

# Function and transmission of accessory chromosomes in *Zymoseptoria tritici*

Dissertation

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## Author's contributions

This thesis consists of six chapters; each represented by a manuscript. Michael Habig developed original ideas and wrote the manuscripts with major contributions.

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### Chapter 1

The origin, function and transmission of accessory chromosomes  
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Chapter 6

The transcription factor Zt107320 affects growth and virulence of the fungal wheat pathogen *Zymoseptoria tritici*

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## Summary

Accessory chromosomes are non-essential genetic elements and defined by a presence/absence polymorphism within a population. These particular chromosomes are widespread across plants, animals and fungi. In fungi, they often contain genes encoding virulence factors and confer a fitness advantage under specific conditions (e.g. on hosts). In plant pathogenic fungi, these chromosomes are considered to be genomic compartments that can mediate rapid evolution of genes directly involved in the interaction with the host. During meiosis a number of fungal accessory chromosomes are transmitted in a non-Mendelian way. The involved exact processes are as yet unclear. The objectives of this PhD thesis are to improve our understanding of the function of the accessory chromosomes of the haploid fungus *Zymoseptoria tritici* and the mechanisms underlying their mitotic and meiotic transmission. This ascomycete fungus is an important pathogen of wheat (*Triticum aestivum* and *T. durum*) causing a diphasic, hemibiotrophic infection. Although *Z. tritici* contains one of the largest known complements of up to eight distinct accessory chromosomes, their function and their meiotic and mitotic transmission has to date been unknown.

For the functional characterization, I used a forward genetics approach by generating isogenic whole chromosome deletion strains for each of the eight distinct accessory chromosome in the reference isolate IPO323. I could show that in contrast to other fungal accessory chromosomes, most accessory chromosomes in *Z. tritici* confer a fitness cost during infection of the host. Deletion of the accessory chromosomes 14, 16, 18, 19 or 21 resulted in increased virulence *in planta*, highlighting that the presence of these chromosomes decreases virulence. This fitness cost varied in infections of different host genotypes. Fitness costs should lead to rapid elimination of these chromosomes from the population. I therefore hypothesized that the continued maintenance of the accessory chromosomes is due to a transmission advantage during meiosis. Using the isogenic whole chromosome deletion strains I thus compared the meiotic transmission of the accessory chromosomes in the presence or absence of a homolog (i.e. being paired or unpaired in the diploid zygote, respectively). My results revealed that unpaired accessory chromosomes of *Z. tritici* have a transmission advantage, as they are inherited by more than the expected 50% of the progeny. Using tetrad analysis, I further demonstrated that this effect is caused by the transmission of female-inherited unpaired chromosomes to all meiotic progeny instead of the expected half. In contrast, male-inherited and paired accessory chromosomes follow Mendelian segregation. This meiotic drive is most likely based on an additional DNA replication step that is restricted to the female-inherited unpaired chromosomes and requires a feedback between pairing of chromosomes and DNA replication during meiosis. This mechanism represents a completely novel aspect of meiosis and could have broader implications for the transmission of unpaired accessory chromosomes in general. I could also show that the accessory chromosomes of *Z. tritici* are frequently lost during vegetative growth *in planta*. Therefore, these frequent losses appear to prevent fixation of the accessory chromosomes as would have been expected due to their meiotic drive. Furthermore, the mitotic loss rate is affected by posttranslational histone modifications which possibly change the nuclear localisation of the chromosomes. Finally, I was able to dissect the interaction between *Z. tritici* and its host by identifying the transcription factor Zt107320 which discriminates compatible from incompatible infections.

In conclusion, this PhD thesis shows for the first time that the accessory chromosomes of *Z. tritici* confer a fitness cost but are maintained in the population by a meiotic drive similar to a selfish genetic element. The frequent losses during mitosis could be causal to the observed presence/absence polymorphism of the accessory chromosomes in the population of *Z. tritici*.



## Zusammenfassung

Akzessorische Chromosomen sind nicht-essentielle genetische Elemente und werden durch einen An-/Abwesenheitspolymorphismus innerhalb einer Population definiert. Diese speziellen Chromosomen sind bei Pflanzen, Tieren und Pilzen weit verbreitet. In Pilzen enthalten sie oft Gene für Virulenzfaktoren und führen unter bestimmten Bedingungen (z.B. bei bestimmten Wirten) zu einem Fitnessvorteil. Bei pflanzenpathogenen Pilzen gelten diese Chromosomen als genomische Kompartimente, die eine schnelle Evolution von Genen ermöglichen. Während der Meiose werden viele akzessorischen Chromosomen von Pilzen auf nicht-mendelsche Weise übertragen. Ziel dieser Dissertation ist es, die Funktion der akzessorischen Chromosomen des haploiden Pilzes *Zymoseptoria tritici* sowie die Mechanismen ihrer mitotischen/meiotischen Übertragung besser zu verstehen. Dieser Schlauchpilz ist ein wichtiges Pathogen von Weizen (*Triticum aestivum* und *T. durum*). Obwohl *Z. tritici* ein großes Komplement von bis zu acht akzessorischen Chromosomen enthält, war ihre Funktion und meiotische/mitotische Übertragung bisher unbekannt.

Für die funktionelle Charakterisierung habe ich einen klassischen genetischen Ansatz verwendet, bei dem ich isogene Stämme mit kompletten Deletionen für jedes der acht verschiedenen akzessorischen Chromosomen im Referenzisolat IPO323 generiert habe. Ich konnte zeigen, dass im Gegensatz zu anderen akzessorischen Chromosomen in Pilzen die meisten akzessorischen Chromosomen in *Z. tritici* einen Fitnessnachteil während der Infektion des Wirtes verursachen. Jedoch variiert der Fitnessnachteil der akzessorischen Chromosomen in Abhängigkeit von den infizierten Wirtsgenotypen. Fitnessnachteile sollten zu einer raschen Eliminierung dieser Chromosomen aus der Population führen. Ich postulierte daher, dass die weitere Präsenz der akzessorischen Chromosomen auf einen Übertragungsvorteil während der Meiose zurückzuführen ist. Ich konnte die meiotische Übertragung der akzessorischen Chromosomen in An- oder Abwesenheit eines homologen Chromosoms (d.h. gepaart bzw. ungepaart in der diploiden Zygote) vergleichen. Meine Ergebnisse belegen, dass ungepaarte akzessorische Chromosomen von *Z. tritici* einen Übertragungsvorteil haben, da sie an mehr als die erwarteten 50% der Nachkommen vererbt werden. Mit Hilfe der Tetrad-Analyse konnte ich zudem zeigen, dass dieser Effekt durch die Übertragung von weiblich vererbten ungepaarten Chromosomen auf alle meiotischen Nachkommen verursacht wird. Im Gegensatz dazu folgen männlich vererbte und gepaarte akzessorische Chromosomen der Mendelschen Segregation. Wahrscheinlich basiert dieser meiotische *Drive* auf einem zusätzlichen DNA-Replikationsschritt, der auf die weiblich vererbten ungepaarten Chromosomen beschränkt ist und ein Feedback zwischen Chromosomenpaarung und DNA-Replikation während der Meiose erfordert. Dieser Mechanismus stellt einen völlig neuen Aspekt der Meiose dar und könnte von genereller Bedeutung bei der Übertragung ungepaarter akzessorischer Chromosomen sein. Weiterhin konnte ich zeigen, dass die akzessorischen Chromosomen von *Z. tritici* häufig während des vegetativen Wachstums *in planta* verloren gehen. Diese häufigen Verluste scheinen eine Fixierung der akzessorischen Chromosomen durch den meiotischen *Drive* zu verhindern. Darüber hinaus wird der mitotische Verlust durch posttranslationale Histonmodifikationen beeinflusst, die möglicherweise die nukleare Lokalisation der Chromosomen verändern. Schließlich konnte ich den Transkriptionsfaktor Zt107320 als wichtige Komponente der Interaktion *zwischen Z. tritici* und seinem Wirt identifizieren, der kompatibel von inkompatiblen Infektionen unterscheidet. Zusammenfassend zeigt diese Dissertation zum ersten Mal, dass die akzessorischen Chromosomen von *Z. tritici* einen Fitnessnachteil verursachen und dank eines meiotischen *Drive* in der Population erhalten bleiben.



## General Introduction

Pathogen-host interactions are highly dynamic. They are characterized by frequent changes in both, the genetic composition of host and pathogen as well as the resulting selective forces. In agriculture natural selection of the host is replaced by managed changes in agricultural methods such as choice of host genotype and use of antimicrobial compounds, which can differ substantially from one year to the next. Hence, pathogens must frequently and rapidly adapt to new environments. In plant pathogenic fungi the adaptation to new host genotypes or fungicides can occur over short time periods [1–4]. This highly dynamic interaction between plant crops and pathogens is reflected on the genomic level of the pathogen. Genes encoding for factors directly interacting with the host are often under positive diversifying or directional selection [5]. However, selection can only act on sequence variation. In order to allow the generation of such sequence variation, genes for effector proteins (small secreted proteins that manipulate the host defences) and other virulence factors are often located in genomic compartments that have a higher rate of sequence evolution [6–10]. Accessory chromosomes are considered to be such genomic compartments that allow the rapid evolution of genes that interact with host processes [9]. These compartments also reduce the risk of potentially disadvantageous changes in essential genes with core functions [9].

In this thesis I have addressed the function and transmission of the accessory chromosomes of the wheat pathogen *Zymoseptoria tritici*. In contrast to other well-known examples of fungal accessory chromosomes, which are directly involved in pathogen-host interactions, the functional relevance of the accessory chromosomes of *Z. tritici* was unknown. Similarly, the transmission mechanisms of the accessory chromosomes was not yet characterized and, thus, it was unclear which processes are responsible for their previously observed presence/absence polymorphism.

### Incidence and function of fungal accessory chromosomes

Accessory chromosomes are present in some but not all members of a population and are not required for normal growth and development. This class of chromosomes is also described as supernumerary, lineage-specific, conditionally-dispensable or B chromosomes and differ from core or A chromosomes that are found in all members of a species. Accessory chromosomes are widespread and have been described in animals and plants - where they are called B chromosomes - and more recently in fungi [11–13]. The first accessory chromosome in a fungus was described in 1991 in *Nectria haematococca* [14]. Since then accessory chromosomes were found in a number of fungal species, the majority of which are associated with plants – either as symbionts or as plant pathogens [12–19].

Considering that accessory chromosomes are dispensable for normal growth and development, their maintenance within populations is unusual. Two possible mechanisms could account for their continued presence: i) genes on accessory chromosomes confer a fitness advantage under certain conditions or ii) accessory chromosomes have a transmission

advantage, i.e. they propagate themselves like selfish elements. For approx. 60% of all plant B chromosomes a transmission advantage or non-Mendelian mode of transmission has been described [20]. In addition, B chromosomes in plants and animals often have a neutral or negative fitness effect on the organism [21]. Therefore, B chromosomes are commonly categorized as selfish genetic elements [11]. In contrast, fungal accessory chromosomes often encode genes that directly confer a fitness advantage under certain conditions [12,13]. In *N. haematococca* a cluster of genes for pea pathogenicity (PEP) is located on the conditionally-dispensable chromosome 14 [22] and is required for the detoxification of the pea phytoalexin pisatin [23]. The effect of genes from accessory chromosomes on host specificity is highlighted by lineages of the genus *Alternaria* [24]. Here, a number of genes that encode host specific toxins are located on conditionally-dispensable chromosomes [18,25–29]. Loss of such a chromosome by an *Alternaria alternata* lineage infecting apple rendered it non-pathogenic on this specific host [18]. Moreover, the ability of *Fusarium oxysporum* f.sp. *lycopersici* to infect its host plant depends on Secreted-in-xylem (SIX) proteins [30]. The majority of known *Six* genes are located on accessory chromosomes, which are called lineage-specific in this fungus [31,32]. In summary, a number of fungal accessory chromosomes contain genes that directly affect host specificity and confer a fitness advantage during certain conditions, e.g. during interaction with hosts. Yet, for many other fungal accessory chromosomes a functional effect has yet to be shown. It is striking that the vast majority of the known accessory chromosomes are from fungi that are closely associated with plants. It is currently unclear whether this is due to the high interest in and intensive research of this group of fungi or has a causal basis, such that plant hosts allow for the maintenance of larger genomes in the associated fungi as proposed by Taylor and colleagues [33].

## Meiotic and mitotic transmission as a source of variation of accessory chromosomes

The presence/absence polymorphism is a defining characteristic of accessory chromosomes. Why do we observe strains that lack accessory chromosomes? Why are accessory chromosomes not fixed (i.e. present in all members of a population) or lost completely from the population? Both fitness and/or transmission advantage should lead to the fixation of accessory chromosomes over time. The widespread presence/absence polymorphism of accessory chromosomes should therefore be a consequence of either i) fitness costs under specific conditions (e.g. non-host conditions), ii) the accessory chromosomes not having reached fixation yet, and/or iii) additional processes that reduce the frequency of the accessory chromosomes over time. Only limited data supports a fitness cost of fungal accessory chromosomes under non-host conditions. The presence of avirulence factors on the dispensable chromosome of *Leptosphaeria maculans* [34] and the accessory chromosome of *Magnaporthe oryzae* [7] directly reduces strain fitness during infection of hosts that recognize these factors leading to effector triggered immunity (ETI) [35]. Therefore, fitness costs under certain conditions could contribute to presence/absence polymorphism. The question whether fixation has yet to be reached, can be addressed using population genomic analysis

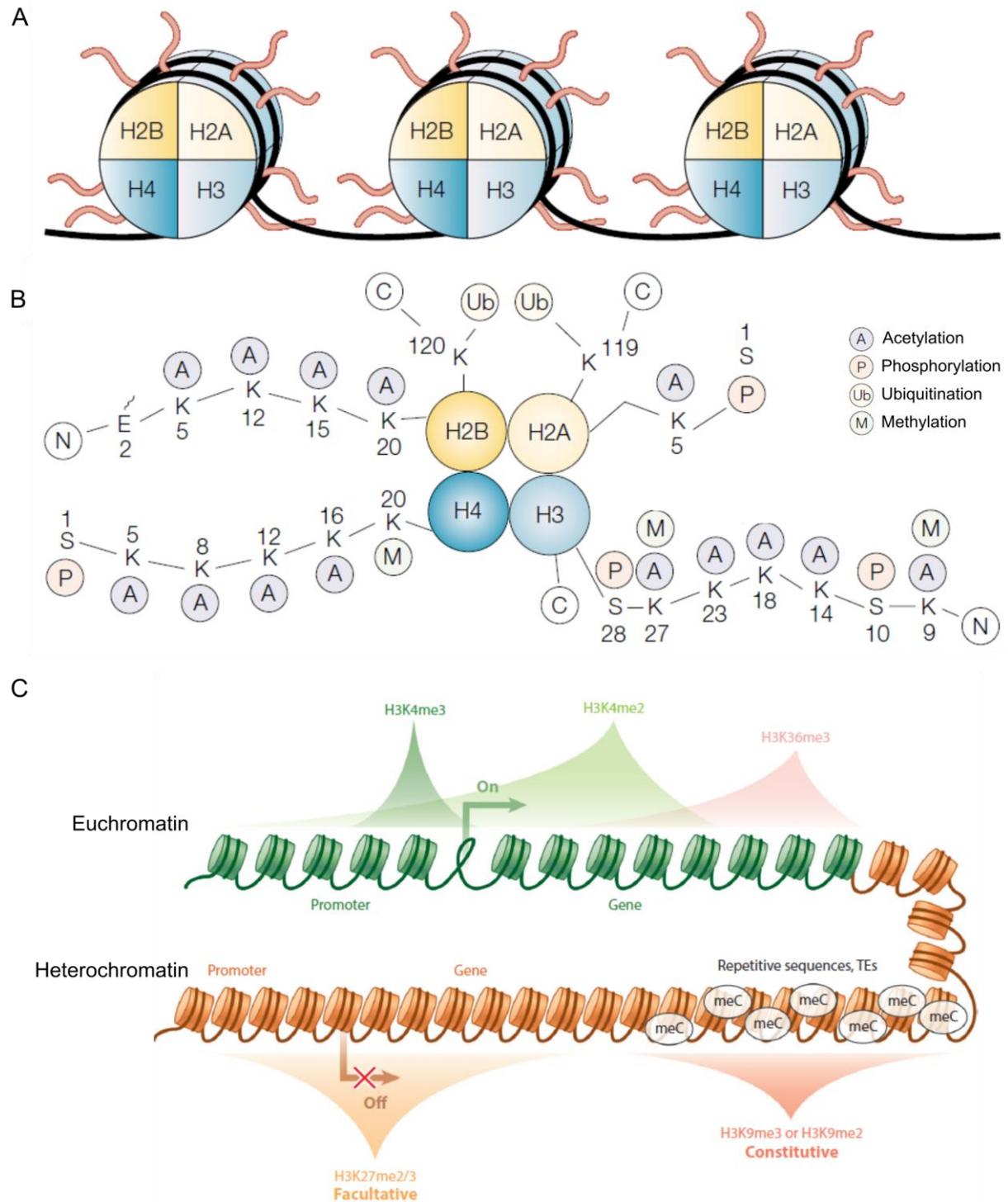
of accessory chromosomes over time, yet this study approach has thus far been used in only a few cases. In *L. maculans* the frequency of isolates lacking the accessory chromosomes remained stable over a ten year period which does not appear to indicate that the presence/absence polymorphism is transient [34]. However, for most fungi data on the presence of accessory chromosomes over time is missing. Furthermore, accessory chromosomes are found and thus transmitted in sexual and asexual fungal species. Their transmission dynamics during meiosis and mitosis has attracted little attention. I will focus on the transmission pattern of accessory chromosomes during both meiotic and mitotic division as a possible mechanistic explanation of the observed presence/absence polymorphism.

Accessory chromosomes are transmitted less faithfully during meiosis and mitosis than core chromosomes. For many fungal accessory chromosomes a non-Mendelian segregation pattern was observed during meiosis, which was characterised by either i) meiotic progenies lacking the accessory chromosome although both parental strains contained the accessory chromosome and/or ii) more than 50% of the meiotic progeny receiving the accessory chromosome although only one of the parental strains contained the chromosome [16,31,34,36,37]. In cases where both parental strains contained homologous accessory chromosomes their absence in the progeny could either be due to non-disjunction or due to the loss of the accessory chromosome. While non-disjunction does not change the absolute frequency of the accessory chromosomes but rather their distribution among the progeny, the loss of accessory chromosomes impacts the absolute frequencies of accessory chromosomes. Both non-disjunction and losses will result in presence/absence polymorphism. For different fungal accessory chromosomes meiotic progenies lacked an accessory chromosome, although both parental strains carried a homolog of the chromosome, as for example reported for *L. maculans*, *M. oryzae*, and *Z. tritici* [16,34,36]. In *Z. tritici* up to 20% of sexually produced ascospores lacked one or more accessory chromosomes, despite both parental strains carrying a homolog [37,38]. Ascospores disomic for accessory and interestingly also core chromosomes were observed at a frequency of approx. 1-2% [38]. In several instances, more than 50% of the progeny received an accessory chromosome although only one parental strain contained the chromosome and therefore the accessory chromosome was unpaired in the diploid zygote. In *L. maculans*, 83 % of the meiotic progeny received an unpaired accessory chromosome [34] and in *Cochliobolus heterostrophus*, a plant pathogen that causes southern corn leaf blight, two thirds of ascospores received the unpaired accessory chromosome 16 [19]. Similarly, in *Z. tritici* transmission of unpaired accessory chromosomes to more than 50% of the progeny was frequently observed [17,37,38]. Although the meiotic transmission of accessory chromosomes is often non-Mendelian, the underlying mechanism of how they are transmitted during meiosis is unclear. Their transmission to more than 50% of the progeny should increase their frequency, thereby possibly compensating for their decrease in abundance due to fitness costs or chromosome losses. This would be similar to the mechanism that maintains the B chromosomes in plants and animals which are often maintained in the population by a transmission advantage despite a fitness cost [11,20].

Fidelity of mitotic transmission of accessory chromosomes will also affect presence/absence polymorphism if these mitotic divisions will eventually result in the production of spores. In purely asexual fungi such as members of the genus *Fusarium* and *Alternaria*, mitotic transmission is the only mode of accessory chromosome inheritance [39,40]. Spontaneous loss of accessory chromosomes during vegetative growth has been observed in *A. alternata* [18] and the insect-pathogenic *Metarhizium anisopliae* [41]. To date, however, an approach to quantify mitotic losses of accessory chromosomes has been carried out only once in the asexual plant pathogenic fungus *F. oxysporum* f. sp. *lycopersici* on the lineage-specific chromosomes 14 [31,42]. Here, 1 in 35,000 spores had lost the lineage-specific chromosome. Interestingly a core chromosome appeared to be non-required for growth *in vitro* and was also lost, albeit at a much lower frequency [42]. More general information on the exact contribution of mitotic transmission to the presence/absence polymorphism is still missing. Moreover, accessory chromosome loss has usually been quantified in the presence of selection which might have biased the observed rates. Therefore, we also still lack exact data on the neutral loss rate of accessory chromosomes.

In eukaryotes chromosomes are organised in chromatin comprising histones. The repeating unit of chromatin is the nucleosome which consists of an octamer of histones comprising two copies each of core histone A2 (H2A), histone 2B (H2B), histone 3 (H3) and histone 4 (H4) around which the DNA is wrapped (Fig 1A). The C- and N-terminal tails of these core histones protrude from the nucleosome and can be covalently modified. These histone modifications regulate aspects of genome maintenance like DNA replication, DNA repair and chromosome and chromatid segregation during mitosis and meiosis [43–45]. Indeed, the chromatin condensation state and the transcriptional state are influenced by posttranslational histone modifications which include acetylation, phosphorylation, ubiquitination and methylation (Fig 1 B) [44,45]. The methylation of lysines in the N-terminal tail of the core histone H3 is of particular interest as it affects chromatin structure and transcription. The di/trimethylation of lysine 9 of histone H3 (H3K9me3) is associated with constitutive heterochromatin and the suppression of transcription and transposition of transposable elements often found in these regions in filamentous fungi [46]. In contrast, transcriptionally active euchromatin is characterised by the presence of trimethylation of lysine 4 of histone H3 (H3K4me3) which localizes to the transcription start site (TSS) or the 5' regions of genes (Fig 1 C) [45,47]. In addition genes located in euchromatin are enriched in H3K4me2 and H3K36me3 [47]. Facultative heterochromatin, in turn, is characterised by the presence of H3K27me2 and H3K27me3 [45,47].

Next to their role in transcriptional regulations and chromatin condensation, histone modifications are also involved in DNA replication and repair, recombination, chromatid cohesions and segregation and thereby in maintaining the integrity of the genome and its faithful transmission to daughter cells [47–52]. Interestingly, the accessory chromosomes of *Z. tritici* and several other plant pathogenic fungi show a higher abundance of H3K9me3 and in particular H3K27me3 methylations compared to the essential core chromosome [53,54]. H3K27me3 is enriched throughout the entire accessory chromosomes, whereas on core chromosomes H3K27me3 is enriched only in the subtelomeric regions [55]. H3K27me3 appears to be involved in tethering these subtelomeric regions to the nuclear envelope [56]. In *Neurospora crassa* the elimination of H3K27me3 resulted in the partial displacement of telomere clusters from the nuclear periphery [57]. Therefore the different posttranslational modifications on core and accessory chromosomes may lead to a different localisation of these chromosomes within the nucleus which in turn could affect DNA replication as well as the transmission of chromosomes in mitosis and meiosis. The methyltransferases KMT1 and KMT6 are responsible for the H3K9me3 and the H3K27me3 methylation, respectively, and deletion of *kmt1* and *kmt6* results in the complete loss of these histone modifications in the genome of *Z. tritici* (Moeller *et al.*, in preparation). However, the role of histone modifications in maintaining the integrity of genomes in plant pathogenic fungi and in particular their role in the maintenance of accessory chromosomes is unclear.



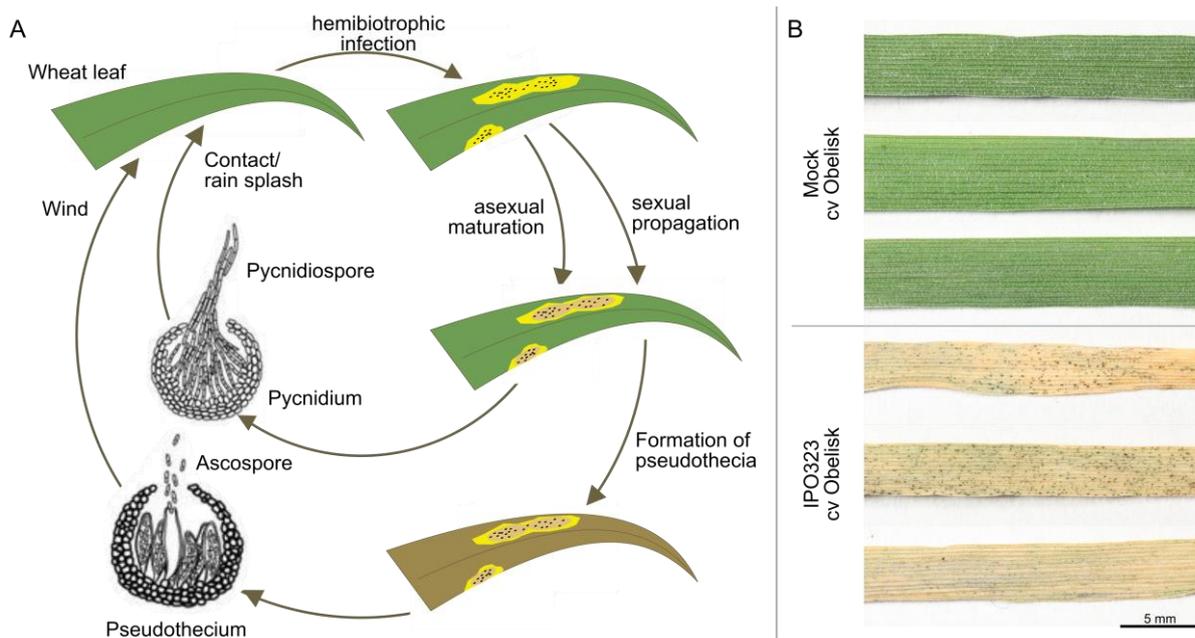
**Fig 1. Nucleosome structure and effect of posttranslational modifications of histones on chromatin condensation and transcription.** A) Schematics of association of histones and DNA in nucleosomes. DNA (black) is wrapped around an octamer of core histones comprising two copies each of H2A, H2B, H3 and H4. C- and N-terminal tails of histones are protruding from the histone octamer. B) Schematics of the C- and N-terminal tails of the core histones with indications of possible posttranslational histone modifications. C) Effect of methylation of histone H3 at lysine residues affects chromatin organisation and transcription. In euchromatin H3K4me3 is often associated with the TSS (green arrow) whereas H3K4me2 and H3K36me3 are associated with genes. In constitutive heterochromatin H3K9me2 or H3K9me3 are enriched and DNA is often methylated on cytosines (meC). H3K27 is di- or trimethylated (H3K27me2/3) in facultative heterochromatin controlling gene expression. (A & B are adapted from [58], C is adapted from [47])

### Model system: *Zymoseptoria tritici*

*Z. tritici* is an ascomycete and a pathogen of bread and durum wheat (*Triticum aestivum* and *T. durum*) [59]. *Z. tritici* speciated approximately 11,000 years ago in the region of the Fertile Crescent which coincided with the domestication of wheat in this region [60,61]. Since then *Z. tritici* has spread to all regions of the world where wheat is grown and it is considered to be one of the most important pathogens of wheat [62].

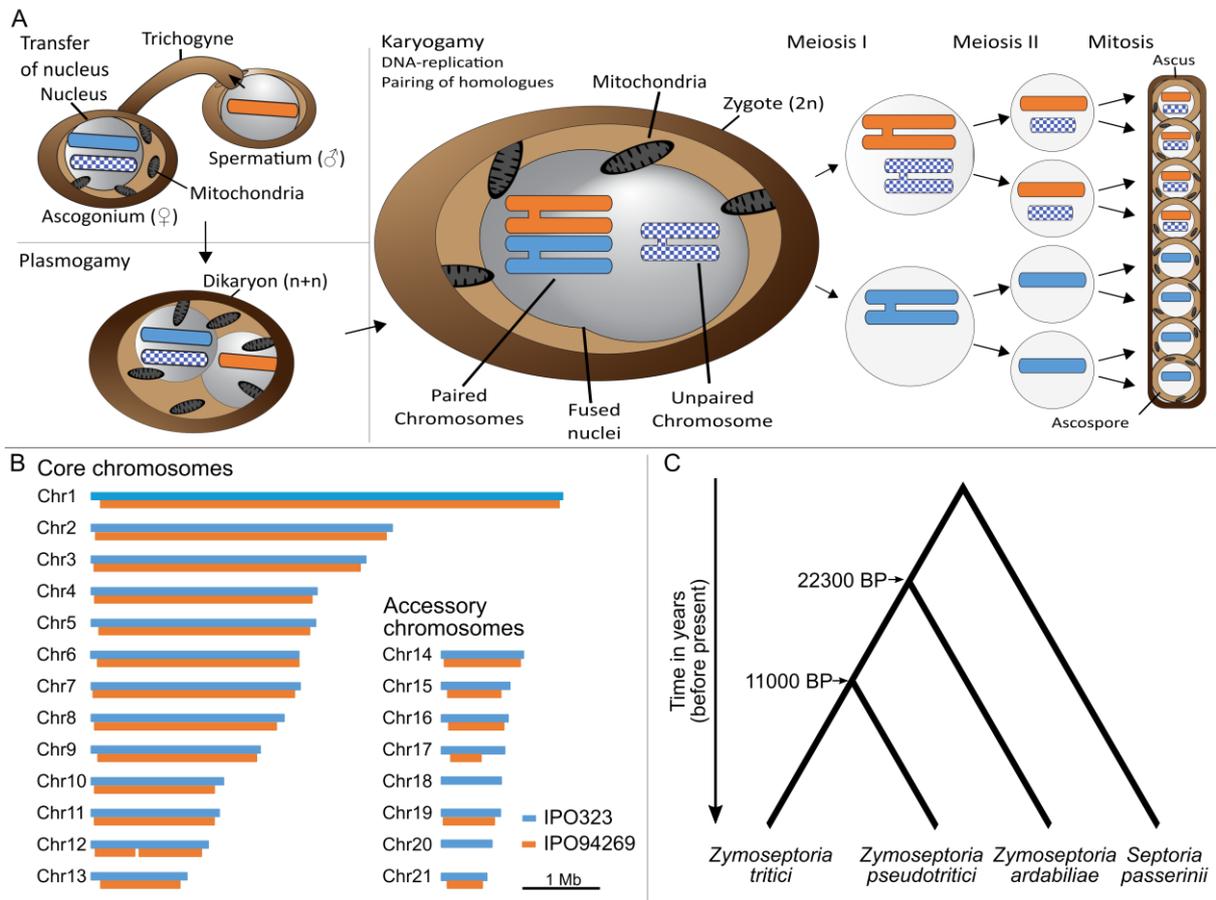
*Z. tritici* is a hemibiotroph that causes a biphasic infection of its host characterised by a long non-symptomatic phase and a rapid switch to necrotrophy. The life cycle of *Z. tritici* includes the production of asexual pycnidiospores and sexually produced ascospores (Fig 2) [63]. Primary inoculum during fall or spring is considered to be mainly composed of windborne ascospores originating from infected wheat debris [64–67]. Spores germinate on the leaf surface, produce hyphae and gain access to the apoplast via the plant stomata [68,69]. Interestingly, the fungus appears to grow slowly in the apoplast with close contact to the plant cell walls but fails to elicit symptoms in the plant host [68]. This symptomless period is considered to be the biotrophic phase of the infection [68,69]. 8-14 days after inoculation growth rates rapidly increase which coincides with the development of necrotic symptoms and the generation of asexually produced pycnidiospores in pycnidia on the host leaves [68,69]. Pycnidiospores, the main source of inoculum during the growth season of wheat, can be transferred to non-infected leaves of the same or neighbouring wheat plants by contact or rain splash and reinitiate an infection [67,70].

Next to the asexually produced pycnidiospores *Z. tritici* releases sexually produced ascospores. These ascospores are generated in pseudothecia with eight ascospores per ascus [71,72]. Ascospores are forcefully ejected from the ascus, windborne and able to disperse over long distances [67]. Ascospores are produced by sexual crosses between two individuals of the heterothallic *Z. tritici*, i.e. the individuals must be of different mating type (*mat1-1* or *mat1-2*) to be able to form a diploid zygote. Mating types and sexual roles are not correlated, meaning that individuals of both types can form female (ascogonia) as well as male structures (spermatia) [73]. The male nucleus is transferred via the trichogyne to the ascogonium followed by plasmogamy resulting in a dikaryon. Subsequent karyogamy and fusion of the two nuclei results in the diploid zygote that will undergo meiosis to produce ascospores (Fig 3A) [74]. Transmission of the mitochondria in *Z. tritici* is uniparental and associated with the female structure [73]. Interestingly, avirulent strains of *Z. tritici* can contribute to zygotes and ascospores when a compatible, virulent strain is also present [73]. Since this process allows for the transmission of avirulent genotypes it likely contributes to the high standing genetic variation found in *Z. tritici* [73,75]. This high standing variation might be one of the reasons why to date only quantitative resistant cultivars are available although a total of 21 major genes for resistance were identified in wheat [59,76,77]. Despite the importance of sexually produced ascospores, the mechanisms and processes essential for crosses and meiosis are poorly understood in this fungus.



**Fig 2. The life cycle of *Z. tritici*.** A) Primary infection in fall/spring by windborne, sexually-produced ascospores which germinate on the leaf surface and gain access to the leaf via stomata where they initiate a hemibiotrophic infection. Asexual propagation results in the production of pycnidia and pycnidiospores. Pycnidiospores are transferred to nearby leaves by either contact or rain splash, where they can initiate a secondary infection. In the presence of a suitable mating partner sexual reproduction results in the formation of pseudothecia and ascospores. Ascospores are forcefully ejected and can be distributed further by wind. B) Example of symptoms of mock and IPO323 infection of the highly susceptible wheat cultivar Obelisk, 28 days after inoculation with  $1 \times 10^7$  cells/mL on the leaf surface. Infected leaf areas are fully necrotic and show pycnidia (dark structures) at high density. (A is adapted from [63]).

The genome of *Z. tritici* was the first genome of a filamentous fungus to be fully sequenced from telomere to telomere in 2011 and shows a remarkable dichotomy [17]. In the sequenced IPO323 reference isolate the genome size is 39.7 Mb distributed among 21 chromosomes. The eight smallest but distinct chromosomes (chr14 to chr21, ranging in size from 0.409 to 0.773 Mb) show a presence/absence polymorphism and are considered to be accessory (Fig 3B) [17]. This is the highest number of reported accessory chromosomes to date in a filamentous fungus, representing 12% of the genome [17]. The accessory chromosomes of *Z. tritici* show homology and synteny with chromosomes in closely related sister species *Z. pseudotritici* and *Z. ardabiliae* indicating their presence already in the last common ancestor and therefore their maintenance in *Z. tritici* since the speciation event 11,000 and 20,300 years ago, respectively (Fig 2C) [60,61,78]. 727 genes were identified on the accessory chromosomes in the reference isolate IPO323 [79] but these are on average expressed less than genes located on the core chromosomes [80,81]. Several individual genes on the accessory chromosomes are however, highly expressed or show specific expression patterns *in planta* vs *in vitro* which could indicate a functional relevance [80–82]. However, no functional analysis of genes located on the accessory chromosomes has been conducted to date.



**Fig 3. Meiosis, genome organisation and phylogeny of *Z. tritici*.** A) Schematic overview of a sexual cross leading to a diploid zygote and subsequent meiosis between two parental strains of *Z. tritici*. Exemplarily, the strains have one accessory chromosome with a homolog in both strains which will be paired in the zygote (blue/orange) and one accessory chromosome unique to one strain (blue checkered) and therefore unpaired in the zygote. The spermatial nucleus is transferred from the male partner via the trichogyne to the ascogonium of the female partner, resulting in plasmogamy and a dikaryon with two separate nuclei. Upon karyogamy, the chromatids are replicated, and meiosis is initiated by pairing of homologous chromosomes. In meiosis I, homologous chromosomes are segregated, followed by chromatid separation in meiosis II. Assuming Mendelian segregation the unpaired accessory chromosome will be transmitted to half of the meiotic progeny. Please note: For the sake of clarity all recombination events have been omitted. B) Genome organisation and syntenic plot of two isolates of *Z. tritici*. The genome of the reference isolate IPO323 (blue) contains 13 core chromosomes (chr1-13) and eight distinct accessory chromosomes (chr14-21). The genome of the Dutch isolate IPO94269 (orange) shares 13 core and six of the eight accessory chromosomes with IPO323 but lacks homologs of accessory chromosome 18 and 20. C) Species tree of *Z. tritici* and its closely related sister species *Z. pseudotritici* and *Z. ardabiliae* using *Septoria passerinii* as an outgroup. Divergence times between *Z. tritici* and *Z. pseudotritici* and *Z. ardabiliae* are indicated. (C is adapted from [60]).

The accessory chromosomes are enriched in repetitive DNA with an abundance of these sequences of 33.6% in comparison to 16.6% on the core chromosomes. Yet, the relative distribution of the major transposable element families does not differ between core and accessory chromosomes [79]. The repetitive DNA in *Z. tritici* show signs of repeat-induced point mutations (RIP) [79,83] a fungal-specific genome defence mechanism against repetitive

sequences [84,85]. RIP often induces point mutations into sequences in the vicinity of repeated sequences, therefore the high frequency of repetitive DNA on the accessory chromosomes could lead to a higher mutation rate on the accessory chromosomes. Although *Z. tritici* has a very high general recombination rate, it is lower on the accessory chromosomes, which together with the higher assumed mutation rate might be the reason why the accessory chromosomes appear to evolve faster than the core chromosome [78,86,87]. The accessory chromosomes also show very high genomic plasticity, whereby different isolates vary in presence/absence of homologous chromosomes and carry a highly differentiated gene content due to numerous insertions and deletions [88–90].

Where did the accessory chromosomes of *Z. tritici* originate? Currently two models are considered: i) the accessory chromosomes originated from within the genome or ii) they were acquired horizontally from another species. The origin of accessory chromosomes from within the genome is supported by the observation of a novel accessory chromosome, which arose through non-allelic recombination resulting in a breakage-fusion-bridge cycle [88,91]. In addition many repeat families are shared between the accessory chromosomes and the core chromosomes [79]. At the same time, the hypothesis of a horizontal acquisition of these chromosomes is also supported by the absence of elevated rates of paralogy [80] and codon usage differences of genes located on the accessory compared to genes on the core chromosome [17]. However, these sequence composition differences between core and accessory chromosomes are no proof of horizontal acquisition but could also be explained as a consequence of relaxed evolutionary constraints, TE content and recombination rate.

In contrast to accessory chromosomes in other fungal plant pathogens, which can contain genes that confer a fitness benefit under certain conditions (e.g. hosts), similar functions have not yet been identified for those from *Z. tritici*. Three studies attempted to dissect the effect of accessory chromosomes on the *in planta* phenotype, using meiotic progeny that lacked one or more accessory chromosomes. All studies showed none or only small effects on fitness-related traits [37,38,92]. Since these studies compared the effect of the presence of accessory chromosomes in non-isogenic meiotic progeny these studies may be biased by additional differences in genomic background. In consideration of the lack of function for the accessory chromosomes, they were instead proposed to yield a repertoire of genes for rapid adaptation to novel environments [93]. Yet, natural selection acts at the individual level and a repertoire of genes would only be advantageous at the population level. Thus, we lack an evolutionary model to explain the maintenance of accessory chromosomes as a repertoire for coming generations. Alternatively, the accessory chromosomes may encode genes that only provide a selective advantage under specific conditions, but again conclusive evidence for this idea is still missing [54].

The accessory chromosomes in *Z. tritici* appear to be less faithfully transmitted during meiotic cell divisions than core chromosomes with frequent absences and disomies in the resulting ascospores [17,37,88]. In cases where both parental strains contained a homolog of an accessory chromosome up to 20% of the progeny lacked a copy of this chromosome and

associated disomies are frequent [17,37,38,88]. In contrast, in crosses where one of the parental strains lacked a homolog of an accessory chromosome present in the other parental strains, these are often found in more than the expected 50% of the meiotic progeny [37,38,88]. Therefore, the accessory chromosomes of *Z. tritici* may additionally have a transmission advantage (i.e. being transmitted to more progeny than Mendelian segregation would predict). However whether this transmission advantage is due to a segregation distortion, a higher viability of ascospores containing an accessory chromosome or a meiotic drive mechanism is not known. A systematic approach dissecting the meiotic transmission of all eight accessory chromosomes of the reference isolate IPO323 is still missing.

*Z. tritici* is a pathogen and thus depends on the interaction with the host and host resources for propagation. How the fungus acquires nutrients during biotrophic growth is unknown but appears to involve either stored or acquired fatty acids or lipids [81]. After the switch to necrotrophy the nutrients of the dead host cells are available for the fungal metabolism and the increase in fungal biomass [94]. How the switch towards necrotrophy is regulated is currently unknown but appears to depend on fungal density (M. Habig, unpublished results). Many aspects of the regulation of the fungal-plant interaction are currently unknown but affecting host responses using effectors is critical for *Z. tritici*. Fungal effectors are secreted molecules that modulate the interaction between the fungus and its host [95]. This class of molecules can include secreted proteins that shield the fungus (e.g. by masking chitin or glucans), suppress the host immune response or manipulate host cell physiology. Effectors have been described for a highly diverse set of fungal pathogens [35,95]. However, to date, only two effectors of *Z. tritici* have been identified, Mg3LysM and Mg1LysM. These prevent the recognition of *Z. tritici* by sequestering chitin or protecting the fungal hyphae against plant derived hydrolytic enzymes [96,97]. Several studies attempted to identify effector candidate genes by assessing loci under diversifying selection or differential gene expression under host conditions [80–82,98]. Surprisingly, to date, only few genes of *Z. tritici* could be shown to influence the phenotype and deletion of many candidate genes had no detectable effect. This result may indicate a high level of redundancy among effectors [98–102]. The majority of genes for which a clear phenotype was identified encode global regulators of metabolism and cell signalling [100] including for example two transcription factors, ZtWor1 and ZtVf1 [99,103]. Transcription factors are pivotal elements of regulatory networks. Hence, analysing regulatory networks by focussing on transcription factors might allow the dissection of the fungal-plant interaction despite a high level of functional redundancy.

## Objectives

The major objective of this doctoral project is to understand the function and transmission of the accessory chromosomes of *Z. tritici*. I specifically assessed how the presence/absence polymorphism, which we observe in field isolates, is generated and which functional consequences result from this polymorphism. In addition, I also characterized the role of transcription factors in mediating the interaction with the host.

## Thesis content

### Chapter 1: Origin, Function and Transmission of Accessory Chromosomes.

Michael Habig and Eva Holtgrewe Stukenbrock. – Manuscript prepared for publication in *The Mycota*, 3<sup>rd</sup> edition - edited by Kerstin Schipper.

In this review, we provide an overview of fungal accessory chromosomes and their possible origin and function. The main focus of this review is the meiotic and mitotic transmission of accessory chromosomes. During meiosis several fungal accessory chromosomes show non-Mendelian transmission and we propose that this particular transmission pattern might contribute to the maintenance of accessory chromosomes in several fungal species. We use the mechanistically much better understood meiotic and mitotic transmission pattern of accessory chromosomes in plants and animals in order to suggest mechanisms for the transmission of fungal accessory chromosomes. In addition, we propose that fungal accessory chromosomes are an ideal tool to study meiosis and the effect of unpaired chromosomes on core meiotic processes like recombination and chromosome segregation.

### Chapter 2: Forward Genetics Approach Reveals Host Genotype-Dependent Importance of Accessory Chromosomes in the Fungal Wheat Pathogen *Zymoseptoria tritici*.

Michael Habig, Jakob Quade and Eva Holtgrewe Stukenbrock. – Article published in 2017 in *mBio* 8 (6). DOI: 10.1128/mBio.01919-17.

In this research article we described the generation of isogenic whole chromosome deletion strains for each of the eight accessory chromosome of the *Z. tritici* reference isolate IPO323. We compared the phenotype of these deletion strains with the phenotype of the reference isolate *in vitro* and *in planta* on different wheat cultivars. For the first time we could show that several of the accessory chromosomes of *Z. tritici* confer a fitness cost and that these fitness costs differ between wheat cultivars. In addition, the timing of the switch from biotrophic to necrotrophic growth is affected by several accessory chromosomes. This is the first study to dissect the phenotypic effect of the accessory chromosomes in *Z. tritici in planta*.

### Chapter 3: Meiotic drive of female-inherited supernumerary chromosomes in a pathogenic fungus.

Michael Habig, Gert H.J. Kema and Eva Holtgrewe Stukenbrock. – Manuscript submitted for publication in *Nature Communications*.

In this research article we address how accessory chromosomes are transmitted during meiotic cell divisions. We use haploid isogenic whole chromosome deletion strains in order to compare the meiotic transmission of an accessory chromosome when it is paired (i.e. with a homolog) in the diploid zygote with the transmission of the same chromosome when it is unpaired. Paired accessory chromosomes show a Mendelian transmission to the meiotic progeny. However, when the same chromosome is unpaired it is transmitted to all instead of

the expected half of the meiotic progeny. But, this is restricted to those unpaired chromosomes that are inherited from the female parent (i.e. the parent that also provided the mitochondria). In contrast, when the unpaired chromosome is inherited via the male parent it follows Mendelian segregation. We propose that an additional DNA replication of unpaired female-inherited accessory chromosomes occurs during meiotic S-phase and suggest a feedback between pairing of chromosomes and initiation of DNA replication. This would represent a previously unknown aspect of meiosis.

**Chapter 4: Extraordinary genome instability and widespread chromosome rearrangements during vegetative growth.**

Mareike Möller, Michael Habig, Michael Freitag, Eva Holtgrewe Stukenbrock. – Manuscript accepted for publication in GENETICS.

In this research article we dissect the mitotic transmission of the accessory chromosomes of *Z. tritici*. We find that during growth *in vitro* and *in planta* chromosome losses occur at a rate of up to 2% during four weeks of propagation. Elevating the temperature during *in vitro* propagation led to substantial increase in losses of accessory chromosomes to more than 50%. Elevated temperatures also increased instability of core chromosomes and induced chromosome rearrangements. This study is the first to report this high level of genome instability in *Z. tritici* which may contribute to the observed presence/absence polymorphism and high structural plasticity of this fungus' accessory chromosomes.

**Chapter 5: Histone posttranslational modifications affect chromosome stability in a plant pathogenic fungus.**

Michael Habig, Hannah Justen, Eva Holtgrewe Stukenbrock. – Manuscript prepared for submission.

In this research article we dissect factors that influence the mitotic transmission of the accessory chromosomes of *Z. tritici*. In order to reduce the effect of selection on the observed frequency of spontaneous chromosome losses we employ a mutation accumulation approach that includes a random one cell bottleneck. We generate 40 replicates for each of six strains and detected the loss of accessory chromosome during asexual propagation *in vitro* over the course of one year. We find that epigenetic histone modifications have a substantial effect on the loss of accessory chromosomes while three different field isolates of *Z. tritici* differ significantly in genome stability. In addition the rate of losses of the accessory chromosomes was constant over time indicating that genome stability is not affected by previous losses of accessory chromosomes. This is the first study to dissect factors that influence the mitotic stability of accessory chromosomes demonstrating that epigenetic histone modifications affect the transmission fidelity of accessory chromosomes in the absence of natural selection.

## Chapter 6: The transcription factor Zt107320 affects growth and virulence of the fungal wheat pathogen *Zymoseptoria tritici*.

Michael Habig, Sharon Marie Bahena-Garrido, Friederike Barkmann, Janine Haueisen and Eva Holtgrewe Stukenbrock. – Manuscript prepared for resubmission in Molecular Plant Pathology.

In this research article we generated deletion and complementation strains of the gene encoding the transcription factor Zt107320. We can show that this transcription factor is differentially expressed during host vs non-host infections and regulates the growth of the fungus *in vitro* and *in planta*. We propose that this transcription factor is involved in discriminating compatible from non-compatible infections and thereby regulates the interaction of the fungus with the host. This study is one of few that could show a direct effect of a single gene of *Z. tritici* on the *in planta* phenotype.

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## Chapter 1

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# Origin, function and transmission of accessory chromosomes

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### Introduction

Accessory chromosomes are found in some individuals of a population, in contrast to essential chromosomes that are present in all individuals. Presence/absence polymorphism of entire chromosomes was first observed in 1907 in a dipteran species using classical karyotyping [1]. Since then accessory chromosomes, also known as B, supernumerary, lineage-specific, or (conditionally) dispensable chromosomes, have been described in a large variety of plant, animal and fungal species, including an estimated 14% of karyotyped orthopteran insect species [2] as well as 8% of monocots and 3% of eudicots [3]. Across this wide range of taxa, accessory chromosomes share the following defining characteristics: i) they are not essential for growth and development, ii) they do not recombine with the essential chromosomes, and iii) they do not follow Mendelian inheritance [2]. Historically, the amenability of many accessory chromosomes in plants and animals (called B chromosomes in these species) to light microscopy allowed for easier and therefore earlier detection and analysis in these kingdoms. In fungi however, the relative small size of accessory chromosomes hindered their detection using conventional light microscopy [4]. The development of pulsed-field gel electrophoresis in 1984 [5] enabled the separation of fungal chromosomes and by allowing the comparison of karyotypes between isolates led to the first discovery of fungal accessory chromosomes in *Nectria haematococca* in 1991 [6]. The advent of next generation sequencing and availability of whole genomes sequences have since fuelled detailed analyses including population genomic analyses of accessory chromosomes in some species [7].

What is the origin of accessory chromosomes? Two non-exclusive models have been proposed to explain the presence of these chromosomes. These chromosomes may originate from essential core chromosomes and diverge or degenerate over time [8], or they may be acquired by horizontal chromosome transfer [4,9]. Both models are supported by current data: In some species, high similarity in sequence composition between core and accessory chromosomes suggests an origin within the same genome, while in other species significant differences in sequence composition may indicate horizontal transfer [10,11]. Phylogenetic analysis

indicates that the accessory chromosomes of *Fusarium oxysporum* f.sp. *lycopersici* have a different origin than the core chromosome [10]. Similarly, the accessory chromosomes in asexual *Alternaria alternata* strains appear to have been horizontally transferred between strains [11]. The presences of mechanisms that allow horizontal transfer of entire chromosomes between distinct lineages have furthermore been experimentally demonstrated [10–14]. It is therefore possible that mechanisms of both models are involved in the origin of new accessory chromosomes in different taxa, although their relative importance appears to vary between species.

In plants and animals B chromosomes are widespread and no unifying pattern of taxonomic distribution is apparent. In contrast, accessory chromosomes in fungi are restricted almost exclusively to plant symbionts with a large fraction of these being plant pathogens [4,15,16]. Why are fungal accessory chromosomes mainly found in plant pathogens? Fungal plant pathogens represent a very intensively studied group of organisms and notably genetic and genomic variation has been a focus of research [17]. It is therefore possible that the excess of examples in this group of organisms reflects the research coverage. However, John Taylor and colleagues proposed that the ability of plant-associated fungi to draw on the abundant resources of photosynthetic organisms enables these species to support enlarged genomes including accessory chromosomes [18]. Determinants of host specificity of several plant-associated fungi locate on accessory chromosomes [10,19–22]. Possibly, this particular genomic location provides an advantage for virulence-related traits as they can be changed readily without affecting core processes. In this way, accessory chromosomes may represent specific genome compartments that allow these fungi to rapidly respond to changes in host plant defences [18].

However, the function of accessory chromosomes appears to be highly variable. B chromosomes in plants and animals often have no known function and the fitness effect is assumed to be neutral or even negative [23]. However, recently few examples demonstrate that B chromosomes in plants and animals can also confer a fitness advantage [2,24,25]. In several pathogenic fungi, accessory chromosomes directly influence pathogenicity as they are required for the infection of specific hosts and hence provide a benefit [10,19–22]. There, these chromosomes encode traits that confer a fitness advantage and natural selection should favour individuals carrying these chromosomes. However, for many fungal accessory chromosomes function and fitness effect have yet to be determined and the influence of selection on the presence of these chromosomes remains still unclear [15]. In contrast to the fitness advantages assumed for many fungal accessory chromosomes, the accessory chromosomes of the wheat pathogen *Zymoseptoria tritici* were shown to cause a fitness cost [26], highlighting the variety of fitness effects associated with accessory chromosomes.

How are accessory chromosomes transmitted during cell divisions? Based on cytological studies, much more is known about the transmission of accessory chromosomes during mitosis and meiosis in plants and animals compared to fungi. Many B chromosomes in plant and animals appear to offset their negative fitness effect by increasing their relative frequency during cell divisions compared to the essential A chromosomes. This increase in frequency has been described as chromosome drive that results from segregation distortion before, during or after meiosis [27]. Therefore, many B chromosomes of plants and animals are considered to be selfish genetic elements propagating themselves at a cost for the organism [27]. Similarly, in fungi, the accessory chromosomes in *Colletotrichum gloeosporioides*, *Gibberella fujikuroi* mating population A, *Leptosphaeria maculans*, *Magnaporthe oryzae*, *Nectria haematococca* mating population VI and *Z. tritici* show non-Mendelian inheritance during meiosis (Table 1). This non-Mendelian inheritance involves either frequent losses or transmission advantages, i.e. transmission of an accessory chromosome to more progeny than Mendelian segregation would suggest [4,12,15,20,28–31]. However, it is likely that accessory chromosomes also are propagated by a drive mechanism in fungi. In support of this, we recently demonstrated a meiotic chromosome drive of accessory chromosomes in *Z. tritici* (Habig *et al.*, submitted).

For fungi without a sexual cycle only mitotic transmission of accessory chromosomes is relevant. Again, fungal accessory chromosomes can show distinct mitotic transmission patterns with extremely high frequencies of chromosome losses and chromosome rearrangements [17,32]. We argue that the distinct transmission patterns of accessory chromosomes during mitosis and meiosis determine the distribution and frequency of accessory chromosomes in fungal populations. A plausible explanation for this chromosome variation is the ability of fungi to rapidly generate new phenotypes in changing environments. Direct evidence of this hypothesis has however not been shown in natural populations of fungi.

We here address the origin, function and transmission of accessory chromosomes in fungi. A main focus of our review is the mitotic and meiotic transmission of the accessory chromosomes, which clearly differs in many fungi from the transmission of core chromosomes. Since the accessory chromosomes of fungi, plants and animals share characteristics, we first briefly discuss the B chromosomes of plants and animals where previous findings can complement the knowledge available on the accessory chromosomes in fungi. We first give an overview on the occurrence and characteristics of fungal accessory chromosomes with a focus on their function and possible origin. We use the term accessory chromosome as a general term and only mention the specific names used in distinct species when referring to examples in detail. Similarly, we will use core chromosomes to term the chromosomes that are shared by all members of a population.

## Accessory chromosomes are widespread and share specific characteristics despite a high level of diversity

In plants, accessory chromosomes are often described as B chromosomes contrasting to the essential A chromosomes shared by all individuals of a species. One of the best studied examples is the rye (*Secale cereale*) B chromosome of which up to eight copies can be found within a single cell. In animals, B chromosomes have been described, among others, in fish, amphibia and insects including a recent example from *Drosophila melanogaster* [33]. These plant and animal B chromosomes share some characteristics: They usually comprise highly repetitive DNA sequences which show sequence similarities to either A chromosomes within the same genome or A chromosomes in closely related species [34]. These sequences can be derived from mobile genetic elements, satellite DNA or ribosomal DNA [27,34–38], and are usually found to be heterochromatic [39,40] and gene poor [41].

Due to their small size, accessory chromosomes in fungi were discovered much later than those in plants and animals. Fungal accessory chromosomes were described first in two plant pathogenic species: *N. haematococca* [6] and *Cochliobolus heterostrophus* [42]. Today we know that the genomes of a wide range of fungal species, in particular plant pathogenic fungi, comprise accessory chromosomes [4,15,16]. Here, we will focus on a few cases that more generally exemplify properties of accessory chromosomes in fungi.

*N. haematococca* can be found in diverse habitats: as a soil saprophyte, as a commensal organism in the rhizosphere, as a pathogen of many different plant species and an opportunistic pathogen in humans [43,44]. The ability to thrive in this diverse set of habitats is partly determined by the presence of accessory chromosomes which are called conditionally-dispensable chromosomes in this fungus [6,45]. Three of the 17 chromosomes found in *N. haematococca* mating population VI (MPVI), namely chromosome (chr) 14, chr15, and chr17, were shown to be dispensable [20]. Similar to the B chromosomes in plant and animals, the conditionally-dispensable chromosomes of *N. haematococca* MPVI contain more repetitive sequences and show a lower GC content in comparison to the core chromosomes [20]. The conditionally-dispensable chromosomes contain more unique genes and these unique genes reflect adaptation to different environmental niches of individual isolates [20].

The accessory chromosomes of the economically important wheat pathogen *Z. tritici* comprise 12% of the genome in the reference isolate IPO323 with eight accessory chromosomes that range in size from 409-773 kb [46]. This represents one of the largest complements of accessory chromosomes described to date. In comparison to the core chromosomes, the accessory chromosomes of *Z. tritici* are relatively gene poor [46,47] and the genes located on these chromosomes show a distinct codon usage [46]. Interestingly, only few genes on the accessory chromosomes encode proteins of known function and most of the predicted genes do not comprise any known functional domains [47]. In contrast to accessory chromosomes of other fungal pathogens, the accessory chromosomes of *Z. tritici* contain a significantly lower number of genes encoding secreted proteins and putative virulence determinants [32,47,48].

Only few genes on the accessory chromosomes belong to gene families that include members on the core and accessory chromosomes and a detailed survey of gene duplicates in the genome of *Z. tritici* showed a low number of paralogous sequences on the accessory chromosome [49]. The accessory chromosomes of *Z. tritici* are enriched in repetitive DNA and transposable elements [47] and, similar to the B chromosomes in plants, show histone modifications associated with heterochromatin [50]. In comparison to the core chromosomes, the accessory chromosomes are highly enriched in trimethylation of lysine27 posttranslational modification of the histone H3 (H3K27me3) [50]. An interesting hypothesis that was proposed when the genome of IPO323 was sequenced was that the accessory chromosomes in this organism originated from horizontal chromosome transfer from another organism [46]. However, the fact that closely related species of *Z. tritici*, *Zymoseptoria pseudotritici*, *Zymoseptoria ardabiliae* and *Zymoseptoria brevis* also harbour accessory chromosomes including regions syntenic to regions of the accessory chromosomes of *Z. tritici* suggest that these chromosomes represent an ancient trait in the genus [51,52] (Eschenbrenner *et al.*, in prep).

The characteristic structural features that distinguish accessory from core chromosomes are also present in *L. maculans*, a pathogen that causes stem canker (blackleg) of oilseed rape (*Brassica napus*) and related crucifers [53,54]. *L. maculans* contains a dispensable mini-chromosome of 700-950kb in size [55] which is mostly comprised of AT-enriched isochores (>60% AT). This dispensable mini-chromosome is AT-rich, gene-poor [56,57] and heterochromatic [58]. Interestingly, the AT-isochores are enriched in transposable elements which are affected by repeat-induced point mutation (RIP), a fungal-specific genome defence mechanism against repetitive sequences [58,59]. RIP can affect neighbouring sequences [60] and therefore genes located within the AT-isochores, are subject to a higher mutation rate [57]. Among the genes located within the AT-isochores many encode effectors - small secreted proteins that manipulate the host defences [61,62] - and their location within these AT-isochores could promote rapid sequence diversification of these genes.

Genome comparison between *F. oxysporum* f.sp. *lycopersici* strain Fol4287 and *F. verticillioides* showed that Fol4287 contains chromosomes 3, 6, 14 and 15 and part of the chromosomes 1 and 2 which do not have syntenic chromosomes/regions in *F. verticillioides* [10]. These accessory chromosomes and regions of chromosomes are called lineage-specific in this tomato pathogen. Again, the lineage-specific regions are rich in transposons, show a lower gene density, a higher proportion of unique genes, a different codon usage and may have a distinct phylogenetic history compared the essential chromosomes [10]. In several additional fungal species accessory chromosomes have been described. These include the plant pathogens *M. oryzae* [63,64], many host-specific lineages of *Alternaria alternata* [19,65–67], *Cochliobolus carbonum* [21], *C. gloeosporioides* [12,68] and *G. fujikuroi* mating population A [29,69]. Recently, an accessory chromosome has also been described in *Botrytis cinerea* [70]. It is striking that accessory chromosomes have been found mostly in plant pathogenic fungi. One exception is the insect pathogenic fungus *Metarhizium anisopliae* which harbours an accessory chromosome [71] and is used as a biocontrol agent [72].

In conclusion, the accessory chromosomes in fungi show similar characteristics to accessory chromosomes found in other organisms as they are enriched in repetitive elements, are mainly heterochromatic and often show a different GC content and codon usage compared to the core chromosomes. However, these characteristics are not exclusive and thus do not allow unequivocal identification of accessory chromosomes because sequences with similar characteristics can also be found within the essential core chromosomes [73].

### Fungal accessory chromosomes are generally associated with function

The dispensability of accessory chromosomes for growth and development raises questions on their functional role. B chromosomes in plants and animals are mostly heterochromatic and therefore gene expression has been assumed to be absent or repressed. Recent studies however, report active transcription of coding sequences located on a number of plant and animal B chromosomes indicating a functional role of genes encoded on these chromosomes [24,25,74–79]. However, transcription is in general found to be lower compared to genes located on the core chromosomes [41]. To date, a fitness benefit of individuals carrying B chromosomes has only been reported for a small number of plant and animal species [2,24]. One of these few cases is the exemplary rye B chromosome, which protects meiocytes against heat stress-induced damage [25].

In contrast, genes located on fungal accessory chromosomes are often associated with a function (see Table 1 and references therein). Some genes on the accessory chromosomes directly influence pathogenicity and host range. One of the conditionally-dispensable chromosomes of *N. haematococca*, the PDA1-CD chromosome, carries a cluster of genes required for pea pathogenicity (PEP) [45]. *N. haematococca* isolates with the PDA1-CD chromosome are highly virulent on pea plants due to the presence of the PDA1 gene which is part of the PEP cluster and codes for a cytochrome P450 enzyme that detoxifies the pea phytoalexin pisatin [80], whereas *N. haematococca* isolates lacking the PDA1-CD chromosome are highly attenuated in the ability to cause lesions on pea [45].

The dispensable chromosome of *L. maculans* also contains genes that affect the host range. The avirulence gene *AvrLm11* that confers avirulence on *Brassica rapa* [56] is located on one of the dispensable chromosomes of *L. maculans* as well as five additional putative effector genes [57]. The loss of the dispensable chromosome of *L. maculans* leads to susceptibility of *B. rapa* carrying the resistance gene *Rlm11* [81]. However, why the accessory chromosome with the avirulence gene *AvrLm11* is present in the population of *L. maculans* remains unclear to date. Individuals with this accessory chromosome are avirulent on *Rlm11* carrying hosts and therefore would have a substantial fitness disadvantage. We speculate that a counteracting selection, possibly a transmission advantage during meiotic or mitotic reproduction, might maintain this chromosome in the population.

In *Z. tritici*, a functional role of the accessory chromosomes is less clear. Based on quantitative trait loci mapping small but significant fitness benefits for certain accessory chromosomes were identified [82]. However, isogenic *Z. tritici* strains with deletions of whole accessory chromosomes could produce more pycnidia than the wildtype and therefore the accessory chromosomes 14, 16, 18, 19 and 21 confer a fitness cost [26]. Interestingly, this fitness cost was dependent on the host wheat cultivar suggesting an interaction of host genotype-specific traits and traits encoded by the accessory chromosomes. *Z. tritici* switches to necrotrophic growth after a prolonged period of symptomless, biotrophic growth. Several accessory chromosomes of *Z. tritici* influence the timing of this lifestyle switch [26] but yet it is unclear how this affects the fitness of the pathogen in the field.

Host-specificity is also influenced by the accessory chromosomes in lineages of the genus *Alternaria*. These plant-pathogenic fungi infect a remarkably large range of hosts [83]. Accessory chromosomes, which are called conditionally-dispensable chromosomes in *Alternaria*, are carried by several *Alternaria* lineages [19,65,66]. These chromosomes are generally smaller than 2.0 MB in size [14,84,85] and represent a prominent example how host specificity is determined by traits encoded by accessory chromosomes. Several genes that are encoding host-specific toxins (HSTs) or are involved in the biosynthesis of toxins are located within gene clusters on the conditionally-dispensable chromosomes. These genes include those producing AF-toxin from the strawberry pathotype [86], AK-toxin from the Japanese pear pathotype [87], ACT-toxin from the tangerine pathotype [88], AM-toxin from the apple pathotype [19] and the AAL-toxin produced by *A. alternata* f.sp. *lycopersici*, a tomato pathogen [89]. In conclusion, genes located on the accessory chromosomes in lineages of the genus *Alternaria* confer a strong selection advantage during host-pathogen interaction and this selection appears to maintain these chromosomes in the populations.

*F. oxysporum* reproduces asexually and consists of many pathogenic clonal lineages which are grouped into host-specific *formae speciales* [90–93]. The ability of *F. oxysporum* to infect and avoid recognition in its host plant depends on small Secreted-in-xylem (SIX) proteins that are secreted into the host xylem during the infection [48]. In the tomato-pathogenic *F. oxysporum* f.sp. *lycopersici* the lineage-specific chromosome 14 harbours six SIX genes and the lineage-specific chromosomes are enriched in secreted effectors and virulence factors [10,94]. Moreover, in the pea-pathogen *F. oxysporum* f.sp. *pisi*, homologs of the pisatin-detoxifying PDA and PEP genes of *N. haematococca* are located on chromosomes considered to be dispensable [95–97]. Furthermore, in *F. oxysporum* f.sp. *medicaginis*, and *F. oxysporum* f.sp. *ciceris* genes with possible pathogenicity-related functions are located on dispensable sequences [95].

**Table 1:** Summary of properties of fungal accessory chromosomes

Species	Number of accessory chromosomes	Size range of accessory chromosomes (Mb)	Genes on accessory chromosomes influencing pathogenicity	Phenotype when present	Mitotic transmission	Meiotic transmission	References
<i>Alternaria alternata</i>	1	< 2	Tox genes	Host-specific pathogenicity	nd (spontaneous loss observed in apple pathotype)	na	[14,19,19,65–67,84–89]
<i>Botrytis cinerea</i>	3	0.22-0.58	None identified	None identified	nd	Non-Mendelian	[70,98]
<i>Cochliobolus carbonum</i>	2	0.75-3.5	TOX2	High virulence on certain genotypes of maize	nd	Mendelian	[21,22]
<i>Cochliobolus heterostrophus</i>	1	0.75-1.2	None identified	None identified	nd	Non-Mendelian	[22,42]
<i>Colletotrichum gloeosporioides</i>	2	1.2-2	None identified	None identified	nd	na	[13,68]
<i>Fusarium oxysporum</i> f.sp. <i>lycopersici</i>	4	< 3.5	Secreted in xylem (Six) genes	Pathogenicity on tomato	Loss in 1 of 35000 spores	na	[10,32,48]
<i>Gibberella fujikuroi</i> MP A	1	0.7	None identified	None identified	nd	Non-Mendelian	[29,69]
<i>Leptosphaeria maculans</i>	2	0.7-0.95	AvrLm11, five effector candidates	Avirulence on hosts containing <i>Rlm11</i>	nd	Non-Mendelian	[56,57,81]
<i>Magnaporthe oryzae</i>	2	0.5-2	AVR-Pita	Unknown	nd	Non-Mendelian	[28,64,99,100]
<i>Nectria haematococca</i> MP VI	3	0.53-1.6	Pea pathogenicity (PEP) cluster	Pathogenicity on pea	nd	Non-Mendelian	[6,20]
<i>Zymoseptoria tritici</i>	8	0.409-0.773	None identified	Lower virulence	Loss at high frequency (up to 1 of 2 spores)	Non-Mendelian	[17,26,30,31,46]

Abbreviations: nd not determined, na not applicable, MP mating population

Although a direct link between pathogenicity and host specificity is lacking for *M. oryzae* it is remarkable that the accessory sequences in this fungus harbour 316 candidate effector genes, defined as genes encoding secreted proteins [99]. In addition, the avirulence gene *AVR-Pita* was found to be located at multiple genomic locations including loci on the accessory chromosome [64,99]. Hence, the presence/absence of the accessory chromosome may influence the ability to infect different host genotypes. Polymorphic localization of *AVR-Pita* (including on the accessory chromosomes) was proposed to be adaptive by allowing loss and rapid recovery of this avirulence gene, consistent with the idea that the accessory chromosomes might serve as a particular genomic environment allowing the rapid evolution of new adaptive traits [64].

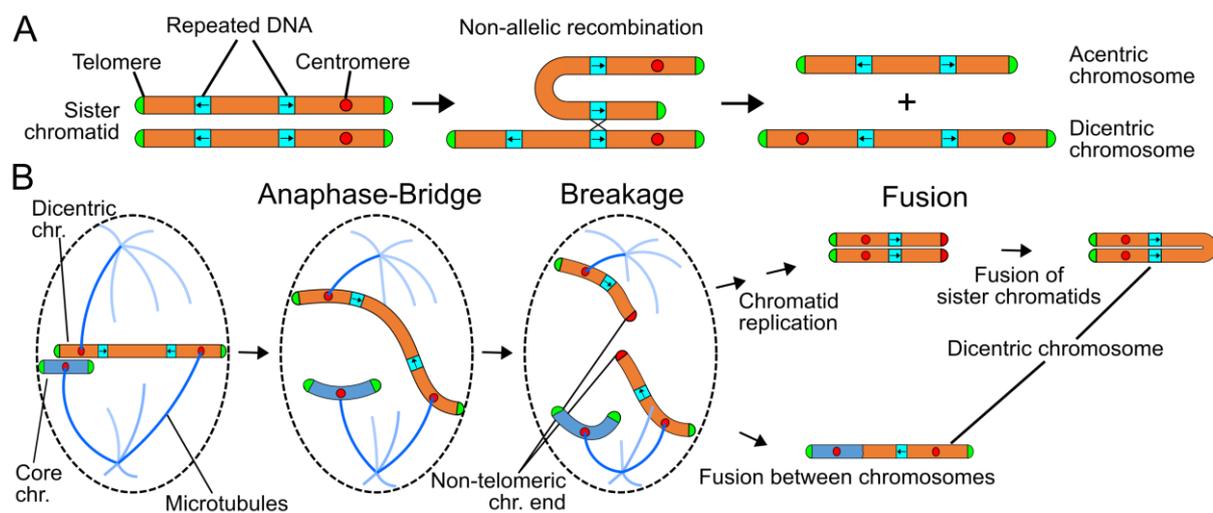
In conclusion, genes located on accessory chromosomes in fungi have often been associated with a fitness benefit - in many cases by encoding traits that directly influence pathogenicity and host specificity. In contrast to the B chromosomes in plants and animals, fungal accessory chromosomes are rarely associated with a fitness cost. Nevertheless, accessory chromosomes can confer a fitness cost under some conditions as demonstrated in *Z. tritici* and the potential effects of avirulence factors present on the accessory chromosomes in *L. maculans* and *M. oryzae*.

### The origin of fungal accessory chromosomes

How have accessory chromosomes originated in fungal genomes? Genome data in particular has shed light on the possible origin of accessory chromosomes and experimental studies have revealed mechanisms of de novo chromosome acquisition. Thereby evidence for two distinct scenarios accumulated: i) accessory chromosomes are derived from essential core chromosomes and ii) they are acquired by horizontal chromosome transfer from closely or distantly related taxa.

Initially, plant and animal B chromosomes were thought to be non-homologous to the essential A chromosomes, but this view later changed. Due to their mosaic-like composition of sequences derived from essential A chromosomes and organelles, they are now mainly considered to originate from A chromosomes [23,41,101]. The origin of B chromosomes has been addressed using genomic data in rye, barley, dogs and several fish species [34,41,101–104]. These analyses suggested a multi-step model for the origin of accessory chromosomes from several core chromosomes where an initial genome duplication (either partial or comprising the whole genome) is followed by reductive chromosome translocations decreasing the size of the B chromosome [23,41]. Chromosomes that have evolved in this way, diverged over time by the accumulation of repetitive sequences and further structural variation that impair homologous recombination with the original core chromosome [8,23]. Alternatively, small accessory chromosomes could be generated by mis-segregation or Robertsonian translocations at or near the centromeres of two acrocentric A chromosomes. This could result in the duplication of a short region of A chromosomes that, if it contains a functional centromere and origin of replication, could be considered a very small B chromosome and later may grow in size by acquiring additional sequences [8].

In some fungal species there is evidence that the accessory chromosomes have originated from the core chromosomes. In others species, it is possible that fusion of hyphae mediate horizontal transfer of chromosomes between lineages or even species. So far, little is known about the exact mechanism that transforms core chromosome copies into accessory chromosomes. In *Z. tritici* experimental mating and karyotyping of progeny strains revealed the evolution of a new accessory chromosome by a process called “breakage-fusion-bridge” (BFB)-cycles that involves chromosomal fusions followed by degenerative breakage [31,105]. BFB-cycles can be initiated by non-allelic homologous recombination between repeats, whereby dicentric and acentric chromosomes can be generated [31,105]. Acentric chromosomes are lost but dicentric chromosomes can form a bridge at anaphase and undergo BFB-cycles (see Fig 1) [31,106]. In *Z. tritici* many repeat families are shared between core and accessory chromosomes, possibly facilitating an origin of the accessory chromosomes within the genome [47]. Size variation due to non-allelic sister chromatid recombination was also observed in *M. oryzae*, [100]. The high content of repetitive DNA on many accessory chromosomes may indeed favour the occurrence of non-allelic homologous recombination.



**Figure 1:** Non-allelic recombination between sister chromatids of an accessory chromosome initiates a breakage-fusion-bridge cycle. The generation of dicentric chromosome followed by BFB-cycles might explain the genomic plasticity of the accessory chromosomes and the bidirectional transfer of sequences between accessory and core chromosomes. A) Non-allelic recombination between repeated sequences on an accessory chromosomes results in an acentric chromosome which is lost during subsequent cell divisions and a dicentric chromosome (adapted from [31]). B) The dicentric chromosome is subject to a breakage-fusion-bridge cycle when the two centromeres are pulled to opposite poles of the dividing cell during anaphase. Thereby, the chromatid breaks and two non-telomeric chromosome ends are generated. These ends can either fuse between the sister-chromatids after sister chromatid replication or fuse to other chromosomes creating a translocation on, in this example, a core chromosome. In both cases, dicentric chromosomes are generated which might again be subjected to a breakage-fusion-bridge cycle. Note: For the sake of clarity sister-chromatids were omitted when possible.

In *F. oxysporum* f.sp. *lycopersici*, the lineage-specific chromosomes appear to have a different origin than the core chromosomes. These chromosomes have a high proportion of unique genes and vary in codon usage as well as GC content and are believed to have been acquired from another *Fusarium* species [10]. Interestingly, under experimental conditions transfer of the lineage-specific chromosome 14 of *F. oxysporum* can occur between asexual lineages of the pathogen by vegetative hyphal fusion [10,107]. For the analysis, strains were used that carried two selection markers (neomycin and hygromycin), located on the chromosome 14 of the donating strain (tomato-pathogenic) and on a core chromosome of the receiving strain (tomato-non-pathogenic), respectively. Co-inoculating these two strains on agar plates allowed for the isolation of colonies resistant to both antibiotics. These double resistant strains were pathogenic on tomato and contained large portions of the lineage-specific chromosome 14. This experimentally validates chromosomal transfers by vegetative fusion (in this case between different strains of the same species) as a possible mechanism for the acquisition of accessory chromosomes [10]. Further examples of chromosome transfers include the asexual plant pathogen *C. gloeosporioides* that infects a wide variety of crops. The supernumerary chromosome of *C. gloeosporioides* is considered to have been transferred by vegetative fusion which can also be observed under laboratory conditions [12,13]. Similarly, chromosome transfer occurs in *Alternaria* spp. Here, pathogenic strains cause leaf spots and blights on different host plants [83]. Phylogenetic analysis of toxin-encoding, conditionally-dispensable chromosomes supports chromosome transfer between different strains [11], which could be confirmed under laboratory conditions [14]. In addition, transfer of accessory chromosomes was suggested by comparative analyses of transposon or repeat content and codon usage of the accessory and the core chromosomes of *A. arborescens* [67]. Although the origin of the conditionally-dispensable chromosomes in *Alternaria* spp. is still unknown, these potential horizontal transfers would be an example for a possible mechanism by which pathogens could acquire novel determinants of host specificity [9].

It is however important to note that the observed differences in sequence composition between core and accessory chromosomes are not proof of a horizontal chromosome transfer. Different recombination and mutation rates, the differences in transposable element content and higher frequencies of pseudogenes could likewise explain some of the observed differences in sequence composition [8]. This is exemplified by the wheat pathogen *F. poae* which carries several accessory chromosomes [108]. Here, the accessory genome was shown to be lacking repeat-induced point mutations (RIP) in contrast to the core chromosomes where this defence mechanism appeared to be functional [109]. The sole absence of RIP may confer differences in sequence composition between core and accessory chromosomes. Similarly, sequences on the accessory chromosomes of *Z. tritici* show signatures of accelerated evolution which could also account for the observed sequence differences between the core and the accessory chromosomes [110,111].

Although accessory chromosomes are thought to be non-recombining with the core chromosomes, transfer of genetic material from accessory to core chromosomes appears to happen frequently. In several cases an accessory chromosome became part of the core chromosomes [10,50,109]. In *Z. tritici*, the distal 0.865 Mb segment of the long right arm of the core chromosome 7 shares characteristics like histone modifications, gene content and gene organization with the accessory chromosomes [50]. This chromosome segment is therefore considered to be either a translocated fragment or an entire accessory chromosome fused to a core chromosome [50]. This type of chromosomal fusion could be the result of BFB-cycles as already observed for an accessory chromosome in the genome of *Z. tritici* [31] (Fig 1B). Similarly, the right arms of the core chromosomes 1 and 2 in *F. oxysporum* f.sp. *lycopersici* share sequence characteristics with the lineage-specific chromosomes and hence these chromosomal regions are also considered to be lineage-specific [10]. In addition, the core chromosome of *F. poae* contains large blocks (>200 kb) of sequences that were recently translocated from the accessory genome [109]. Hence, sequence exchange between accessory and core chromosomes occurs but the extent of this exchange and the functional and evolutionary consequences remain to be elucidated.

### Accessory chromosomes are mitotically instable

Accessory chromosomes often show distinct patterns of mitotic transmission compared to the core chromosomes. These transmission differences can also affect their meiotic transmission if cells of the germline are affected (e.g. non-disjunction during first pollen mitosis of rye B chromosomes). Further examples of non-equal transmission during mitosis in plants and animals were described for *Aegilops speltoides* and grasshoppers in which the number of accessory chromosome differs between organs of the organism [112,113].

In fungi, the differentiation between soma and germline is obscure. Therefore, any differences in transmission of accessory chromosomes affect their overall propagation. Indeed, asexual fungi rely solely on mitotic transmission. Accessory chromosomes in several fungi can be lost during mitotic transmission. In *F. oxysporum* f.sp. *lycopersici* the two smallest lineage-specific chromosomes (chr1, chr14) are lost at a frequency of one in 35,000 spores. Interestingly, the core chromosome 12 could also be lost but at a much lower frequency than the accessory chromosomes [32]. Similarly, in *A. alternata* loss of the conditionally-dispensable chromosome occurred spontaneously *in vitro* [19] and rendered the strain non-pathogenic. In *Z. tritici* and its closely related sister species *Z. ardabiliae* spontaneous loss of accessory chromosomes was observed at a very high frequency of up to one in 50 spores [17]. Surprisingly, temperature had a dramatic effect on the loss-rate of accessory chromosomes in *Z. tritici*. A rise in temperature from 18°C to 28°C increased the losses of accessory chromosomes to one in two spores [17]. Importantly, during mitotic growth *in planta*, which results in the production of asexual pycnidiospores, losses of accessory chromosomes were also observed at a similar rate to those *in vitro* at 18°C [17]. This demonstrates that accessory chromosome losses occur throughout the entire life-cycle of *Z. tritici*. Interestingly, accessory chromosome 18, which was frequently lost during *in planta* experiments is also often absent in field isolates of *Z. tritici* [31,114]. Hence,

the presence/absence polymorphism for the accessory chromosomes appears to be – at least partially – generated by frequent losses during mitotic divisions. Interestingly, in addition to frequent chromosome losses the same study reported frequent chromosome fusions, chromosome breakages and accessory chromosome duplications at elevated temperatures [17]. Chromosomal fusions involved core as well as accessory chromosomes what further highlights the potential plasticity of the *Z. tritici* genome and the potential for bidirectional sequence exchange between core and accessory chromosomes.

Why do the accessory chromosomes show a different transmission during mitosis? Interestingly, the accessory chromosomes in *Z. tritici*, *F. asiaticum*, and *F. fujikuroi* are enriched in the histone modification H3K27me3 [8,50]. This histone modification, which on core chromosomes is restricted to subtelomeric regions, is involved in the localization of these subtelomeric regions to the nuclear envelope [115–117]. Therefore, entire accessory chromosomes might be localized at the nuclear envelope. This different localization would also be supported by the fact that in *Z. tritici* centromeres do not co-localize in one focus, as observed in many fungi, plants and animals [118–120] but to several distinct foci [50]. We hypothesize that the different epigenetic marks on the accessory chromosomes affect their location in the nucleus and thereby influencing their DNA replication and transmission. Recently, we could show that removal of the H3K27me3 histone modification leads to an increased transmission fidelity of the accessory chromosomes supporting the pivotal role for histone modifications in distinguishing core and accessory chromosomes (Moeller *et al.*, in preparation, Habig *et al.*, in preparation).

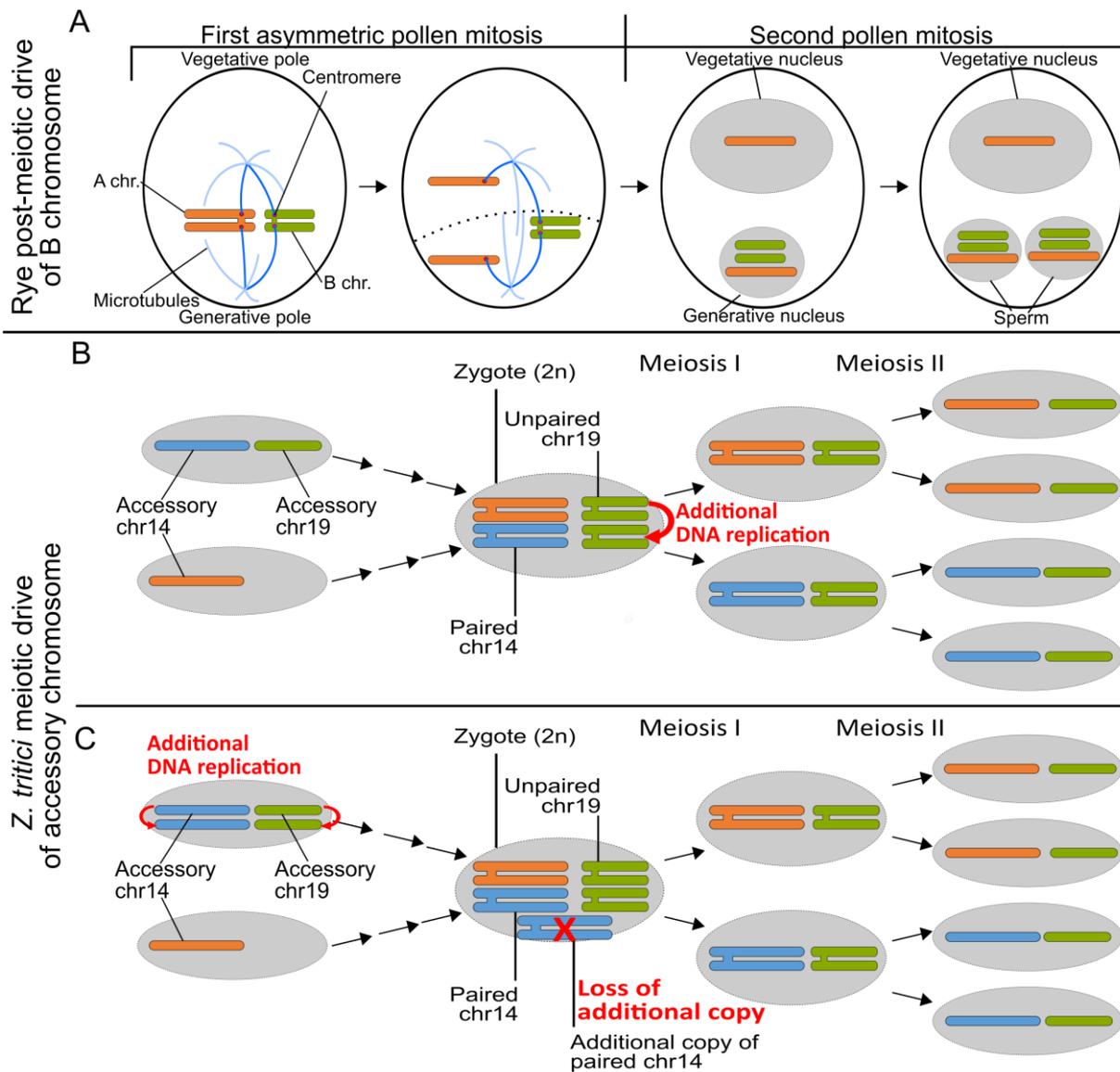
### Accessory chromosomes are frequently transmitted in a non-Mendelian way during meiosis

By definition, accessory chromosomes are present in some but not all members of a population. During sexual mating this presence/absence polymorphism can result in unpaired (i.e. univalent) accessory chromosomes during meiosis if the two parental strains have different chromosome complements (Fig 2). How are univalent accessory chromosomes transmitted during meiotic divisions? In animals and plants, the widespread occurrence of accessory chromosomes is generally attributed to their selfish mode of transmission [27,121], which increases their frequency by preferential segregation during cell divisions including meiosis. However, little is known about the meiotic transmission of fungal accessory chromosomes. For several, a non-Mendelian mode of inheritance has been reported, which appears to be similar to the mode in plants and animals. We will therefore first describe some of the well-understood examples of B chromosome transmission in plants and animals, which may direct our research of accessory chromosome inheritance in fungi.

B chromosome accumulation mechanisms, also termed chromosome drive, involve non-Mendelian modes of transmission and have been described in approx. 60% of the plant species that carry B chromosomes [121]. Chromosome drive can occur either at the pre- or post-meiotic stages or during meiosis [27,122–124]. The B chromosome of rye is a well characterized

example for a chromosome drive mechanism acting post-meiotically [27,125–127]. Following meiosis the pollen development involves two mitotic divisions resulting in tri-cellular pollen. One pollen consists of two sperm cells and a vegetative nucleus, which does not provide genetic material to the offspring [128]. During the first pollen mitosis the two sister-chromatids of B chromosomes show non-disjunction [125] and will in most cases become part of the generative nucleus. Thereby the resulting sperm cells will contain two B chromosomes instead of the expected one (Fig 2A) [27,125]. What are the underlying mechanism that can lead to the exploitation of asymmetric cell divisions by B chromosome? The different segregation of A versus B chromosomes during mitotic or meiotic cell divisions predicts functional differences between their centromeres (or the loci that mediate attachment of the chromosomes to the spindle). Indeed, the centromeres of A and B chromosomes in rye differ in their repeat composition, with the B chromosome centromeres including additional classes of repeats [127]. Here, a non-disjunction control region (NCR) acting in trans, controls chromosome segregation of the B chromosome, possibly by long non-coding RNAs, which affect the centromere organization [27,77,126].

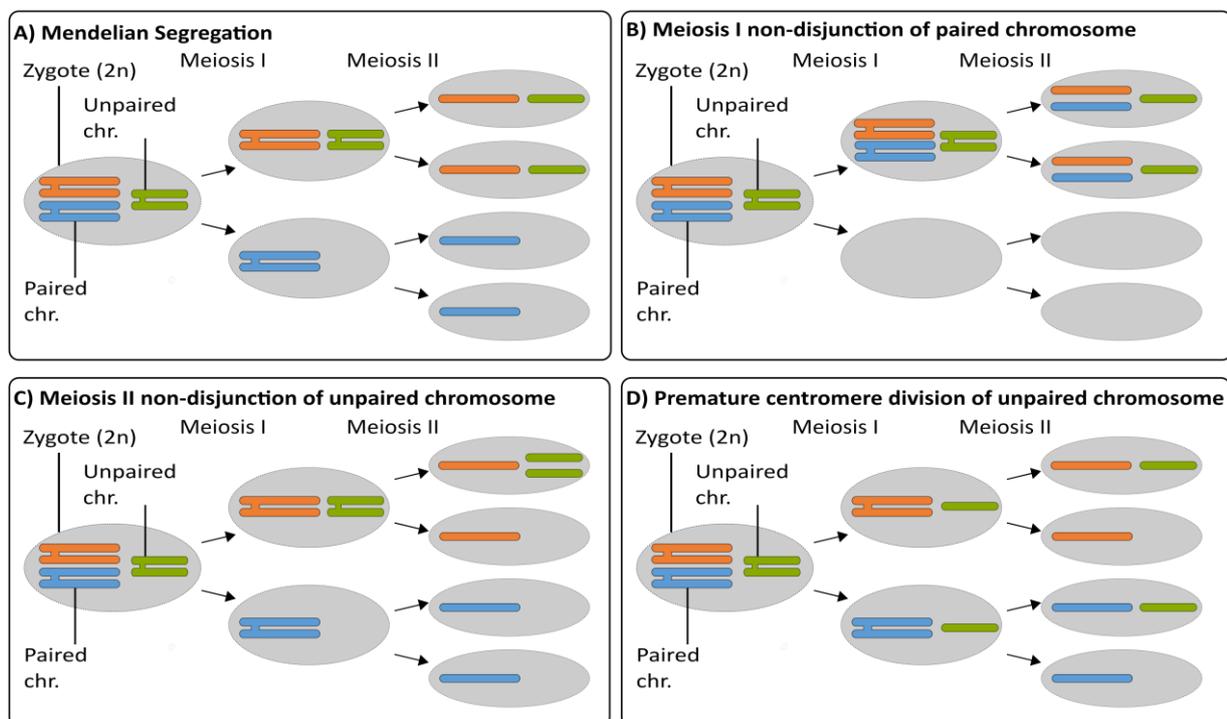
In general, centromeric sequences and the centromere-associated histone CenH3 have been shown to evolve rapidly (reviewed in [129]), leading to the “centromere drive” hypothesis [130,131]. Accordingly, centromeric DNAs act as selfish genetic elements promoting their own transmission. Originally, this hypothesis considered this preferential transmission to only occur in asymmetrical female meiosis. However, every asymmetrical cell division that eventually results in germline cells should be susceptible to a non-random segregation of chromosomes as seen in the example of the rye B chromosome. B chromosomes are often considered selfish genetic elements. Other selfish genetic elements have also been shown to increase their relative frequency by killing or disabling gametes or embryos that lack the selfish element [132]. To date, no example of such a killing mechanism has been described for an accessory chromosome. Yet, it is worth noting that drive of entire chromosomes has been described for the sex chromosomes in several species within the order Rodentia and Diptera. Here these drives are indeed caused either by asymmetrical segregation during mitotic or meiotic cell divisions or by the selective killing of gametes that do not carry the sex chromosomes (reviewed in [132–134]).



**Figure 2:** Potential drive mechanisms for accessory chromosomes in plants and fungi. A) Suggested mechanism for a post-meiotic drive affecting the B chromosome in rye. The centromeres of the B chromosome are not separating during the first pollen mitosis. Due to the non-symmetric cell division the B chromosome sister-chromatids are more likely to become part of the generative nucleus of which the two sperm cells are generated in the second pollen mitosis. This mechanism leads to an increase in the B chromosome frequency in the progeny without additional DNA replication. B & C) Two possible mechanisms for a meiotic chromosome drive in *Z. tritici* involving additional replication of accessory chromosomes by the example of a paired (i.e. homologs in both parental strains) accessory chromosome 14 and an unpaired chromosome 19. B) Unpaired accessory chromosomes undergo an additional round of DNA replication initiated after the pairing of homologues within the zygote suggesting an additional feedback mechanism between pairing of homologs and DNA replication. C) Alternatively, additional DNA replication of unpaired chromosomes takes place prior to meiosis affecting all accessory chromosomes. Since no additional copies of the paired accessory chromosomes are found these must be lost during the zygote stage where pairing occurs. Please note that for the sake of clarity the subsequent mitotic cell division resulting in eight ascospores is not displayed and recombination events were omitted. (B&C adapted from Habig *et al.*, in review).

In fungi, little is known about the meiotic transmission of accessory chromosomes. Those few cases for which the inheritance of the accessory chromosome was analysed have shown i) loss of accessory chromosomes during meiotic and mitotic cell divisions and ii) non-Mendelian segregation with an increase of chromosome frequencies [6,20,28,30,42,46,56,100,135,136].

Loss of accessory chromosomes during meiosis was observed for many fungal accessory chromosomes and may, in part, explain the observed presence/absence polymorphism. Examples are the accessory mini-chromosome of *M. oryzae* that fails to segregate during crosses [28] and the dispensable chromosome of *L. maculans* which is lost in approx. 5% of the progeny following meiosis [55,56]. In these cases, the frequent losses and disomies found in progenies indicate non-disjunction during the meiotic divisions I or II (Fig 3). In *Z. tritici*, accessory chromosomes are missing in up to 20% of the progeny of meiotic crosses where both parental strains contain the respective accessory chromosome [30,136]. Importantly, in *Z. tritici* meiotic progeny disomic of accessory chromosomes are frequent, which also indicates non-disjunction during one of the meiotic divisions [136]. While non-disjunction does not change the absolute frequency of the accessory chromosomes but leads to a re-distribution of the copies among the progeny, losses will cause a reduction in the frequency. Currently, the relative importance of these two processes is unknown. However, by using tetrad analysis, we could recently show that both losses as well as non-disjunction of accessory chromosomes do occur in *Z. tritici* (Habig *et al.*, in review).



**Figure 3:** Modes of accessory chromosome transmission during the cell divisions of meiosis. None of these mechanism leads to a change in the absolute frequency of the accessory chromosome within the progeny. Three accessory chromosomes are illustrated, two of which are homologues to each other (orange/blue) and one accessory chromosome (green) lacking a homolog. Recombination events were omitted to increase clarity. A) During Mendelian segregation, the homologous chromosomes are segregating during Meiosis I and sister-chromatids during Meiosis II. B) Non-disjunction of a paired

chromosome during Meiosis I leads to two disomic meiotic progeny and the loss of the paired accessory chromosome in the remaining two meiotic products. C) Non-disjunction of sister-chromatids of an unpaired chromosome during Meiosis II leads to one meiotic product disomic for the unpaired chromosome. D) Premature centromere division of unpaired chromosome during Meiosis I generates single chromatid chromosome that is segregated during Meiosis II.

If chromosome losses reduce the number of accessory chromosomes a counteracting mechanism must be in place, which increases the frequency of the accessory chromosomes to avoid their complete loss. Maintenance of chromosomes could either occur if natural selection would favour individuals carrying a particular accessory chromosome under some conditions. As described above, many fungal accessory chromosomes confer a fitness advantage on certain hosts. However, a few examples demonstrate that chromosome maintenance may also be conferred by a meiotic transmission advantage [30,42,46,56,136]. In *C. heterostrophus* two thirds of randomly selected ascospores contained the accessory chromosome 16, although only one of the parental strains carried the chromosome [42]. Similarly, in *L. maculans* 83% of the progeny received an accessory chromosome that was unpaired during meiosis [56]. However, the mechanistic basis for these transmission advantages has not been described.

Transmission rates higher than 50% for unpaired accessory chromosomes have also been observed for *Z. tritici* with frequent observation of meiotically produced ascospores containing disomic accessory chromosomes [30,46,136]. Tetrad analysis, in which all meiotic products of a single meiosis were isolated and analysed, revealed that the *Z. tritici* accessory chromosomes are subject to a meiotic drive mechanism (Habig *et al.* in review). Interestingly, the meiotic drive appears to be restricted to those chromosomes that are inherited from the female parent (considered to be the parent that also provides the mitochondria to the offspring). Inheritance of the accessory chromosome from the male parent does not lead to a chromosome drive. Moreover, the meiotic drive only affected those accessory chromosomes without a homolog resulting in transmission of the unpaired chromosomes to all meiotic products when inherited from the female. If the chromosomes were inherited from the male or had a homolog they showed Mendelian-inheritance. This unique transmission pattern of unpaired accessory chromosomes might be explained by a feedback mechanism during meiosis that initiates an additional round of DNA replication for unpaired accessory chromosomes inherited from the female parent (Fig 2B). Alternatively, a pre-meiotic DNA replication is followed by a regulated loss of the redundant copies of paired accessory chromosomes (Fig 2C). The centromeres of the accessory and the core chromosomes of *Z. tritici* do not show significant differences in size, location and sequence characteristics [50]. Therefore, it appears that, in contrast to the rye B chromosome, the centromeres are not be involved in the chromosome drive in *Z. tritici*.

Although other mechanisms could also be responsible, this particular transmission pattern of the *Z. tritici* accessory chromosomes may explain some of the observed characteristics of the accessory chromosomes. The meiotic drive may cause the observed lower recombination rate on the accessory chromosomes [52,137]. The lower recombination rate would in turn account for the accumulation of transposable elements on these chromosomes [46] and the observed

lower efficacy of selection in removing non-adaptive mutations from the coding sequences on the accessory chromosomes [111]. Most importantly, the additional amplification of the accessory chromosome could explain the continued maintenance of the accessory chromosomes in this fungus despite their fitness costs [26]. *Z. tritici* therefore represents the first example for fungal accessory chromosomes that show meiotic chromosome drive similar to the transmission pattern of B chromosomes in plants and animals.

Tetrad analyses to decipher the meiotic transmission of accessory chromosomes have also been performed in *N. haematococca* MP VI which carries the accessory chromosome PDA1-CDC [6]. In contrast to the meiotic drive observed in *Z. tritici*, tetrad analysis of *N. haematococca* MP VI showed Mendelian segregation of unpaired and paired PDA1-CDC. Interestingly, when both parental strains contained one PDA1-CDC, losses were frequent (10-19% of the progeny) but also progenies disomic for PDA1-CDC were highly abundant [138]. Moreover, when a strain with four copies of the accessory chromosome was crossed with a strain lacking PDA1-CDC only about 50% of the progeny inherited the accessory chromosome [138]. Therefore, even when several copies of an accessory chromosome are present in the zygote their transmission varies between cases where all copies are inherited from one parent and cases where each parent contributed one copy of the accessory chromosome.

In conclusion, meiotic instability and non-Mendelian inheritance appear to be common among accessory chromosome in fungi. Losses and disomies are frequent and for *Z. tritici* there is evidence for a chromosome drive resembling the scenarios in plants and animals. Frequently observed aneuploidy of accessory chromosomes in fungi and the particular meiotic inheritance of these chromosomes could represent interesting starting points to further dissect the mechanism of meiotic transmission of fungal accessory chromosomes. In general, the non-essential fungal accessory chromosomes represent interesting models for studies of meiotic mechanisms. The presence/absence polymorphism allows for the comparative analysis of transmission studying the same chromosomes when paired and when unpaired. Hence pairing of homologous chromosomes – which is pivotal for meiosis and other closely related mechanisms like recombination and DNA repair – can be addressed using fungal accessory chromosomes as model systems.

## Concluding remarks

Accessory chromosomes are a diverse set of genomic entities that are widespread in plants, animals and fungi. There is no single sequence characteristic that is shared among all accessory chromosomes between these kingdoms nor a unifying sequence characteristic that has been identified among the accessory chromosomes in fungi. However, fungal cells appear to be able to differentiate between core and accessory chromosomes. Many fungal accessory chromosomes show differences in their mitotic and meiotic transmission compared to core chromosomes. In particular, the transmission of accessory chromosomes during meiosis and its effect on the maintenance of these chromosomes is not well understood. The meiotic drive identified for the accessory chromosomes of *Z. tritici* appears to be responsible for the maintenance of these chromosomes despite conferring a fitness costs during host interaction. This could be indicative for other fungal systems where accessory chromosomes confer fitness costs, like the accessory chromosomes of *L. maculans* and *M. oryzae* which contain avirulence genes. Interestingly, in both organisms the accessory chromosomes also show a non-Mendelian transmission during meiosis [28,56,57,81,100] why a more detailed analysis of their meiotic transmission pattern could be highly relevant.

Moreover, the underlying mechanisms responsible for the distinctly different meiotic and mitotic transmission of accessory chromosomes are unknown. How does the fungal cell distinguish between accessory and core chromosomes? In the absence of common sequence characteristics of fungal accessory chromosomes epigenetic marks could be promising subjects of future studies. The involvement of the histone mark H3K27me3 in the transmission fidelity of the accessory chromosomes of *Z. tritici* could be indicative of a mechanism that connects the transmission pattern to the localization of the accessory chromosomes in the nucleus. To date, the epigenetic landscapes for a very limited number of accessory chromosomes have been determined. In those cases the histone mark H3K27me3 has been identified as being enriched on the accessory chromosomes compared to the core chromosome [8,15,50]. However, the functional effect of this H3K27me3 enrichment on the meiotic and mitotic transmission of the accessory chromosomes is unknown for most species.

In conclusion, we propose that fungal accessory chromosomes provide an intriguing opportunity to dissect novel chromosome drive mechanisms and may serve as models to improve our understanding of the underlying mechanisms of meiotic and mitotic transmission of accessory as well as core chromosomes.

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## Chapter 2

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# Forward Genetics Approach Reveals Host Genotype-Dependent Importance of Accessory Chromosomes in the Fungal Wheat Pathogen *Zymoseptoria tritici*

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Running head: Function of accessory chromosomes in *Z. tritici*

Keywords: genome plasticity, functional dissection, host-pathogen interactions, induced chromosome deletions, virulence

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### Abstract

The fungal wheat pathogen *Zymoseptoria tritici* possesses a large complement of accessory chromosomes showing presence/absence polymorphism among isolates. These chromosomes encode hundreds of genes; however, their functional role and why the chromosomes have been maintained over long evolutionary times are so far not known. In this study, we addressed the functional relevance of eight accessory chromosomes in reference isolate IPO323. We induced chromosome losses by inhibiting the  $\beta$ -tubulin assembly during mitosis using carbendazim and generated several independent isogenic strains, each lacking one of the accessory chromosomes. We confirmed chromosome losses by electrophoretic karyotyping and whole-genome sequencing. To assess the importance of the individual chromosomes during host infection, we performed *in planta* assays comparing disease development results in wild-type and chromosome mutant strains. Loss of the accessory chromosomes 14, 16, 18, 19, and 21 resulted in increased virulence on wheat cultivar Runal but not on cultivars Obelisk, Titlis, and Riband. Moreover, some accessory chromosomes affected the switch from biotrophy to necrotrophy as strains lacking accessory chromosomes 14, 18, 19, and 21 showed a significantly earlier onset of necrosis than the wild type on the Runal cultivar. In general, we observed that the timing of the lifestyle switch affects the fitness of *Z. tritici*. Taking the results together, this study was the first to use a forward genetics approach to demonstrate a cultivar-dependent functional relevance of the accessory chromosomes of *Z. tritici* during host infection.

## Importance

*Zymoseptoria tritici* is a prominent fungal pathogen of wheat of worldwide distribution. This fungus shows a remarkable genome organization, with a large number of chromosomes that are present in only some isolates and therefore considered to be “accessory” chromosomes. To date, the function of these accessory chromosomes in *Z. tritici* has been unknown, although their maintenance in the species over evolutionary times suggests a functional relevance. Here we deleted whole accessory chromosomes to test the effect of these chromosomes on host specificity and virulence of the fungus. We show for the first time that some accessory chromosomes of *Z. tritici* affect the fitness of the fungus during host infection in a cultivar-dependent manner. These results show that the accessory chromosomes encode host-specific virulence determinants having a negative effect on fitness. Understanding the population dynamic of the accessory chromosomes and the molecular interaction of pathogen and plant traits is crucial to improve wheat-breeding strategies.

## Introduction

The fungus *Zymoseptoria tritici* (synonym *Mycosphaerella graminicola*) is the causative agent of the wheat disease Septoria tritici blotch. The pathogen is distributed worldwide where wheat is grown and greatly impacts crop yields. Experimental and genomic approaches have shed light on the host-associated lifecycle of *Z. tritici*, and yet the underlying determinants of pathogenicity and host specificity are largely unknown (see, e.g., references 1–4). The fungus establishes an intercellular hyphal network upon infection through open stomata. It propagates without causing disease symptoms during a biotrophic phase of 7 to 14 days. For asexual sporulation, *Z. tritici* induces host cell death allowing necrotrophic feeding and the development of pycnidia in substomatal cavities (5). Population genetic studies have confirmed that sexual mating occurs frequently in the field (6, 7), and yet the sexual stage and conditions required for sexual development are not known.

The haploid fungus is well suited for genetic and genomic studies as its genome is one of the best-assembled fungal genomes, with 21 fully sequenced chromosomes of the IPO323 reference isolate (1). These chromosomes can be classified into 13 core chromosomes common to all isolates and a set of supernumerary or accessory chromosomes showing presence/absence polymorphism among isolates (8). Reference strain IPO323 has eight accessory chromosomes, representing the highest number of reported accessory chromosomes in a filamentous fungus and comprising 12% of the genome of this isolate (1).

Accessory chromosomes have been described for many eukaryotes (see, e.g., references 9–11). Prominent examples among filamentous fungi include the plant-pathogenic species *Fusarium oxysporum*, *Nectria haematococca*, and *Leptosphaeria maculans*, in which accessory genomic elements were shown to contribute to host specificity and virulence (12–14). A similar role has not yet been described for the accessory chromosomes of *Z. tritici*. Similarly to reports in other fungi, the accessory chromosomes in *Z. tritici* vary in chromosomal organization from the core chromosomes by a lower gene density (1), a higher proportion of unique genes (15), and an

enrichment in transposable elements (1). Recently, we were able to show that the telomeres, subtelomeric regions, and centromeres do not differ between accessory and core chromosomes and yet that the accessory chromosomes are greatly enriched in nucleosomes with H3K27 trimethylation (16), which is known to be associated with gene silencing (15). The accessory chromosomes are frequently lost during meiosis (1, 8, 17) and show a high level of structural variation, whereby isolates differ in the presence/absence of homologue chromosomes and can carry highly differentiated gene content due to numerous insertions and deletions (17–19). In the IPO323 reference isolate, more than 800 genes are located on the accessory chromosomes (20) and show, on average, lower expression than genes located on the core chromosomes during growth in axenic culture and infection of the wheat host (3, 15). However, among the genes located on the accessory chromosomes, 174 were classified as being highly expressed and 22 showed host-specific regulation (15). In a separate study, 79 of the genes located on the accessory chromosomes differed in expression during colonization and asexual reproduction (3). However, no functional analyses of these genes have been performed to date.

Intriguingly, synteny among regions of the accessory chromosome of *Z. tritici* and its sister species *Zymoseptoria ardabiliae* and *Zymoseptoria pseudotritici* indicates that the accessory chromosomes were inherited from a common ancestor and therefore appear to have been maintained since the speciation event ~11,000 years ago (21, 22). The accessory chromosomes evolve faster than the core chromosomes due to a relaxation of selection (23). Yet it is still unknown why the accessory chromosomes are maintained in the species. They were previously proposed to provide a repertoire of genes for adaptive evolution (24). Yet natural selection acts at the individual level, and a repertoire of genes would be advantageous only at the population level. Thus, we lack an evolutionary model to explain the maintenance of accessory chromosomes as a repertoire of genes for future generations of the pathogen. Alternatively, the accessory chromosomes may encode genes that provide a selective advantage only under specific conditions (11). In a heterogeneous environment, the accessory chromosomes may have been maintained by balancing selection, sometimes conferring a fitness advantage and sometimes not.

To understand the presence and evolutionary dynamics of these chromosomes, we need to determine their functional relevance. A functional genetic assessment should ideally use isogenic strains that differ only in the presence and absence of the gene or genetic element of interest. In the past, functional studies of the accessory chromosomes of *Z. tritici* relied on phenotyping of progenies with chromosome losses (8, 25). However, meiotic recombination additionally introduces variation throughout the genome of the progeny, making a comparison between accessory chromosome-containing and chromosome-lacking strains difficult. Such additional variation may be particularly strong in *Z. tritici* because of its high genomic plasticity, as highlighted in a recent study where more than 200 isolate-specific genes were described (19). Here, we circumvented these problems by inducing whole chromosome loss during mitosis to generate isogenic strains that then differed only in the presence/absence of an accessory chromosome. The fungicide carbendazim was used to manipulate mitosis and to

disrupt  $\beta$ -tubulin assembly of the mitotic spindle (26). This approach allowed us to obtain at least one strain for each of the eight accessory chromosomes of the IPO323 reference isolate that had lost that one particular accessory chromosome. We demonstrate for the first time that accessory chromosomes of *Z. tritici* have a functional effect on the *in planta* phenotype of *Z. tritici* in a host cultivar-specific manner. In addition, we show that the wheat cultivar influences disease progression and that *Z. tritici* fitness is affected by the timing of the switch from biotrophy to necrotrophy. Taken together, these results provide the first evidence that a variety of host genotypes have different fitness effects on the accessory chromosomes in *Z. tritici* and may play a role in the distribution and dynamics of these small chromosomes.

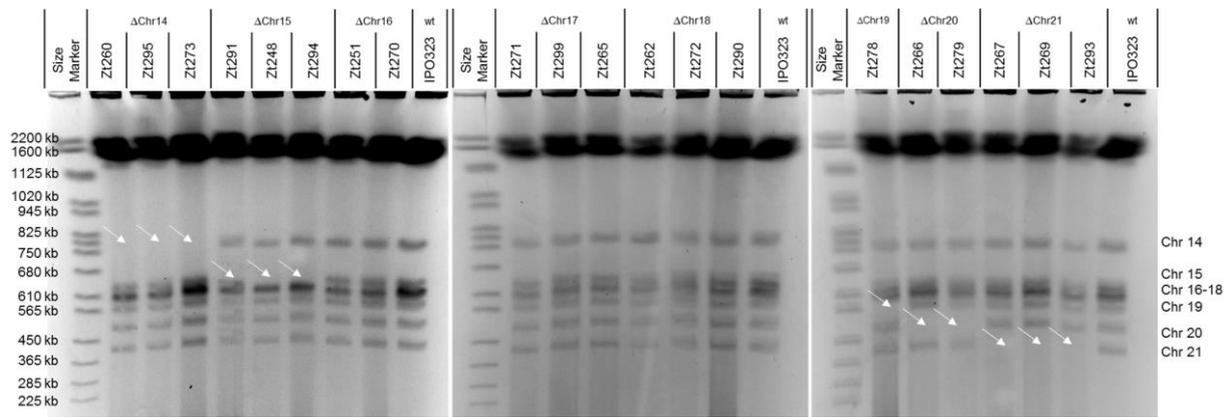
## Results

**Variation in the rate of chromosome losses among eight accessory chromosomes.** To induce aneuploidy and chromosome losses in *Z. tritici*, we treated cells with carbendazim during vegetative growth. In total, 188 independent liquid cultures of *Z. tritici* were inoculated in the presence and, as a control, in the absence of carbendazim for up to 2 weeks before being streaked onto yeast-malt-sucrose (YMS) (4 g/liter yeast extract, 4 g/liter malt extract, 4 g/liter sucrose) agar plates. We screened 2,892 colonies for the loss of accessory chromosomes by a multiplexed PCR targeting the right subtelomeric region of each of the eight accessory chromosomes (see Fig. S1 in the supplemental material). The loss of a chromosome was confirmed by at least three independent PCRs for each accessory chromosome, two targeting the left and right subtelomeric regions, respectively, and one targeting the centromeric region. In total, we could confirm 37 chromosome losses (relative frequency, 1.28%) by PCR screen (Table 1). The total number of colonies characterized using PCR-based karyotyping in the presence of carbendazim was 2,506 ( $\Sigma$ , 33 chromosomal losses; relative frequency, 1.32%). The total number of colonies characterized using PCR-based karyotyping in the absence of carbendazim was 386 ( $\Sigma$ , 4 chromosomal losses; relative frequency, 1.04%). Interestingly, the distribution of chromosome losses varied significantly among chromosomes ( $P = 0.009$ ; chi-square test with Yates correction). A total of 13 *Z. tritici* strains had lost chromosome 15, while none lacked chromosome 19, suggesting different degrees of stability of the eight accessory chromosomes. Four chromosome losses were detected in control experiments without carbendazim (386 tested colonies). While the low number of chromosome losses in the nontreated strains prevented solid statistical inferences, we note that the frequency of spontaneous chromosome losses did not deviate much from the frequency seen with carbendazim-treated strains.

**TABLE 1** Absolute and relative frequencies of unique chromosomal loss events in IPO323 in the presence and absence of carbendazim

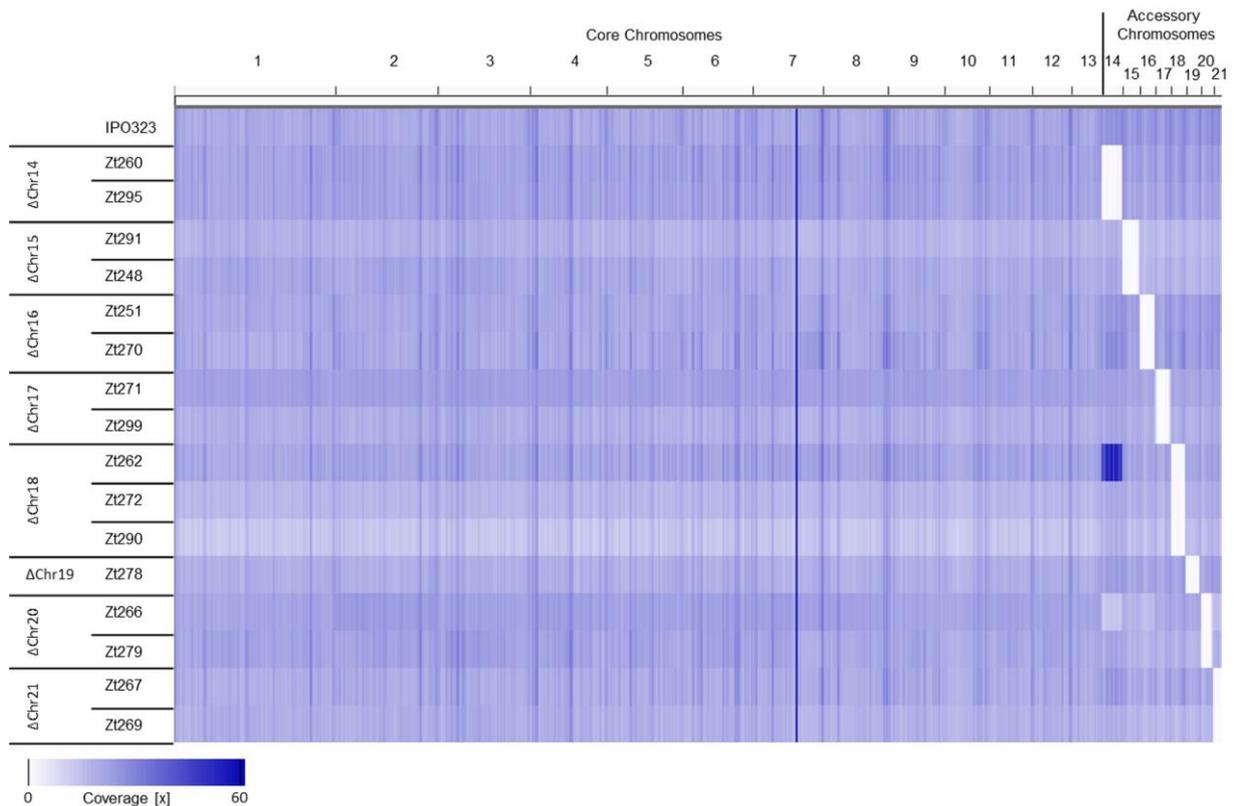
Accessory chromosome	No. of chromosomal losses		Total
	With carbendazim	Without carbendazim	
14	2	2	4
15	12	1	13
16	5	0	5
17	3	1	4
18	4	0	4
19	0	0	0
20	2	0	2
21	5	0	5

**Aneuploid strains are isogenic to IPO322.** We applied two approaches to identify and exclude aneuploid strains that had acquired additional mutational changes, i.e., which did not remain isogenic to the progenitor strain IPO323. First, the chromosome sizes of all strains were analyzed using pulsed-field gel electrophoresis (PFGE). Please note that, in order to verify aneuploid strains that had lost one of each accessory chromosome of IPO323, we included the *Z. tritici* 278 (Zt278) strain derived from a separate study. Strain Zt278 was isolated from a pycnidium formed on a leaf infected with IPO323, and the strain was found to have lost accessory chromosome 19, which we could not delete with carbendazim *in vitro*. The PFGE analyses also allowed us to confirm the loss of accessory chromosomes 14, 15, 19, 20, and 21 for all the respective strains identified in the PCR assay (Fig. 1). Chromosomes 16, 17, and 18 could not be separated due to the small size differences (607 kb, 584 kb, and 574 kb); however, a lower intensity in the chromosome band correlated well with the expected chromosome loss for the respective strains (Fig. 1). Importantly, none of the strains showed any additional chromosomal aberration for accessory or core chromosomes (Fig. S2 and S3).



**FIG 1** Pulsed-field gel electrophoresis for the accessory chromosomes of *Z. tritici*. Pictures of three pulsed-field gel electrophoresis gels for all strains used in this study are shown. Size marker, *Saccharomyces cerevisiae*. White arrows indicate missing chromosome bands. Absence of chromosome 14 is observed for strains Zt260, Zt295, and Zt273. Absence of chromosome 15 is observed for strains Zt291, Zt248, and Zt294; absence of chromosome 16 or 17 or 18 can be inferred for strains Zt251, Zt270, Zt271, Zt299, Zt265, Zt262, Zt272, and Zt290. Absence of chromosome 19 is observed for strain Zt278; absence of chromosome 20 is observed for strains Zt266 and Zt279; and absence of chromosome 21 is observed for strains Zt267, Zt269, and Zt293.

Second, we sequenced whole genomes for a subset of 16 strains, including the progenitor IPO323 strain, using next-generation sequencing technology. On average,  $4 \times 10^6$  paired-end Illumina reads were obtained for each strain, providing an average read coverage for all strains of 20 $\times$  (see Table S1 in the supplemental material). Paired-end reads were mapped to the reference genome of IPO323 to identify rearrangements, deletions, duplications, single nucleotide polymorphisms (SNP), and indels. The resequencing confirmed the loss of accessory chromosomes in the tested aneuploid strains (Fig. 2). Furthermore, for 13 of the 16 resequenced strains, we did not find evidence of additional chromosomal rearrangements or deletions. Those 13 strains showed homogeneous read coverage on all core and accessory chromosomes comparable to the observed coverage for the resequenced IPO323 progenitor strain. Three strains, however, exhibited additional large-scale chromosomal variation in addition to the expected chromosome losses (Fig. 2). Strain Zt262 showed an increase in the read coverage for chromosome 14 consistent with a possible duplication of the chromosome. Strain Zt266 had reduced read coverage for chromosome 14, indicating that the sequenced strain comprised a mixture of cells with and without chromosome 14. For strain Zt248, we observed a deletion of a 6.6-kb fragment in the telomeric region of chromosome 14.



**FIG 2** Genome-wide map of Illumina read coverage of the IPO323 progenitor strain and the aneuploid strains. The absence of reads mapped to the deleted accessory chromosomes confirms chromosome loss in the respective strains. Increased coverage of chromosome 14 in strain Zt262 indicates chromosome duplication, while the lower coverage of chromosome 14 in strain Zt266, on the other hand, suggests a mix of cells with and without this chromosome.

To further identify genomic changes in the aneuploid strains, we identified SNPs and small indels for all resequenced aneuploid strains using the genome of IPO323 as the reference (1). Resequencing of the IPO323 progenitor strain revealed 13 SNPs and two indels compared to the reference genome (Fig. S4). These novel SNPs either originated from sequencing errors in the Sanger-sequenced reference or in the Illumina-resequenced genome or represented mutations derived during laboratory propagation. An average of 3.2 SNP and indels per strain were novel and not present in the IPO323 progenitor (Table 2). Nine of these novel SNPs and indels were located in coding sequences in six strains (Zt248, Zt271, Zt272, Zt291, Zt295, and Zt299); seven of the SNP/indels in five strains (Zt248, Zt271, Zt272, Zt291, and Zt299) were nonsynonymous (Table S2). The remaining 10 strains did not contain any novel SNPs and indels in coding sequences, confirming that there were either no or very few genetic changes in the aneuploid *Z. tritici* strains beside the absence of the distinct accessory chromosomes.

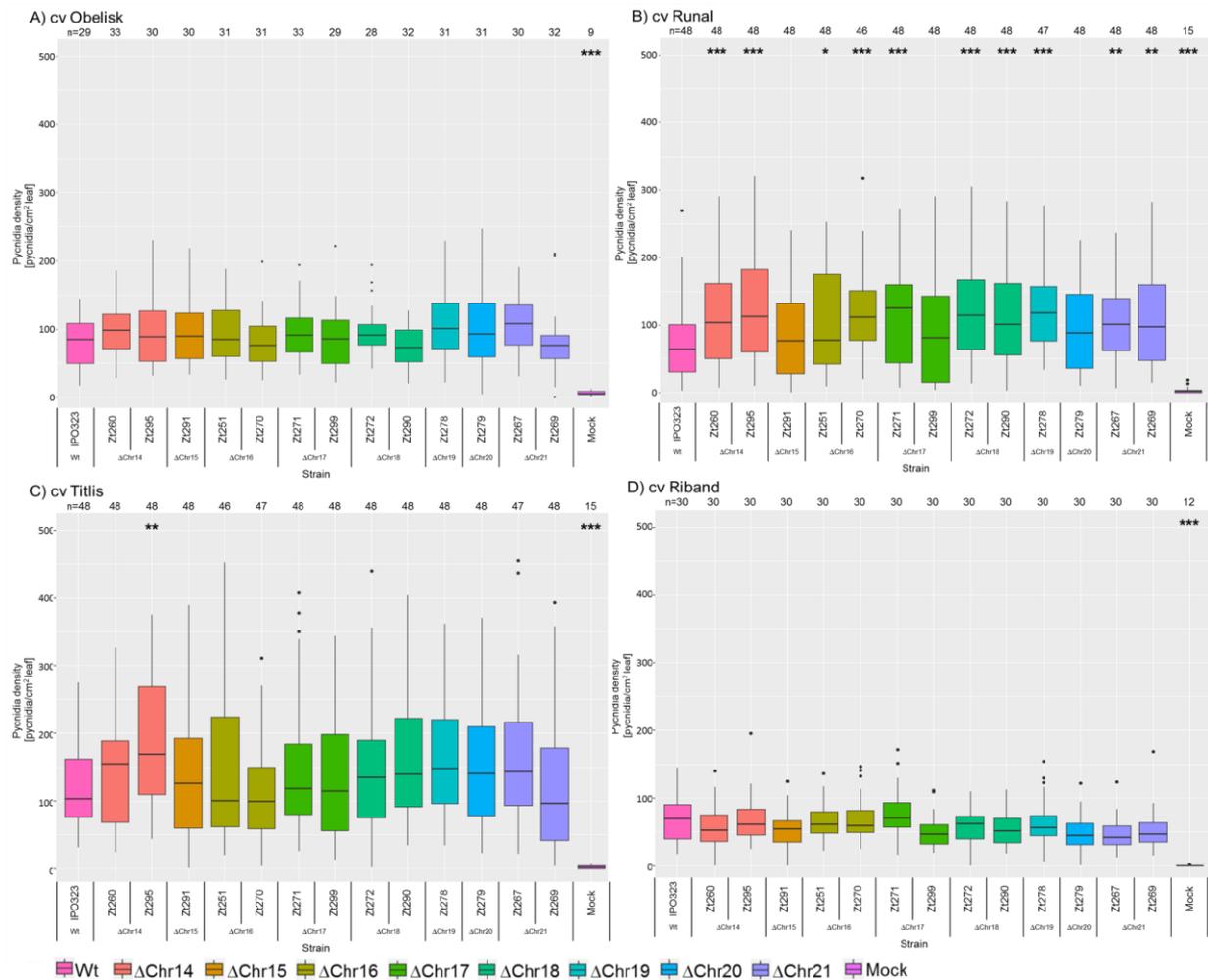
**TABLE 2** Absolute frequencies of novel SNPs and small indels for the aneuploid strains not present in the IPO323 progenitor strain

Strain	Chromosomal status	No. of novel SNPs/indels ( $\Sigma = 51$ )	No. of novel SNPs/indels in coding sequences ( $\Sigma = 9$ )
Zt260	$\Delta$ Chr14	4	0
Zt295	$\Delta$ Chr14	6	3
Zt291	$\Delta$ Chr15	3	2
Zt248	$\Delta$ Chr15	2	1
Zt251	$\Delta$ Chr16	2	0
Zt270	$\Delta$ Chr16	1	0
Zt271	$\Delta$ Chr17	7	1
Zt299	$\Delta$ Chr17	8	0
Zt262	$\Delta$ Chr18	6	0
Zt272	$\Delta$ Chr18	1	1
Zt290	$\Delta$ Chr18	1	0
Zt278	$\Delta$ Chr19	2	0
Zt266	$\Delta$ Chr20	0	0
Zt279	$\Delta$ Chr20	2	0
Zt267	$\Delta$ Chr21	4	0
Zt269	$\Delta$ Chr21	2	1

***In vitro* phenotypic variation does not correlate with loss of accessory chromosomes.** We next asked if the loss of accessory chromosomes conferred a fitness effect in the *Z. tritici* strains. We first compared the morphologies of *Z. tritici* colonies grown *in vitro* under various growth conditions (Fig. S5). The strains used for the phenotypic characterizations were derived from independent experiments and therefore represented truly independent chromosomal loss events. The assay allowed us to test the effect of chromosome deletions on growth under conditions of high temperature (28°C), osmotic stress (2 M sorbitol, 1 M NaCl), and oxidative stress (H<sub>2</sub>O<sub>2</sub>) and in the presence of additional cell wall stress agents, including Congo red and calcofluor (27). In general, growth at 28°C increased the extent of colony melanization. However, the observed variation did not correlate with the absence or presence of a particular accessory chromosome (Fig. S5). Likewise, we observed no differences between wild-type and aneuploid strains in colony morphology or growth under the other tested *in vitro* conditions. The only exception was strain Zt266 (with a chromosome 20 deletion [ $\Delta$ chr20]), which showed reduced growth at 28°C and on plates amended with Congo red, calcofluor, sorbitol, and NaCl (Fig. S5).

**Accessory chromosomes influence the fitness of the aneuploid strains *in planta*.** We next asked to what extent the absence of a particular accessory chromosome affected the fitness of *Z. tritici in planta*. We restricted this analysis to the 13 strains that did not show any large-scale chromosomal aberrations and which we considered to be isogenic. In order to cover a wider range of biotic interactions, four distinct wheat cultivars (Obelisk, Runal, Titlis, and Riband) were included. To quantify disease levels, we measured the density of asexual fruiting bodies at 21 days postinfection (dpi). A minimum of two independent experiments was conducted for each strain under all conditions in a randomized, balanced, and blinded experimental design.

The effect of the experimental factors “cultivar,” “strain,” and “experiment” was tested using rank-transformed data in a one-way analysis of variance (ANOVA). The omnibus test showed a significant effect of the factors “cultivar” ( $P = 2 \times 10^{-16}$ ) and “strain” ( $P = 2 \times 10^{-16}$ ) and “experiment” ( $P = 2 \times 10^{-16}$ ) as well as a significant interaction between the factors “strain” and “cultivar” ( $P = 1.1 \times 10^{-6}$ ). On the basis of these significant interactions, we conclude that the accessory chromosomes affect the phenotype in a cultivar-dependent manner. Further *post hoc* analysis revealed that none of the aneuploid strains varied in the production of pycnidia for the Obelisk cultivar compared to the progenitor IPO323 strain, suggesting that the accessory chromosomes have no effect on the fitness of *Z. tritici* in this wheat cultivar (Fig. 3A). In the Runal cultivar, surprisingly, the wild-type IPO323 strain showed a lower density of pycnidia than most of the tested aneuploid strains (Fig. 3B). These differences were significant for the two strains that had lost chromosome 14 (Zt260 and Zt295), for both strains that had lost chromosome 16 (Zt251 and Zt270), for one of the two tested strains that had lost chromosome 17 (Zt271), for both tested strains that had lost chromosome 18 (Zt272 and Zt290), for the one tested strain that had lost chromosome 19 (Zt278), and for both strains that had lost chromosome 21 (Zt267 and Zt269). For the Titlis cultivar, only strain Zt295, which lacked chromosome 14, showed a significant deviation in pycnidia density from that seen with the progenitor IPO323 strain. For cultivar Riband, none of the tested strains showed a significant difference from the progenitor IPO323 strain. It is interesting that in both cases in which chromosome loss strains, considered to be isogenic, differed in phenotype, we were able to identify an SNP/indel in coding regions of at least one of the strains. Zt271 ( $\Delta$ chr17) shows a nonsynonymous deletion of 20 bp in the coding region of a gene of unknown function (ZT09\_chr\_1\_01551), and ZT295 ( $\Delta$ chr14) contains nonsynonymous SNPs in coding regions of three genes of unknown function. It is possible that these mutations contribute to the observed phenotypic differences between the otherwise isogenic strains (Table S2). Interestingly, the pycnidia density for the aneuploid strains was higher than that for the progenitor IPO323 strain in cultivars Obelisk, Runal, and Titlis, whereas we found a lower pycnidia density (albeit the data were not statistically significant) for the Riband cultivar. Therefore, our results suggest that the independent loss of several of the eight accessory chromosomes confers a gain of fitness in *Z. tritici*, particularly in the Runal wheat cultivar.

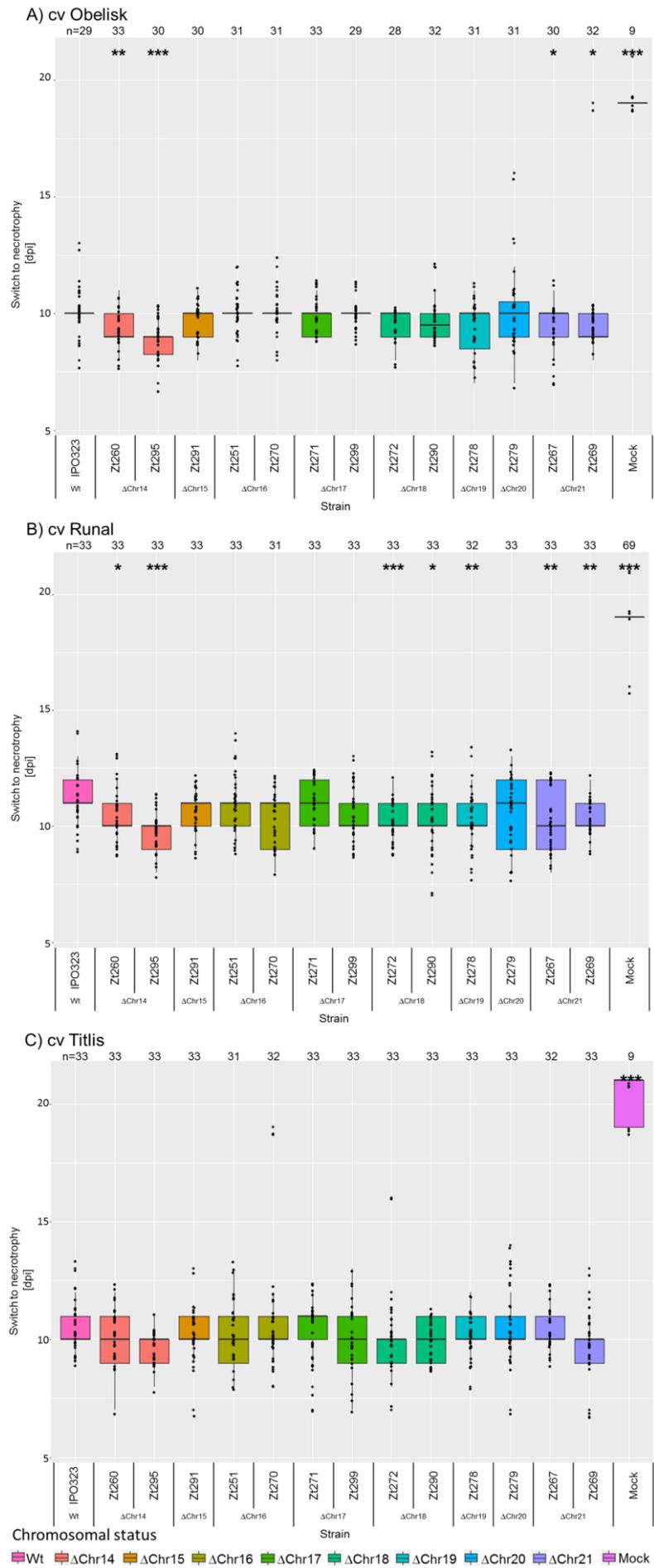


**FIG 3** Pycnidia density of the IPO323 progenitor and the aneuploid strains produced at 21 dpi in different wheat cultivars. Box plots are shown for pycnidia density data from wheat cultivars (cv) (A) Obelisk, (B) Runal, (C) Titlis, and (D) Riband. Statistical significance, inferred through an ANOVA and a subsequent *post hoc* Tukey's HSD test comparing the aneuploid strains to the IPO323 reference, is indicated as follows: \*,  $P < 0.05$ ; \*\*,  $P < 0.005$ ; \*\*\*,  $P < 0.0005$ . Data shown are based on results from (A) three, (B and D) four, or (D) two independent experiments.

**The switch from biotrophy to necrotrophy is influenced by the accessory chromosomes.** The temporal development of disease of *Z. tritici* can vary between isolates and cultivars (M. Habig, unpublished data), and we hypothesized that the temporal development of *Z. tritici in planta* could be influenced by the presence/absence of certain accessory chromosomes. In particular, the timing of the switch from biotrophy to necrotrophy may affect the overall fitness of *Z. tritici* as the switch is crucial for the nutrient availability of the fungus and thus for its ability to produce pycnidia. Therefore, to test if the accessory chromosomes contribute to temporal disease development, we monitored the inoculated leaves daily to compare data corresponding to symptom development for the aneuploid strains on the three wheat cultivars Obelisk, Runal, and Titlis in three independent experiments (Fig. 4). The effects of the experimental factors "cultivar," "strain," and "experiment" were tested using rank transformation in a one-way ANOVA. The omnibus test showed a significant effect of the factors "cultivar" ( $P = 2 \times 10^{-16}$ ) and "strain" ( $P = 2 \times 10^{-16}$ ) and "experiment" ( $P = 2 \times 10^{-16}$ ). However, the interaction between the

factors “strain” and “cultivar” was statistically nonsignificant. Therefore, the effect of the accessory chromosomes on the timing of the switch to necrotrophy is independent of the cultivar. *Post hoc* analysis showed that both independent strains lacking chromosome 14 (Zt260 and Zt295) showed an earlier onset of necrosis than the IPO323 progenitor strain on the Runal wheat cultivar. Similarly, the two strains without chromosome 18 (Zt272 and Zt290) varied significantly from the progenitor IPO323 strain. Strain Zt278 lacking chromosome 19 and the two strains without chromosome 21 (Zt267 and Zt269) caused symptoms significantly earlier than IPO323. On the Obelisk cultivar, the strains lacking chromosomes 14 and 21 showed a significantly earlier onset of necrosis. However, as shown above, the temporal variation of disease development on Obelisk did not translate into differences in the total disease symptoms measured at 21 dpi (Fig. 3A). On the Titlis cultivar, no aneuploid strain showed significant differences from the IPO323 strain results. From our *in planta* assay results, we thereby conclude that the temporal development of fungal infection is influenced by the complement of accessory chromosomes but that this effect is independent of the host cultivar.

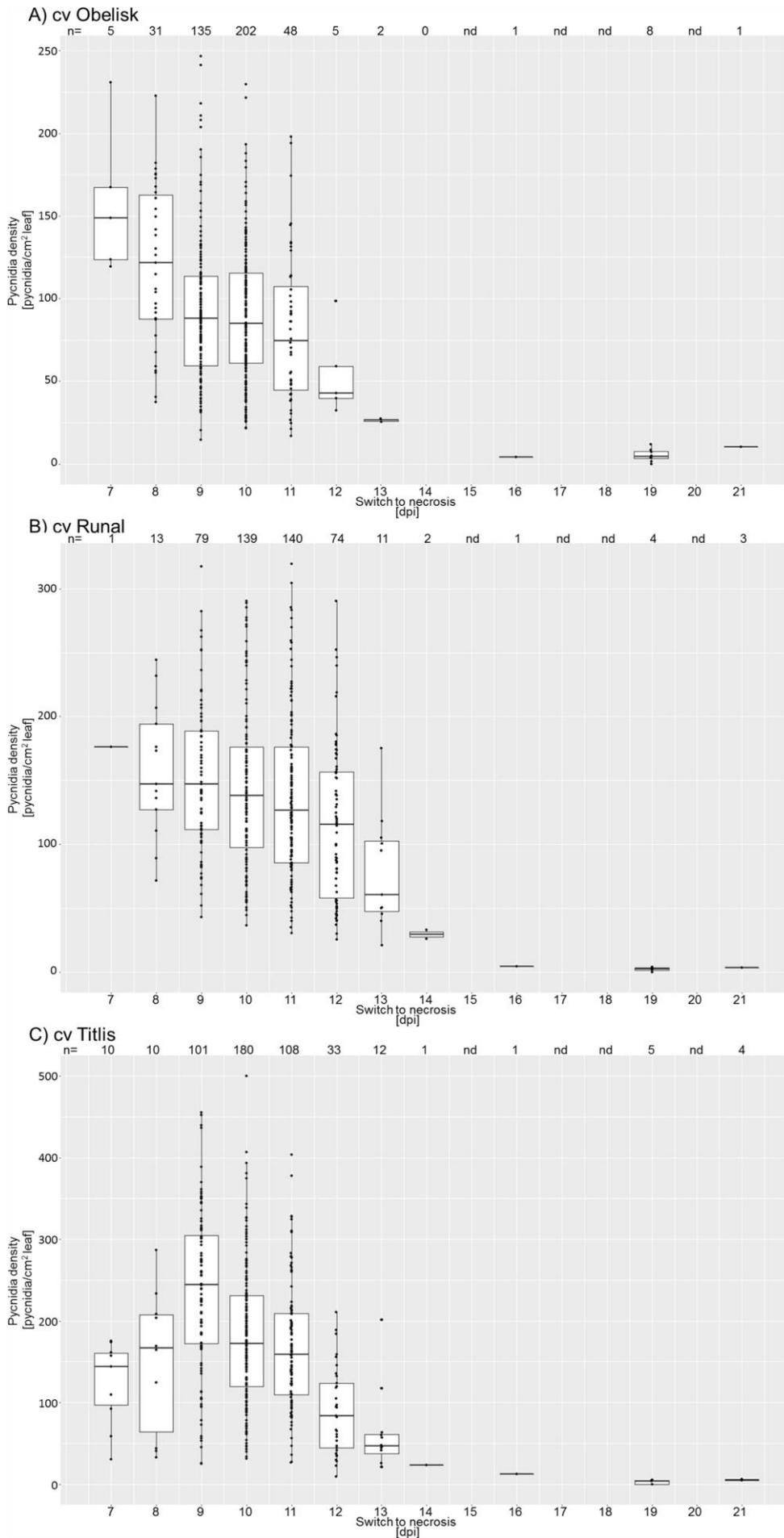
**The timing of the switch to necrotrophy impacts the level of pycnidia formation in a wheat cultivar-dependent manner.** We next addressed the issue of whether the timing of the switch to necrotrophy influences the number of pycnidia produced by the fungus. An earlier switch to necrotrophy should be correlated with a higher number of pycnidia as the fungus would have a longer phase during which to take up nutrients released from dead plant tissue. We also considered the possibility that the timing of the switch could be determined by host genotype. To test the influence of the host genotype, we pooled data for all fungal strains on the three wheat cultivars Obelisk, Runal, and Titlis and correlated the time of appearance of necrotic symptoms on individual leaves with the corresponding pycnidia density obtained for the corresponding leaf at 21 dpi. The balanced experimental design, in which all three cultivars were tested in same three experiments with the same fungal strains, allowed us to pool data for all fungal strains and to compare the effects of the cultivars without the confounding effect of the fungal strains. However, a lack of statistical power prevented performance of the analysis with a nonpooled data set, i.e., on the level of individual accessory chromosomes.



**FIG 4** Timing of the switch to necrotrophy of the IPO323 progenitor and the aneuploid strains. (A to C) Boxplots depicting the day postinfection at which the first necrotic symptoms appeared on the leaf surface for cultivars (A) Obelisk, (B) Runal, and (C) Titlis. Data used in the analyses were pooled from results from 3 independent experiments. Statistical significance, inferred through an ANOVA and subsequent *post hoc* Tukey's HSD test comparing the aneuploid strains to the IPO323 reference, is indicated as follows: \*,  $P < 0.05$ ; \*\*,  $P < 0.005$ ; \*\*\*,  $P < 0.0005$ .

Both "cultivar" and "switch to necrotrophy" were identified as significant factors influencing pycnidia density in a one-way ANOVA performed on rank-transformed data (28) ( $P < 2 \times 10^{-16}$  and  $P < 2 \times 10^{-16}$ , respectively). To test whether the cultivars influenced the timing of the switch to necrotrophy, the interaction of these two factors was also tested. The interaction proved to be highly statistically significant ( $P = 3.5 \times 10^{-6}$ ), indicating that the three cultivars influence the shape of the correlation between the switch to necrotrophy and the pycnidia density. A *post hoc* pairwise comparison revealed that the shapes of the curves were significantly different between the Titlis cultivar and Obelisk ( $P = 7.0 \times 10^{-6}$ ), and we therefore conclude that the timing of the switch impacts the level of pycnidia formation in a cultivar-dependent manner.

In detail, the number of pycnidia seen with the Titlis wheat cultivar was lower on plants where the first necrosis symptoms developed at 7 and 8 dpi than on those plants that developed the first symptoms at 9 dpi (Fig. 5C). In contrast, for the Obelisk wheat cultivar, the pycnidia density was higher on leaves on which the first symptoms appeared at 7 and 8 dpi than on leaves on which the first symptoms appeared at 9 dpi (Fig. 5A). For the Runal wheat cultivar, the pycnidia density showed little variation from 7 to 9 dpi (Fig. 5B). For all three wheat cultivars, however, the density of pycnidia decreased when the first symptoms developed later than 9 dpi. An early switch from biotrophic growth to necrotrophic growth thereby correlated with less pycnidia production and lower fitness on the Titlis wheat cultivar, while earlier disease development correlated with increased pycnidia production and higher fitness of the pathogen on Obelisk. Thus, the host genotype can impact the fitness advantage of different *Z. tritici* strains based on their ability to develop disease early or late after infection.



**FIG 5** The timing of switch to necrotrophy influences the number of pycnidia produced. (A to C) Boxplots of the pycnidia density on leaves at 21 dpi grouped according to the day (postinfection) when the first necrotic symptoms appeared on the respective leaf for cultivars (A) Obelisk, (B) Runal, and (C) Titlis. Data for all tested *Z. tritici* strains were pooled for each of the cultivars. The shapes of the curves as tested by the interaction of the factor cultivar and the switch to necrotrophy proved significantly different for the pairwise comparisons of Obelisk and Titlis ( $P = 7.0 \times 10^{-6}$ ).

## Discussion

The genome of *Z. tritici* can contain a large number of accessory chromosomes; however, a functional relevance of these chromosomes has so far not been directly shown. On the basis of the forward genetics approach applied here, we could demonstrate, for the first time, that the accessory chromosomes affect the phenotype of *Z. tritici in planta*. Most notably, the effects of the accessory chromosomes are dependent on host genotype. The deletion of accessory chromosomes 14, 16, 18, 19, and 21 led to an increase in the pycnidia density in the Runal wheat cultivar, whereas no effect was detected in wheat cultivars Obelisk, Titlis, and Riband. The results were consistent for four of five pairs of independent chromosome loss mutants, i.e., pairs of strains that had lost the same accessory chromosome. On Runal, however, one chromosome loss strain ( $\Delta$ chr17) showed significantly increased production of pycnidia, while the other  $\Delta$ chr17 strain did not. Similarly, on Titlis, only one of the two  $\Delta$ chr14 strains showed increased production of pycnidia. Our comparison of full-genome sequences revealed that these otherwise isogenic strains deviate with additional SNP/indels in coding regions. We hypothesize that these extra mutations may explain the observed differences between the two sets of chromosome loss strains. The switch from biotrophy to necrotrophy was also affected by the accessory chromosomes—those effects, however, proved to be similar for all three tested cultivars and therefore not dependent on the host genotype.

The finding of an increased pycnidia density in the Runal wheat cultivar upon deletion of accessory chromosomes 14, 16, 18, 19, and 21 suggests that these chromosomes reduce the fitness of *Z. tritici* in a cultivar-dependent manner. One explanation could be that chromosomes 14, 16, 18, 19, and 21 express factors that are recognized by the Runal cultivar and induce partial immunity either via pathogen-associated-molecular-pattern-triggered immunity (PTI) or via effector-triggered immunity (ETI). *Z. tritici* was previously shown to induce PTI in wheat upon recognition of chitin in the fungal cell wall. *Z. tritici* secretes Mg3LysM and Mg1LysM, the two LysM effectors that bind chitin with high affinity to prevent PTI (2, 29). However, accessory chromosomes of *Z. tritici* may encode avirulence genes or other secreted molecules that specifically trigger defense reactions in some wheat cultivars.

Interestingly, the observed effects of the accessory chromosomes on the phenotype are quantitative; i.e., they are small but significant. Our findings of quantitative effects of the accessory chromosomes are in agreement with a recent study of *Z. tritici* based on quantitative trait locus (QTL) mapping of virulence-associated loci. In this study, similar small effects of the accessory chromosome 18 on melanization and pycnidia density were demonstrated in the Titlis cultivar and of chromosome 21 on pycnidia size in the Runal cultivar (25). These small

effects contrast with the scenario in *N. haematococca* and *F. oxysporum* (f. sp. *lycopersici*), where accessory genetic elements are the determining factors for genotype-specific pathogenicity (12–14). However, even though the effects of accessory chromosomes in *Z. tritici* are small, our results show that they can contribute to fitness differences on different hosts. Surprisingly, the accessory chromosomes seem to have a negative fitness effect that should lead to a complete loss of the accessory chromosomes unless they also provide a fitness advantage under other conditions or at other stages of the life cycle.

The localization of pathogenicity-related genes on the accessory chromosomes may be a route by which to rapidly generate phenotypic variation on which natural selection can act. The localization of pathogenicity-related genes on accessory chromosomes may reflect past adaptations to more-diverse host populations in natural ecosystems. In this context, it is interesting that the accessory chromosomes of *Z. tritici* show some homology to accessory chromosomes of the sister species *Z. pseudotritici* and *Z. ardabiliae* infecting wild grasses (22, 30, 31). The finding of conserved accessory chromosome fragments among different *Zymoseptoria* species shows that the accessory chromosomes represent an ancient trait that predates the diversification of species in the *Zymoseptoria* genus. *Z. pseudotritici* and *Z. ardabiliae* infect wild grass species and have a wider host range than *Z. tritici* (31). It is conceivable that the accessory chromosomes not only determine wheat cultivar specificity in *Z. tritici* but also play a role in determining the host range of *Z. pseudotritici* and *Z. ardabiliae*.

An intriguing finding of this study is that the accessory chromosomes affect the timing of the switch from biotrophy to necrotrophy. In addition, we find pronounced differences between the three cultivars with respect to the correlation between the onset of necrosis and the number of pycnidia produced. An early switch to necrotrophy is correlated with a higher density of pycnidia in the Obelisk cultivar but with a lower pycnidia density in Titlis. The early onset of necrosis in the Titlis wheat cultivar might be associated with an earlier recognition of the fungus by the plant, resulting in plant defenses that in turn reduce the number of pycnidia that the fungus can produce. The differences in the correlations between the first symptoms of necrosis and the density of pycnidia for the three cultivars indicate that plant factors influence the temporal development of the disease symptoms in combination with an effect of accessory chromosome complements.

Although the accessory chromosomes are relatively gene poor (1), comprise mainly heterochromatic DNA (16), and in general are transcribed at a considerably lower level than the core chromosomes, several genes are strongly upregulated during infection (3, 15). Such differential gene expression levels support the idea of the functional importance of genes on the accessory chromosomes as also suggested by our study results. A total of nine effector candidates can be identified on the accessory chromosomes by computational predictions from secretome data (32). Among the effector candidates, one locates on chromosome 14, two on chromosome 16, two on chromosome 18, and one on chromosome 21. These and the differentially expressed genes from chromosomes 14 and 19 identified in previous studies (3, 15) are promising candidates for further genetic and functional analyses.

In conclusion, we demonstrate here for the first time that the large complement of accessory chromosomes in the wheat pathogen *Z. tritici* plays a role in fitness during host infection. The effects were found for five of the eight accessory chromosomes and affected two fitness-related traits. Surprisingly, we identified significant fitness costs of the accessory chromosomes only, suggesting that other factors or selective traits act to maintain the chromosomes in the pathogen populations. One of the fitness traits found to be affected in this study, pycnidia density, is affected in a wheat cultivar-dependent manner, strongly suggesting an interaction between host genes and genes harbored by the accessory chromosomes of *Z. tritici*.

## Materials and Methods

**Fungal and plant material.** The Dutch IPO323 isolate was kindly provided by Gert Kema (Wageningen, the Netherlands) and is available from the Centre of the Royal Netherlands Academy of Arts and Sciences (Utrecht, the Netherlands) under accession number CBS 115943. Strains were maintained in either liquid yeast-malt-sucrose (YMS) broth (4 g/liter yeast extract, 4 g/liter malt extract, 4 g/liter sucrose) at 18°C on an orbital shaker or on solid YMS (with 20 g/liter agar added) at 18°C. The *Triticum aestivum* cultivar Obelisk was obtained from Wiersum Plantbreeding BV (Winschoten, the Netherlands). Wheat cultivars Runal and Titlis were obtained from DSP AG (Delley, Switzerland). Wheat cultivar Riband was kindly provided by Jason Rudd (Rothamsted Research, Harpenden, United Kingdom).

**Induction of aneuploidy.** IPO323 (local identifier [ID] Zt244) maintained on YMS solid medium was inoculated at 200 cells/ml into YMS liquid media with 0 to 3 µg/ml carbendazim (Sigma-Aldrich, Munich, Germany) at 18°C with shaking at 200 rpm. At 7 days postinoculation, the cell suspension was transferred into a new test tube and the incubation continued for another 7 days. At 7 and 14 days postinoculation, cells were streaked onto YMS solid plates and incubated for 7 days at 18°C. If available, eight colonies from each treatment were karyotyped by PCR using multiplexed primers targeting subtelomeric regions of the accessory chromosomes (see below). If necessary, the loss of a specific accessory chromosome was confirmed by additional PCR along the chromosome.

**PCR karyotyping.** We initially karyotyped carbendazim-treated colonies using a PCR approach. DNA of a single colony was isolated by resuspending cells in 50 µl 25 mM NaOH followed by 10 min at 98°C and a neutralization step performed by adding 150 µl 13.3 mM TrisHCl (pH 5.5). Two multiplexed PCRs were developed using eight primer pairs. Each of the eight primer pairs was designed to bind in the subtelomeric region of chromosome 14, 15, 16, 17, 18, 19, 20, and 21, respectively. PCR was performed using Phusion DNA polymerase (New England Biolabs, Frankfurt, Germany) (0.04 units/µl) with 0.2 mM deoxynucleoside triphosphates (dNTPs), 3% dimethyl sulfoxide (DMSO), and a 1.6 µM concentration of each primer in a total volume of 7 or 10 µl. If a colony showed no amplicon for one accessory chromosome, the colony was propagated and DNA was isolated using a standard phenol-chloroform protocol (33). To confirm the loss of an accessory chromosome, at least three independent PCRs for each accessory chromosome were performed. To verify the performance of each of these PCRs, a

primer pair amplifying a section of the housekeeping GAPDH (glyceraldehyde-3-phosphate dehydrogenase) gene was included as an internal control. An accessory chromosome was considered lost only if all PCRs specific for the accessory chromosomes confirmed the absence of the accessory chromosome while showing amplification of the GAPDH locus. See Table S3 in the supplemental material for a list of all primers used in this study.

**Pulsed-field gel electrophoresis (PFGE).** Verification of the karyotype was conducted using a pulsed-field gel electrophoresis system (DRII; Bio-Rad, Munich, Germany). Plugs containing intact chromosomes were produced from a *Z. tritici* cell suspension by centrifugation ( $3,500 \times g$ , 10 min) and resuspension of the cell pellet in 1 ml double-distilled water (ddH<sub>2</sub>O). Whole cells were embedded in 1 ml 2.2% Low-Range Ultra Agarose (Bio-Rad, Munich, Germany)–0.5× Tris-borate-EDTA (TBE) buffer and incubated twice for 24 h each time at 55°C in 5 ml lysis buffer (1.5 mg/ml proteinase K, 1% SDS, 0.45 M EDTA, pH 8.0). Small chromosomes were separated in 1.2% pulsed-field agarose (Bio-Rad, Munich, Germany)–0.5× TBE buffer and were processed for 48 h at 14°C using an angle of 120°, 5 V/cm, and a switching time of 50 to 150 s. Midsize chromosomes were separated in 1% pulsed-field agarose–1× TBE buffer and were processed for 72 h at 14°C using an angle of 106°, 3 V/cm, and a switching time of 250 to 1,000 s. Large chromosomes were separated in 0.8% pulsed-field agarose–1× TRIS-acetate-EDTA (TAE) buffer and were processed for 92 h at 13°C using an angle of 106°, 2 V/cm, and a switching time of 1,000 to 2,000 s. Gels were stained for 30 min in a 0.5 µg/ml ethidium bromide solution followed by 10 min of destaining in H<sub>2</sub>O.

**Genome sequencing.** For sequencing, a minimum of 2 µg DNA was isolated using a previously described phenol-chloroform extraction protocol (33). Sequencing was performed using an Illumina HiSeq 3000 system and included only two rounds of PCR-based amplification in order to minimize the number of PCR artifacts. Paired-end reads of 150 bp were sequenced for each strain. Sequencing was performed at the Max Planck-Genome-centre Cologne, Cologne, Germany. The reads determined in this work were deposited in the Sequence Read Archive (see below).

**Data handling.** Specific parameters for Illumina read processing are summarized in Text S1 in the supplemental material. In short, the program Trimmomatic (34) was used for quality filtering of the read data (using the following parameter settings: headcrop, 2; crop, 149; leading, 3; trailing, 3; slidingwindow, 4:15; Minlen, 50). Reads were mapped to the reference genome of IPO323 using bowtie2 (35). Reads mapping two or more times were removed using Picard (<http://broadinstitute.github.io/picard>). Major rearrangements and whole-chromosome deletions were assessed by inspection of the genome-wide read coverage. SNPs and indel mutations were identified using SAMtools software (36) with the function mpileup, applying a minimum base quality threshold of 20 and a ploidy level of 1 for all chromosomes. SNPs and indels with a quality value of <20 and a read depth threshold (DP) value of <10 were discarded. According to the haploid genome structure of *Z. tritici*, we further discarded sites with an allele frequency of <0.8%.

**Determination of the *Z. tritici* phenotypes *in vitro* and *in planta*.** The *Z. tritici* strains were grown on YMS solid medium for 5 days at 18°C before the cells were scraped from the plate surface. The cell number was adjusted to  $10^7$  cells/ml in ddH<sub>2</sub>O, and the reaction mixture was serially diluted to  $10^3$  cells/ml. A 3- $\mu$ l volume of each cell dilution was transferred onto YMS agar that included the test compounds, and the reaction mixture was incubated for 7 days at 18°C or 28°C. For the tests using high osmotic stresses, 1 M NaCl and 2 M sorbitol (obtained from Carl Roth GmbH, Karlsruhe, Germany) were added to the YMS solid medium. For the tests using cell wall stresses, 500  $\mu$ g/ml Congo red and 200  $\mu$ g/ml calcofluor (obtained from Sigma-Aldrich Chemie GmbH, Munich, Germany) were added to the YMS solid medium. To test the effect of reactive oxygen species, 2 mM H<sub>2</sub>O<sub>2</sub> (obtained from Carl Roth GmbH, Karlsruhe, Germany) was added to the YMS solid medium.

For the *in planta* phenotypic assays, we germinated seeds of the four wheat cultivars Obelisk, Runal, Titlis, and Riband on wet sterile Whatman paper for 4 days before potting was performed using Fruhstorfer Topferde soil (Hermann Meyer GmbH, Rellingen, Germany). Wheat seedlings were further grown for 7 days before inoculation. *Z. tritici* strains were grown on YMS solid medium for 5 days at 18°C before the cells were scraped from the plate surface. The cell number was adjusted to  $10^7$  cells/ml in H<sub>2</sub>O with 0.1% Tween 20. The cell suspension was brushed onto approximately 5 cm on the abaxial and adaxial sides of the second leaf of each seedling. Inoculated plants were placed in sealed bags containing water for 48 h to facilitate infection through stomata. Plants were grown under constant conditions with a day/night cycle of 16 h of light ( $\sim 200$   $\mu$ mol/m<sup>2</sup>/s) and 8 h of darkness in growth chambers at 20°C. Plants were grown for 21 days postinoculation at 90% relative humidity (RH). For determining the onset of necrosis, the leaves were visually inspected for the appearance of symptoms at 7, 8, 9, 10, 11, 12, 13, 14, 16, 19, and 21 dpi within three experiments using the wheat cultivars Obelisk, Runal, and Titlis. The day of the first appearance of symptoms was recorded for each plant. Upon completion of the experiment, the infected leaves were cut and taped to sheets of paper and pressed for 5 days at 4°C before being scanned at a resolution of 2,400 dpi using a flatbed scanner (HP Photosmart C4580; HP, Böblingen, Germany). Scanned images were analyzed using automated image analysis and ImageJ (37) and a method adapted from reference 38. The readout (number of pycnidia per square centimeter of leaf surface) was used for all subsequent analyses. See Table S4 for a summary of all *in planta* results.

Statistical analyses were conducted in R (version r3.4.1) (39) using the suite RStudio (version 1.0.143) (40). Data inspection showed a nonnormal distribution for all data sets, including the measured pycnidia density (number of pycnidia per square centimeter) and the data for the switch to necrotrophy and thus also the relationship between the two. Therefore, we performed an omnibus analysis of variance using rank transformation of the data (28). For the three data sets, we performed calculations using the following models: (i) pycnidia density  $\sim$  strain \* cultivar \* experiment; (ii) switch to necrotrophy  $\sim$  strain \* cultivar \* experiment; and (iii) pycnidia density  $\sim$  switch to necrotrophy \* strain \* cultivar \* experiment. In all cases, *post hoc* tests were performed using Tukey's honestly significant difference (HSD) test (41).

**Accession number(s).** The reads determined in this work were deposited in the Sequence Read Archive with BioProject ID PRJNA371572.

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## Supplemental Material

**FIG S1** Gel electrophoresis for PCR karyotyping of strains used in this study of (A) multiplexed PCR targeting the subtelomeric region of chromosomes 14-21 (B-I) of PCR (B) along chromosome 14, (C), along chromosome 15, (D) along chromosome 16, (E) along chromosome 17, (F) along chromosome 18, (G) along chromosome 19, (H) along chromosome 20, (I) along chromosome 21.

See attached PDF file.

**FIG S2** Pulsed-field gel electrophoresis for midsize core chromosomes of *Z. tritici*. Pictures of three pulsed-field gel electrophoresis gels for all strains used in this study. Size marker: *Hansenula wingei* chromosomes

See attached PDF file.

**FIG S3** Pulsed-field gel electrophoresis for large core chromosomes of *Z. tritici*. Pictures of three pulsed-field gel electrophoresis gels for all strains used in this study. Size marker: *Schizosaccharomyces pombe* chromosomes

See attached PDF file.

**FIG S4** Illustration of the location of all single nucleotide polymorphisms (SNPs) and indels identified in all sequenced strains. Genome-wide distribution of SNPs across the thirteen core and eight accessory chromosomes of the reference strain IPO323.

See attached PDF file.

**FIG S5** *In vitro* phenotype of IPO323 and aneuploid strains. *In vitro* growth phenotypes were assessed for the chromosome deletion mutants. Cells were grown using different conditions and on different media including temperature stress and cell wall stress agents.

See attached PDF file.

**TABLE S1** Summary of next generation sequencing data and read mapping to the IPO323 reference genome

See attached Excel file.

**TABLE S2** Summary of identified SNP and indels in the resequenced genomes of the aneuploid strains

See attached Excel file.

**TABLE S3** List of all primers used in this study

See attached Excel file.

**TABLE S4** Summary of *in planta* results

See attached Excel file.

**TEXT S1** Protocol of read processing, reference assembly and SNP calling

See attached PDF file.



## Chapter 3

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# Meiotic drive of female-inherited supernumerary chromosomes in a pathogenic fungus

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### Abstract

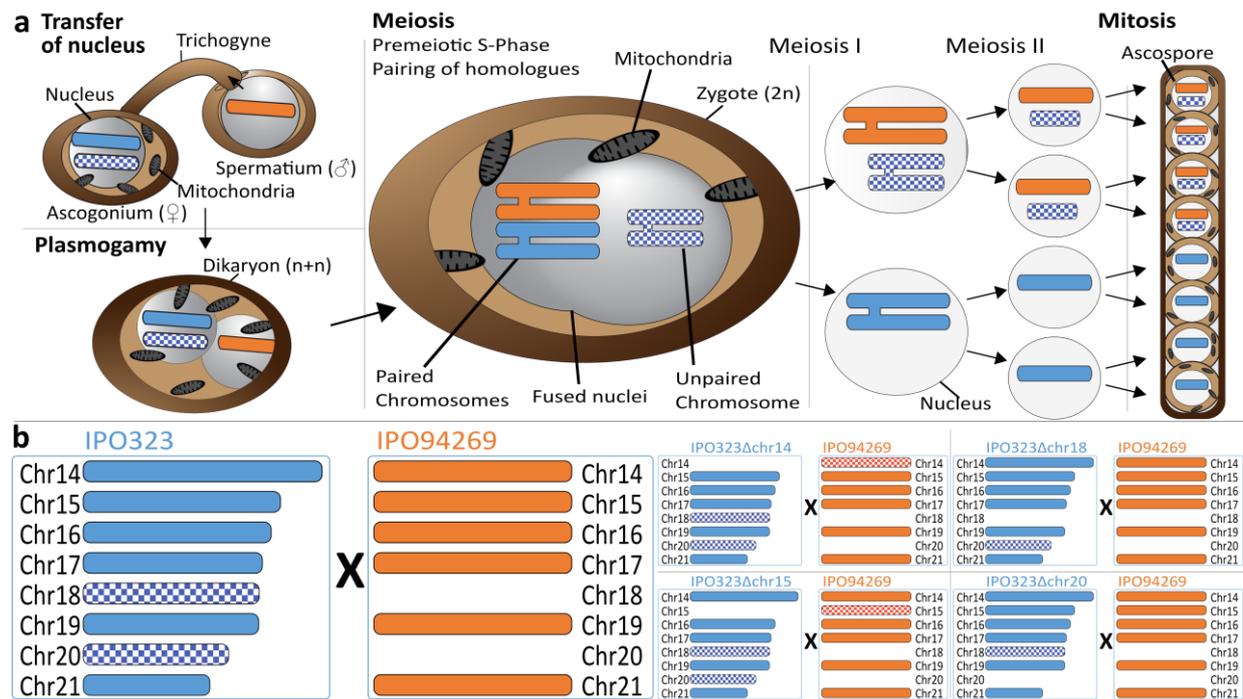
Meiosis is a key cellular process of sexual reproduction involving the pairing of homologous sequences. In many species however, meiosis can also involve the segregation of supernumerary chromosomes which can lack a homolog and can therefore be unpaired. How these unpaired chromosomes undergo meiosis is largely unknown. In this study we investigated chromosome segregation during meiosis in the haploid fungus *Zymoseptoria tritici* that possesses a large complement of supernumerary chromosomes. We used isogenic whole chromosome deletion strains to compare meiotic transmission of chromosomes when paired and unpaired. Unpaired chromosomes inherited from the male parent as well as paired supernumerary chromosomes showed Mendelian inheritance. In contrast, unpaired chromosomes inherited from the female parent showed non-Mendelian inheritance but were amplified and transmitted to all meiotic products. We conclude that the supernumerary chromosomes of *Z. tritici* show a meiotic drive and propose an additional feedback mechanism during meiosis which initiates amplification of unpaired female-inherited chromosomes.

In eukaryotes meiosis is a highly conserved mechanism that is critical for gamete formation and involves recombination between chromosomes. Meiosis combines one round of DNA replication with two subsequent rounds of chromosome segregation (reviewed in (1, 2)). DNA replication during the meiotic S-phase progression is coupled directly to interactions between homologous sequences and results in the pairing of chromosomes and recombination (3). The initial pairing of homologous chromosomes is important for meiosis and proper chromosome segregation (reviewed in (4)). However, it is less clear how meiosis proceeds when pairing of homologous chromosomes does not take place due to unequal sets of chromosomes, as is the case in organisms with non-essential supernumerary chromosomes.

Supernumerary chromosomes, also known as B chromosomes, conditionally-dispensable, lineage-specific or accessory chromosomes, are present in some but not all members of a population. They are estimated to be present in 14% of karyotyped orthopteran insect species (5), 8% of monocots, and 3% of eudicot species (6). These chromosomes commonly show non-Mendelian modes of inheritance, leading to segregation distortion during meiosis and a change in the frequency of the supernumerary chromosome in the progeny – a process that has been described as a chromosome drive (7, 8). Segregation advantage of supernumerary chromosomes can be due to drive mechanisms at the pre-meiotic, meiotic, or post-meiotic stages of gamete formation (9–13) and has been demonstrated in animals and plants (11, 14). In fungi, supernumerary chromosomes have been characterized in several species and notably studied in fungal pathogens where their presence in some cases is associated with virulence (15, 16). The underlying mechanisms causing non-Mendelian inheritance of the fungal supernumerary chromosomes are however poorly understood.

The genomic composition of the fungal plant pathogen *Zymoseptoria tritici* provides an attractive model to analyze supernumerary chromosome transmission. The genome of this fungus contains one of the largest complements of supernumerary chromosomes reported to date (17). The eight distinct supernumerary chromosomes (chr14 to chr21) of the reference isolate IPO323 show presence/absence polymorphisms among isolates and differ in their genetic composition compared to the essential chromosomes (17, 18). The supernumerary chromosomes in *Z. tritici* are enriched in repetitive elements (17, 19, 20) and are mainly heterochromatic (21). They are frequently lost during mitosis (22) and meiosis (17, 23–25) and they show a considerably lower recombination rate compared to the core chromosomes (26, 27). However, core and supernumerary chromosomes share many repetitive element families and their subtelomeric regions contain the same transposable element families (21, 20, 19). In contrast to many gene-poor supernumerary chromosomes described in plants and animals, those in *Z. tritici* possess a relatively high number of protein-coding genes (727, corresponding to 6% of all genes) (19). Recently, we demonstrated that the supernumerary chromosomes of *Z. tritici* confer a fitness cost: Isogenic strains lacking distinct supernumerary chromosomes produce higher amounts of asexual spores during host infection when compared to wild type with the complete set of supernumerary chromosomes (28). Despite the instability and fitness cost of the supernumerary chromosomes, they have been maintained over long evolutionary

times (29, 27) and it is therefore intriguing to address the mechanisms of supernumerary chromosome maintenance in the genome in *Z. tritici*.

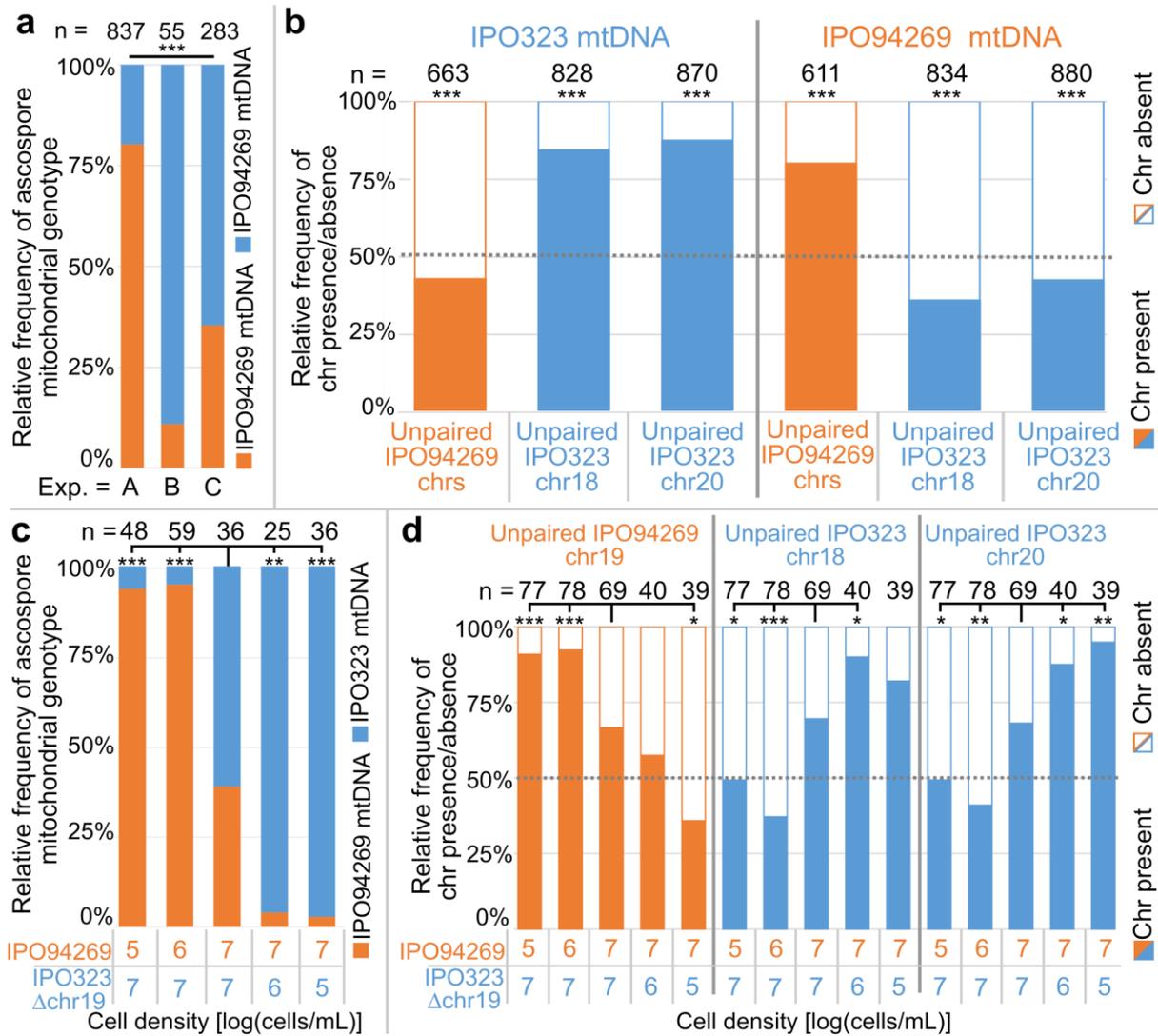


**Fig. 1.** Meiosis and chromosome segregation in *Z. tritici*. a) Schematic overview of the assumed sexual process between two parental strains of *Z. tritici* (30, 31) with one supernumerary chromosome shared and therefore paired (blue/orange) and one supernumerary chromosome unique to a strain (blue checkered) and unpaired in the zygote. The spermatial nucleus is transferred from the male partner via the trichogyne to the ascogonium of the female partner, resulting in plasmogamy and a dikaryon with two separate nuclei. Prior to karyogamy, the chromosomes are replicated and comprise each two chromatids and meiosis is initiated by pairing of homologous chromosomes. In meiosis I, homologous chromosomes are segregated, followed by chromatid separation in meiosis II. A subsequent mitosis results in the production of eight ascospores contained within one ascus. The expected segregation of chromosomes according to Mendelian law of segregation is shown - which for unpaired chromosomes is 4:0. b) Schematic illustration of the distribution of supernumerary chromosomes present in the parental strains exemplified for five of nine different crosses performed in this study. Parental strain IPO323 contains eight supernumerary chromosomes (chr14-21, blue). Parental strain IPO94269 contains six supernumerary chromosomes with a homolog in IPO323 (chr14, chr15, chr16, chr17, chr19, and chr21 in orange). The IPO323 chromosomes chr18 and chr20 are not present in IPO94269. We crossed a set of IPO323 chromosome deletion strains with IPO94269 to generate an additional unpaired chromosome (illustrated examples include IPO323Δchr14 X IPO94269, IPO323Δchr15 X IPO94269, IPO323Δchr18 X IPO94269, IPO323Δchr20 X IPO94269). Orange and blue indicate chromosomes that are shared between both strains. Checkered orange and checkered blue indicate chromosomes that are unique to one parent and therefore unpaired in the zygote.

Here, we used *Z. tritici* with its unique set of supernumerary chromosomes as a model to study the dynamics of unpaired chromosomes during meiosis. *Z. tritici* is a heterothallic, haploid ascomycete (i.e. two individuals of different mating type [*mat1-1* and *mat1-2*] are required to form a diploid zygote) (32, 30). If two haploid cells of opposite mating types contain a different complement of supernumerary chromosomes, the resulting diploid zygote consequently contains unpaired chromosomes. Upon Mendelian segregation during meiosis (segregation of the homologous chromosomes during meiosis I followed by chromatid segregation during meiosis II), four (50%) of the eight produced ascospores are predicted to contain the unpaired chromosomes (Fig. 1A). To test this prediction, we performed crosses between isolates with different subsets of supernumerary chromosomes (Fig. 1B). Based on controlled experiments and tetrad analyses, we surprisingly found that the supernumerary chromosomes of *Z. tritici* are subject to a meiotic drive restricted to unpaired chromosomes inherited from the female parent. Our results suggest that this drive mechanism is due to an additional, female-specific amplification of unpaired chromosomes during meiosis, a process that can ensure the maintenance of these chromosomes over long evolutionary times.

## Results

**Unpaired supernumerary chromosomes show drive correlated with mitochondrial transmission.** To test the transmission of supernumerary chromosomes during meiosis we used the reference strain IPO323 (mating type *mat1-1*) and eight isogenic chromosome deletion strains (IPO323 $\Delta$ chr14-21, mating type *mat1-1*) generated in a previous study (28). Each of the chromosome deletion strains differs in the absence of exactly one supernumerary chromosome, thereby allowing us to compare the transmission of individual chromosomes in a paired and an unpaired state. We crossed these strains *in planta* with the isolate IPO94269 (*mat1-2*) (Fig. 1B) in three separate experiments (A, B and C) and used a combination of PCR assays, electrophoretic karyotyping and whole genome sequencing to assess the segregation of chromosomes during meiosis. IPO94269 contains six supernumerary chromosomes homologous to the IPO323 chromosomes 14, 15, 16, 17, 19, and 21 (Fig. S1)(17). The experiments included a total of 39 crosses of IPO323/IPO323 chromosome deletion strains with IPO94269 resulting in different complements of paired and unpaired supernumerary chromosomes in the diploid zygote (Table 1, Table S1, Text S1). We hypothesized that the inheritance of the unpaired supernumerary chromosomes could be linked to the female or male role of the parental strain. Sexual mating of heterothallic fungi of the genus *Zymoseptoria* involve a female partner that produces a sexual structure called the ascogonium. The ascogonium receives the spermatium with the male nucleus from the fertilizing male partner through a particular structure called the trichogyne (33) (Fig. 1A). Importantly, the same strain can act as either the female or male partner (34). We used specific mitochondrial PCR based markers to distinguish the mitochondrial genotype in the progeny and thereby determined which of the two parental strains (in this case IPO323 or IPO94269) acted as a female partner in a cross.



**Fig. 2.** Unpaired supernumerary chromosomes show a segregation advantage only when inherited from the female parent. a) Relative frequencies of mitochondrial genotypes in ascospores in experiments A, B and C. The mitochondrial transmission varied significantly between the three experiments. Statistical significance was inferred by Fisher's exact test. b) Relative frequencies of the presence and absence of unpaired supernumerary chromosomes in all progeny ascospores pooled for experiments A, B and C according to the mitochondrial genotype of the ascospore. Orange and blue indicate unpaired chromosomes originating from IPO94269 or IPO323, respectively. For simplification the frequencies of unpaired chromosomes 14, 15, 16, 17, 19, and 21 originating from IPO94269 are pooled, while data for both unpaired chromosome 18 and 20 originating from IPO323 are depicted separately. Unpaired chromosomes inherited from the parent that provided the mitochondrial genotype (i.e. the female parent) are overrepresented in the progeny, while the same chromosomes when originating from the male parent are not. Statistical significance was inferred by a two-sided binomial test with a probability of  $p=0.5$ . c) Cell density affects the sexual role during mating and thereby the transmission of the mitochondria. Relative frequencies of mitochondrial genotype in ascospores isolated from crosses of IPO94269 and IPO323 $\Delta$ chr19 that were co-inoculated on wheat at different cell densities. The resulting progeny shows a correlation between cell-density and mitochondria transmission. Strains inoculated at lower density in general take the female role as observed by the mitochondrial transmission. Statistical significance was inferred by a two-sided Fisher's exact test compared to the co-inoculation with equal cell densities of both strains. d) The cell density affects the transmission of unpaired chromosomes. Relative frequencies in all ascospores of the presence and absence of unpaired supernumerary chromosomes 19, inherited from parent IPO94269 and unpaired chromosomes 18 and 20, inherited from IPO323 $\Delta$ chr19 are indicated according to the cell density of the parental strains IPO94269 and

IPO323 $\Delta$ chr19 at inoculation. Statistical significance was inferred by a two-sided Fisher's exact test compared to the co-inoculation with equal cell density of both strains. (\* =  $p < 0.05$ , \*\* =  $p < 0.005$ , \*\*\* =  $p < 0.0005$ ).

In all three experiments the ascospore progeny showed either the mitochondrial genotype of IPO94269 or IPO323, highlighting that both strains can act as the female and male partner during crosses (Table S2, S3, S4). However, transmission of the mitochondrial genotype varied significantly between experiments whereby the relative frequency of the IPO94269 mitochondrial genotype in the progeny was 80%, 11% and 65% in experiment A, B and C, respectively (Fig. 2A). Interestingly, the transmission of unpaired chromosomes correlated with the sexual role (female/male) of the parent from which the unpaired chromosome was inherited. Unpaired chromosomes inherited from IPO94269 were underrepresented among ascospores with the IPO323 parent mitochondrial genotype (Fig. 2B). In contrast, the unpaired supernumerary chromosomes 18 and 20, which were always inherited from the parent IPO323, were highly overrepresented among ascospores with the IPO323 mitochondrial genotype (Fig. 2B). For ascospores with the mitochondrial type of the IPO94269 parent, this segregation distortion was reversed (Fig. 2B).

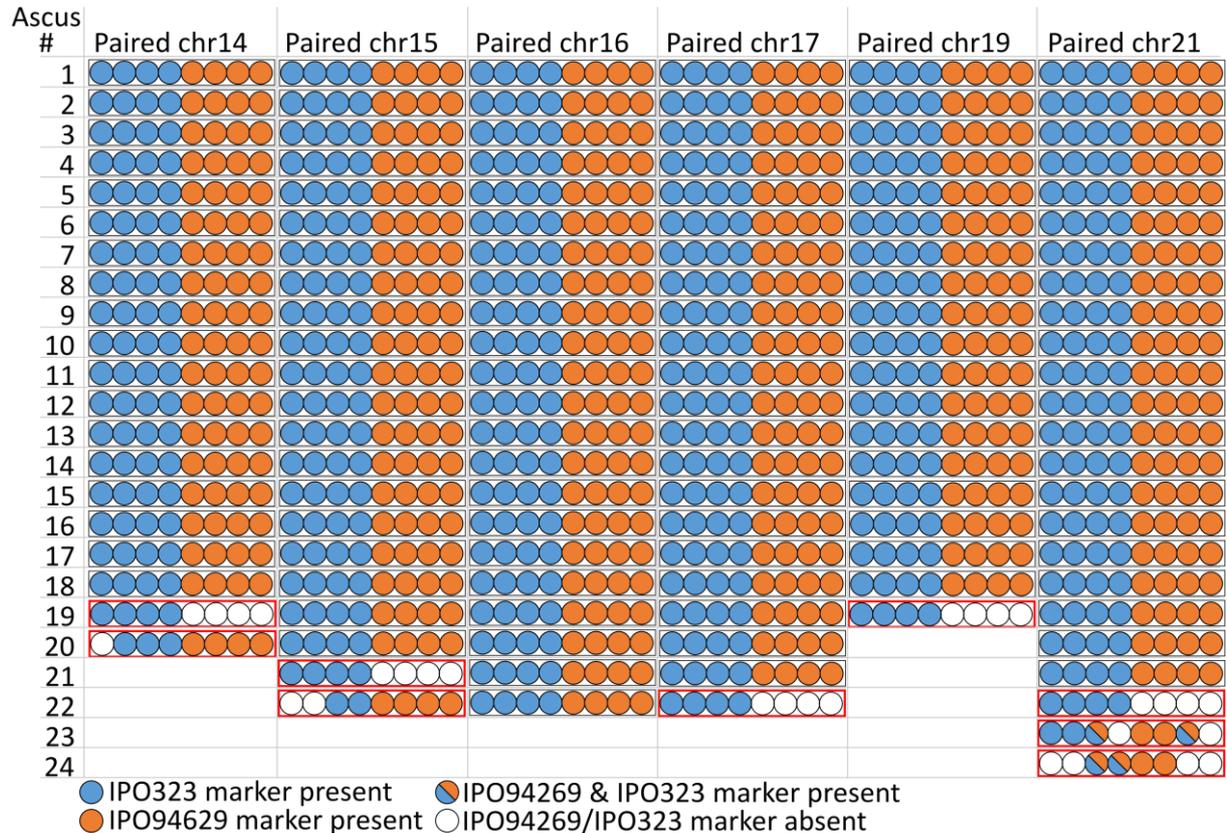
Although the transmission of the supernumerary chromosomes was highly similar between experiments A, B and C when the mitochondrial genotype was used to group the data (Fig. S2A), the overall transmission of the supernumerary chromosomes varied considerable between the experiments due to the highly divergent mitochondrial genotype inheritance in the three experiments (Fig. S2B). In spite of these variations, we consistently find a transmission advantage for supernumerary chromosomes, except chromosome 14, when pooling data across the experiments (Fig. S2C). Based on these observations, we conclude that unpaired supernumerary chromosomes show a chromosome drive mechanism, but this drive is restricted to chromosomes inherited from the mitochondria-donating female parent.

**Transmission of mitochondria is affected by cell density.** We next asked which factors determine the sexual role and thereby the mitochondrial inheritance in the sexual crosses of *Z. tritici*. To this end, we considered the cell density of the two parental strains as well as the relative timing of infection as determining factors of the sexual role. To test this, we set up crosses between the strains IPO323 $\Delta$ chr19 and IPO94269 in which the cell density varied from  $10^5$  cells to  $10^7$  cells/mL of each of the parental strains. Furthermore, we set up crosses in which we varied the relative timing of the infection of the two parental strains to each other by inoculating one parental strain 6 or 12 days later than the other parental strain. To distinguish the female and male partner in the crosses we again assessed the mitochondrial transmission frequencies. Interestingly, we find that the cell density of the two parental strains strongly correlates with the transmission of the mitochondrial genotype. Crosses with a lower cell density of IPO323 $\Delta$ chr19 resulted in a higher proportion of the progeny carrying the IPO323 mitochondrial genotype (Fig. 2C). Similarly, a lower cell density of IPO94269 resulted in a higher proportion of the progeny carrying the IPO94269 mitochondrial genotype. This finding strongly

suggests that a density-dependent mechanism affects the sexual role of the *Z. tritici* strains during sexual mating. In consistency with our first experiments, variation in the sexual role predictably affected the transmission of unpaired supernumerary chromosomes. The unpaired chromosome 19 inherited from the parent IPO94269 increased in frequency in the meiotic progeny with increasing frequency of the IPO94269 mitochondrial genotype. Unpaired chromosome 18 and chromosome 20 inherited from the parent IPO323 $\Delta$ chr19 increased in frequency in the meiotic progeny with an increase in frequency of the IPO323 mitochondrial genotype (Fig. 2D). Moreover, we find that the relative timing of the infection of the two strains affected the transmission of the *Z. tritici* strains. In crossing experiments where one strain was inoculated with six or twelve days delay, the later-inoculated strain more frequently exhibited the female role (Fig. S2D). This could be either due to the later inoculated strain having a growth disadvantage compared to the earlier inoculated strain therefore producing a lower density of cells. This scenario would be in agreement with our observation that the parental strain with lower cell density develops the female structure (Fig. 2C). Alternatively, the timing of maturation of the male and female structures might differ and possibly the female and male structures of different age could be incompatible. However, a clear effect of cell density and timing is discernible and we therefore conclude that environmental factors that affect the infection density and timing of different *Z. tritici* strain also strongly affect the sexual role of strains and thereby the transmission of supernumerary chromosomes.

**Paired supernumerary chromosomes show Mendelian segregation with frequent losses.** In *Z. tritici*, as in other ascomycetes, one meiosis produces eight ascospores by an additional mitosis following meiosis (34, 23). The outcome of single meiotic events can be analyzed by tetrad analyses whereby the eight ascospores of a tetrad - in ascomycetes an ascus - are isolated and genotyped. We used tetrad analysis to address how paired supernumerary chromosomes segregate during meiosis. For a total of 24 separate asci, we verified that all eight ascospores originated from the same ascus and were the products of a single meiosis using six segregating markers located on the essential chromosomes (Table 1, Text S1, Table S1). With these 24 asci we characterized segregation patterns while eliminating post-meiotic effects on the observed chromosomal frequencies. Each tetrad allowed the analysis of the segregation pattern for both unpaired and paired chromosomes. First, we focused on the segregation of paired chromosomes within these 24 tetrads. We observed the transmission of paired supernumerary chromosomes in 129 instances. We then determined the segregation of each paired supernumerary chromosome using specific segregating markers for each of the supernumerary chromosomes from both parental strains. In general, the paired supernumerary chromosomes showed Mendelian segregation (Fig. 3). Of the 129 instances of paired supernumerary chromosome, 120 (93%) showed Mendelian segregation with the expected 4:4 ratio (Fig 3) and only nine instances (7%) showed a deviation from the 4:4 ratio (Fig. 3, red outline). In no instances did the number of ascospores with a segregating marker for a paired supernumerary chromosome exceed the four ascospores predicted by Mendelian segregation. In two of the nine instances however, we found ascospores with two copies of supernumerary chromosome 21, representing one copy from IPO323 and another from IPO94269. Whole genome

sequencing validated the presence of two copies of chromosome 21 in the genomes of these ascospores. In the two additional ascospores of the tetrad analysis the chromosome 21 was missing (Fig. S3A-C) suggesting that the deviations from non-Mendelian segregation are due to loss of chromosomes, non-disjunction of sister chromatids, or non-disjunction of homologous chromosomes during meiosis (23).

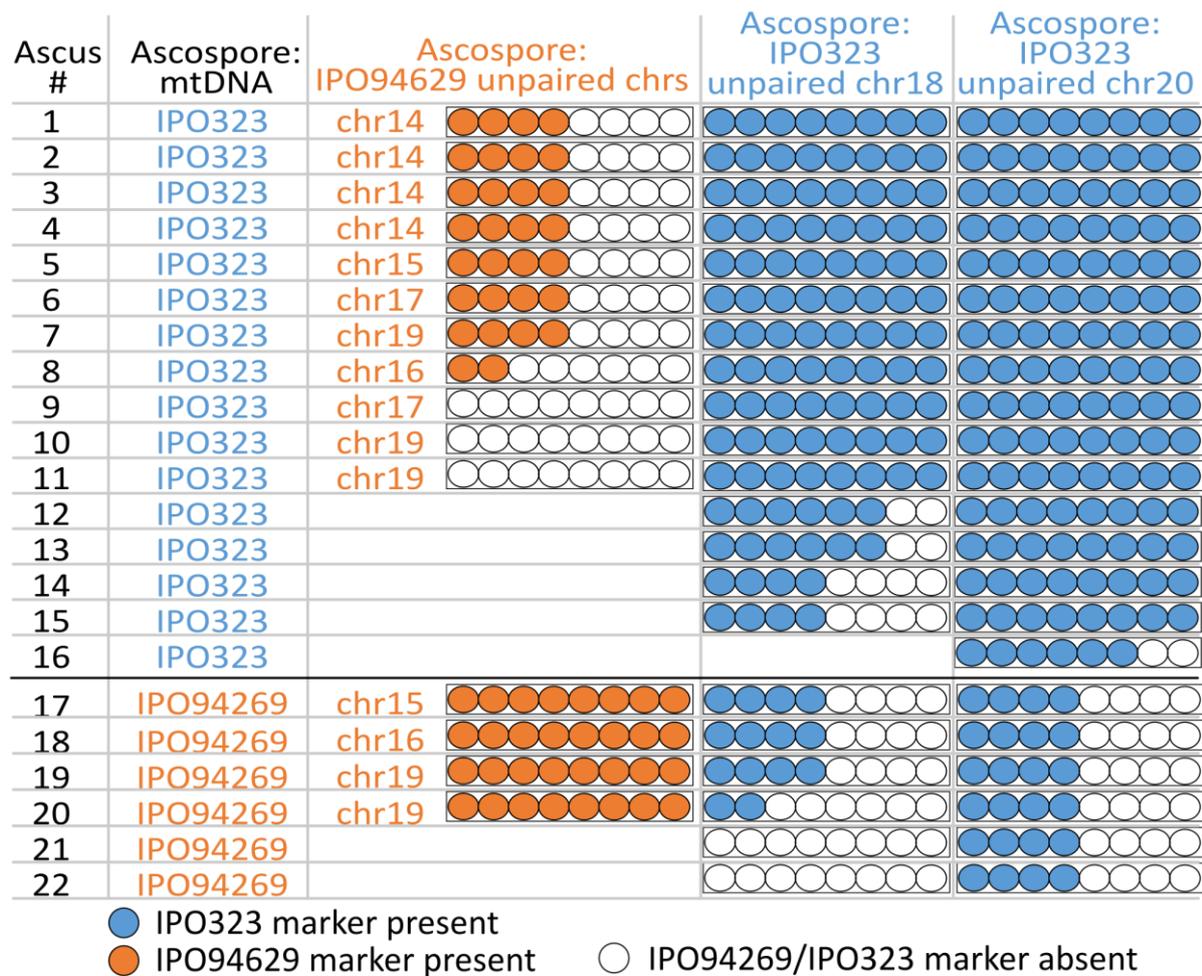


**Fig. 3.** Paired supernumerary chromosomes show Mendelian segregation. Analysis of segregation of paired supernumerary chromosomes in 24 complete tetrads. The transmission of chromosomes 14, 15, 16, 17, 19, and 21 with homologs in both parental strains IPO94269 and IPO323 was detected using segregating markers for chromosomes inherited from the parental strains IPO94269 (orange) and IPO323/IPO323 $\Delta$ chr14-21 (blue) in the eight ascospores originating from 24 asci. In 120 of the 129 cases we observed a 4:4 ratio in the progeny. Note: for crosses/chromosome combinations where no paired chromosome was present no ascus is shown, which reduces the number of shown asci from the 24 asci that were analyzed in both Fig. 3 and Fig. 4.

We next extended the analysis of transmission fidelity to all ascospores isolated in experiments A, B and C to compare the rate of loss of paired supernumerary chromosomes. We assessed the rate of chromosome loss from 10078 instances of paired supernumerary chromosomes in isolated meiotic progenies. In 377 cases (3.7%) we found evidence for supernumerary chromosome loss in the ascospores based on the absence of specific chromosome markers (Table S5). Interestingly, the frequency of loss of paired supernumerary chromosomes varied significantly between the individual chromosomes ( $\chi^2$ -Test: exp. A:  $p=1.96 \times 10^{-06}$ , exp. B:  $p=1.72 \times 10^{-09}$ , exp. C:  $p=4.18 \times 10^{-4}$ ) (Table S5) with chromosome 16 showing the lowest loss rate in all three experiments. The frequency of chromosome loss, however, shows no correlation to particular chromosome characteristics like chromosome size or the extent of homology between the chromosomes from IPO323 and IPO94269 (Fig S1B).

**Unpaired supernumerary chromosomes inherited from the female show meiotic drive.** Using the same 24 complete tetrads we dissected the fate of unpaired chromosomes during single meiotic events. Each tetrad contained between one to three unpaired chromosomes with chromosome 18 and 20 being solely inherited from IPO323 and chromosome 14, 15, 16, 17, and 19 being solely inherited by the IPO94269 in crosses performed with the five IPO323 whole-chromosome-deletion strains. In contrast to paired supernumerary chromosomes, unpaired supernumerary chromosomes show distinct segregation distortion (Fig. 4) that correlates with mitochondrial transmission; unpaired chromosomes originating from the female parent show a strong meiotic chromosome drive. On the other hand, unpaired chromosomes originating from the male parent (i.e. the parent that did not provide the mitochondria) show Mendelian segregation and are more frequently lost.

In the 24 tetrads dissected here, all eight ascospores originating from the same ascus showed the same mitochondrial genotype (Table S3-S4) confirming previous results on the uniparental inheritance of mitochondria in *Z. tritici* (30). Isolated ascospores had the mitochondrial genotype of the parent IPO323 in 18 asci, while the ascospores of the remaining six asci showed the IPO94269 genotype, confirming that both parental strains, IPO323 and IPO94269, can act as the female parent during sexual mating with no significant difference in the frequency between the two strains (two sided binomial ( $p=0.5$ ),  $p=0.25$ ) (30). In 18 asci that showed the IPO323 mitochondrial genotype, supernumerary chromosomes 18 and 20, inherited from IPO323 (the female), were unpaired in 15 and 16 meioses, respectively (Fig. 4). Chromosome 18 was present in all eight ascospores in 11 of the 15 asci (ascus #1-11) instead of the expected four ascospores. In two asci, the chromosome was present in six ascospores (ascus #12-13). Chromosome 20 was present in all eight ascospores (ascus #1-15) in 15 of the 16 asci and in one ascus in six ascospores (ascus #16). This transmission pattern was reversed for the six asci exhibiting the IPO94269 mitochondrial genotype (Fig. 4). Here, the female-inherited unpaired chromosomes from IPO94269 show meiotic drive while the male-inherited unpaired chromosomes from IPO323 show Mendelian segregation or were lost (Fig. 4). We validated our PCR-karyotyping by sequencing the genomes of 16 ascospore isolates originating from two asci and mapped the resulting reads to the reference genome of IPO323. For all 16 ascospores we find similar coverage for all essential chromosomes and supernumerary chromosomes (Fig. S3) and all 16 ascospores show similar coverage for chromosome 18 and 20 verifying the transmission of these chromosomes to the eight ascospores of each ascus instead of the expected four ascospores.



**Fig. 4.** Unpaired supernumerary chromosome show meiotic drive if inherited from the female parent. Analysis of segregation of unpaired supernumerary chromosomes in 24 complete tetrads according to the mitochondrial genotype. The transmission of chromosomes unique to one of the parental strains and the mitochondrial genotype was detected using chromosomal or mitochondrial markers originating from IPO94269 (orange) and IPO323 (blue) in eight ascospores derived from 24 asci. When IPO323 was the female parent (i.e. the ascospores inherited the mitochondrial genotype of the IPO323 parent) unpaired chromosomes 18 and 20 originating from IPO323 show a strong chromosome drive and are overrepresented in the ascospores. When IPO94269 was the female parent unpaired chromosomes originating from IPO94269 show a strong chromosome drive. Unpaired chromosomes originating from the male parent (i.e. the one not donating the mitochondrial genotype) show Mendelian segregation or are lost. Note: for crosses/chromosome combinations where no unpaired chromosome was present no ascus is shown, which reduces the number of shown asci from the 24 asci that were analyzed in both Fig. 3 and Fig. 4.

The meiotic drive of the unpaired supernumerary chromosome could imply an additional amplification step that only affects unpaired chromosomes derived from the female parent. We found however that in one cross, this additional amplification of an unpaired chromosome was incomplete: In the ascus A08-1, unpaired chromosome 18 was transmitted to all eight ascospores, but four of the ascospores contained only a partial chromosome 18 (Fig. S3F). The partial chromosomes 18 showed Mendelian segregation, indicating that the additional amplification step of the unpaired chromosome 18 occurred prior to the first meiotic division, which in this rare case was incomplete.

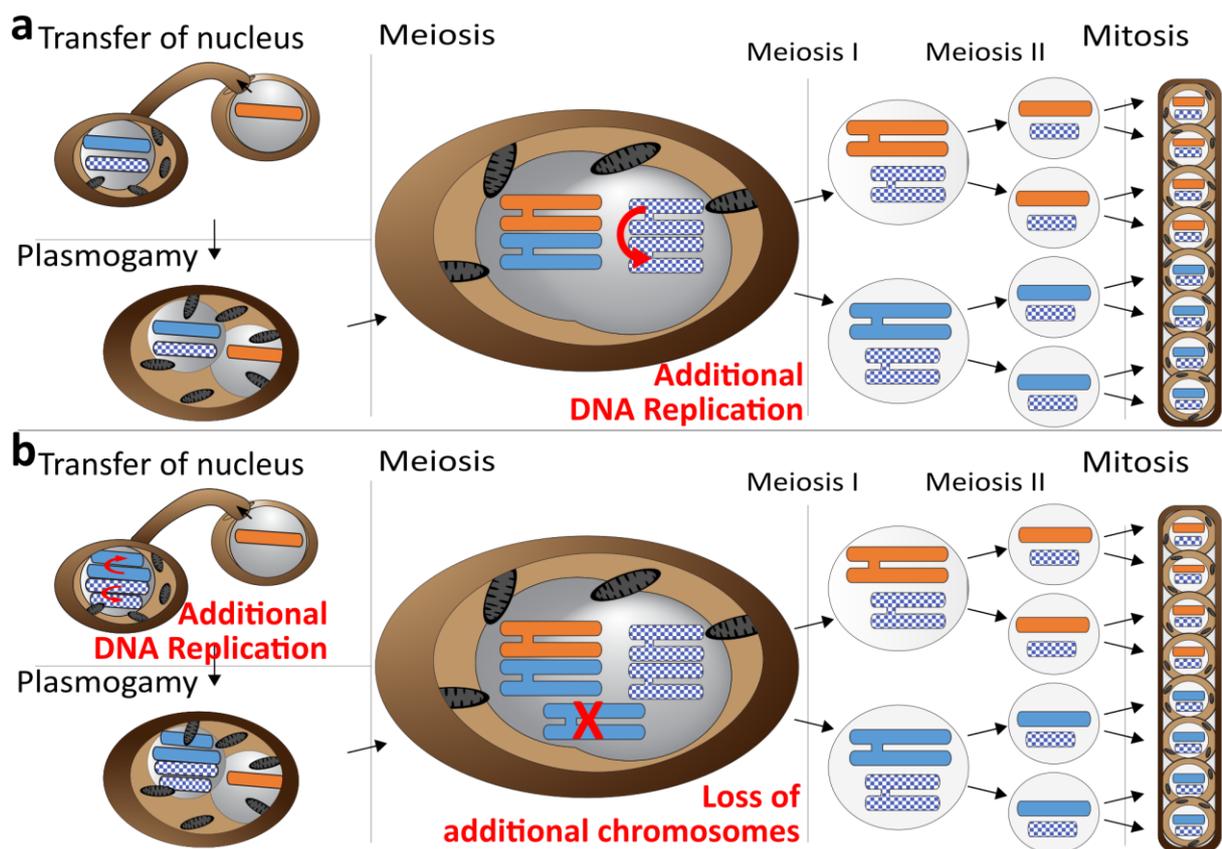
## Discussion

The fate of supernumerary chromosomes during meiosis is poorly understood despite the widespread occurrence of this type of chromosome in different taxa. Here, we show that unpaired chromosomes of the fungal plant pathogen *Z. tritici* are transmitted and amplified by a meiotic drive which acts only on chromosomes inherited from the female parent (Fig. 5A). Crossing experiments of haploid individuals of opposite mating types document this as: i) unpaired supernumerary chromosomes show chromosome drive only when inherited from the female parent; ii) unpaired supernumerary chromosomes show Mendelian segregation with frequent losses when inherited from the male parent; and iii) paired supernumerary chromosomes show Mendelian segregation, but with frequent losses during meiosis.

Our data strongly suggest that this chromosome drive does not result from pre- or post-meiotic mechanisms but occurs during meiosis. First, we exclude the occurrence of a post-meiotic chromosome drive, e.g. killing of ascospores that did not contain the drive element, as this would make it impossible to isolate complete tetrads, which we however succeeded to do. Second, we also exclude any pre-meiotic mechanisms, which could consist of either a premeiotic amplification (Fig. 5B) or preferential segregation of the supernumerary chromosomes. However, both mechanism would affect all supernumerary chromosomes in the haploid nucleus prior to karyogamy, because at this stage it is not yet defined which of the supernumerary chromosomes will become paired or unpaired. If such a pre-meiotic amplification or preferential segregation would occur, the diploid zygote resulting after karyogamy would be trisomic for all paired supernumerary chromosomes and disomic for the unpaired chromosomes. This trisomy would result in a non-Mendelian inheritance of paired supernumerary chromosomes. We did however, observe Mendelian segregation for the paired supernumerary chromosomes and importantly did not find any indication of additional copies of paired supernumerary chromosomes in the tetrad analysis. This segregation pattern would only be possible if the additional copies of the female-inherited paired supernumerary chromosomes would be lost during meiosis (Fig. 5B). Tightly regulated chromosome loss has been described for sex chromosomes in several insect species during embryonic development, where maternal and paternal imprinting determine the elimination of chromosomes (35). For *Z. tritici*, a similar mechanism would imply that all additional copies of all female supernumerary chromosomes would be eliminated during meiosis - except for the two copies of the female-inherited unpaired supernumerary chromosomes - while male supernumerary chromosomes would be unaffected. We consider this mechanism to be unlikely. In general, any pre-meiotic mechanism should affect both unpaired and paired chromosomes and therefore would require a counteracting mechanism after karyogamy that restores the Mendelian segregation pattern observed in the paired supernumerary chromosomes.

We here propose that an additional amplification affecting only female-inherited supernumerary chromosomes underlies the observed meiotic drive of supernumerary chromosomes in *Z. tritici* during sexual mating. The additional amplification of unpaired supernumerary chromosomes would require an additional initiation of DNA replication

restricted to unpaired chromosomes and therefore a feedback between pairing of homologous chromosomes and DNA replication. Although DNA replication is a highly regulated process and any feedback from chromosome pairing is currently unknown, we suggest that this model is the most likely to explain the observed pattern. Feedback between meiotic S-phase and the pairing of homologous chromosomes has been proposed based on the effects of interchromosomal interaction proteins like Spo11 - a key mediator of interhomolog interactions that is responsible for the initiation of meiotic recombination - on the progression of meiotic DNA replication (3). Although Spo11 cannot explain the additional amplification of unpaired chromosomes it illustrates a feedback process between pairing of homologs and DNA replication. In addition, pairing of homologous chromosomes or sequences has also been described for somatic cells in *Saccharomyces cerevisiae*, *Schizosaccharomyces pombe* and *Drosophila melanogaster* highlighting the possible existence of homolog pairing prior to DNA replication (36–40). We therefore consider it possible that meiosis in *Z. tritici* involves an additional feedback mechanism that induces an additional round of amplification based on the unpaired chromosome status.



**Fig. 5.** Meiotic chromosome drive in *Z. tritici*. Schematic illustration depicting two possible mechanisms for the observed meiotic chromosome drive of female-inherited unpaired supernumerary chromosomes. Light blue/orange: paired supernumerary chromosome. Checkered blue: unpaired supernumerary chromosome. a) Chromosome drive occurring during meiosis, only those unpaired supernumerary chromosomes in the zygote that originated from the female parent are subject to an additional round of DNA replication, allowing for pairing of the two copies of the chromosome. b) Alternative scenario under which the chromosome drive occurs prior to meiosis. All supernumerary

chromosomes are amplified to double the copy number during development of the female ascogonium. The supernumerary chromosomes are paired in the zygote during meiosis. Only additional copies of the supernumerary chromosomes inherited from the female are lost while the supernumerary chromosomes inherited from the male are unaffected.

A meiotic chromosome drive can explain the continued maintenance of supernumerary chromosomes in *Z. tritici* despite the negative effects on fitness during host infection (28). However, it is unclear how this type of chromosome drive can act simultaneously on several separate chromosomes. In our experiments, we found that seven of the eight supernumerary chromosomes showed drive, and we hypothesize that the drive mechanism (i.e., the additional amplification of the unpaired female-inherited supernumerary chromosomes) depends on a general characteristic of the supernumerary chromosomes. Interestingly, we observed the meiotic drive of unpaired accessory chromosomes for seven of the eight accessory chromosomes of the reference isolate IPO323. Chr14 for which no such transmission advantage was observed, is the largest of the accessory chromosomes (773 kb) in IPO323 (17). A large insertion spanning approx. 400 kb in chr14 shows presence/absence polymorphism in *Z. tritici* resulting in isolates that contain a much smaller chr14 (24). Interestingly the smaller chr14 showed a transmission advantage when present in one of the parental strains in a previous study (24). This observation indicates that chromosome size might be a factor influencing the observed drive. Currently, we cannot explain why the hypothesized additional amplification of the supernumerary chromosome is restricted to unpaired chromosomes inherited from the female parent. In ascomycetes, plasmogamy and karyogamy are separated by a dikaryon state, in which the female and male nuclei are separate (34). Consequently, there is a temporal separation of the processes determining uniparental inheritance of the mitochondria, nuclear inheritance, and the proposed additional amplification of the unpaired supernumerary chromosomes. Female chromosome drive in the fungus would depend on a female-derived signal that persists through plasmogamy to karyogamy when DNA amplification takes place. We currently do not know the nature of this signal, but speculate that it could be mediated by epigenetic mechanisms similar to genomic imprinting.

In this study, we have shown that supernumerary chromosomes of *Z. tritici* are subject to a meiotic drive, which is probably dependent on an additional meiotic amplification of unpaired chromosomes. This mechanism may explain the continued maintenance of supernumerary chromosomes in *Z. tritici* over long evolutionary times in spite of their frequent loss during mitosis and their fitness cost during plant infection and asexual propagation.

## Materials and Methods

**Fungal and plant material.** The Dutch isolates IPO323 and IPO94269 are available from the Westerdijk Institute (Utrecht, The Netherlands) with the accession numbers CBS115943 and CBS115941. *Triticum aestivum* cultivar Obelisk used for the *in planta* fungal mating was obtained from Wiersum Plantbreeding BV (Winschoten, The Netherlands). Sexual crosses were conducted as described in (32, 30).

**Crosses:** Detailed descriptions of the experimental procedures for crosses, ascospore isolation and karyotyping are provided in Text S1. Table 1 provides a summary of the crosses performed and the verified progeny obtained within these crosses.

**Table 1.** Summary of crosses and progeny generated in this study

#	Parental strain 1	Parental strain 2	Unpaired chr from IPO94269	Unpaired chr from IPO323	Condition	Ascospores (random/all)			Verified tetrads (mtIPO323/mtIPO94269)		
						Exp A	Exp B	Exp C	Exp A	Exp B	Exp C
1	IPO323	IPO94269	-	chr18, chr20	Coinoculation 10 <sup>7</sup> cells/mL	96/96	12/96	51/88	-	2/2	0
2	IPO323 Δchr14	IPO94269	chr14	chr18, chr20	Coinoculation 10 <sup>7</sup> cells/mL	96/96	8/64	38/72	-	3/0	1/0
3	IPO323 Δchr21	IPO94269	chr21	chr18, chr20	Coinoculation 10 <sup>7</sup> cells/mL	89/89	4/32	52/78	-	0	0
4	IPO323 Δchr16	IPO94269	chr16	chr18, chr20	Coinoculation 10 <sup>7</sup> cells/mL	96/96	6/48	38/115	-	1/0	0/1
5	IPO323 Δchr17	IPO94269	chr17	chr18, chr20	Coinoculation 10 <sup>7</sup> cells/mL	96/96	2/16	15/59	-	0	2/0
5	IPO323 Δchr19	IPO94269	chr19	chr18, chr20	Coinoculation 10 <sup>7</sup> cells/mL	96/96	9/72	38/77	-	3/1	0/1
7	IPO323 Δchr20	IPO94269	-	chr18	Coinoculation 10 <sup>7</sup> cells/mL	96/96	4/32	19/31	-	2/0	0
8	IPO323 Δchr18	IPO94269	-	chr20	Coinoculation 10 <sup>7</sup> cells/mL	96/96	4/32	30/67	-	1/0	2/0
9	IPO323 Δchr15	IPO94269	chr15	chr18, chr20	Coinoculation 10 <sup>7</sup> cells/mL	96/96	6/48	14/54	-	1/0	0/1
10	IPO323 Δchr19	IPO94269	chr19	chr18, chr20	IPO323 10 <sup>6</sup> cells/mL	-	-	25/42	-	-	0
11	IPO323 Δchr19	IPO94269	chr19	chr18, chr20	IPO323 10 <sup>5</sup> cells/mL	-	-	38/43	-	-	0
12	IPO323 Δchr19	IPO94269	chr19	chr18, chr20	IPO323 10 <sup>4</sup> cells/mL	-	-	2/16	-	-	0
13	IPO323 Δchr19	IPO94269	chr19	chr18, chr20	IPO94269 +6dpi	-	-	40/46	-	-	0
14	IPO323 Δchr19	IPO94269	chr19	chr18, chr20	IPO94269 +12dpi	-	-	11/11	-	-	0
15	IPO323 Δchr19	IPO94269	chr19	chr18, chr20	IPO94269 10 <sup>6</sup> cells/mL	-	-	60/84	-	-	0
16	IPO323 Δchr19	IPO94269	chr19	chr18, chr20	IPO94269 10 <sup>5</sup> cells/mL	-	-	48/80	-	-	0
17	IPO323 Δchr19	IPO94269	chr19	chr18, chr20	IPO94269 10 <sup>4</sup> cells/mL	-	-	28/28	-	-	0
18	IPO323 Δchr19	IPO94269	chr19	chr18, chr20	IPO323 +6dpi	-	-	5/29	-	-	0
19	IPO323 Δchr19	IPO94269	chr19	chr18, chr20	IPO323 +12dpi	-	-	51/71	-	-	1/0
Σ						761/761	55/440	603/1091	-	13/3	5/3

\* Includes random and randomized ascospores. Randomized ascospores were generated by randomly selecting one ascospore per tetrad.

**Karyotyping.** Ascospores were karyotyped for the presence of supernumerary chromosomes using primer pairs specifically designed to test for the presence of chromosomes 14, 15, 16, 17, 18, 19, 20, and 21 derived from IPO323 and from IPO94269 (Table S1 and Text S1) using standard conditions (28). For unpaired chromosomes that were overrepresented in the ascospores of experiment B and C, we validated their presence by two additional PCRs that amplify a sequence in the right subtelomeric and left subtelomeric regions of the chromosome (Table S1 and Text S1). For paired supernumerary chromosomes, we designed the primers to reveal a size difference in the amplification products of IPO323-derived and IPO94269-derived chromosomes (Table S1 and Text S1). In addition, verification of the karyotype was conducted using a pulsed-field gel electrophoresis system as described previously (28) (Fig. S4).

**Statistical analysis.** All statistical analyses were conducted in R (version R3.4.1) (41) using the suite R Studio (version 1.0.143) (42). Two-sided Fisher's exact tests were performed at a confidence level of 0.95. Due to the co-dependency of mitochondrial data of ascospores isolated from a potential or verified ascus, due to the fact that all ascospores of an ascus will receive the same mitochondrial genotype, one ascospore from each potential and verified ascus was randomly selected and included in the statistical analysis of the mitochondrial genotype transmission. For large datasets the Fisher's exact test was replaced by the Pearson's Chi-squared test. Two-sided binomial tests were performed with a hypothesized probability of  $p=0.5$  at a confidence level of 0.95 on all statistical analysis on a deviation of an assumed Mendelian segregation of unpaired supernumerary chromosomes which would be predicted to be present in 50% of the progeny. All statistical analysis on transmission of supernumerary chromosomes included all randomly selected ascospores as well as all ascospores selected from potential and verified asci.

**Genome sequencing.** For sequencing, DNA of IPO94269 and 16 ascospores was isolated using a phenol-chloroform extraction protocol as described previously (43). Library preparation and sequencing using a Pacific Biosciences Sequel for IPO94269 and Illumina HiSeq3000 machine for 16 ascospore-derived colonies were performed at the Max Planck Genome Centre, Cologne, Germany. Reads have been deposited in the Sequence Read Archive and are available under the BioProject PRJNA438050. Assembly of the IPO94269 genome was conducted at the Max Planck Genome Center, Cologne using the software suite HGAP 4 (44) from Pacific Biosciences using the default settings. Synteny analysis was conducted with SyMAP version 4.2 (45, 46) (Fig. S5). Illumina reads of the ascospores were filtered and mapped to the reference genome of IPO323 (17) as previously described (28) in which the transposable elements were masked (19).

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## Author contribution

M.H. designed and performed all experiments and analyzed the data. E.H.S directed the project. G.H.J.K. and E.H.S. helped designing the study. M.H. wrote the initial draft of the manuscript with support of E.H.S. All authors commented on the manuscript.

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## Supplementary Information

See attached Word file.





## Chapter 4

Manuscript accepted in GENETICS

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# Extraordinary genome instability and widespread chromosome rearrangements during vegetative growth

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### Abstract

The haploid genome of the pathogenic fungus *Zymoseptoria tritici* is contained on “core” and “accessory” chromosomes. While 13 core chromosomes are found in all strains, as many as eight accessory chromosomes show presence/absence variation and rearrangements among field isolates. The factors influencing these presence/absence polymorphisms are so far unknown. We investigated chromosome stability using experimental evolution, karyotyping and genome sequencing. We report extremely high and variable rates of accessory chromosome loss during mitotic propagation *in vitro* and *in planta*. Spontaneous chromosome loss was observed in 2 to >50 % of cells during four weeks of incubation. Similar rates of chromosome loss in the closely related *Z. ardabiliae* suggest that this extreme chromosome dynamic is a conserved phenomenon in the genus. Elevating the incubation temperature greatly increases instability of accessory and even core chromosomes, causing severe rearrangements involving telomere fusion and chromosome breakage. Chromosome losses do not impact the fitness of *Z. tritici* *in vitro*, but some lead to increased virulence suggesting an adaptive role of this extraordinary chromosome instability.

## Introduction

Pathogenic fungi pose global threats to agriculture, human, and animal health. Pathogens infecting plants and animals have been shown to rapidly adapt to changing environments, including industrial agriculture and medical treatments, usually in response to adaptive pressure caused by the widespread use of fungicides and pharmaceuticals (Selmecki *et al.*, 2010; Bennett *et al.*, 2014). The genomes of many prominent fungal plant pathogens exhibit high levels of structural variation including isolate- or lineage-specific regions often characterized by high repeat contents, and accessory chromosomes varying in frequency among individual isolates (De Jonge *et al.*, 2013; Persoons *et al.*, 2014; Plissonneau & Stürchler, 2016). Structural variation is often associated with meiotic recombination, but highly variable regions are also found in asexually and mostly asexually reproducing species (Wang *et al.*, 2006; Chuma *et al.*, 2011; Faino *et al.*, 2016). In several plant pathogenic fungi, determinants of virulence, so called effector proteins, are located in dynamic regions of the genome (Miao *et al.*, 1991a; Coleman *et al.*, 2009; Ma *et al.*, 2010). Accessory chromosomes often carry genes that encode virulence determinants and are linked to pathogenicity. Therefore, the absence of a particular chromosome in some species results in avirulent phenotypes (Ma *et al.*, 2010; Tsuge *et al.*, 2016; Vlaardingerbroek *et al.*, 2016; van Dam *et al.*, 2018). Little is known about the mechanisms that drive the dynamics of accessory chromosomes and highly variable regions in genomes of eukaryotic pathogens.

The plant pathogenic fungus *Zymoseptoria tritici* reproduces both, sexually and asexually and causes disease on wheat, especially in Northern Europe and North America, resulting in extensive annual yield loss (Fones & Gurr, 2015; Torriani *et al.*, 2015). The *Z. tritici* reference isolate IPO323 contains eight accessory chromosomes with sizes ranging from 0.4 to 1 Mb, accounting for 12% of the entire genome (Goodwin *et al.*, 2011). Chromosome rearrangements, including complete loss of accessory chromosomes, are a frequent phenomenon in this fungus and have been demonstrated to occur during meiosis (Wittenberg *et al.*, 2009; Croll *et al.*, 2015; Fouché *et al.*, 2018). Infection experiments with *Z. tritici* strains in which single or multiple accessory chromosomes had been deleted showed that at least some accessory chromosomes encode virulence factors that determine host specificity (Habig *et al.*, 2017). In contrast to the core chromosomes, accessory chromosomes are enriched with transposable elements and have low gene density (Goodwin *et al.*, 2011). Chromatin of accessory chromosomes shows hallmarks of constitutive and facultative heterochromatin, consistent with the observed transcriptional silencing of genes present on these chromosomes (Kellner *et al.*, 2014; Rudd *et al.*, 2015; Schotanus *et al.*, 2015). The centromeres, subtelomeric regions and telomeric repeats of accessory chromosomes are indistinguishable from those of core chromosomes (Schotanus *et al.*, 2015), suggesting that accessory chromosomes contain all the required regions for proper chromosome segregation. The unusual high number of accessory chromosomes, in combination with the extreme variability in chromosome content among field isolates or progeny from controlled crosses, make *Z. tritici* an excellent eukaryotic model to study accessory chromosomes and their dynamics (Mehrabi *et al.*, 2007; Wittenberg *et al.*, 2009; Stukenbrock *et al.*, 2010; Goodwin *et al.*, 2011). Here, we provide evidence for unexpectedly

high rates of chromosome loss and changes during asexual propagation, both *in vitro* and *in planta*. We describe the types of structural rearrangements and overall variation in chromosome stability in this important crop pathogen.

## Materials and Methods

### Short term *in vitro* growth experiment in liquid culture

*Zymoseptoria* strains were diluted from glycerol stocks (-80°C), plated on YMS agar (4 g yeast extract, 4 g malt extract, 4 g sucrose, and 20 g agar per 1 liter) plates and grown for seven days at 18°C to obtain single colonies. One single colony was picked and suspended in 100 µL of YMS. Three replicate cultures were inoculated with 20 µL of cells from the single colony (~50,000 cells). Cells were grown in 25 mL YMS medium at 18°C or 28°C shaking at 200 rpm and 900 µL of the cultures were transferred to fresh medium after 3-4 days of growth. In total eight transfers were conducted, for a total time course of four weeks.

### Short term *in vitro* growth experiment on agar plates

A single colony derived directly from a plated dilution of frozen stock for Zt09 (IPO323Δ18) was resuspended in 1000 µL YMS including 25% glycerol by 2 min vortexing on a VXR basic Vibrax at 2,000 rpm, and 10-50 µL were replated onto a YMS agar plate. Forty replicates were produced. Cells were grown for seven days at 18°C whereby a random colony (based on vicinity to a prefixed position on the plate) derived from a single cell was picked and transferred to a new plate as described above. The transfer was conducted for a total of four times before a randomly chosen colony of each replicate was PCR screened and their complement on accessory chromosomes characterized as described below.

### Screening by PCR

Cells from transfer eight of liquid cultures were diluted and plated on YMS-agar plates to obtain single colonies. To extract DNA, single colonies were suspended in 50 µL of 25 mM NaOH and boiled at 98°C for 10 min; 50 µL of 40 mM Tris-HCl, pH 5.5, were added and 4 µL were used as template for the PCR. Primers for the right and left subtelomeric regions and close to the centromere were used for the chromosome loss screening in our *Z. tritici* isolates, for *Z. ardabiliae* primers in the center of candidate accessory chromosome unitigs were used. Primers and expected fragment lengths are listed in Table S1. Primers were designed with Clonemanager (Sci-Ed Software, Denver, USA) and MacVector and ordered from eurofins Genomics (Ebersberg, Germany).

### Plant experiments and pycnidia isolation

Growth conditions for plants (*Triticum aestivum*) were 16 h at light intensity of ~200 µmol/m<sup>2</sup>s<sup>-1</sup> and 8 h darkness in growth chambers at 20°C with 90% humidity. Seeds of the *Z. tritici* susceptible wheat cultivar Obelisk (Wiersum Plantbreeding BV, Winschoten, The Netherlands) were germinated on wet sterile Whatmann paper for four days at growth conditions before potting. Following potting, plants were further grown for seven days before infection.

For the phenotypic analyses, three independent experiments were conducted and 21 plants were used per strain per experiment. An ~5 cm long section on the second leaf of each plant was infected by brushing a cell suspension with  $10^7$  cell/ml in H<sub>2</sub>O + 0.1% Tween 20 on the abaxial and adaxial side of the second leaf. Plants were placed into sealed bags containing ~1 L of water for 48 h to facilitate infection at maximum air humidity. Infected leaf areas were harvested 21 days post infection for further phenotypic analysis or prepared for pycnidia isolation by surface sterilization with 1.2% NaClO for 2 min followed by 70% ethanol for a few seconds and washed twice with H<sub>2</sub>O. Leaves were placed into a sterile environment with maximum air humidity and incubated for 7-14 days at plant growth conditions. Spores that had been pressed out from pycnidia were isolated using a sterile syringe needle and resuspended into 50  $\mu$ L YMS medium with 50% glycerol. Cells were suspended by 30 min vortexing on a VXR basic Vibrax (IKA, Staufen, Germany) at 2,000 rpm, plated on YMS agar, and incubated for seven days at 18°C. Colonies arising from single cells were PCR screened and their complement on accessory chromosomes characterized as described below.

### **Phenotypic characterization of infected leaves**

Harvested leaves were taped to a sheet of paper and scanned using a flatbed scanner at a resolution of 2,400 dpi. The scanned leaves were analyzed using ImageJ (Schneider *et al.*, 2012) and a plug-in described previously (Stewart *et al.*, 2016). The measurement of pycnidia/cm<sup>2</sup> was used for further statistical analyses in R (Ihaka & Gentleman, 1996). The Wilcoxon-Rank-Sum test and the Holm's correction for multiple testing were applied to assess statistical differences between the samples.

### ***In vitro* phenotype assay**

A spore solution containing  $10^7$  cells/mL and tenfold dilution series to 1,000 cells/mL was prepared. To test for responses to different stress conditions *in vitro*, YMS plates containing NaCl (0.5 M and 1 M), sorbitol (1 M and 1.5 M), Congo Red (300  $\mu$ g/mL and 500  $\mu$ g/ml), H<sub>2</sub>O<sub>2</sub> (1.5 mM and 2 mM), MMS (methyl methanesulfonate, 0.01 %), a H<sub>2</sub>O-agar plate and two plates containing only YMS were prepared. Three  $\mu$ L of the spore suspension dilutions were pipetted on the plates and incubated at 18°C for seven days. One of the YMS plates was incubated at 28°C to test for thermal stress responses.

### ***In vitro* growth assay**

YMS liquid cultures containing  $10^5$  cells/mL of either Zt09 or the chromosome-loss strains Zt09 $\Delta$ 14 and Zt09 $\Delta$ 21 were prepared. Three replicates per strain were used in each experiment. The spores were grown for four days at 18°C at 200 rpm in 25 mL YMS and the OD<sub>600</sub> was measured at different time points throughout the experiment. The data was fitted using the R package growthcurver (Sprouffske & Wagner, 2016) and the *r* values of each replicate used for statistical comparison. Wilcoxon rank-sum test was used to compare the *r* values of the different strains.

**Pulsed-field gel electrophoresis (PFGE) and Southern blotting**

Fungal strains were grown in YMS medium for five days. Cells were harvested by centrifugation for 10 min at 3,500 rpm.  $5 \times 10^8$  cells were used for plug preparation. The cells were resuspended in 1 mL H<sub>2</sub>O and mixed with 1 mL of 2.2 % low range ultra agarose (Bio-Rad, Munich, Germany). The mixture was pipetted into plug casting molds and cooled for 1 h at 4°C. The plugs were transferred to 50 mL screw cap Falcon tubes containing 5 mL of lysis buffer (1 % SDS; 0.45 M EDTA; 1.5 mg/ml proteinase K, Roth, Karlsruhe, Germany), and incubated in lysis buffer for 48 h at 55°C, replacing the buffer once after 24 h. Chromosomal plugs were washed three times for 20 min with 1 x TE buffer before storage in 5 mL of 0.5 M EDTA at 4°C. PFGE was performed with a CHEF-DR III pulsed field electrophoresis system (BioRad, Munich, Germany). To separate the small accessory chromosomes, the following settings were applied: switching time 50 s – 150 s, 5 V/cm, 120° angle, 1 % pulsed field agarose (BioRad, Munich, Germany) in 0.5 x TBE (TRIS/borate/EDTA) for 48 h. Separation of mid-size chromosomes was conducted with the settings: switching time 250 s – 1000 s, 3 V/cm, 106° angle, 1 % pulsed field agarose in 0.5 x TBE for 72 h. *Saccharomyces cerevisiae* chromosomal DNA (BioRad, Munich, Germany) was used as size marker for the accessory chromosomes, and *Hansenula wingei* chromosomal DNA (BioRad, Munich, Germany) for mid-size chromosomes. Gels were stained in ethidium bromide staining solution (1 µg/ml ethidium bromide in H<sub>2</sub>O) for 30 min. Detection of chromosomal bands was performed with the GelDoc™ XR+ system (Bio-Rad, Munich, Germany). Southern blotting was performed as described previously (Southern, 1975) using DIG-labeled probes generated with the PCR DIG labeling Mix (Roche, Mannheim, Germany) following the manufacturer's instructions.

**Sequencing and genome comparison**

DNA for whole genome sequencing was prepared as described in (Allen *et al.*, 2006). Library preparation with a reduced number of PCR cycles (four cycles) and sequencing were performed by the Max Planck Genome Center, Cologne, Germany (<http://mpgc.mpipz.mpg.de/home/>) using an Illumina HiSeq3000 machine, obtaining ~30x coverage with 150-nt paired-end reads. Raw reads were quality filtered using Trimmomatic and mapped to the reference genome of *Z. tritici* or *Z. ardabiliae* using bowtie2. SNP calling was conducted with samtools and bcftools and further quality filtered. The filtered SNPs were validated by manual inspection. The data was visualized in IGV (Integrative Genomics Viewer, <http://software.broadinstitute.org/software/igv/>) (Thorvaldsdóttir *et al.*, 2013). Detailed commands for each step of the data processing pipeline can be found in the Supplementary Text.

## Results

### Accessory chromosomes are lost at a very high rate *in vitro*

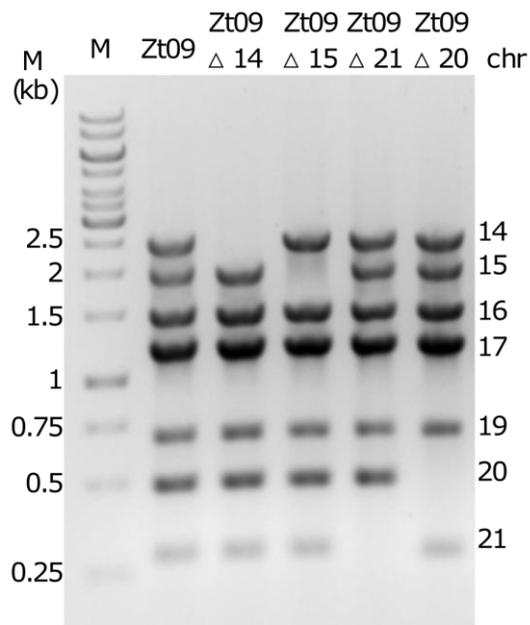
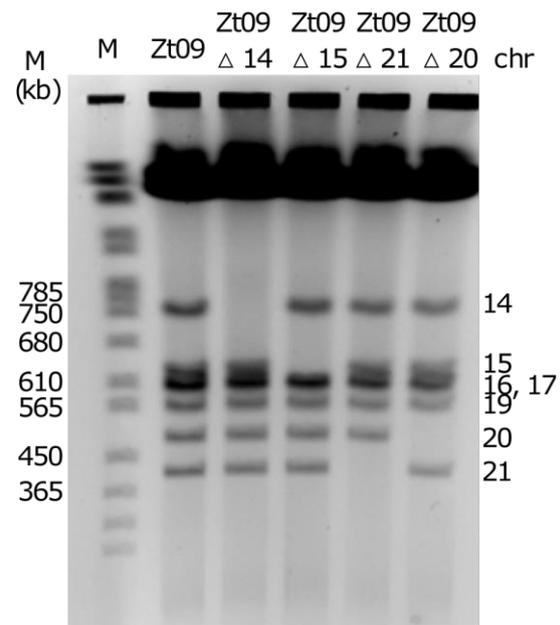
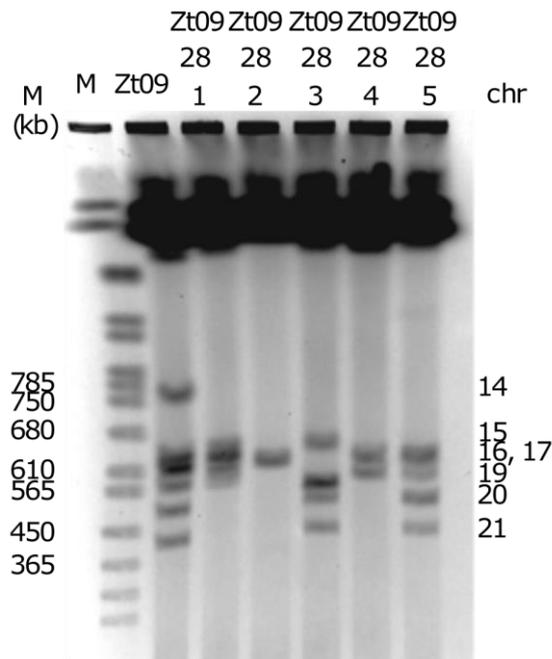
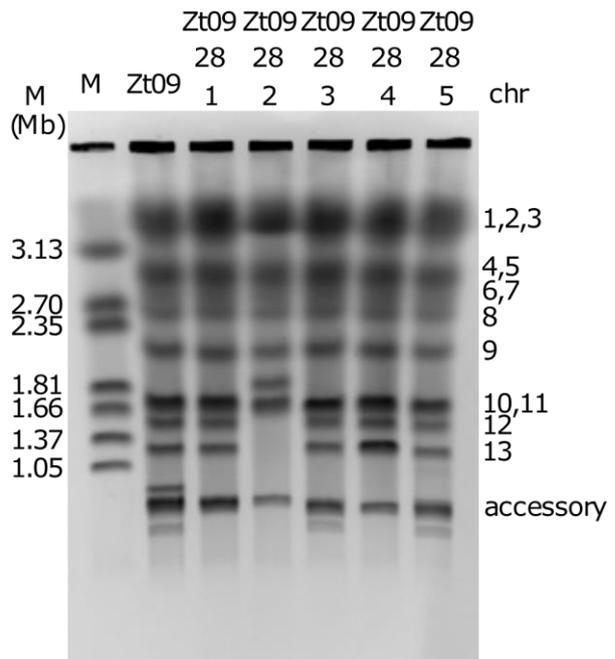
To assess the stability of accessory chromosomes in *Z. tritici* we conducted an *in vitro* long-term growth experiment. We used the *Z. tritici* isolate IPO323 $\Delta$ chr18, derived from the reference strain IPO323 for which there is a completely assembled genome (Goodwin *et al.*, 2011). IPO323 $\Delta$ chr18 lost chromosome 18 during previous *in vitro* propagation, and is here referred to as Zt09 (Kellner *et al.*, 2014).

We propagated fungal cells in liquid culture at 18°C, including eight transfers to fresh medium. After four weeks (representing ~80 cell divisions per cell), 576 single strains originating from three replicate cultures were tested for the presence of the seven accessory chromosomes from the Zt09 progenitor by a PCR assay. The presence of marker regions, located close to the centromere, and the right and left telomere repeats of each accessory chromosome, were tested (Table S1). When the screening by PCR suggested absence of an accessory chromosome, we further validated the result by electrophoretic separation of the fungal chromosomes by pulsed field gel electrophoresis (PFGE) (Figure 1 A and B). Thirty eight of the 576 (~7 %) tested strains lacked one accessory chromosome. We did not find strains that lacked more than one chromosome, but observe a clear trend where accessory chromosomes 14, 15 and 16 were lost more frequently than smaller accessory chromosomes (Table 1). For example, chromosome 14 was absent in eighteen strains, chromosome 15 in eight and 16 in nine strains. The small chromosomes 20 and 21 were lost only in two and one strain, respectively, whereas chromosomes 17 and 19 were never absent following the *in vitro* propagation (Table 1 and Table S2).

**Table 1: Summary of chromosome losses in *Z. tritici* during evolution experiments *in vitro* and *in planta*.** Listed are the number of strains that lost an accessory chromosome and which accessory chromosomes were lost. Sizes of the accessory chromosomes are listed next to the chromosome number. Strains with more than one chromosome lost are listed in brackets.

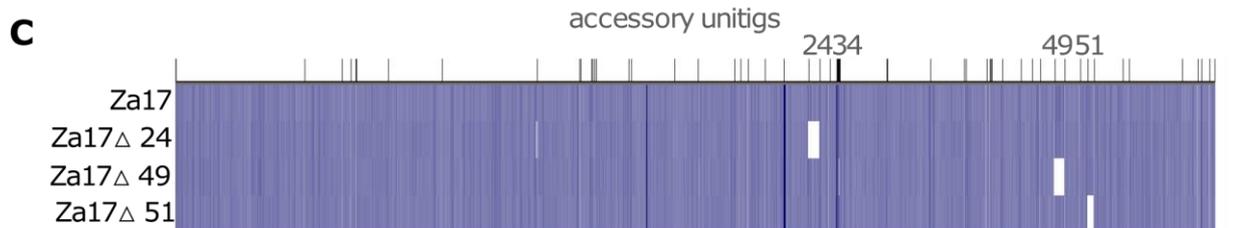
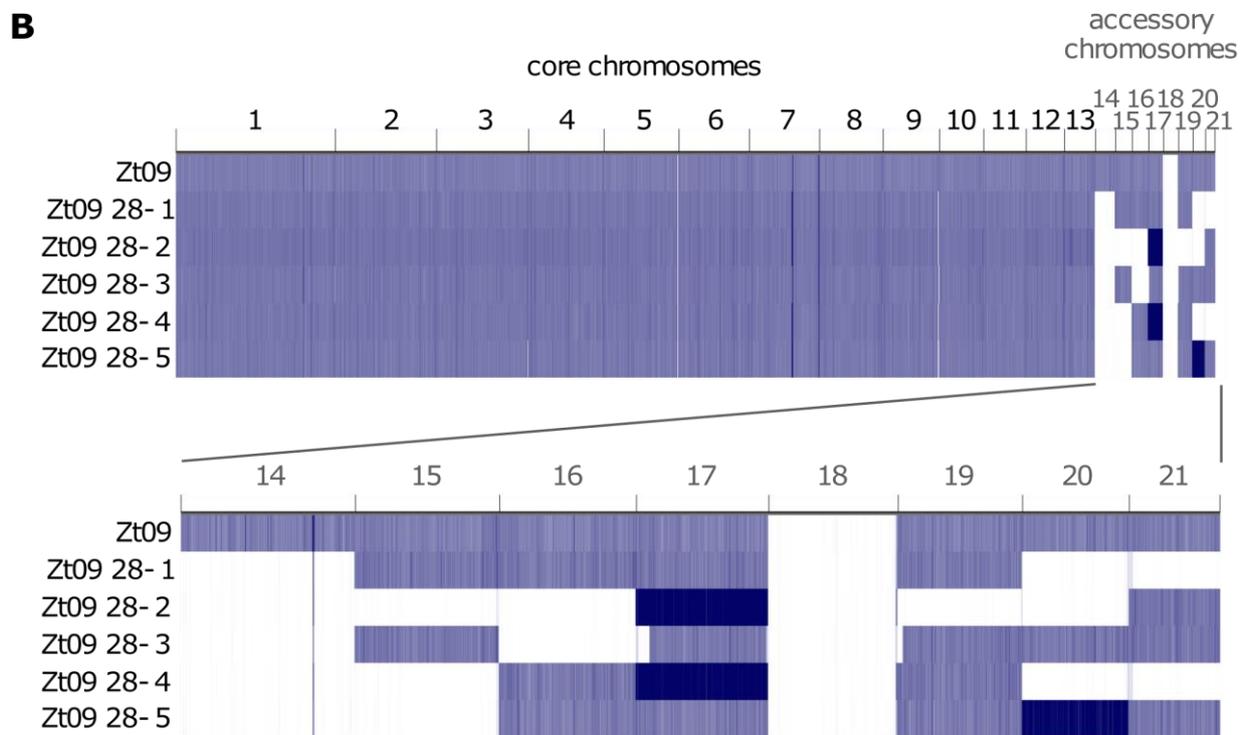
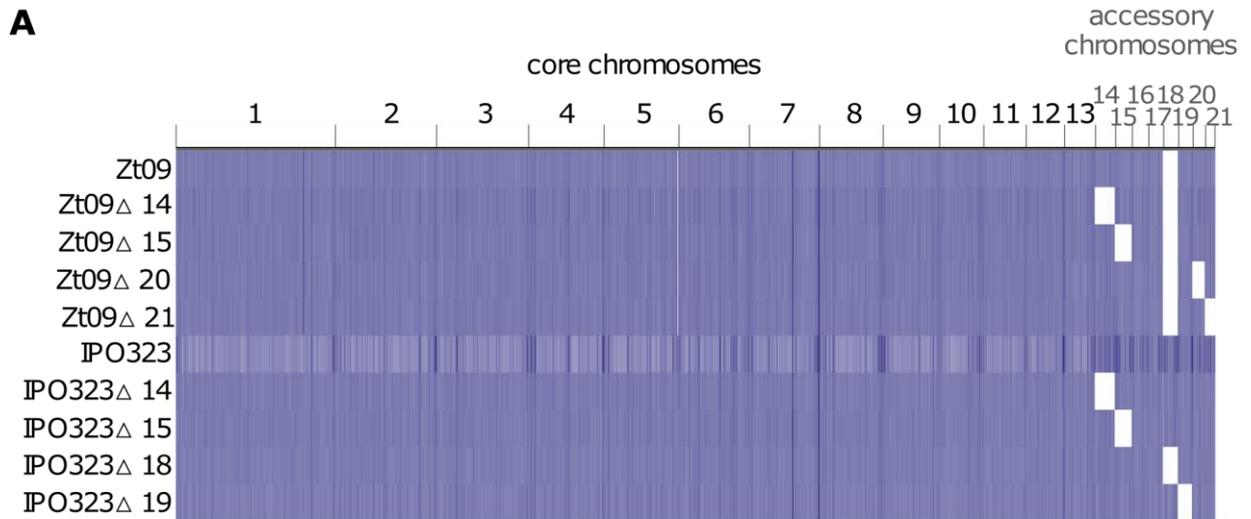
Chromosome (kb)	Zt09 <i>in vitro</i> culture	Zt09 <i>in vitro</i> plate	IPO323 <i>in planta</i>	Zt09 <i>in vitro</i> temperature stress
14 (773)	18	0	1	108 (33)
15 (639)	8	0	6	2 (8)
16 (607)	9	4	1	0 (9)
17 (584)	0	0	0	0
18 (574)	N/A	N/A	7	N/A
19 (550)	0	0	2	2 (2)
20 (472)	2	0	0	1 (17)
21 (409)	1	1	0	1 (7)
<b>Total chr loss strains</b>	<b>38</b>	<b>5</b>	<b>17</b>	<b>114 (34)</b>
<b>Total strains tested</b>	<b>576</b>	<b>40</b>	<b>986</b>	<b>188</b>

We considered the possibility that natural selection acted on a large population of cells in the liquid *Z. tritici* cultures and thereby contributed to the observed non-random chromosome losses. To test this, we designed a second evolution experiment to propagate individual cell lineages in the absence of selection. For this experiment, 40 strains originating from one common progenitor (Zt09) were cultivated on plates. Every week, randomly selected colonies derived from single cells were transferred to a fresh plate. These repeated strong bottlenecks allowed us to propagate single lineages of *Z. tritici* without an effect of natural selection (Lynch *et al.*, 2008). After four weeks of growth (including four transfers to new plates), the 40 evolved strains were tested for the presence of accessory chromosomes as described above. Five out of 40 (~13 %) strains, twice as many as in the previous experiment, lacked an accessory chromosome suggesting that selection in a larger population of cells indeed had removed some of the spontaneously occurring chromosome losses. Interestingly, in this experiment we observe the loss of only two different chromosomes: Chromosome 16 was lost in four strains, and chromosome 21 in one strain. Our two *in vitro* experiments suggest that chromosome loss is not entirely random, and that specific chromosomes are lost at a higher rate (Table 1), depending on the *in vitro* growth conditions. The frequency of chromosome losses during asexual growth *in vitro* is extremely high in *Z. tritici* and exceeds previously reported spontaneous chromosome losses in *F. oxysporum* f. sp. *lycopersici* by far (Vlaardingerbroek *et al.*, 2016).

**A PCR screening****B Pulsed-field gel electrophoresis****C Pulsed-field gel electrophoresis****D Pulsed-field gel electrophoresis**

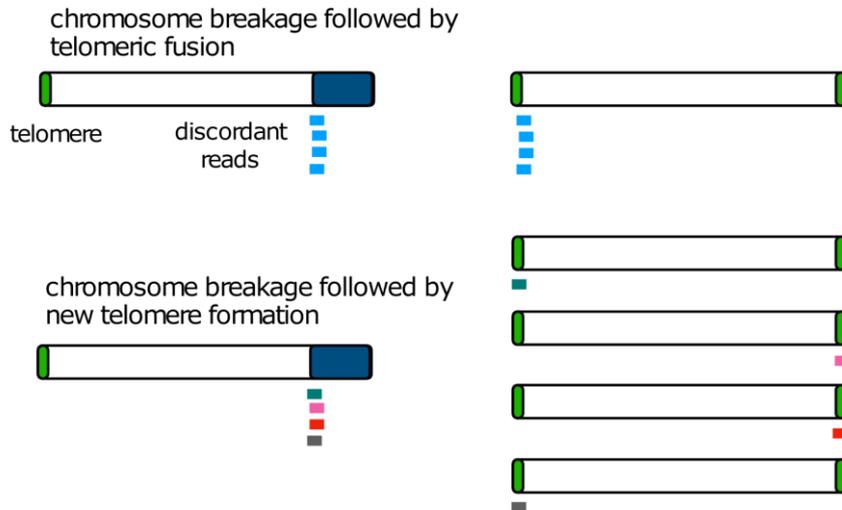
**Figure 1: Screening by PCR and pulsed-field gel electrophoresis to identify accessory chromosome-loss strains in *Z. tritici*.** (A) Screening by PCR of the progenitor Zt09 strain and evolved strains to assess chromosome losses using primer pairs located close to the centromeric region of the respective accessory chromosome. Multiplex PCR was used to simultaneously screen for the presence or absence of all accessory chromosomes in one strain. Absence of a PCR product (black arrows) indicates the absence of the chromosome; chromosome 18 is not present in Zt09. (B) Pulsed-field gel electrophoresis (PFGE) was conducted to validate the absence of accessory chromosomes in chromosome loss candidates identified by the initial PCR. Here, the separation of small accessory chromosomes of the progenitor strain Zt09 and four chromosome-loss strains is shown. The corresponding chromosome to each band is indicated on the right. The absence of chromosomal bands (black arrows) confirms the loss of the respective chromosome; chromosome 18 is similar in length to 16 and 17 but is absent in Zt09. PFGE of the accessory (C) and mid-size (D) chromosomes of strains originating from the *in vitro* 110

temperature stress experiment confirms multiple accessory chromosome losses, size alterations and chromosome fusions (see also Figures 3 and S1 and Table S3). The bands for chromosomes 3, 12, 13 and 21 are absent in strain 28-2, however, genome sequencing confirms that the chromosomes are still present in the genome (Figure 2C). The absence of bands on the PFGE can be explained by chromosome size changes whereby chromosomes 3 and 13 and 12 and 21 have experienced chromosome fusions (Figure 3 and S1). Note a new chromosome band with a size of ~1.8 Mb resulting from the fusion of chromosomes 12 and 21. In the strain 28-3, the accessory chromosomes 17 and 19 are shorter than the reference chromosomes due to chromosome breakage (Figure 2C and Table S3). Strain 28-4 has an additional band of ~1.2 Mb, representing the fusion of a duplicated chromosome 17 (Figure S1). All images of stained gels are color-inverted to make differences more obvious.



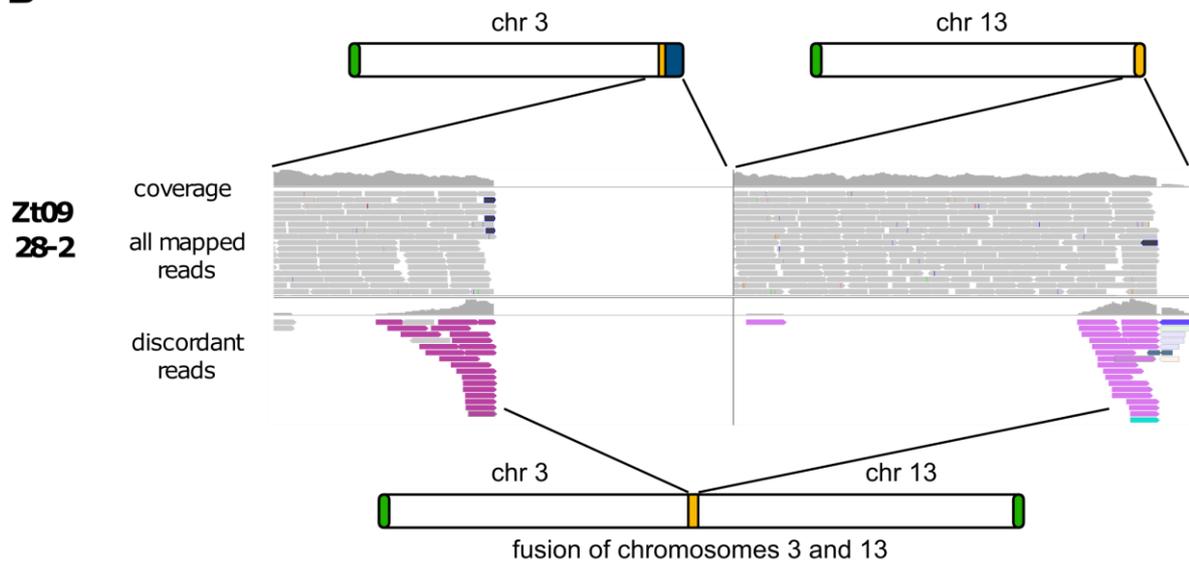
**Figure 2: Whole genome sequencing confirms loss of accessory chromosomes in *Z. tritici* and *Z. ardabiliae*.** The genomes of chromosome-loss strains derived from the *Z. tritici* *in vitro* evolution experiment and the *in planta* pycnidiospore isolations, and the *Z. ardabiliae* *in vitro* evolution experiment and the respective progenitor strains were sequenced by paired-end Illumina sequencing and mapped to the respective reference isolates, IPO323 (*Z. tritici*) A and B) or Za17 C) (*Z. ardabiliae*). The losses of entire chromosomes (white boxes) were verified for each strain, but few mutations in form of SNPs, INDELS or copy number variation were detected (Table S4). The *Z. tritici* reference genome consists of whole chromosomes, while the *Z. ardabiliae* reference genome is composed of unitigs obtained from the SMRT sequencing assembly (ordered by name of the unitig). The read depth is here normalized to 1 X per strain. B) Genome sequencing of strains derived from the temperature stress experiment revealed, besides verification of accessory chromosome losses, chromosome breaks at the ends of core and accessory chromosomes and accessory chromosome duplications. A zoom in on the coverage of accessory chromosomes highlights the duplications of chromosomes 17 (Zt09-28-2, Zt09-28-4) and 20 (Zt09-28-5), and the chromosome breakage of chromosomes 17 and 19 (Zt09-28-3). Darker blue shading indicates higher coverage. Most repetitive sequences have a higher coverage than single copy regions resulting in different shades of blue in the coverage graph. The intense dark blue lines indicate high coverage regions on rDNA clusters or repetitive DNA due to underestimation of repeats in the reference assembly (for example, the thin black line in chromosome 7 indicates the location of several rDNA cluster repeats, the only such repeats in the annotated genome sequence, but the expected true number of rDNA repeats is ~ 50).

**A**



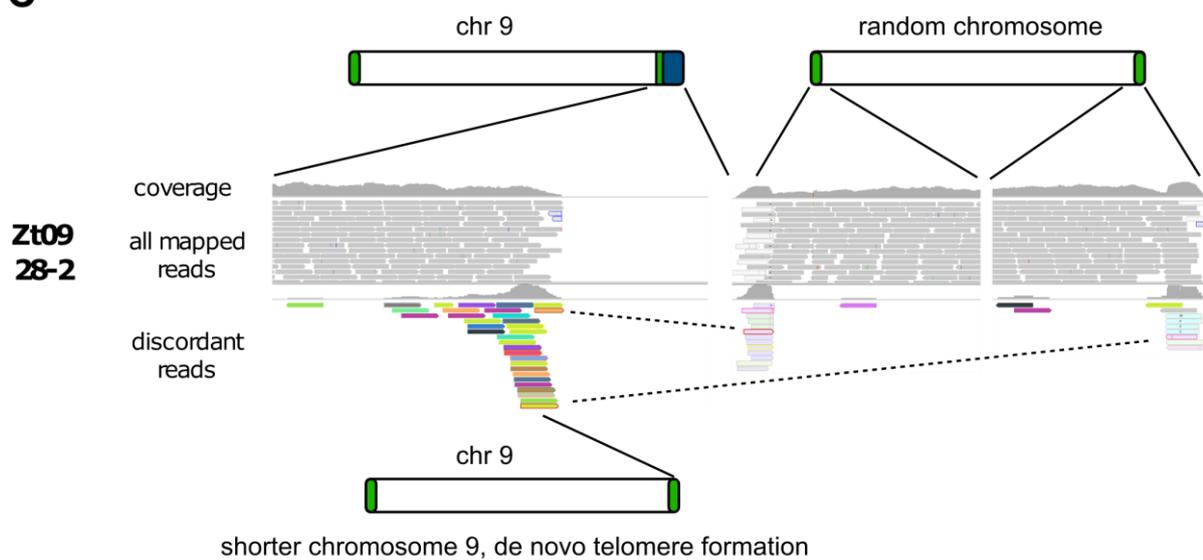
**B**

**chromosome breakage of chr 3 followed by telomeric fusion to chr 13**



**C**

**chromosome breakage of chr 9 followed by new telomere formation**



■ deletion    ■ fusion    ■ telomere

**Figure 3: Discordant read analysis reveals chromosome fusion and chromosome breakage.** A) Schematic illustration of discordant read mapping in the case of chromosome breakage and fusion. If chromosome breakage is followed by fusion to a different chromosome, the discordantly mapping reads at the breakpoint will have their respective read mate on the chromosome that is fused to the breakpoint. If breakage is followed by *de novo* telomere formation, the discordant reads have their respective read mates on telomeric repeats on random chromosomes. B and C) Read mapping to the genome of the reference isolate IPO323. B) In the experimentally evolved strains of *Z. tritici* at 28°C, the fusion of chromosomes 3 and 13 in the strain Zt09 28-2 is indicated by the increased occurrence of discordant reads at the chromosome breakpoint of the right arm of chromosome 3 and the right arm of chromosome 13. The color of the reads represents the chromosome their respective mate is mapping to. C) New telomere formation at the breakpoint, here, as example, shown for chromosome 9 in the strain Zt09 28-2. Telomere formation is indicated by a high number of discordant reads, where the read mates are mapping to the telomeric repeats of different random chromosomes (dashed line). A total of twelve breakage events that were followed by *de novo* telomere formation were detected in the five analyzed strains derived from the experiment at elevated temperature.

### Accessory chromosomes are unstable *in planta*

The growth of *Z. tritici* in rich medium *in vitro* is highly distinct from growth of the fungus in its natural environment. *Zymoseptoria tritici* is a hemi-biotrophic pathogen infecting the mesophyll of wheat leaves (Ponomarenko *et al.*, 2011; Goodwin *et al.*, 2011), where it forms asexual fruiting bodies, pycnidia, containing presumably clonal conidiospores (pycnidiospores). Pycnidia develop in the sub-stomatal cavities, where the environment and nutrient availability differ from our tested *in vitro* conditions (Rudd *et al.*, 2015; Haueisen *et al.*, 2017). To test whether chromosome loss occurs only *in vitro* or also during the natural lifecycle of *Z. tritici*, we assessed the loss of accessory chromosomes *in planta*. We collected pycnidia from wheat leaves infected with the *Z. tritici* strain IPO323 and tested single pycnidiospores for the presence of accessory chromosomes. A total number of 968 pycnidiospores originating from 42 separate pycnidia were screened by PCR. In total, 17 strains missing an accessory chromosome were identified (~1.7%). Chromosomes 15 and 18 were the most frequently lost chromosomes, while chromosomes 17, 20 and 21 were not lost in any of the strains (Table 1 and Table S2). As for the *in vitro* experiments, no strains lacking more than one chromosome were found. Interestingly, chromosome 18, which was found to be lost at high rates in our plant experiments, was shown to be frequently absent in field isolates of *Z. tritici* (Croll *et al.*, 2013; McDonald *et al.*, 2016). In summary, our findings show that chromosome loss in *Z. tritici* not only results from the non-disjunction of homologous chromosomes during meiosis II, as proposed previously (Wittenberg *et al.*, 2009), but also from frequent chromosome losses during asexual growth and mitotic spore formation *in planta*. The observed frequency of chromosome losses *in planta* is not as high as during *in vitro* growth, however, the number of mitotic divisions to develop pycnidia is presumably lower than during four weeks of *in vitro* growth. Therefore, we propose that the reduced number of chromosome losses *in planta* rather reflects the reduced number of cell divisions than possible fitness effects of chromosome losses.

**Chromosome instability is greatly increased during exposure to heat stress**

In their natural environments, pathogens are exposed to various kinds of biotic and abiotic stresses. For example, the local environment on the leaf surface can fluctuate severely in temperature and humidity conditions (Zhan & McDonald, 2011). We assessed the impact of an increase in temperature on chromosome stability of *Z. tritici*. To this end, we cultivated Zt09 *in vitro* at elevated temperatures, namely an increase of ten degrees Celsius from 18° to 28°C during four weeks of incubation. Subsequent screening by PCR for accessory chromosomes revealed severe chromosome losses in the tested strains. Out of 188 evolved and tested strains, 148 (~80 %) were missing at least one accessory chromosome. Chromosome 14 was the most frequently lost chromosome and 34 evolved strains were lacking more than one chromosome. The maximum number of missing chromosomes was six in one strain (Table 1 and Table S2). PFGE revealed that karyotype alterations were not restricted to simple chromosome loss as observed in the *in vitro* experiments at 18°C and the *in planta* experiments. In contrast, we observed frequent size variation of both core and accessory chromosomes as a consequence of chromosome breakage, fusions and duplications (see below) (Figure 1C and 1D, Figure S1, Table S3).

**Genome sequencing of chromosome-loss strains reveals chromosome breakage and fusion, but a low number of SNPs**

In total, we sequenced 19 genomes of strains that had lost different chromosomes or displayed rearrangements of chromosomes as revealed by PFGE. The strains represented derived strains from the different *in vitro* and the *in planta* experiment and the respective progenitor strains; all sequencing results have been deposited in the Sequence Read Archive (SRA) under BioProject ID PRJNA428438. Overall, we confirmed the absence of complete accessory chromosomes (Figure 2A), and we found very few additional changes. After filtering to exclude reads of poor quality and coverage (see Materials and Methods, Supplementary Text) we identified a total number of nine SNPs in eight sequenced strains originating from the *in vitro* experiment in liquid culture at 18°C and the *in planta* experiment. Of these, three SNPs were located in coding regions (Table S4). Besides the complete loss of accessory chromosomes and the identified SNPs, we found no other mutations when comparing the progenitor and evolved strains. However, we identified SNPs distinguishing Zt09 from our IPO323 reference strain, and the published genome sequence; most of these SNPs were in non-coding sequences (Table S4). As we show chromosome losses in different strains, we conclude that the few point mutations do not have measurable impact on chromosome loss.

Whole genome sequencing of five strains evolved at 28°C verified the losses of several accessory chromosomes. Furthermore, comparison of the re-sequenced genomes of the progenitor and the evolved strains revealed substantial size variation resulting from chromosome breakage of core and accessory chromosomes, and duplications of accessory chromosomes (Figure 2B). Chromosome breaks located close to the ends of chromosomes resulted in shortened chromosomes caused by subtelomeric deletions of ~0.2-60 kb (Table S3). Detailed analyses of the distribution of discordant paired-end reads mapping to different chromosomes revealed a high number of reads at the chromosome breakpoints with the

respective read mates mapping to telomeric repeats (Figure 3). This suggested that most chromosome breaks resulted in shorter chromosomes to which telomeres were added *de novo*. Besides *de novo* telomere formation, we found evidence for fusion of core chromosomes in strain Zt09 28-2, where discordant read mapping indicated fusion of the right arms of chromosomes 3 and 13 (Table S3 and Figure 3). PFGE and Southern blots further showed fusion of chromosomes 12 and 21 in Zt09 28-2 (Figure S1). In a previous study, we reported evidence for the likely fusion of an ancestral accessory chromosome and core chromosome 7 in the reference isolate IPO323 and Zt09 (Kellner *et al.*, 2014; Schotanus *et al.*, 2015). Here, we deduced a fusion of the duplicated chromosome 17 in strain Zt09 28-4 (Figure 1C and D, Figure S1). Similar fusions of the same accessory chromosome 17 following meiosis had been suggested previously, and “breakage-fusion bridge” cycles (McClintock, 1938, 1941) were invoked as a mechanism to form a new accessory chromosome (Croll *et al.*, 2013). Based on the position of centromeres in *Z. tritici* (Schotanus *et al.*, 2015), all three chromosome fusion events that we detected, resulted in the formation of dicentric chromosomes. On two chromosomes involved in the fusion events, we identified the “internal” breakpoints (Table S3) likely reflecting the consequences of chromosome breakage of a dicentric chromosome during mitosis. This indicates that breakage-fusion bridge cycles also occur during mitosis in *Z. tritici* and can be a mechanism involved in the structural variation detected in our experiments. In total, in the five analyzed genomes derived from strains grown at 28°C, we identified spontaneous changes: (1) fifteen chromosome breakages (twelve with *de novo* telomere formation and three without evidence for new telomeres), (2) three chromosome fusions, (3) three chromosome duplications, and (4) 16 chromosome losses (Table S3). The breakpoints of the structural rearrangements often co-localize with annotated transposons, predominantly class I elements (Table S5). Transposable elements are often associated with structural rearrangements (Faino *et al.*, 2016; Plissonneau & Stürchler, 2016) and their activation under stress conditions might contribute to the observed genome instability (Chadha & Sharma, 2014). A key observation from our genome data analyses is the frequent involvement of *de novo* telomere formation and chromosomal fusion as a mechanism to heal broken chromosome ends. While this has been reported for cancer cells (Murnane, 2012), it is generally considered a rare event in normal, non-transformed cells. Here we show that *de novo* telomere formation and chromosome fusion readily occur in a filamentous fungus during growth at temperature stress.

### **Accessory chromosome instability also occurs in *Zymoseptoria* sister species**

To assess whether the high frequency of mitotic chromosome loss is specific to *Z. tritici*, we conducted a short-term *in vitro* evolution experiment on the fungus *Z. ardabiliae*, a closely related sister species of *Z. tritici* that infects wild grasses. We used the previously characterized isolate STIR04 1.1.1 (Stukenbrock *et al.*, 2011), here called Za17, in our experiments.

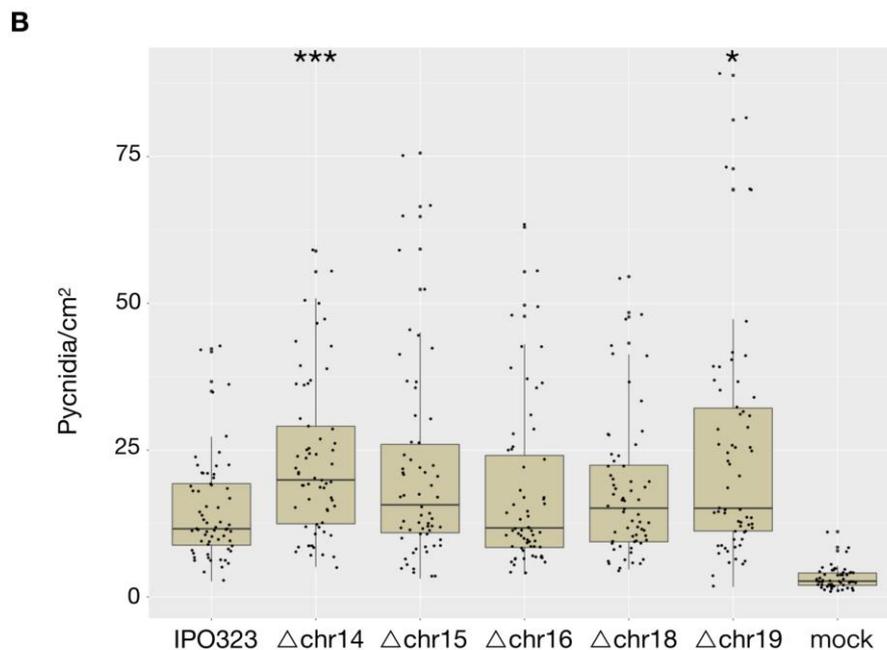
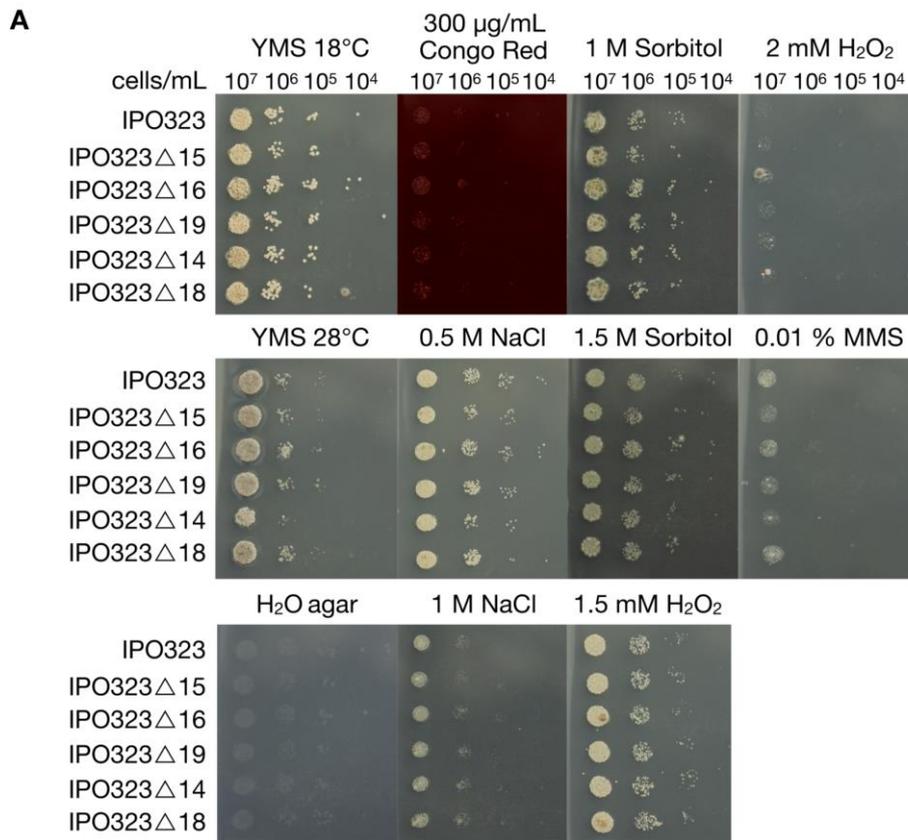
We first generated a high-quality reference genome based on long-read SMRT sequencing (Supplementary Text). Analyses of a population genomic dataset revealed at least four accessory chromosomes varying in their frequency among 17 *Z. ardabiliae* isolates (Stukenbrock *et al.* 2011; Stukenbrock & Dutheil 2018). Based on PFGE and homology of *Z. ardabiliae* unitigs to fragments of *Z. tritici* IPO323 accessory chromosomes (Soderlund *et al.*,

2011), we designed primers to amplify six loci located on putative accessory chromosomes of Za17 (Table S1).

Next, we conducted an *in vitro* evolution experiment at 18°C for four weeks in liquid culture with Za17 as the progenitor strain. Screening of 288 single clones by PCR identified five strains lacking one of the accessory chromosomes (~1.7%). We further verified the absence of accessory chromosomes by PFGE for all five strains and whole genome sequencing for three of the five *Z. ardabiliae* strains (Figure 2C). By combining PFGE and genome sequencing of chromosome loss strains we could confirm unitigs 24, 34, 49 and 51 to be accessory sequences pertaining to three accessory chromosomes (Figure 2C). These findings resemble the rapid loss of chromosomes in *Z. tritici* and suggest a common phenomenon, most likely related to mitotic cell division in the two *Zymoseptoria* species.

### **Accessory chromosome losses affect *in planta* phenotypes**

We next addressed the impact of spontaneous chromosome loss by comparing the fitness of the progenitor and evolved *Z. tritici* strains under different *in vitro* growth conditions, and during infection of a susceptible wheat variety by comparing growth and pycnidia formation. *In vitro*, the growth rate of strains lacking an additional accessory chromosome (Zt09Δ14 and Zt09Δ21) was comparable to the growth rate of the progenitor, Zt09, but with a slight tendency to slower growth of the derived chromosome-loss strains (Figure S2). For a more detailed phenotypic characterization, we used the five chromosome-loss strains isolated from pycnidia from the *in planta* experiment (Table S2). Several stress conditions including osmotic, oxidative, temperature, and cell wall stresses were tested *in vitro*. We compared the fungal phenotypes of the chromosome-loss strains and the progenitor strain IPO323 and observed no difference in growth rate or colony morphology between any chromosome-loss strain and IPO323 (Figure 4A). *In planta*, however, we observed phenotypic differences between the evolved chromosome-loss strains and their progenitor. We measured fitness by counting the number of asexual fruiting bodies formed in stomata on infected wheat leaves and found a slightly higher fitness of the chromosome-loss strains compared to the progenitor strain (Figure 4B). The difference was most pronounced for the strains lacking chromosome 14 and chromosome 19 where the density of pycnidia was found to be significantly higher (*p* values of 0.0003 and 0.015, Wilcoxon-Rank-Sum test and Holm's correction for multiple testing) compared to the IPO323 progenitor (Figure 4B).



**Figure 4: *In vitro* and *in planta* phenotype assays of the *Z. tritici* reference isolate IPO323 and the *in planta* chromosome-loss strains. A)** Several *in vitro* stress conditions were tested to assess the effect of accessory chromosome losses on fitness. Five *in planta* chromosome-loss strains were tested, IPO323 was used as the reference strain. We observed no noteworthy differences in growth rate or colony morphology between reference and chromosome-loss strains. B) To investigate the effect of accessory chromosome losses on *Z. tritici* infection of wheat, three independent experiments with the reference

strain IPO323 and five chromosome-loss strains were conducted. The fitness of each strain was measured by counting the number of pycnidia per cm<sup>2</sup> on the leaf surface. Statistical analysis (Wilcoxon-rank-sum test and Holms correction, p-values 0.0003 and 0.015) identified two chromosome-loss strains (Chr. 14 and 19) that show a significantly higher number of pycnidia compared to the reference strain IPO323 under the conditions used in this experiment.

## Discussion

### Consequences for evolution of accessory chromosomes

Accessory chromosomes were proposed to serve as an 'evolutionary cradle' for creating novel virulence genes without risking the disruption of essential genes on the core chromosomes (Croll & McDonald, 2012). Indeed, signatures for accelerated evolution were shown by overall higher dN/dS ratios of genes on the accessory chromosomes compared to the core chromosomes (Stukenbrock *et al.*, 2010). However, natural selection acts at the level of individuals and cannot maintain "a playground" for future beneficial effects. Rather, we hypothesize that the dynamic of accessory chromosome loss reflects a dynamic in the selective environment of *Z. tritici*, where under certain conditions these small chromosomes confer a fitness advantage, while under different conditions they cause decrease in fitness. A fitness advantage of chromosome-loss strains *in planta* may indicate the presence of avirulence factors on at least some accessory chromosomes. Avirulence factors can be recognized by the host to induce a resistance response resulting in reduction in virulence or even abortion of infection (Petit-Houdenot & Fudal, 2017; Zhong *et al.*, 2017). Our previous findings support this notion, as we found small but significant negative effects of accessory chromosomes during host infection (Habig *et al.*, 2017). In contrast, Fouché and colleagues (Fouché *et al.*, 2018) detected small, quantitative increases of virulence traits if specific accessory chromosomes were present. However, so far no avirulence factors and very few potential effectors were identified on accessory chromosomes in *Z. tritici* and the vast majority of annotated genes on accessory chromosomes do not have a predicted function (Goodwin *et al.*, 2011; Grandaubert *et al.*, 2015). In other pathogenic fungi, however, the opposite effect has been demonstrated in several cases. A strong positive impact on virulence of accessory chromosomes have been observed in *Fusarium oxysporum* f. spp. and *Nectria haematococca* (Coleman *et al.*, 2009; Ma *et al.*, 2010; van Dam *et al.*, 2018). In these pathogens chromosome losses resulted in complete loss of pathogenicity. While these cases are currently considered the norm, our observations show that responses to accessory chromosome loss can be more varied. Even though this fungus is an important pathogen in all wheat-growing countries, the biology of *Z. tritici* is still not completely understood. Mating and overwintering in the soil or litter layer remain largely unknown lifecycle stages, and we postulate that they impose different selection pressures on the fungus. During these stages, accessory chromosomes may confer significant fitness advantages that have not been demonstrated yet. Nevertheless, any effects on fitness found to date are rather small. While beneficial effects, even if they are comparably small, might explain the maintenance of accessory chromosomes in *Z. tritici* populations, negative effects as observed in this study and reported by Habig *et al.* (Habig *et al.*, 2017) raise the question why these chromosomes have not been lost over time, especially as they can easily be lost during

meiosis and mitosis. Accessory chromosome can be lost during meiosis (Wittenberg *et al.*, 2009), but interestingly accessory chromosomes have also been shown to follow non-Mendelian segregation and to be transmitted at a significantly higher rate than expected, if one of the two mating partners lacks an accessory chromosome (Fouché *et al.*, 2018, Habig *et al.*, submitted). While there is no comprehensive mechanistic explanation yet, chromosome conservation by re-replication during meiosis may counteract the chromosome loss during vegetative growth and therefore maintain accessory chromosomes in pathogen populations. In *Alternaria alternata*, the spontaneous loss of a conditionally dispensable chromosome during sub-culturing has been described for one isolate (Johnson *et al.*, 2001). Furthermore, loss of a conditionally dispensable chromosome after meiosis has been observed in *Nectria haematococca* (aka *Fusarium solani* MPVI) (Miao *et al.*, 1991b). A recent study of the plant pathogenic fungus *Fusarium oxysporum* forma specialis *lycopersici* describes the spontaneous loss of dispensable chromosomes *in vitro* in one cell out of 35,000 (Vlaardingerbroek *et al.*, 2016). This rate is much lower than the chromosome loss rate that we observe in *Z. tritici* and *Z. ardabiliae*, where, depending on the growth conditions, between ~ 2 and ~ 80 % of the tested strains, lack an accessory chromosome. In combination, these results indicate that chromosome instability is a common phenomenon in fungi, but the stability of chromosomes can vary substantially between different species. Studies in *Saccharomyces* and *Candida* species have shown that chromosome loss resulting in aneuploidy is a relatively frequent phenomenon in diploid cells and is advantageous under certain conditions (Selmecki *et al.*, 2010; Kumaran *et al.*, 2013; Bennett *et al.*, 2014; Zhu *et al.*, 2014). The loss of chromosomes in haploid organisms, such as *Z. tritici*, is expected to have more severe consequences, as genetic information is lost from the cell. We considered, however, that the effect of rapid chromosome loss or instability may be advantageous if these traits are selected for in pathogens like *Zymoseptoria*.

The temperature stress experiment *in vitro* showed that genome instability in *Z. tritici*, at this temperature, is not restricted to the accessory chromosomes, but also involves the core genome. New chromosome formation induced by telomere to telomere fusion followed by breakage of the dicentric chromosome was reported in *Cryptococcus neoformans* (Fraser *et al.*, 2005) but likely occurred during meiosis rather than mitosis. In the asexual *Candida glabrata*, clinical isolates frequently inhabit chromosomal rearrangements indicating that structural variation acts as a virulence mechanism (Poláková *et al.*, 2009) and might be a response to stresses such as antifungal treatment or elevated temperature as observed here. The unstable regions close to the chromosome ends on the core chromosomes, however, show structural characteristics similar to the accessory chromosomes, such as higher repeat content, lower gene density and specific histone modification patterns (Dhillon *et al.*, 2014; Grandaubert *et al.*, 2015; Schotanus *et al.*, 2015).

The accessory chromosomes of *Z. tritici* do not differ in terms of centromere or telomere organization from the core chromosomes, but the chromatin is largely transcriptionally inactive and potentially more condensed due to an enrichment with the histone H3 that is trimethylated at lysine 27 (H3K27me<sub>3</sub>), which covers almost all of the accessory chromosomes (Schotanus *et al.*, 2015). Enrichment of the facultative heterochromatin mark H3K27me<sub>3</sub> seems to be a

common feature of accessory chromosomes in several fungal pathogens but the functional relevance for stability and transcriptional regulation of these chromosomes has not been addressed so far (Galazka & Freitag, 2014; Seidl *et al.*, 2016). H3K27me3 is also enriched in subtelomeric regions of core chromosomes, that we show here as prone to instability. High-resolution microscopic analyses indicate an unusual localization of centromeres in the *Z. tritici* nuclei, suggesting a distinct spatial organization of different chromosomes (Schotanus *et al.*, 2015). Heterochromatin has been found close to the nuclear periphery, and H3K27me3 has been shown to be involved in facilitating lamina-proximal positioning (Harr *et al.*, 2015). We hypothesize that the heterochromatic structure reflects a distinct physical organization of core and accessory chromosomes and likely the subtelomeric regions in the nucleus of *Z. tritici*, and that this is correlated to the instability of heterochromatic regions and chromosomes. A Hi-C study on *Neurospora crassa* showed that absence of H3K27me3 resulted in movement of subtelomeric regions and centromeres from the nuclear periphery into the nuclear matrix (Klocko *et al.*, 2016). Further experiments focusing on localization of specific *Zymoseptoria* chromosomes in the nucleus by cytology or Hi-C (Galazka *et al.*, 2016) should be conducted to address this hypothesis.

In conclusion, using experimental evolution, electrophoretic karyotyping and genome sequencing we showed that the accessory chromosomes of *Z. tritici* are highly unstable during asexual growth across numerous mitotic cell divisions (“mitotic growth”) *in vitro* as well as *in planta*. Surprisingly, increasing the temperature from 18 to 28°C dramatically increased overall genome instability in *Z. tritici*. Besides chromosome losses we observed structural variation in form of chromosome breakage, duplication, and fusion involving both core and accessory chromosomes, and all events were increased near telomeric sequences. In *Z. tritici*, all of these chromosome abnormalities have thus far been associated with meiosis (Wittenberg *et al.*, 2009; Croll *et al.*, 2013), however, our study highlights an important role of mitotic growth in generating genetic diversity. The variability in chromosome number and structure obtained over a relatively short period of time (during one infection event, or four weeks of vegetative growth) correlates with the genomic diversity observed in field populations of this important plant pathogen (McDonald & Martinez, 1991; Linde *et al.*, 2002; Zhan *et al.*, 2003; Mehrabi *et al.*, 2007). Our findings demonstrate that even sexual fungal pathogens can accelerate the generation of new genetic diversity by mitosis-associated structural variation. The mitotic events responsible for these rearrangements are still to be characterized.

Lastly, chromosome instability has been observed in various organisms and is a frequent phenomenon in cancer cells (McGranahan *et al.*, 2012). Epigenetic factors and heterochromatin in particular have been shown to impact chromosome stability in human cancer cells (Slee *et al.*, 2012). The accessory chromosome dynamics correlating with distinct chromatin patterns suggest that *Z. tritici* may provide a new model for mechanistic studies of chromosome instability in cancer cells.

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## Competing interests

The authors declare no competing interests.

## Availability of Data and Materials

The sequencing data generated for this project were deposited in the Sequence Read Archive under BioProject ID PRJNA428438.

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## Supplement Tables and Figures

**Table S1:** List of primers used for screening for accessory chromosomes in *Z. tritici* and *Z. ardabiliae*.

See attached Word file

**Table S2:** List of chromosome-loss strains identified in the *in vitro* and *in planta* experiments.

See attached Word file

**Table S3:** Chromosome variation of sequenced Zt09-derived strains grown at 28°C.

See attached Word file

**Table S4:** Location and annotation of SNPs and INDELS found in the sequenced chromosome-loss strains.

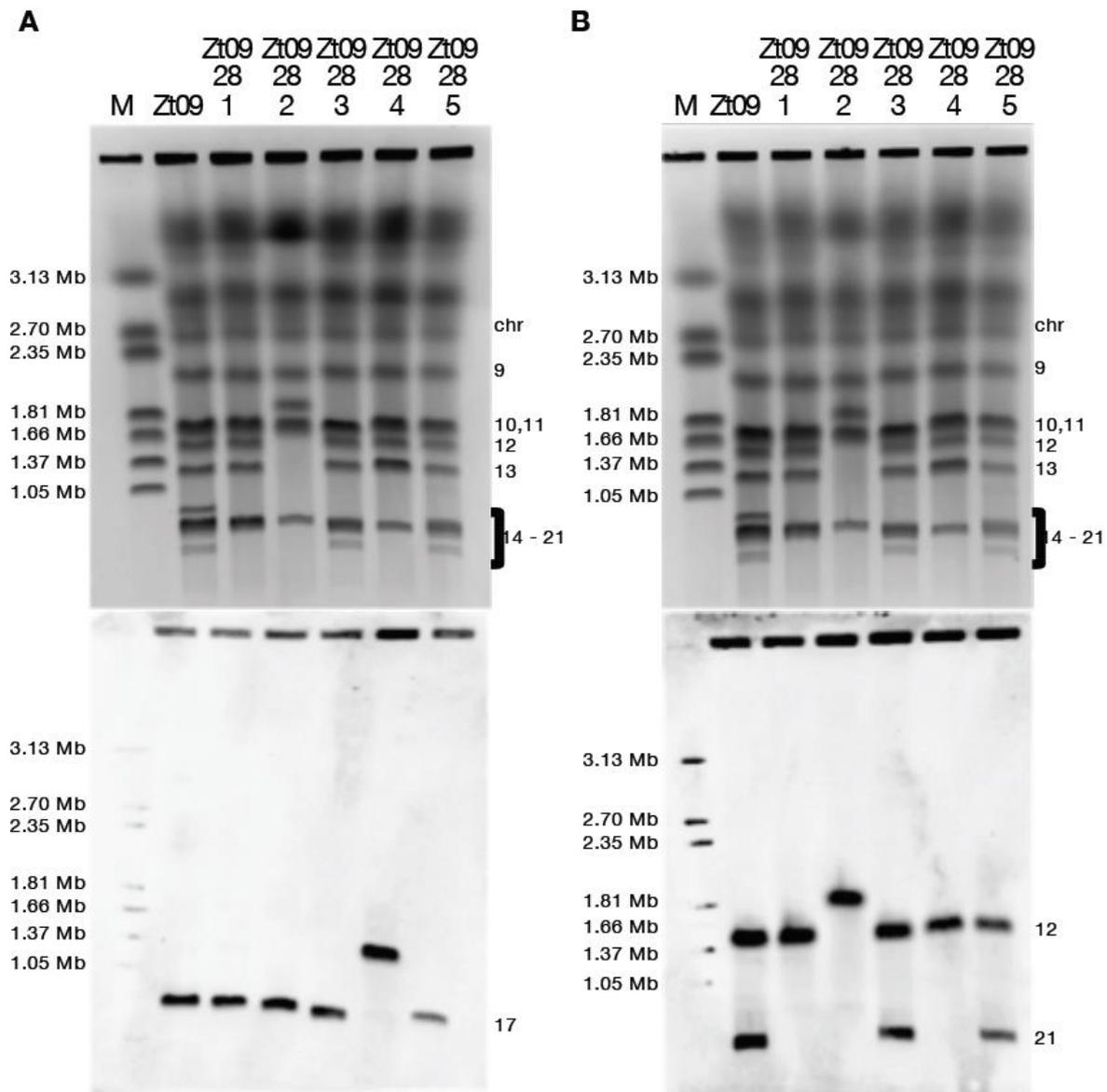
See attached Word file

**Table S5:** Sequence annotation of chromosomal breakpoints detected in the sequenced chromosome-loss strains derived from the temperature stress experiment.

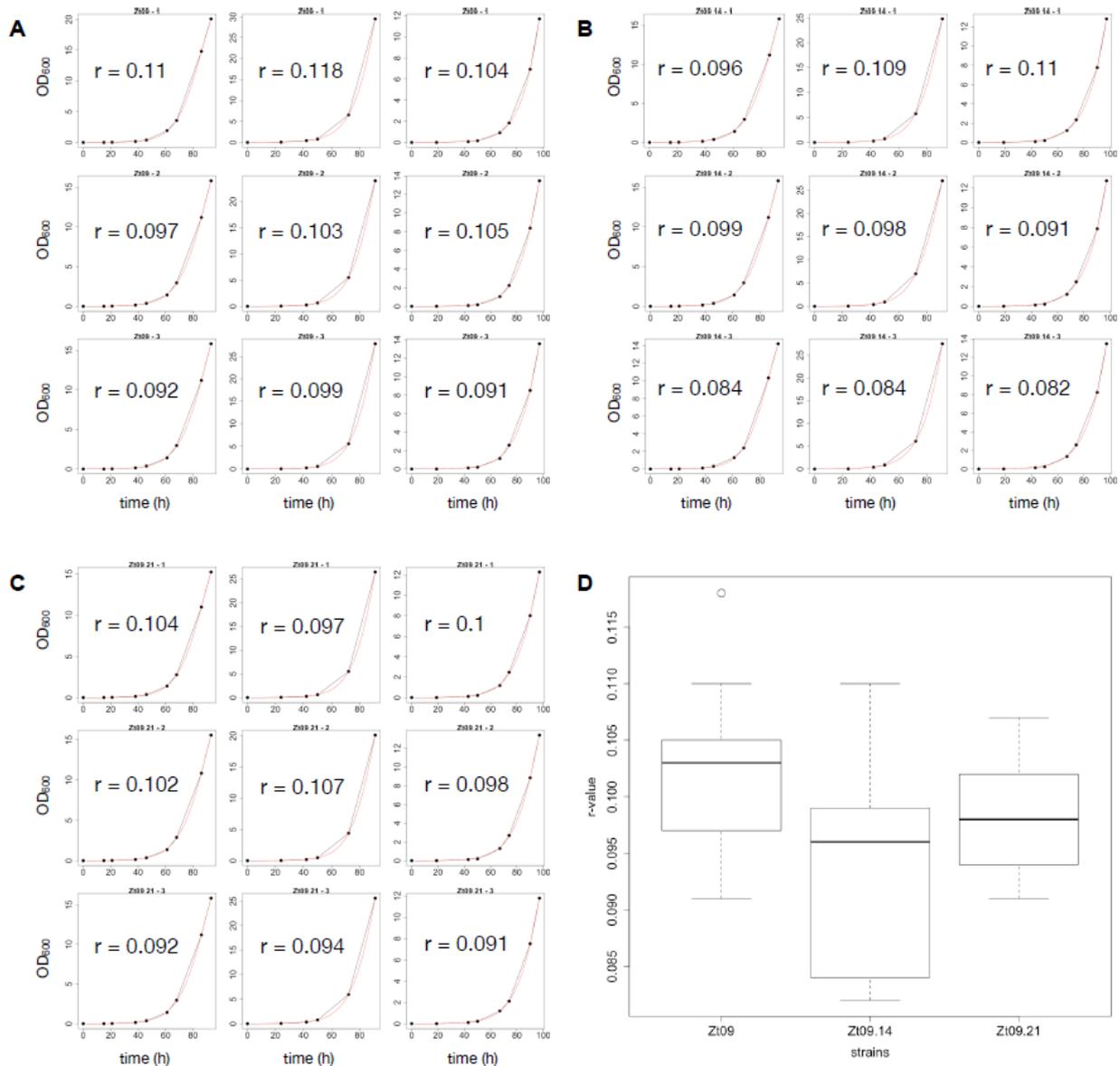
See attached Word file

**Supplementary Text:** Detailed commands for each step of the data processing pipeline.

See attached Word file



**Figure S1: Southern blotting of pulsed-field gels using chromosome-specific probes confirms chromosome fusions.** To prove and visualize chromosome fusions in the strains derived from the temperature stress experiment, we conducted PFGE followed by Southern blots with probes specific to the chromosomes, which based on genome data and karyotype analyses, were found to have fused. Panel (A) shows a color-inverted image of the pulsed-field gel (top) and Southern blot (bottom) using a probe specific for chromosome 17. The genomes of all tested strains have chromosome 17, and the genomes of strains Zt09 28-2 and Zt09 28-4 contain a duplicated chromosome 17 (Figure 2C). While in Zt09 28-2 the duplication resulted in two separate copies of the chromosome, the two chromosomes 17 in Zt09 28-4 fused and formed a new ~ 1.2 Mb chromosome. (B) For the second Southern blot, we used probes for chromosomes 12 and 21. Chromosome 21 is absent in the strains Zt09 28-1 and Zt09 28-3 (PFGE, top and Southern analyses, bottom). Genome sequencing shows that chromosome 21 is present in Zt09 28-2 (Figure 2C), however the respective band on the pulsed-field gel is missing. Similarly, chromosomes 12 and 13 are not present in this strain (PFGE, top), while the chromosome sequences are present in the whole genome sequence data. Apparent loss of chromosome 13 can be explained by the fusion of chromosomes 3 and 13, as indicated by the sequence analysis (Figure 3). The probes for chromosomes 12 and 21 both hybridize to one band in the size of ~1.8 Mb (Southern, bottom) matching the size expected if a chromosome fusion occurred and explaining the absence of their respective 'original' chromosomal bands.



**Figure S2: Growth assay of Zt09 and chromosome-loss strains Zt09Δ14 and Zt09Δ21.** Three independent growth assays were conducted with the progenitor strain Zt09 (A) and two chromosome-loss strains Zt09Δ14 (B) and Zt09Δ21 (C), representing the loss of the largest and smallest accessory chromosome. Plotted are the fitted growth curves of all replicates and experiments generated with the R package growthcurver. The growth curves are based on OD<sub>600</sub> measurements. Panel (D) displays a boxplot of all r values of the different strains. There are no significant differences between the growth curves of the three tested strains (Wilcoxon rank-sum test Zt09 – Zt09Δ14: p-value = 0.1443, Zt09 – Zt09Δ21: p-value = 0.3762).



## Chapter 5

Manuscript prepared for submission

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# Histone posttranslational modifications affect chromosome stability in a plant pathogenic fungus

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### Abstract

Segregation of sister chromatids during mitosis ensures transmission of genetic material to daughter cells and is pivotal for genome stability. Covalent posttranslational modification of histones regulate chromatid segregation as well as chromatin function and nuclear localisation of chromosomes. The genome of the haploid wheat pathogenic fungus *Zymoseptoria tritici* comprises up to eight accessory chromosomes that show presence/absence polymorphism within populations. These accessory chromosomes are frequently lost during mitotic divisions which might enable the fungus to adapt rapidly to changing environments. We hypothesized that the histone modifications H3K9me3 and H3K27me3, which are enriched on the accessory chromosomes of *Z. tritici*, affect the segregation of sister chromatids and thereby cause losses of chromosomes during mitosis. We used a mutation accumulation approach with random one cell bottlenecks to estimate the frequency of chromosome losses without confounding interference of selection over a total of approx. 244800 mitotic divisions. We found that the histone modification H3K27me3 increased the rate of chromosome losses, indicating an adaptive role of this histone modification in regulating chromosome losses, whereas H3K9me3 caused stability of the accessory chromosome. The rate of chromosome losses showed high variation among three field isolates from The Netherland, Denmark and Iran, which may be indicative of adaptation. Finally, we observed that the rate of chromosome loss is an inherent property of the genome, which has not change over the course of the experiment. We conclude that histone modifications regulate the chromosome loss rate and speculate that the losses of the accessory chromosome of *Z. tritici* are adaptive.

## Author Summary

In eukaryotes DNA is wrapped around histones and organized in chromatin. Posttranslational modifications of histones regulate gene expression and genome maintenance including chromatid segregation during mitosis. Our model organism *Z. tritici* is an important wheat pathogen with a global distribution that contains a large complement of accessory chromosomes. These chromosomes are found in some but not all members of a population and influence the interaction of *Z. tritici* with the host wheat. Previously, we demonstrated that the accessory chromosomes of *Z. tritici* are frequently lost during mitosis and are enriched in two specific histone modifications. Here, we showed that the histone modification H3K27me3 increased whereas H3K9me3 reduced loss of accessory chromosomes. In addition, we found high variation in the rate of chromosome losses among field isolates of *Z. tritici* and showed that the rate of chromosome losses remains constant over time and therefore appear to be an inherent characteristic of the genome. We hence speculate that the rate of chromosome losses is adaptive and enables the fungus to rapidly adapt to changing environments.

## Introduction

Fungal pathogens can adapt rapidly to changing environments. In agriculture various fungal plant pathogens have become resistant to fungicide treatments and overcome plant resistance mechanisms [1,2]. High levels of genetic variation at the population level is considered to be one of the factors allowing fungal pathogens to adapt quickly. Next to variation at the gene level, structural variation is widespread in plant pathogenic fungi and often involved in pathogenicity [3–6]. Such structural variation can consist of large genome regions or entire chromosomes, including for example presence/absence polymorphisms of accessory chromosomes. The main determinants of host specificity are located on such accessory genomic elements in *Fusarium oxysporum* f.sp. *lycopersici*, *Nectria haematococca* and members of the genus *Alternaria* [3–6] highlighting their importance for pathogenicity in these species. In contrast to their importance for host-specificity little is known on the mechanisms responsible for generating and maintaining these structural variations.

The accessory chromosomes of the plant pathogenic ascomycete *Zymoseptoria tritici* are an ideal model to dissect structural genome variation and its causes and consequences. *Z. tritici* is a hemibiotrophic fungus that infects wheat, *Triticum aestivum* and *T. durum*, causing dramatic losses in yield [7]. The reference isolate IPO323 contains eight distinct accessory chromosomes, which show a lower gene density and are enriched in transposable elements when compared to the essential core chromosomes [8]. Interestingly, the presence of these accessory chromosomes causes a small but significant fitness cost which importantly depends on the host genotype [9]. The accessory chromosomes of *Z. tritici* show a pronounced presence/absence polymorphism within populations [10], and are often lost, translocated or disomic following meiosis [10–12]. In addition, unpaired accessory chromosomes show a meiotic drive, i.e. they are transmitted to all meiotic products instead of the expected half (Habig *et al.*, submitted). This meiotic drive is considered to be essential for the maintenance of the accessory

chromosomes despite their fitness cost (Habig *et al.*, submitted). In contrast, during mitosis the accessory chromosomes are lost frequently during growth *in vitro* and *in planta* [13]. The presence/absence polymorphism of accessory chromosomes might therefore be the results of frequent losses during mitosis in combination with an increase in frequency during meiosis by a meiotic drive. The factors affecting the loss of accessory chromosomes are unknown but might include epigenetic marks, like histone modifications, which affect the conformation of chromosomes.

In eukaryotes, DNA is organized in chromatin which regulates transcription as well as DNA replication, DNA repair, recombination and also chromosome and chromatid segregation during mitosis and meiosis [14]. Posttranslational histone modifications affect the chromatin condensation state from euchromatin to heterochromatin [15]. In filamentous fungi the trimethylation of lysine 9 of histone H3 (H3K9me3) is associated with heterochromatin and the suppression of transcription and transposition of transposable elements [16]. In contrast, the trimethylation of the lysine 27 of histone H3 (H3K27me3) is enriched in the subtelomeric regions [17] and appears to be involved in tethering these subtelomeric regions to the nuclear envelope [18]. Interestingly, the accessory chromosomes of *Z. tritici* show a higher abundance of H3K9me3 and H3K27me3 methylations compared to the essential core chromosomes [19,20]. The methyltransferases KMT1 and KMT6 are responsible for the H3K9me3 and the H3K27me3 methylation, respectively, and deletion of *kmt1* and *kmt6* results in the complete loss of these histone modifications in the genome of *Z. tritici* (Moeller *et al.*, in preparation). Next to their role in transcriptional regulations, histone modifications are also involved in DNA replication and repair, recombination, chromatid cohesions and segregation and thereby in maintaining the integrity of the genome and its faithful transmission to daughter cells [21–26]. However, the role of histone modifications in maintaining the integrity of genomes in plant pathogenic fungi and in particular their role in the maintenance of accessory chromosomes is unclear.

Here, we aimed at understanding the neutral rate of accessory chromosome loss as a factor affecting the distribution of these genetic elements. Moreover, we specifically tested the role of histone modification on chromosome loss rates. In particular we focussed on the effect of two histone modifications H3K9me3 and H3K27me3 and evaluated how these might affect the rate by which the accessory chromosomes were lost over time. We specifically assessed the neutral rate of chromosomal losses by excluding natural selection using a mutation accumulation (MA) approach. MA experiments have previously been used with various model organisms such as *Arabidopsis thaliana*, *Caenorhabditis elegans*, *Drosophila melanogaster* and *Saccharomyces cerevisiae* to estimate the neutral rate of point mutations and small indels [27–30]. However, the neutral rate of larger structural rearrangements and losses of chromosomes is currently unexplored. Since losses of accessory chromosomes in plant pathogenic fungi affect their pathogenicity [6,31] we were interested in the rate of chromosomal losses and on the mechanisms of these chromosome losses, including the role of epigenetic marks. We demonstrated the strong influence of histone modifications on the neutral loss rate of accessory chromosomes during mitotic cell divisions.

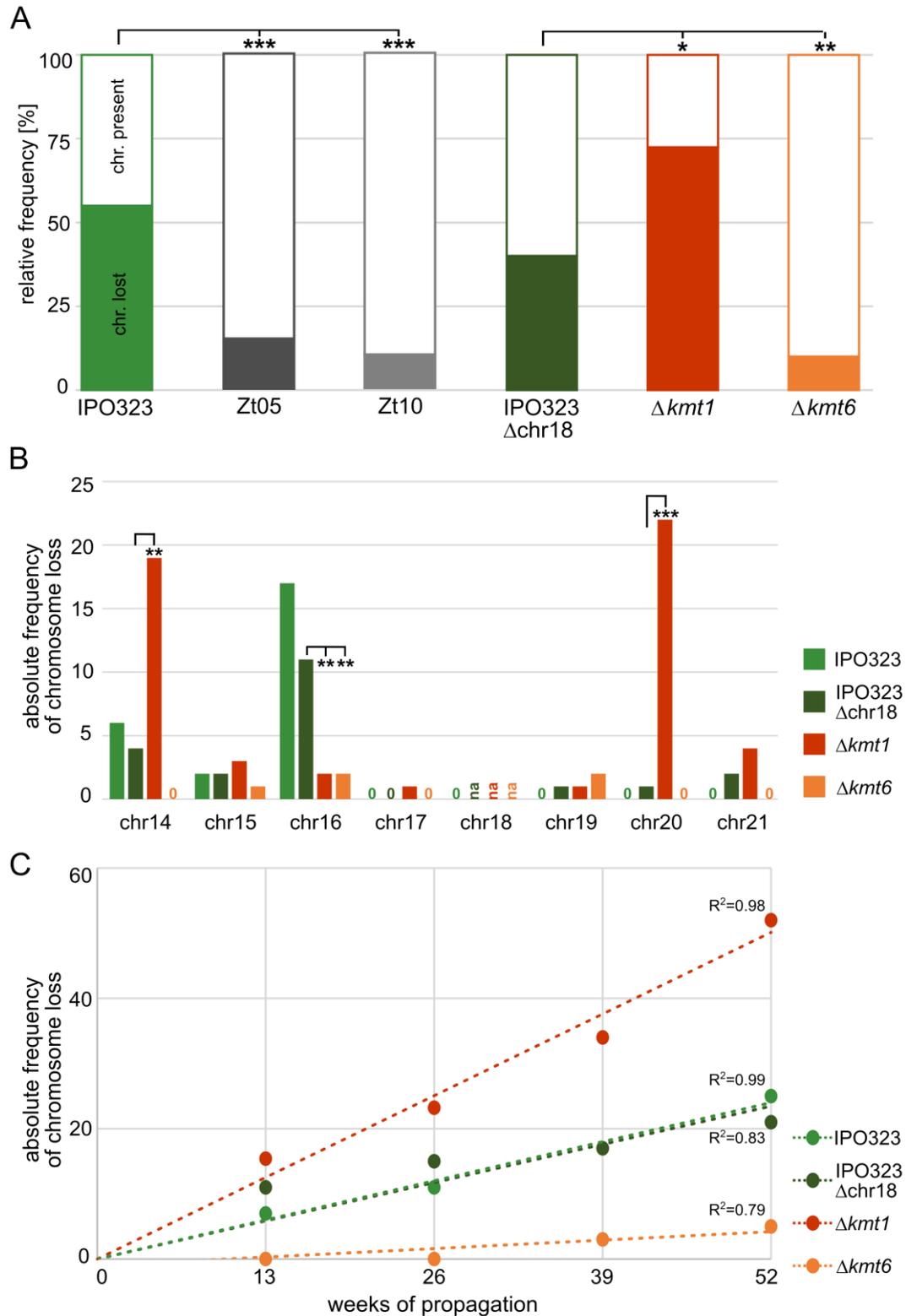
## Results

To assess the rate of chromosome losses in the absence of natural selection we used a MA approach employing 40 independent replicates per each strain for a total of six strains. These strains included three different field isolates from the Netherland, Denmark and Iran (IPO323, Zt05 and Zt10, respectively), one strain that had lost one accessory chromosome (IPO323 $\Delta$ chr18) as well as two deletion mutants lacking the histone methyltransferase KMT1 ( $\Delta$ kmt1) or KMT6 ( $\Delta$ kmt6). Every seven days a randomly chosen single colony was transferred to a new petridish and further propagated for seven days before the process was repeated. Importantly, this procedure introduced single cell bottlenecks, thereby preventing any effect of natural selection on losses of accessory chromosomes. We propagated each replicate for 52 weeks. *In vitro*, *Z. tritici* divides every approx. 8.4h [13] which translates into 20 cell divisions per week of propagation. Therefore, we can, due to the one cell bottleneck, calculate the exact number of mitotic divisions for every replicate with: 52 weeks x 20 divisions/week = 1040 mitotic divisions per replicate (resulting in approx. 40800 mitotic divisions for each strain with a total of 244800 mitotic division for all strains). We further determined the karyotype for the accessory chromosomes using PCR amplification of regions in both (left and right) subtelomeric regions of each accessory chromosome for replicates at 13, 26, 39 and 52 weeks of propagation. This allowed us to test for the effect of chromosome number, histone modification and natural variation on the structural plasticity of the accessory chromosome of *Z. tritici*.

### **The loss rate of accessory chromosomes varied little over time and was not affected by the number of lost chromosomes**

We first hypothesized that the loss of accessory chromosomes could change over time and be dependent on previous losses of accessory chromosomes. A loss of one chromosome could potentially increase or decrease the likelihood of an additional chromosome being lost. To test this we used two approaches: i) we included the reference isolate IPO323 of *Z. tritici* containing eight accessory chromosomes and compared the loss of chromosomes to an IPO323 $\Delta$ chr18 strain and ii) we tracked the loss of accessory chromosomes over the course of the experiment. In detail on i), many field isolates have lost chromosome 18 [10,32] and in order to test whether the number of accessory chromosomes and in particular chromosome 18 affects the loss of other accessory chromosomes we also included an IPO323 derived strain that spontaneously lost chromosome 18 and compared the rate of the chromosome losses after 52 weeks of propagation (Fig 1A). Of the 40 replicates of IPO323 22 replicates (55%) had lost at least one accessory chromosome after 52 weeks of propagation. The chromosome loss rate was very similar for IPO323 $\Delta$ chr18. Here, 16 of the 40 replicates (40%) had lost at least one accessory chromosome. Moreover, both strains show very similar results for the loss rate of individual chromosomes (Fig 1B, Table 1), with a total of 7.8% or 7.5% of all accessory chromosomes being lost in IPO323 and IPO323 $\Delta$ chr18, respectively. Most chromosome losses were detected for chromosome 16 for both IPO323 (17 of 40 strains) and IPO323 $\Delta$ chr18 (11 of 40 strains). With all or the majority of chromosome losses occurring for the three largest accessory chromosome 14, 15 and 16 in IPO323 or IPO323 $\Delta$ chr18, respectively. For none of the accessory chromosomes

a significant difference between the loss rate in IPO323 and in IPO323 $\Delta$ chr18 was obtained (Fig 1B).



**Fig 1. Rate of losses of accessory chromosome varied between isolates, were affected by histone modifications but remained constant over time.** A) Histogram of relative frequencies of replicates for each strain having lost at least one chromosome (fully coloured) or having retained all accessory chromosomes present in the progenitor strain (blank) after 52 weeks of propagation (n=40 replicates

per strain). The field isolates IPO323, Zt05, Zt10 differed in their chromosome loss as well as the deletion mutants  $\Delta kmt1$  and  $\Delta kmt6$  differ from IPO323 $\Delta$ chr18 which was used to create these deletion strains. B) Histogram of absolute frequencies of chromosome losses for IPO323, IPO323 $\Delta$ chr18,  $\Delta kmt1$  and  $\Delta kmt6$  according to individual accessory chromosome. IPO323 and IPO323 $\Delta$ chr18 showed a similar pattern of chromosome losses with chr16 having the highest frequency of chromosome losses.  $\Delta kmt1$  produced the highest frequencies of losses for chr14 and chr20 but a lower frequency for chr16 compared to the IPO323 $\Delta$ chr18. C) The rate of chromosomal losses showed little variation over time. Chromosomal losses as determined at 0, 13, 26, 39 and 52 weeks of propagation and the resulting linear regression including the corresponding coefficient of determination are depicted. A&B) Significance as determined by the Fisher's exact test is denoted as \* =  $p < 0.05$ , \*\* =  $p < 0.005$ , \*\*\* =  $p < 0.0005$ .

Table 1. Summary of loss of accessory chromosomes after 52 weeks of propagation.

	IPO323					
	IPO323	$\Delta$ chr18	$\Delta kmt1$	$\Delta kmt6$	Zt05	Zt10
Replicates	40	40	40	40	40	40
Number of accessory chromosomes per genome	8	7	7	7	8	6
Total number of accessory chromosomes at start	320	280	280	280	320	240
Accessory chromosome lost after 52 weeks of propagation	25	21	52	5	6	4
Loss rate of chromosomes per chromosome after 52 weeks	7.8%	7.5%	18.6%	1.8%	1.9%	1.7%
Loss rate of chromosomes per chromosome per mitotic division	$7.7 \times 10^{-5}$	$7.4 \times 10^{-5}$	$1.8 \times 10^{-4}$	$1.8 \times 10^{-5}$	$1.8 \times 10^{-5}$	$1.6 \times 10^{-5}$

In detail on ii), to test whether the loss of chromosomes affected the likelihood of losing additional chromosomes we tracked the loss of accessory chromosomes over the course of the experiment. We determined the complement of accessory chromosomes at week 13, 26, 39 and 52 weeks of propagation. Both IPO323 and IPO323 $\Delta$ chr18 showed a similar loss of chromosomes over time, and thus no indication of an increase over time (Fig 1C). Therefore, we conclude that the number of accessory chromosomes does not influence the rate at which accessory chromosomes are lost and the loss rate of accessory chromosomes appears to be an intrinsic characteristic of the genome of *Z. tritici* but constant over time. Interestingly, we did not observe losses of the accessory chr18 in IPO323, which is absent in many field isolates [10,32]. Therefore the presence/absence polymorphism of this chromosome in the field might be influenced by other factors such as losses during meiotic transmissions and/or selection. Over the course of the whole 52 weeks of propagation the rate of chromosomal losses/per chromosome IPO323 and IPO323 $\Delta$ chr18 was very similar with  $7.7$  and  $7.4 \times 10^{-5}$  loss per chromosome per mitotic division, respectively (Table 1).

### Histone modifications affected the loss rate of chromosomes

We next asked whether histone modifications, which influence the chromatid organisation, also affect the loss rate of accessory chromosomes. The accessory chromosomes of IPO323 are highly enriched on the whole chromosome in the histone modification H3K27me<sub>3</sub>. But this histone modification is restricted to the subtelomeric regions of the essential core chromosomes in *Z. tritici* [20]. H3K27me<sub>3</sub> could be involved in tethering the subtelomeric regions of chromosomes to the nuclear envelope [18,33,34]. In addition, the accessory chromosomes of *Z. tritici* contain a higher proportion of transposable elements which are in turn enriched in the silencing H3K9me<sub>3</sub> histone modification [8,20,35]. To test whether these two histone modifications affect the chromosome stability we assessed the rate of chromosome loss in two deletion mutants, in which either the histone methyltransferase KMT1, responsible for the trimethylation at the H3K9 position, or the histone methyltransferase KMT6, responsible for the trimethylation at the H3K27 position, was deleted. Both deletions were constructed in the IPO323Δchr18 genomic background.

Interestingly, the removal of the H3K9me<sub>3</sub> histone modification significantly increased the number of replicates that lacked at least one accessory chromosome compared to IPO323Δchr18 (Fisher's exact test,  $p = 3.6 \times 10^{-3}$ ) (Fig 1A). 29 of the 40 replicates had lost at least one accessory chromosome. A total of 52 chromosomes were lost after 52 weeks of propagation. This represents a loss of 18.6% of all accessory chromosomes (Table 1). Chromosomes were affected differently. Chromosome 14 and 20 showed a significantly higher loss rate within the Δkmt1 than compared to the wildtype (Fisher's exact test,  $p = 3.8 \times 10^{-4}$ ,  $p = 8.9 \times 10^{-9}$ , respectively). However, the loss rate of chromosome 16 was highly reduced in the Δkmt1 mutant (Fisher's exact test,  $p = 0.012$ ) (Fig 1B). No significant differences were observed for the other accessory chromosomes.

In contrast, the removal of the H3K27me<sub>3</sub> histone modification resulted in a significantly reduced chromosome loss rate compared to the IPO323Δchr18 (Fisher's exact test,  $p = 1.6 \times 10^{-3}$ ). A total of 5 chromosomes were lost after 52 weeks of propagation. This reduction in the rate of chromosome losses is due to the significant reduction of the loss of chromosome 16. For this chromosome the loss rate in the Δkmt6 mutant is significantly lower (Fisher's exact test,  $p = 0.013$ ). Again, for both the Δkmt1 and Δkmt6 mutants the rate of chromosome losses appears to be stable during the course of the experiment, with no indication that the rate of chromosome losses increases over time (Fig 1C, Table S1).

**Rate of chromosome losses varied among natural isolates**

We next asked whether the rate of chromosomal losses is similar for different *Z. tritici* isolates. We therefore included two additional isolates Zt05 and Zt10, from Denmark and Iran, respectively. These isolates have been fully sequenced and genome assemblies on chromosome levels are available [36]. Based on synteny Zt05 contains seven unitigs that have a homolog among the accessory chromosomes of IPO323 (chr14-chr20). In addition Zt05 carries one unitig (unitig26) that has no homolog in IPO323 and that we therefore consider to be an accessory chromosome (Fig S1A). Similarly, the Iranian isolate Zt10 has homologs for five of the accessory chromosome of IPO323 (chr14, 15, 16, 17, 19) and carries one unitig (unitig15) that has no homolog in IPO323 and that we therefore also consider to represent an accessory chromosome (Fig S1B).

The rate of chromosome loss is much lower for both Zt05 and Zt10 compared to the Dutch isolate IPO323 (Fig 1A). Six and four replicates had lost at least one chromosome in Zt05 and Zt10, respectively. This loss rate is significantly smaller than that inferred for the IPO323 reference isolate (Fisher's exact test,  $p = 3.3 \times 10^{-4}$ ,  $p = 2.4 \times 10^{-5}$ ). Zt05 and Zt10 showed a similar loss rate of  $1.8$  and  $1.6 \times 10^{-5}$  per chromosome per mitotic divisions, respectively. Surprisingly, the homologs of chromosome 16 in Zt05 and Zt10, Unitig21 and Unitig13, respectively, were not lost over the course of 52 weeks, which contrasted with the high loss rate observed for chromosome 16 in IPO323 and IPO323 $\Delta$ chr18 (Table S2-S3).

**Chromosome loss can proceed via a partial chromosome loss**

We next asked by which mechanism a chromosome is lost. We considered two scenarios possible: i) loss of the whole chromosome in one event or ii) a sequential loss via partial chromosome loss. For this analysis we pooled all data for IPO323 and IPO323 $\Delta$ chr18 and considered a chromosome to be partially lost if only one of the subtelomeric regions was present as detected by PCR. After 13, 26, and 39 weeks of propagation we detected a total of eleven partial chromosome losses (Table 2). This is lower than the 33 complete chromosome losses we detected within the same time frame. This indicates that partial losses do occur but are less frequently observable compared to whole chromosome losses. This finding could be due to a less frequent occurrence and/or a lower stability of these partial losses. We next asked whether these partial losses eventually lead to whole chromosomes becoming lost at a later time point. Indeed, we find that of the eleven partial chromosome losses seven resulted in the whole chromosome being lost at a later time point, indicating that once a chromosome is partially lost it is significantly more likely that this chromosome will become lost completely compared to the loss of a whole chromosome in one event (Fisher's exact test,  $p = 5.4 \times 10^{-6}$ ). Interestingly, the partial transient stage appears less stable for chr16 compared to the other accessory chromosomes. For seven partial deletions of chr16 six resulted in a full chromosome deletion – whereas of the four partial deletions observed for all other accessory chromosomes, only one resulted in the loss of the whole chromosome. Although the low number prevents any statistical inference, we speculate that the higher instability of the partially lost chr16 might be responsible for the higher total loss rate of chromosome 16.

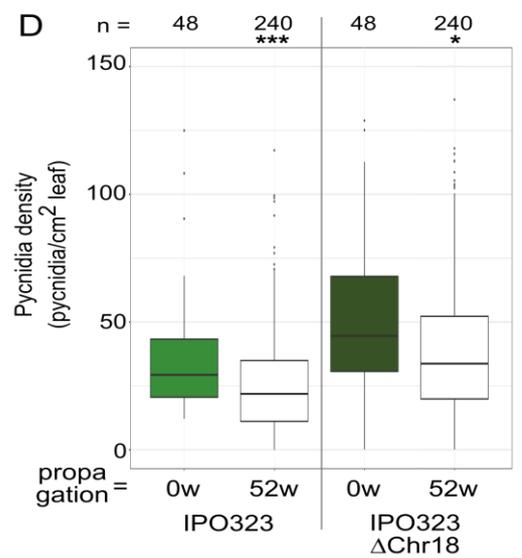
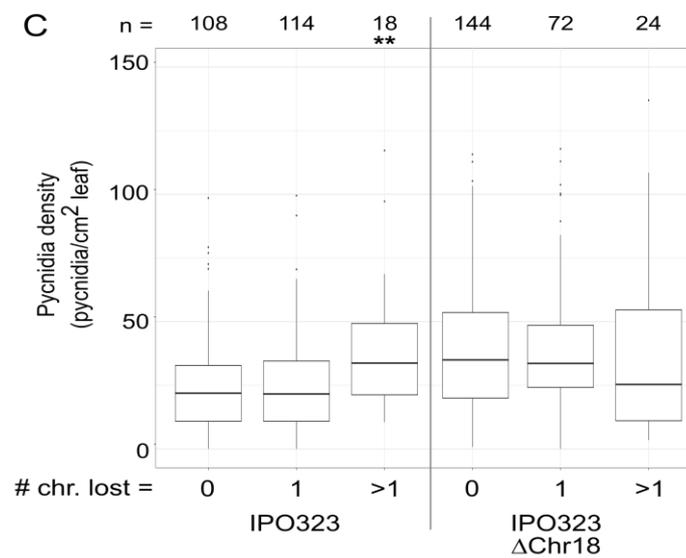
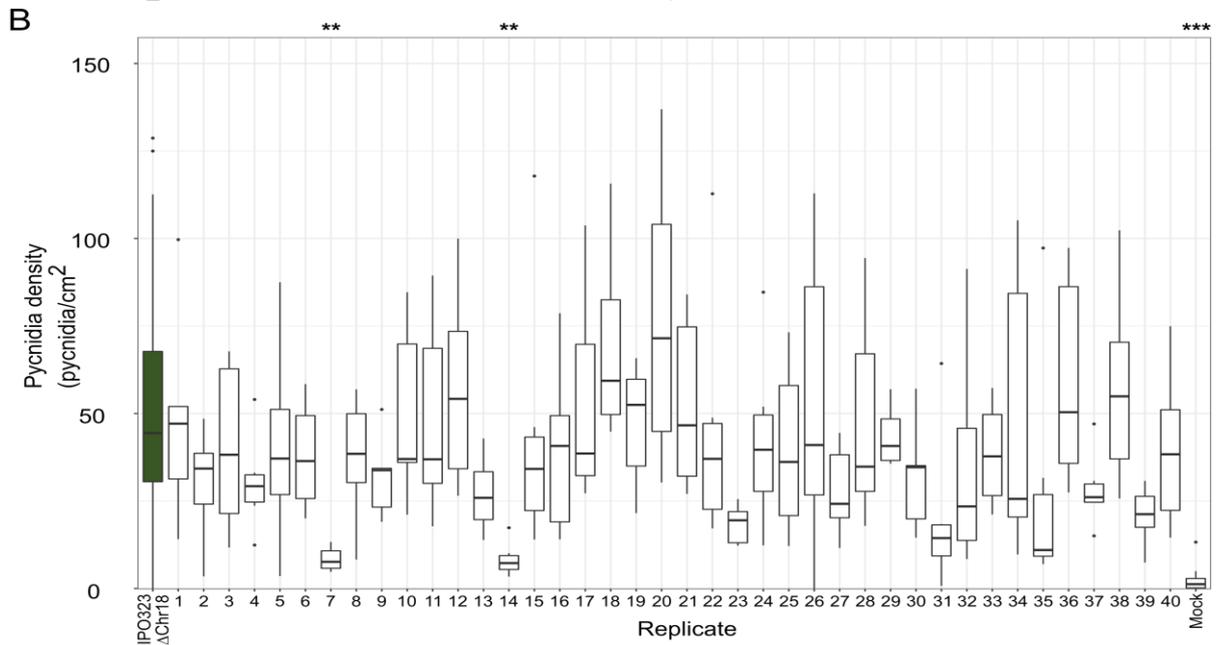
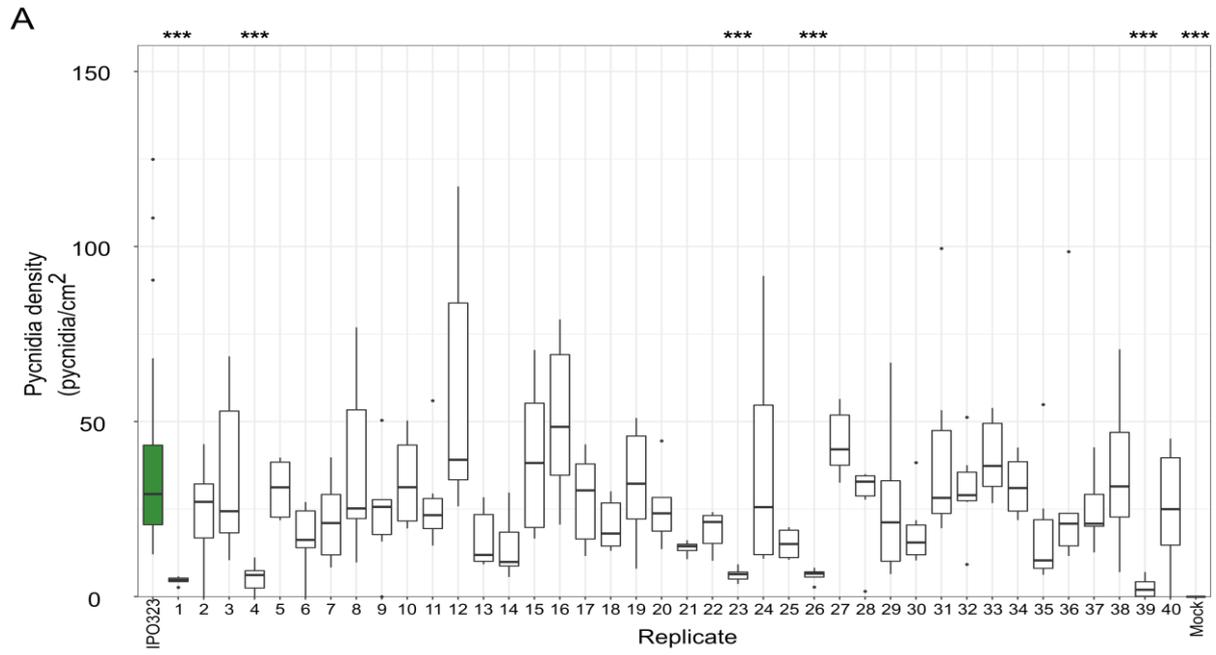
Table 2: Summary of partial chromosome losses and the fate of partially lost chromosomes.

	Partial chromosome losses at 13, 26 and 39 weeks of propagation	Of these later resulting in complete chromosome loss	relative frequency
chr16	7	6	86%
All other chromosomes	4	1	25%
Total	11	7	64%

### Mutation accumulation causes phenotypic variation

We next asked whether the mutations that accumulated over 52 weeks of propagation influenced the phenotype of the replicates *in planta*. We measured the ability of the replicates to produce pycnidia, the asexual fructifications for the IPO323 and the IPO323 $\Delta$ chr18, as this parameter has been widely used to determine the fitness of individual strains [9,13,37,38]. Five replicates of IPO323 after 52 weeks of propagation (replicate 1, 4, 23, 26 and 39) produced significantly less pycnidia compared to the progenitor IPO323 (Fig 2A) whereas two replicates of IPO323 $\Delta$ chr18 after 52 weeks of propagation (replicate 7, 14) produced less pycnidia than the progenitor IPO323 $\Delta$ chr18 strain (Fig 2B). Interestingly, the loss of more than one chromosome increased the fitness of IPO323 significantly in comparison to those replicates that did not lose any accessory chromosomes (ANOVA,  $p=0.001$ ). But no significant difference was observed for replicates that had lost a single accessory chromosome (Fig 2C). Similarly, the loss of one or more than one accessory chromosome did not significantly alter the fitness of the replicates of IPO323 $\Delta$ chr18 (Fig 2C).

Commonly, the fitness effect of mutations is assumed to be slightly deleterious on average [39,40]. The one cell bottleneck applied here caused fixation of all mutations. We therefore hypothesized that the propagated replicates should have a lower fitness compared to the progenitor strain. We pooled all *in planta* data for the propagated strains and compared their ability to produce pycnidia with the progenitor strain. For all propagated IPO323 and IPO323 $\Delta$ chr18 replicates, we see a significant (ANOVA,  $p= 1.1 \times 10^{-4}$ ,  $p=0.021$ ) reduction in the ability to produce pycnidia *in planta* compared to the progenitor strain (Fig 2D). Therefore, the assumption that the majority of mutations are slightly deleterious holds true for *Z. tritici*.



**Fig 2. Mutation Accumulation caused phenotypic differences between replicates and progenitor strains.** A) Box plot depicting the observed pycnidia density *in planta* of the progenitor strain IPO323 (light green) and the 40 replicates after 52 weeks of propagation. Replicates 1, 4, 23, 26 and 39 differed significantly from the phenotype observed for the progenitor strain. Plots show pooled data of two experiments with a total n=48 for progenitor strain and n=6 for each of the replicates. B) Box plot depicting the observed pycnidia density *in planta* of the progenitor strain IPO323Δchr18 (dark green) and the 40 replicates after 52 weeks of propagation. Replicates 7 and 14 differed significantly from the phenotype observed for the progenitor strain. Plots show pooled data of two experiments with a total n=48 for progenitor strain and n=6 for each of the replicates. C) Effect of chromosome losses on the fitness of the fungus. Boxplot comparing all propagated replicates of IPO323 and IPO323Δchr18 in their ability to produce pycnidia dependent on number of chromosomes lost. Loss of more than one chromosome in IPO323 leads to a significant increase in the ability to produce pycnidia, whereas no significant differences are observed for the different number of chromosome losses in IPO323Δchr18. Note: Data for all replicates with more than one accessory chromosome lost are pooled. D) On average the fixed mutations, which accumulated during the propagation, reduced the fitness. Boxplot comparing the pycnidia density of the progenitor strains IPO323 (light green) and IPO323Δchr18 (dark green) with the pooled pycnidia density data of all 40 replicates for each strain after 52 weeks of propagation. The propagated replicates of both IPO323 and IPO323Δchr18 are less able to produce pycnidia. Significance was inferred using ANOVA on ranked pycnidia density using the model pycnidia density ~ replicate \* experiment (A&B) or pycnidia density ~ number of chromosome lost \* replicate \* experiment (C). Significance as determined by Tukey's HSD is denoted as \* = p<0.05, \*\* = p<0.005, \*\*\* = p<0.0005.

## Discussion

The accessory chromosomes of plant pathogenic fungi show substantial presence/absence polymorphisms. The exact processes contributing to such variations represent an unsolved puzzle in our understanding of the biology of these fungi. The maintenance of these chromosomes is considered to be either due to selection [41] or their transmission advantage during meiosis (Habig *et al.*, submitted). At the same time, little is known on the processes causing chromosome loss. It is possible that the presence/absence polymorphism is due to an interplay between selection of the accessory chromosomes during certain conditions (e.g. infection of certain host genotypes) and their loss under other conditions (e.g., on other host genotypes). The accessory chromosomes of *F. oxysporum* f.sp. *lycopersici*, *Z. tritici* and *Z. ardabiliae* show a high rate of mitotic loss [13,42]. In *F. oxysporum* f.sp. *lycopersici* 1/35000 had lost a lineage specific chromosome after *in vitro* propagation [42] whereas in *Z. tritici* one in 50 cells had lost at least one chromosome after four weeks of *in vitro* propagation [13]. Here, we extended these studies and assessed specific factors that could affect the mitotic loss rate. In our experiment, we used a mutation accumulation approach to exclude any confounding natural selection that additionally contributes to the presence/absence polymorphism. Surprisingly, we found contrasting effects of two types of histone modification on the presence/absence polymorphism with H3K9me3 increasing and H3K27me3 decreasing chromosome stability.

In fungi, the histone modification H3K9me3 is involved in the regulation of chromatin structure and associated with constitutively silent heterochromatin [25] but can also be involved in the regulation of expression of small secreted proteins [43] or fungal alkaloids [25,44,45]. In *Z. tritici* H3K9me3 is associated with repetitive sequences [20]. The increase in mitotic instability upon loss of H3K9me3, achieved through deletion of *kmt1*, could be due to an increase in transposable element activity or a change in the chromatid formation to a more open conformation, which then decreases chromosome stability. *kmt6*, the second histone modification gene considered in this study, is responsible for the H3K27me3 methylation. Its deletion increased chromosome stability. H3K27me3 is associated with the subtelomeric regions of chromosomes and believed to anchor these regions to the nuclear envelope [17,18]. In *Neurospora crassa* the elimination of H3K27me3 resulted in the partial displacement of telomere clusters from the nuclear periphery [46]. In *Z. tritici*, H3K27me3 is not only associated with the subtelomeric regions of all chromosomes but enriched throughout the accessory chromosomes [20]. Therefore, complete accessory chromosomes might be tethered to the nuclear periphery [20]. Interestingly, the centromeres in *Z. tritici* are not located within one centre but appear to be distributed in several distinct centres [20], which could also be caused by the localization of the accessory chromosomes in the nuclear periphery versus a nuclear matrix location of the core chromosomes. This different spatial localization of the accessory chromosomes has been suggested to affect the transmission of the accessory chromosomes during meiosis (Habig *et al.*, submitted) and mitosis [20]. The localization at the nuclear envelope might result in frequent mis-segregations during mitotic division resulting in losses and disomies. Therefore, we propose that the removal of the H3K27me3 histone modification might result in a nuclear matrix localization of the accessory chromosomes, similar to the localization of the core chromosomes and that this matrix location results in more faithfully segregating accessory chromosomes.

Our observed loss rates of  $7.4 \times 10^{-5}$  per mitotic division and per chromosome for IPO323 $\Delta$ chr18 appears to be low compared to the previously described loss rate of one in 50 cells lacking an accessory chromosome after four weeks of propagation [13]. However, when using the loss rate established here and applying it to the set-up of the previous study we would have predicted to find 23.7 chromosomes being lost in the previous study ( $7.4 \times 10^{-5}$  chromosome losses/per chromosomes/per mitotic division  $\times$  576 cells  $\times$  80 mitotic divisions  $\times$  seven chromosomes). This is lower than the observed rate of 38 chromosomal losses reported suggesting that in the previous study other processes contributed to the observed loss rate. In contrast, in our study, we can exclude natural selection affecting the observable rate of chromosome losses and can therefore precisely infer the neutral rate of chromosome losses. The loss rate of  $7.4 \times 10^{-5}$  per chromosomes and mitotic division is high compared to the observed two losses of one copy of a diploid chromosome in *S. cerevisiae* [47] which translates to  $4 \times 10^{-7}$  chromosome losses per chromosome per mitotic division. Populations of *Z. tritici* have high standing genetic variability enabling *Z. tritici* to adapt rapidly to environmental changes [48,49]. The accessory chromosomes of *Z. tritici* are considered to be a cradle for adaptive evolution [50]. We therefore suggest that the high neutral loss rate of the accessory chromosomes is, in combination with the observed meiotic drive, contributing to this high

standing variability allowing frequent losses and gain of the information on the accessory chromosomes.

Interestingly, the loss rate of accessory chromosomes in natural isolates varied considerably. Zt05 and Zt10, isolated in Denmark and in Iran, respectively, have much lower loss rates compared to IPO323. IPO323 was isolated in the Netherlands in 1981 [51] and has been propagated *in vitro* since. Adaptation to laboratory conditions might have increased the genome instability as also seen in various human cell lines [52,53]. Zt05 and Zt10 were isolated later, in 2004/2005 and 2001 [54,55], respectively. Therefore it is conceivable that the observed differences between the natural isolates of *Z. tritici* are due to the differences in their propagation history. However, in a previous study on the closely related sister species *Z. ardabiliae* the observed losses of accessory chromosomes translates to a loss rate of  $3.2 \times 10^{-5}$  [13]. This loss rate is intermediate of the observed loss rates for IPO323 and Zt05 and Zt10 and therefore the observed variation in loss rate likely to lie within the range of natural variation. How this natural variation is generated is currently unknown. We speculate that differences in the epigenetic histone modifications in Zt05 and Zt10, in particular the H3K9me3 and H3K27me3, might be involved. The high variation between field isolates may be adaptive and allows the fungus to lose accessory chromosomes that incur fitness costs dependent on different environmental or host parameters. For example, local climate conditions as well as the range of wheat cultivars differ between the geographic locations at which IPO323, Zt05 and Zt10 were isolated.

We found that the fitness benefit of losing more than one accessory chromosome in IPO323 is similar to previous reports where fitness costs of the accessory chromosomes were observed [9,13]. In contrast to previous studies we did not observe a fitness benefit when losing one accessory chromosome. This might be due to the accumulation of mutations also on the core chromosomes in this study which might mask the phenotypic effects of losing one accessory chromosome. Dissecting the effect of losing accessory chromosome from effects of additional mutations accumulating in the genome will require analysis of whole genome sequences.

In conclusion, we were able to estimate the rate of accessory chromosomes loss in *Z. tritici* without confounding effects of natural selection. With our mutation accumulation approach we found that chromosomes are lost at a substantially higher rate than has been described for other fungi. We further demonstrated that epigenetic marks are affecting the transmission fidelity of the accessory chromosomes in *Z. tritici*. Interestingly, the removal of H3K27me3 increased stability of accessory chromosomes indicating a destabilizing role of this histone mark. The mutation accumulation replicates will further enable us to dissect the processes leading to chromosomal losses and by employing whole genome sequencing will allow for precise estimates of mutation rates and chromosomal aberrations in this important plant pathogenic fungus.

## Materials and Methods

**Fungal strains and plant material.** The Dutch isolates IPO323 are available from the Westerdijk Institute (Utrecht, The Netherlands) with the accession numbers CBS115943. *Z. tritici* strains Zt09 is a derivative of IPO323 that had spontaneously lost chromosome 18. *Z. tritici* strains Zt05 (ID: MgDk09\_U34) [54] and Zt10 (ID: Mg\_STIR04\_A26b) [55] were isolated in Denmark and Iran, respectively and are available on request. The generation of deletion mutants of *kmt1* (IPO323 $\Delta$ chr18 $\Delta$ Mgr107709, *hyg*) and *kmt6* (IPO323 $\Delta$ chr18 $\Delta$ Mgr92660, *hyg*) is described elsewhere (Moeller et. al, in preparation) *Triticum aestivum* cultivar Obelisk used for the *in planta* phenotypes was obtained from Wiersum Plantbreeding BV (Winschoten, The Netherlands).

**Mutation accumulation experiment.** For each of the six strains (IPO323, IPO323 $\Delta$ chr18, Zt05, Zt10,  $\Delta$ *kmt1*,  $\Delta$ *kmt6*) 40 replicates were created all originating from a single colony. Each replicate was grown on a separate petri dish containing YMS agar (4 g yeast extract, 4 g malt extract, 4 g sucrose, and 20 g agar per 1 litre) for 7 days at 18°C before a single colony was selected based on the vicinity to a predetermined and fixed position on the petri dish. The colony was transferred into 1000  $\mu$ L YMS medium including 25% glycerol and vortexed to separate individual cells for 2 min on a VXR basic Vibrax (IKA, Staufen, Germany) at 2,000 rpm. Of this cell suspension 10 to 15  $\mu$ L were distributed onto a separate petri dish and again grown for seven days at 18°C before another colony was picked at random as described above. This process was repeated over the course of 52 weeks. The remaining cell suspensions of each transfer were stored at -80°C and replicates were regrown on YMS agar-plate and cells isolated for DNA-isolation and phenotypic characterisation.

**PCR-based karyotyping.** PCR based karyotyping was conducted as described in [9]. Briefly: For each replicate of IPO323, IPO323 $\Delta$ chr18, Zt05, Zt10,  $\Delta$ *kmt1*,  $\Delta$ *kmt6* frozen cell suspension was placed on YMS agar and grown for 7-14 days. DNA was extracted from the cells using standard DNA isolation procedures [56]. We developed a total of six multiplexed PCRs. For IPO323 and IPO323 derived strains (IPO323 $\Delta$ chr18,  $\Delta$ *kmt1*,  $\Delta$ *kmt6*) two multiplex primer sets were used each amplifying short (150bp -900bp) regions in either the right or the left subtelomeric region of the accessory chromosome 14, 15, 16, 17, 18, 19, 20, and 21, respectively. For the generation of the multiplex primer sets for Zt05 and Zt10 *de novo* assemblies described in a previous study were used [36]. For Zt05 two multiplex primer sets were used each amplifying short (150-900bp) regions in either the right or the left subtelomeric regions of the accessory unitigs 14, 19, 20, 21, 23, 24, 25, and 26. For Zt10 two multiplex primer sets were used each amplifying short (250-800bp) regions in either the right or the left subtelomeric regions of the accessory unitigs 13, 14, 15, 16, 18, and 19 (see Table S4 for a list of all primers in this study). A chromosome was considered missing if both subtelomeric PCR showed no amplification. Previously, we have shown that the lack of both subtelomeric marker faithfully represents the loss of an accessory chromosome [13].

***In planta* phenotypic characterisation.** Infection of wheat was conducted with the 40 replicates of *Z. tritici* strains IPO323 and IPO323 $\Delta$ chr18 after 52 weeks of propagation and the respective progenitor strains (after 0 weeks of propagation) as described in [9]. A total of two experiments were conducted with a total of six leaflets being infected for each of the replicate and 48 leaflets infected for the progenitor strain. Briefly: Seeds of *Z. tritici* cultivar Obelisk were germinated on wet Whatmann paper for four days and before potting. Growth conditions for plants were 16h at a light intensity of  $\sim 200 \mu\text{mol}/\text{m}^2\text{s}^{-1}$  and 8 h darkness in growth chambers at 20°C with 90% humidity. Following potting, plants were further grown for seven days before infection. An  $\sim 5$  cm long section on the second leaf of each plant was infected by brushing a cell suspension with  $10^7$  cells/ml in  $\text{H}_2\text{O} + 0.1\%$  Tween 20 on the abaxial and adaxial side of the second leaf. Plants were placed into sealed bags containing  $\sim 1$  L of water for 48 h to facilitate infection at maximum air humidity. Infected leaf areas were harvested 21 days post infection for further phenotypic analysis and the pycnidia density was determined by scanning and automated image analysis [9,37].

**Data Analysis.** Statistical analyses were conducted in R (version R3.4.1) [57] using the suite R Studio (version 1.0.143) [58]. Absolute frequency tables of chromosome losses were analysed using the Fisher's exact test [59]. Plant phenotypic data was obtained for strain IPO323 and IPO323 $\Delta$ chr18. Data inspection of *in planta* phenotypic data showed a non-normal distribution of pycnidia density (pycnidia/cm<sup>2</sup>). Therefore, we performed an omnibus analysis of variance using rank-transformation of the data [60]. For the data sets, we defined with the following models: (i) pycnidia density  $\sim$  replicate \* experiment and ii) pycnidia density  $\sim$  number of chromosomes lost \* replicate \* experiment. The Shapiro-Wilk test [61] showed normal distribution of the residuals using this model (IPO323:  $p=0.3464$ , IPO323 $\Delta$ chr18:  $p=0.2186$ ). Post hoc tests were performed using Tukey's HSD [62]. Synteny analysis was conducted with SyMAP version 4.2 [63] using a minimum contig size of 10.000 for Zt05 and Zt10 and 100.000 for IPO323.

## Acknowledgments

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## Supporting Information

S1 Table: All PCR based karyotyping results for progenitor and replicates of IPO323, IPO323 $\Delta$ chr18,  $\Delta$ *kmt1*,  $\Delta$ *kmt6* at 13, 26, 39 and 52 weeks of propagation

See attached Excel file

S2 Table: All PCR based karyotyping results for progenitor and replicates of Zt05 at 52 weeks of propagation

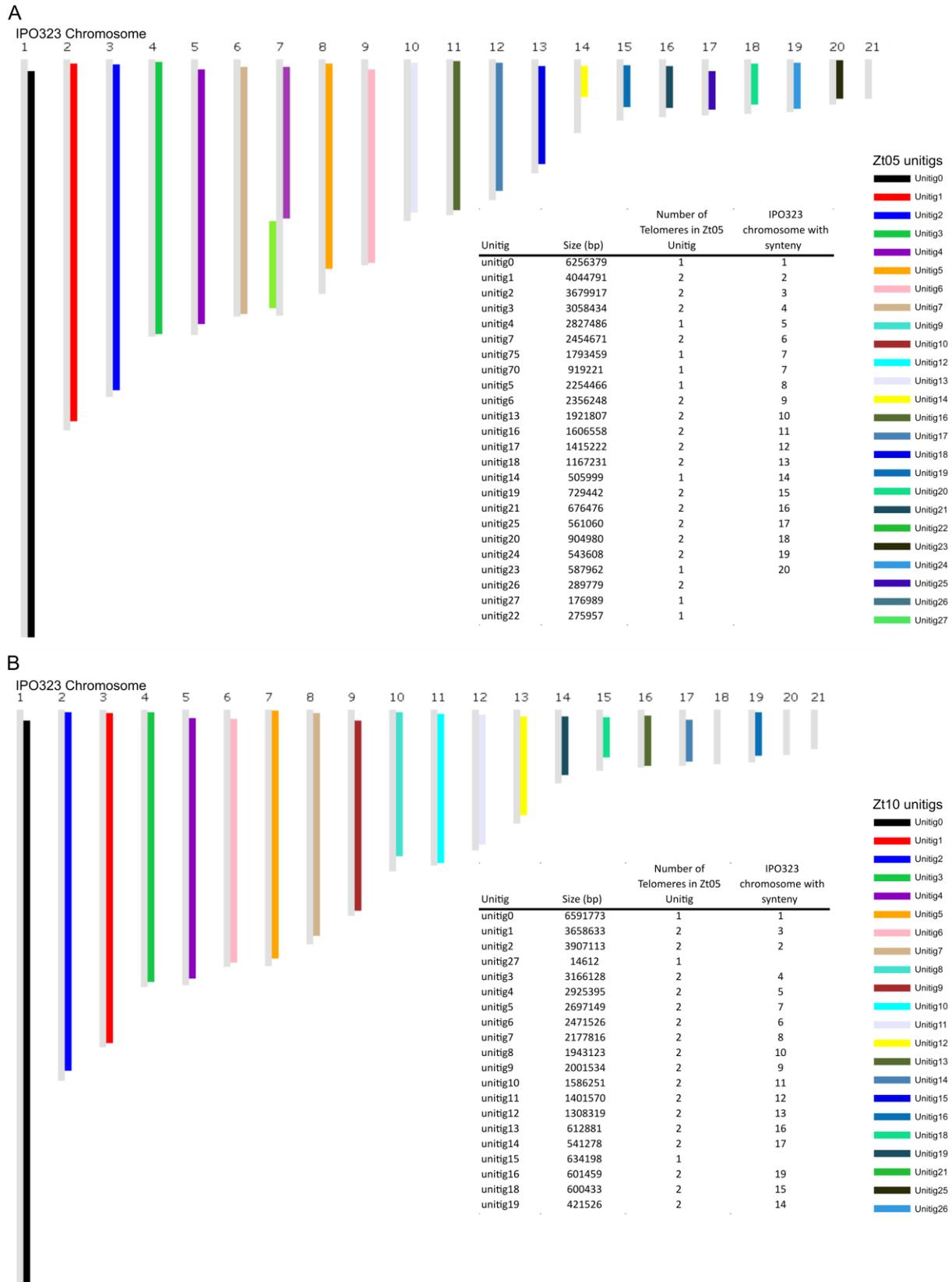
See attached Excel file

S3 Table: All PCR based karyotyping results for progenitor and replicates of Zt10 at 52 weeks of propagation

See attached Excel file

S4 Table: List of all primers used within this study

See attached Excel file



**S1 Figure: Graphical display and table summarizing synteny information of A) Zt05 and B) Zt10 on IPO323 reference genome. Synteny was constructed using SyMAP. Table includes unitigs that included at least one telomere.**





## Chapter 6

Manuscript prepared for resubmission in Molecular Plant Pathology

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# The transcription factor Zt107320 affects growth and virulence of the fungal wheat pathogen *Zymoseptoria tritici*

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Running head: Zt107320 regulates virulence in *Z. tritici*

Keywords: incompatible host, pycnidia formation, septoria tritici blotch

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### Summary

*Zymoseptoria tritici* is a filamentous fungus causing Septoria tritici blotch in wheat. The pathogen has a narrow host range and infections of other grasses are blocked early after stomatal penetration. During these abortive infections the fungus shows a markedly different expression pattern. However, the underlying mechanism of this differential gene expression between host and non-host is largely unknown, but likely includes transcriptional regulators to induce an infection program in a compatible host. In the rice blast pathogen *Magnaporthe oryzae*, MoCOD1, a member of the fungal Zn(II)<sub>2</sub>Cys<sub>6</sub> transcription factor family, has been shown to directly affect pathogenicity. Here we analyse the role of the putative transcription factor Zt107320, a homolog of MoCOD1, during the compatible and non-compatible infection of *Z. tritici*. We show for the first time that Zt107320 is differentially expressed in host versus non-host infections and that the lower expression corresponds to an abortive infection in non-hosts. Using deletion and complementation strains we further show that Zt107320 regulates the growth rate of the fungus and affects the cell wall composition *in vitro*. Moreover,  $\Delta$ Zt107320 strains showed reduced pycnidia production during the compatible infections of wheat. We conclude that Zt107320 directly influences the fitness of the fungus and propose that Zt107320 regulates the growth processes and pathogenicity during infection. Our results suggest that this putative transcription factor is involved in discriminating compatible and non-compatible infections.

## Introduction

The fungus *Zymoseptoria tritici* (synonym *Mycosphaerella graminicola*) infects wheat and causes the disease Septoria tritici blotch. The fungus is found worldwide where wheat is grown and can cause severe yield reduction (Fones and Gurr, 2015). Upon infection the fungus enters the leaf through stomata and establishes a hyphal network in the mesophyll. It propagates without causing visual symptoms for 7-14 days before inducing necrosis and producing pycnidia – asexual fructifications (Kema, G. H. J. *et al.* 1996). Under experimental conditions, the fungus has a narrow host range infecting wheat and shows abortive infections on closely related non-host grass species like *Triticum monococcum* (Jing *et al.* 2008) and *Brachypodium distachyon* (Kellner *et al.* 2014; O'Driscoll *et al.* 2015). However, the underlying determinants of host specialisation of *Z. tritici* are largely unknown.

A previous study comparing the expression profiles of *Z. tritici* during early infection (4 days post infection) of the compatible host *T. aestivum* and the non-host *B. distachyon* revealed a set of 289 genes that were similarly expressed in the two hosts, but differentially expressed compared to growth in axenic culture (Kellner *et al.* 2014). These genes are likely crucial for *Z. tritici* during stomatal penetration that occurs in same way in both hosts. However, 40 genes showed differential expression between host and non-host infections (Kellner *et al.* 2014) and are possibly involved in the discrimination of compatible and non-compatible host-pathogen interactions. The signalling and regulatory networks responsible for these differential expression patterns are unknown. One of the differentially expressed genes encodes a putative transcription factor Zt107320. Expression of *Zt107320* was significantly increased during infection of *T. aestivum* compared to the early infection of *B. distachyon* (Kellner *et al.* 2014) suggesting a host-dependent regulation of the gene.

*Zt107320* encodes a putative transcription factor belonging to the Zn(II)<sub>2</sub>Cys<sub>6</sub> family. This family of transcription factors is exclusive to fungi (MacPherson *et al.* 2006; Pan and Coleman, 1990) and many members of this gene family play an important role in the regulation of fungal physiology. For example, Zn(II)<sub>2</sub>Cys<sub>6</sub> transcription factors in *Magnaporthe oryzae*, *Fusarium oxysporum* and *Leptosphaeria maculans* are involved in the regulation of fungal growth and pathogenicity (Fox *et al.* 2008; Galhano *et al.* 2017; Imazaki *et al.* 2007; Lu *et al.* 2014). Interestingly, the homolog of *Zt107320* in the rice blast pathogen *M. oryzae*, MoCOD1, was shown to affect conidiation and pathogenicity (Chung *et al.* 2013). *MoCOD1* was shown to be upregulated during conidiation, appressorium formation and at 72h post infection. Furthermore,  $\Delta$ *MoCOD1* strains showed defects in conidial germination and appressorium formation. *In planta* the deletion strain  $\Delta$ *MoCOD1* was attenuated in extending growth from the first-invaded cells and caused markedly reduced symptoms when compared to the wildtype (Chung *et al.* 2013).

Transcription factors regulate expression by integrating various signalling pathways and represent interesting targets for dissecting causes and mechanism of pathogenicity and host specificity. In *M. oryzae*, a systemic approach was applied to characterise all 104 members of the Zn(II)<sub>2</sub>Cys<sub>6</sub> family of transcription factors. Of these, 61 were shown to be involved in fungal

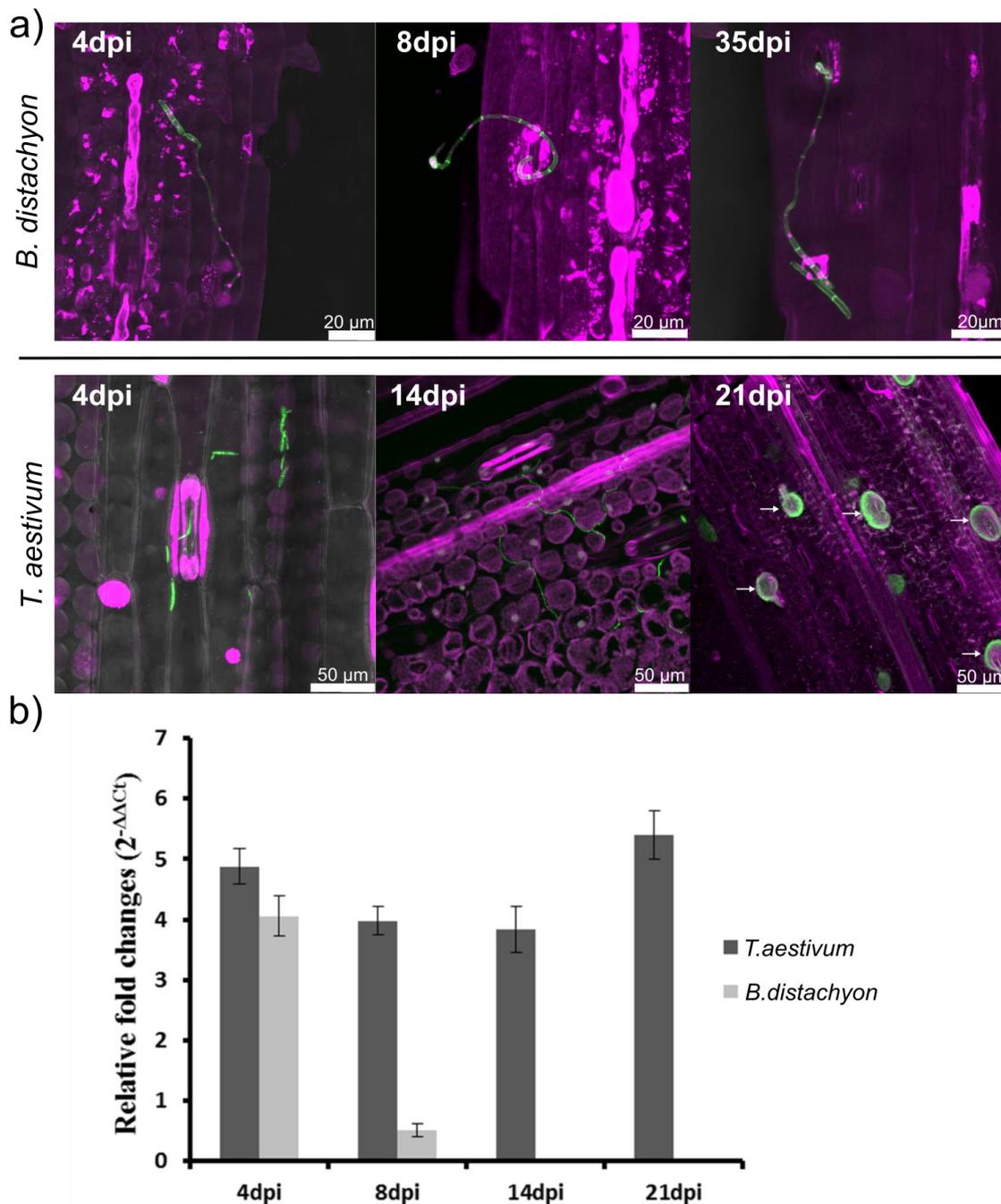
development and pathogenicity (Lu *et al.* 2014). In contrast, in *Z. tritici* only few regulatory genes have been characterised. Recently, two transcription factors ZtWor1 (Mirzadi Gohari *et al.* 2014) and ZtVf1 (Mohammadi *et al.* 2017) were shown to be important regulators of development and virulence of *Z. tritici* during compatible infections of its wheat hosts, highlighting how transcription factors can be used to identify and dissect aspects of pathogenicity.

Based on the close homology of *Zt107320* to *MoCOD1* and the differential expression during host and non-host infections, we hypothesized that *Zt107320* plays an important role during the early stages of infection of *Z. tritici* in its host wheat. Our results confirm that *Zt107320* affects the virulence of *Z. tritici* during compatible infections and regulates the growth rate and cell wall properties of this important plant pathogen.

## Results

### Infections of *Z. tritici* are blocked in the substomatal cavities during non-compatible interaction with *B. distachyon*

To describe the compatible and non-compatible infections of *Z. tritici* in more detail, we inoculated leaves of 11 days old seedlings of *T. aestivum* (cultivar Obelisk) and *B. distachyon* (ecotype Bd21), and analysed the infection development by confocal microscopy. Fungal cells germinated upon contact with the leaf surface and developed infection hyphae. In contrast to previous observations (O'Driscoll *et al.* 2015), we found that *Z. tritici* infection hyphae entered into open *B. distachyon* stomata at four days post inoculation (dpi). However, further infection development of *Z. tritici* was blocked in the substomatal cavities of *B. distachyon* leaves (Fig. 1a) similar to phenotypes previously observed in other incompatible interactions with einkorn wheat (Jing *et al.* 2008, 2008). Consequently, *Z. tritici* hyphae did not colonize the mesophyll tissue of *B. distachyon*, no necrotic lesions developed, and no asexual pycnidia formed. Interestingly, the fungal growth was completely halted and no further growth was observed, even when leaves were observed for up to five weeks post infection (Fig 1a). In contrast, compatible infection of *Z. tritici* on the wheat cultivar Obelisk was characterised by infection of the leaf stomata by 4 dpi, followed by the establishment of an extensive hyphal network in the mesophyll tissue of the leaf until 7-11 dpi followed by a switch to necrotrophy and a substantial increase in biomass resulting in the formation of pycnidia (Haeisen *et al.* 2017).



**Figure 1. *Z. tritici* infection hyphae are blocked in the substomatal cavities of *B. distachyon* which coincides with reduced expression of *Zt107320*.** a) Micrographs showing *Z. tritici* cells and emerging hyphae during penetration of *B. distachyon* stoma and *T. aestivum*. On *T. aestivum* *Z. tritici* germinated and penetrated the stomata at 4 dpi. Widespread growth of *Z. tritici* within the leaf mesophyll occurred until 14 dpi resulting in the formation of pycnidia (indicated by arrows) at 21 dpi. In contrast *Z. tritici* germinated on *B. distachyon* and penetrated stomata, but growth was halted at the stomata with no further growth (8dpi - 35dpi). Maximum projections of confocal image z-stacks. Nuclei and grass cells displayed in purple and fungal hyphae or septae, respectively in green. b) Transcription of *Zt107320* in compatible (*T. aestivum*) and non-compatible (*B. distachyon*) hosts. *In planta* expression of *Zt107320* relative to expression during axenic growth. Values are normalized to the expression of the gene encoding the housekeeping protein GAPDH. Error bars indicate the standard error of the mean (SEM) of three independent biological replicates per sample.

### *Zt107320* is expressed specifically during the compatible infection of wheat

Previously, differential expression between infection of the host *T. aestivum* and the non-host *B. distachyon* was detected for 40 genes at 4 dpi using an RNAseq based approach (Kellner *et al.* 2014). Building on this expression analysis, we focused on the transcription factor *Zt107320*. The expression kinetics of the gene *Zt107320* was further validated by RT-qPCR. We confirmed the previously described differential expression at 8 dpi (Fig. 1b). Expression in the non-host *B. distachyon* was greatly reduced at 8 dpi compared to expression in axenic cultures, whereas the expression of *Zt107320* during a compatible infection of the host *T. aestivum* was strongly upregulated during the course of the infection until 21 dpi.

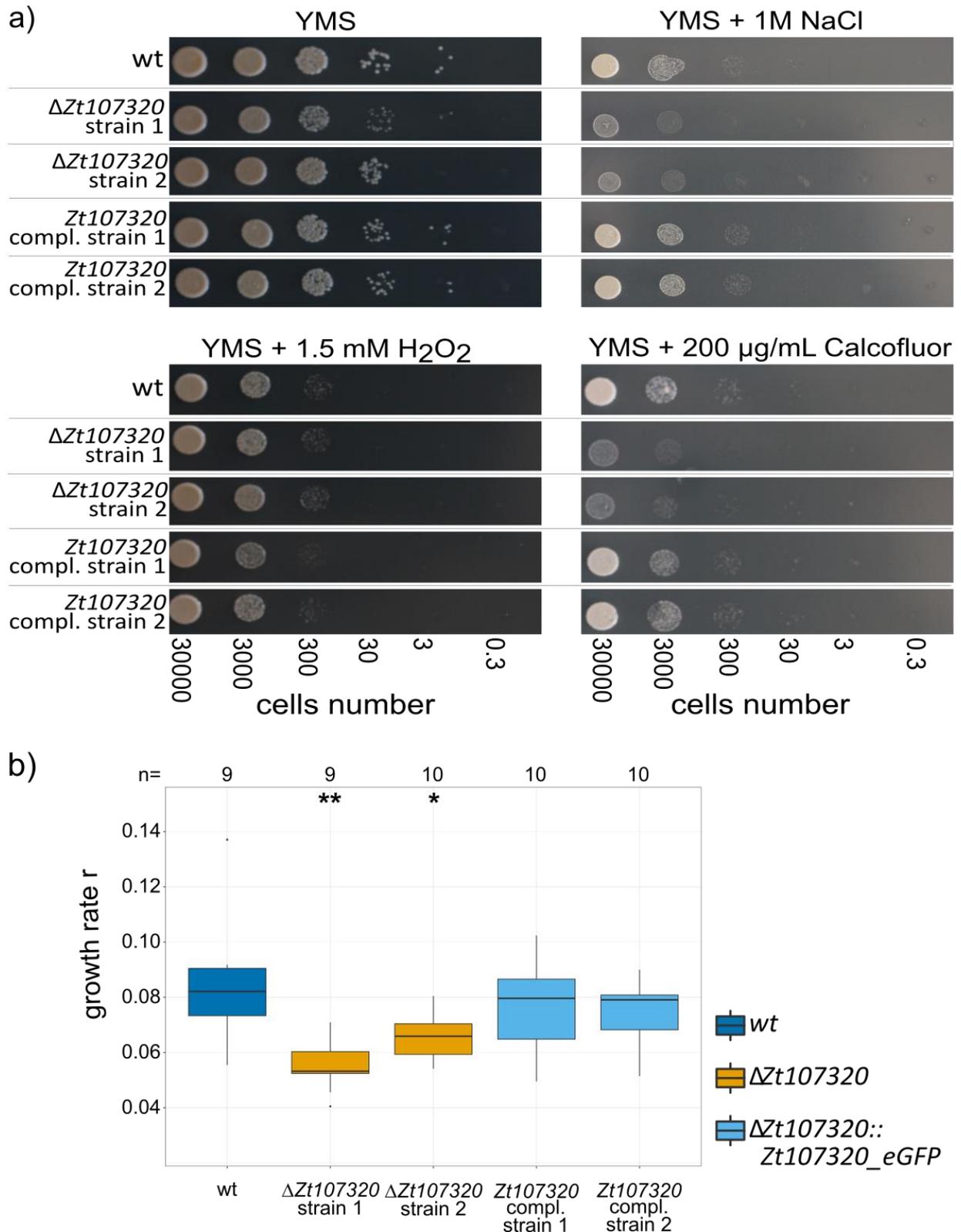
### *Zt107320* regulates growth and affects cell-wall properties

Based on the coinciding growth reduction and reduced expression of *Zt107320* in non-compatible infections, we next asked whether *Zt107320* is involved in the regulation of growth of *Z. tritici* *in vitro*. We determined the maximum growth rate  $r$  in liquid cultures of *Z. tritici* assuming a logistic growth curve model. For both independent deletion mutants, we observed a significant reduction in the maximum growth rate compared to the wildtype. In contrast, both tested complementation strains showed no significant difference in the growth rate compared to the wildtype (Fig. 2a).

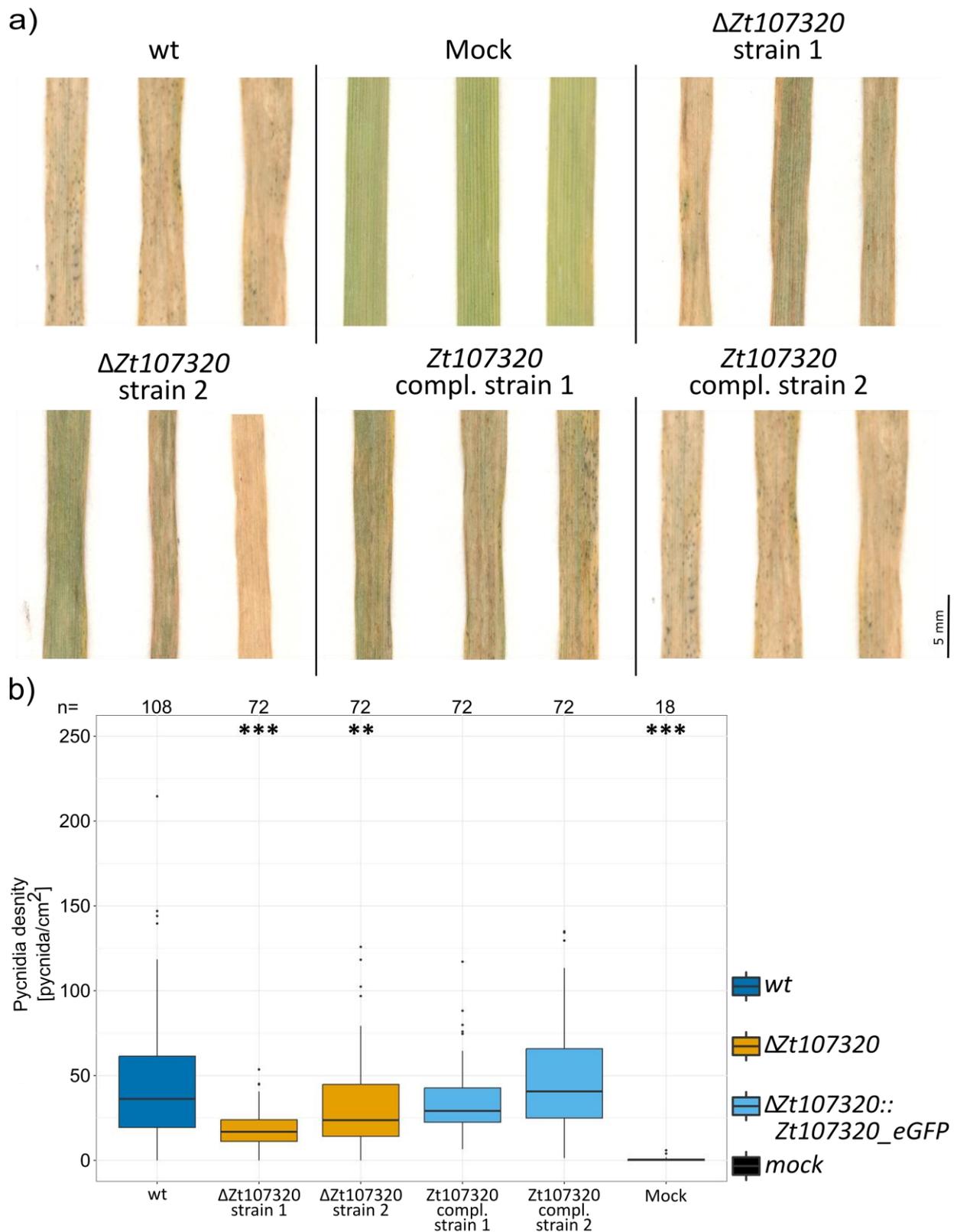
The effect of *Zt107320* deletion was not restricted to the growth rate but also included cell-wall properties. Compared to the wildtype we observed reduced growth *in vitro* when challenging the deletion mutants with high osmotic stresses (0.5 M and 1M NaCl, 1 M, 1.5 M and 2 M Sorbitol) as well as cell wall stressors (300  $\mu\text{g}/\text{mL}$  and 500  $\mu\text{g}/\text{mL}$  Congo red). Again, the wildtype phenotype was restored in both complementation strains (Fig. 2b and Fig. S1). Interestingly, temperature stress did not affect the wildtype and the deletion mutants differently, as well as the addition of  $\text{H}_2\text{O}_2$ . This indicates a specific effect of *Zt107320* on the cell wall properties but not on the ability of the fungus to counteract reactive oxygen species which are produced by the plant during the hypersensitive cell death response (Jones and Dangl, 2006).

### *Zt107320* influences the fitness of *Z. tritici* by affecting the number of pycnidia produced

We next addressed whether the effect of *Zt107320* deletion on the cell growth rate and the cell wall properties also affected the ability of *Z. tritici* to infect its host, *T. aestivum*. We infected a predefined area of the second leaf of the susceptible wheat cultivar Obelisk and determined the number of pycnidia 21 days post infection. We observed a pronounced reduction in the production of pycnidia. The density of pycnidia per  $\text{cm}^2$  of infected leaf area was reduced significantly for both independent deletions of *Zt107320* (ANOVA,  $p < 1 \cdot 10^{-7}$ ,  $p = 0.002$ ) (Fig. 3 a, b) compared to the wildtype. Complementation of the *Zt107320* in-locus restored the wildtype phenotype *in planta*. Pycnidia are the asexual fructifications containing pycnidiospores and the density of pycnidia is considered to be a quantitative measure for virulence (Stewart *et al.* 2016). Thus we conclude that the predicted transcription factor *ZT107320* regulates genes that determine fitness of *Z. tritici* during infection of the compatible wheat host.



**Figure 2. *Zt107320* regulates growth and affects cell wall properties of *Z. tritici*.** a) *In vitro* growth of the wildtype (wt), two deletion strains ( $\Delta Zt107320$ ), two complementation strains ( $\Delta Zt107320::Zt107320\_eGFP$ ) on YMS media including the indicated compounds to assess the role of osmotic stress (1M NaCl), reactive oxygen species (1.5 mM  $H_2O_2$ ) and cell wall stressors (200 mg/mL Calcofluor). b) Maximum growth rate in liquid culture. Statistical significance inferred through an ANOVA and subsequent post hoc Tukey's HSD comparing the deletion and complementation strains to the wildtype, is indicated as \* =  $p < 0.05$ ; \*\* =  $p < 0.005$ ; \*\*\* =  $p < 0.0005$ .



**Figure 3. *Zt107320* deletion affects the pycnidia production during a compatible infection of wheat.** a) Representative pictures of leaves infected with the *Z. tritici* wildtype (wt), two deletion strains ( $\Delta Zt107320$ ), two complementation strains ( $\Delta Zt107320::Zt107320\_eGFP$ ) and mock infected leaves. b) Boxplot depicting the pycnidia density (pycnidia per  $\text{cm}^2$  leaf) pooled for two independent experiments. Number of leaves (n) for each strain is indicated on top. Statistical significance, inferred through an ANOVA and subsequent post hoc Tukey's HSD comparing the deletion and complementation strains to the wt, is indicated as \*=  $p < 0.05$ ; \*\*=  $p < 0.005$ ; \*\*\*=  $p < 0.0005$ .

## Discussion

Here we show that the putative transcription factor Zt107320, belonging to the fungal Zn(II)<sub>2</sub>Cys<sub>6</sub> family, is involved in the infection program of *Z. tritici* during a compatible interaction with the host plant *T. aestivum*. Transcription of *Zt107320* is specifically induced during infection of wheat and not during the infection of the non-host *B. distachyon*. This suggests a functional role of this gene in the regulation of the infection program that allows the fungus to overcome host defences and propagate in the mesophyll tissue.

To date only a small number of genes of *Z. tritici* have been shown to attenuate virulence in this important wheat pathogen (Orton *et al.* 2011; Poppe *et al.* 2015; Rudd, 2015), among which are two chitin binding LysM effectors Mg3LysM and Mg1LysM (Lee *et al.* 2014; Marshall *et al.* 2011), two transcription factors ZtWor1 and ZtVf1 (Mirzadi Gohari *et al.* 2014; Mohammadi *et al.* 2017) and three rapidly evolving small proteins (Hartmann *et al.* 2017; Poppe *et al.* 2015; Zhong *et al.* 2017). The deletion of *Zt107320* results in a relatively small, nevertheless significant, reduction in the pycnidia density in contrast to the full or partially avirulent phenotype for the two previously deleted transcription factors ZtWor1 and ZtVf1, respectively (Mirzadi Gohari *et al.* 2014; Mohammadi *et al.* 2017). Similar to our observation in the *Zt107320* deletion mutant, deletion of *MoCOD1*, the rice blast homolog of *Zt107320*, led to a quantitative reduction in lesion size and number (Chung *et al.* 2013). This partial, but not complete reduction in virulence of  $\Delta Zt107320$  strains, corresponds with the reduced, but still substantial, growth rate of these deletion strains *in vitro*. Therefore, other mechanisms, independent of *Zt107320* are involved in the regulation of growth and development *in vitro* and *in planta*. Similarly, a partial, but not complete reduction of invasive growth was observed in  $\Delta MoCOD1$  (Chung *et al.* 2013). The effect of *Zt107320* on fungal growth and pathogenicity is similar to the effect of other members of the Zn(II)<sub>2</sub>Cys<sub>6</sub> transcription factor family; TPC1 regulates invasive polarized growth and virulence in the rice blast fungus *M. oryzae* (Galhano *et al.* 2017), and the FOW2 transcription factor is known to control the ability of *F. oxysporum* to invade roots and colonize plant tissue (Imazaki *et al.* 2007).

In conclusion, we showed that *Zt107320* affects the fitness of *Z. tritici*. *Zt107320* is differentially expressed between host and non-host, being upregulated during the early stages of infection on hosts and down-regulated on non-hosts. This down-regulation corresponds to a starkly reduced and halted growth of *Z. tritici* on the non-host *B. distachyon*. We therefore hypothesize that the putative transcription factor *Zt107320* belongs to the regulatory network that regulates growth, integrating signals that differ between compatible and non-compatible infections. Future studies should address the specific targets of *Zt107320* and their expression pattern during compatible and non-compatible interactions. Furthermore, the transcriptional regulation of *Zt107320* suggests that specific signals in the compatible host-pathogen interaction in wheat are responsible for the up-regulation of this particular transcription factor. Identification of these signals will provide fundamental insight into the molecular basis of host specificity in *Z. tritici*.

## Experimental Procedures

### Fungal and plant strains

The Dutch isolate IPO323 was kindly provided by Gert Kema (Wageningen, The Netherlands) and is available from the Westerbijk Institute (Utrecht, The Netherlands) with the accession number CBS 115943. The strain used in our experiments lacked accessory chromosome 18, presumably lost during culture maintenance in vitro. Strains were maintained in either liquid Yeast Malt Sucrose (YMS) broth (4 g/L Yeast extract, 4 g/L Malt extract, 4 g/L sucrose) at 18°C on an orbital shaker or on solid YMS (+20 g/L agar) at 18°C. The *T. aestivum* cultivar Obelisk was obtained from Wiersum Plantbreeding BV (Winschoten, The Netherlands). *B. distachyon* inbred line Bd21 was kindly provided by Thierry Marcel (Bioger, INRA, France).

### Analysis of *Z. tritici* during its compatible and non-compatible infections by confocal microscopy

The morphology of *Z. tritici* within and on the surface of leaves of *B. distachyon* inbred and *T. aestivum* were analysed by confocal laser-scanning microscopy (CLSM) as described previously (Hauelsen et al. 2017). For infection, distinct areas of the second leaf of 11 days old seedlings were brush-inoculated with  $1 \times 10^7$  cells/ml in 0.1 % Tween 20. Plants were incubated at 22 °C [day] and 20 °C [night] and 100 % humidity with a 16-h light period for 48 h. Subsequently, humidity was reduced to 70 %. Microscopy was conducted using a Leica TCS SP5 and analyses of the image z-stacks were done using the Leica Application Suite Advanced Fluorescence (Leica Microsystems, Wetzlar, Germany).

### RNA isolation and quantitative RT-PCR

We analysed gene expression patterns of *Zt107320* of *Z. tritici* employing a qRT-PCR experiment. Total RNA was extracted from fungal axenic cultures (grown for 72 h in YMS medium at 18°C and 200 rpm) and from snap-frozen leaf tissue infected with *Z. tritici* (4, 8, 14 and 21 dpi) using the TRIZOL reagent (Invitrogen, Karlsruhe, Germany), following the manufacturer's instructions. Three biological replicates were included in the experimental set up. The cDNA samples were used in a qRT-PCR experiment employing the iQ SYBR Green Supermix Kit (Bio-Rad, Munich, Germany). PCR was conducted in a CFX96 RT-PCR Detection System (Bio-Rad, Munich, Germany) with the constitutively expressed control gene Glyceraldehyde-3-phosphate dehydrogenase (GAPDH).

### Generation of *Zt107320* deletion mutants and complementation by gene replacement

Fungal transformations and the creation of knock-out and complementation mutants of *Zt107320* were conducted as previously described (Poppe *et al.* 2015). In brief: Gene deletions were created by amplifying an approximately 1 kb region of the 5' and 3' flanking regions of *Zt107320* using PCR. The amplified flanking sequences were fused to a hygromycin resistance (*hygR*) cassette and an *EcoRV* cut vector backbone (pES61) using Gibson assembly (Gibson *et al.* 2009). Electro-competent cells of the *Agrobacterium tumefaciens* strains AGL1 were transformed using standard protocols. These transformed *A. tumefaciens* cells were used for the transformation of *Z. tritici* as previously described (Zwiers and De Waard, Maarten A. 2001).

The same strategy was applied for the creation of the complementation strains by a C-terminal fusion of the *Zt107320* gene with an eGFP tag and a geneticin resistance cassette as a selection marker (Fig. S2). After transformation and homologous recombination in the  $\Delta Zt107320$  strain 1, the hygromycin resistance cassette was replaced by *Zt107320-eGFP* and the geneticin resistance cassette (NeoR) (Fig. S2). Homologous recombination and integration was confirmed using PCR and Southern blot analysis by standard protocols. In short, genomic DNA was isolated using Phenol-Chloroform isolation (Sambrook and Russell, 2001). Restriction digestion was performed using PvuII, followed by gel-electrophoresis, blotting and detection using Dig-labelled probes binding to the upstream and downstream flank of *Zt107320* (Fig. S2). In total four  $\Delta Zt107320$  strains, and two  $\Delta Zt107320::Zt107320$ -eGFP strains were created.

#### Isolation of fungal DNA and Southern blot analysis

DNA was isolated using phenol/chloroform applying a protocol previously described (Sambrook and Russell, 2001). Transformants were first screened using a PCR based approach detecting the resistance cassette and the endogenous locus. Candidate transformants were further confirmed by Southern blot analysis using a standard protocol (Southern, 1975). Probes were generated using the PCR DIG labelling Mix (Roche, Mannheim, Germany) according to the manufacturer's instructions (Table S1).

#### *In vitro* phenotyping

The *Z. tritici* strains were grown on YMS solid medium for 5-7 days at 18°C before the cells were scraped from the plate surface. For the determination of the growth rate, the cells were resuspended and counted. The cell density was adjusted to 50000 cells/mL and 175µl of the cell suspension was added to a well of a 96 well plate. Plates were inoculated at 18°C at 200 rpm with the OD600 being measured twice daily on a Multiskan Go plate reader (Thermo Scientific, Dreieich, Germany) (Table S2). Estimation of the growth rate was done by employing the logistic growth equation as implemented in the growthcurver package (version 0.2.1) in R (version R3.4.1) (R Core Team, 2015). For the determination of the *in vitro* phenotypes, the cell number was adjusted to 10<sup>7</sup> cells/mL in ddH<sub>2</sub>O and serially diluted to 10<sup>3</sup> cells/mL. 3 µL of each cell dilution was transferred onto YMS agar including the tested compounds and incubated for seven days at 18°C or 28°C. To test high osmotic stresses 0.5 M NaCl, 1 M NaCl, 1M Sorbitol, 1.5 M Sorbitol, 2 M Sorbitol (obtained from Carl Roth GmbH, Karlsruhe, Germany) were added to the YMS solid medium. To test cell wall stresses 300 µg/mL and 500 µg/mL Congo red and 200 µg/mL Calcofluor (obtained from Sigma-Aldrich Chemie GmbH, Munich, Germany) were added to the YMS solid medium. Finally, to determine the effect of reactive oxygen species on the mutant growth morphology 2 mM H<sub>2</sub>O<sub>2</sub> (obtained from Carl Roth GmbH, Karlsruhe, Germany) was added to the YMS solid medium.

### *In planta* phenotyping

For the *in planta* phenotypic assays, we germinated seeds of the wheat cultivar Obelisk on wet sterile Whatman paper for four days before potting using the soil Fruhstorfer Topferde (Hermann Meyer GmbH, Rellingen, Germany). Wheat seedlings were further grown for seven days before inoculation. *Z. tritici* strains were grown in YMS solid medium for five days at 18°C before the cells were scraped from the plate surface. The cell number was adjusted to 10<sup>8</sup> cells/mL in H<sub>2</sub>O + 0.1% Tween 20, and the cell suspension was brushed onto approximately five cm on the abaxial and adaxial sides of the second leaf of each seedling. Inoculated plants were placed in sealed bags containing water for 48 h to facilitate infection through stomata. Plants were grown under constant conditions with a day night cycle of 16h light (~200µmol/m<sup>2</sup>\*s) and 8h darkness in growth chambers at 20°C. Plants were grown for 21 days post inoculation at 90% relative humidity (RH). At 21 dpi the infected leaves were cut and taped to sheets of paper and pressed for five days at 4°C before being scanned at a resolution of 2400 dpi using a flatbed scanner (HP photosmart C4580, HP, Böblingen, Germany). Scanned images were analysed using an automated image analysis in Image J (Schneider *et al.* 2012) adapted from (Stewart *et al.* 2016). The read-out pycnidia/cm<sup>2</sup> leaf surface was used for all subsequent analyses. See Table S3 for summary of *in planta* results.

### Statistical analysis

Statistical analyses were conducted in R (version R3.4.1) (R Core Team, 2015) using the suite R Studio (version 1.0.143) (RStudio Team, 2015). Data inspection showed a non-normal distribution for all data sets, including the measured pycnidia density (pycnidia/cm<sup>2</sup>). Therefore, we performed an omnibus analysis of variance using rank-transformation of the data (Conover and Iman, 1981) using the model: pycnidia density ~ strain \* experiment and  $r \sim$  strain, respectively. Post hoc tests were performed using Tukey's HSD (Tukey, 1949).

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## Supplementary Information

**Figure S1. In vitro phenotype of Zt107320 mutants.** *In vitro* growth of the wildtype (wt), two independent deletion strains ( $\Delta Zt107320$ ), two independent complementation strains ( $\Delta Zt107320::Zt107320\_eGFP$ ) on YMS media including the indicated compounds to assess the effect of osmotic stress (NaCl, Sorbitol), reactive oxygen species ( $H_2O_2$ ), cell wall stressors (Calcofluor, Congo Red) and increased temperature (28°C) on growth and morphology of *Z. tritici*.

See attached PDF file.

**Figure S2. Generation of Zt107320 mutants in Z. tritici.** a) Schematic illustration of the gene replacement strategy of *Zt107320*. Generation of the  $\Delta Zt107320$  strains by homologous recombination between the upstream (UF) and downstream flanking regions (DF) of *Zt107320* in its genomic locus and a plasmid carrying the hygromycin resistance cassette (*hygR*) located between the UF and DF. Homologous recombination results in the integration of the hygromycin-resistance gene cassette (*hygR*)

in the locus of *Zt107320*.  $\Delta Zt107320::Zt107320\_eGFP$  were generated by homologous recombination between UF and DF of the  $\Delta Zt107320$  strains and a transformed plasmid containing a c-terminal fusion of *Zt107320* and eGFP and a geneticin resistance cassette (NeOR) located between the UF and DF Yellow bars indicate the position of the probes used in the Southern blot analyses. b) Confirmation of *Zt107320* mutants by Southern blot analyses.

See attached PDF file.

**Table S1: List of all primers used within this study**

Primer Number	Description	Sequence
oES1856_u2_107320	$\Delta Zt107320$	TACGAATTCTTAATTAAGATCGAATTGTGGACTGAACC
oES1857_u3_107320	$\Delta Zt107320$	TGCTCCTCAATATCAAAGCATTGTGCTGTCAACTC
oES1858_k1_107320	$\Delta Zt107320$	TGAGTGGTGACAGCACAATGCTTTGATATTGAAGGAGC
oES1859_k2_107320	$\Delta Zt107320$	GGATAAAGGCGGTCCACCTGGCTAGCAGATCTCTATTC
oES1860_d1_107320	$\Delta Zt107320$	GGAATAGAGATCTGCTAGCCAGGTGGACCGCTTTATC
oES1861_d2_107320	$\Delta Zt107320$	TCGAGGGTACCGAGCTCGATGCCATGTAGCTGGGATTG
oeS2513_eGFP_Fw	$\Delta Zt107320::Zt107320\_eGFP$	AAGTATGATTTCGGCTGGAGGAGGCGCGGAGGAGGCATGGTG AGCAAGGGCGA
oeS2514_eGFP_Rv	$\Delta Zt107320::Zt107320\_eGFP$	AAAATGCTCCTCAATATCAGGTTTACTTGTACAGCTCGTCC
oeS2515_K1_Gen_ovehang	$\Delta Zt107320::Zt107320\_eGFP$	GGACGAGCTGTACAAGTAAACCTGATATTGAAGGAGCATTTT
oeS2516_ORF1_Fw	$\Delta Zt107320::Zt107320\_eGFP$	TTTCTTTCCCATCTTCAGTATGCTGATCTCCACGCTGTC
oeS2517_ORF1_Rv	$\Delta Zt107320::Zt107320\_eGFP$	TCGCCCTTGCTCACCATGCCTCCTCCGCCGCTCCTCCAGCCGAA TCATACTCG
oeS2518_P1_gpdA_Fw	$\Delta Zt107320::Zt107320\_eGFP$	CTGGTCATGACATATCACTGGTACCCGGGGATCTTTCGAC
oeS2519_P2_gpdA_Rv	$\Delta Zt107320::Zt107320\_eGFP$	CAGCGTGGAGATCAGCATACTGAAGATGGGAAAAGAAAGA
oeS2520_U3_ovehang	$\Delta Zt107320::Zt107320\_eGFP$	CGAAAGATCCCCGGGTACCAGTGATATGTCATGACCAG
oeS2521_107320_Rv_ovehang	$\Delta Zt107320::Zt107320\_eGFP$	TCGCCCTTGCTCACCATGCCTCCTCCGCCGCTCCTCCAGCCGAA TCATACTT
oeS2521_107320_Rv_ovehang	$\Delta Zt107320::Zt107320\_eGFP$	TCGCCCTTGCTCACCATGCCTCCTCCGCCGCTCCTCCAGCCGAA TCATACTT
oES2557	$\Delta Zt107320::Zt107320\_eGFP$	CGGATAAAGGCGGTCCACCTCAGAAGAAGTCAAG
oES2558	$\Delta Zt107320::Zt107320\_eGFP$	CTTGACGAGTCTTCTGAAGGTGGACCGCTTTATCCG
oES879	<i>GAPDH</i> _qPCR	CCGAGAAGGACCCAGCAAAC
oES880	<i>GAPDH</i> _qPCR	TGACGGGAATGTCGGAGGTG
oES950	<i>Zt107320</i> qPCR	GCCGTACCCGATCATCAAAC
oES951	<i>Zt107320</i> qPCR	GGCTGCCGTCGTAATTTCC

**Table S2: Summary of *in vitro* growth data**

See attached Excel file

**Table S3: Summary of *in planta* phenotype**

See attached Excel file





## General Discussion

Accessory chromosomes are widespread and can be found in plants, animals and fungi [1,2]. In plants and animals accessory chromosomes (called B chromosomes in these species) often have a neutral or negative effect on the fitness of the organism. In contrast, some fungal accessory chromosomes provide a fitness benefit under certain conditions (e.g. specific host infections) while for others a function is yet to be determined [3–5]. Many of these chromosomes in plants, animals, and fungi show a non-Mendelian segregation during meiosis or a segregation advantage during mitosis. This transmission advantage is considered to account for the continued maintenance of most plant and animal B chromosomes, which thus are considered selfish genetic elements [6]. However, in fungi the relevant transmission processes are largely unknown. Here I used the wheat-pathogenic ascomycete *Z. tritici* which contains a large complement of accessory chromosomes as a model for analysis of their function and transmission dynamics. My main findings are the following:

- i) The accessory chromosomes of *Z. tritici* confer fitness costs *in planta*, in dependence of the host genotype.
- ii) Accessory chromosomes, which lack a homolog in the diploid zygote (i.e. being unpaired), are transmitted to all meiotic progeny when inherited from the female parental strain, thereby showing meiotic drive.
- iii) Transmission of accessory chromosomes during mitosis is associated with very high genome instability and frequent losses of accessory chromosomes.
- iv) The rate of these chromosome losses is affected by epigenetic histone modifications and varies between isolates.
- v) The interaction between *Z. tritici* and wheat is regulated by transcription factor Zt107320, which influences the growth of *Z. tritici in planta* and *in vitro* and could be a signal integration point that discriminates compatible from non-compatible infections.

Based on these main findings, I conclude that the presence/absence polymorphism of the accessory chromosomes of *Z. tritici* is determined on the one hand by their frequent loss during mitosis and meiosis and also as a consequence of selection imposed by the fitness costs *in planta*, and on the other hand, by the meiotic drive mechanism that increases the frequency of the accessory chromosomes during sexual crosses. Hence, the accessory chromosomes in *Z. tritici* resemble B chromosomes in transmission and functional effect.

The meiotic drive we identified for the accessory chromosomes of *Z. tritici* is likely to include an additional amplification of female-inherited unpaired accessory chromosomes. This represents a novel mode of a meiotic drive and importantly could have implications not only for other fungal accessory chromosomes but also for our general understanding of meiosis. A feedback between pairing of homologs and DNA replication is currently not considered in the canonical meiosis although for several model organism pairing of homologs can precede DNA replication [7–11]. The ability to compare the meiotic transmission of the accessory chromosomes when these are unpaired or paired in *Z. tritici* represents a unique opportunity

to dissect this mechanism in more detail and determine the underlying genetic and epigenetic factors. This is not possible with many of the popular genetic model organisms as these either lack accessory chromosomes or the here described unusual meiotic drive mechanism. Considering the wide distribution of accessory chromosomes and non-Mendelian transmission, I therefore propose the use of *Z. tritici* as a model for meiosis and meiotic transmission of unpaired chromosomes. The results of this doctoral thesis are testimony to the potential of this model system to identify novel aspects of basic cellular processes.

In general, the accessory chromosomes of *Z. tritici* allow us to follow transmission of whole chromosomes during all cell divisions and to determine neutral rates of changes on eukaryotic chromosomes. Since the accessory chromosomes are not required for normal growth and development, selection should not constrain or bias the observable changes. As shown in this doctoral thesis the analysis of genome instability and the effect of histone modifications can be highly informative. The findings may be insightful for our understanding of processes where chromosome stability or genomic changes can cause disease. Cancer is often characterized by either aneuploid or polyploid cells with frequent genomic rearrangements. Interestingly, expression from meiosis-specific genes is considered oncogenic in cancer genesis [12,13]. Therefore, the results of this doctoral thesis could combine these different aspects of cancer genesis and may be helpful in identifying shared mechanisms underlying aneuploidy and genome rearrangements and/or generally conserved processes enhancing cancer.

In addition, the accessory chromosomes of *Z. tritici* can serve as a model for other fungal accessory chromosomes. For many fungal accessory chromosomes a function has yet to be identified and many show a non-Mendelian meiotic transmission similar to *Z. tritici*. The approach used within this doctoral thesis might therefore help the dissection of the function and transmission of accessory chromosomes in other fungal species. The generation of whole chromosomes deletion strains and their use for the functional dissection as well as for the analysis of meiotic transmission should be applicable to sexual fungi, like *Leptosphaeria maculans* and *Cochliobolus heterostrophus*, for which a transmission advantage of the accessory chromosomes was reported [14,15].

The above overview highlights that the results of this doctoral thesis may be of broader interest, yielding new insights in diverse fields ranging from accessory chromosome biology, dynamics of selfish genetic elements and their influence on disease to basic processes governing meiosis. In the following, I will recapitulate and briefly discuss the specific results of the individual chapters and conclude with a perspective on the most important open questions and resulting opportunities for future research.

In chapter 2 I focussed on the function of the accessory chromosomes of *Z. tritici*. By disrupting the mitotic spindle I was able to induce aneuploidy and to generate whole chromosome deletion strains. Interestingly, deletion of accessory chromosome 14, 16, 18, 19 or 21 resulted in increased virulence *in planta*. This effect was influenced by the host genotype. Previous studies were based on meiotic progeny to compare strains with and without specific accessory chromosomes [16] or to map QTLs for specific traits [17,18]. Either no or only small functional effects of the accessory chromosomes were detected. It is worth noting that these studies did not compare isogenic lines that exclusively differed in the presence/absence of the accessory chromosome and, hence, they cannot exclude any bias due to the genomic background. This is different in the current study, in which I could identify significant fitness costs of the accessory chromosomes. These costs should lead to rapid elimination of these chromosomes from the population unless a counteracting selective force or transmission advantage of the accessory chromosome acts to maintain them. Since the accessory chromosomes of *Z. tritici* show homology and synteny with accessory chromosomes of closely related sister species *Z. ardabiliae* and *Z. pseudotritici* which indicates their maintenance in *Z. tritici* since at least the speciation event [19–21] such counteracting dynamics seems to be highly likely.

Since no fitness advantage but rather a fitness cost was associated with the accessory chromosomes of *Z. tritici* I next asked in chapter 3 whether the accessory chromosomes of *Z. tritici* are maintained by a transmission advantage similar to the mechanisms previously found to maintain B chromosomes in plants and animals. Using the previously established isogenic whole chromosome deletion strains, I compared transmission of an accessory chromosome while being paired (i.e. having a homolog within the diploid zygote) with its transmission when being unpaired (i.e. having no homolog in the diploid zygote). Interestingly, how unpaired chromosomes are transmitted during meiosis is unknown despite the widespread occurrence of supernumerary chromosome which are likely to be unpaired during meiotic divisions. For most model organisms pairing between chromosomes is essential for the proper synapsis and segregation of the two homologues during the first meiotic division (reviewed in [22]). Therefore, it is fascinating to explore how meiosis affects unpaired chromosomes.

I demonstrated that female-inherited unpaired chromosomes are transmitted to all meiotic progeny, whereas when the same chromosome is paired or inherited from the male parent the chromosomes follow Mendelian segregation. These findings suggest that female-inherited unpaired chromosomes undergo an additional amplification, which requires a feedback between the pairing of the chromosomes and the meiotic S-phase. In canonical meiosis the meiotic S-phase is concluded prior to the establishment of double-stranded-breaks (DSB) by Spo11. DSBs are in turn required for the pairing of homologous sequences [22] making such a feedback unlikely. However, the deletion of *SPO11* decreases the length of meiotic S-phase by ~25% which indicates that S-phase progression is directly linked to the development of meiotic interhomolog interactions [23,24]. In addition, pairing of homologous chromosomes or sequences occurs in somatic cells in *Saccharomyces cerevisiae*, *Schizosaccharomyces pombe* and *Drosophila melanogaster* [7–11]. Indeed several mechanisms exist in which homology can be recognized independently of DSB and recombination. Examples are repeat induced point

mutations (RIP) and methylation-induced premeiotically (MIP) mechanisms in haploid nuclei preparing for karyogamy in fungi (reviewed in [25]) and somatic or Spo11-independent homology-dependent pairing or interactions between sequences in *D. melanogaster*, *S. cerevisiae*, *S. pombe*, *Sordaria macrospora*, *Mus musculus* and *Caenorhabditis elegans* (reviewed in [22,26,27]). Hence, it is conceivable that unpaired chromosomes are recognized during meiosis and DNA replication is re-initiated.

Currently, it is unknown why this additional amplification of DNA is restricted to those unpaired chromosomes inherited from the female parent. We speculate that this could involve an epigenetic imprinting mechanism. Importantly, the meiotic drive first described here, will increase the frequency of the accessory chromosomes and might therefore be responsible for their long-term maintenance in *Z. tritici*. Intriguingly, the meiotic drive mechanism is mechanistically different from all previously reported mechanisms of B chromosome drive in plants and animals. Here, B chromosomes show a transmission advantage based on preferential segregation in non-symmetric cell division ensuring their transmission to cells that will give rise to the germline [6]. In contrast, meiotic drive in *Z. tritici* is most likely based on an additional round of DNA replication.

What regulates the meiotic drive of unpaired accessory chromosomes? Interestingly, we observed the meiotic drive of unpaired accessory chromosomes for 7 of the 8 accessory chromosomes of the reference isolate IPO323. Chr14 for which no such transmission advantage was observed, is the largest of the accessory chromosomes (773 kb) in IPO323 [28]. A large insertion spanning approx. 400 kb in chr14 shows presence/absence polymorphism in *Z. tritici* resulting in isolates that contain a much smaller chr14 [29]. Interestingly these smaller chr14 are transmitted to more than 50% of the progeny when present only in one of the parental strains [29]. I therefore hypothesize that the additional replication of the accessory chromosomes that leads to their transmission advantage might be influenced by the size of the chromosome. Chromosome size could possibly also affect the localisation of accessory chromosomes in the nucleus and in turn impact their replication and transmission but support for this model is still missing.

*Z. tritici* propagates via sexually produced ascospores as well as via asexually generated pycnidiospores. Hence, I was also interested in how accessory chromosomes are transmitted during mitotic divisions. In chapter 4 we were able to show that the loss of accessory chromosomes occurs during somatic growth *in vitro* and most importantly also *in planta* and that elevating the temperature caused a significant increase in the instability of the genome with losses of accessory chromosomes increasing in frequency within short periods of time. Therefore, the loss of accessory chromosomes during vegetative growth *in planta* contributes to the presence/absence polymorphism in *Z. tritici*. Based on these results I became interested in the factors that influence the transmission of the accessory chromosomes in chapter 5. As previous studies failed to eliminate the effect of selection on the observed rate of chromosome loss I here determined the neutral rate of chromosome loss by using a mutation accumulation approach with random one cell bottlenecks. These mutation accumulation approaches have been used for a number of haploid and diploid model organisms but failed to address how

accessory chromosomes are subject to and influenced by genome stability [33–36]. I determined the effect of the number of accessory chromosomes and histone modifications on the neutral loss rate of accessory chromosomes as well as the natural variation in the neutral loss rate of accessory chromosomes between three field isolates of *Z. tritici*. The neutral loss rate of accessory chromosomes varied significantly between the three isolates but did not change over the course of one year and does not appear to be influenced by the number of accessory chromosomes. Interestingly, the chromosome loss rate was influenced by the posttranslational histone modification. Deleting *kmt1*, which encodes a methyltransferase that catalyses the trimethylation of H3 at lysine 9 residue led to a significant increase in the chromosome loss rate. In contrast, the deletion of *kmt9*, which encodes a methyltransferase that catalyses the trimethylation of histone 3 at lysine 27 residue decreased the loss rate of the accessory chromosome. We speculate that the nuclear localization of the accessory chromosomes, which is influenced by histone modifications, could affect the mitotic transmission of these chromosomes. We further speculate that the differences in loss rate between the different field isolates might be adaptive. Furthermore we can confirm previous results that losing one or more accessory chromosomes in the reference isolate IPO323 increases fitness [37]. Moreover, the accumulated mutations produced slightly deleterious phenotypic effects, thereby confirming for the first time for a fungal plant pathogen that the majority of mutations are slightly deleterious [38,39].

Finally, I became interested in understanding the determinants of host specificity of *Z. tritici*. I hypothesized that transcription factors, which are integrating various signals and determine the response of the pathogen, could be interesting candidates to dissect the genetic basis of host specificity. Indeed, two transcription factors have recently been shown to regulate the interaction of *Z. tritici* with its host plant [40,41]. I could show that the transcription factor Zt107320 regulates growth *in planta* and *in vitro* as well as cell wall properties of *Z. tritici*. Hence, it might be involved in discriminating compatible from incompatible infections. These effects of Zt107320 are similar to the effects previously observed for the transcription factor MoCOD1, a homologue of Zt107320 in the rice blast fungus *Magnaporthe oryzae*, and other fungal transcription factors belonging to the same family of transcription factors [42–44]. However, the deletion of *Zt107320* led to partial loss of virulence and therefore a Zt107320 independent regulation of growth must exist in *Z. tritici*. In conclusion, transcription factors might represent interesting targets that could help dissect the molecular basis of the interaction between plant pathogens and hosts despite the generally high redundancy on the functional level.

## Perspective

Based on the novel meiotic drive of unpaired accessory chromosomes identified in the current work I propose several new questions and hypotheses which are currently being addressed or will be studied in the near future.

I hypothesized that a feedback mechanism between pairing and DNA replication exists which results in the additional amplification of unpaired DNA. I am currently addressing both aspects of this hypothesis: i) pairing of chromosomes and ii) DNA replication. During canonical meiosis DSB are introduced by Spo11 and are required for pairing and synapsis of homologous chromosomes [45]. Therefore Spo11 may also be involved in pairing of the accessory chromosomes as long as a homolog is present and that therefore these chromosomes would fail to pair in a *SPO11* deletion mutant and would then also undergo the additional round of DNA replication during meiosis, which we would be able to detect using tetrad analysis. We are currently generating the *SPO11* deletion mutants and determine its effect on the meiotic transmission of paired and unpaired chromosomes. To address DNA replication I aim at identifying the origins of replications. I hypothesize that the localisations and/or density of the origin of replications on the accessory chromosomes are different from that of the core chromosomes. The origin of replication sequences do not show sequence homology between *S. cerevisiae*, *S. pombe* and other eukaryotes but are bound by a highly conserved protein machinery [46–51]. The highly conserved Origin Recognition Complex (ORC) is a heterohexameric protein that binds to the origin of replication sequence and mediates the loading of the replicate helicase complex MCM2-7 (reviewed in [46]). Similar to previous studies, we will use GFP-fusion proteins of ORC2 and MCM2, subunits of the ORC and MCM2-7 complex, respectively, in a chromatin immunoprecipitation combined with sequencing (ChIP-seq) approach to identify the origin of replication [51]. This should help us to identify the origin of replication in the genome and compare the origin of replication between core and accessory chromosomes

During meiosis DSBs are formed by Spo11 and are required for recombination, chromosome pairing, synapsis and segregation [45]. DSBs are resolved by either crossover (CO) or noncrossover (NCO) events, both of which are associated with gene conversion, a process by which sister chromosome sequences are usually used to repair double-strand breaks (reviewed in [52]). Due to their importance in chromosome segregation the number of COs is highly regulated [53–55]. If COs are repressed on one chromosome, e.g. by a large, heterozygous inversion, the rate of CO and recombination increases for the other homozygous chromosome pairs - a process called interchromosomal effect [53,56]. I therefore hypothesize that the unpaired chromosomes in *Z. tritici*, which cannot pair and form CO affect the rate of CO, NCO and thereby gene conversion and recombination of paired chromosomes. Detailed recombination analysis has previously been performed using natural isolates or meiotic progeny, revealing that *Z. tritici* has a high recombination rate [21,57]. However, these studies have not addressed the effect of unpaired accessory chromosome on recombination rates. Within chapter 3 we have isolated a total of 24 complete tetrads – which arose from isogenic

zygotes that varied only in the number of unpaired chromosomes with a minimum of one and a maximum of three unpaired chromosomes. We will determine the impact of unpaired chromosomes on the recombination rate and moreover the effect of gene conversion on *Z. tritici* genome evolution, which previously was not possible due to the lack of isolated complete tetrads.

We further hypothesized that the localisation of the accessory chromosomes within the nucleus is different for the accessory chromosomes compared to the core chromosomes. We can show that the histone H3K27me3, which is linked to a localisation of subtelomeric sequences to the nuclear envelope [31,32], increases the loss of accessory chromosomes during mitosis. Currently we are testing whether this histone modification also influences the transmission of the accessory chromosomes during meiosis. In addition we plan to determine the localisation of the accessory chromosomes within the nucleus. Moreover, we are trying to establish a strain without any accessory chromosomes in order to compare the nuclear organisation between strains with and without accessory chromosomes using oligopaint FISH probes in mitotic cells [58,59]. In addition, we will fluorescently label one accessory and one core chromosome using TetO/TetR-mCherry and the LacO/LacI-GFP system, thereby visualizing chromosome localization during the cell cycle in living cells [60,61].

Combining these approaches will allow us to dissect the mechanism of the meiotic drive and the mitotic losses and dissect the mechanisms responsible for both the maintenance and the presence/absence polymorphism of the large complement of the accessory chromosomes in *Z. tritici* as a basis for evolution of this important wheat pathogen.

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## List of abbreviations

BFB	Breakage-fusion-bridge
ChIP-seq	Chromatin immunoprecipitation combined with sequencing
chr	Chromosome
CO	Crossover
DNA	Deoxyribonucleic acid
DSB	Double-strand break
eGFP	Enhanced green fluorescent protein
ETI	Effector triggered immunity
FISH	Fluorescence <i>in situ</i> hybridization
H3K27me3	Trimethylation of the histone 3 histone at lysine residue 27
H3K9me3	Trimethylation of the histone 3 histone at lysine residue 9
HSD	Honestly significant difference
HST	Host -specific toxin
MCM	Minichromosome maintenance complex
NCO	Non-crossover
ORC	Origin Recognition Complex
PFGE	Pulsed-field gel electrophoresis
QTL	Quantitative trait locus
RNA	Ribonucleic acid
SNP	Single nucleotide polymorphism
WGS	Whole genome sequencing
YMS	Yeast malt sucrose
Zt	Zymoseptoria tritici

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## Publications

1. **Habig M**, Quade J, Stukenbrock EH. 2017. Forward genetics approach reveals host-genotype dependent importance of accessory chromosomes in the fungal wheat pathogen *Zymoseptoria tritici*. *mBio*, <https://doi.org/10.1128/mBio.01919-17>
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## Patents

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3. Müller-Hartmann, H. und **Habig, M.** (2005) Method for treating small volumes with electrical current. EP05014758,
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5. Müller-Hartmann, H., **Habig, M.**, Hoffmann, P. und Siebenkotten, G. (2003) Container with at least one electrode. EP1476537.

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## Affidavit

I, **Michael Habig**, declare that:

Apart from my supervisor's guidance the content and design of the thesis is all my own work;

Specific aspects of my thesis were supported by colleagues; their contribution is specified in detail in the section "Author's contributions";

This thesis has not been submitted elsewhere partially or wholly as part of a doctoral degree to another examining body, and no other materials are published or submitted for publication than indicated in the thesis.

Chapter 4 has been submitted as part of a doctoral degree by Mareike Möller to the University of Kiel. The contribution of Mareike Möller to chapter 4 is detailed in the section "Author's contributions";

Additional details on methods and supporting data are provided as supplementary materials in the provided files, as specified in the individual chapters;

The thesis has been prepared subject to the Rules of Good Scientific Practice of the German Research Foundation (DFG).

Signature: \_\_\_\_\_