological replicates from IPO323Δchr18 WT (Zt09) were used as a positive control. As a negative control mock (H_2O) infected leaves were included in the infection assay to take other factors into account that could have influenced the infection and symptoms on the leaf. Spore suspension of respective *Z. tritici* replicates was applied on defined areas of the second leaf of each plant. For each treatment 39 plants/ leaves were infected. Symptoms of fungal infection were evaluated 21 days post infection (dpi). Leaves were observed daily to date the first occurrence of necrotic lesions and pycnidia. In the end of the observation period the infection phenotype was evaluated manually, therefore abundance of pycnidia and the percentage of necrotic leaf area per infected leaf region was estimated. To support and extend the results of manual visual evaluation, the leaves were harvested, dried and then scanned and evaluated with the image analyses program ImageJ. For statistical support of the data the significance was tested with the Wilcoxon signed-rank test. The results of plant infection can vary between the respective biological replicates. To visualize

this variation, data of the individual replicates will be listed separately.

4.2.1 Results of the manual visual evaluation of *Z. tritici* infected leaves

The plant infection assay was conducted for 21 days. Originally observation was planned for 28 dpi, but even plants of the negative control started to show signs of senescence (necrosis). The leaves were harvested, followed by manual evaluation for pycnidia coverage and necrotic lesions under the binocular. Therefore a scoring pattern representing the percentage of leaf area covered with necrosis or pycnidia was applied on the leaves (scoring: 0, 1-20, 21-40, 41-60, 61-80, 81-100). The evaluation of symptoms was performed by Mareike Möller. Leaves infected with the respective deletion strains developed different symptoms, thereby the fungal strains can be described for their different virulence phenotypes.

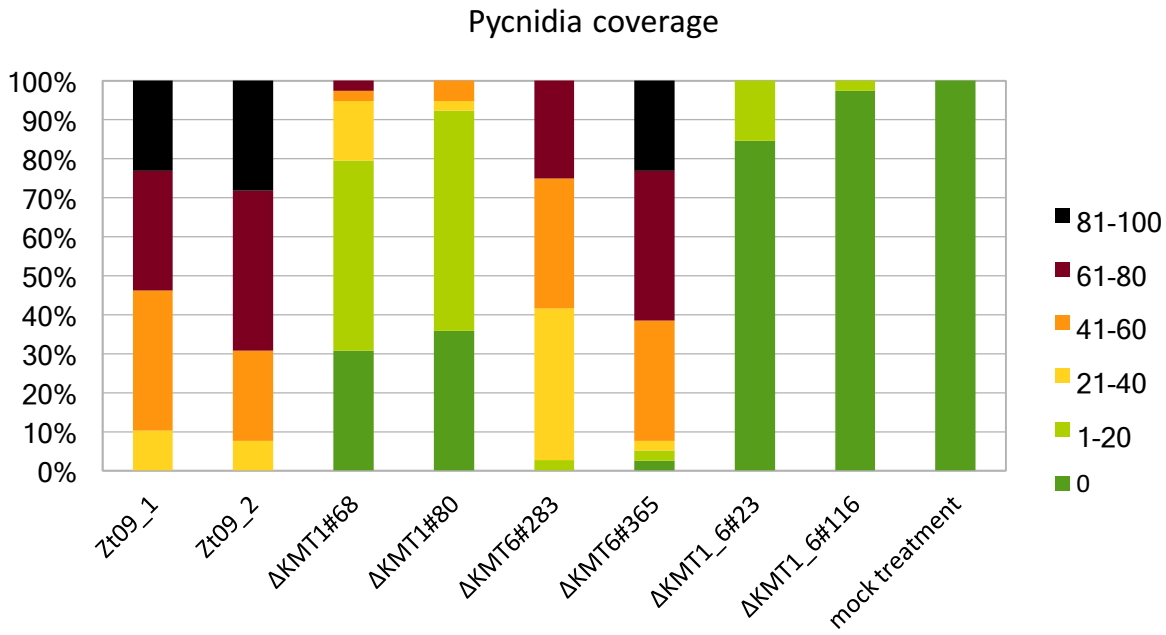


Figure 10. Plant infection assay comparing the virulence phenotype of *kmt1*, *kmt6* and a double deletion mutant to WT infection. Two biological replicates were used each for plant infection. 39 leaves were inoculated per treatment, for candidate #283 only 36 leaves could be analysed. The *kmt6* deletion candidates caused slightly decreased pycnidia coverage on the infected wheat leaves. In kmt1 deletion mutants the pycnidia formation was strongly decreased, though even more in the double deletion mutant.

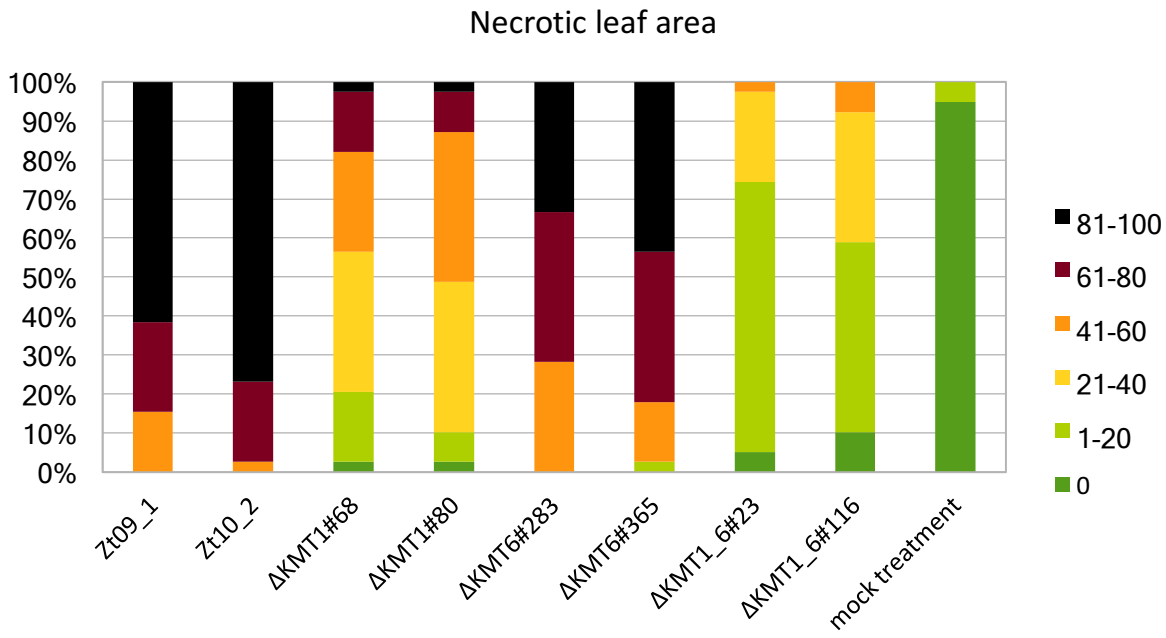


Figure 11. Plant infection assay comparing the virulence phenotype of *kmt1*, *kmt6* and a double deletion mutant to WT infection. Two biological replicates were used each for plant infection. 39 leaves were inoculated per treatment. Compared to WT infection all *Z. tritici* deletion mutants caused less necrotic lesions. Candidates missing the histone methyltransferase KMT6 showed decreased symptoms, though necrotic area was decreased stronger in *kmt1* deletion mutants and the double deletion mutant showed the least infection symptoms.

In Figure 10 and 11 the different virulence phenotypes on planta are represented. The WT (Zt09) infected leaves showed pycnidia coverage from 21 to 100 percent and most leaves showed necrosis coverage from 81 to 100 percent. Infection with Δ*kmt6* spore suspension also caused strong infection symptoms. Overall high pycnidia coverage was found, data from transformant #365 showed similar pycnidia scoring as the WT infection. Leaves infected with #283 showed fewer pycnidia, here pycnidia coverage from 81-100 percent could not be detected. In general a necrotic leaf area between 41-100 percent was found. Δ*kmt1* infected leaves showed a strongly decreased pycnidia coverage compared to leaves infected by the WT. Mainly the values add up between 0 to 20 percent. Necrotic leaf area values between 0-100 could generally be detected, but mainly between 21-60. Manual visual evaluation of leaves infected with the double deletion mutant *Δkmt1_6* revealed even less pycnidia than Δ*kmt1* infected leaves. Most of them showed no pycnidia coverage at all. Six leaves of transformant #23 and only one from transformant #116 infection showed pycnidia coverage of 1 to 20 percent. Necrosis coverage between 0 to 60 percent was detected, but mainly between 0 to 20 percent. Leaves infected with mock

treatment (H_2O) showed no pycnidia, though two of them showed a necrosis coverage between 1 to 20 percent.

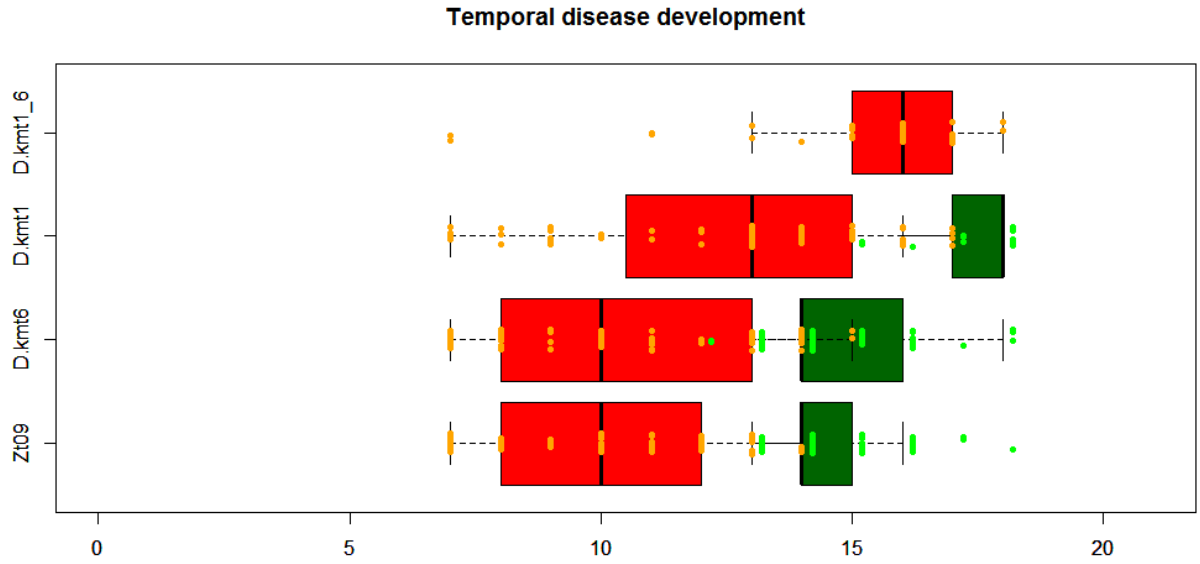


Figure 12. Temporal disease development of the infected wheat leaves inoculated with different strains of *Z. tritici*. The x-axis indicates the days post infection. The red boxes represent the first occurrence of necrotic lesions. The green boxes indicate first pycnidia. Each of the small dots represents infection symptoms on one leaf.

To evaluate infection pattern of the different mutant strains, the leaves were screened manually for first occurrence of pycnidia and necrotic lesions for 21 dpi. The WT strain Zt09 and the *kmt6* deletion mutant induced infection symptoms earlier on the infected wheat leaves than the *Δkmt1* mutant. Leaves infected with the double deletion mutant developed first necrosis even later. At this point in time the *Δkmt6* mutant and Zt09 infected leaves already developed first pycnidia. During the screening of *Δkmt1_6* no pycnidia were found (Figure 12).

4.2.2 Results of automated image analyses of *Z. tritici* infected leaves

To collect more precise information about the infected *T. aestivum* leaves and support the manual visual evaluation, an automated analysis was conducted using the image processing software ImageJ. The output was the percentage of necrotic leaf area and the amount of pycnidia per cm² on the leaf areas infected with *Z. tritici* spore suspension. For statistical evaluation the Wilcoxon signed-rank test was applied to the data. Thereby two samples can be tested for statistical significance to each other. Statistical significance is given when the p-value is smaller than 0.05.

Table 1. Average pycnidia count per cm^2 and percentage of necrotic area per infected leaf area.

treatment used for leaf infection	ø pycnida count per cm^2	ø percentage of necrotic area
Zt09.1	86.9	98.4
Zt09.2	122.7	98.4
Δ*kmt1*#68	28.2	99.1
Δ*kmt1*#80	27.7	98.3
Δ*kmt6*#283	51.4	98.4
Δ*kmt6*#365	85.2	98.4
Δ*kmt1_6*#23	23.1	98.1
Δ*kmt1_6*#116	25.4	97.6
mock treatment	10.9	80.3

The average number of pycnidia per cm^2 varies drastically between leaves infected with the different strains of *Z. tritici* (Table 1). The lowest amount of pycnidia was found on leaves infected with Δ*kmt1_6* deletion mutants, followed by Δ*kmt1* and Δ*kmt6* infected leaves (Figure 14). The WT strain Zt09 showed the strongest virulence phenotype on planta. The amount of pycnidia induced by the individual candidates of Δ*kmt6* and WT Zt09 varied between each other. The candidate Zt09.1 showed lower virulence on planta than Zt09.2 and the candidate Δ*kmt6*#283 induced less pycnidia development on the leaves than Δ*kmt6*#365 (Figure 13). ImageJ also recognized a small amount of pycnidia on leaves of the negative control. When the data of the respective two candidates is pooled and compared to the WT, all results are statistically significant based on p-values from the conduced Wilcoxon signed-rank test (Table 2). All leaves showed values of a necrotic leaf area around 98%, unaffected by the treatment used for infection (Figure 15). Only the mock infected leaves showed a necrosis coverage of 80.3 percent, but even here the median value is 98.3 percent. The different virulence phenotypes on planta are represented in Figure 16.

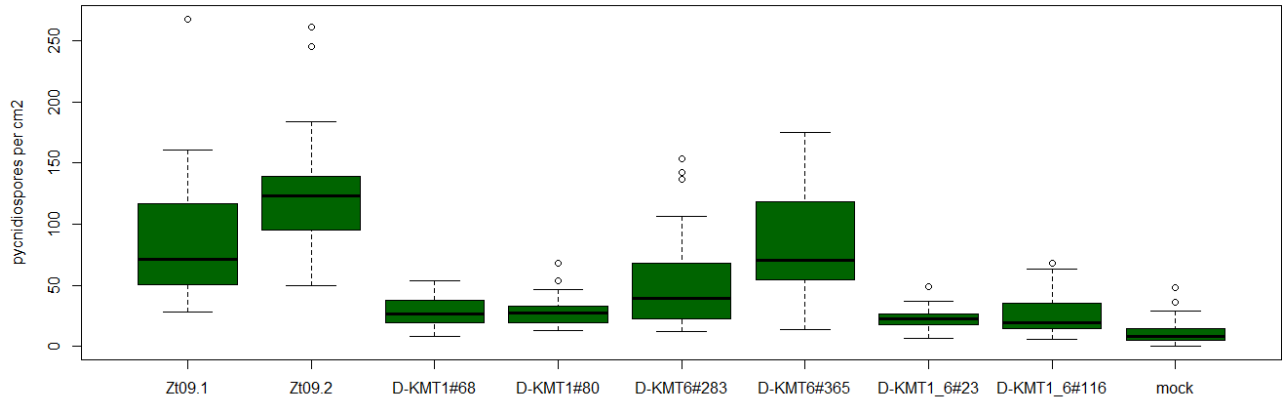
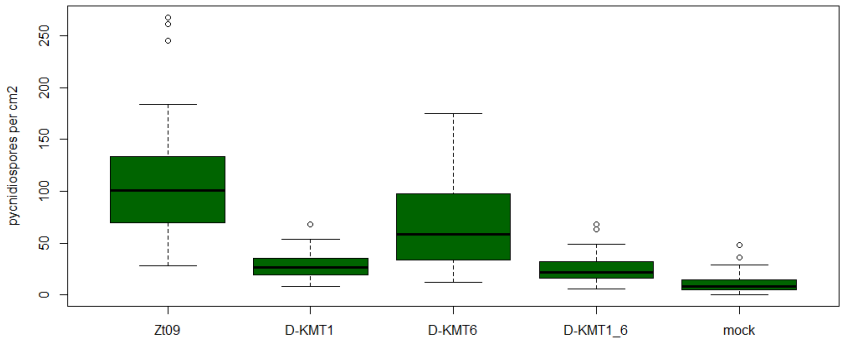


Figure 13. Amount of pycnidia found per cm^2 infected leaf. The boxplot represents the virulence of all tested candidates. The infection success differs between the respective replicates of one strain,



especially between WT Zt09 and kmt6 deletion strains.

Figure 14. Amount of pycnidia found per cm^2 of the infected leave area. The data of the respective candidates was merged. The WT infected leaves showed the highest pycnidia amount. Δ*kmt6* infected leaves showed less and Δ*kmt1* as well as Δ*kmt1_6* showed the least amount of pycnidia.

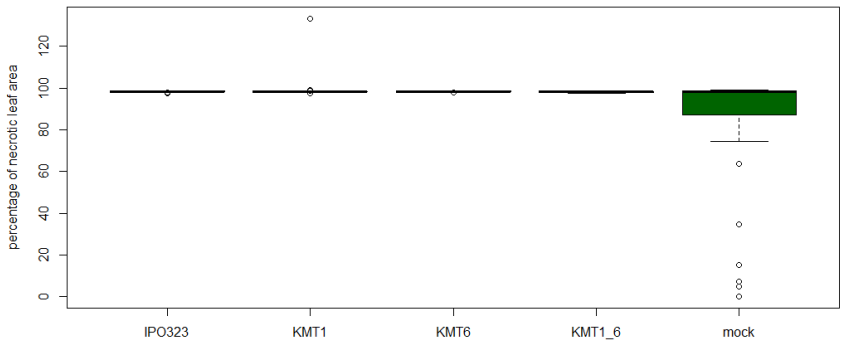


Figure 15. Percentage of necrotic leaf area on the infected leaf areas. All leaves showed similar infection symptoms. Only mock infected leaves showing fewer necrotic area.

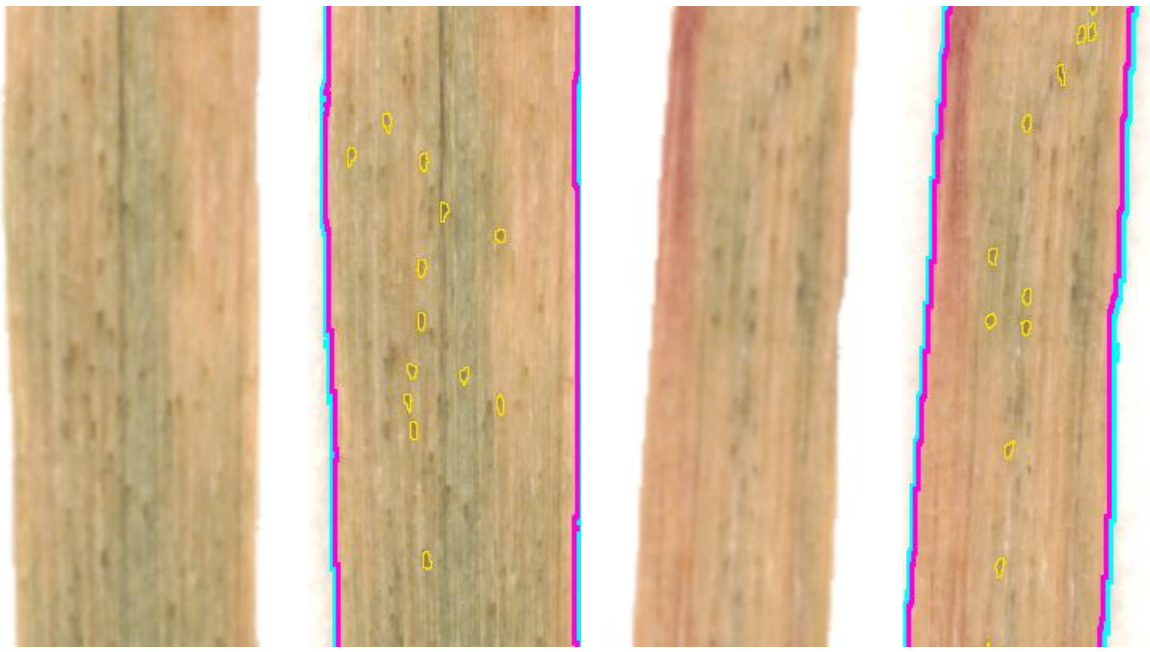
Table 2. The Wilcoxon signed-rank test was conducted on the results for pycnidia count per cm^2. The data of the respective two biological candidates was pooled and compared to WT infection. All results are statistically significant.

Z. tritici strain used for infection	p-values from Wilcoxon test relating to WT infection
Δ*kmt1*	$< 2.2e^{-16}$
Δ*kmt6*	$2.245e^{-6}$
Δ*kmt1_6*	$< 2.2e^{-16}$

Table 3. Results of all leaf infections was tested separately for statistical significance, as the respective biological replicates differ in their virulence. The Wilcoxon signed-rank test showed that all results are significant except one. When data of kmt6#365 is compared to WT Zt09.1 significance can not be given, as the number of pycnidia per cm^2 is very similar for both candidates.

treatment used for leaf infection	p-values relating to pooled Zt09 data	p-values relating to Zt09.1	p-values relating to Zt09.2
Δ*kmt1*#68	$< 2.2e^{-16}$	2.918^{e-15}	$< 2.2^{e-16}$
Δ*kmt1*#80	$< 2.2e^{-16}$	3.876^{e-15}	$< 2.2^{e-16}$
Δ*kmt6*#283	$1.171e^{-8}$	6.917^{e-05}	7.491^{e-11}
Δ*kmt6*#365	0.045	0.9446	0.0005419
Δ*kmt1_6*#23	$< 2.2e^{-16}$	$< 2.2^{e-16}$	$< 2.2^{e-16}$
Δ*kmt1_6*#116	$< 2.2e^{-16}$	6.761^{e-15}	$< 2.2^{e-16}$

In this bachelor thesis we could confirm decreased virulence in planta for all deletion candidates. Comparing the Δ*kmt6* and WT infection success, a small but still significant effect on virulence was found. The double deletion mutant and the candidates lacking KMT1 showed a strongly decreased ability for plant infection. Results of manual evaluation and the automated image analysis are showing the same tendencies.



A: Zt09 WT infected leaf

B: Δkm6 infected leaf

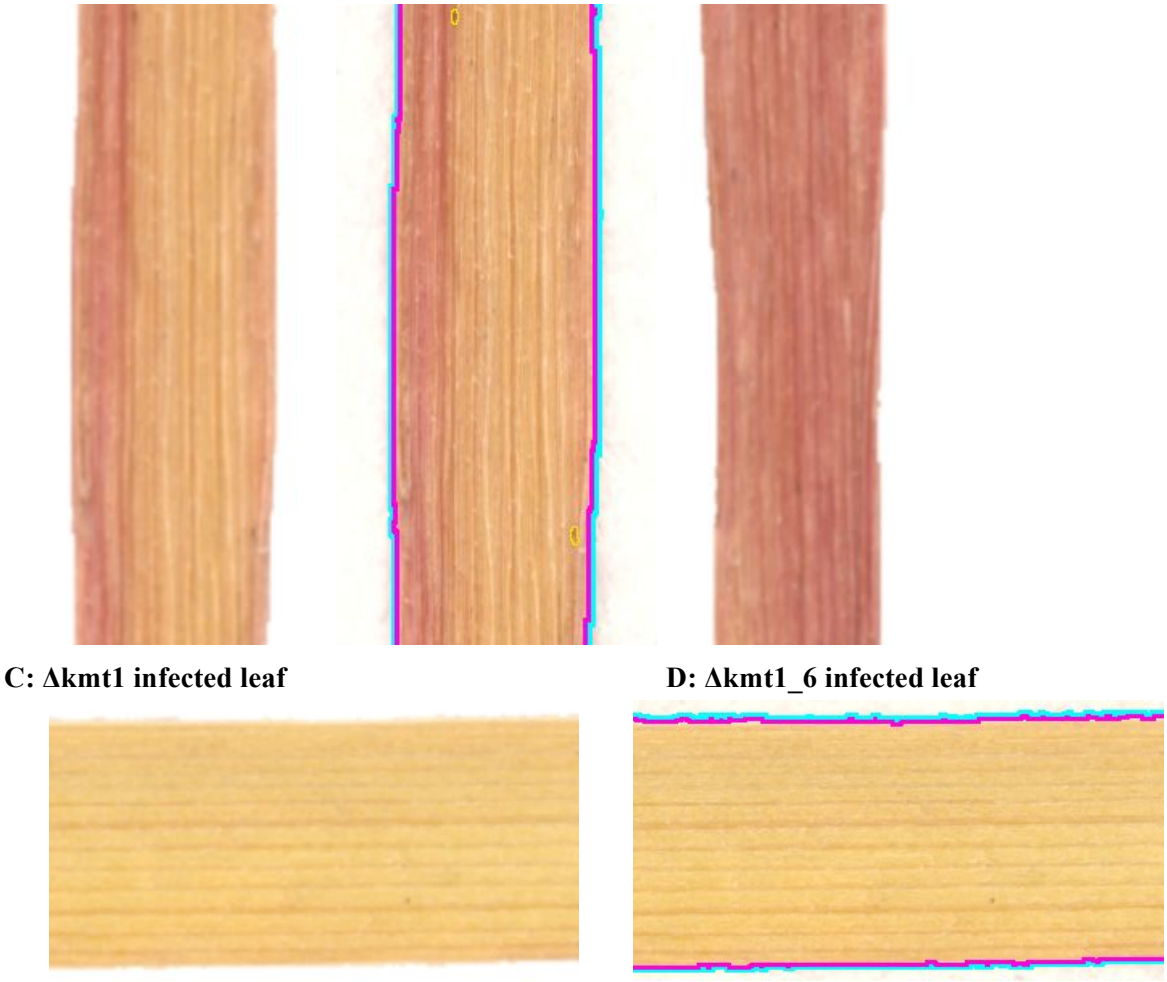


Figure 16. Original scans and overlay images of wheat leaves infected with different *Z. tritici* strains. Areas that ImageJ recognized as pycnidia are labelled with yellow dots. The program classified pycnidia based on size and grey values, which lead to false positive identifications but also not all pycnidia were recognized.

5 Discussion

5.1 *Z. tritici* strains with modified DNA methylation

In this thesis the role of DNA methylation in *Z. tritici* was studied. The objective was to establish strains with altered DNA methylation systems. The *Z. tritici* strain Zt09 lacks DNA methylation, because the gene *dim2* responsible for DNA methylation is not functional. In the strain Zt10 this gene is still functional and though DNA methylation is present in the genome of Zt10. The aim was to integrate a functional *dim2* gene from Zt10 into the genome from Zt09

and thereby restore the DNA methylation. Also the functional *dim2* gene in Zt10 should be replaced by a resistance cassette for the antibiotic hygromycin to inactivate DNA methylation in Zt10. For integration of the respective inserts into the genomes of the *Z. tritici* strains the plasmids pES188 and pES189 were constructed by using Gibson Assembly. *A. tumefacies* mediated transformation was used for integration via homologous recombination. The transformation worked only for Zt10 x pES188, here the construct could be integrated into the genome of Zt10. By PRC and Southern Blot seven candidates with a correctly integrated insert could be verified.

To re-integrate a functional *dim2* gene into the *Z. tritici* strain Zt09 the plasmid pES189 was assembled. The gene should be integrated at its original locus at chromosome 6. In Zt09 a *dim2* gene is present at this locus, but due to repeat induced point mutations (RIP) it is non functional. *Z. tritici* transformation was conducted tree times, yet we were not able to confirm a positive transformed candidate. After the first transformation only five colonies were able to grow on YMS medium containing hygromycin, so only five *Z. tritici* cells might have integrated the construct correctly. Thus it was not probable that the transformation worked correctly, when only five cells were able to establish a hygromycin resistance (in comparison: after Zt10 transformation with pES 188 168 colonies grew).
For the second transformation the wrong antibiotics were used. To eliminate the *A. tumefaciens* cells, the Hybond-N+ membrane should be transferred to YMS medium containing the antibiotic cefotaxime, tree days after transformation. Hygromycin is included as well to select only the positivly transformed *Z. tritici* cells (which gained a hygromycin resistance). Cefotaxime was accidentally used instead of carbenicillin. The *A. tumefaciens* cells carry a resistance against this antibiotic. Since the *A. tumefaciens* cells had a longer time frame to infect the *Z. tritici* cells, it is likely that *A. tumefaciens* infected the cells more than once and the insert was integrated more than once into the genome of Zt09. This was the reason why we decided not to further test these cells for integration of the insert. After a third transformation an unusual phenotype appeared: the plate was filled with a cell layer, but no single colonies appeared. All cells were hygromycin resistant. So there might be a possibility, that they had integrated the insert correctly. Because we were not able to select single colonies and because of the unusual phenotype we did not work further with these cells. A possible mistake could have been a false calculation or counting of cells under the "Neubauer improved" cell chamber. In this case too many *Z. tritici* cells would have been used for transformation.

Besides possible manual mistakes the size of the integration construct mainly due to the *dim2*

gene (4723bp) could have made it more difficult to be integrated into the genome of Zt09 via homologous recombination, than the smaller *dim2* deletion construct of pES188 into Zt10. Even though there are numerous non functional copies of *dim2* throughout the Zt09 genome, integration at chromosome 6 should be possible cause the respective upstream and downstream flanking regions are specific for that locus. Thereby the possibility that the insert is integrated into another region of the genome is slight. But even if an incorrect integration would occur, the hygromycin resistance cassette would be integrated somewhere into the genome and the cells should be growing on the YMS hygromycin plates.

To establish a Zt09 mutant strain with a functional *dim2* gene would be a step in understanding the role of DNA methylation in *Z. tritici* in more than one context. In *Z. tritici* (Zt09) methylation is absent because of a RIP induced inactivation of the gene *dim2* (Dhillon et al., 2010). It is known that sister species of *Z. tritici* (*Z. ardabiliae*, *Z. pseudotritici* and *Z. brevis)* as well as the isolate Zt10 still feature DNA methylation (Möller, unpublished data). Re-integration of *dim2* into Zt09 could contribute to understand the role of this gene and the resulting DNA methylation for *Z. tritici* as successful pathogen of wheat. The isolate Zt10 may also play a role in understanding the success of *Z. tritici* compared to its sister species, since Zt10 was collected in Iran, part of a geographical area where *Z. tritici* emerged as a highly specified pathogen of wheat (Stukenbrock et al. 2007). Here the successful deletion of *dim2* can be a first step to address the question, if DNA methylation contributed to the evolution of this pathogen.

There are many possibilities to further elucidate the role of DNA methylation in *Z. tritici.* Phenotypic analysis in vitro could help to understand if DNA methylation has an influence on growth, fertility, cell shape and under various stress conditions. A plant infection assay could help to learn more about the influence of DNA methylation on the virulence phenotype and modulation of host-pathogen interaction in *Z. tritici.* ChIP (Chromatin-Immuno-Precipitation) sequencing could be performed for Zt09 and Zt10 with and without a functional *dim2* gene, to study if DNA methylation changes the pattern of histone modification. Especially the connection from DNA methylation to changes in tri-methylation of H3K9 and H3K27 could be of interest. In plants and mammals it was shown that histone methylation patterns shift, when DNA methylation is removed by mutating DNA methyltransferase genes. Histone H3 lysine 27 tri-metiylation then covers areas that are normally under influence of H3K9 tri-methylation (Deleris et al., 2012, Saksouk et al., 2014). Since DNA methylation in Zt09 got inactivated due

to RIP it would be interesting to see, if regions covered with H3K9me3 in Zt10 are covered with H3K27me3 in the homologous regions of Zt09. In *N. crassa* it was found that relicts of repeat induced mutations direct heterochromation formation (Lewis et al., 2009). In *Z. tritici* it could be investigated if a stronger heterochromatin formation could be found in the respective areas in Zt09 compared to Zt10. Further studies could include RNA sequencing to evaluate the influence of DNA methylation on gene expression. To study the involvement of *dim2* in chromosome stability experimental evolution approaches could be used.

5.2 Role of KMT1 and KMT6 in virulence of *Z. tritici*

To study the role of the histone methyltransferases KMT6 and KMT1 in virulence of *Z. tritici* a plant infection assay on its host plant wheat was conducted. Thereby the Zt09 WT ability to infect its host plant was compared to a *kmt1* deletion mutant, a *kmt6* deletion mutant and a double deletion mutant, which is lacking both genes encoding for the histone methyltransferases. Two biological replicates were each tested for their virulence. Thereby the different virulence phenotypes on planta could be compared.

The histone methyltransferase KMT6 is responsible for the threefold methylation of lysine 27 on histone 3 (H3K27). This epigenetic mark leads to formation of facultative heterochromatin and thereby to silencing or low transcription activity of the underlying genes (Cao et al., 2002). In this bachelor study *Z. tritici* candidates lacking the histone methyltransferase KMT6 showed a slightly decreased ability to form pycnidia and necrotic lesions on the infected leaves compared to WT infection. A small but still statistically significant differences to the virulence phenotype could be detected in planta. In different plant pathogenic fungi loss of the

methyltransferase KMT6 leads to expression of genes, that are normally silenced because of H3K27 tri-methylation. KMT6 was shown to be involved in regulation of secondary metabolites and species specific genes that are potentially involved in pathgenicity in *F. graminearum*. *A*lso in the rice pathogen *F. fujikuroi* the absence of KMT6 leads to de-repression of genes encoding for secondary metabolites (Studt et al., 2016). In *F. graminearum kmt6* deletion mutants showed growth defects and were sterile (Connolly et al., 2013). However in the conducted plant infection assay with *Z. tritici* the ability to infect the plants was just slightly reduced in *kmt6* deletion candidates. This may indicate that deletion of *kmt6* and thereby lack of histone H3 lysine 27 tri-methylation is not a determinate factor for virulence of *Z. tritici*. Genes that may be de-repressed by the absence of H3K27me3 can not be of major importance for pathogen-host interaction. In a prior study in *Z. tritici* (master thesis M. Möller) *kmt6* deletion mutants showed no significant difference in virulence compared to the WT infection in a plant infection assay on wheat.

These results also correspond to the hypothesis that the histone methyltransferase KMT6 can not be the determinant factor for virulence in *Z. tritici*. Nevertheless, the respective study showed a connection of histone methyltransferase KMT6 to secondary metabolite production in *Z. tritici*. When grown under temperature stress conditions, *kmt6* deletion mutants showed a stronger melanization compared to the WT.

In this thesis the biological replicates of *kmt6* deletion mutants #283 and #365 showed a difference in their ability of leaf infection. Candidate #283 infected leaves developed less pycnidia compared to the other replicate. A PCR confirmed a partial loss of chromosome seven for #283. The long right arm of core chromosome seven shows characteristics for accessory chromosomes, like poor transcription and a low recombination rate (Kellner et al. 2014). It is enriched with heterochromatin due to tri-methylation of H3K27, which is a strong characteristic for the accessory chromosomes in *Z. tritici* as well. Genes encoding for putative secreted proteins are also enriched in this arm compared to the other parts of chromosome seven. Probably it shows these characteristics, cause its origin may be an accessory chromosome that was fused to the core chromosome seven (Schotanus et al., 2015).
With these part of chromosome seven missing candidate #283 showed fewer infection symptoms on planta, which may indicate that the long right arm of chromosome seven plays a

role in plant infection. It could be possible that genes important for pathogenicity of *Z. tritici* are located here. To investigate if there is a role in virulence a further plant infection assays could be conducted, comparing *kmt6* deletion mutants with candidates who lack *kmt6* as well as the long arm of chromosome seven.

The histone methyltransferase KMT1 is responsible for tri-methylation histone H3 lysine 9 (H3K9me3), which leads to formation of constitutive heterochromatin and silencing of gene transcription (Nakayama et al., 2001). In *S. pombe* it was found to be important for chromosome stability (Allshire et al. 1995).
In this bachelor study a plant infection assay was conducted to evaluate, if the virulence phenotype changes, when *kmt1* is deleted. It revealed a significantly different virulence phenotype on planta compared to WT infection. Pycnidiospores and the necrotic lesions were strongly decreased. Studies of the histone methyltransferse KMT1 in different fungal species revealed its importance for normal growth, development and in virulence. In *N. crassa* KMT1 is involved in regulation of normal growth and fertility (Tamaru and Selker 2001). A reduction of the mitotic conidia and delayed conidiation after development, as well as growth abnormalities were found for the sapotrophic fungi *A. fumigatus* in context to the missing histone mark (Palmer et al., 2008). For the *Z. tritici kmt1* deletion candidates in this bachelor study an effect on virulence could be confirmed in the plant infection assay. A prior study in *Z. tritici* (master thesis M. Möller) also confirmed effects on growth, not only on planta but also in axenic culture. Deletion mutants lacking *kmt1* showed delayed growth compared to WT cells. In the respective study a change of phenotype in axenic culture could also be confirmed, the mutant cells developed long hyphae, while the WT cells were shaped like yeast cells. The delayed growth of *kmt1* deletion candidates raises the question, if the cells were potentially able to develop infection structures and a stronger virulence phenotype on planta, if they had more time to grow.

It would be necessary to conduct a plant infection assay over a longer time period to evaluate this hypothesis. Yet it would be challenging to design a plant infection assay, that could be conducted for more than 21 days dpi. The plants (also the negative control group) started to show signs of natural senescence after this period of time, which could be confused with infection induced necrosis. A possible method to evaluate if *kmt1* deletion mutants were potentially able to infect the plant at a later point in time could be microscopic analysis in the end of a plant infection assay. If the fungus started to develop hyphae that grow into the stomata and if early pycnidia structures are visible, it could be possible that the *kmt1* deletion mutant is

able to develop a stronger virulence phenotype in planta. Without these infection structures the fungus would probably not be able to infect the leaves, even when it would be given more time to develop.

Still there are is another hypothesis for what may causes the reduced infection symptoms. In the plant pathogenic fungi *L. maculans* the histone methyltransferase KMT1 was shown to be involved in the transcriptional regulation of effector genes, which indicates a role of KMT1 in plant infection and virulence (Soyer et al., 2014). If KMT1 in *Z. tritici* is also involved in transcriptional regulation of effector genes and thereby production of substances that are necessary for plant infection, the deletion of *kmt1* would change patterns in effector gene expression and thereby may causing changes in the pathogen-host interaction, which could lead to decreased virulence. In this case microscopy analysis would probably reveal no infection structures.

For discussion of the results in a broader context, the effect of KMT1 on TEs elements may play a role. Tri-methylation of H3K9 leads to formation of constitutive heterochromatin and thereby to a constant repression of genes (Nakayama et al., 2001). The formation of constitutive heterochromatin may prevent TEs from further spreading into the genome. In *D. melanogaster* involvement of heterochromation formation in silencing of TEs could be shown (Carmena and Gonzales, 1995). A low GC content seems characteristic for regions with TEs (Lerat et al, 2000). In *Z. tritici* areas covered with H3K9me3 were also found to have a low GC. A relation between enriched H3K9me3 and occurrence of TEs could be shown (master thesis M. Möller). These findings can not be directly linked to the virulence phenotype evaluation in this study. Nevertheless, it would be possible that also the phenotype on planta would change, if the absence of H3K9me3 would de-repress transcription of TEs, that could potentially spread further into the genome.

Another factor that could potentially influence virulence in *Z. tritici* is, that a lack of H3K9 tri-methylation leads to a redistribution of H3K27me3. (E. Stukenbrock and M. Möller, unpublished).There are also similar findings for *N. crassa*, which show that a lack of H3K9me3, established by deletion of KMT1, leads to a redistribution of double- and tri-methylation of H3K27 (Jamieson et al., 2013). Regions organized as constitutive heterochromation may become more dynamic facultative heterochromation, if they are covered with H3K27me3 instead of H3K9me3. Genes rendered by facultative heterochromatin may be expressed in some

stages of cell development. This could have an influence on virulence as well.

A further deletion mutant tested on its virulence was a double deletion mutant who is lacking both histone methyltransferases KMT1 and KMT6. The candidates also showed a strongly different virulence phenotype compared to WT infection. Here the infected plants were showing comparable infection symptoms to Δ*kmt1* infected plants. With both histone methyltransferases missing, there probably is a wider de-repression of genes that are normally silenced due to formation of facultative heterochromatin (H3K27me3) and constitutive heterochromatin (H3K9me3). There can not be a redistribution of H3K27me3 like in the candidates missing only KMT1, because *kmt6* was deleted as well. Candidates lacking KMT1 and KMT6 also showed a slower growth in vitro than WT cells.

For a final assessment also the methods need to be discussed. The plant infection assay was conduced as a double blind experiment. During the infection process the plants were randomized and during the daily manual screening it was not known which group of plants was infected with which fungal candidate. These arrangements were set up to prevent a biased evaluation of symptoms. The results were evaluated manually as well as automatically by utilizing the software ImageJ. Both methods had some advantages and disadvantages. During the manual leaf screening it was possible to differ between infection induced necrosis and natural senescence or injuries that appeared due to mechanical stress. The program ImageJ detects necrotic area based on grey values. This means that darker shaded leaf areas will always be evaluated as necrotic, also if there is a chlorosis, injuries or a contamination on the leaf surface. For all treatments except the negative control a value of 98 percent necrotic leaf area was measured by ImageJ. This value does not represent the necrotic areas on the leafs correctly. This could be assured by manual symptom screening. For evaluation of the necrotic leaf areas manual visual screening was clearly the better choice. It opens the possibility to distinguish between infection symptoms and changes in leaf colour with other origins.

For evaluation of pycnidia ImageJ was able detect the correct tendencies for the amount of pycnidiospores per cm^2. The program recognizes pycnidia based on grey values and their shape. Thereby pycnidia which do not fit into this mask would not be counted. There is also the challenge to choose the best fitting settings for the macro used to run the program. It will define the sensitivity and thereby the amount of pycnidia found on the leaf surfaces. To find out which macro to use, a test evaluation with scans that represent distinct virulence phenotypes was

conduced before. The chosen setting (V15_default) is highly sensitive. Thus even small pycnidia could be found, but also impurities on the leaf surface with the given characteristics were recognized for pycnidia. The infection process partially led to grey residues on the leaves, probably caused by the substance Tween. The high sensitivity resulting in many false positive counted data points. Nevertheless, automated image analysis is a highly valuable tool to measure the pycnidia count. The tendencies in amount of pycnidia between the different strains were represented correctly and correlated with manual evaluation. For automated leaf analysis the number of pycnidia needs to be the main indicator for infection, because detection of necrotic area does not represent the given data correctly. Event though a plant experiment with *kmt1* and *kmt6* deletion mutants was conducted prior to this study and showed similar tendencies, the plant infection assay would need to be repeated again under the same conditions to verify the seen results.

6 Literature

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7 Appendix

8 Declaration/ Eidesstattliche Erklärung

Declaration

I herewith declare, that I have written this thesis independently and by myself. I used no other sources, than those listed. I have indicated where I used quotations. I assure that this thesis has not been submitted for examination elsewhere.

Eidesstattliche Erklärung

Hiermit erkläre ich, dass ich die vorliegende Arbeit selbständig und ohne fremde Hilfe angefertigt und keine anderen als die angegebenen Quellen und Hilfsmittel verwendet habe. Die eingereichte schriftliche Fassung der Arbeit entspricht der auf dem elektronischen Speichermedium. Weiterhin versichere ich, dass diese Arbeit noch nicht als Abschlussarbeit an anderer Stelle vorgelegen hat.

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