Characterisation of the sensitivity of *Zymoseptoria tritici* to demethylation inhibitors in Europe

Dissertation zur Erlangung des Doktorgrades der Agrarwissenschaften (Dr. sc. agr.)

Fakultät Agrarwissenschaften

Universität Hohenheim

Institut für Phytomedizin

vorgelegt von

M. Sc. Anna Huf

aus Bad Kreuznach

2020

Diese Arbeit wurde unterstützt und finanziert durch die BASF SE, Unternehmensbereich Pflanzenschutz, Forschung Fungizide, Limburgerhof.

Datum der mündlichen Prüfung:	16.03.2021
Dekan:	Prof. Dr. Ralf Vögele
1. Prüfer:	Prof. Dr. Ralf Vögele
2. Prüfer:	Prof. Dr. Philipp Schlüter
3. Prüfer:	Apl. Prof. Dr. Thomas Miedaner
Leitung des Kolloquiums:	Prof. Dr. Martin Hasselmann

Teile dieser Arbeit wurden bereits in den Veröffentlichungen Huf et al., 2018 und Huf et al., 2020 beschrieben.

Table of contents

Abbreviations

SI units and common abbreviations are not listed. Abbreviations for different European countries were used according to ISO 3166 standard (except United Kingdom that was abbreviated with UK).

a.i	active ingredient
BcMFSM2	Major facilitator superfamily gene involved in MDR2 of *Botrytis cinerea*
bp	base pair
CbCYP51	sterol 14α-demethylase gene of *Cercospora beticola*
CYP51	sterol 14α-demethylase gene of *Z. tritici*
CYP51	sterol 14α-demethylase enzyme of *Z. tritici*
*CYP51*p	promotor region of sterol 14α-demethylase gene of *Z. tritici*
CYTB	cytochrome bc_1 complex
d.c.	double concentrated
Del	deletion of an amino acid
DMI	demethylation inhibitor
DMSO	dimethylsulphoxide
dpi	days post inoculation
EC_{50}	effective concentration of 50% inhibition
FRAC	Fungicide Resistance Action Committee
kb	kilo base pair
MAMA	mismatch amplification mutation assay
MDR	multidrug resistance
MfCYP51	sterol 14α-demethylase gene of *Monilinia fructicola*
MFS1	major facilitator superfamily 1 gene of *Z. tritici*
MFS1	major facilitator superfamily 1 transporter of *Z. tritici*
*MFS1*p	promotor region of major facilitator superfamily 1 gene of *Z. tritici*
MOA	Mode of action

NCBI	National Center for Biotechnology Information
NTC	no-template control
OD	optical density
PCR	polymerase chain reaction
PdCYP51	sterol 14α-demethylase gene of *Penicillium digitatum*
qPCR	quantitative PCR
QoI	quinone outside inhibitor
rpm	revolution per minute
ScCYP51	sterol 14α-demethylase gene of *Saccharomyces cerevisiae*
ScCYP51	sterol 14α-demethylase enzyme of *Saccharomyces cerevisiae*
SDH	succinate dehydrogenase enzyme
SNP	single nucleotide polymorphism
spp.	species pluralis
STB	Septoria tritici blotch
T_m	melting temperature
TAE	tris-acetate-EDTA
TE	transposable element
UAA	upstream activation sequence
ViCYP51	sterol 14α-demethylase gene of *Venturia inaequalis*
WT	wild type

Figures

Tables

Abstract

The fungal pathogen *Zymoseptoria tritici* (formerly *Septoria tritici*) causes Septoria tritici blotch (STB), one of the most yield reducing diseases of wheat worldwide. In addition to cultural control measures and the cultivation of wheat varieties with a level of disease resistance, STB control relies heavily on the application of foliar fungicides with different modes of action. The demethylation inhibitors (DMIs) have been one of the most widely applied fungicides for many decades and belong to one of the most important fungicide modes of action in STB management. DMIs inhibit the sterol 14α-demethylase, an essential enzyme in the ergosterol biosynthesis pathway, encoded by the *CYP51* gene of fungi. Widespread and intensive use of the DMIs over time has led to a continuous negative shift in the sensitivity of *Z. tritici* towards DMIs that have been used for a long time. This shift in sensitivity is mainly driven by the accumulation of mutations in the *CYP51* gene resulting in the selection of various *CYP51* haplotypes. More recently, *CYP51* overexpression and an increased efflux activity, based on the overexpression of the MFS1 transporter, have been shown to be additional mechanisms affecting DMI sensitivity of *Z. tritici*. Inserts in the *CYP51* promotor (*CYP51*p) and *MFS1* promotor (*MFS1*p) were observed to be responsible for *CYP51* and *MFS1* overexpression. The prevalence and contribution of different DMI resistance mechanisms to a reduced DMI sensitivity of *Z. tritici* were investigated in isolates from across Europe in 2016 and 2017. The *CYP51* gene of all isolates was sequenced and the *CYP51*p and *MFS1*p was investigated for inserts in order to determine the character of the *CYP51* haplotypes as well as to identify *CYP51* overexpression or if an increased efflux activity was occurring in these isolates.

Detection of new mutations in *CYP51*, although not necessarily correlated with distinct changes in DMI sensitivity of *Z. tritici*, demonstrated that evolution of *CYP51* is still ongoing. Additionally, new combinations of existing alterations resulted in the emergence and identification of new *CYP51* haplotypes not previously described in the literature. As a result of the identification of numerous new haplotypes, it was decided to establish a new nomenclature in order to describe all 57 *CYP51* haplotypes found in *Z. tritici* across 2016 and 2017 as well as to facilitate the future designation of newly emerging haplotypes. Thirteen of the 57 *CYP51* haplotypes were observed to represent around 89% of all isolates and were designated as ‘frequently found *CYP51* haplotypes’. Among these thirteen most frequent *CYP51* haplotypes, isolates carrying haplotypes C8, G1, and F3 showed highest sensitivity to epoxiconazole, followed by haplotypes E4 and F2 with moderate adaptation and haplotypes F4, E3, D13, E5, F8, H4, and H6, all carrying the alteration S524T, conferring the highest level of adaptation. *In vitro* sensitivity data for these haplotypes and different DMIs indicated limited cross-resistance between epoxiconazole as well as prothioconazole compared to mefentrifluconazole. These results were supported by *in vivo*

selection studies that indicated overall a different trend in the selection pattern for *CYP51* haplotypes by the two DMI fungicides prothioconazole and mefentrifluconazole.

A heterogenous distribution of 'frequently found *CYP51* haplotypes' was observed across Europe demonstrating an east-west gradient in DMI adaptation of *Z. tritici*. In the Western European countries Ireland, UK, and the Netherlands, the majority of the *Z. tritici* populations were composed of haplotypes expressing a moderate or high adaptation to epoxiconazole. In Central Europe, for example in Germany and Poland, the majority of the populations were composed of haplotypes conferring highest sensitivity or moderate adaptation among all 'frequently found *CYP51* haplotypes'. In Eastern Europe, a high frequency of haplotypes with the highest sensitivity among all 'frequently found *CYP51* haplotypes' was observed. Nevertheless, compared to past studies, an ongoing shift towards a reduced DMI sensitivity was observed across Europe, especially in intensive wheat growing areas.

Further investigation of the isolates in this study found 121, 863, 868, and 300 bp inserts in *CYP51*p. The 121, 863, and 868 bp inserts were assumed to be equivalent to the already published 120, 862, and 866 bp inserts. Therefore, the 121 bp insert was named as 120 bp, the 863 bp as 862 bp, and the 868 bp as 866 bp insert in the course of this work. Previously, the 120 bp insert in *CYP51*p was shown to be associated with *CYP51* overexpression, unlike the 862 and 866 bp inserts which are probably not. On average, a 5- to 8-fold reduction in epoxiconazole sensitivity was correlated to the 120 bp insert in this work, whereas the 300, 862, and 866 bp inserts were only associated with a small impact on sensitivity, if at all. In 2016, *CYP51* overexpression based on the 120 bp insert was detected, with the exception of Denmark, in isolates from every investigated country in a low to moderate frequency. However, an increase in the frequency was observed for some countries in 2017. The 120 bp insert was mainly detected in isolates with the F2 haplotype, however, for the first time, this insert was also detected in conjunction with other *CYP51* haplotypes further demonstrating an ongoing evolution of *CYP51* overexpression and *CYP51*p.

Across all isolates, seven different inserts with a length of 150, 267, 308, 338, 369, 377, or 519 bp were detected in *MFS1*p. The 150, 338, 369, and 519 bp inserts have been published before and are associated with *MFS1* overexpression. Among those, the 519 bp insert is correlated with highest *MFS1* expression in literature. In this work, *in vitro* sensitivity data for epoxiconazole revealed that the 519 bp insert in *MFS1*p was correlated with up to an 8-fold reduction in sensitivity, whereas the remaining inserts only conferred a minor impact on sensitivity to epoxiconazole. In the 2016 isolates, the 519 bp insert was only detected at a low frequency in Germany, Poland, and in countries of Western Europe. Shorter *MFS1*p inserts were mainly detected in Ireland and UK. In contrast, the prevalence of smaller *MFS1*p inserts increased in the 2017 isolates although their frequency was

generally still at a low level (with the exception of Ireland and the Netherlands). The frequency of the 519 bp insert was relatively stable in isolates from both years.

Overall, it was shown that the occurrence of *CYP51* haplotypes was still the most frequent and important mechanism conferring a reduction in sensitivity to DMIs by *Z. tritici* in Europe. Nevertheless, an increase in the frequency of isolates exerting *CYP51* overexpression and those exhibiting increased efflux activity was observed compared to earlier studies. Glasshouse data demonstrated that DMIs can still contribute to disease control, and in some cases give full control, of STB even if isolates expressed *CYP51* overexpression and/or an increased efflux in addition to also carrying moderately or highly adapted *CYP51* haplotypes. However, in order to prevent the further increase and spread of further adapted *CYP51* haplotypes plus additional resistance mechanisms in the *Z. tritici* population across Europe, anti-resistance-management strategies should be a high priority in the use of DMIs. In addition, especially integrated disease management strategies, such as the appropriate choice of cultivars, should be applied in order to keep STB disease pressure low and consequently reduce the number of fungicide applications. Moreover, resistance-management strategies may exploit the limited cross-resistance between different DMIs, for example, by the use of mixtures or alternation of different DMI fungicides. However, control strategies should also incorporate the use of fungicides with different MOAs. The aim of all these strategies is to reduce selection of adapted *Z. tritici* isolates and consequently to prolong the efficacy of DMIs in STB management.

1 Introduction

1.1 Wheat cultivation

Domestication of wheat is thought to have started 10.000 years ago in the Fertile Crescent (Lev-Yadun et al., 2000; Peng et al., 2011). Today, wheat is the most widely grown crop in the world and the most important cereal cultivated in the EU (Fones and Gurr, 2015). Wheat is grown from the far North from 67°N in Norway, Finland, and Russia to 45°S in Argentina and Chile (Gustafson et al., 2009). Modern wheat varieties belong to two species. Around 95% of the world wheat production is derived from hexaploid bread wheat (*Triticum aestivum*) and the remaining 5% from tetraploid hard or durum wheat (*T. durum*) (Peng et al., 2011). Wheat is mainly produced for human consumption and animal feed, however, it is also used as a raw material for biofuels and for alcoholic distillation. Additionally, wheat straw serves for livestock bedding, roof thatching and basket-making (Gustafson et al., 2009; Fones and Gurr, 2015).

In 2017, wheat was grown on around 220 million hectares worldwide and a global harvest of 771 million metric tons was achieved (FAO, 2019). The largest quantities of wheat were produced in Asia followed by Europe and America. The top 5 countries with the highest wheat production within Europe were Russia, France, Ukraine, Germany and UK (FAO, 2019). Since the green revolution, a significant increase in grain production was achieved based on the development of high yielding wheat- and rice varieties (Khush, 1999). Due to a steadily increasing global population and changing diets, the demand for food is predicted to increase by 70% by 2050 (FAO, 2009; Beddington et al., 2011; O'Driscoll et al., 2014). At the same time agricultural production is challenged by adverse effects through climatic change and is facing the need for climate-resilient production systems that are sustainable (Beddington et al., 2011). Additionally, abiotic environmental factors such as salinity, water- and nutrient supply as well as biotic factors such as animal pests, plant pathogens, and weeds threaten agricultural production (Oerke, 2006; Haggag et al., 2015). At a worldwide scale, potential losses of wheat yields due to pests have been estimated to be close to 50% (Oerke, 2006). Oerke (2006) described that highest potential yield losses are due to competition with weeds, that can be managed mechanically or chemically, followed by plant pathogens and animal pests that are mainly managed based on synthetic chemicals. The importance of plant pathogens increases with increasing crop productivity. Therefore, in Western Europe, under conditions of intensive agricultural production, the potential losses due to plant pathogens were as high as that of weeds (Oerke, 2006).

Rust diseases (*Puccinia striiformis*, *Puccinia triticina*) and powdery mildew (*Blumeria graminis*) are known as serious diseases of wheat (Jørgensen et al., 2014). Due to mycotoxin production also Fusarium head blight (*Fusarium* spp.) is considered as an increasing problem in wheat production (Bottalico and Perrone, 2002; Jørgensen et al., 2014). Nevertheless, in Europe, the most prevalent and yield reducing fungal disease of wheat remains Septoria tritici blotch (STB) caused by the fungal pathogen *Zymoseptoria tritici* (Jørgensen et al., 2014; Fones and Gurr, 2015).

1.2 Septoria tritici blotch and its causal agent *Zymoseptoria tritici*

1.2.1 Relevance

The ascomycetous fungal pathogen *Zymoseptoria tritici* (Desm.) Quaedvlieg and Crous is the causal agent of Septoria tritici blotch (STB), an important disease of wheat grown in temperate climates worldwide (Orton et al., 2011; Fones and Gurr, 2015; Torriani et al., 2015). In fields cultivated with STB susceptible wheat varieties, severe epidemics of STB can cause yield losses of up to 50% (Eyal et al., 1987). Within the European Union (EU), STB is the most prevalent and devastating foliar disease of wheat (Jørgensen et al., 2014; Fones and Gurr, 2015; Torriani et al., 2015). In the EU, approximately 70% of fungicides applied annually in cereal production target the control of *Z. tritici* in wheat demonstrating its high economic relevance (Ponomarenko et al., 2011; Torriani et al., 2015). Annual losses in Europe caused by STB are estimated to be $400 million (Ponomarenko et al., 2011).

1.2.2 Taxonomy

Z. tritici was named *Septoria tritici* Roberge in Desmaz. until 2011 and is the anamorph of the fungus (Desmazières, 1842; Quaedvlieg et al., 2011; Eyal et al., 1987). The teleomorph of *Z. tritici* is *Mycosphaerella graminicola* (Fuckel) J Schroeter in Cohn (Sanderson, 1976). In 2011, a new genus named *Zymoseptoria* was founded to accommodate *Septoria*-like species found on graminicolous hosts (Quaedvlieg et al., 2011). In their work, Quaedvlieg et al. (2011) identified that species in the *Zymoseptoria* genus can be separated from the *Septoria* genus based on molecular phylogeny studies. Additionally, species of the *Zymoseptoria* genus are characterised morphologically by their yeast-like growth in culture and the formation of up to three different conidial types, namely pycnidial conidia, phragmospores on aerial hyphae, and yeast-like growth proliferation via microcyclic conidiation (Quaedvlieg et al., 2011). *Z. tritici* belongs to the class of the Dothideomycetes, the order Capnodiales and the family Mycosphaerellaceae (EPPO, 2019).

1.2.3 Disease cycle, infection process and epidemiology

Z. tritici has developed various opportunities to survive the intercrop period in winter wheat cultivation and thus to provide primary inoculum at wheat seedling emergence. Ascospores, pycnidiospores as well as mycelium can all overwinter on plant material such as wheat seeds, stubble, debris, wheat volunteers and grass species (Suffert et al., 2011). Under field conditions seed transmission of *Z. tritici* is believed to be unlikely (Jones and Cooke, 1969; Suffert et al., 2011). *Z. tritici* is able to infect a few grass species as alternative hosts which may play a role in long-term conservation of inoculum. However, so far, the precise role of alternative hosts in epidemiology of STB is not fully clear (Brokenshire, 1975; Ao and Griffiths, 1976; Suffert et al., 2011). Wind-dispersed ascospores are thought to be the main source for primary infection of wheat seedlings in autumn (Shaw and Royle, 1989a; Eriksen and Munk, 2003). Ascospores of *Z. tritici* are produced in fruiting bodies of the sexual stage, named pseudothecia, ascocarps or perithecia (Eyal et al., 1987; Suffert et al., 2011). A bipolar and heterothallic mating system with two opposite mating types (MAT1-1 and MAT1-2), that are needed for sexual reproduction and generation of ascospores, was identified for *Z. tritici* (Kema et al., 1996a). A positive correlation between the frequency of pseudothecia and the infection density is assumed due to a higher chance of opposite mating types to encounter under high infection densities compared to lower densities (Cowger et al., 2002). Pseudothecia were found regularly during the growing season but appear around 30 to 55 days later than pycnidia. Pseudothecia remain active on senescent leaves as well as on plant debris and act as ascospore source for later infections (Hunter et al., 1999; Eriksen and Munk, 2003; Suffert et al., 2011). In the Northern Hemisphere, release of ascospores follows a seasonal pattern with a first peak in late autumn and a second peak at the end of the growing season in summer (Hunter et al., 1999; Eriksen and Munk, 2003; Suffert et al., 2011). Although ascospores are thought to be the main source for primary infection of wheat seedlings in autumn, also rain-splash dispersed pycnidiospores from wheat debris could act as primary inoculum when wheat is stubble sown or later sown than the main period of ascospore release (Shaw and Royle, 1989a; Holmes and Colhoun, 1975; Brown et al., 1978; Shaw, 1987; Eriksen and Munk, 2003).

The disease cycle of STB caused by *Z. tritici* is shown in Figure 1 (extracted from Ponomarenko et al., 2011).

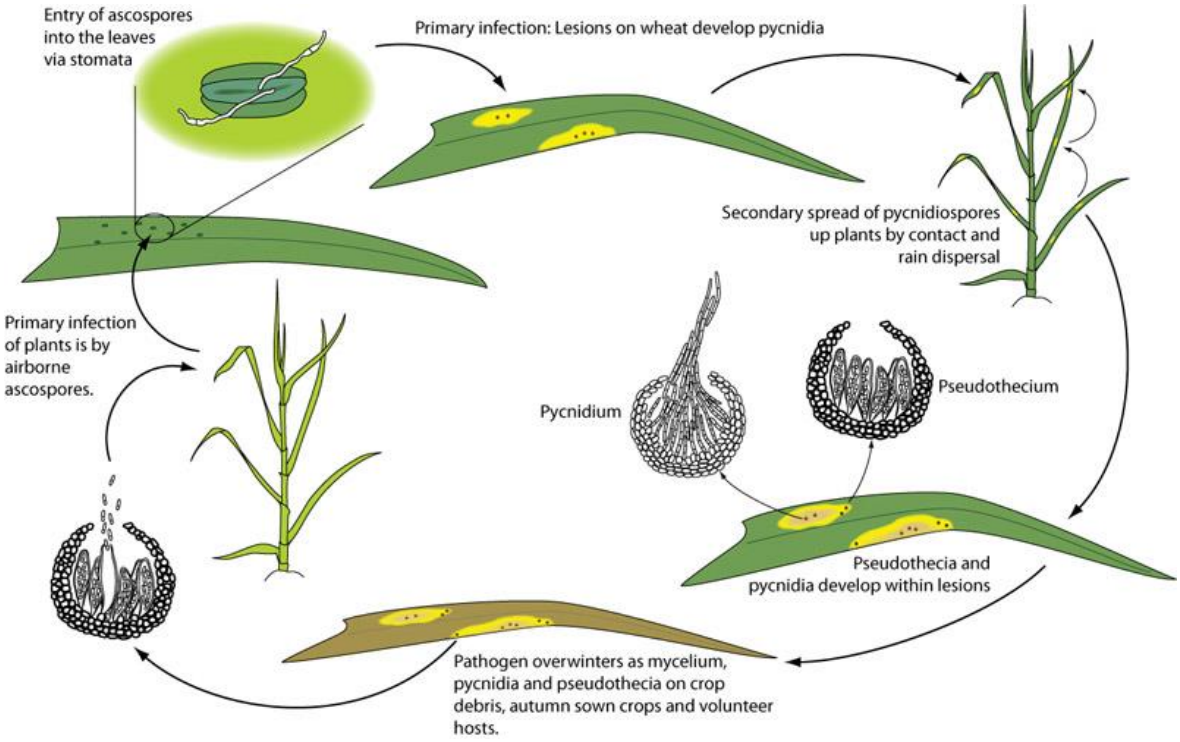


Figure 1: Disease cycle of Septoria tritici blotch caused by *Zymoseptoria tritici* on wheat. (Figure was extracted from Ponomarenko et al., 2011).

Primary infection starts with germination of asco- or pycnidiospores at the leaf surface and penetration of the host via stomata (Kema et al., 1996b). A latent phase of 10-14 days without visible symptoms follows in which the fungus grows slowly in the apoplast in close contact to wheat mesophyll cells (Kema et al., 1996b; Keon et al., 2007). Based on this latent phase *Z. tritici* has often been referred to as a hemibiotrophic pathogen (Keon et al., 2007; Brunner et al., 2013). However, proof of the acquisition of nutrients by *Z. tritici* during the asymptomatic growth phase remains unclear, as it does not develop haustoria or other intracellular feeding structures (Kema et al., 1996b; Rudd et al., 2008). Compared to biotrophic fungal pathogens growth of *Z. tritici* appears more similar to endophytes (Joosten et al., 1990; Solomon and Oliver, 2001; Thomma et al., 2005; Keon et al., 2007; Rudd et al., 2015; Sánchez-Vallet et al., 2015). Sánchez-Vallet et al. (2015) suggested that nutrients stored in the spore may serve as a food source during the asymptomatic phase. Additionally, some cell wall degrading enzymes are specifically expressed possibly indicating that host cell wall components may be degraded and serve as nutrition for the fungus without causing major damage to the plant (Sánchez-Vallet et al., 2015). The expression profile of plant cell wall degrading enzymes indicates an important role of these enzymes in plant colonization, although the number of cell wall degrading enzymes is smaller in *Z. tritici* compared to other fungal pathogens (Goodwin et al., 2011; Brunner et al., 2013). Uptake of released nutrients is believed to be mediated by sugar and amino acid transporters expressed during the asymptomatic phase (Keon et al., 2007; Yang et al., 2013; Brunner et al., 2013; Kellner et al., 2014). In *Z. tritici* only two effectors *Mg1*LysM and

*Mg3*LysM have been observed to be essential for the successful infection process. Both effectors prevent activation of chitin-triggered-immunity and consequently, prevent growth inhibition of *Z. tritici* during the asymptomatic phase (de Jonge et al., 2010; Marshall et al., 2011; Sánchez-Vallet et al., 2013, 2015). After the latent phase, *Z. tritici* switches to a necrotrophic lifestyle and disease symptoms appear as irregular chlorotic lesions that develop later into necrotic lesions carrying brown pycnidia (Eyal et al., 1987; O'Driscoll et al., 2014; Keon et al., 2007). Typical symptoms of *Z. tritici* in a wheat field are shown in Figure 2.

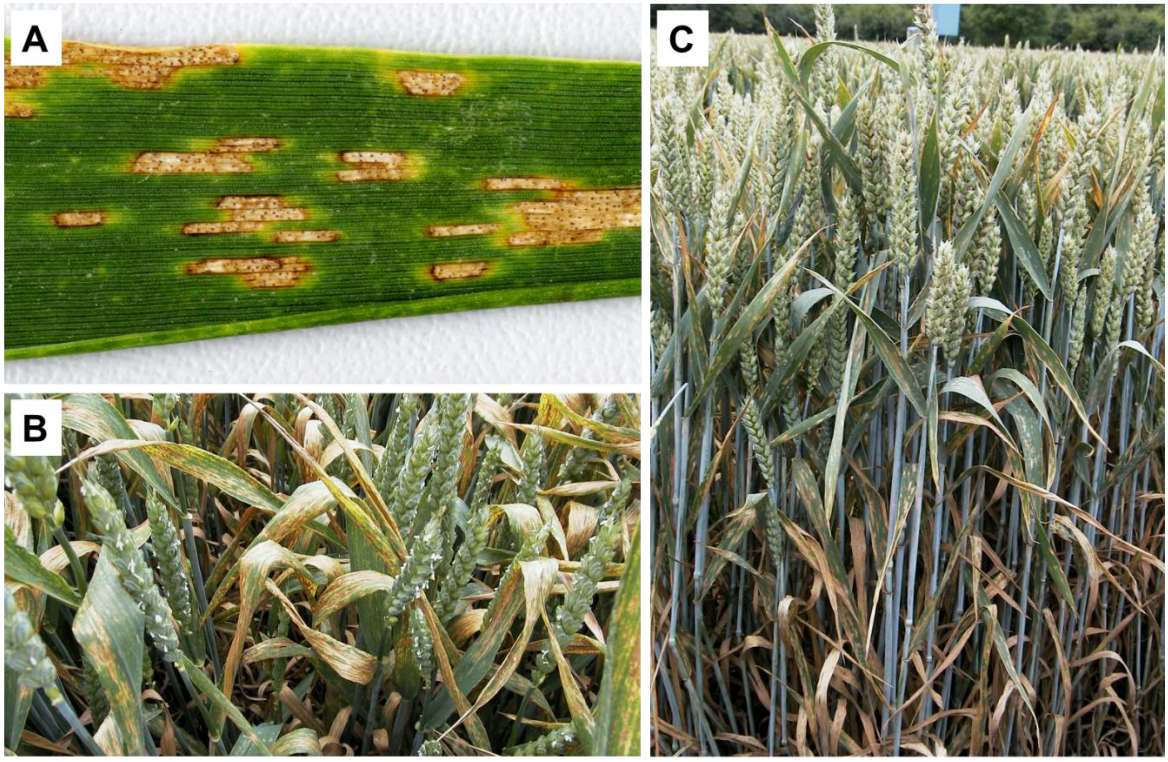


Figure 2: Disease symptoms of *Z. tritici*. A: Symptoms of *Z. tritici* on a wheat leaf showing black conidia within necrotic lesions. This picture was kindly provided by Bill Clark, Technical Director, NIAB. **B:** Top view of *Z. tritici* infection on wheat. **C:** Front view of *Z. tritici* infection on wheat. Pictures B and C were kindly provided by Dieter Strobel, technical manager, BASF SE.

The factors that trigger the switch from asymptomatic to necrotrophic growth are still unknown (Sánchez-Vallet et al., 2015). Environmental factors as well as several pathogen related mechanisms, including the production of protein effectors are supposed to play a role in the induction of necrosis (Keon et al., 2007; Rudd et al., 2008; Brunner et al., 2013; Mirzadi Gohari et al., 2015; Rudd et al., 2015; Sánchez-Vallet et al., 2015). During the necrotrophic phase the growth of the fungus becomes more rapid and asexual sporulation structures, the pycnidia that form pycnidiospores are produced (Keon et al., 2007; O'Driscoll et al., 2014). Pycnidiospores are dispersed by rain-splash and are considered to be the main source of inoculum for secondary infections throughout the growing season (Holmes and Colhoun, 1975; Shaw, 1987; Suffert et al., 2011). Pycnidiospores lead to local secondary infections on uninfected leaves of the same plant and spread the disease

upwards through the canopy as well as to plants nearby. Therefore, several cycles of multiplication, even within one leaf layer, can occur during the growing season leading to rapid development of epidemics under favourable conditions (Shaw and Royle, 1993; Ponomarenko et al., 2011). Pycnidiospores in pycnidia were found to stay viable for several month on infected wheat debris, thus also serving as an additional source for inoculum overwinter (Hilu and Bever, 1957; Suffert et al., 2011).

Temperature and humidity are important factors influencing STB infection of wheat (Holmes and Colhoun, 1974). Wet weather and temperatures between 17 to 25°C are favourable for *Z. tritici* infections (Holmes and Colhoun, 1974; Eyal et al., 1987). Under artificial conditions 7°C and a period of only 24 h in a moisture chamber with high humidity were not sufficient to produce infections (Holmes and Colhoun, 1974; Eyal et al., 1987). It was observed that temperatures below -2°C in the early stages of growth reduce the risk of a STB infection on winter wheat (Fones and Gurr, 2015). As secondary infections are driven by pycnidiospores dispersed by rain splash, the occurrence of rain events (5-10 mm rainfall at one day or 10 mm rain in total over two or three consecutive days with at least 1 mm rain) is a crucial factor influencing the speed of STP epidemics (Holmes and Colhoun, 1975; Shaw, 1987; Thomas et al., 1989; Suffert et al., 2011). Consequently, *Z. tritici* is favoured by cool, humid climates often found in EPPO´s “Maritime Zone” including Northern France, Germany, and UK (Holmes and Colhoun, 1974; Bouma, 2005; Fones and Gurr, 2015; Goodwin, 2019).

1.2.4 Genetics

The genome of *Z. tritici* was the first and most complete sequence of a filamentous fungus to be studied in 2011 (Goodwin et al., 2011). The complete genome of *Z. tritici* (isolate IPO323) has a total size of 39.7 Mb and consists of 21 chromosomes that carry 10,933 predicted genes (Goodwin et al., 2011). The genome was shown to consist of 13 core chromosomes and eight dispensable chromosomes that can be lost without visible effects on the fitness of the fungus (Wittenberg et al., 2009; Goodwin et al., 2011). On the core chromosomes, which contain 88% of the genome, close to 59% of the genes could be annotated, whereas only 10% of the genes could be annotated on the dispensable chromosomes, which contain 12% of the genome (Goodwin et al., 2011; Dhillon et al., 2014). The dispensable chromosomes contain redundant genes that represent copies of genes present on core chromosomes and the majority of the annotated genes are coding for putative transcriptome factors or otherwise function in gene regulation or signal transduction (Goodwin et al., 2011). Variation in the chromosome number of the *Z. tritici* genome is meiotically generated during the sexual cycle of the fungus (Wittenberg et al., 2009; Goodwin et al., 2011). It was believed that *Z. tritici* shows a high level of sexual reproduction leading to a high degree of genetic variation (Chen and McDonald, 1996; Schnieder et al., 2001). Indeed, a high genetic variability within *Z. tritici* populations, even

within a single wheat field, resulting in many different molecular phenotypes was observed (McDonald and Martinez, 1990; Schnieder et al., 2001; reviewed in Simón et al., 2012).

Dhillon et al. (2014) described in their studies that at least 17% of the *Z. tritici* genome was estimated to be repetitive. These repeated elements did not contain tandem repeats and low-copy repetitive families with fewer than ten repeats per family. Additionally, 5.7% of the genome was shown to consist of these low-copy repetitive families. In their studies, Dhillon et al. (2014) also identified that the largest fraction of all repetitive elements was built by class I transposable elements (transposable elements that amplify via an RNA intermediate). Dispensable chromosomes showed a significantly higher frequency of transposable- and repetitive elements compared to the core chromosomes (Dhillon et al., 2014). Translocations in the genome due to transposable elements provide a broad range in genetic variation and may lead to chromosome length polymorphisms often found in the *Z. tritici* genome (McDonald and Martinez, 1991; Goodwin et al., 2001; Daboussi and Capy, 2003; Wittenberg et al., 2009). A high plasticity of the *Z. tritici* genome based on polymorphisms in length and number of chromosomes is supposed to play an important role in strategies of *Z. tritici* populations to adapt to adverse biotic and abiotic conditions in wheat fields (Wittenberg et al., 2009; Orton et al., 2011).

1.3 Disease management of Septoria tritici blotch in wheat

In wheat, control of STB is achieved by an integrated disease management approach including cultural practices, breeding of resistant wheat varieties and the application of chemical fungicides (Orton et al., 2011; Jørgensen et al., 2014). As yield losses are mainly driven by STB infections on the flag, second and third leaf of wheat plants, it is of major relevance in STB control to prevent infections on these top three leaves (Shaw and Royle, 1989b; Thomas et al., 1989).

1.3.1 Cultural methods

Cultural control measures comprising the adjustment of the sowing date, the crop density, the input of fertilizer, the crop rotation and tillage can affect severity of fungal diseases in wheat (Jørgensen et al., 2014). Compared to other fungal pathogens disease severity of STB is less affected or unaffected by crop rotation or tillage systems (Schuh, 1990; Gladders et al., 2001; Brown and Hovmøller, 2002). However, widening the crop rotation to 2-5 years between wheat crops or implementing a crop rotation with non-cereal crops were demonstrated to reduce STB severity compared to fields of consecutive wheat cultivation (Pedersen and Hughes, 1992; Bailey et al., 2001). This observation may be explained by a reduced availability of inoculum in the early growing stages (Suffert et al., 2011).

Nevertheless, in areas with a high intensity of wheat production such as Northern Europe, wind-dispersed ascospores are estimated to be available in all growing regions serving as the main source of primary inoculum in the early season (Eriksen and Munk, 2003). Another factor influencing STB severity is the sowing date. Early sowing of wheat increases the risk for severe STB epidemics due to a longer development of early-sown crops. This longer development favours initial infections and multiplication resulting in a higher amount of inoculum at the end of winter compared to late-sown crops (Shaw and Royle, 1993; Jørgensen et al., 1997). Consequently, later drilling can reduce disease incidence of STB, however, very late sowing can also reduce yields (Gladders et al., 2001; Jørgensen et al., 1997). Additionally, STB severity may be affected by crop density. Low seed rates result in a more open crop canopy that favours secondary infections of STB by splash-born pycnidiospores (Bjerre et al., 2006; Jørgensen et al., 1997). On the other hand, high seed rates may increase the risk of lodging associated with reduced yields (Fischer and Stapper, 1987; Berry et al., 2000). Under favourable weather conditions development of *Z. tritici* was also demonstrated to be promoted by high nitrogen fertilization (Simón et al., 2003). All in all, the application of cultural control measures alone in STB management is challenging because a method reducing the risk of one disease may enhance the risk of another (Jørgensen et al., 2014). Additionally, some of the described measures may have a negative impact on yield resulting in reluctance of the farmers to adopt cultural control measures in their cropping systems (Jørgensen et al., 2014; O'Driscoll et al., 2014).

1.3.2 Variety resistance and mixtures

Cultivation of resistant wheat varieties is a relevant approach in STB management (Orton et al., 2011; Jørgensen et al., 2014). Breeding for *Z. tritici* resistance has become a major focus of wheat breeding programmes and considerable progress has been achieved in understanding the genetics of STB resistance during the last 20 years (Arraiano and Brown, 2006; Brown et al., 2015). Two types of STB resistance are known in wheat. The first type is specific (qualitative) resistance that is near-complete and oligogenically controlled by major genes with large effects. This resistance type is isolate dependent and follows a gene-for-gene relationship (Brading et al., 2002; Arraiano and Brown, 2006; Brown et al., 2015). In contrast, the second type, quantitative/partial resistance, is incomplete and polygenically controlled by several to many genes with small to moderate effects (Jlibene et al., 1994; Zhang et al., 2001; Brown et al., 2015). This type of resistance is isolate independent (Chartrain et al., 2004). So far, 21 resistance genes conferring qualitative STB resistance were identified and mapped in wheat (Brown et al., 2015). Growing of resistant varieties with qualitative resistance, however, led to selection of adapted *Z. tritici* isolates that were able to overcome the resistance (Cowger et al., 2000; reviewed in Brown et al., 2015). Consequently, breeding for qualitative resistance traits alone probably does not represent an approach for durable STB resistance (Brown et al., 2015). In contrast to this,

quantitative/partial resistance is more durable but less studied and harder to breed for (Orton et al., 2011). Moreover, breeding for STB resistance was observed to be associated with a reduced yield potential of wheat varieties. Therefore, modern breeding strategies aim to identify quantitative trait loci for STB resistance that are not associated with yield losses (Torriani et al., 2015). Breeding strategies that include numerous partial resistance genes, pyramiding of several R genes, mutating susceptibility genes, or combination of all three components may represent a more durable approach in STB control (Orton et al., 2011; O'Driscoll et al., 2014; Brown et al., 2015). Only a handful of wheat varieties moderately resistant to STB are cultivated across the majority of wheat fields in Europe (Fones and Gurr, 2015). As STB resistance usually appears as a quantitative trait (reviewed in Brown et al., 2015) cultivar resistance alone is often not sufficient to keep disease levels low enough at a level that does not impair yield and fungicides also have to be applied (Jørgensen et al., 2014; Heick et al., 2017a). It was observed that fungicide applications had a positive effect on yield even in cultivars ranked as the most STB resistant (Jørgensen et al., 2014). This finding may also be attributed to the fact that disease resistance seldomly covers all relevant fungal diseases of wheat and fungicide treatments may have additional positive physiological effects on the crop (Bartlett et al., 2002). Nevertheless, it was demonstrated that growing resistant cultivars reduced potential yield losses and the expected fungicide costs in field trials in Denmark, France, Sweden and UK (Jørgensen et al., 2008). Therefore, in order to provide an integrated approach in STB management, further breeding and cultivation of disease-resistant and high-yielding cultivars is recommended for the future (Jørgensen et al., 2014).

Another approach in integrated STB management was proposed to be cultivation of cultivar mixtures that consist of susceptible and resistant wheat varieties. The utility of cultivar mixtures in STB control, however, is discussed controversially. On the one side it was shown that compared to their pure stand the severity of STB was reduced in cultivar mixtures (Mille et al., 2006; Gigot et al., 2013; Vidal et al., 2017). On the other side, it was demonstrated that the ability of cultivar mixtures to reduce disease severity was variable between years and influenced by environmental conditions (Cowger and Mundt, 2002). Moreover, efficiency in disease control and overall performance of a cultivar mixture cannot be predicted based solely on the performance of the single cultivars and little information is available how to select cultivars to mix (Finckh and Mundt, 1992; Mille et al., 2006).

1.3.3 Chemical control of *Z. tritici* and introduction to fungicides

Today, as cultural control measures can have negative impact on wheat yields (Jørgensen et al., 2014; O'Driscoll et al., 2014) and growing of resistant cultivars does often lead to insufficient suppression of the disease, management of *Z. tritici* still relies to a major extent on the application of chemical fungicides (Cools et al., 2013; Heick et al., 2017a; Omrane et al., 2017; Kildea et al., 2019). Since more than 200 years fungicides have been applied

to plants in order to protect them against- and/or eradicate established fungal infections (Brent and Hollomon, 2007a; Oliver and Hewitt, 2014). Due to the application of fungicides, diseases can be controlled and the productivity of crop production is increased (Oerke, 2006).

Over one century ago, chemical disease control started with the application of basic agents such as copper, sulphur and organic mercury. Today, fungicides are a major element in crop cultivation and help to protect crop yields and quality (Oerke, 2006; Oliver and Hewitt, 2014; Lucas et al., 2015). In 2019, around 235 molecules, that belong to several chemical classes, exist for fungal disease control (FRAC, 2019). Modern fungicides are characterised according to their chemical structure, their mode of action (MOA) and their properties for crop protection (Oliver and Hewitt, 2014). Differentiation is made between single-site fungicides (single-site MOA) that target a single cellular process and multi-site inhibitors (multi-site MOA) that attack a range of cellular processes (Lucas et al., 2015). Moreover, fungicides are differentiated between systemic and not systemic fungicides, as well as between fungicides with protectant, curative or eradicative activity (Oliver and Hewitt, 2014).

Non-systemic fungicides are surface-acting molecules with protectant activity, as they are not taken up by the plant (Oliver and Hewitt, 2014). A protectant fungicide is applied before fungal infections occur and inhibits early stages in the infection process. Multi-site inhibitors are often non-systemic fungicides with protectant activity (Oliver and Hewitt, 2014). A disadvantage of these fungicides is that their action is limited to treated foliage and they need to be used in a preventative approach. Therefore, repeated applications are required for complete spray coverage of the target crop during the growing season (Oliver and Hewitt, 2014; Hollomon, 2015). In contrast to this, systemic fungicides penetrate the plant tissue and are distributed within the entire plant. This feature enables not only protection of plant parts that were directly sprayed with fungicides, but additionally other plant parts as newly formed tissues are protected (Oerke, 2006). Systemic fungicides often show protectant and curative (after infection) activity and act as single-site inhibitors (Brent and Hollomon, 2007a, 2007b; Oliver and Hewitt, 2014). Compared to non-systemic fungicides, systemic fungicides often provide a longer period of disease control (Oliver and Hewitt, 2014). Their introduction has enabled a greater flexibility in fungicide application timing and displayed the prerequisite for application of disease thresholds and forecast systems (Oerke, 2006; Brent and Hollomon, 2007a; Oliver and Hewitt, 2014). Different approaches have been developed in STB management based on combinations of variable parameters such as the estimate of current disease severity, key weather variables, and crop development in order to optimize fungicide usage and to support treatment decisions (Hansen et al., 1994; Verreet et al., 2000; Moreau and Maraite, 2000; Milne et al., 2003; Newe et al., 2003; Jørgensen et al., 2014, 2017a).

Across different European countries, the number of fungicide applications in STB control and in winter wheat in general, varies between one to four applications per season with an average of two treatments (Jørgensen et al., 2014; Lucas et al., 2015; Heick et al., 2017a; Kildea et al., 2019). Thereby, the intensity of fungicide usage does not only differ between countries but also within countries (Jørgensen et al., 2014). In their work, Jørgensen et al. (2014) compared the pesticide use in winter wheat between Germany, France, the United Kingdom and Denmark based on sold amounts of pesticides and national surveys from 2006 and 2007. Their results revealed that usage of fungicides in Denmark is much lower compared to the other countries. A marked difference in the fungicide usage between Northern France and Southern France was observed with a lower treatment frequency in the South. Compared to France, fungicide usage in Germany was higher, but overall, the highest fungicide usage was observed in UK. These differences may be explained due to different disease severities, diverse climatic conditions, differences in the attitude for economic optimization, different operating policy plans that aim to reduce pesticide use, diverse organisation of advice to farmers, and variable pesticide prices (Jørgensen et al., 2014).

Currently, four classes of fungicides are mainly used in management of STB. These consist of multi-site fungicides such as chlorothalonil and the single-site classes of the C-14 demethylation inhibitors (DMIs), the quinone outside inhibitors (QoIs) and the succinate dehydrogenase inhibitors (SDHIs) (Jørgensen et al., 2018; Heick et al., 2017a; Kildea et al., 2019).

The multi-site inhibitor chlorothalonil forms chlorothalonil-reduced glutathione derivates in cells. As enzyme activity and cell viability is supposed to be controlled by the glutathione concentration in cells, the loss of glutathione by derivate formation with chlorothalonil and the lack of regeneration of the reduced thiol could explain its fungicidal activity (Tillman et al., 1973). Chlorothalonil was introduced in the mid-1960s, mainly for the control of *Septoria* spp. in cereals, *Phytophthora infestans* in potatoes and *Botrytis* spp. in vegetables as well as ornamentals. Chlorothalonil is an important and widely used mixing partner for QoI, DMI, and SDHI fungicides. Mixtures of chlorothalonil with single-site fungicides should protect the latter ones against fungicide resistance (Oliver and Hewitt, 2014). Nevertheless, due to revocation of its registration (European Commission, 2019), chlorothalonil will be removed for the European market by the season 2021.

The QoIs, commonly named strobilurins, inhibit the electron transport chain in complex III (bc1 complex) of mitochondrial respiration (Becker et al., 1981) and first launches were in 1996 for control of cereal diseases (Ypema and Gold, 1999; Bartlett et al., 2002). QoIs show a broad spectrum of activity against several fungal pathogens from the ascomycetes, basidiomycetes and oomycetes. On the other side, they have no or low effects on non-target organisms, low acute toxicity to animals and are rapidly degraded in soil resulting in

a beneficial environmental profile (Oliver and Hewitt, 2014; Lucas et al., 2015). The two first synthetic QoIs were kresoxim-methyl and azoxystrobin (Ammermann et al., 1992; Godwin et al., 1992; Sauter et al., 1999) which were followed by several other QoIs, for example, pyraclostrobin, trifloxystrobin, and picoxystrobin (Bartlett et al., 2002).

SDHIs inhibit the succinate dehydrogenase (SDH, also known as complex II), a relevant enzyme of the citric acid cycle and the mitochondrial respiratory chain (Hägerhäll, 1997). Inhibition of SDH results in energy deficiency and in release of active oxygen that explain the fungicidal activity of SDHIs (Oliver and Hewitt, 2014). In 1966, the first SDHIs, oxycarboxin and carboxin were released (von Schmeling and Kulka, 1966; Oliver and Hewitt, 2014). Several SDHIs followed between the 1970s and the end of the 1990s, but their activity was limited to basidiomycetes (Glättli et al., 2011). In 2002, due to further structural changes, boscalid, the first SDHI with a broad range of activity against fungal pathogens of the basidiomycota and ascomycota, was released (Stammler et al., 2007, 2008a; Glättli et al., 2011). The launch of boscalid initiated the release of a series of new SDHI compounds by several agrochemical companies including for example the compounds bixafen, fluopyram, fluxapyroxad, isopyrazam, benzovindiflupyr and penthiopyrad (Glättli et al., 2011; Stammler et al., 2015). The relevance of SDHIs in crop protection increased quickly making them rank with QoIs and DMIs in importance and market share (Oliver and Hewitt, 2014; Torriani et al., 2015).

DMIs belong to the fungicide class of the sterol biosynthesis inhibitors (SBIs). The SBI fungicide class consists of four sub-groups that all inhibit different target enzymes in the sterol biosynthesis pathway. These sub-groups are represented by the DMIs, the amines, the keto-reductase inhibitors, and the squalene-epoxidase inhibitors of which the latter ones are not used commercially in agriculture. The DMI group shows the largest number of fungicidal compounds and the broadest spectrum of activity across all SBI groups (FRAC, 2019). DMIs have been the most widely used fungicides in the management of STB in the last decades (Parker et al., 2011; Cools and Fraaije, 2013). As DMIs are the investigated fungicide class in the present work, their mode of action and other properties are introduced in more detail in the following chapter.

1.3.4 Demethylation inhibitors (DMIs)

The first DMIs were introduced around the 1970s and 40 DMI compounds followed until 2015 (Ziogas and Malandrakis, 2015). DMIs show a broad spectrum of activity against a range of economically important plant pathogenic ascomycetes and basidiomycetes on arable crops, viticulture, and plantation crops. An exception is represented by oomycetes pathogens because they do not have an ergosterol biosynthesis pathway (Oliver and Hewitt, 2014; FRAC, 2019). DMIs belong to several chemical groups, consisting of piperazines, pyridines, pyrimidines, imidazoles, triazoles, and triazolinthiones. A few

pyrimidines (e.g. fenarimol) and imidazoles (e.g. prochloraz) have gained market relevance in agriculture. The triazole group, however, is commercially the most important and consists of the highest number of compounds across all DMIs (Oliver and Hewitt, 2014; FRAC, 2019). The present work focuses on compounds of the triazole group.

Within the triazole group, marked relevant fungicides are represented by epoxiconazole (BASF SE), cyproconazole (formerly Sandoz, today Syngenta), triadimenol (Bayer), tebuconazole (Bayer), propiconazole (Janssen Pharmaceutica), and the triazolinthione prothioconazole (Bayer) (Oliver and Hewitt, 2014). In 2019, a new DMI, mefentrifluconazole (BASF SE), was registered and became available on the European market in 2020. As shown in Figure 3, the molecular structure of the triazoles is characterised by a nitrogen containing heterocycle with a lipophilic moiety (Mercer, 1993; Lamb et al., 1999). The structure of the azole theme of the triazolinthione, prothioconazole is different compared to the triazoles and shows a thio-group in the nitrogen-heterocycle. This thio-group, however, is lost during the activation process of the compound after exposure to plants (Oliver and Hewitt, 2014).

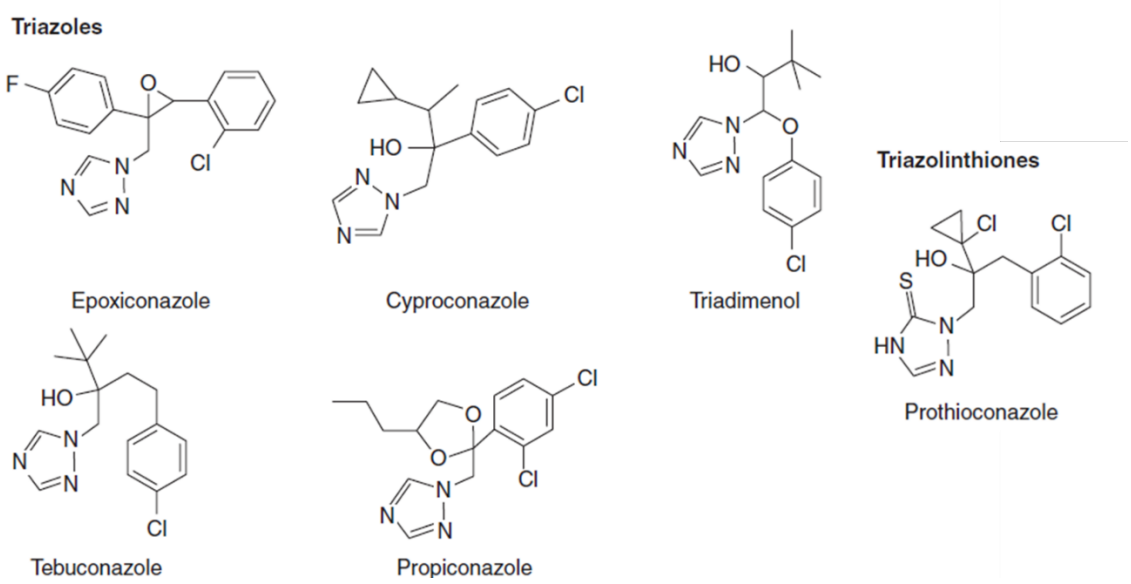


Figure 3: Chemical structure of important triazoles and prothioconazole, a triazolinthione. Figure was extracted and modified from Oliver and Hewitt, 2014.

The target of DMI fungicides is the sterol 14α-demethylase (CYP51 enzyme), a cytochrome P450 monooxygenase catalysing the removal of the C14-methyl group from 24-methylendihydrolanosterol (eburicol) in ergosterol biosynthesis pathway (Figure 4) (Yoshida and Aoyama, 1984, 1987; Gadher et al., 1983; Bossche et al., 1984; Oliver and Hewitt, 2014). The CYP51 enzyme is suggested to be a membrane bound protein localised in the endoplasmatic reticulum in close association with a flavoprotein (NADPH) that is necessary for the electron transfer in sterol 14-demethylation (Mercer, 1993; Parker et al., 2011; Kelly and Kelly, 2013). Inhibition of sterol 14-demethylation results in the accumulation of precursor sterols and in reduced ergosterol synthesis (Yoshida and

Aoyama, 1984, 1987; Baldwin, 1983, 1990; Gadher et al., 1983; Baloch et al., 1984; Kelly et al., 1995; Lamb et al., 1996). Ergosterol is the major sterol in fungi and serves as an important structural component in the architecture of cell membranes (Siegel, 1981; Mercer, 1993). Therefore, reduced ergosterol availability and the accumulation of precursor sterols lead to membrane disfunction (Siegel, 1981; Lamb et al., 1999; Becher and Wirsel, 2012; Oliver and Hewitt, 2014). Incorporation of precursor sterols was shown to result in decreased rigidity and increased fluidity of the plasma membrane (Abe et al., 2009). Furthermore, it is proposed that 14-methylated intermediates are toxic, and accumulation may also be responsible for growth inhibition (Kelly et al., 1995; Akins, 2005).

The number of *CYP51* genes within different fungal species was observed to vary between one, two or even three genes that encode CYP51 proteins falling into three different clades named CYP51A, CYP51B and CYP51C (Becher et al., 2011; Becher and Wirsel, 2012). CYP51A and CYP51B were identified, for example, in species of *Magnaporthe*, *Pyrenophora*, *Penicillium*, and *Fusarium*. Some species show only one CYP51A and one CYP51B protein, however, also others exist that show an additional duplication of CYP51A or CYP51B (Becher et al., 2011; Becher and Wirsel, 2012; Fan et al., 2013). The CYP51C clade was only detected in species of *Fusarium* that show three *CYP51* genes falling into all three clades (Becher et al., 2011). *Z. tritici* carries a single *MgCYP51* gene (hereafter referred to as *CYP51*) falling into clade CYP51B (Becher and Wirsel, 2012; Lucas et al., 2015).

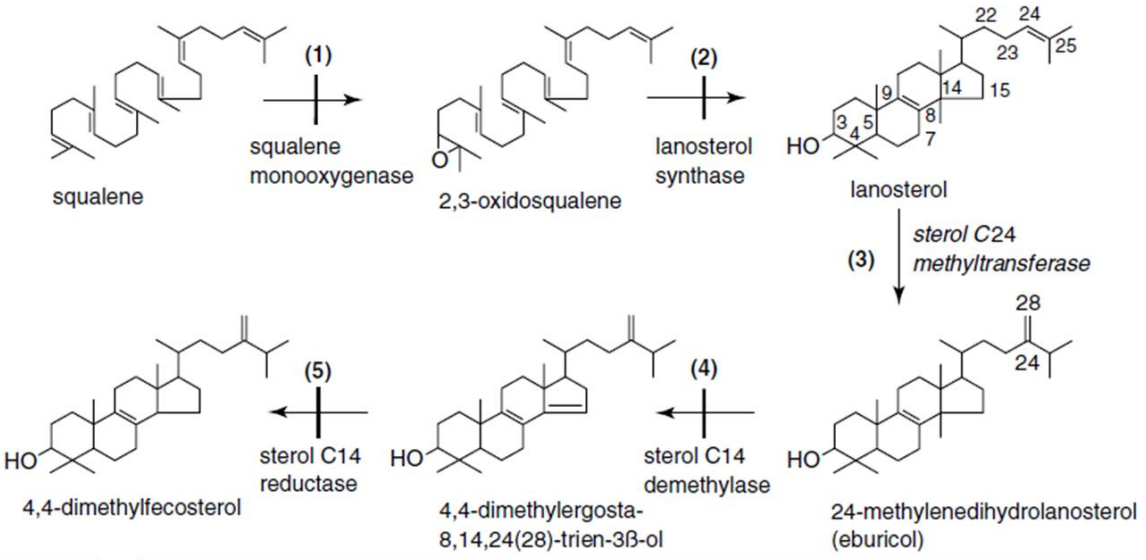


Figure 4: Part of the ergosterol biosynthesis pathway in fungi. Picture was extracted from Kuck et al., 2012 (modified). The first five reactions of ergosterol biosynthesis pathway and enzymes involved in these reactions are shown.

DMIs show protective as well as curative activity in control of *Z. tritici* (Jørgensen et al., 2018). As MgCYP51 from *Z. tritici* (hereafter referred to as CYP51) has not been crystallised so far, homology models based on the crystal structure of other organisms are used to

investigate the CYP51 protein structure and binding of triazoles in *Z. tritici*. It is expected that CYP51 of *Z. tritici* forms a typical P450 fold with a usually conserved structural core around the prosthetic haem group in the active site of the protein (Mullins et al., 2011). DMIs bind to CYP51 through direct interaction with the prosthetic haem group. Within this interaction the nitrogen atom (N-4) of the triazole ring forms a sixth ligand with the haem and the lipophilic moiety of N-1 binds to the amino acid tertiary structure of the active site and substrate channel (Jefcoate, 1978; Bossche et al., 1984; Lamb et al., 2003). The latter interaction probably explains differences in inhibition of CYP51 by different DMIs and their selectivity for different organisms (Mercer, 1993). The triazolinthione, prothioconazole, is suggested to bind not through direct coordination to the haem (Parker et al., 2011). However, the observed effectiveness of prothioconazole *in vivo* cannot be explained by inhibition that is expected from binding characteristics. Therefore, it is suggested that metabolization of prothioconazole to the desthio metabolite, a triazole, *in planta* and/or *in vivo* in the fungus may be necessary for the antifungal activity (Parker et al., 2011; Fraaije et al., 2012; Parker et al., 2013). Further studies, however, are needed to explore these hypotheses (Parker et al., 2011, 2013).

1.4 Fungicide resistance

Evolution is an ongoing process in all organisms comprising the ability of all species to evolve continuously in order to adapt to changes in their environment (Seidl and Thomma, 2014). In crop cultivation fungicides are part of this environment and exert selection pressure on fungal populations, hence forcing them to evolve mechanisms to overcome the lethal effects of a fungicide treatment (Hollomon, 2015; Lucas et al., 2015). An acquired, stable and heritable genetic alteration that enables a fungus to overcome harmful effects of a fungicide is defined as fungicide resistance (Delp and Dekker, 1985).

In fungal pathogens, four mechanisms have been documented that may confer fungicide resistance. These mechanisms include the alteration of the target site of the fungicide, the overexpression of the target site, the exclusion of the fungicide from the cell and, in rare cases, detoxification of the fungicide (Oliver and Hewitt, 2014; Lucas et al., 2015; reviewed FRAC, 2019).

In phytopathogenic fungi the most common resistance mechanism is the alteration of the target site due to mutations in the encoding genes (Ma and Michailides, 2005; Brent and Hollomon, 2007a; Jørgensen et al., 2019). Random mutations in the genome occur in all living organisms in a low natural rate (Ma and Michailides, 2005; Brent and Hollomon, 2007a). These mutations can result in changes of the amino acid sequence and consequently in an altered shape of the target protein that reduces the binding affinity of a fungicide (Brent and Hollomon, 2007a; FRAC, 2019). Alteration of the target site often

results in cross-resistance between fungicides of the same MOA group but not exclusively (Oliver and Hewitt, 2014). Compared to the sensitive population a resistant individual has a competitive advantage under fungicide application (van den Bosch et al., 2014; Oliver and Hewitt, 2014). Whereas application of the fungicide removes the majority of the sensitive population, the resistant mutant is selected and may become dominant under continued selection pressure of the fungicide (Ma and Michailides, 2005; Brent and Hollomon, 2007a; Oliver and Hewitt, 2014; Lucas et al., 2015). In the absence of fungicide application, the fitness of resistant individuals determines their persistence in a fungal population. Target site alterations are often associated with a reduced fitness in resistant individuals compared to the sensitive population. If a so-called fitness penalty/fitness cost exists, the frequency of resistant individuals may decrease in the absence of fungicide selection pressure (Ma and Michailides, 2005; Oliver and Hewitt, 2014; Ishii, 2015).

The second known resistance mechanism is the overexpression of the target enzyme based on the upregulation of the encoding gene (Lucas et al., 2015; FRAC, 2019). A higher number of target enzymes may increase the likelihood of an interaction with the natural substrates. Consequently, cellular processes can occur to some degree despite the presence of a fungicide (FRAC, 2019). In phytopathogenic fungi, overexpression of the target enzyme is not a widespread mechanism. Nevertheless, it was reported in some cases (Lucas et al., 2015). *CYP51* overexpression was detected for example in *Penicillium digitatum*, *Venturia inaequalis* and in *Z. tritici* (Hamamoto et al., 2000; Schnabel and Jones, 2001; Cools et al., 2012).

Increased active efflux by membrane bound transporters, in literature commonly referred to as multidrug resistance (MDR), was shown to be the third mechanism contributing to fungicide resistance (de Waard et al., 2006; Coleman and Mylonakis, 2009; Hahn and Leroch, 2015). Thereby, overexpression of ABC- or MFS-type multidrug transporters confer an increased efflux activity that prevents the accumulation of toxic fungicide concentrations at the target site inside fungal cells (de Waard et al., 2006; Hahn and Leroch, 2015). MDR is well known from bacteria as well as from human cancer cells and was described for human fungal pathogens such as *Candida albicans* and *C. glabrata* (Grkovic et al., 2002; Morschhäuser, 2010; Wu et al., 2014). So far, the importance of increased efflux activity in plant pathogenic fungi is still under evaluation (Omrane et al., 2015; Jørgensen et al., 2019) and differently discussed.

The fourth known resistance mechanism is the detoxification of fungicides based on metabolic enzymes as glutathione transferases, esterases or cytochrome P450s (Jabs et al., 2001; Leroux et al., 2002; Lucas et al., 2015; Sevastos et al., 2017). Enhanced metabolism conferring detoxification of pesticides is a widespread mechanism in herbicide resistance of weeds (Yu and Powles, 2014) and in insecticide resistance of insects (Li et al., 2007). In contrast to this, metabolization of fungicides is rare in fungi (Oliver and Hewitt,

2014; Lucas et al., 2015) and was only reported in a few species for example in *V. inaequalis* (Jabs et al., 2001), *Botrytis cinerea* (Leroux et al., 2002), and *Fusarium graminearum* (Sevastos et al., 2017).

In literature, two patterns of fungicide resistance emergence are described, 'qualitative' (also named 'single-step' or 'disruptive') resistance and 'quantitative' (also named 'multi-step' or 'continuous') resistance (Brent and Hollomon, 2007a). 'Qualitative' resistance is often accompanied by a sudden, considerable loss of fungicide efficacy and the emergence of a clearly distinguishable sensitive and resistant population. This resistance pattern is mainly caused by single mutations in major genes and therefore controlled monogenically (Brent and Hollomon, 2007a; de Miccolis Angelini et al., 2015). Development of QoI resistance in several pathogens based on the single amino acid alteration G143A in CYTB is an example of 'qualitative' resistance emergence (Gisi et al., 2002; Fraaije et al., 2002, 2005; Lesniak et al., 2011). 'Quantitative' resistance is characterised through a slow, gradual and continuous shift of a sensitive fungal population towards increased resistance levels. Usually, this resistance pattern relies on mutations in several genes (polygenic) with a low individual effect on the phenotype (minor genes). Additionally, accumulation of several mutations within a single target-site encoding gene was reported (polyallelic). Additive effects of multiple mutated minor genes or mutations within a single gene can result in an increased and varying level of resistance and consequently in a gradual emergence of resistance. As a result, a continuous range of sensitivity across adapted sub-populations is observed (Sanglard et al., 1998; Brent and Hollomon, 2007a; Cools and Fraaije, 2008; de Miccolis Angelini et al., 2015). Quantitative resistance emergence was described for example for ethirimol resistance in *B. graminis* (Hollomon, 1981) and for DMI resistance in *Z. tritici* (Stergiopoulos et al., 2003; Cools et al., 2005; Cools and Fraaije, 2008).

The risk of fungicide resistance development is influenced by the biology of the pathogen, the chemical factors of the fungicide and the agricultural measures (treatment measures) (Brent and Hollomon, 2007a; Hollomon, 2015). For example, pathogens with a short life cycle, high spore production, a sexual stage and rapid, long-distance dispersal of spores are more prone to emergence of fungicide resistance than pathogens without these properties (Brent and Hollomon, 2007a, 2007b). The fungicide associated risk is affected by the chemical class of the fungicide, the mode of action, and properties of action. Systemic fungicides with a single target site show a higher risk for resistance development compared to non-systemic fungicides and fungicides with multi-site action (Brent and Hollomon, 2007b; Oliver and Hewitt, 2014; Hollomon, 2015). Single-site fungicides have one biochemical target. Hence, a single gene mutation can rapidly lead to resistance. In contrast, multi-site inhibitors target multiple biochemical processes and a combination of several mutations would be required for resistance. Therefore, resistance emergence for multi-sites is generally much slower and less likely (Hollomon, 2015). The pathogen and fungicide associated risk are inherent and mostly out of the growers control, however, the

treatment measures can vary and can be adjusted to reduce the risk of resistance development (Brent and Hollomon, 2007a, 2007b; Hollomon, 2015). In general, the risk can be reduced by reducing the fungicides use and by employing cultural measures or appropriate choice of cultivars that do not favour disease development. Additionally, frequency and dose of a fungicide application as well as the agricultural system (protected or arable cropping) can affect development of fungicide resistance (Brent and Hollomon, 2007a, 2007b; Grimmer et al., 2015).

Since the 1970s, occurrence of fungicide resistance has increased and has become an important issue in a broad range of phytopathogenic fungi associated with the introduction and widespread use of fungicides with specific MOAs (Brent, 2012; Lucas et al., 2015). As a result, in 1981 the Fungicide Resistance Action Committee (FRAC), an industry run group of specialists from agrochemical manufacturers, was founded. In this organisation resistance issues are discussed, and fungicide resistance management guidelines are published annually. These guidelines provide information for various crops and pathogens about how to apply fungicides and at the same time maintain a good anti-resistance management (FRAC, 2019). Strategies of anti-resistance management will be discussed at a later point in the present work.

1.4.1 Resistance to demethylation inhibitors in phytopathogenic fungi

In many fungal plant pathogens, resistance to DMIs evolved gradually and slowly over the last 20 years (Oliver and Hewitt, 2014) based on emergence of different resistance mechanisms. In several pathogens single target-site alterations within CYP51 and/or the accumulation of different alterations was shown to be responsible for DMI adaptation (Délye et al., 1997; Wyand and Brown, 2005; Cools and Fraaije, 2008; Stammler et al., 2008b; Rehfus et al., 2019). Interestingly, some alterations could be detected at homologous positions across different plant pathogenic fungi and human pathogens, for example, Y137F in *Z. tritici* that was also identified at homologous positions in *Erysiphe necator*; *Phakopsora pachyrhizi*, *B. graminis* and *C. albicans*. Instead, others occurred solely in one species or genus (Becher and Wirsel, 2012; Schmitz et al., 2014; Mair et al., 2016). In contrast to most target site alterations that confer resistance against single-site fungicides of other MOAs, mutations in *CYP51* often affect only sensitivity to a specific or a limited group of DMIs. Therefore, they confer only incomplete cross-resistance across all DMI fungicides (Stammler et al., 2008b; Cools et al., 2013; Ziogas and Malandrakis, 2015).

The second mechanism contributing to DMI resistance is the overexpression of *CYP51*. This phenomenon was detected in different plant pathogens for example *V. inaequalis* (Schnabel and Jones, 2001), *Cercospora beticola* (Bolton et al., 2012), *P. triticina* (Stammler et al., 2009), *P. pachyrhizi* (Schmitz et al., 2014) and *Z. tritici* (Cools et al., 2012).

CYP51 overexpression is often based on alterations (e.g. insertions) in the predicted regulatory region of the *CYP51* gene (Schnabel and Jones, 2001; Cools et al., 2012; reviewed in Cools et al., 2013). This mechanism was shown to confer lower resistance levels than CYP51 target site alterations in many pathogens, however, affects sensitivity to all DMIs (reviewed in Cools et al., 2013).

The third mechanism affecting DMI resistance is an enhanced efflux activity. Whereas this mechanism is well established in human pathogens, its importance remains less clear in plant pathogens (Morschhäuser, 2010; Cools et al., 2013). So far, in plant pathogens it has only been described for example in *B. cinerea* (Kretschmer et al., 2009), *Oculimacula yallundae* (Leroux et al., 2013) and *Z. tritici* (Stammler and Semar, 2011; Leroux and Walker, 2011; Omrane et al., 2015). Overexpression of ABC- or MFS-type multidrug transporters conferring an increased efflux activity (de Waard et al., 2006; Hahn and Leroch, 2015) was shown to rely on mutations in transcription factors or on insertions in the promotor region regulating the expression of transporter genes (Coste et al., 2006; Kretschmer et al., 2009; Omrane et al., 2015, 2017). Compared to the relatively high fungicide resistance levels conferred by target site mutations, MDR confers low to medium resistance levels (Hahn and Leroch, 2015).

The presence of several *CYP51* paralogues may be advantageous under DMI selection pressure as costs of changes in the enzyme structure or an increased gene expression may be compensated by the presence of an unchanged *CYP51* paralogue (Cools et al., 2013). Nevertheless, solely a few studies exist about the function of multiple *CYP51* paralogues and further research is necessary to elucidate this topic (Ziogas and Malandrakis, 2015).

Despite a long-term and widespread use of DMIs, control failures of these fungicides are rare in the field (Cools et al., 2013). The time period until fungicide resistance was detected after the market launch of DMI fungicides varied for several pathogens. For example, compared to other pathogens, emergence of DMI resistance was relatively fast in *B. graminis* f. sp. *graminis* and *O. yallundae* where the first detection of resistance was only 3 to 5 years after introduction. In many other pathogens, for example, in *B. graminis* f. sp. *tritici*, *Z. tritici*, *V. inaequalis*, and *P. triticina*, the first detection of resistance took a lot longer, with up to 10 years or more after introduction (Stanis and Jones, 1985; reviewed in Jørgensen et al., 2019). The occurrence of resistant strains does not necessarily result in field failure of fungicides as the resistance levels are often low and incomplete cross-resistance across DMIs can exist (Stammler et al., 2008b; Cools et al., 2013). Nevertheless, the continued intensive use of DMIs has led to the development of resistance levels that can now reach more critical levels in some regions accompanied with marked reduction in pathogen sensitivity increasing the need for regular resistance monitoring (Jørgensen et al., 2018, 2019).

1.4.2 Fungicide resistance in *Z. tritici*

The pathogen associated risk for the development of fungicide resistance in *Z. tritici* is classified as being medium (Brent and Hollomon, 2007b). For all three single-site fungicide classes mainly used in the control of STB (QoIs, SDHIs, DMIs) resistant isolates can be detected in the field, however their frequencies and distribution vary across Europe (Fraaije et al., 2005; Lucas and Fraaije, 2008; Cools and Fraaije, 2013; Rehfus, 2018; FRAC, 2019).

Resistance of *Z. tritici* to QoI fungicides occurred soon after their introduction and was shown to rely on the alteration G143A in CYTB leading to high resistance levels against QoIs (Gisi et al., 2002; Fraaije et al., 2005). A rapid increase in the frequency of isolates carrying the G143A was observed even two seasons after its initial retrospective detection in 2001 in UK (Fraaije et al., 2005). Already in 2006, more than 90% of STB isolates in Northwest Europe carried alteration G143A (Jørgensen et al., 2019) making QoIs inefficient in the control of *Z. tritici* in these regions (Lucas and Fraaije, 2008).

The first SDHI resistant *Z. tritici* isolates were detected in 2012 (FRAC, 2019). SDHI resistance is based on different SDH alterations, for example, B-N225I, B-T268I/A, C-T79N, C-N86S, C-W80S, and C-H152R. It was observed that some alterations confer high resistance levels, whereas others only lead to a lower resistance level *in vitro* and *in planta* (Rehfus, 2018; FRAC, 2019). In 2018, frequency of isolates with low resistance levels increased in Northern Germany, Ireland, the Netherlands, and the United Kingdom (UK) with alterations C-T79N and C-N86S being the most frequent ones. A low frequency of isolates with low resistance factors were found in other countries. The overall frequency of isolates showing moderate resistance factors (C-H152R) was low. So far, no reduced field performance of SDHIs is reported (FRAC, 2019).

The first isolates with a reduced DMI sensitivity were identified in a time period from 1993 to 2002 by Mavroeidi and Shaw (2005). Since then a gradual shift in the sensitivity of *Z. tritici* populations towards a reduced DMI sensitivity was observed for DMIs currently on the European market (Fraaije et al., 2007; Cools et al., 2011; Lucas et al., 2015). Until 2008, no reduced field performance of DMIs in control of *Z. tritici* was reported, although different *CYP51* haplotypes were detected in field populations and a shift towards a reduced DMI sensitivity was measured *in vitro*. Nevertheless, a decline in the field efficacy of DMIs was observed in recent years, however, with varying levels of DMI efficacy across different DMIs and countries of Europe (Clark, 2006; Heick et al., 2017a; Jørgensen et al., 2017b; Strobel et al., 2017; Blake et al., 2018; Jørgensen et al., 2018). The underlying resistance mechanisms were observed to be complex and diverse (Cools and Fraaije, 2013; Leroux and Walker, 2011) and will be deciphered in the next chapter.

1.4.3 DMI resistance mechanisms in *Z. tritici*

All three resistance mechanisms known to confer reduced DMI sensitivity in several plant pathogens (described in chapter 1.4.1) were identified in *Z. tritici*. The most common mechanism is the alteration of CYP51 (Cools and Fraaije, 2013; Cools et al., 2013). Additionally, *CYP51* overexpression and an increased efflux through overexpression of membrane bound transporters was shown to reduce DMI sensitivity of *Z. tritici* (Leroux and Walker, 2011; Cools et al., 2012; Omrane et al., 2015).

So far, more than 30 alterations in CYP51 based on mutations and deletions in the *CYP51* gene have been described (Cools and Fraaije, 2013). These alterations were observed to accumulate and to occur in several combinations resulting in distinct *CYP51* haplotypes (Brunner et al., 2008; Cools and Fraaije, 2013). A heterogenous frequency of CYP51 alterations was observed over time and across Europe (Leroux et al., 2007; Brunner et al., 2008; Stammler et al., 2008b; Stammler and Semar, 2011). Brunner et al. (2008) suggested that new *CYP51* mutations and new combinations of mutations conferring increasing DMI adaptation arose locally in North-West Europe either through *de novo* mutations or through intragenic recombination. Subsequently, they were spread eastwards through wind-dispersed ascospores (Brunner et al., 2008). Alterations L50S, D134G, V136A/C, S188N, A379G, I381V, Y459D, Y461N, N513K, and S524T as well as deletion of Y459 and G460 were most commonly found in modern European *Z. tritici* populations (Stammler et al., 2008b; Cools and Fraaije, 2013). As most of these alterations occur in combination, it is difficult to assess their individual effect on triazole sensitivity and CYP51 enzyme activity (Lucas et al., 2015). Nevertheless, heterologous expression of *ScCYP51* mutations in a *Saccharomyces cerevisiae* mutant and comparison of the sensitivity of resistant phenotypes only differing in specific CYP51 alterations allowed the characterisation of individual mutations (Fraaije et al., 2007; Leroux et al., 2007; Cools et al., 2010, 2011; Leroux and Walker, 2011). It was observed that some alterations such as L50S, S188N, and N513K confer no effect on DMI sensitivity (Leroux et al., 2007; Cools and Fraaije, 2013), whereas alterations V136A, I381V, S524T and alterations or deletions at positions 459-461 result in a decreased sensitivity to DMIs (Fraaije et al., 2007; Leroux et al., 2007; Cools et al., 2010, 2011; Leroux and Walker, 2011; Cools and Fraaije, 2013). Some of the CYP51 alterations have contrasting effects on sensitivity to different DMIs and lead to *CYP51* haplotypes showing incomplete cross-resistance between different DMIs (Leroux et al., 2007; Fraaije et al., 2007; Cools et al., 2011). Alteration V136A, for example, causes a reduced sensitivity to epoxiconazole, prothioconazole, prochloraz, and propiconazole, whereas sensitivity to tebuconazole, triadimenol, and difenoconazole remains high (Cools et al., 2011; Leroux and Walker, 2011; Lucas et al., 2015). In contrast to this, alteration I381V affects sensitivity to most triazoles, especially tebuconazole, whereas sensitivity to prochloraz is increased (Fraaije et al., 2007; Leroux et al., 2007). It is suggested that the *Z. tritici* population is able to continuously adapt to changing patterns of fungicide use based on the fact that resistance

first evolved for older DMIs as triadimenol and tebuconazole and afterwards for each newly introduced DMI. This circumstance has led to the emergence of increasingly complex *CYP51* haplotypes based on the accumulation of additional alterations in CYP51 (Cools and Fraaije, 2013; Lucas et al., 2015; Jørgensen et al., 2019). Emergence of alteration S524T (Stammler et al., 2008b) is the latest confirmation of this assumption as this alteration is now often found in *CYP51* haplotypes that show increased adaptation to the two currently most effective and widely used triazoles epoxiconazole and prothioconazole (Cools et al., 2011; Lucas et al., 2015).

Different approaches were used to classify *CYP51* haplotypes, among them the most commonly used R-type system that categorises different *CYP51* haplotypes into triazole resistant types ('tri R-types') and additionally follows the putative evolution of *CYP51* (Leroux et al., 2007; Leroux and Walker, 2011). This R-type system has been a valid tool in the past, however, has not been updated recently, whereas increasingly complex *CYP51* haplotypes continued to occur (Stammler and Semar, 2011; Cools and Fraaije, 2013; Buitrago et al., 2014). To date, many *CYP51* haplotypes without a R-type description exist. Due to the accumulation of several alterations in *CYP51*, it is no longer a convenient approach to describe resistant isolates simply by listing of all alterations as it has been done for other pathogens. Therefore, a new approach to describe *CYP51* haplotypes was developed in the course of the present work.

The second mechanism contributing to DMI resistance in *Z. tritici* is the overexpression of *CYP51* (Stergiopoulos et al., 2003; Cools et al., 2012). A constitutive 10- to 40- fold *CYP51* overexpression was observed in isolates which showed a 7- to 16-fold reduction in DMI sensitivity *in vitro* (Cools et al., 2012). As *CYP51* overexpression is known to rely in many cases on alterations (e.g. insertions) in the predicted regulatory region (Schnabel and Jones, 2001; Cools et al., 2012, 2013), the *MgCYP51* promotor region (hereafter referred to as *CYP51*p) was investigated for *Z. tritici* (Chassot et al., 2008; Cools et al., 2012; Omrane et al., 2015). A high polymorphism in *CYP51*p and prevalence of small inserts (8 to 30 bp) were identified across field isolates of *Z. tritici* (Omrane et al., 2015). Nevertheless, overexpression of *CYP51* could only be correlated to a 120 bp insert in *CYP51*p of *Z. tritici* (Cools et al., 2012). Another *CYP51*p insert of around 1000 bp was identified in isolates showing a reduced DMI sensitivity compared to isolates with the same *CYP51* haplotype without a *CYP51*p insert (Chassot et al., 2008). However, more recent studies revealed that the precise size of this insert is either 862 bp or 866 bp and that these two inserts have no, or at least not a standalone, distinct impact on *CYP51* expression (Omrane et al., 2015; Kildea et al., 2019). In contrast to CYP51 alterations that can affect several DMIs differently, *CYP51* overexpression affects sensitivity to all DMIs (complete cross-resistance) (Cools et al., 2012; Cools and Fraaije, 2013).

The third mechanism contributing to DMI resistance of *Z. tritici* is an increased active efflux of membrane bound transporters. This assumption was made after the detection of *Z. tritici* isolates that showed a reduced sensitivity to triazoles (cross-resistance) and simultaneously to some unrelated compounds as tolnaftate and terbinafine as well as to some SDHIs (Leroux and Walker, 2011). Although several transporter genes were identified to be overexpressed in isolates which showed cross-resistance between unrelated compounds, the overexpression of the *Mg*MFS1 transporter (hereafter referred to as MFS1 transporter) was strongest indicating that this transporter plays a major role in increased efflux activity of *Z. tritici* (Omrane et al., 2015, 2017). As observed for *CYP51*p, a high polymorphism in the *MgMFS1* promotor region (hereafter referred to as *MFS1*p), including small insertions between 1 to 29 bp, was observed in isolates expected to exert increased efflux (Omrane et al., 2015). However, constitutive overexpression of the *MgMFS1* gene (hereafter referred to as *MFS1* gene) was shown to rely on different larger insertions in *MFS1*p, namely a 150, 338, 369, and 519 bp insert (Omrane et al., 2015, 2017). Based on their results, Omrane et al. (2017) proposed a classification for different *MFS1*p inserts. The 519 bp insert was labelled as type I insert, the 338 and 369 bp inserts were labelled as type II, and the 150 bp insert was labelled as type III. The 519 bp insert is associated with the highest *MFS1* overexpression, whereas type II and type III inserts confer significantly lower *MFS1* overexpression compared to isolates with the 519 bp insert (Omrane et al., 2015, 2017). Research into the frequency and distribution of *MFS1*p inserts across European *Z. tritici* populations and knowledge about the effect of an increased efflux on DMI sensitivity is in its infancy.

In this work, *CYP51* genes and *CYP51*p or *MFS1*p regions of pathogens other than *Z. tritici* will be designated with the respective abbreviation of the name of the pathogen.

2 Objectives

Fungicide resistance monitoring and characterisation of resistance mechanisms is essential for the establishment of anti-resistance management strategies, thereby ensuring the sustainable use of fungicides in the European market. A shift in DMI sensitivity of *Z. tritici* has been reported over many years associated with the emergence and combination of CYP51 alterations. Distinct *CYP51* haplotypes developed and occurred over time and space in Europe. More recently, it has been shown that *CYP51* overexpression and an increased efflux activity additionally affect DMI sensitivity in *Z. tritici*. In this study, isolates from the European DMI sensitivity monitoring of 2016 and 2017 were analysed. The aim was to identify the frequency of different DMI resistance mechanisms in *Z. tritici* across Europe and to characterise their effect on DMI adaptation. Therefore, the following topics were investigated and discussed in this work:

- Detection of *CYP51* haplotypes, *CYP51* overexpression and an increased efflux activity in *Z. tritici*
- Development of a new nomenclature for labelling *CYP51* haplotypes
- Determination of the effect on DMI sensitivity associated with new CYP51 alterations
- *In vitro* sensitivity evaluation of isolates carrying different DMI resistance mechanisms
- Detection of the frequency and distribution of different DMI resistance mechanisms across Europe
- Analysis of cross-resistance between different DMIs
- *In vivo* sensitivity evaluation of isolates carrying different DMI resistance mechanisms
- Estimation about further evolution and spread of different DMI resistance mechanisms
- Discussion of consequences for efficacy of DMIs in the field and implication for future DMI field performance

3 Material and Methods

3.1 Technical equipment

In Table 1, technical equipment used during the present study is listed. Devices such as centrifuges, incubators, thermomixer and pipettes, typically used in laboratories are not included.

Table 1: Technical equipment used.

Equipment	Company
Airbrush (nozzle size 0.8 mm), SATAminijet® 3000 B HVLP	SATA GmbH & Co. KG, Kornwestheim, DE
Analytical balance, MC 410S	Sartorius AG, Göttingen, DE
Application chamber SPK08 (applying 400 L/ha)	BASF SE, Ludwigshafen, DE
Blue light transillumination, UVT-28 BE	Herolab GmbH Laborgeräte, Wiesloch, DE
Gel documentation system, EasyDoc plus	Herolab GmbH Laborgeräte, Wiesloch, DE
Gel electrophoresis, Sub-Cell GT Basic System	Bio-Rad Laboratories Inc., Hercules, US
Inoculation station	BASF SE, Ludwigshafen, DE
KNF Laboport® vacuum pump	KNF Neuberger GmbH, Freiburg, DE
Macherey-Nagel™ NucleoVac 96 Vacuum Manifold	Macherey-Nagel GmbH & Co. KG, Düren, DE
Macherey-Nagel™ NucleoVac Vacuum Regulator	Macherey-Nagel GmbH & Co. KG, Düren, DE
Microscope, Leica DMLB	Leica Mikrosysteme Vertrieb GmbH, Wetzlar, DE
PowerPac™ Basic Power Supply	Bio-Rad Laboratories Inc., Hercules, US
Real-Time PCR Thermocycler, q-Tower^3	Analytik Jena AG, Jena, DE
Spectrophotometer, NanoDrop 2000	Thermo Fisher Scientific Inc., Waltham, US
Sunrise™ absorbance reader	TECAN Group AG, Männerdorf, CH
Thermal cycler, DNA Engine DYAD	Bio-Rad Laboratories Inc., Hercules, US
Thermal cycler, Mastercycler gradient	Eppendorf AG, Hamburg, DE
Thermal cycler, peqSTAR 96X Universal Gradient	VWR International GmbH, Darmstadt, DE
Thermal cycler, MJ Research PTC-200 Gradient	Bio-Rad Laboratories Inc., Hercules, US
Tissue Lyser, Mixer Mill MM200	Retsch GmbH, Haan, DE
UV light transillumination, UVT-28 ME-HC	Herolab GmbH Laborgeräte, Wiesloch, DE
XC10 Color Camera	Olympus Deutschland GmbH, Hamburg, DE

3.2 Chemicals and consumables

Chemicals and consumables used in this study are shown in Table 2.

Table 2: Chemicals and consumables used.

Chemicals/Consumables	Company
0.2 mL Thermo Strips	Thermo Fisher Scientific Inc., Waltham, US
6x Orange DNA loading dye	Fermentas GmbH, St. Leon-Rot, DE
8 cm plant pots	Pöppelmann GmbH und Co. KG, Lohe, DE
96-well microtiter plates	VWR International GmbH, Darmstadt, DE
96-well PCR plates	4titude® Ltd., Wotton, UK
ABgene™ 48-deep well plates	Thermo Fisher Scientific Inc., Waltham, US
Aceton	Bernd Kraft GmbH, Duisburg, DE
Adhesive PCR Seal	4titude® Ltd., Wotton, UK
Bacto™ Peptone	Becton, Dickinson and Company, Franklin Lakes, US
Biozym LE Agarose	Biozym Biotech Trading GmbH, Wien, AT
C-Chip disposable haemocytometer	NanoEnTek Inc., Seoul, KR
ClipTip™ pipette tips	Thermo Fisher Scientific Inc., Waltham, US
Combitips advanced	Eppendorf AG, Hamburg, DE
DEPC-water	Ambion Inc., Austin, US
Deltalab cotton swabs (sterile)	Deltalab, Barcelona, ES
Difco™ Agar	Becton, Dickinson and Company, Franklin Lakes, US
Difco™ ISP medium 2	Becton, Dickinson and Company, Franklin Lakes, US
Dimethylsulfoxid (DMSO)	AppliChem GmbH, Darmstadt, DE
EDTA	Calbiochem, Merck KGaA, Darmstadt, DE
Eppendorf Safe-Lock Tubes	Eppendorf AG, Hamburg, DE
Ethanol	Sigma Aldrich, St. Louis, US
Ethidium bromide (10 mg/mL)	Thermo Fisher Scientific Inc., Waltham, US
Falcon tubes	Sarstedt AG & Co. KG, Nümbrecht, DE
Gauze	Lohmann & Rauscher GmbH & Co. KG, Neuwied, DE
GelGreen nucleic acid stain (10.000x)	Biotium, Inc., Hayward, US
Glacial acetic acid	Honeywell Specialty Chemicals Seelze GmbH, Seelze, DE
Glycerol	VWR International GmbH, Darmstadt, DE
Meliseptol® Foam pure	B. Braun Melsungen AG, Melsungen, DE
O´GeneRuler, 1 kb DNA Ladder	Fermentas GmbH, St. Leon-Rot, DE
Petri dish (Ø 92 mm)	Greiner Bio-One International GmbH, Frickenhausen, DE
Invitrogen™ UltraPure™ DNA Typing Grade™ 50x TAE Buffer	Thermo Fisher Scientific Inc., Waltham, US

Chemicals/Consumables	Company
TipOne® RPT filter tips	Starlab GmbH, Ahrensburg, DE
RNase Away	Molecular Bio-Products Inc., San Diego, US
Streptomycin sulphate	Sigma Aldrich, St. Louis, US
Tris Base, Molecular biology grade	Merck KGaA, Darmstadt, DE
Tween® 80	Merck KGaA, Darmstadt, DE
Ultra Clear Cap Strips	Thermo Fisher Scientific Inc., Waltham, US
Yeast Extract	Merck KGaA, Darmstadt, DE

3.3 Enzymes and kits

Enzymes and kits used in the present study can be seen in Table 3.

Table 3: Enzymes and kits used.

Name	Company
DreamTaq™ Hot Start 2x PCR Master Mix	Thermo Fisher Scientific Inc., Waltham, US
Maxima™ Hot Start 2x PCR Master Mix	Thermo Fisher Scientific Inc., Waltham, US
NucleoSpin® Gel and PCR Clean-up	Macherey-Nagel GmbH & Co. KG, Düren, DE
NucleoSpin® 96 PCR Clean-up	Macherey-Nagel GmbH & Co. KG, Düren, DE
NucleoSpin® Plant II	Macherey-Nagel GmbH & Co. KG, Düren, DE
NucleoSpin® 8 Plant II	Macherey-Nagel GmbH & Co. KG, Düren, DE
NucleoSpin® 96 Plant II	Macherey-Nagel GmbH & Co. KG, Düren, DE
Phusion™ Hot Start, High-Fidelity DNA Polymerase Master Mix	Thermo Fisher Scientific Inc., Waltham, US
Takyon™ No ROX Probe 2x MasterMix dTTP	Eurogentec, Seraing, BE

3.4 Buffers and solutions

In Table 4, buffers and solutions used during this work are listed.

Table 4: Buffers and solutions used.

Name	Composition	Notes
10% aceton	10% (v/v) acetone	
TAE buffer (50x)	2 M Tris Base 1 M acetic acid 5 mM EDTA	dilution of stock solution to 1x working solution with deionized water

Name	Composition	Notes
TAE buffer (1x)	TAE buffer (50x) Invitrogen™ UltraPure™ DNA Typing Grade™	dilution of stock solution to 1x working solution with deionized water
Tris-HCl buffer (10x)	0.5 M Tris base	pH 8 (with HCl), autoclaved, dilution of stock solution to 1x working solution with sterile water

3.5 Growth media

Growth media used for cultivation of *Z. tritici* are shown in Table 5.

Table 5: Growth media used.

Name	Composition	Notes
Inoculation medium	0.02% Tween 80 (v/v)	
ISP2-agar with streptomycin	3.8% (w/v) ISP medium 2 (0.003% (w/v) streptomycin sulphate)	autoclaved, pH 7.2, cooled to 60°C before addition of streptomycin (100 mg/mL stock solution, sterile filtered, to 100 mg/L)
Malt medium with glycerol	2% (w/v) malt extract 15% (v/v) glycerol	autoclaved
YBG medium d.c.	2% (w/v) Bacto™ Peptone 2% (w/v) yeast extract 4% (v/v) glycerol	autoclaved, pH 6.8

3.6 Oligonucleotides

Table 6 lists oligonucleotides used as primers for PCR reactions and for Sanger sequencing in the present work. In addition, the used polymerase, annealing temperature, and target/purpose are shown. Oligonucleotides were synthesized internally at BASF SE with exception of probe St-Cyp51-524 that was purchased from Eurogentec.

Table 6: Oligonucleotides used. The sequence in 5'→ 3' orientation, annealing temperature (T_m), and target/purpose are shown.

Name	Sequence (5'->3')	T_m (°C)	Target/purpose
KES 540 (fw)	ATGGGTCTCCTCCAGGAAGTC	69	amplification and sequencing *CYP51*
KES 541 (rv)	TCAGTTCTTCTCCTCCTTCTCCTC		
KES 2192 (fw)	CCTCGACAACATCCTCTG	63.5	amplification and sequencing *CYP51*
KES 2193 (rv)	GCATAAGATCCACCTATGG		
KES 2223 (rv)	TGAGCTCCTTGATACTGCCGTCG	50	sequencing *CYP51*
KES 2283 (fw)	ATCCTCTGTGCCAATTTCTC	50	sequencing *CYP51*

Name	Sequence (5‘->3‘)	T_m (°C)	Target/purpose
KES 2136 (fw)[1]	GTGGCGAGGGCTTGACTAC	64	amplification *CYP51*p
KES 2188 (rv)	GTCTGGCCGAATTGCGCGTC		
KES 2143 (fw)[2]	ACATGATCCCTGATCCGTTC	58	amplification *MFS1*p
KES 2144 (rv)[2]	CGGCGACTTCTTGCTGAA		
KES 2334 (fw)[3]	GCAAGGATTCGGACTTGACG	63	amplification *MFS1*p
KES 2335 (rv)[3]	CTGCCGGTATCGTCGATGAC		
KES 1139 (fw)	GTACGGATTACAGCAGTTTGTTCGG	60	MAMA primer S524 in CYP51
KES 1144 (rv)	CCAAGTCTTCCACTTCAGTTCTTCT		qPCR for S524 or T524 in CYP51
KES 1138 (fw)	GTACGGATTACAGCAGTTTGTTCTC		MAMA primer T524 in CYP51
St-Cyp51-524	TAAAGZGGGEGAGPAGGPA		probe (6´FAM) for detection S524T in CYP51

[1] sequence is extracted from Cools et al., 2012, KES 2136 = Mg51-proF

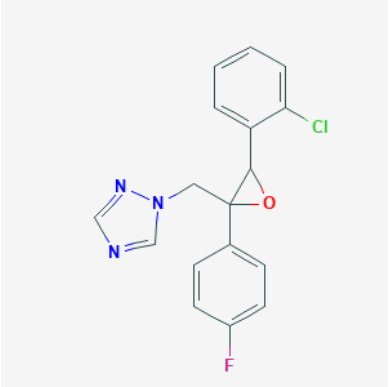
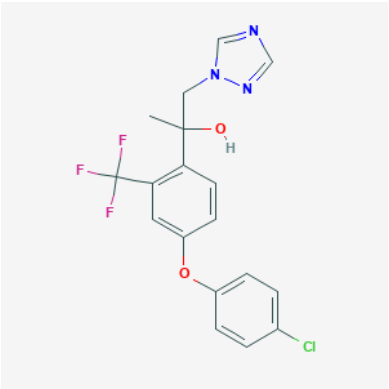
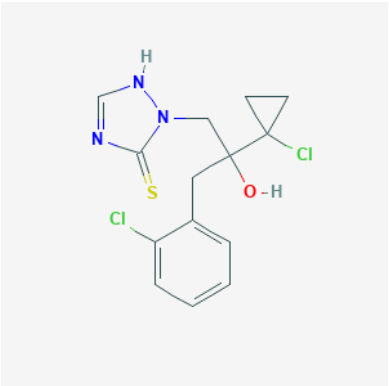
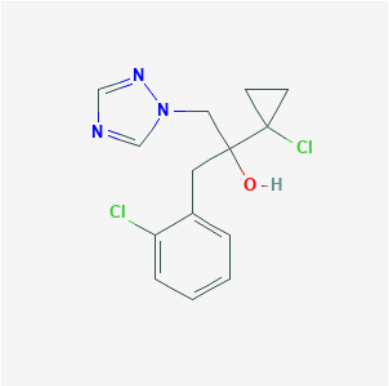
[2] sequence is extracted from Omrane et al., 2015, KES 2143 = Z4_110044_FW, KES 2144 = Z4_110044_RV

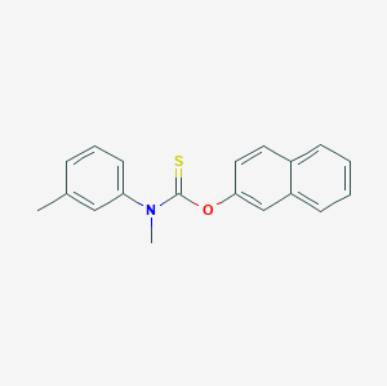
[3] sequence is extracted from Omrane et al., 2017, KES 2334 = MFS1_2F, KES 2335 = MFS1_4R

3.7 Fungicides

The sensitivity of *Z. tritici* isolates was tested with different DMIs that are available on the European market with the exception of tolnaftate that is not registered in agriculture (Table 7). Microtiter tests were performed by EpiLogic (Freising, Germany) with the commercially available formulation of epoxiconazole and mefentrifluconazole. Instead of prothioconazole, the metabolite prothioconazole-desthio (technical active ingredient (a.i.) purchased from Sigma Aldrich) was used as it is suggested that this metabolite confers antifungal activity of prothioconazole (Parker et al., 2011; Fraaije et al., 2012; Parker et al., 2013). In glasshouse studies performed in this work only commercially available formulations of epoxiconazole, mefentrifluconazole and prothioconazole were used. The technical active ingredient tolnaftate was purchased from Sigma Aldrich.

Table 7: Fungicides used.

Technical a.i. (trade name)	Company (market launch in Europe/further information)	Structural formula[1]
Epoxiconazole (Opus®)	BASF SE (1993)	
Mefentrifluconazole (Revysol®)	BASF SE (2019)	
Prothioconazole (Proline®)	Bayer Crop Science (2004)	
Prothioconazole-desthio	Bayer Crop Science (metabolite of prothioconazole, shown above)	

Technical a.i. (trade name)	Company (market launch in Europe/further information)	Structural formula[1]
Tolnaftate	Sigma Aldrich (not registered in agriculture)	

[1]Structural formulas extracted from PubChem, 2019

3.8 Software

Software used during the present work is listed in Table 8.

Table 8: Software used.

Name	Company
BLAST	BASF SE, Ludwigshafen, DE
Geneious R11	Biomatters, Auckland, NZ
EditSeq	DNASTAR Inc., Madison, US
E-workbook suite/Labbook	IDBS, Guildford, UK
Magellan™	TECAN Group AG, Männerdorf, CH
MegAlign™ Pro	DNASTAR Inc., Madison, US
Molecular Operating Environment (MOA)	Chemical Computing Group Inc., Montreal, CA
Primer3Plus	BASF SE, Ludwigshafen, DE
qPCRsoft® 3.4	Analytik Jena AG, Jena, DE
RESLAB	BASF SE, Ludwigshafen, DE
RStudio 3.6.1	RStudio Inc., Boston, US
SeqMan™ Pro	DNASTAR Inc., Madison, US

3.9 Fungal isolates

Fungal isolates used in this study were obtained from BASF European internal routine sensitivity monitoring or from a monitoring programme on trial sites.

3.9.1 European routine sensitivity monitoring

STB-infected leaves were collected during European internal routine sensitivity monitoring of BASF SE at commercially treated fields across different countries in Europe in 2016 and 2017, in order to receive an overview about the DMI sensitivity of regional *Z. tritici* populations. Sampling was planned and conducted by BASF SE and subsequently samples were shipped to the company EpiLogic (Freising, Germany). Afterwards, EpiLogic generated single pycnidia isolates and tested their sensitivity to different DMIs in microtiter tests. All microtiter tests described in this chapter for isolates from routine sensitivity monitoring were performed from EpiLogic. The routine sensitivity monitoring in 2016 and 2017 was performed with epoxiconazole. In 2016, a representative collection of 331 isolates was selected out of all isolates of the routine monitoring and additionally tested for their sensitivity to mefentrifluconazole and prothioconazole-desthio in microtiter tests. These isolates were transferred to BASF SE and further analysed in this work. In 2016, isolates from the Western European countries Ireland, UK, France, and the Netherlands were analysed as well as from the Central European countries Germany, Poland and Czech Republic. Additionally, isolates from the North European countries Denmark and Sweden were tested. In 2017, 512 isolates were tested for their sensitivity towards epoxiconazole. A subset of 90 isolates of these 512 isolates was additionally tested for their sensitivity to mefentrifluconazole and prothioconazole-desthio. All isolates from routine sensitivity monitoring in 2017 were transferred to BASF SE and further analysed in this work. In 2017, isolates from the Western European countries Ireland, UK, France and the Netherlands were tested as well as from the Central European countries Germany, Poland, Czech-Republic and Slovakia. Moreover, isolates were analysed from the Eastern European countries Russia and Ukraine, the South-East European country Bulgaria as well as from the Southern European countries Italy and Spain. Isolate details are given in supplementary Table 22.

3.9.2 Trial site monitoring

Infected *Z. tritici* leaf samples were collected in 2016 from a field trial in Ireland, Germany, France, and Denmark, respectively, in order to analyse changes in the occurrence and frequency of *CYP51* haplotypes after solo-treatment of DMIs. Samples were taken in untreated, prothioconazole (Proline®) and mefentrifluconazole (Revysol®) treated plots, respectively, resulting in 3 samples per field trial. These samples were sent to the company EpiLogic and they generated 10 isolates per sample resulting in a total number of 120

isolates. These isolates were analysed for their *CYP51* haplotypes in the current work. Isolate details are shown in supplementary Table 23.

3.9.3 Cultivation of *Z. tritici* isolates

Isolates of *Z. tritici* were cultivated at ISP2 streptomycin agar plates at 18°C with a 12 h dark and 12 h light cycle and were transferred to fresh plates every 7-14 days with a sterile cotton swab. Isolates were cultivated for 7 days before they were used in microtiter or glasshouse tests. Long-term storage was performed in 2% (w/v) malt medium with 15% (v/v) glycerol at -80°C.

3.10 Cultivation of wheat plants for glasshouse tests

Fungicide sensitivity studies with *Z. tritici* in the glasshouse were performed with the wheat cultivar 'Riband'. Inoculation with fungal spores was performed on wheat plants at growth stage BBCH 11. For this reason, around 10 wheat seedlings per pot were cultivated in Universal perlite soil (BASF SE) at 20°C with 16 h light in the glasshouse until BBCH 11.

3.11 Molecular biological methods

3.11.1 Isolation of genomic DNA

Genomic DNA was extracted from fungal material (conidia, mycelia) of *Z. tritici* that was transferred from ISP2 streptomycin agar plates to a 2 mL Eppendorf tube. After addition of a metal bead, the fungal material was frozen for 20-30 min on dry ice and subsequently homogenized in an oscillating mill for 1 min at 20 Hz. DNA was extracted using NucleoSpin® Plant II (single column extraction) or NucleoSpin® 8/96 Plant II Kits (48-/96-well vacuum processing) according to the manufacturers' protocol for the extraction from plant material utilizing lysis buffer PL1. If necessary, purity and concentration of DNA was determined at a Nanodrop2000 photometrically. DNA was stored for short-time at 4°C and for long time storage at -20°C.

3.11.2 Polymerase chain reaction (PCR)

Polymerase chain reaction (PCR) was performed to amplify target DNA sequences. In Table 6 (chapter 3.6), the pathogen specific oligonucleotides that were used are listed. PCR reactions, as shown in Table 9, were prepared in a clean bench to avoid contaminations and a sample with DEPC-water instead of target DNA served as a negative control (NTC). Volumes given in Table 9 were doubled for a PCR reaction with a final volume of 50 µl.

Table 9: Components of PCR reactions.

Component	Volume (µl) in a final volume of 25 µl
2x Master Mix (Maxima/DreamTag/Phusion Flash)	12.50
DEPC-H_2O	7.50
Primer forward (10 pmol/µl)	1.25
Primer reverse (10 pmol/µl)	1.25
Template DNA	2.5

Depending on the purpose of the experiment, different DNA polymerases were used. Maxima™ Hot Start and DreamTaq™ Hot Start *Taq* polymerases were used for identification and sequencing of inserts in *CYP51*p and *MFS1*p, whereas Phusion™ High-Fidelity polymerase, that shows a proofreading activity, was used for sequencing of *CYP51*. A schematic procedure of a PCR reaction is provided in Table 10. Annealing temperatures and elongation time were adjusted in each PCR reaction according to Table 6 in chapter 3.6.

Table 10: Schematic procedure of a PCR reaction programme. Maxima™ Hot Start, DreamTaq™ Hot Start *Taq* polymerases or Phusion™ High-Fidelity polymerase were used. Annealing temperature and elongation time were adjusted depending on the pair of oligonucleotides and DNA polymerase used.

Function	Temperature (°C) Maxima or DreamTaq/Phusion	Time (min) Maxima or DreamTaq/Phusion	Cycles
Initial denaturation	95/98	4:00/0:30	1
Denaturation	95/98	0:15/0:10	35-39
Annealing	50-72	0:30/0:05	
Elongation	72	1:00 per kb/0:15 per kb	
Final elongation	72	5:00	1
Cooling	4	∞	

Amplified DNA fragments were subsequently used for PCR clean-up, sequencing, SNP detection by qPCR or for identification of insertions in *CYP51*p or *MFS1*p.

3.11.3 Gel electrophoresis

Agarose gel electrophoresis was used to separate amplified DNA fragments in order to visualise the exact size or for subsequent clean-up. If the exact size of the DNA fragments needed to be identified, for example, in promotor insertion studies, ethidium bromide was added to 1% TAE agarose gels and the DNA fragments were visualised under UV-light. 1%

TAE agarose gels without ethidium bromide but GelGreen in samples and loading dye were used if DNA fragments needed to be excised from the gel for subsequent PCR clean-up. DNA fragments on these gels were visualised with blue light (430-490 nm). Gels were run at 80 V for 60-120 min and the results were analysed with the EasyDoc plus gel documentation system. Amplified DNA fragments with correct size were excised from the gels and transferred to a 2 mL Eppendorf tube for subsequent clean-up and Sanger sequencing.

3.11.4 PCR clean-up

For subsequent sequencing, amplified DNA fragments were cleaned-up from excised agarose gel slices using the NucleoSpin® Gel and PCR Clean-up kit according to the manufacturers' protocol. In contrast to the protocol, DNA fragments were solved using 400 µl NTI buffer under shaking at 800 rpm at a ThermoMixer. NucleoSpin® 96 PCR Clean-up kit was used for large scale clean-up of 96-well PCR plates directly after PCR (without previous separation of DNA fragments on an agarose gel). PCR products were eluted in 90 µl elution buffer NE after following the manufacturers' protocol for manual vacuum processing.

3.11.5 Sequencing

Sequencing of purified PCR products was performed via Sanger sequencing internally in BASF SE or by the external companies SeqLab (Göttingen, Germany) and Eurofins MWG Operon (Ebersberg, Germany). *CYP51*p and *MFS1*p were sequenced with oligonucleotides used for the respective PCR reaction. Details for sequencing of the *CYP51* gene are described in chapter 3.13. Sequence analysis was conducted with the programmes DNASTAR Lasergene (DNASTAR, Madison, US) and Geneious (version R11) from Biomatters (Auckland, New Zealand). Contigs of two or three sequence reads were generated with SeqMan Pro. MegAlign or Geneious were used to compare nucleotide and protein sequences among themselves. Internal BLAST search of BASF SE or BLAST search of NCBI was applied for verification of the sequence origin and for research purposes.

3.11.6 SNP detection via quantitative real-time PCR (qPCR)

Quantitative real-time PCR was used to detect the presence of alteration S524T in CYP51 of single isolates for which sequencing of *CYP51* stopped before this position. The qPCR assay was already established internally in BASF SE before this work and is based on TaqMAMA genotyping. The TaqMAMA assay combines a 5' nuclease polymerase chain reaction utilizing a fluorogenic DNA probe (TaqMan®) with a mismatch amplification mutation assay (MAMA). The TaqMan probe is an oligonucleotide labelled with a reporter

dye (5' end) and a quencher dye (3' end). During elongation DNA polymerase degrades the probe and the reporter dye is liberated from the quencher dye resulting in measurable fluorescence (Glaab and Skopek, 1999). The increase of fluorescence is proportional to the amplified PCR product in each cycle. The MAMA assay enables quantitative discrimination of SNPs between alleles by exploiting MAMA primers that are either specific for the wild type or for the mutated sequence. Specificity and discrimination between alleles are increased by an additional mismatch in both sequences besides the nucleotide exchange of the investigated SNP (Cha et al., 1992; Glaab and Skopek, 1999).

QPCR reactions were prepared in a clean bench as described in Table 11 to avoid contamination and a sample with DEPC water instead of target DNA served as a negative control (NTC). Light conditions were reduced as much as possible during preparation of qPCR reactions, as TaqMan probes are sensitive to light.

Table 11: Components of a qPCR reaction with MAMA primers and TaqMan probe.

Component	Volume (µl) in a final volume of 25 µl
Takyon NoROX Probe MasterMix dTTP (2x)	12.50
DEPC-H_2O	7.25
KES 1138 or 1139 forward (10 pmol/µl)	1.25
KES 1144 reverse (10 pmol/µl)	1.25
St-Cyp51-524 TaqMan probe (10 pmol/µl)	0.25
Template DNA	2.50

Oligonucleotides KES 1139 and KES 1144 were used for detection of the wild type allele and oligonucleotides KES 1138 and KES 1144 were used for detection of the mutated allele (oligonucleotides shown in Table 6). QPCR reactions were run on the qTower^3 from Qiagen with the programme shown in Table 12.

Table 12: Programme used for qPCR reactions.

Function	Temperature (°C)	Time (min)	Cycles
Initial denaturation	95	5:00	1
Denaturation	95	0:10	40
Annealing & Elongation	60	0:45	

Fluorescence was measured at the end of elongation after each cycle and the C_t values were determined with the software qPCRsoft 3.4 from Analytik Jena (Jena, Germany). The number of qPCR cycles that are required until a predefined threshold is exceeded is described by the C_t value. Allele frequencies of sensitive and resistant alleles were calculated according to the formula of Germer et al. (2000):

Frequency of allele$_1$ = 1/($2^{\Delta Ct}$ + 1),

where ΔC_t = (C_t of allele$_1$-specific PCR) – (C_t of allele$_2$-specific PCR).

A DNA sample with 100% sensitive allele and a DNA sample with 100% mutated allele served as verification of each run.

3.12 Fungicide sensitivity tests

Microtiter tests were performed in the laboratory in order to test the *in vitro* sensitivity of *Z. tritici* isolates towards different compounds. Sensitivity studies in the glasshouse were performed with *Z. tritici* isolates in order to test efficacy of DMIs *in vivo*, under conditions that are more realistic to field conditions compared to *in vitro* studies.

3.12.1 Microtiter tests

Microtiter tests were performed by EpiLogic (for DMI sensitivity) and during this work (for tolnaftate sensitivity) to calculate the EC_{50} of *Z. tritici* isolates for different compounds. The EC_{50} value describes the effective concentration that leads to 50% inhibition of fungal growth compared to growth in the untreated control. Fungicides used are shown in Table 7. Sensitivities of *Z. tritici* isolates towards DMIs were tested *in vitro* with the commercial formulations of epoxiconazole and mefentrifluconazole and the technical grade of prothioconazole-desthio. These microtiter tests were performed by EpiLogic. Microtiter tests with technical grade of tolnaftate were performed for identification of an increased efflux activity in *Z. tritici* isolates. These microtiter tests with tolnaftate were part of this work. Fungicides were tested in 7 concentrations which were selected based on intrinsic activity of the fungicides and are given in Table 13. Technical grades were dissolved in DMSO, whereas formulations were dissolved in sterile deionized water.

Table 13: Tested fungicide concentrations in microtiter tests.

Fungicide	Tested concentrations (mg/L)
Epoxiconazole	0, 0.003, 0.01, 0.03, 0.1, 0.3, 1, 3
Mefentrifluconazole	0, 0.001, 0.003, 0.01, 0.03, 0.1, 0.3, 1
Prothioconazole-desthio	0, 0.00064, 0.0032, 0.016, 0.08, 0.4, 2, 10
Tolnaftate	0, 0.01, 0.03, 0.1, 0.3, 1, 3, 10

Microtiter tests were performed in 96-well microtiter plates as described in the “SEPTTR microtiter monitoring method BASF 2009 V1” on the website of FRAC (FRAC, 2019) with slight modifications. The procedure is explained in the following by means of tolnaftate as these microtiter tests were performed during this work. The microtiter method of EpiLogic

is also based on this procedure. A 10,000 mg/L stock solution was prepared with technical grade of tolnaftate in DMSO. This stock solution was used to prepare a double concentrated dilution series in sterile deionized water. At first, intermediate dilutions of 200 mg/L and 60 mg/L were prepared in 25 mL Falcon tubes. Afterwards, these dilutions were used to prepare a fungicide dilution series in a 48-deep-well plate as shown in Figure 5. Fifty µl of double concentrated fungicide dilution was mixed with 50 µl of spore suspension in the 96-well microtiter plates resulting in the final test concentration.

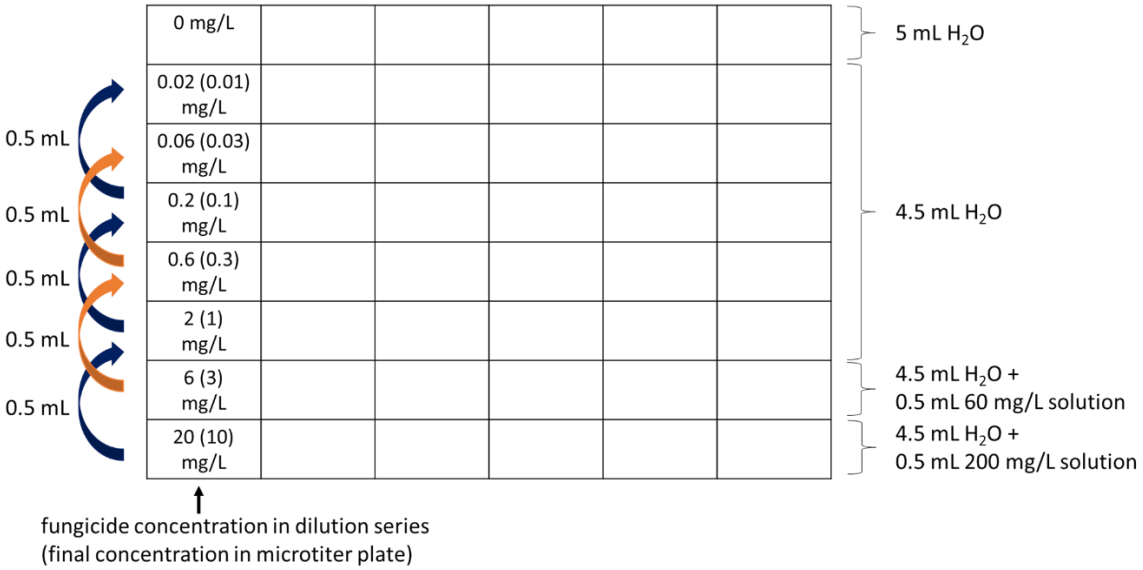


Figure 5: Preparation of fungicide dilution series of tolnaftate for microtiter tests. Dilution series was prepared double concentrated and afterwards mixed with spore suspension in a ratio of 1:1.

Spore suspensions were prepared with *Z. tritici* isolates that have been cultivated for 7 days on ISP2 streptomycin agar plates. Spores of *Z. tritici* were transferred to 4 mL of YBG d.c. medium with a sterile cotton swab. Spore density was determined by counting the spores in a haemocytometer (C-Chip) under the light microscope. Spore density was adjusted to a final concentration of 1.6 x 10^4 spores/mL in YBG d.c. medium and stored at 4°C until they were added to the fungicide dilution series in the microtiter plates. Each isolate was tested in 4 replicates. Additionally, a blank containing media and fungicide solution without spores was also tested in 4 replicates at each microtiter plate. Consequently, 2 isolates could be tested at one 96-well microtiter plate. The schematic set-up of a microtiter plate is shown in Figure 6.

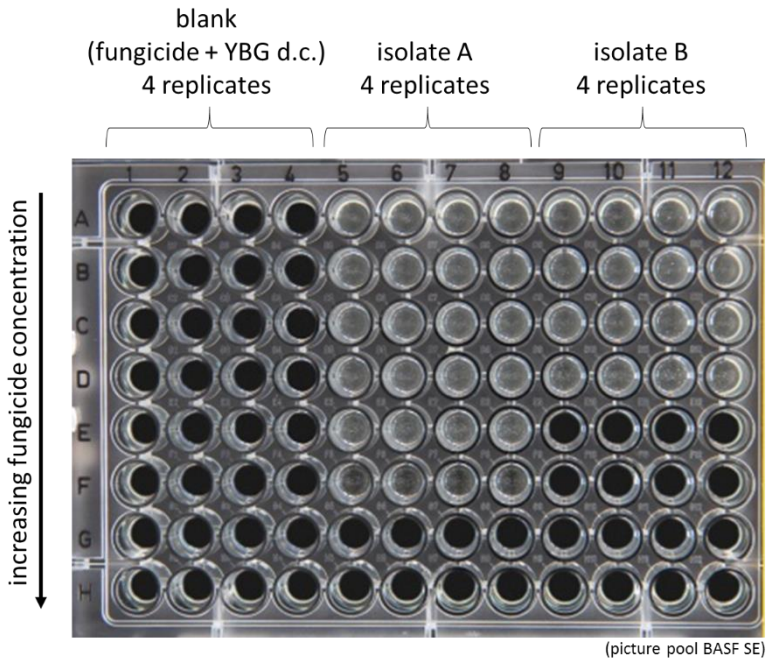


Figure 6: Schematic set-up of a 96-well microtiter plate in microtiter test. Row A serves as water control and no fungicide is added. Over rows B-H fungicide concentration is increasing. Columns 1-4 are composed of fungicide and media without fungal spores and serve as blank. Columns 5-8 contain spores in four replicates of isolate A and columns 9-12 contain spores in four replicates of isolate B. The picture was taken from picture pool of BASF SE.

After addition of the fungal spores, microtiter plates were put into plastic bags and incubated at 18°C in darkness for 7 days. Afterwards, fungal growth was measured in a photometer at 405 nm and optical density (OD) values were transferred into an excel spreadsheet using the software Magellan™ (version 7.2). EC_{50} values were calculated with E-WorkBook Suite/Labbook (IDBS, Germany) based on the following formula:

$f(x) = 100\% * (1-1/(1 + \exp(b(\log(x) - \log(e)))))$

100% = maximum, b = slope, e = EC_{50}

3.12.2 Fungicide sensitivity tests in the glasshouse

Fungicide sensitivity tests in the glasshouse were performed to test the efficacy of different DMIs for *Z. tritici* isolates that show a DMI adaptation in microtiter tests under conditions that are more realistic to field conditions compared to *in vitro* studies.

3.12.2.1 Fungicide application

Fungicides were applied as a one-day preventative treatment in a spray chamber (SPK08) of BASF SE with flat fan nozzles and a water volume which corresponds to 400 L/ha. Wheat seedlings cultivated according to chapter 3.10 were sprayed with the full registered field dose rate of commercially available formulations of epoxiconazole (Opus®), prothioconazole (Proline®) and mefentrifluconazole (Revysol®). Full registered field dose rates are

125 g a.i./ha epoxiconazole, 200 g a.i./ha prothioconazole and 150 g a.i./ha mefentrifluconazole. In total, 100 mL of fungicide solution was applied to a maximum of 16 plant pots simultaneously. Per treatment and isolate 3 replicates were conducted. The test was performed in a fully randomised experimental design.

3.12.2.2 Inoculation

One day after fungicide application the treated wheat seedlings were inoculated with spore suspensions of different *Z. tritici* isolates. Four replicates of fungicide untreated wheat seedlings were used to determine growth of fungal isolates under untreated conditions. *Z. tritici* isolates were cultivated for 7 days on ISP2 streptomycin agar plates before inoculation. Spores were transferred with a sterile cotton swab into 20 mL of 0.02% Tween80-water. The spore suspension was filtered through two layers of gauze. Subsequently, a 1:10 dilution was prepared and spore density was determined by counting spores in a haemocytometer under the light microscope. The spore concentration was adjusted to a final density of $2.5x10^6$ spores/mL in a final volume of 100 mL of 0.02% Tween80-water. Inoculation of wheat plants was performed with an airbrush (0.8 mm nozzle size). The plants were inoculated until a homogenous coverage of spore suspension droplets was visible without a run-off. The bottom of the glasshouse trolleys was covered with wet fleece and with a semipermeable foil above. Each isolate was placed on its own glasshouse trolley to avoid cross contamination. Inoculated plants were covered with a light permeable plastic box at the glasshouse trolley to allow *Z. tritici* penetration and were cultivated for 20-21 days in a glasshouse chamber with 80% humidity, 20°C and a light period of 15 h. Four days after inoculation the plastic surroundings were removed.

3.12.2.3 Evaluation

Diseased leaf area (%) was assessed visually 20-21 days post inoculation (dpi). All wheat leaves that were available at the time of fungicide application and *Z. tritici* inoculation were evaluated, but no leaves that occurred afterwards. Finally, the data of diseased leaf area were used to calculate the fungicide efficacy according to Abbott (1925) with the software RESLAB or E-WorkBook Suite:

$$\% \text{ efficacy} = (\% \text{ disease untreated} - \% \text{ disease treated}) \times 100\% \ (\% \text{ disease untreated})^{-1}$$

3.13 Determination of *CYP51* haplotypes

In this work, the *CYP51* haplotypes of all isolates were determined. For this purpose, the *CYP51* was amplified in a PCR and subsequently sequenced. Isolates from the routine sensitivity monitoring of 2016 and the isolates from the trial site monitoring of 2016 were used in a PCR (described in 3.11.2) with oligonucleotides KES 2192 and KES 2193 (shown in Table 6). The amplified PCR fragments were separated on an agarose gel, excised from

the gel (described in 3.11.3) and PCR clean-up from the agarose gel was performed (described in 3.11.4). Sanger sequencing was performed with oligonucleotides KES 2192, KES 2193 and an additional sequencing primer, KES 2223 (shown in Table 6). As this procedure was very labour-intensive, it was slightly changed for isolates from the routine monitoring of 2017. These isolates were used in a PCR with oligonucleotides KES 2192 and KES 2193. The PCR products were directly cleaned-up after PCR without previous separation on an agarose gel. Sequencing was subsequently performed with oligonucleotides KES 2283 (shown in Table 6), KES 2193 and KES 2223. Oligonucleotide KES 2283 was used instead of KES 2192 to obtain specific sequencing results. Sequences were analysed as described in chapter 3.11.5 and SNPs in *CYP51*, corresponding amino acid exchanges, and consequently *CYP51* haplotypes were identified. In a few cases the sequencing reaction stopped before the position of the known amino acid exchange S524T. The presence of this alteration was tested via qPCR in these cases as described in chapter 3.11.6.

3.14 Detection of *CYP51* overexpression of *Z. tritici*

An insert in *CYP51*p of *Z. tritici* was correlated to *CYP51* overexpression (Cools et al., 2012). All isolates from routine sensitivity monitoring of 2016 and 2017 were investigated for *CYP51*p inserts, to identify *CYP51* overexpression in these isolates. A slightly modified version of a PCR method described by Cools et al. (2012) was used. Sequence of oligonucleotide KES 2136 was extracted from Cools et al. (2012), whereas KES 2188 was designed during this work and PCR conditions were adjusted (shown in Table 6). In this PCR, the regulatory region of *CYP51* was amplified and the size of the PCR products was subsequently determined on agarose gels with ethidium bromide (shown in 3.11.3). To check, whether the inserts found during this work showed the same sequence as inserts already published from Cools et al. (2012), Omrane et al. (2015, 2017) and Kildea et al. (2019), a selection of amplified fragments of each size was sequenced. PCR products were excised from agarose gels, cleaned-up and sequencing was performed with oligonucleotides KES 2136 and KES 2188.

3.15 Detection of increased efflux activity of *Z. tritici*

In *Z. tritici*, it was shown that constitutive overexpression of the *MFS1* gene coding for an MFS1 transporter confers increased efflux activity. Inserts in *MFS1*p were identified to be responsible for this overexpression (Omrane et al., 2015, 2017). All isolates from routine sensitivity monitoring of 2016 and 2017 were investigated for inserts in *MFS1*p with a PCR method extracted from Omrane et al. (2015) in order to identify an increased efflux activity. *MFS1*p was amplified with oligonucleotides KES 2143 and KES 2144 and the size of PCR

products was subsequently determined on agarose gels with ethidium bromide. Isolates that did not show a PCR product were used in a second PCR extracted from Omrane et al. (2017) with oligonucleotides KES 2334 and KES 2335. Subsequently, the size of amplified PCR products was analysed again. Sequences of used oligonucleotides are shown in Table 6.

The size of some amplified fragments differed only slightly and could not be exactly determined with agarose gel electrophoresis. Therefore, these fragments were excised from agarose gels, cleaned-up and sequenced with oligonucleotides of the respective PCR method. Moreover, a selection of amplified DNA fragments of each size was additionally sequenced to check whether inserts found in this work show the same sequence as inserts already published from Omrane et al. (2015, 2017).

The phenotypical effect of different *MFS1*p inserts on an increased efflux activity of *Z. tritici* isolates was determined by microtiter tests with tolnaftate. Tolnaftate sensitivity was tested for a selection of isolates (when available) for each identified *MFS1*p insert. Tolnaftate is a thiocarbamate that inhibits squalene epoxidase and is used against human fungal infections (Barrett-Bee et al., 1986), but not in agriculture. It was shown that an adaptation of *Z. tritici* isolates towards tolnaftate can indicate an increased efflux activity (Leroux and Walker, 2011; Omrane et al., 2015). Microtiter tests with tolnaftate were performed as described in chapter 3.12.1.

3.16 Homology modelling

Homology modelling of the CYP51 protein of *Z. tritici* was performed by Dr. Ian Craig of BASF SE. The software Molecular Operating Environment (MOE) with standard setting for homology modelling was used. As no crystal structure of the CYP51 of *Z. tritici* is available so far, the ScCYP51 of *Saccharomyces cerevisiae* bound to R-desthioprothioconazole (PBD 5EAE) with a resolution of 2.1 Å was deployed as a structural template. The sequence identity of the template compared to CYP51 of *Z. tritici* amounts to 49%. Positions of amino acids at which alterations occur were manually highlighted in the wild type model.

3.17 Bioinformatic analysis

The programme Primer3Plus at an internal bioinformatics site from BASF SE (Bioinformatics@BASF) was used to design oligonucleotides for standard PCR and Sanger sequencing. The software EditSeq and SeqMan™ Pro were used for editing of DNA sequences. The software MegAlign™ Pro and Geneious R11 were used to generate alignments of DNA and amino acid sequences. BLAST search was performed at the internal bioinformatics site of BASF SE or with BLAST search of NCBI. The qPCR was evaluated

using the software qPCR Soft® 3.4. The software used in this work and corresponding manufacturers are shown in Table 8.

EC_{50} values in microtiter tests were calculated using the software Magellan™ and E-WorkBook Suite/Labbook. Fungicide efficacy in sensitivity tests in the glasshouse were calculated using the software RESLAB or E-WorkBook Suite.

The software RStudio (version 3.6.1) was used for statistical analysis. Significant differences between tolnaftate sensitivity obtained from microtiter tests were calculated with Kruskal-Wallis-Test ($p = 0.05$) followed by the post-hoc Nemenyi Test ($p = 0.05$) for data that are not normally distributed. Significant differences between fungicide efficacy in glasshouse tests were calculated between the grouped efficacies for adapted isolates and the efficacy for the reference isolate using Kruskall-Wallis-Test ($p = 0.05$) for data that are not normally distributed.

4 Results

In this thesis, isolates derived from across Europe in annual routine sensitivity monitoring of 2016 (chosen sub-sample) and 2017 were investigated for different DMI resistance mechanisms. The *CYP51* gene of all isolates was sequenced in order to identify *CYP51* haplotypes and, if present, new mutations in *CYP51*. New *CYP51* mutations were tested for their effect on DMI sensitivity based on sensitivity comparisons between isolates differing in the presence or absence of a new *CYP51* mutation. Additionally, protein homology modelling was used to assess the effect of *CYP51* mutations that lead to amino acid alterations in the CYP51 enzyme in order to predict their effect on DMI binding. The sensitivities of all *Z. tritici* isolates were correlated to their respective *CYP51* haplotypes to evaluate the impact on DMI sensitivity. Additionally, the frequency as well as distribution of *CYP51* haplotypes across Europe was analysed. Isolates were investigated for *CYP51* overexpression and an increased efflux activity via promotor insertion studies and the effects on DMI adaptation were evaluated. Frequency and distribution of *CYP51* overexpression and increased efflux across Europe were analysed. *CYP51* haplotypes were investigated for cross-resistance between different DMIs based on *in vitro* sensitivity studies and on the composition of *CYP51* haplotypes after DMI treatment in field trials. The impact of different *CYP51* haplotypes, *CYP51* overexpression and increased efflux on DMI efficacy *in vivo* was tested in glasshouse studies in order to predict their impact on the field efficacy of DMIs.

4.1 Identification of *CYP51* haplotypes in *Z. tritici*

During routine sensitivity monitoring of BASF, *Z. tritici* isolates were collected across different countries in Europe and tested for their sensitivity towards epoxiconazole in 2016 and 2017. The sensitivity of isolates was tested in microtiter tests by EpiLogic. Afterwards, a collection of isolates (n=331) from sensitivity monitoring of 2016 and the complete set of isolates (n=512) from 2017 were analysed for mutations in *CYP51* and the resulting *CYP51* haplotypes were determined in this work. The *CYP51* gene was PCR-amplified and subsequently sequenced. Splicing of DNA sequences was virtually performed based on alignments with the cDNA sequence from the genome database. *CYP51* haplotypes were determined based on mutations in the *CYP51* gene that lead to amino acid alterations in the CYP51 enzyme. Therefore, *CYP51* haplotypes will be described hereafter based on amino acid alterations in CYP51 (CYP51 alterations). Amino acid alterations found in CYP51 across all investigated *Z. tritici* isolates are listed in Table 14.

Table 14: CYP51 alterations found in *Z. tritici* isolates investigated from routine sensitivity monitoring of 2016 and 2017. Wild type (WT) codon and codon of *CYP51* mutations are shown. A deletion of an amino acid is described as 'Del'. Amino acid alterations that have been already described for *Z. tritici* are categorised as 'existing'. Amino acid alterations identified for the first time during this work are categorised as 'new'.

CYP51 alteration	Codon WT → codon *CYP51* mutant	Occurrence (existing or new)
L50S	TTG → TCG	existing
K95M	AAG → ATG	new
D134G	GAT → GGT	existing
V136A	GTT → GCT	existing
V136C	GTT → TGT	existing
V136G	GTT → GGT	existing
Y137F	TAT → TTT	existing
S188N	AGC → AAC	existing
L197V	CTC → GTC	new
N284H	AAC → CAC	existing
A318G	GCG → GGG	new
I377V	ATT → GTT	new
A379G	GCT → GGT	existing
I381V	ATC → GTC	existing
A410T	GCT → ACT	existing
I452V	ATC → GTC	new
Del K456	AAA → ---	new
Del E457	GAA → ---	new
Del D458	GAC → ---	new
Del Y459	TAT → ---	existing
Del G460	GGC → ---	existing
Del Y461	TAC → ---	existing
Del G462	GGC → ---	existing
Del L463	CTG → ---	new
Y459D	TAT → GAT	existing
Y459P	TAT → CCT	existing
Y459S	TAT → TCT	existing
G460D	GGC → GAC	existing
Y461S	TAC → TCC	existing
Y461H	TAC → CAC	existing
Y461N	TAC → AAC	new
N507S	AAT → AGT	new
N513K	AAC → AAG	existing
S524T	AGC → ACC	existing

In total, 34 CYP51 alterations were identified across all investigated isolates. Twenty-three of those alterations have been already described for *Z. tritici* in literature. In contrast, 11 new CYP51 alterations were detected that have not been described for *Z. tritici* so far.

A single amino acid alteration was found in CYP51 of a few isolates, but in most cases combinations of more than one CYP51 alteration were identified. A new nomenclature was established during this work to enable description of all *CYP51* haplotypes identified across all isolates. This nomenclature is composed of a combined letter and number code. The letter describes the total number of amino acid alterations in CYP51, whereas the number describes the specific combination of a defined number of amino acid alterations. For example, *CYP51* haplotype C7 contains three specific amino acid alterations in CYP51 (L50S, V136A, Y461H; shown in Table 15), whereas haplotype C8 contains another combination of three specific amino acid alterations in CYP51 (L50S, I381V, Y461H; shown in Table 15). The wild type *CYP51* is described as WT. A list with *CYP51* haplotypes detected in the past and assignment of the new nomenclature was published in the course of this work in Huf et al. (2018).

All *CYP51* haplotypes identified in isolates from routine sensitivity monitoring of 2016 and 2017 are shown in Table 15 and the number of isolates that carried a respective haplotype is given.

Table 15: All *CYP51* haplotypes identified in *Z. tritici* isolates investigated from routine sensitivity monitoring of 2016 and 2017. *CYP51* haplotypes are shown in the caption and the table is consecutive over several pages. *CYP51* haplotypes marked with an asterisk '*' are described for the first time for *Z. tritici*. The wild type amino acid and the position is given in the first column of the table. The sign '-' indicates no change of the wild type amino acid. The abbreviation 'del' indicates a deletion of the respective amino acid. The year of detection and total number of isolates (No. of isolates) identified across both years is shown. These results have been previously published partially in Huf et al., 2020.

Wild type amino acid and position	***CYP51* haplotype**											
	WT	**A2**	**A3**	**A14***	**A15***	**B8**	**B15***	**C2**	**C4**	**C6**	**C7**	**C8**
L50	-	S	-	-	-	S	-	S	S	S	S	S
K95	-	-	-	-	-	-	-	-	-	-	-	-
D134	-	-	-	-	-	-	-	-	-	-	-	-
V136	-	-	-	-	-	-	-	-	-	A	A	-
Y137	-	-	F	-	-	-	-	-	-	-	-	-
S188	-	-	-	N	-	-	-	-	-	-	-	-
L197	-	-	-	-	-	-	-	-	-	-	-	-
N284	-	-	-	-	-	-	-	-	-	-	-	-
A318	-	-	-	-	-	-	-	-	-	-	-	-
I377	-	-	-	-	-	-	-	-	-	-	-	-
A379	-	-	-	-	-	-	-	-	-	-	-	-
I381	-	-	-	-	-	-	V	V	V	-	-	V
A410	-	-	-	-	-	-	-	-	-	-	-	-
I452	-	-	-	-	V	-	-	-	-	-	-	-
K456	-	-	-	-	-	-	-	-	-	-	-	-
E457	-	-	-	-	-	-	-	-	-	-	-	-
D458	-	-	-	-	-	-	-	-	-	-	-	-
Y459	-	-	-	-	-	-	P	S	D	-	-	-
G460	-	-	-	-	-	-	-	-	-	-	-	-
Y461	-	-	-	-	-	S	-	-	-	S	H	H
G462	-	-	-	-	-	-	-	-	-	-	-	-
L463	-	-	-	-	-	-	-	-	-	-	-	-
N507	-	-	-	-	-	-	-	-	-	-	-	-
N513	-	-	-	-	-	-	-	-	-	-	-	-
S524	-	-	-	-	-	-	-	-	-	-	-	-
Year of detection	'17	'17	'17	'17	'17	'17	'17	'16 '17	'16 '17	'17	'16 '17	'16 17
No. of isolates	14	1	1	2	1	3	1	5	4	1	2	140

Wild type amino acid and position	*CYP51* haplotype											
	C9*	**C18**	**D2**	**D7**	**D9**	**D10**	**D13**	**D26***	**D27***	**E3**	**E4**	**E5**
L50	S	S	S	S	S	S	-	S	S	S	S	S
K95	-	-	-	-	-	-	-	-	-	-	-	-
D134	-	-	-	-	G	-	-	-	-	-	G	-
V136	A	-	-	A	A	A	C	-	-	A	A	A
Y137	-	-	-	-	-	-	-	-	-	-	-	-
S188	-	N	N	-	-	-	-	-	-	-	-	-
L197	-	-	-	-	-	-	-	-	-	-	-	-
N284	-	-	-	-	-	-	-	-	-	-	-	-
A318	-	-	-	-	-	-	-	G	-	-	-	-
I377	-	-	-	-	-	-	-	-	V	-	-	-
A379	-	-	-	-	-	-	-	-	-	-	-	-
I381	-	-	-	-	-	V	V	V	V	V	V	V
A410	-	-	-	-	-	-	-	-	-	-	-	-
I452	-	-	-	-	-	-	-	-	-	-	-	-
K456	-	-	-	-	-	-	-	-	-	-	-	-
E457	-	-	-	-	-	-	-	-	-	-	-	-
D458	-	-	-	-	-	-	-	-	-	-	-	-
Y459	-	-	-	-	-	-	-	-	-	-	-	-
G460	-	-	D	-	-	-	-	-	-	-	-	-
Y461	N	-	-	S	H	H	H	H	H	S	H	H
G462	-	-	-	-	-	-	-	-	-	-	-	-
L463	-	-	-	-	-	-	-	-	-	-	-	-
N507	-	-	-	-	-	-	-	-	-	-	-	-
N513	-	K	K	-	-	-	-	-	-	-	-	-
S524	-	-	-	T	-	-	T	-	-	T	-	T
Year of detection	‘16	‘17	‘17	‘16 ‘17	‘16 ‘17	‘16	‘16 ‘17	‘17	‘17	‘16 ‘17	‘16 ‘17	‘16 ‘17
No. of isolates	1	1	1	4	2	1	12	1	1	15	231	24

Wild type amino acid and position	*CYP51* haplotype											
	E7*	**E8**	**E9***	**E21**	**E25***	**E26***	**E27***	**E28***	**E29***	**F1**	**F2**	**F3**
L50	S	S	S	-	-	S	S	S	S	S	S	S
K95	-	-	-	-	-	-	-	M	-	-	-	-
D134	-	-	G	G	-	G	-	G	-	-	-	-
V136	C	C	A	A	A	A	-	G	C	-	-	C
Y137	-	-	-	-	-	-	-	-	-	-	-	-
S188	-	N	-	N	-	-	N	-	-	N	N	N
L197	-	-	-	-	-	-	-	-	-	-	-	-
N284	-	-	-	-	-	-	-	-	-	-	-	-
A318	-	-	-	-	-	-	-	-	-	-	-	-
I377	-	-	-	-	-	-	-	-	-	-	-	-
A379	-	-	-	-	G	-	-	-	-	G	-	-
I381	V	V	V	-	V	V	V	-	V	V	V	-
A410	-	-	-	-	-	-	-	-	-	-	-	-
I452	-	-	-	-	-	-	-	-	-	-	-	-
K456	-	-	-	-	-	-	-	-	-	-	-	-
E457	-	-	-	-	-	-	-	-	-	-	-	-
D458	-	-	-	-	-	-	-	-	-	-	-	-
Y459	-	-	S	-	-	Del	Del	-	-	D	Del	Del
G460	-	-	-	Del	-	-	Del	-	-	-	Del	Del
Y461	H	H	-	Del	S	-	-	S	S	-	-	-
G462	-	-	-	(Del)[1]	-	-	-	-	-	-	-	-
L463	-	-	-	-	-	-	-	-	-	-	-	-
N507	-	-	-	-	-	-	-	-	-	-	-	-
N513	-	-	-	-	-	-	-	-	-	-	K	K
S524	T	-	-	-	T	-	-	-	T	T	-	-
Year of detection	‘16	‘16	‘16	‘17	‘17	‘17	‘17	‘17	‘17	‘16	‘16 ‘17	‘16 ‘17
No. of isolates	2	1	1	1	1	1	1	1	1	1	100	11

[1]This deletion occurs either at position G460 or G462. The position could not be clarified with certainty in sequence alignment.

Wild type amino acid and position	***CYP51* haplotype**											
	F4	**F5**	**F6**	**F7**	**F8**	**F9***	**F10***	**F19***	**F20***	**G1**	**G2**	**G6***
L50	S	S	S	S	S	-	S	S	S	S	S	S
K95	-	-	-	-	-	-	-	-	-	-	-	-
D134	-	-	-	-	G	-	G	-	G	-	-	G
V136	C	A	A	A	A	C	A	A	A	-	-	A
Y137	-	-	-	-	-	-	-	-	-	-	-	-
S188	N	N	N	-	-	-	-	-	-	N	N	-
L197	-	-	-	-	-	-	-	-	-	-	-	-
N284	-	-	-	-	-	-	-	-	-	-	-	-
A318	-	-	-	-	-	-	-	-	-	-	-	-
I377	-	-	-	-	-	-	-	-	-	-	-	-
A379	-	-	-	G	-	G	-	-	-	G	G	-
I381	V	-	-	V	V	V	V	-	V	V	V	V
A410	-	-	-	-	-	-	-	-	-	-	-	-
I452	-	-	-	-	-	-	-	-	-	-	-	-
K456	-	-	-	-	-	-	-	-	-	-	-	-
E457	-	-	-	-	-	-	-	-	-	-	-	-
D458	-	-	-	-	-	-	-	-	-	-	-	-
Y459	-	Del	Del	-	-	Del	-	Del	-	Del	Del	Del
G460	-	Del	Del	-	-	Del	-	Del	-	Del	Del	Del
Y461	H	-	-	S	H	-	S	-	H	-	-	-
G462	-	-	-	-	-	-	-	-	-	-	-	-
L463	-	-	-	-	-	-	-	-	-	-	-	-
N507	-	-	-	-	-	-	-	-	S	-	-	-
N513	-	K	-	-	-	-	-	K	-	K	-	K
S524	T	-	T	T	T	T	T	T	-	-	T	-
Year of detection	‘16 ‘17	‘16 ‘17	‘16 ‘17	‘16 ‘17	‘16 ‘17	‘16	‘16 ‘17	‘17	‘17	‘16 ‘17	‘17	‘16 ‘17
No. of isolates	13	4	9	5	38	1	5	1	1	101	2	2

Wild type amino acid and position	*CYP51* haplotype								
	G7	**H3**	**H4**	**H5**	**H6**	**H9**	**I1**	**I2***	**I4***
L50	S	S	S	S	S	S	S	S	-
K95	-	-	-	-	-	-	-	-	-
D134	-	-	-	-	-	-	-	G	-
V136	A	-	A	-	C	-	A	A	-
Y137	-	-	-	-	-	-	-	-	-
S188	N	N	N	N	N	N	N	-	-
L197	-	-	-	-	-	-	-	-	V
N284	-	H	-	-	-	-	-	-	-
A318	-	-	-	-	-	-	-	-	-
I377	-	-	-	-	-	-	-	-	-
A379	G	G	G	G	G	G	G	G	-
I381	V	V	V	V	V	V	V	V	-
A410	-	-	-	T	-	-	-	-	-
I452	-	-	-	-	-	-	-	-	-
K456	-	-	-	-	-	-	-	-	Del
E457	-	-	-	-	-	-	-	-	Del
D458	-	-	-	-	-	-	-	-	Del
Y459	-	Del	Del	Del	Del	Del	Del	Del	Del
G460	-	Del	Del	Del	Del	Del	Del	Del	Del
Y461	S	-	-	-	-	-	-	-	Del
G462	-	-	-	-	-	-	-	-	Del
L463	-	-	-	-	-	-	-	-	Del
N507	-	-	-	-	-	-	-	-	-
N513	-	K	-	K	-	K	K	K	-
S524	T	-	T	-	T	T	T	T	-
Year of detection	‘16	‘16 ‘17	‘16 ‘17	‘17	‘16 ‘17	‘17	‘16	‘16	‘17
No. of isolates	3	2	22	3	27	1	5	2	5

Across all isolates, 57 *CYP51* haplotypes, including the *CYP51* wild type (WT), were identified. Twenty of those haplotypes (marked with an asterisk) were described for the first time for *Z. tritici*, whereas remaining *CYP51* haplotypes have been already described in literature. The alignment of the amino acid sequence of each *CYP51* haplotype (including haplotypes with new CYP51 alterations) is given in the supplements in Figure 36. Among all isolates, it was observed that the majority of the *Z. tritici* population was composed of

only a few *CYP51* haplotypes. Most *CYP51* haplotypes were identified in a low frequency or even in a single isolate. Across both years, 13 *CYP51* haplotypes were identified in more than 10 isolates, respectively. Haplotype E4 was identified most frequently (n=231 isolates), followed by haplotypes C8 (n=140 isolates), G1 (n=101 isolates) and F2 (n=100 isolates). The remaining haplotypes identified in more than 10 isolates have been F8 (n=38 isolates), H6 (n=27 isolates), E5 (n=24 isolates), H4 (n=22 isolates), E3 (n=15 isolates), WT (n=14 isolates), F4 (n=13 isolates), D13 (n=12 isolates) and F3 (n=11 isolates). These 13 *CYP51* haplotypes were found in a total of 748 isolates resulting in 88.7% of all investigated isolates. Consequently, the remaining 44 *CYP51* haplotypes accounted for 11.3% of all isolates. Therefore, results in the current thesis focus in most cases on the 13 most frequent found *CYP51* haplotypes hereafter described as 'frequently found *CYP51* haplotypes'. The remaining haplotypes will be described as 'rarely found *CYP51* haplotypes' hereafter. In the following chapter, the impact of new CYP51 alterations on DMI sensitivity was evaluated, followed by the evaluation of DMI sensitivity of all *CYP51* haplotypes.

4.2 Impact of new amino acid alterations in CYP51 of *Z. tritici*

To analyse if new CYP51 alterations show an impact on sensitivity of *Z. tritici* towards DMIs, the position of new amino acid alterations in the CYP51 enzyme was determined and epoxiconazole sensitivity was evaluated for isolates that only differ in the presence or absence of a new CYP51 alteration.

4.2.1 Molecular characterisation of amino acid alterations in the CYP51 enzyme

The location of new amino acid alterations within the CYP51 protein structure of *Z. tritici* was examined in order to predict a possible effect of these alterations on triazole binding. For this purpose, a homology model was used. Enzyme modelling was performed by Dr. Ian Craig (BASF SE). As no crystal structure of the CYP51 of *Z. tritici* is available so far, protein modelling was based on the X-ray from *S. cerevisiae* (PDB 5EAE). The CYP51 wild type enzyme was modelled and wild type amino acids identified as positions of new alterations were manually highlighted (Figure 7).

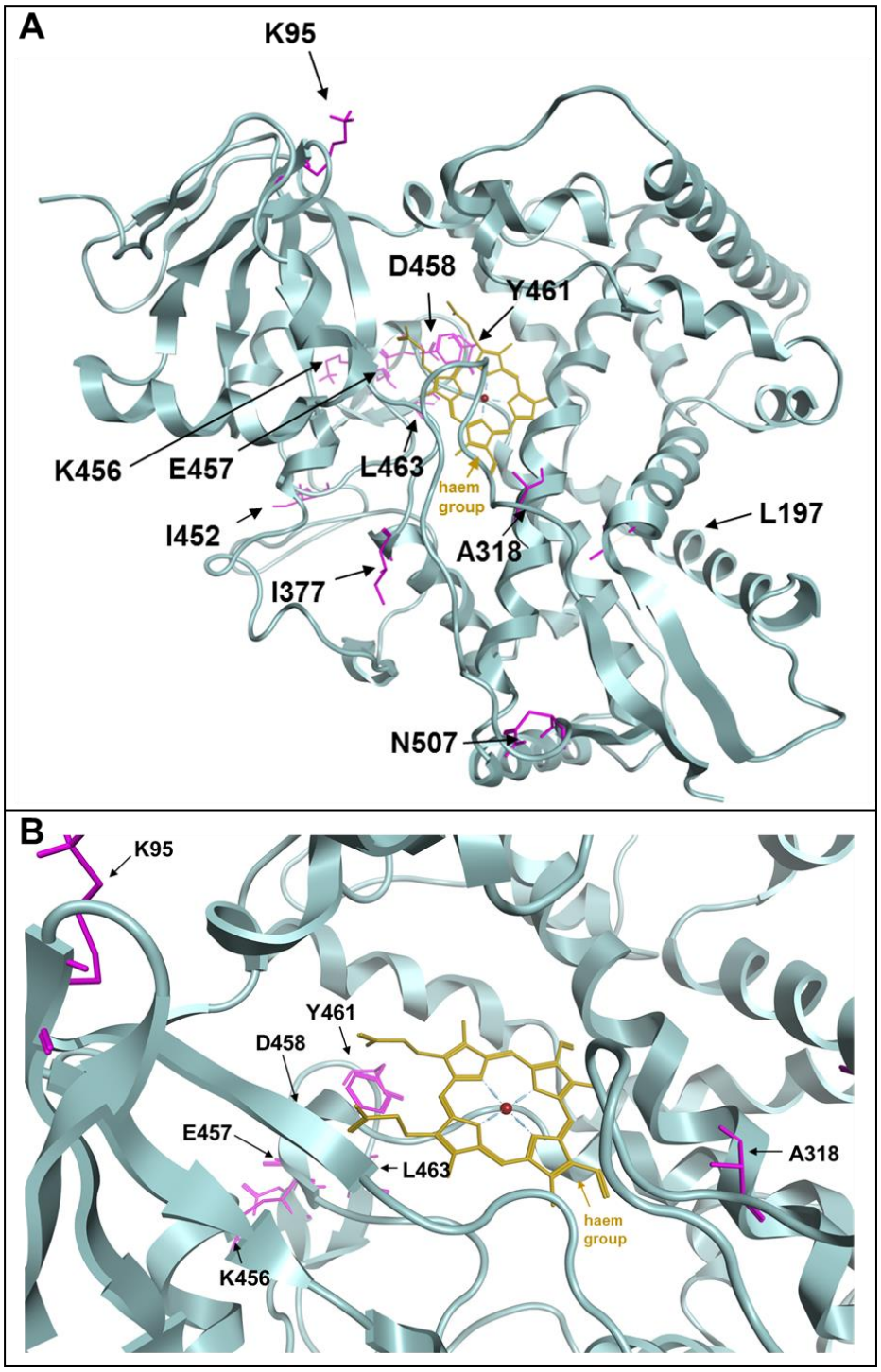


Figure 7: Homology model of the sterol 14-α demethylase enzyme (CYP51) of *Z. tritici* depicting the location of wild type amino acids identified as positions of new alterations. The homology model is based on the X-ray from *S. cerevisiae* (PDB 5EAE). The structure of the enzyme is shown in cartoon style (grey). The haem group (yellow, stick model) indicates the DMI binding site. Wild type amino acids observed to be subject of new alterations K95M, L197V, A318G, I377V, I452V, Del K456, Del E457, Del D458, Y461N, Del L463, N507S were highlighted in pink (stick model). **A:** Overview about all positions at which new amino acid alterations were identified. **B:** Closer view on the surrounding of the haem group.

Locations of amino acids identified as subject of new alterations were observed at different positions in the CYP51 enzyme. The prosthetic haem group (yellow) indicates the binding site of triazoles. Positions of amino acids N507 (30.1 Å), K95 (25.4 Å), and I452 (20.1 Å) are located far away from the haem group. Positions of amino acids L197 (17.6 Å), A318 (12.9 Å), I377 (16.7 Å), K456 (18.5 Å), E457 (19.5 Å), D458 (17.2 Å), and L463 (16.2 Å) are localised in closer proximity to the haem. All distances between amino acid residues that were observed as positions of new or existing CYP51 alterations and the haem are listed in Table 24 in the supplements. Alteration Y461N emerged at position Y461 at which already existing alterations were identified. Residue Y461 is located 13.6 Å away from the haem group. Based on results of the enzyme modelling, a direct impact of new alterations at positions N507, K95, and I452 on triazole binding seems less likely compared with remaining new alterations due to the larger distances of N507, K95, and I452 to the haem group. Epoxiconazole sensitivity was evaluated in correlation to new CYP51 alterations in the following chapter to analyse if DMI sensitivity is affected by one of the new CYP51 alterations.

4.2.2 Impact of new CYP51 alterations on DMI sensitivity of *Z. tritici*

The EC_{50} values of *Z. tritici* isolates carrying a new CYP51 alteration for epoxiconazole (determined in microtiter tests by EpiLogic) were plotted compared to EC_{50} values of wild type isolates in order to analyse the phenotypical impact of new CYP51 alteration on DMI sensitivity. As most of the new alterations only occurred in combination with other CYP51 alterations, the sensitivity of isolates with new CYP51 alterations was compared to the sensitivity of isolates carrying *CYP51* haplotypes that show the same combination of CYP51 alterations and only differ in the presence of a new alteration. If an isolate with such a *CYP51* haplotype was not available, the sensitivity of isolates with the new alterations was compared to isolates with a relatively similar CYP51 alteration background. An overview about *CYP51* haplotypes that were compared in order to analyse the impact of respective CYP51 alterations are given in Table 16.

Table 16: Overview about comparisons of *CYP51* haplotypes to analyse the impact of new CYP51 alterations on DMI sensitivity. *CYP51* haplotypes marked with an asterisk '*' are described for the first time for *Z. tritici*.

New CYP51 alteration to test	*CYP51* haplotype comparison
I452V	WT A15*: I452V
L197V, Del K456, Del E457, Del D458, Del L463	WT I4*: L197V, Del456-Del463
A318G	C8: L50S, I381V, Y461H D26*: L50S, A318G, I381V, Y461H
I377V	C8: L50S, I381V, Y461H D27*: L50S, I377V, I381V, Y461H
Y461N	C7: L50S, V136A, Y461H C9*: L50S, V136A, Y461N
K95M	D6: L50S, D134G, V136G, Y461S E28*: L50S, K95M, D134G, V136G, Y461S
N507S	E4: L50S, D134G, V136A, I381V, Y461H F20*: L50S, D134G, V136A, I381V, Y461H, N507S

Comparison of EC_{50} values of epoxiconazole for all isolates carrying a *CYP51* haplotype with a new alteration are shown in Figure 8. *CYP51* haplotypes with a new alteration are marked in black and with an asterisk as they also represent new *CYP51* haplotypes.

Compared to the sensitivity of the wild type or already existing *CYP51* haplotypes, isolates with alterations I377V (haplotype D27*), I452V (haplotype A15*), Y461N (haplotype C9*), or N507S (haplotype F20*) showed comparable sensitivities. These results indicate that alterations I377V, I452V, Y461N and N507S do not exert an additive effect on epoxiconazole adaptation. Moreover, only a small impact on epoxiconazole sensitivity (1.4-fold higher adaptation) was correlated to alteration K95M (haplotype E28*) compared to isolates carrying haplotype D6.

A 2.4-fold higher adaptation was observed in the isolate carrying alteration A318G (haplotype D26*) compared to the mean EC_{50} of haplotype C8. At this point, however, it should be noted that the isolate carrying alteration A318G additionally carry an insert in the *MFS1*p region that may confer a slight increased efflux activity (150 bp insert, shown in chapter 4.6.2 and 4.6.3). Therefore, it cannot be clarified at this point, if slightly higher adaptation of this isolate is conferred by the new CYP51 alteration A318G or by an increased efflux.

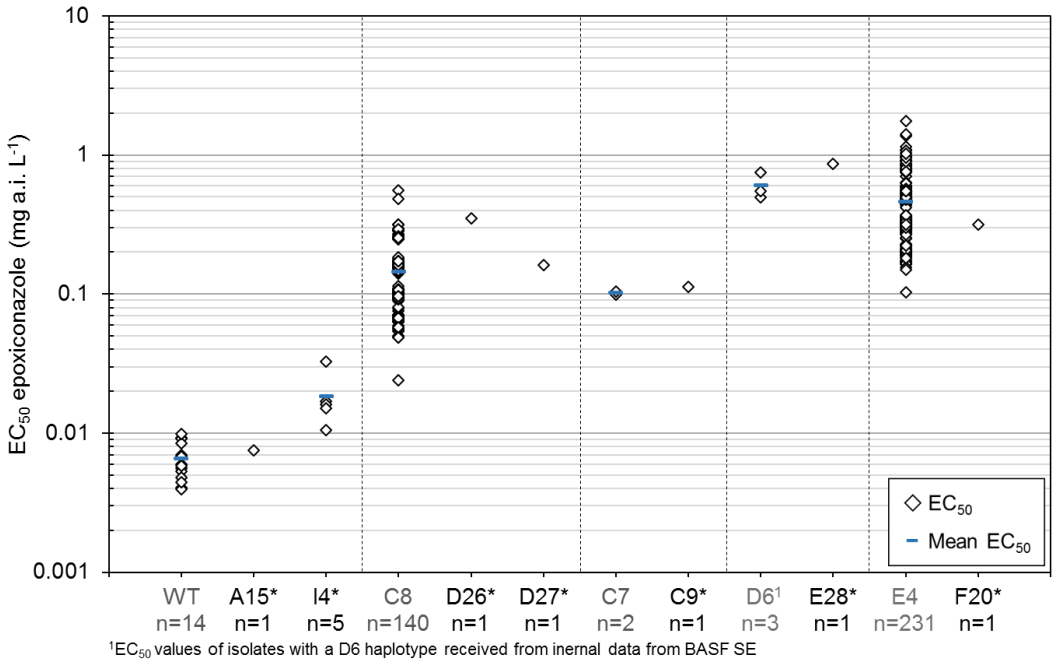


Figure 8: DMI sensitivity of *Z. tritici* isolates carrying new CYP51 alterations. Sensitivity of *CYP51* haplotypes carrying a new CYP51 alteration (marked in black and with an asterisk) were compared to the sensitivity of the *CYP51* wild type or existing *CYP51* haplotypes (marked in grey). EC_{50} values were determined in microtiter tests by the company EpiLogic and are shown for each tested isolate. The number of tested isolates per haplotype is shown in the diagram. Mean EC_{50} values were calculated across all isolates carrying the same *CYP51* haplotype if more than one isolate was identified.

Isolates carrying alteration L197V combined with Del K456, Del E457, Del D458, and Del L463 (haplotype I4*) showed around a 3-fold higher adaptation to epoxiconazole compared to the wild type. Consequently, among all new CYP51 alterations identified in this work, L197V combined to these deletions showed the highest impact on epoxiconazole sensitivity. Nevertheless, the resistance level of isolates carrying alteration L197V, and Del 456-463 was low compared to more adapted *CYP51* haplotypes, for example, haplotype E4. Moreover, as alteration L197V and deletion of amino acid positions 456, 457, 458, and 463 (haplotype I4*) occur in combination with deletion of positions 459, 460, 461, and 462, their individual effect cannot be assessed. Consequently, it cannot be clarified if adaptation of haplotype I4* is driven by alteration L197V, Del K456, Del E457, Del D458, or Del L463 or by alteration of positions 459-462.

All in all, results indicate that none of the new CYP51 alterations could be correlated to distinct effects on epoxiconazole sensitivity that result in high resistance levels or in a pronounced additive effect on epoxiconazole adaptation compared to already existing *CYP51* haplotypes. Therefore, these new CYP51 alterations were not investigated further for their own. Nevertheless, in the following chapter, *CYP51* haplotypes with a new CYP51 alteration were also used for sensitivity evaluation of epoxiconazole for all *CYP51* haplotypes found in this work.

4.3 DMI sensitivity of *Z. tritici* isolates carrying different *CYP51* haplotypes

Sensitivity of epoxiconazole was determined for all isolates obtained from the routine sensitivity monitoring of 2016 and 2017. EC_{50} values were gained from microtiter tests that were performed by the company EpiLogic. EC_{50} values of microtiter tests were plotted against the respective *CYP51* haplotypes determined in the current work in order to investigate their impact on DMI sensitivity. Results are shown for the 13 'frequently found *CYP51* haplotypes' in Figure 9 and for the 'rarely found *CYP51* haplotypes' in Figure 10.

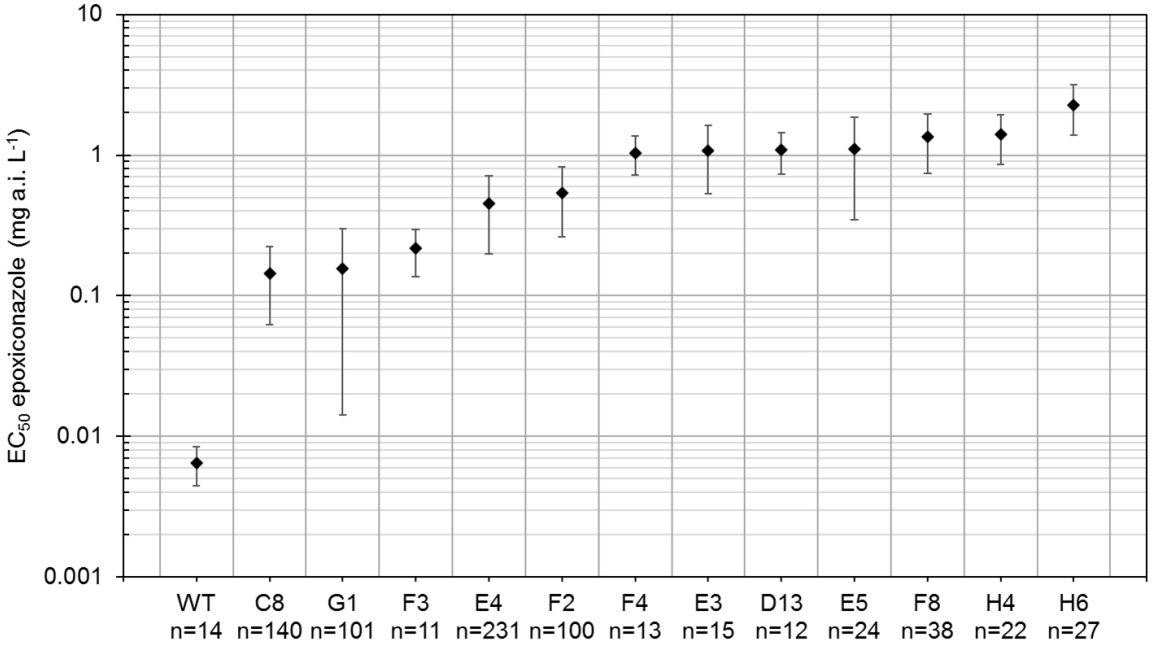


Figure 9: Epoxiconazole sensitivity of *Z. tritici* isolates with 'frequently found *CYP51* haplotypes' across 2016 and 2017. Isolates were obtained from BASF routine sensitivity monitoring. EC_{50} values were determined in microtiter tests by the company EpiLogic. The *CYP51* haplotypes, number of tested isolates, mean EC_{50} values, and standard deviations are shown. Mean EC_{50} and standard deviations were calculated over all EC_{50} values of isolates with the same *CYP51* haplotype. *CYP51* haplotypes were arranged according to increasing adaptation.

EC_{50} values of all isolates (n=748) carrying 'frequently found *CYP51* haplotypes', including the wild type, ranged from 0.004 to 4.630 mg epoxiconazole L^{-1}. Wild type isolates showed EC_{50} values ranging from 0.004 to 0.010 mg epoxiconazole L^{-1}, thus represented a 2.5-fold difference in sensitivity. All other isolates with 'frequently found *CYP51* haplotypes' showed a reduced sensitivity compared to the wild type with EC_{50} values ranging from 0.024 to 4.630 mg epoxiconazole L^{-1}. Among these, isolates carrying a C8 haplotype showed highest sensitivity for epoxiconazole with a mean EC_{50} of 0.143 mg a.i. L^{-1}, followed by haplotypes G1 and F3 with a mean EC_{50} of 0.157 and 0.217 mg a.i. L^{-1}, respectively. Isolates with a E4 or F2 haplotype showed moderate adaptation to epoxiconazole with a mean EC_{50} of 0.454 and 0.542 mg a.i. L^{-1}, respectively. Comparisons between the sensitivity of different haplotypes enable predictions about the effect of single alterations. For example, haplotype

C8 and E4 only differ in alteration D134G and V136A. Higher EC_{50} values of most of the isolates with a E4 haplotype compared to isolates with a C8 haplotype demonstrate that these two alterations (D134G, V136A) lead to a further reduction in epoxiconazole sensitivity of *Z. tritici*. Adaptation of haplotype E4 and F2 was followed by higher adaptation of haplotypes F4, E3, D13, and E5 that showed similar mean EC_{50} values of 1.041, 1.082, 1.093 and 1.104 mg a.i. epoxiconazole L^{-1}, respectively. Isolates with haplotype F8 showed the third highest adaptation for epoxiconazole with a mean EC_{50} of 1.350 mg a.i. L^{-1} and isolates with haplotype H4 showed the second highest adaptation with a mean EC_{50} of 1.400 mg a.i. L^{-1}. Isolates with haplotype H6 showed highest adaptation for epoxiconazole with a mean EC_{50} of 2.266 mg a.i. L^{-1}. Haplotypes F4, E3, D13, E5, F8, H4, and H6 have in common that they carry alteration S524T. This indicates that further reduction in epoxiconazole sensitivity compared to moderately adapted haplotypes E4 and F2 is probably driven by alteration S524T combined with other alterations.

EC_{50} values of isolates carrying the 44 'rarely found *CYP51* haplotypes' are shown in comparison to the *CYP51* wild type as a reference (Figure 10). EC_{50} values of all isolates (n=95) with 'rarely found *CYP51* haplotypes' ranged from 0.006 to 3.055 mg epoxiconazole L^{-1}. Across these haplotypes, EC_{50} values of isolates with an A2, A3, and A15* haplotype were located in the range of the sensitivity of wild type isolates. Ten *CYP51* haplotypes exert a low adaptation to epoxiconazole with EC_{50} or mean EC_{50} values between 0.01 and 0.1 mg a.i. L^{-1}. These haplotypes were C18 < A14* < I4* < B8 < D2 < B15* < E21 < E27* < D9 < C4 (ranked according to increased adaptation). Twenty-five *CYP51* haplotypes showed EC_{50} or mean EC_{50} values between 0.1 and 1 mg epoxiconazole L^{-1}. These haplotypes were C7 < C6 < C9* < F5 < D27* < C2 < E9* < F20* < F19* < F6 < E26* < D26* < D7 < E25* < D10 < F9* < E29* < H5 < H3 < G7* < G2 < F1 < H9 < E28* < G6* (ranked according to increased adaptation). Among all 'rarely found *CYP51* haplotypes', 6 haplotypes showed highest adaptation with EC_{50} or mean EC_{50} values larger than 1 mg epoxiconazole L^{-1}. These haplotypes were E7* < E8 < F7 < I1 < F10* < I2* (ranked according to increased adaptation). It was observed that new *CYP51* haplotypes (marked with an asterisk) showed varying sensitivities to epoxiconazole across the whole range of sensitivities of all 'rarely found *CYP51* haplotypes'. For example, epoxiconazole sensitivity of isolates carrying haplotype A14* or B15* was only slightly reduced compared to the wild type, whereas haplotypes E7*, F10*, and I2* belonged to the six haplotypes leading to the highest reduction in sensitivity.

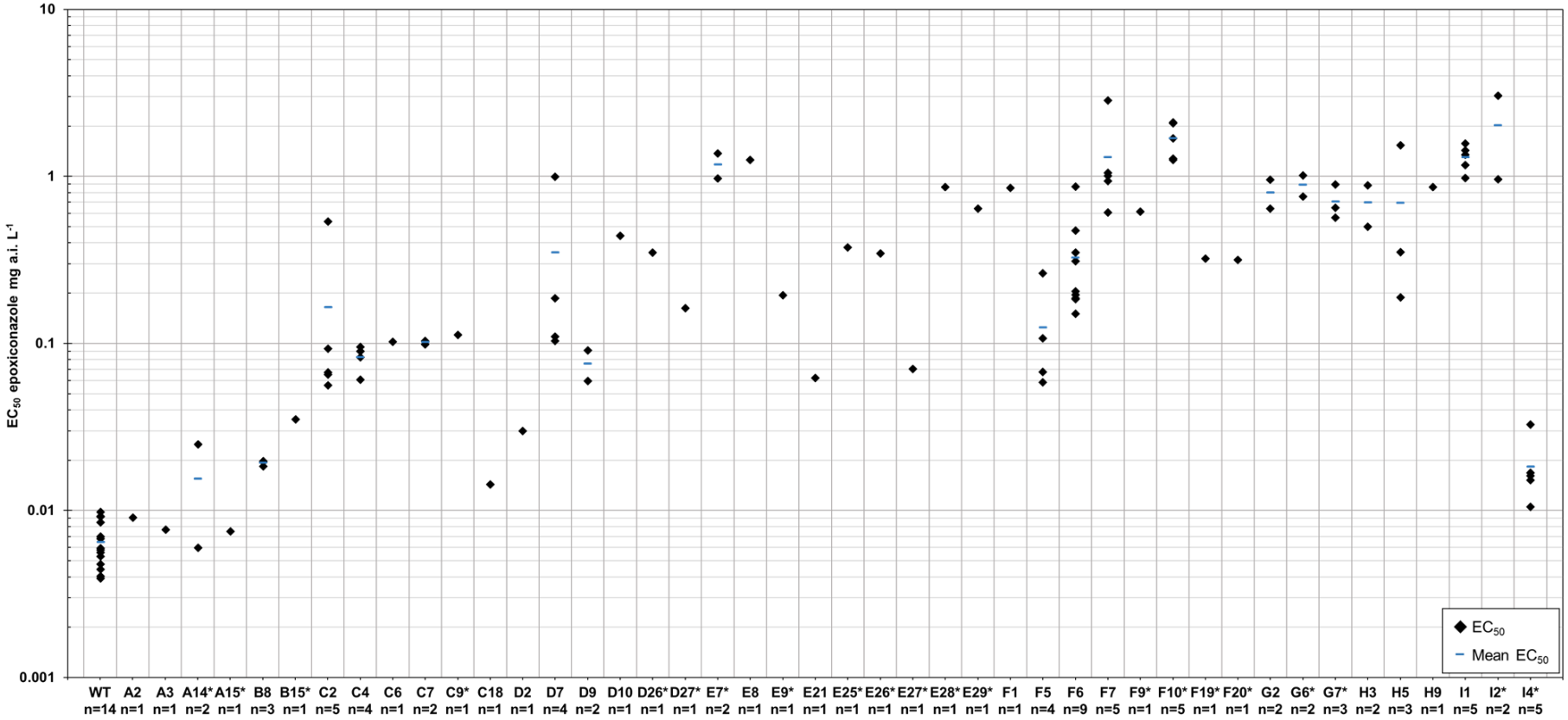


Figure 10: Epoxiconazole sensitivity of *Z. tritici* isolates with 'rarely found *CYP51* haplotypes' identified across 2016 and 2017. Isolates were obtained from BASF routine sensitivity monitoring. EC_{50} values of wild type isolates are shown as a reference. EC_{50} values were determined in microtiter tests by the company EpiLogic. The EC_{50} values of each tested isolate per *CYP51* haplotype is shown and the number of tested isolates is given in the diagram. Haplotypes that were described for the first time are marked with an asterisk. Mean EC_{50} values are shown for *CYP51* haplotypes that were found in more than one isolate and were calculated over all isolates with the same haplotype.

Despite the observed variation of epoxiconazole sensitivity between different *CYP51* haplotypes, that is suggested to rely on the accumulation of different CYP51 alterations, a relatively large variation in epoxiconazole sensitivity was also identified among isolates within a *CYP51* haplotype group. This variation in sensitivity may be attributable to additional resistance mechanisms such as *CYP51* overexpression or an increased efflux activity. These mechanisms will be investigated and reported further in the course of this work. Beforehand, DMI adaptation in regional *Z. tritici* populations was investigated based on occurrence of *CYP51* haplotypes and the knowledge about their epoxiconazole sensitivity obtained in this chapter.

4.4 Composition and evolution of *CYP51* haplotypes in regional *Z. tritici* populations across Europe

Frequency and distribution of *CYP51* haplotypes was analysed in different European countries to get an overview about the DMI adaptation of regional *Z. tritici* populations in 2016 and 2017. In this chapter, frequency of each 'frequently found *CYP51* haplotype' from 2016 and 2017 is shown. Frequencies of 'rarely found *CYP51* haplotypes' were summed up and referred to as 'others'. Changes in haplotype frequency and occurrence between 2016 and 2017 was analysed for chosen countries which were tested in both years.

4.4.1 Frequency and distribution of *CYP51* haplotypes in 2016

In 2016, *Z. tritici* isolates gained from BASF routine sensitivity monitoring from 9 different European countries were investigated for their *CYP51* haplotypes. The resulting distribution and frequencies of 'frequently found *CYP51* haplotypes' are shown in Figure 11. The order of 'frequently found *CYP51* haplotypes' in the legend of the diagram is arranged according to their increasing epoxiconazole adaptation (from WT: no adaptation to H6: highest adaptation), as observed in the previous chapter 4.3.

In 2016, a heterogenous distribution and frequency of 'frequently found *CYP51* haplotypes' was observed across the investigated countries in Europe. In that year, no isolate carrying a wild type *CYP51* (*CYP51* WT) was identified. Frequencies of haplotype C8, that showed highest sensitivity to epoxiconazole among the 'frequently found *CYP51* haplotypes', ranged from 12.3 to 50% across Czech Republic, Denmark, France, Germany, Poland, and Sweden. In UK, only a single isolate with a C8 haplotype was observed and no isolate with this type was detected in Ireland and the Netherlands. Haplotype G1 was detected in all countries, with exception of Czech Republic and the Netherlands, in frequencies ranging from 3.2% in UK to 42.9% in Denmark. Only single isolates with a F3 haplotype were detected in France but in no other country. The most frequent haplotype across all tested countries was haplotype E4, expressing moderate adaptation to epoxiconazole among all

'frequently found *CYP51* haplotypes'. This haplotype was found in frequencies ranging between 18.4 to 48% in all countries with exception of Denmark and the Czech Republic. Haplotype F2 was found in frequencies ranging between 11 to 50% in Czech Republic (50%), UK (19%), the Netherlands (18.2%), Poland (14.3%), Germany (13.6%), and Sweden (11.1%). This haplotype was found in lower frequencies in France (5.5%) and in Ireland (4.3%). It should be noted at this point that the number of tested isolates in Denmark, Czech Republic, the Netherlands and Sweden was overall lower and consequently single isolates can result in high percentages of haplotypes compared to the remaining countries.

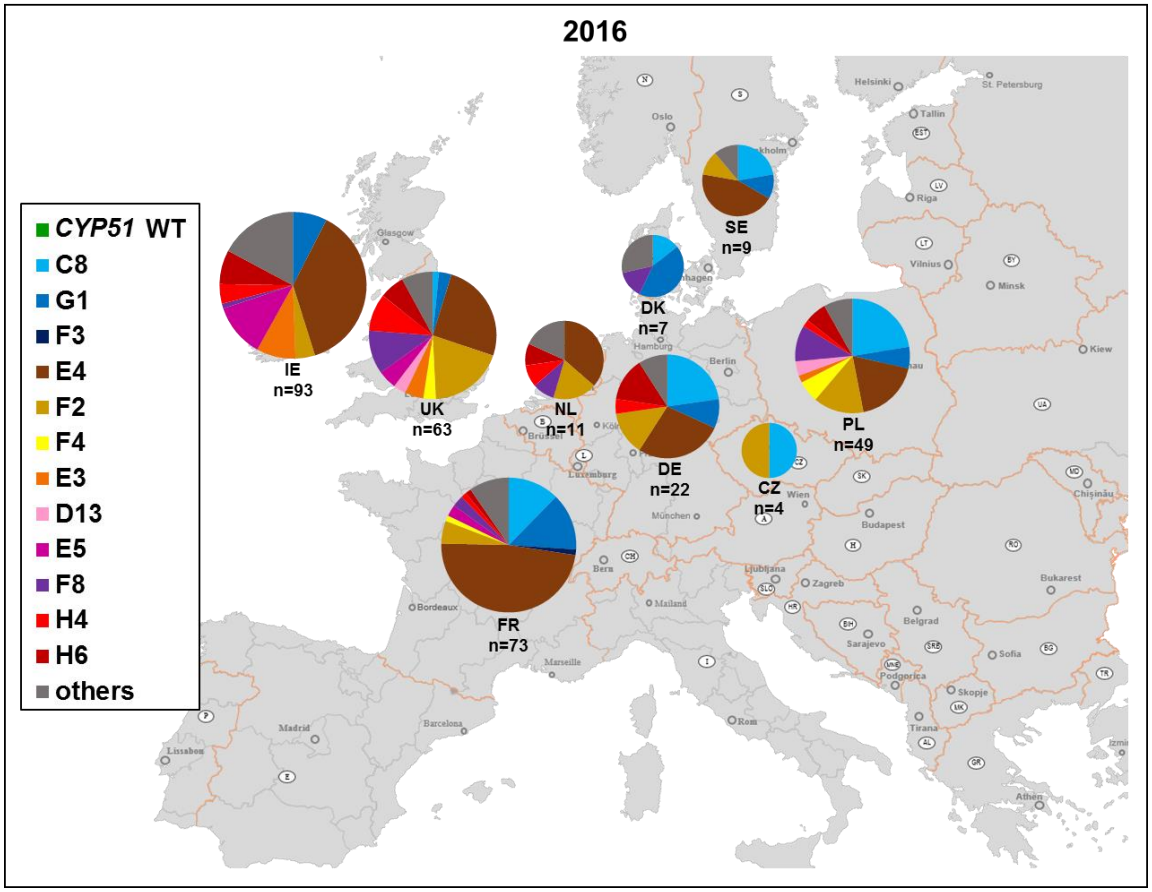


Figure 11: Frequency and distribution of *CYP51* haplotypes of *Z. tritici* across different European countries in 2016. *Z. tritici* isolates were obtained from BASF routine sensitivity monitoring. Frequency and distribution of each 'frequently found *CYP51* haplotype' is shown. Frequencies of all 'rarely found *CYP51* haplotypes' are summarised as others. The order of the 'frequently found *CYP51* haplotypes' in the diagram is arranged according to their increasing mean EC_{50} values for epoxiconazole, from *CYP51* wild type (*CYP51* WT, no adaptation) to haplotype H6 (highest adaptation). The colour code for *CYP51* haplotypes is given in the legend in the figure. The number of tested isolates is given under the respective pie chart. The size of the pie chart is increasing with the number of tested isolates per country. These results have been previously published partially in an altered figure (Figure 2) in Huf et al., 2018.

Across all 'frequently found *CYP51* haplotypes', haplotypes F4, E3, D13, E5, F8, H4, and H6 carry alteration S524T and were associated with the highest adaptation to epoxiconazole (described in chapter 4.3). Haplotype F4 was only found in Poland (6.1%) and UK (3.2%) and in a single isolate in France. Haplotypes E3 and E5 were mainly

detected in Ireland (E3: 8.6%, E5: 11.8%), followed by UK (E3: 4.8%, E5: 4.8%) and in single isolates in Poland and France but in no other country. Haplotype D13 was identified in UK (3.1%) and Poland (4.1%). Frequencies of haplotype F8 ranged from 9.1 to 14.3% across the Netherlands, Poland, UK, and Denmark. Single isolates with a F8 haplotype were found in France and Ireland, but no isolate with this haplotype was detected in Czech Republic, Germany, and Sweden. Highest frequency of the H4 haplotype was identified in UK (9.5%), followed by the Netherlands (9.1%), Germany (4.5%), and Ireland (4.3%). A single isolate with this type was found in France and Poland. The H6 haplotype was observed in Germany (13.6%), the Netherlands (9.1%), Ireland (7.5%), UK (6.3%), and Poland (6.1%). Only a single isolate with a H6 type was detected in France. Both haplotypes H4 and H6 were not found in Czech Republic, Denmark, or Sweden.

In summary, these results show that in 2016 the highest frequency of haplotypes with alteration S524T was found in UK, Ireland, and the Netherlands. In these countries around 27 to 43% of all isolates were composed of a 'frequently found *CYP51* haplotype' with alteration S524T. Moreover, in these countries also 'rarely found *CYP51* haplotypes' with alteration S524T were detected. Therefore, 45 to 50% of the *Z. tritici* populations in these countries carried a haplotype with alteration S524T (data not shown). The remaining *Z. tritici* populations were mainly composed of haplotypes expressing moderate adaptation to epoxiconazole. Compared to this, frequencies of alteration S524T were lower in France, the Central European countries Germany, Poland, and Czech Republic as well as in the North European countries Denmark and Sweden. In these countries the majority of the *Z. tritici* populations were composed of haplotypes C8, G1, E4, and F2 expressing lower or moderate adaptation to epoxiconazole across all 'frequently found *CYP51* haplotypes'. In Ireland, UK, and the Netherlands, haplotype C8 was only detected at low frequencies or was not detected at all. These results indicate that in 2016 an east-west gradient in the adaptation of *Z. tritici* across Europe could be observed with higher epoxiconazole adaptation in regional *Z. tritici* populations from UK, Ireland and the Netherlands compared to the remaining countries.

4.4.2 Frequency and distribution of *CYP51* haplotypes in 2017

In 2017, *Z. tritici* isolates from 13 European countries obtained from BASF routine sensitivity monitoring were analysed for their *CYP51* haplotypes. In Figure 12, the frequencies and distribution of 'frequently found *CYP51* haplotypes' in 2017 are shown. The order of the 'frequently found *CYP51* haplotypes' in the diagram is arranged according to their increasing epoxiconazole adaptation (from WT: no adaptation to H6: highest adaptation) observed in chapter 4.3.

Similar to 2016, a heterogenous distribution and frequency of 'frequently found *CYP51* haplotypes' was observed across the investigated countries in Europe in 2017. In that year,

isolates carrying the *CYP51* WT were detected, namely in Bulgaria, Russia, and Slovakia in frequencies ranging from 9.1 to 15.8%. Isolates with a *CYP51* WT were absent in the remaining countries.

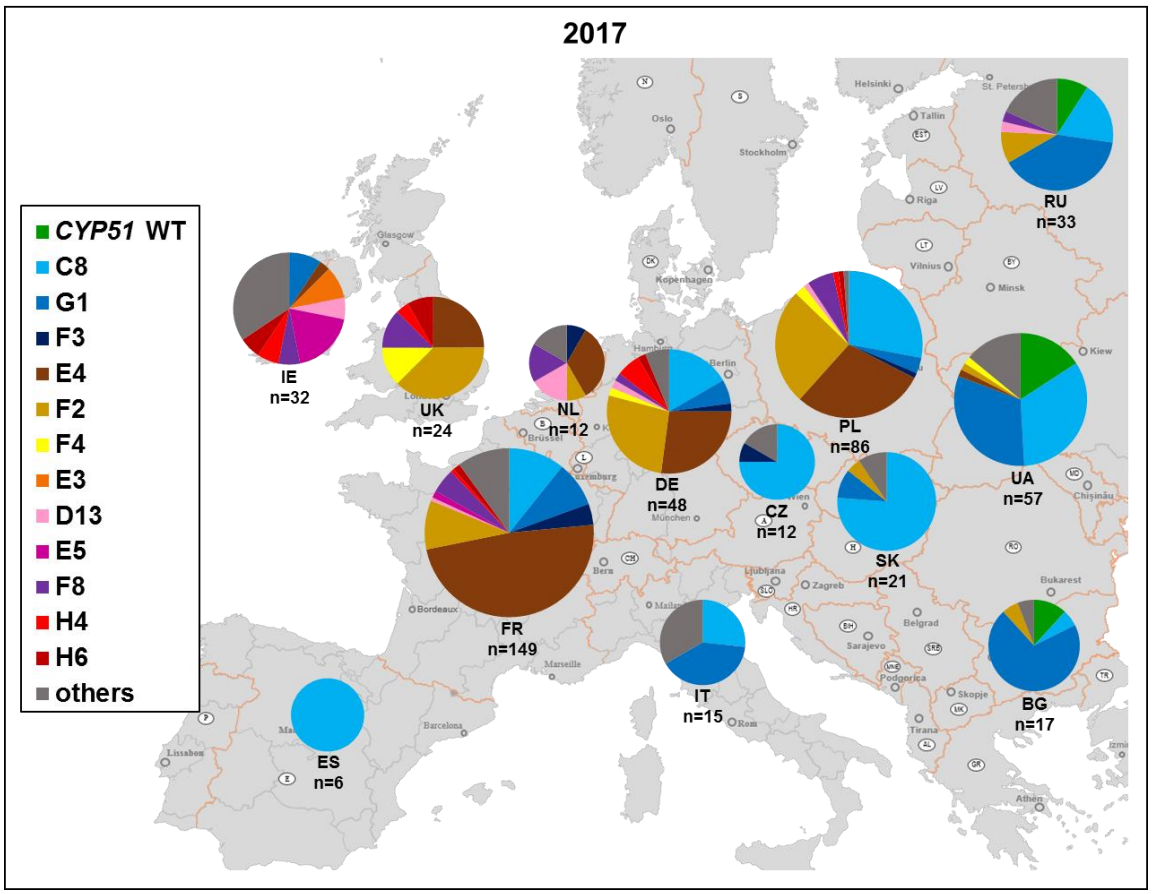


Figure 12: Frequency and distribution of *CYP51* haplotypes of *Z. tritici* across different European countries in 2017. *Z. tritici* isolates were obtained from BASF routine sensitivity monitoring. Frequency and distribution of each 'frequently found *CYP51* haplotype' is shown. Frequencies of all 'rarely found *CYP51* haplotypes' are summarised as others. The order of the 'frequently found *CYP51* haplotypes' in the diagram is arranged according to their increasing mean EC_{50} values for epoxiconazole, from *CYP51* wild type (*CYP51* WT, no adaptation) to haplotype H6 (highest adaptation). The colour code for *CYP51* haplotypes is given in the legend in the figure. The number of tested isolates is given under the respective pie chart. The size of the pie chart is increasing with the number of tested isolates per country.

Haplotype C8 was found in each tested country with exception of Ireland, UK, and the Netherlands. A high frequency of the C8 haplotype was found in Spain (100%), Slovakia (76.2%), and Czech Republic (75%). Frequencies ranging from 10.7 to 33.3% were found in Germany, France, Italy, Poland, Russia and Ukraine. In Bulgaria, 5.9% of all isolates showed a C8 haplotype. Highest frequencies of haplotype G1 were identified in Bulgaria (70.6%), Russia (39.4%), Ukraine (31.6%), and Italy (40%). In Germany, France, Ireland, Poland, and Slovakia frequencies of haplotype G1 ranged between 3.5 and 9.5%. In UK, the Netherlands, Spain, and Czech Republic, the G1 haplotype was not observed. Haplotype F3 was detected in Czech Republic, France, Germany, the Netherlands, and Poland in frequencies ranging from 1.2 to 8.3%. In 2017, similar to 2016, the most frequent

haplotype across all countries was haplotype E4, however, it was mainly detected in UK, the Netherlands, France, Germany, and Poland. Frequencies of this haplotype ranged from 25 to 33.3% across Germany, the Netherlands, Poland, and UK and was highest in France (48%). A single isolate with an E4 haplotype was found in Ireland and Ukraine, but no isolate with an E4 haplotype was found in Russia, Bulgaria, Slovakia, Italy, Czech Republic, and Spain. Haplotype F2 was detected in higher frequencies in UK (37.5%), Germany (27.1%), and Poland (25.6%) compared to lower frequencies between 4.8% and 9.4% in Bulgaria, France, the Netherlands, Russia, and Slovakia. A single isolate with a F2 haplotype was identified in Ukraine. Isolates with haplotypes carrying alteration S524T such as F4, E3, D13, E5, F8, H4, and H6 were mainly found in Ireland, UK, and the Netherlands and in lower frequencies in some of the remaining countries. Haplotype F4 was found in 12.5% of all isolates in UK and in single isolates in Germany, Poland, and Ukraine. Haplotypes E3 and E5 were mainly detected in Ireland with frequencies of 9.4% and 18.8%, respectively. A single isolate with a E5 haplotype was identified in France, however, none of these two types were found in any other country. Haplotype D13 was detected most frequently in the Netherlands (16.7%), followed by Ireland (6.3%). Again, also in 2017, it has to be pointed out that the number of tested isolates is lower for some countries such as Spain, the Netherlands, Czech Republic as well as Italy and consequently single isolates can result in high percentages of *CYP51* haplotypes in these countries. Single isolates with a D13 haplotype were found in Germany, France, Poland, and Russia. Frequencies of haplotype F8 was highest in UK and the Netherlands, followed by Ireland, Poland, and France. A single isolate with this haplotype was found in Germany and Russia. Frequencies of the H4 haplotype accounted to 4.3% in UK and 6.3% in Germany and Ireland, respectively. The H6 haplotype was most frequently found in UK (8.6%), followed by Ireland (6.3%). Single isolates with this haplotype were found in Germany. In France and Poland, single isolates with a H4 and H6 haplotype were found, whereas these haplotypes were absent in the remaining countries. In general, no isolate with a 'frequently found *CYP51* haplotype' carrying alteration S524T was found in Spain, Italy, Czech Republic, Slovakia, and Bulgaria. Only single isolates with such a haplotype were found in Russia and Ukraine.

In conclusion, these results show that the majority of the *Z. tritici* populations in Southern and Eastern European countries were composed of *CYP51* haplotypes C8 and G1 (lowest epoxiconazole adaptation among 'frequently found *CYP51* haplotypes'). In 2017, for France, Germany, and Poland, results of 2016 were confirmed, and the majority of the population was composed of the C8 and G1 haplotype and the moderately adapted haplotypes E4 and F2. Again, in these countries, frequencies of haplotypes with alteration S524T expressing highest epoxiconazole adaptation across all 'frequently found *CYP51* haplotypes' were lower compared to the Netherlands, Ireland, and UK. In these 3 countries the highest frequency of haplotypes with alteration S524T were found. These results confirm the east-west gradient in the adaptation of regional *Z. tritici* populations to

epoxiconazole which was already observed in 2016. In 2017, highest sensitivity of *Z. tritici* was observed in Eastern as well as Southern European countries. Increasing adaptation was observed in Germany, Poland as well as France and highest adaptation was observed in the Western European countries Ireland and UK as well as the Netherlands.

Comparison of *CYP51* haplotype frequencies between 2016 and 2017 across all isolates and all countries is not reasonable as the scope of tested countries differed between these two years. Changes in *CYP51* haplotype frequencies over this period will be examined in the next chapter for some chosen countries that were tested in both years with a representative number of isolates.

4.4.3 Comparison of *CYP51* haplotype occurrence and frequency between 2016 and 2017

The frequency and occurrence of *CYP51* haplotypes was compared in selected countries between 2016 and 2017 to analyse changes of *Z. tritici* populations in composition of *CYP51* haplotypes over a short period of time. Occurrence and frequency of *CYP51* haplotypes in Ireland, UK, Germany, Poland, and France in 2016 and 2017 are shown in Figure 13.

In Ireland, a strong decrease (about ~34%) in the frequency of haplotype E4 and increase (about ~17%) of isolates carrying 'rarely found *CYP51* haplotypes' was observed. The majority of these isolates showed alteration S524T (data not shown). Additionally, the frequency of 'frequently found *CYP51* haplotypes' carrying alteration S524T increased in 2017 compared to 2016 indicating a further shift towards a reduced DMI sensitivity in Ireland. In contrast to this, a slight decrease (about ~5%) of 'frequently found *CYP51* haplotypes' with alteration S524T and mainly an increase (about ~19%) of moderately adapted haplotype F2 was observed in UK. However, it should be noted that the sampling in Ireland and UK was restricted in 2017. A lower number of isolates was tested compared to 2016 in both countries and especially in UK, samples derived from a lower number of locations. The limited sampling in 2017 may contribute to the overall composition of *CYP51* haplotypes in these two countries.

In Germany, Poland, and France, the composition of *CYP51* haplotypes of the *Z. tritici* populations stayed relatively stable between 2016 and 2017. In these countries *Z. tritici* populations were mainly composed of the 'frequently found *CYP51* haplotypes' in both years. Nevertheless, especially in Germany and Poland, changes in the frequency of these haplotypes were observed. In Germany, mainly the frequency of haplotype F2 increased (about ~14%), whereas in Poland both haplotypes E4 and F2 increased (both about ~11%, respectively). Frequency of isolates carrying haplotypes C8 and G1 slightly decreased in Germany (C8 around ~6%, G1 around ~3%). In Poland, only frequency of haplotype G1 slightly decreased (about ~3%), whereas frequency of haplotype C8 slightly increased

(about ~6%) in 2017. In France, a slight decrease in the frequency of haplotype G1 (about ~5%) was observed and frequency of isolates with haplotype F2 slightly increased (about ~4%) indicating a slight further shift towards a reduced sensitivity. However, changes were small across both years in France. The frequency of isolates carrying a haplotype with alteration S524T stayed relatively stable in Germany and France and even decreased in Poland (data not shown). Moreover, the frequency of other haplotypes ('rarely found *CYP51* haplotypes') decreased in Poland in 2017. A slight decrease in the frequency of other haplotypes was also detected in Germany, whereas their total frequency stayed stable in France.

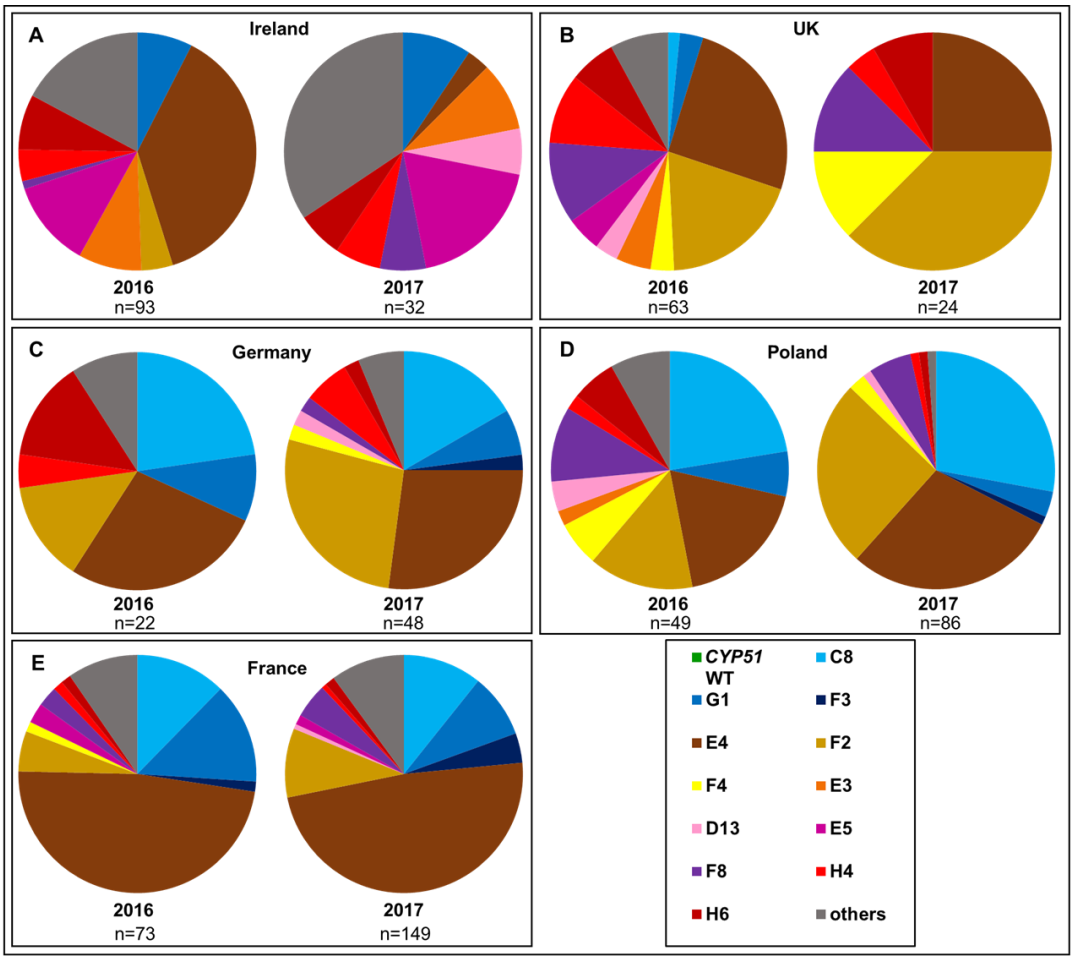


Figure 13: Comparison of the composition of *CYP51* haplotypes of *Z. tritici* between 2016 and 2017 in different European countries. *Z. tritici* isolates were obtained from BASF routine sensitivity monitoring. Frequency and distribution of each 'frequently found *CYP51* haplotype' is shown. Frequencies of all 'rarely found *CYP51* haplotypes' are summarised as others. The order of the 'frequently found *CYP51* haplotypes' in the diagram is arranged according to their increasing mean EC_{50} values for epoxiconazole, from *CYP51* wild type (*CYP51* WT, no adaptation) to haplotype H6 (highest adaptation). The colour code for *CYP51* haplotypes is given in the figure and the number of tested isolates is shown under the respective pie chart. Composition of *CYP51* haplotypes from 2016 and 2017 is shown for **A:** Ireland, **B:** United Kingdom (UK), **C:** Germany, **D:** Poland, and **E:** France. These results have been previously published in an altered figure (Figure 1) in Huf et al., 2020.

All in all, the results demonstrate that, over a short period of time, development of *CYP51* haplotype composition of *Z. tritici* populations between 2016 and 2017 was dependent on the country. In Ireland, a further shift towards a reduced epoxiconazole sensitivity is indicated, whereas no clear further shift was observed for UK. In Germany, a slight decrease of isolates with haplotypes conferring highest sensitivity to epoxiconazole across all 'frequently found *CYP51* haplotypes' and an increase of haplotypes expressing moderate adaptation indicate a gradual further shift towards a reduced sensitivity. Minor changes in the frequency of *CYP51* haplotypes were observed in France which indicate a slight further shift towards a reduced sensitivity between 2016 and 2017. Changes in the frequency of *CYP51* haplotypes in Poland indicate no further shift towards a reduced sensitivity between 2016 and 2017.

4.5 *CYP51* overexpression in *Z. tritici*

In addition to the accumulation of CYP51 alterations, *CYP51* overexpression was shown to affect sensitivity of *Z. tritici* towards DMIs (Leroux and Walker, 2011; Cools et al., 2012). A 10- to 40-fold *CYP51* overexpression was associated with a 120 bp insert in *CYP51*p of *Z. tritici* (Cools et al., 2012). Additionally, an 862 bp and 866 bp *CYP51*p insert was identified. However, these inserts were assumed not to alter *CYP51* expression (Chassot et al., 2008; Leroux and Walker, 2011; Omrane et al., 2015; Kildea et al., 2019). In the current work, all *Z. tritici* isolates from routine sensitivity monitoring were tested for inserts in *CYP51*p in order to identify an increased *CYP51* expression. The effect of identified inserts on the sensitivity of *Z. tritici* to epoxiconazole was analysed and finally, occurrence and distribution of these inserts in Europe was evaluated.

4.5.1 Identification of *CYP51* overexpression in *Z. tritici* isolates

All *Z. tritici* isolates obtained from the routine sensitivity monitoring of 2016 and 2017 were investigated for inserts in *CYP51*p, based on a PCR detection method, in order to identify *CYP51* overexpression. Additionally, amplified *CYP51*p fragments were sequenced for a selection of isolates (*CYP51*p wild type and isolates with a *CYP51*p insert) to check whether inserts identified in the current work show similar sequences compared to inserts previously described in literature.

Amplification of *CYP51*p resulted in four different sizes of DNA fragments across all investigated isolates. Isolates without an insert showed a *CYP51*p fragment of ~350 bp. Additionally, fragments of ~500, ~700, and ~1200 bp were detected indicating the presence of a *CYP51*p insert in these isolates.

Sequencing of different wild type fragments (n=15) with ~350 bp revealed that all tested isolates showed small *CYP51*p inserts with a length varying from 8 to <30 bp compared to the reference strain IPO323 (alignment shown in Figure 37 in the supplements). As described in the introduction (chapter 1.4.3), a high polymorphism and occurrence of small inserts in *CYP51*p is known for *Z. tritici*, but only a 120 bp insert was associated with *CYP51* overexpression. Therefore, in this work, it was focused on detection of inserts >50 bp and remaining isolates were regarded to carry a *CYP51* wild type promotor (WT *CYP51*p). Sequencing of *CYP51*p fragments with a length of ~500, ~700, or ~1200 bp revealed four different inserts with a length of 121, 300, 863, and 868 bp.

The complete sequence of the 121 bp insert in *CYP51*p of *Z. tritici* is shown in the alignment in Figure 38 in the supplementary material. The insertion of this insert is located at position -83 bp upstream (numbering based on WT *CYP51*p of St IPO323) of the *CYP51* start codon (Figure 14).

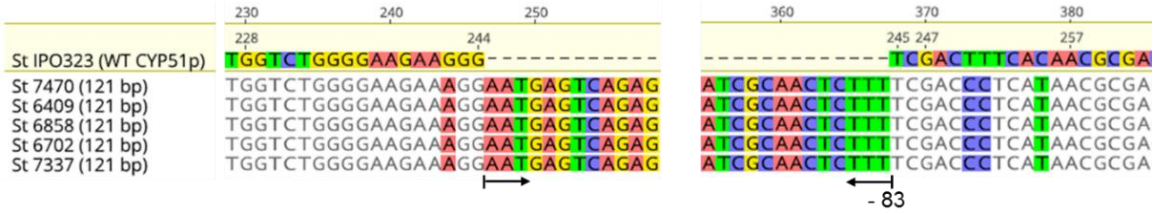


Figure 14: Insertion sites of the 121 bp insert in *CYP51*p of *Z. tritici*. Extract of the complete DNA sequence alignment of an isolate carrying the WT *CYP51*p (St IPO323) and isolates carrying the 121 bp insert is shown. Left and right insertion site of the insert is indicated by the arrows. Nucleobases of the reference sequence of St IPO323 and all disagreements to this sequence are highlighted in colour (adenine = red, guanine = yellow, thymine = green, cytosine = blue). The insertion site of the 121 bp insert is located -83 bp upstream of the *CYP51* start codon (numbering based on WT *CYP51*p of St IPO323). The alignment was performed with MUSCLE algorithm.

The 863 and 868 bp inserts were observed to be almost identical to each other (sequence identity 97%). Complete sequences of the 863 and 868 bp inserts are depicted in Figure 40 and Figure 41 in the supplements. As shown in Figure 15, the insertion site of the 863 bp insert is located -207 bp upstream of the *CYP51* start codon, whereas the 868 bp insert was inserted at position -184 bp (numbering based on WT *CYP51*p of St IPO323). A sequence duplication of respectively 13 or 14 bp was observed at both insertion sites in isolates carrying the 863 or 868 bp insert.

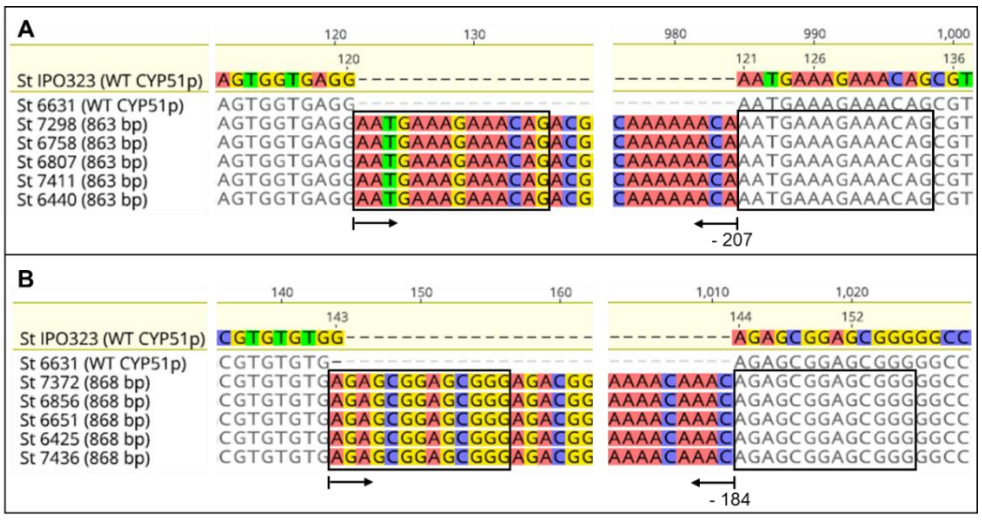


Figure 15: Insertion sites of the 863 and 868 bp inserts in *CYP51*p of *Z. tritici*. Extract of the complete DNA sequence alignment of isolates carrying a WT *CYP51*p (St IPO323, St 6631) and isolates carrying either the 863 or 868 bp insert. The second isolate with a WT *CYP51*p (St 6631) was aligned for correct alignment. Nucleobases of the reference sequence of St IPO323 and all disagreements to this sequence are highlighted in colour (adenine = red, guanine = yellow, thymine = green, cytosine = blue). Left and right insertion sites of the inserts are indicated by the arrows. Alignments were performed with MUSCLE algorithm. **A:** Extract of the DNA sequence alignment of the WT *CYP51*p and isolates carrying the 863 bp insert. The insertion site of the 863 bp insert is located -207 bp upstream of the *CYP51* start codon (numbering based on WT *CYP51*p of St IPO323). A sequence duplication with a length of 14 bp (boxed) was observed in isolates carrying the 863 bp insert. **B:** Extract of the DNA sequence alignment of the WT *CYP51*p and isolates carrying the 868 bp insert. The insertion site of the 868 bp insert is located -184 bp upstream of the *CYP51* start codon (numbering based on WT *CYP51*p of St IPO323). A sequence duplication with a length of 13 bp (boxed) was observed in isolates carrying the 868 bp insert.

Due to the high sequence similarity between the 863 and 868 bp inserts, no distinction was made between those two inserts in the current work. They were grouped together and will be described as inserts of around ~900 bp.

In this work, a *CYP51*p insert with a length of 300 bp was detected for the first time. The complete sequence of this insert is shown in supplementary material (Figure 39). The insertion site of the 300 bp insert is located -107 bp upstream of the *CYP51* start codon (numbering based on WT *CYP51*p of St IPO323, Figure 16). A sequence duplication of 12 bp was observed located at both insertion sites in isolates carrying the 300 bp insert.

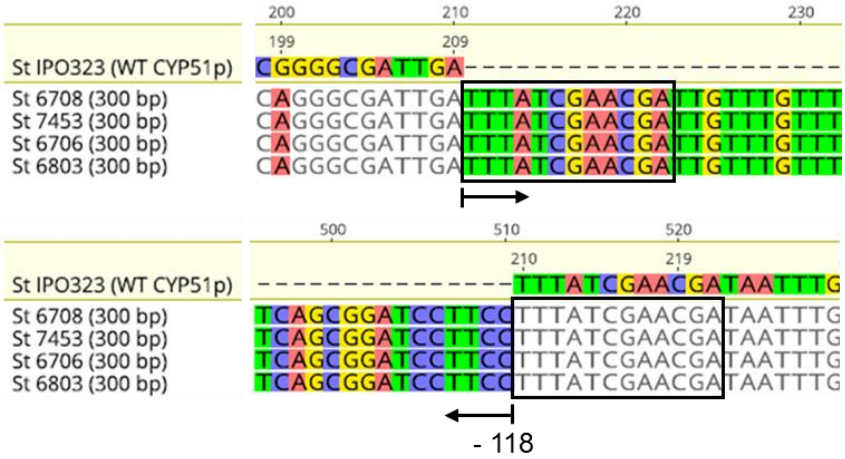


Figure 16: Insertion sites of the 300 bp insert in *CYP51*p of *Z. tritici*. Extract of the complete DNA sequence alignment of an isolate carrying the WT *CYP51*p (St IPO323) and isolates carrying the 300 bp insert is shown. Left and right insertion site of the insert is indicated by the arrows. Nucleobases of the reference sequence of St IPO323 and all disagreements to this sequence are highlighted in colour (adenine = red, guanine = yellow, thymine = green, cytosine = blue). The insertion site is located -118 bp upstream of the *CYP51* start codon (numbering based on WT *CYP51*p of St IPO323). A sequence duplication with a length of 12 bp (boxed) was observed in isolates carrying the 300 bp insert. The alignment was performed with MUSCLE algorithm.

In Table 17, the number of *Z. tritici* isolates with an insert in *CYP51*p is shown for all investigated isolates from routine sensitivity monitoring of 2016 and 2017 (n=843). Additionally, the number of *Z. tritici* isolates with an insert in *CYP51*p is shown separately for isolates with ‘frequently found *CYP51* haplotypes’ and grouped for all ‘rarely found *CYP51* haplotypes’.

Table 17: Number of *Z. tritici* isolates with a wild type *CYP51*p (WT *CYP51*p) or a *CYP51*p insert across all *CYP51* haplotypes (All), summarised for 'rarely found *CYP51* haplotypes' and separated for each 'frequently found *CYP51* haplotype'. Isolates that did not produce an amplicon in PCR test are described with n.a..

	CYP51 haplotype	WT *CYP51*p	Length of *CYP51*p insert (bp)			n.a.
			121	300	~900	
Frequently found	WT	13	-	-	-	1
	C8	9	-	-	130	1
	D13	1	-	-	11	-
	E3	-	-	-	15	-
	E4	1	-	-	229	1
	E5	1	-	-	22	1
	F2	6	92	-	2	-
	F3	11	-	-	-	-
	F4	-	-	-	13	-
	F8	-	-	-	38	-
	G1	78	1	2	20	-
	H4	-	-	-	22	-
	H6	1	-	-	26	-
	Rarely found	48	4	2	40	1
	All	**169**	**97**	**4**	**568**	**5**

*CYP51*p inserts were identified in different frequencies across all tested isolates. In total, 169 isolates showed a WT *CYP51*p. Among all isolates with 'frequently found *CYP51* haplotypes', the highest frequency of the WT *CYP51*p (n=78) was found in isolates with *CYP51* haplotype G1. All isolates with haplotype F3 and, with the exception of one isolate, all isolates with a WT *CYP51* haplotype showed a WT *CYP51*p.

The 121 bp insert in *CYP51*p was detected in 97 isolates. This insert was mainly identified in isolates with *CYP51* haplotype F2 (n=92 isolates). From 100 isolates with a F2 haplotype, 92 carried the 121 bp insert in *CYP51*p. In addition, this insert was found in a single isolate with haplotype F6, C2, D7, H3, and G1, respectively. The number of isolates with a *CYP51*p insert separated for each 'rarely found *CYP51* haplotype' is shown in Table 25 in the supplementary material. The 300 bp insert was only detected in 4 isolates. Two of those isolates showed the 'frequently found *CYP51* haplotype' G1. Additionally, it was found in one isolate with a H9 and a C9 haplotype, respectively. In total, 568 isolates showed an insert of ~900 bp. With the exception of the WT *CYP51* haplotype and haplotype F3, a ~900 bp insert was observed in combination with each 'frequently found *CYP51* haplotype'. Moreover, almost all isolates with a 'frequently found *CYP51* haplotype' additionally showed

a ~900 bp insert in *CYP51*p, with the exception of the WT *CYP51* haplotype and haplotypes F2, F3, and G1.

In summary, across all investigated isolates, the ~900 bp insert in *CYP51*p was found most frequently, followed by the WT *CYP51*p, the 121 bp insert, and the 300 bp insert. The effect of these *CYP51*p inserts on epoxiconazole sensitivity of *Z. tritici* was evaluated in the following chapter.

4.5.2 DMI sensitivity of *Z. tritici* isolates correlated to inserts in *CYP51*p

The EC_{50} values of *Z. tritici* isolates carrying the same *CYP51* haplotype but differing in the presence or absence of a *CYP51*p insert were compared to analyse the impact of different inserts on the sensitivity to epoxiconazole. Thereby, all *CYP51* haplotypes for which EC_{50} values of isolates with and without a *CYP51*p insert were available from routine sensitivity monitoring of 2016 and 2017 were used (Figure 17, Figure 18). Single EC_{50} values were used in cases when only one isolate was detected, and mean EC_{50} values were calculated when more than one isolate was identified with a similar *CYP51* haplotype and with a WT *CYP51*p or a *CYP51*p insert. Only isolates without an additional increased efflux activity (no insert in *MFS1*p, further information is given in chapter 4.6) were included in sensitivity evaluation of this chapter in order not to bias a possible effect that may be associated with *CYP51*p inserts.

In Figure 17, it is shown that *Z. tritici* isolates with a 121 or 300 bp insert in *CYP51*p showed higher adaptation towards epoxiconazole than isolates without an insert. Across all haplotypes (except the wild type), mean EC_{50} values of isolates without an insert ranged from 0.070 to 0.134 mg epoxiconazole L^{-1}, whereas EC_{50} or mean EC_{50} values of isolates with the 121 bp insert ranged from 0.532 to 0.996 mg epoxiconazole L^{-1}. Isolates with the 121 bp insert showed on average a 5- to 8-fold higher adaptation compared to isolates with the same *CYP51* haplotype but without this insert. Among all isolates with haplotype F2 and the 121 bp insert, EC_{50} values ranged from 0.166 to 1.199 mg epoxiconazole L^{-1}. Isolates carrying a F2 haplotype but no *CYP51*p insert showed a mean EC_{50} for epoxiconazole of 0.074 mg a.i. L^{-1}. On average, isolates carrying haplotype F2 and the 121 bp insert showed a 7-fold higher epoxiconazole adaptation than isolates carrying haplotype F2 with the WT *CYP51*p. The most sensitive isolate with a F2 haplotype and the 121 bp insert showed around a 2-fold higher adaptation, whereas adaptation was 16-fold higher in the least sensitive isolate.

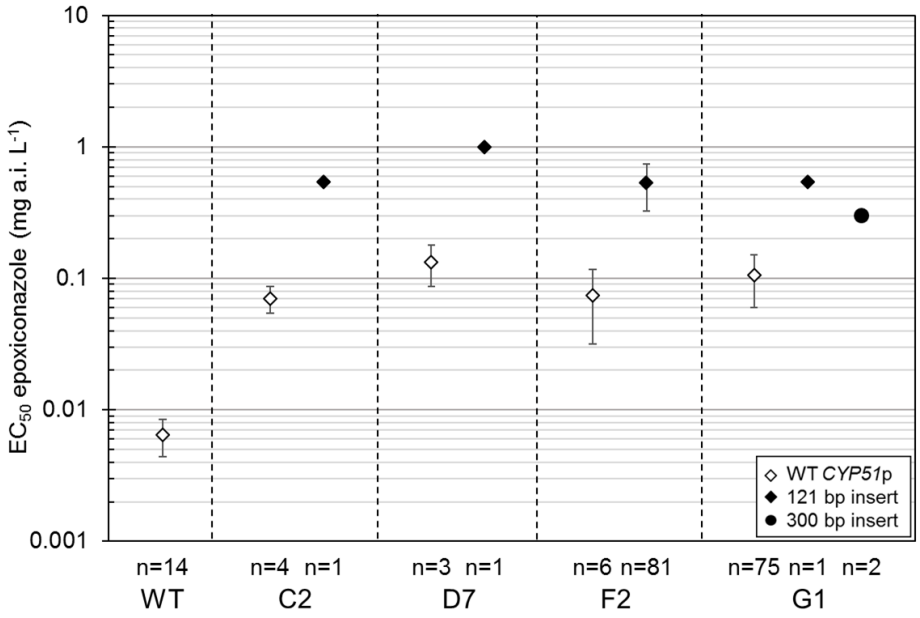


Figure 17: Sensitivity of *Z. tritici* isolates carrying the 121 or 300 bp insert in *CYP51*p. EC_{50} values were determined in microtiter tests by EpiLogic and are shown for isolates carrying the same *CYP51* haplotype but differing in the presence of a wild type *CYP51* promotor (WT *CYP51*p) or either the 121 or 300 bp inserts. Sensitivity of the *CYP51* wild type (WT) is shown as a reference. Single EC_{50} values are shown when only one isolate was detected, and mean EC_{50} values and standard deviations were calculated when more than one isolate was identified per haplotype and with a WT *CYP51*p or a *CYP51*p insert. Mean EC_{50} and standard deviations were calculated over all isolates with the same *CYP51* haplotype and a WT *CYP51*p or a *CYP51*p insert, respectively. Some standard deviations are smaller than the symbol itself. The number of tested isolates per haplotype with a WT *CYP51*p or a *CYP51*p insert is given in the diagram.

Isolates with the 300 bp insert in *CYP51*p showed a mean EC_{50} of 0.301 mg epoxiconazole L^{-1}, whereas isolates with the same *CYP51* haplotype and a WT *CYP51*p showed a mean EC_{50} of 0.105 mg epoxiconazole L^{-1}. Therefore, isolates carrying the 300 bp insert showed around a 3-fold higher adaptation to epoxiconazole compared to isolates with the same *CYP51* haplotype and a WT *CYP51*p.

The effect of the *CYP51*p inserts around ~900 bp on sensitivity to epoxiconazole is shown in Figure 18. The comparison of epoxiconazole sensitivity for isolates differing in the presence or absence of a ~900 bp insert in *CYP51*p could be done for 11 *CYP51* haplotypes. In five *CYP51* haplotype groups (C7, C8, D13, E4, E5), no clear difference was observed between the EC_{50} or mean EC_{50} values of isolates with a ~900 bp insert compared to isolates with the same haplotype and a WT *CYP51*p. In the six remaining haplotype groups (D9, F2, F5, F7, G1, H6), isolates with a ~900 bp insert showed around a 1.5- to 3.5-fold higher adaptation compared to isolates with the same haplotype and a WT *CYP51*p. Across these haplotypes, EC_{50} or mean EC_{50} values of isolates with a WT *CYP51*p ranged from 0.060 to 0.994 mg epoxiconazole L^{-1}, whereas EC_{50} or mean EC_{50} of isolates with a ~900 bp insert ranged from 0.091 to 2.867 mg epoxiconazole L^{-1}.

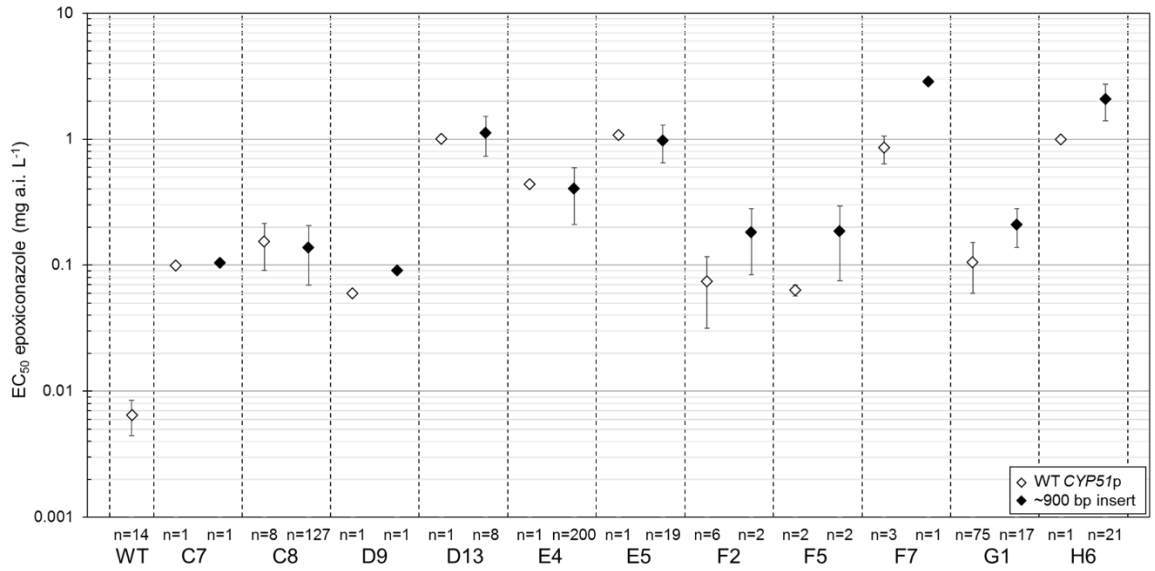


Figure 18: Sensitivity of *Z. tritici* isolates carrying a ~900 bp insert in *CYP51*p. EC_{50} values for epoxiconazole were determined in microtiter tests by EpiLogic and are shown for isolates carrying the same *CYP51* haplotype but differing in the presence of a wild type *CYP51* promotor (WT *CYP51*p) or a ~900 bp insert. Sensitivity of the *CYP51* wild type (WT) is shown as a reference. Single EC_{50} values are shown when only one isolate was detected, and mean EC_{50} values and standard deviations were calculated when more than one isolate was identified per haplotype and with a WT *CYP51*p or a ~900 bp insert. Mean EC_{50} and standard deviations were calculated over all isolates with the same haplotype and a WT *CYP51*p or a ~900 bp insert, respectively. Some standard deviations are smaller than the symbol itself. The number of tested isolates per haplotype with a WT *CYP51*p or a ~900 bp insert is given in the diagram.

In Figure 17 and Figure 18, isolates with different *CYP51* haplotypes and a WT *CYP51*p showed on average a 9- to 166-fold higher epoxiconazole adaptation compared to the sensitivity of wild type isolates (*CYP51* wild type, WT *CYP51*p and WT *MFS1*p). As this higher adaptation is conferred by isolates with different *CYP51* haplotypes but a WT *CYP51*p and WT *MFS1*p, it is probably based on the combination of *CYP51* alterations alone. Additionally, *CYP51* wild type isolates analysed in this work showed a 2.5-fold difference in epoxiconazole sensitivity (described in chapter 4.3). Compared to these values, results shown in this chapter indicate that the impact on epoxiconazole sensitivity associated with the ~900 and 300 bp inserts in *CYP51*p appears of minor relevance. An additive effect due to *CYP51* overexpression, based on the 121 bp insert in *CYP51*p, on adaptation of *Z. tritici* towards epoxiconazole was observed. Therefore, prevalence of *CYP51* overexpression was investigated in *Z. tritici* populations across Europe in the following chapter.

4.5.3 Frequency and distribution of inserts in *CYP51*p of *Z. tritici* across Europe

The occurrence and frequency of inserts in C*YP51*p of *Z. tritici* was analysed for all countries investigated in the routine sensitivity monitoring from 2016 and 2017 to identify the

frequency of *CYP51* overexpression in regional *Z. tritici* populations across Europe. The resulting frequency and distribution of *CYP51*p inserts in *Z. tritici* is shown in Figure 19.

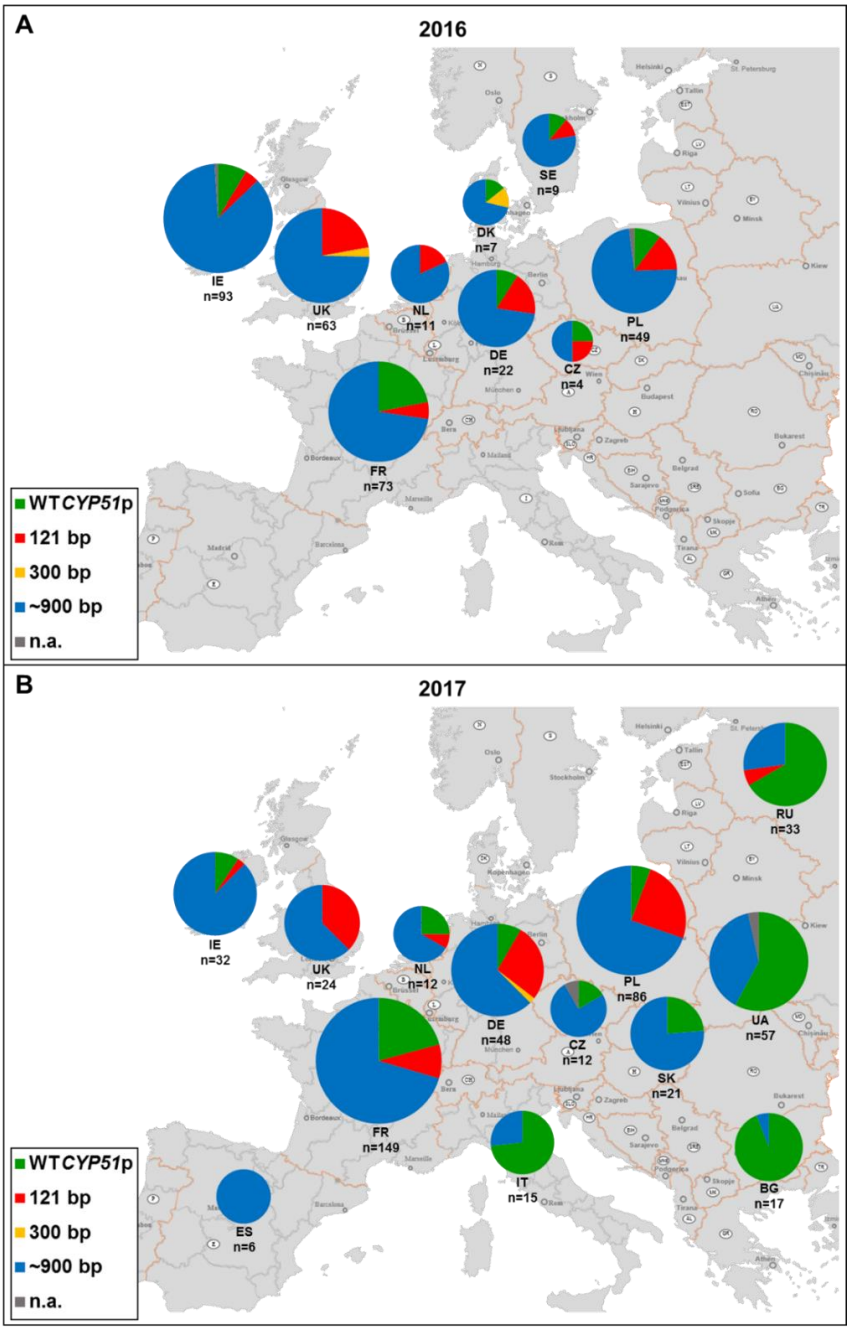


Figure 19: Frequency and distribution of inserts in *CYP51*p of *Z. tritici* across different European countries. Isolates were obtained from BASF routine sensitivity monitoring of 2016 and 2017. Frequency of isolates carrying the wild type *CYP51*p (WT *CYP51*p) and frequencies of isolates with a *CYP51*p insert of 121, 300 or ~900 bp are shown. For some isolates, no amplicon was observed after PCR (n.a.). The colour code for *CYP51*p inserts is given in the legend in the figure. The number of tested isolates is given under the respective pie chart and the size of the pie chart is increasing with the number of tested isolates per country. **A:** Distribution and frequency of inserts in *CYP51*p in 2016. **B:** Distribution and frequency of inserts in *CYP51*p in 2017. These results have been previously published partially in Huf et al., 2020.

In 2016, frequencies of isolates carrying a WT *CYP51*p ranged between 9.1 and 25% across all countries, with the exception of UK and the Netherlands. In these two countries no isolate with a WT *CYP51*p was detected. The majority of investigated isolates showed a ~900 bp insert in *CYP51*p. This insert was identified in each country in high frequencies ranging from 50 to 86%. The 121 bp insert, associated with the highest impact on epoxiconazole sensitivity among all identified inserts (described in chapter 4.5.2), was detected in all investigated countries with the exception of Denmark. However, frequencies of the 121 bp insert were lower than frequencies of the ~900 bp insert. Frequencies of the 121 bp insert were lowest in Ireland (4.3%) and France (5.5%). In Germany, the Netherlands, Poland, and Sweden, the frequency of this insert ranged between 11.1 and 18.2%. The highest frequencies were observed in Czech Republic (25%) and UK (22.2%). High frequencies of the 121 bp insert in Czech Republic may be attributable to the lower sample size in this country because single isolates show a high impact on frequency distribution under these circumstances. The 300 bp insert was only found in 3 isolates. Two of these isolates were detected in UK and one was found in Denmark.

In 2017, high frequencies of isolates carrying a WT *CYP51*p were detected in East and South-East European countries such as Bulgaria (94.1%), Russia (66.7%), and Ukraine (57.9%) as well as in Italy (77.3%). In the remaining countries frequencies of these isolates ranged between 5.8 and 23.8%, with the exception of Spain and UK, in which no isolate with a WT *CYP51*p was detected. Isolates with a ~900 bp insert in *CYP51*p were found in each country tested. High frequencies ranging from 62.5 to 87.5% were detected across Czech Republic, France, Ireland, the Netherlands, Poland, Slovakia, and UK. In Spain, all tested isolates carried a ~900 bp insert. Compared to this, lower frequencies were identified in Bulgaria (5.9%), Russia (27.3%), and Ukraine (38.6%) as well as in Italy (26.7%). The 121 bp insert was mainly detected in countries of Western and Central Europe, with the exception of Czech Republic and Slovakia. Frequency of isolates carrying this insert was highest in UK (37.5%), followed by Germany (27.1%) and Poland (24.4%). In France and Ireland, lower frequencies of 8.7% and 3.1%, respectively were identified. Two isolates with the 121 bp insert were found in Russia. No isolates with this insert were observed in all remaining countries. The 300 bp insert was only detected in a single isolate in Germany.

Changes in the frequency of different *CYP51*p inserts between 2016 and 2017 can be evaluated for important cereal growing countries that were tested in both years. In UK, Germany and Poland, an increase about 9 to 15% in the frequency of the 121 bp insert was observed accompanied with a decrease in the frequency of the ~900 bp insert (UK: ~12%, DE: ~10%, PL:~ 4%) and/or the WT *CYP51*p (PL: ~4%). A slight increase (~3%) of the 121 bp insert and a slight decrease of the ~900 bp insert (~2%) as well as the WT *CYP51*p (~1%) was observed in France. In Ireland, frequencies of the inserts stayed relatively stable. In the Netherlands, frequencies of the 121 bp (~10%) and ~900 bp (~15%) inserts decreased, whereas frequency of the WT *CYP51*p increased (25%). However, it should be

noted that the sample size in the Netherlands was much smaller and single isolates can have a high impact on overall frequency of *CYP51*p inserts. All in all, results presented here indicate that in UK, Poland, and Germany, the frequency of *CYP51* overexpression based on the 121 bp *CYP51*p insert increased in 2017, whereas in France only a slight increase of *CYP51* overexpression was observed. No clear change in the frequency of *CYP51* overexpression was detected in Ireland.

4.6 Increased efflux activity in *Z. tritici*

The third mechanism that has been shown to affect DMI sensitivity of *Z. tritici* is an increased efflux activity of membrane bound transporters (Leroux and Walker, 2011). In *Z. tritici*, an increased efflux activity was shown to rely on overexpression of the *MFS1* gene coding for an MFS1 transporter (Omrane et al., 2015). This overexpression was associated with different *MFS1*p inserts. Highest *MFS1* overexpression was correlated to a 519 bp insert in *MFS1*p. Additionally, inserts with a length of 150, 338, and 369 bp were identified in *MFS1*p of *Z. tritici* field isolates leading to a lower *MFS1* overexpression than the 519 bp insert (Omrane et al., 2015, 2017).

In this work, all isolates from routine sensitivity monitoring were investigated for an increased efflux activity by identification of inserts in *MFS1*p. The effect of different inserts on an increased efflux activity of *Z. tritici* was investigated using tolnaftate. Additionally, the impact on epoxiconazole sensitivity of *Z. tritici* associated with different *MFS1*p inserts was evaluated. Finally, the occurrence and frequency of *MFS1*p inserts was analysed across Europe.

4.6.1 Identification of an increased efflux activity in *Z. tritici* isolates

The promotor region of all *Z. tritici* isolates obtained from routine sensitivity monitoring of 2016 and 2017 was investigated for inserts based on PCR methods described by Omrane et al. (2015, 2017). In order to identify the exact size of inserts slightly differing in length, amplified fragments of the promotor region of these isolates were sequenced. Additionally, *MFS1*p fragments of a subset of isolates without an insert and for each insert were sequenced in order to check whether inserts identified in this work showed similar sequences compared to inserts already described in literature.

PCR-amplification of *MFS1*p resulted in the identification of five different sizes of DNA fragments across all investigated isolates. Isolates without an insert showed a fragment size of ~600 bp (with oligonucleotide set 1: KES 2143 and KES 2144). Additionally, fragments of ~750, ~950, ~1000, or ~1100 bp were detected indicating the presence of inserts in these isolates. All isolates that produced no amplicon during the PCR with oligonucleotide set 1

were additionally tested in a PCR with another oligonucleotide set (oligonucleotide set 2: KES 2334 and KES 2335). Some of these isolates showed an *MFS1*p fragment after PCR with oligonucleotide set 2. The PCR with oligonucleotide set 2 resulted in *MFS1*p amplicon sizes of ~500 bp for isolates without an insert and ~800 as well as ~1000 bp for isolates with an insert. Nevertheless, some isolates still produced no amplicon during PCR with oligonucleotide set 2.

Sequencing of different *MFS1*p fragments with or without an insert revealed a high polymorphism in *MFS1*p between *Z. tritici* isolates. Small insertions and deletions ranging from 3 to 42 bp were observed in each tested isolate compared to the reference sequence of IPO323 (shown in Figure 42 in the supplements). As described in the introduction (chapter 1.4.3), a high polymorphism and the occurrence of small *MFS1*p inserts has already been observed in previous studies, but *MFS1* overexpression was mainly correlated to larger *MFS1*p inserts. Therefore, this work was focused on detection of inserts >50 bp with the remaining isolates regarded to carry a wild type *MFS1*p (WT *MFS1*p).

Sequencing of *MFS1*p fragments revealed the occurrence of seven different inserts with a length of 150, 267, 308, 338, 369, 377, and 519 bp across all tested isolates.

The complete sequence of the 150 bp insert is shown in the alignment in Figure 43 in supplementary material. Insertion of the 150 bp insert occurred -214 bp upstream of the *MFS1* start codon (numbering based on WT *MFS1*p of St IPO323, Figure 20). As reported for inserts in *CYP51*p (shown in chapter 4.5.1), a sequence duplication (10 bp) was observed at both insertion sites in isolates carrying the 150 bp insert.

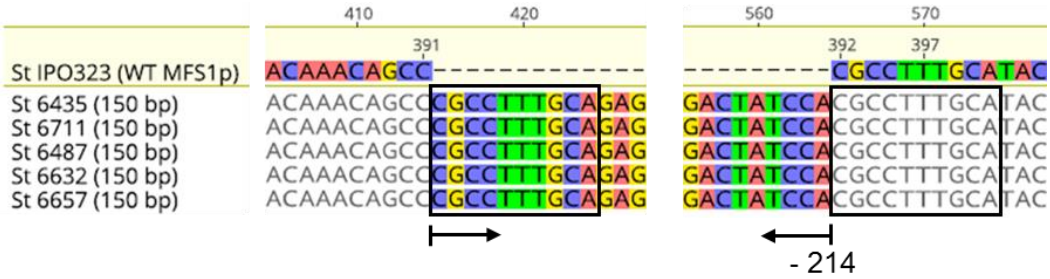


Figure 20: Insertion sites of the 150 bp insert in *MFS1*p of *Z. tritici*. Extract of the complete DNA sequence alignment of an isolate carrying the WT *MFS1*p (St IPO323) and isolates carrying the 150 bp insert is shown. Left and right insertion site of the insert is indicated by the arrows. Nucleobases of the reference sequence of St IPO323 and all disagreements to this sequence are highlighted in colour (adenine = red, guanine = yellow, thymine = green, cytosine = blue). The insertion site is located -214 bp upstream of the *MFS1* start codon (numbering based on WT *MFS1*p of St IPO323). A sequence duplication with a length of 10 bp (boxed) was observed in isolates carrying the 150 bp insert. The alignment was performed with MUSCLE algorithm.

Complete sequences of the 267, 308, 338, 369, or 377 bp inserts are shown in supplementary material Figure 44. Inserts with a length of 267, 308, and 377 bp were identified for the first time in *Z. tritici* in this work. These three new inserts showed a high sequence identity between each other (between 66-80%) and to the already described 338

(sequence identity between 71-76%) and 369 bp inserts (sequence identity between 68-97%). The 338 and 369 bp inserts showed a sequence identity of 77%. Differences in the sequences of these inserts mainly result from small insertions and deletions. If gaps between all sequences of the 267, 308, 338, 369, and 377 bp inserts were removed, a high sequence identity was observed across the remaining 252 bp (92-100%) in pairwise comparisons (data not shown).

The insertion sites of the 267, 308, 338, 369, and 377 bp inserts are shown in Figure 21. Insertion of these inserts occurred at position -468 bp upstream of the *MFS1* start codon (numbering based on WT *MFS1*p of St IPO323). A 3 bp sequence duplication was observed at the insertion sites in isolates carrying either the 267, 308, 369 or 377 bp inserts. Isolates carrying the 338 bp insert showed a 13 bp sequence duplication around the insertion sites of the insert. Based on this sequence duplication, the insertion site of the 338 bp insert could not be assigned with a high level of certainty in the alignment alone. Possibly, the insertion site has to be shifted by a few bases.

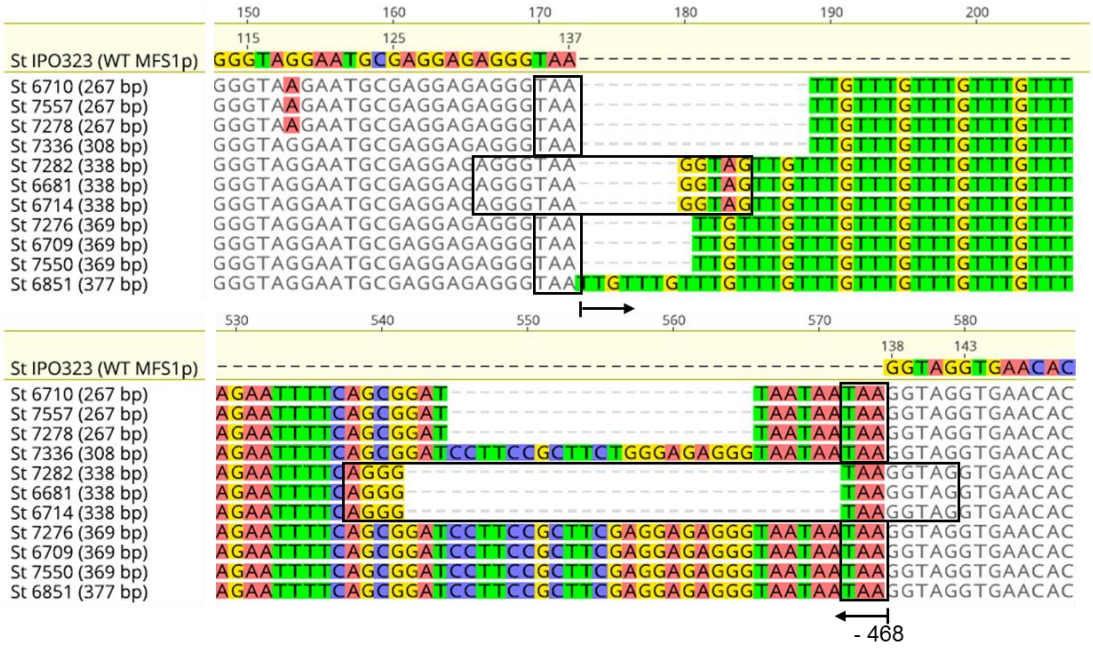


Figure 21: Insertion sites of different inserts with a length of around 300 bp in *MFS1*p of *Z. tritici*. Extract of the complete DNA sequence alignment of an isolate carrying the WT *MFS1*p (St IPO323) and isolates carrying either the 267, 308, 338, 369 or 377 bp insert is shown. Left and right insertion site of the inserts is indicated by the arrows. Nucleobases of the reference sequence of St IPO323 and all disagreements to this sequence are highlighted in colour (adenine = red, guanine = yellow, thymine = green, cytosine = blue). The insertion site is located -468 bp upstream of the *MFS1* start codon (numbering based on WT *MFS1*p of St IPO323). A sequence duplication with a length of 3 bp (boxed) was observed in isolates carrying either the 267, 308, 369 or 377 bp insert and a 12 bp sequence duplication (boxed) was observed in isolates carrying the 338 bp insert. The alignment was performed with MUSCLE algorithm.

The complete sequence alignment of *MFS1*p of isolates carrying the 519 bp insert is depicted in Figure 45 in the supplements. Insertion of the 519 bp insert occurred at position -402 bp (numbering based on WT *MFS1*p of St IPO323) upstream of the *MFS1* start codon

(Figure 22). A sequence duplication of 5 bp was observed at the insertion sites in isolates carrying the 519 bp insert.

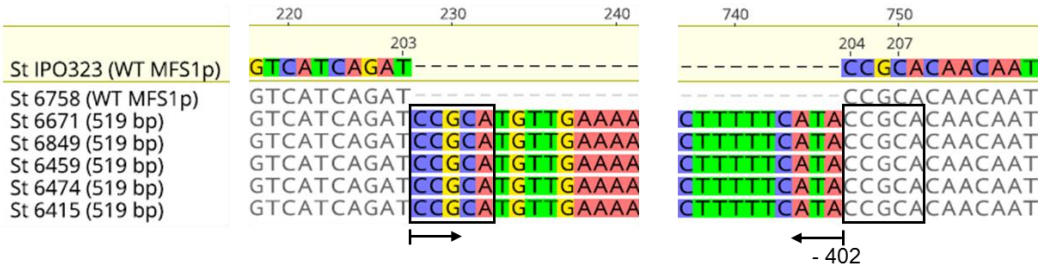


Figure 22: Insertion sites of the 519 bp insert in *MFS1*p of *Z. tritici*. Extract of the complete DNA sequence alignment of isolates carrying the WT *MFS1*p (St IPO323, St 6758) and isolates carrying the 519 bp insert is shown. A second isolate with a WT *CYP51*p was aligned for correct alignment. Left and right insertion site of the insert is indicated by the arrows. Nucleobases of the reference sequence of St IPO323 and all disagreements to this sequence are highlighted in colour (adenine = red, guanine = yellow, thymine = green, cytosine = blue). The insertion site is located -402 bp upstream of the *MFS1* start codon (numbering based on WT *MFS1*p of St IPO323). A sequence duplication with a length of 5 bp (boxed) was observed in isolates carrying the 519 bp insert. The alignment was performed with MUSCLE algorithm.

Besides inserts in *MFS1*p, a 148 and 165 bp deletion was found in one isolate, respectively. Deletion of the 148 bp sequence occurred -426 bp upstream of the *MFS1* start codon, whereas the 165 bp deletion occurred -339 bp. Sequences of the deletions are shown in supplementary material Figure 46.

Inserts in *MFS1*p were found in different frequencies across all investigated isolates (n=843) from routine sensitivity monitoring of 2016 and 2017 (Table 18).

Table 18: Number of *Z. tritici* isolates carrying a wild type *MFS1*p (WT *MFS1*p) or an insert in *MFS1*p. Isolates that did not produce an amplicon in PCR test are described with n.a..

Length of insert (bp) in *MFS1*p	Number of isolates
WT *MFS1*p	702
150	26
267	7
308	1
338	13
369	6
377	1
519	37
n.a.	48

Across all investigated isolates, 702 isolates, thus the majority, carried a WT *MFS1*p. Among all isolates with an insertion, the 519 bp insert was found most frequently (n=37 isolates), followed by the 150 bp insert (n=26 isolates) and the 338 bp insert (n=13 isolates).

The 267 bp insert was identified in 7 isolates and the 369 bp insert was detected in 6 isolates. Only one isolate with a 308 or 377 bp insert was detected, respectively. For 48 isolates no amplicon was observed after PCR, probably due to high sequence polymorphism in *MFS1*p region. *MFS1*p inserts were not observed to be correlated to a specific *CYP51* haplotype (shown in supplementary material Table 26).

4.6.2 Impact of inserts in *MFS1*p on an increased efflux activity of *Z. tritici*

Microtiter tests with tolnaftate were performed to test whether inserts in *MFS1*p impact efflux activity of *Z. tritici*. These tests included isolates with a WT *MFS1*p and isolates carrying different *MFS1*p inserts. Increased EC_{50} values of the thiocarbamate tolnaftate indicate an increased efflux activity in *Z. tritici* isolates. Ten *Z. tritici* isolates, if available, were tested for each *MFS1*p insert identified in this work (except the 308 bp insert). If less than 10 isolates were available, the total number of detected isolates with the respective *MFS1*p insert from 2016 and 2017 was used.

EC_{50} values of *Z. tritici* isolates carrying different *MFS1*p inserts for tolnaftate are shown in Figure 23.

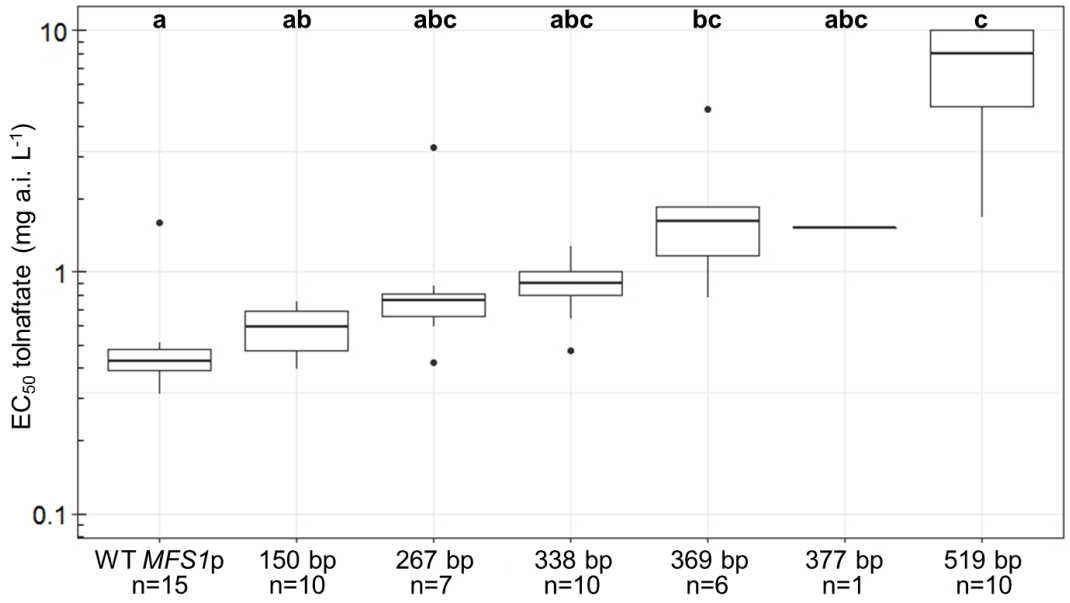


Figure 23: Sensitivity of *Z. tritici* isolates carrying different inserts in *MFS1*p for tolnaftate. Box and Whisker of EC_{50} values of isolates carrying a wild type *MFS1*p (WT *MFS1*p) and of isolates carrying either the 150, 267, 338, 369, 377, or 519 bp insert for tolnaftate are shown. The number of tested isolates (n) is given in the diagram. Tolnaftate is used as an indicator for an increased efflux activity in *Z. tritici*. Different letters above the boxes indicate significant differences according to post-hoc Nemenyi test (significance level: p= <0.05). The highest tolnaftate concentration tested in microtiter tests was 10 mg tolnaftate L^{-1}. In Box and Whisker plots and statistics highest concentration of 10 mg tolnaftate L^{-1} was used for isolates with EC_{50} values larger than 10 mg tolnaftate L^{-1}.

Increasing EC_{50} values for tolnaftate were observed for *Z. tritici* isolates with increasing length of *MFS1*p inserts (Figure 23). Compared to the sensitivity of isolates with a WT *MFS1*p, significant differences in tolnaftate sensitivity were observed for isolates carrying either the 369 or 519 bp insert. Significant differences between tolnaftate sensitivities were calculated with the Kruskall-Wallis-Test (significance level: p = <0.05), followed by the post-hoc Nemenyi test (significance level: p = <0.05) across mean values of the same isolates from two independent experiments. As 10 mg tolnaftate L^{-1} was the highest tested concentration, an EC_{50} value of 10 mg tolnaftate L^{-1} was used for the calculation of statistical differences for isolates that showed a higher EC_{50} value than 10 mg a.i. L^{-1}. Most isolates with the 150, 267, 338, and 377 bp insert showed higher EC_{50} values for tolnaftate than isolates with a WT *MFS1*p, but these differences were not significant. Compared to isolates with a WT *MFS1*p, these results indicate the highest increased efflux activity in isolates carrying the 369 or 519 bp insert, whereas the effects of the 150, 267, 338, and 377 bp inserts were lower. Nevertheless, compared to isolates with a WT *MFS1*p, an increased efflux is also indicated in isolates carrying the 150, 267, 338, and 377 bp inserts. Among all isolates with an *MFS1*p insert, significant differences in tolnaftate sensitivity were detected between isolates carrying the 150 and 519 bp insert, whereas across isolates with remaining inserts no significant differences were observed.

One isolate with a WT *MFS1*p showed an increased EC_{50} value for tolnaftate compared to the remaining isolates with a WT *MFS1*p (Figure 23) indicating an increased efflux activity in this isolate. This isolate showed a small insert with a length of 42 bp in *MFS1*p (isolate St 6781 shown in Figure 42 in the supplements). This finding indicates that for future studies it may be useful to also identify small inserts in *MFS1*p (smaller than <50 bp) and to analyse if they are correlated to an increased efflux.

EC_{50} values of tolnaftate for isolates with the 148 or 165 bp deletion in *MFS1*p were located in the sensitivity range of isolates with a WT *MFS1*p (Figure 47 in supplementary material). These results indicate no effect of these deletions on efflux activity of *Z. tritici*.

Altogether, an increased efflux activity is indicated in all isolates carrying an *MFS1*p insert. Highest impact on increased efflux was correlated to the 369 and 519 bp inserts. Further studies were carried out in the following chapter in order to test whether these effects can be confirmed with the sensitivity of isolates to epoxiconazole.

4.6.3 DMI sensitivity of *Z. tritici* isolates correlated to inserts in *MFS1*p

The EC_{50} values of epoxiconazole for *Z. tritici* isolates carrying the same *CYP51* haplotype but differing in the presence or absence of an *MFS1*p insert were compared to analyse if the impact on tolnaftate sensitivity associated with different *MFS1*p inserts (previous chapter (4.6.2)) is similarly reflected in sensitivity towards epoxiconazole. All *CYP51*

haplotypes for which EC_{50} values of isolates with and without an insert in *MFS1*p were available from routine sensitivity monitoring of 2016 and 2017 were used. Only isolates without *CYP51* overexpression based on the 121 bp insert in *CYP51*p were included in sensitivity evaluation of this chapter in order not to bias a possible effect of *MFS1*p inserts.

Compared to isolates carrying the same *CYP51* haplotype and a WT *MFS1*p, isolates with the 150 bp insert in *MFS1*p showed slightly higher EC_{50} or mean EC_{50} values (Figure 24). Nevertheless, only for 2 haplotypes (F8 and G1) a 2-fold higher adaptation to epoxiconazole was observed associated with the 150 bp insert, whereas isolates carrying remaining haplotypes and the 150 bp insert showed a lower adaptation. These results indicate that the 150 bp insert probably only confers a low effect on epoxiconazole sensitivity.

Sensitivity of isolates carrying either the 267, 308, 338, 369, or 377 bp insert in *MFS1*p to epoxiconazole is presented in Figure 25. The EC_{50} or mean EC_{50} values of most isolates with the 267, 338, 369, or 377 bp insert were slightly higher compared to isolates carrying the same *CYP51* haplotype and a WT *MFS1*p. Nevertheless, at most around a 2- to 2.5-fold higher adaptation to epoxiconazole was observed to be associated with these inserts (in most cases for isolates carrying the 338 bp insert). Compared to this, in many cases the observed adaptation to epoxiconazole associated with these inserts was also lower. These results demonstrate that the 267, 338, 377, and 369 bp inserts probably only confer low impacts on epoxiconazole sensitivity of *Z. tritici*. One isolate with a 308 bp insert in *MFS1*p and a *CYP51* haplotype E4 was identified. This isolate showed around a 2.5-fold higher adaptation to epoxiconazole than isolates with an E4 haplotype and a WT *MFS1*p. This isolate was not tested for tolnaftate sensitivity, however, results for epoxiconazole sensitivity indicate that this insert might exert similar effects than the remaining inserts around 300 bp. As across 2016 and 2017 only one isolate with a 308 bp insert in *MFS1*p was detected, evaluation of tolnaftate and DMI sensitivity of more isolates carrying this insert is recommended to confirm the results of this work.

Single isolates with an insert in *MFS1*p (150 or 338 bp) showed a higher sensitivity to epoxiconazole than isolates with the same *CYP51* haplotype but a WT *MFS1*p. Wild type isolates (*CYP51* wild type, WT *CYP51*p, and WT *MFS1*p) investigated in this work already showed a 2.5-fold difference in epoxiconazole sensitivity (described in 4.3). These results may imply that natural variability in sensitivity of *Z. tritici* isolates to azoles, already described in previous studies (Gisi et al., 1997; Stergiopoulos et al., 2003; de Waard et al., 2006), may interfere with the slight effects on epoxiconazole sensitivity associated with the 150 bp insert and inserts around 300 bp.

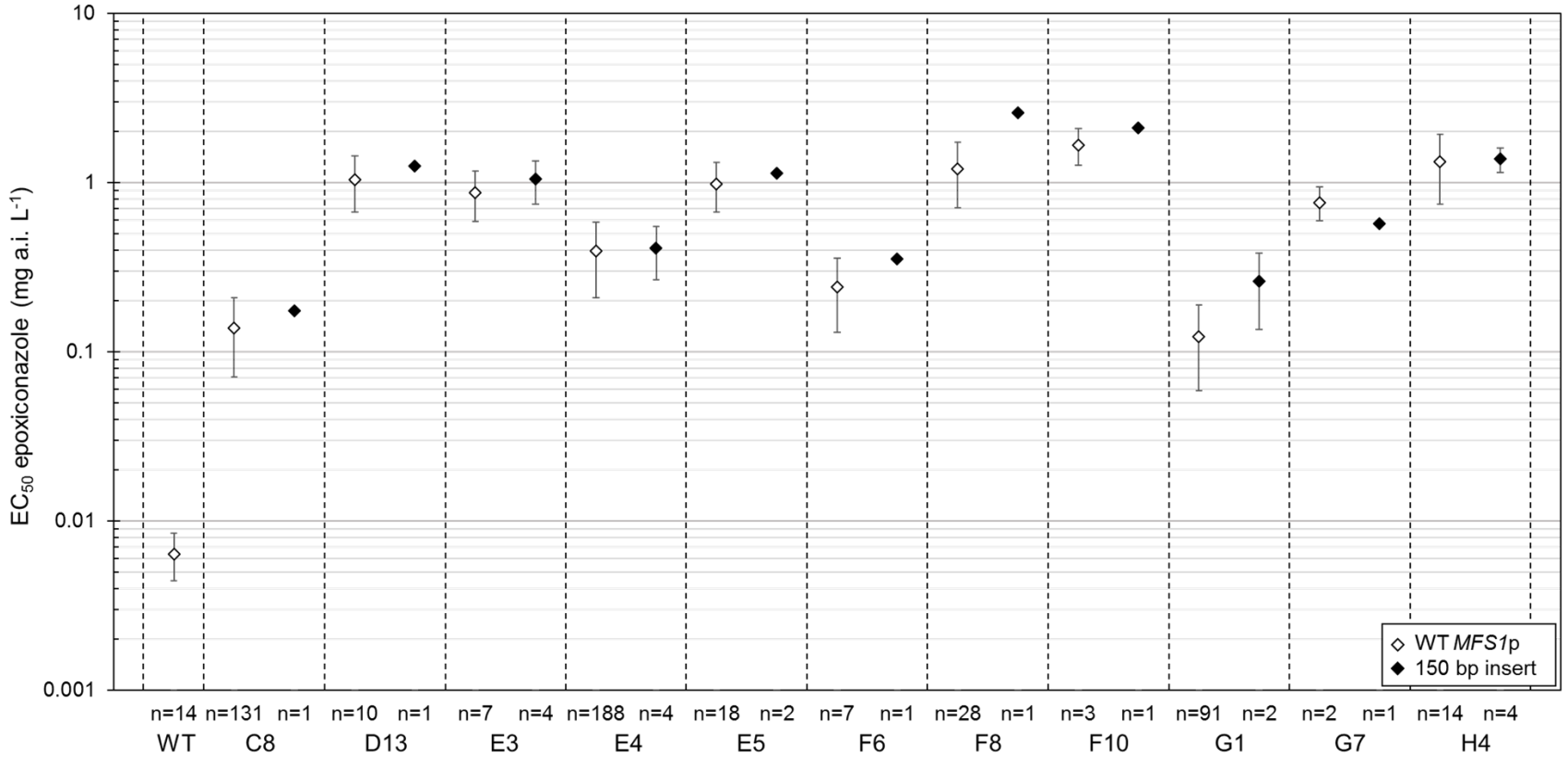


Figure 24: Sensitivity of *Z. tritici* isolates carrying the 150 bp insert in *MFS1*p. EC_{50} values for epoxiconazole were determined in microtiter tests by EpiLogic and are shown for isolates carrying the same *CYP51* haplotype but differing in the presence of a wild type *MFS1* promotor (WT *MFS1*p) or the 150 bp insert. As a reference, sensitivity of *CYP51* wild type isolates (WT) is given. Single EC_{50} values are shown when only one isolate was detected, and mean EC_{50} values and standard deviations were calculated when more than one isolate was identified per *CYP51* haplotype and with a WT *MFS1*p or an *MFS1*p insert. Mean EC_{50} and standard deviations were calculated over all isolates with the same *CYP51* haplotype and a WT *MFS1*p or an *MFS1*p insert, respectively. Some standard deviations are smaller than the symbol itself. The number of tested isolates per *CYP51* haplotype and with a WT *MFS1*p or an *MFS1*p insert is given in the diagram.

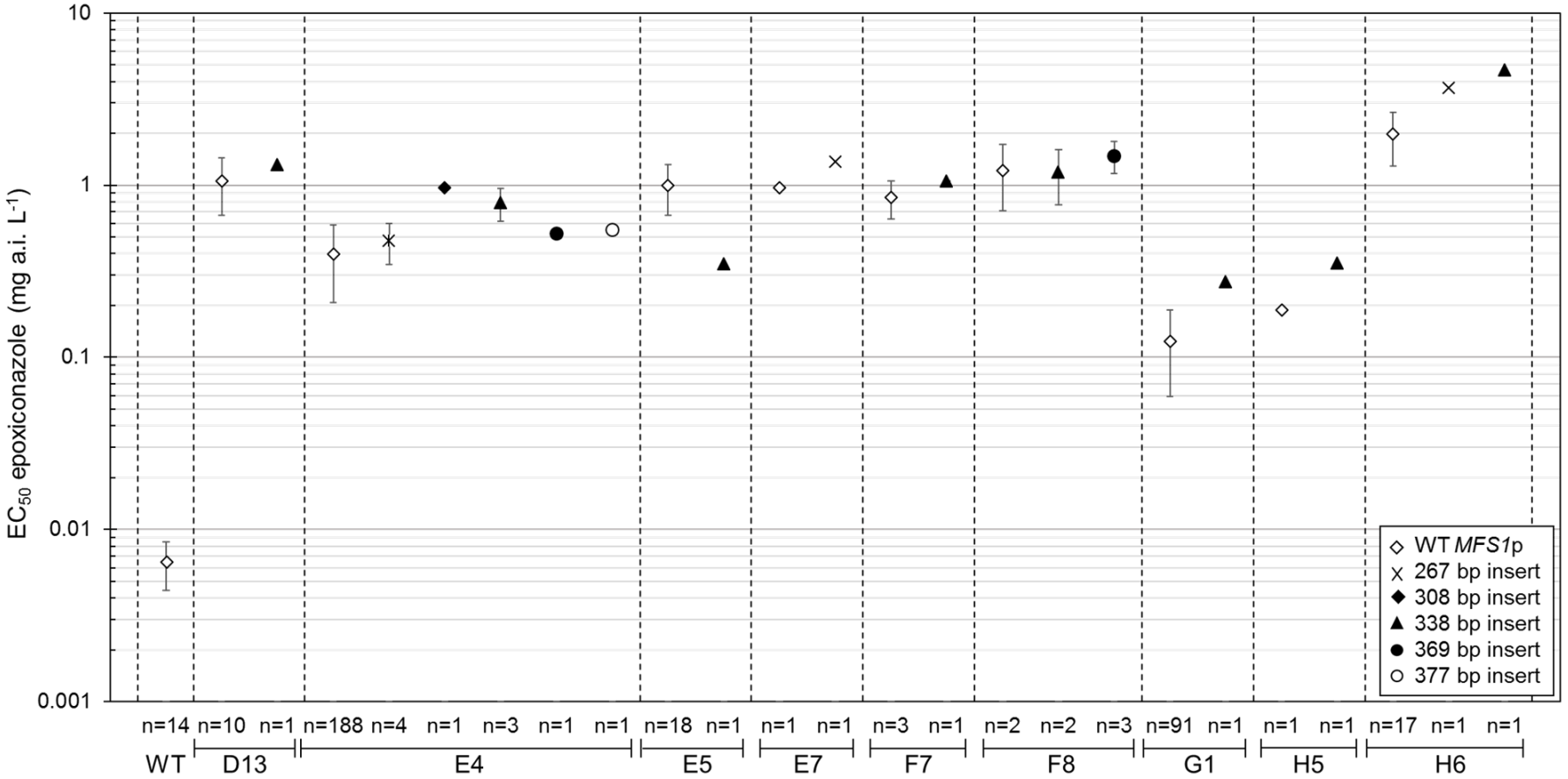


Figure 25: Sensitivity of *Z. tritici* isolates carrying an insert around 300 bp in *MFS1*p. EC_{50} values for epoxiconazole were determined in microtiter tests by EpiLogic and are shown for isolates carrying the same *CYP51* haplotype but differing in the presence of a wild type *MFS1* promotor (WT *MFS1*p) or the 267, 308, 338, 369, or 377 bp insert. As a reference, sensitivity of *CYP51* wild type isolates (WT) is given. Single EC_{50} values are shown when only one isolate was detected, and mean EC_{50} values and standard deviations were calculated when more than one isolate was identified per *CYP51* haplotype and with a WT *MFS1*p or an *MFS1*p insert. Mean EC_{50} and standard deviations were calculated over all isolates with the same *CYP51* haplotype and a WT *MFS1*p or an *MFS1*p insert, respectively. Some standard deviations are smaller than the symbol itself. The number of tested isolates per *CYP51* haplotype and with a WT *MFS1*p or an *MFS1*p insert is given in the diagram.

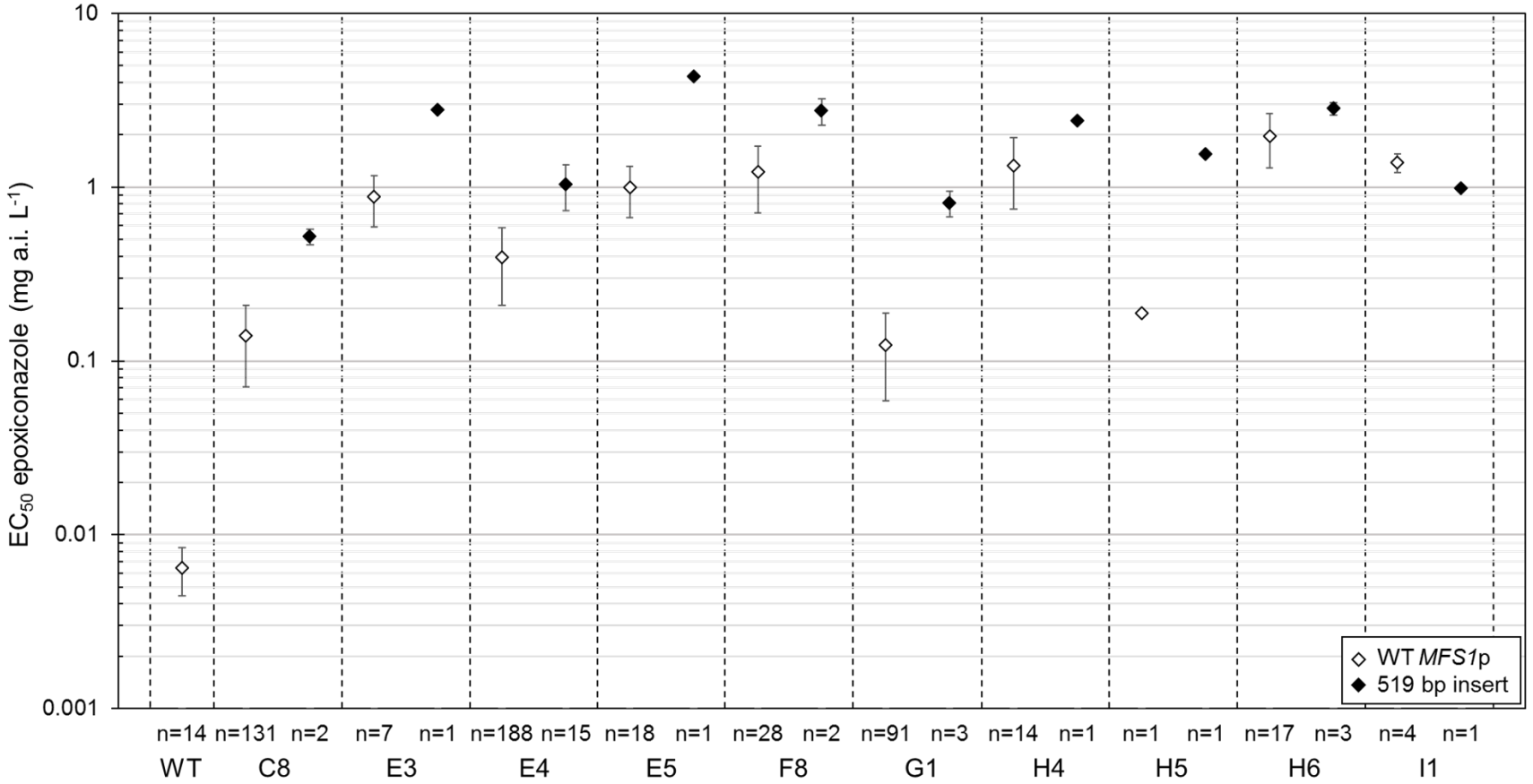


Figure 26: Sensitivity of *Z. tritici* isolates carrying the 519 bp insert in *MFS1*p. EC_{50} values for epoxiconazole were determined in microtiter tests by EpiLogic and are shown for isolates carrying the same *CYP51* haplotype but differing in the presence of a wild type *MFS1* promotor (WT *MFS1*p) or the 519 bp insert. As a reference, sensitivity of *CYP51* wild type isolates (WT) is given. Single EC_{50} values are shown when only one isolate was detected, and mean EC_{50} values and standard deviations were calculated when more than one isolate was identified per *CYP51* haplotype and with a WT *MFS1*p or an *MFS1*p insert. Mean EC_{50} and standard deviations were calculated over all isolates with the same *CYP51* haplotype and a WT *MFS1*p or an *MFS1*p insert, respectively. Some standard deviations are smaller than the symbol itself. The number of tested isolates per *CYP51* haplotype and with a WT *MFS1*p or an *MFS1*p insert is given in the diagram.

Sensitivity of isolates carrying the 519 bp insert in *MFS1*p for epoxiconazole is shown in Figure 26. Isolates carrying the 519 bp insert showed on average around 2- to 8- fold higher EC_{50} or mean EC_{50} values compared to isolates with the same *CYP51* haplotype and a WT *MFS1*p, except isolates with haplotype H6 and I1. Isolates carrying haplotype H6 and the 519 bp insert showed a smaller adaptation of less than 2-times. The isolate carrying haplotype I1 and the 519 bp insert showed a higher sensitivity than isolates carrying haplotype I1 and the WT *MFS1*p. These results indicate that in single cases the impact on epoxiconazole sensitivity associated with the 519 bp insert seems lower compared to previous results described here. On average, among all *MFS1*p inserts, the 519 bp insert was associated with highest effects on epoxiconazole sensitivity.

Sensitivity of isolates for which *MFS1*p could not be characterised (described in chapter 4.6.1) for epoxiconazole is shown in supplementary material in Figure 48. No clear trend in differences of epoxiconazole sensitivity was observed for these isolates compared to isolates with a WT *MFS1*p. In single cases a slightly higher adaptation to epoxiconazole was observed for isolates for which *MFS1*p could not be characterised. If this variation in sensitivity occurs due to natural variability in sensitivity of *Z. tritici* field isolates to azoles (Gisi et al., 1997; Stergiopoulos et al., 2003; de Waard et al., 2006) or due to a specific mechanism correlated to changes in *MFS1*p, needs to be clarified in future studies.

Altogether, the impact on tolnaftate sensitivity conferred by different *MFS1*p inserts was reflected in most cases in the sensitivity to epoxiconazole. Highest impact on tolnaftate sensitivity associated with the 519 bp insert was confirmed for the sensitivity to epoxiconazole. Additionally, lower impacts on tolnaftate sensitivity correlated to the 267, 338, and 377 bp inserts were confirmed for epoxiconazole sensitivity. Significant differences in the tolnaftate sensitivity between isolates that carry the 369 bp insert and isolates with a WT *MFS1*p suggested a strong enhanced efflux activity in these isolates (described in 4.6.2). However, this finding was not reflected in the impact on epoxiconazole sensitivity associated with the 369 bp insert. The number of tested isolates carrying a 267, 369 or 377 bp insert was relatively low, as these inserts were rarely identified in *Z. tritici* across 2016 and 2017. Evaluating the impact associated with these inserts on triazole sensitivity in a higher number of isolates is recommended for future studies to confirm the results obtained here.

Compared to the sensitivity of wild type isolates (WT *CYP51*, WT *CYP51*p and WT *MFS1*p), isolates carrying an altered *CYP51* haplotype and the WT *MFS1*p used in this chapter for sensitivity comparisons (Figure 24, Figure 25, Figure 26) showed on average a 19- to 306-fold higher adaptation to epoxiconazole (calculated with mean EC_{50} values). Additionally, as described in chapter 4.3, investigated wild type isolates showed a 2.5-fold difference in their sensitivity to epoxiconazole. Compared to these values, results presented here indicate that the impact on epoxiconazole sensitivity associated with the 150, 267, 308, 338,

369, and 377 bp inserts in *MFS1*p seems of minor relevance. Additionally, compared to resistance levels of *CYP51* haplotypes alone, results imply that the impact on epoxiconazole sensitivity associated with inserts in *MFS1*p appears lower compared to combination of CYP51 alterations. Nevertheless, an additive effect of an increased efflux activity on adaptation of *Z. tritici* was observed in isolates carrying the 519 bp insert in *MFS1*p. Therefore, prevalence of an increased efflux was investigated for *Z. tritici* across Europe.

4.6.4 Frequency and distribution of inserts in *MFS1*p of *Z. tritici* across Europe

All isolates from routine sensitivity monitoring of 2016 and 2017 were analysed for inserts in *MFS1*p to identify the frequency of an increased efflux activity in regional populations of *Z. tritici* in Europe. The designation of Omrane et al. (2017) for different *MFS1*p inserts (described in 1.4.3) will be used hereafter in order to simplify description. As no major differences were observed for the impact on epoxiconazole sensitivity correlated to the 267, 308, and 377 bp inserts compared to the 338 and 369 bp inserts and a high sequence similarity was observed between all of these inserts, the 267, 308, and 377 bp inserts were also designated as type II inserts. Consequently, the 150 bp insert is referred to as type III insert, the 267, 308, 338, 369, and 377 bp insert as type II and the 519 bp insert as type I.

Figure 27 A shows that in 2016 a heterogenous frequency of the 150 bp insert (type III), 267, 338, 369, and 377 bp inserts (type II) and 519 bp insert (type I) in *MFS1*p was observed across Europe. In Denmark, Czech Republic, and Sweden, only the WT *MFS1*p was detected. The type III insert was mainly detected in Ireland (9.7%) and UK (6.3%) as well as in one isolate in Poland. This insert was not found in any other investigated country. Type II inserts were mainly detected in UK. In this country, type II inserts with a length of 267 bp (6.3%), 338 bp (7.9%), and 369 bp (3.2%) were detected. In Ireland, only the type II insert with 338 bp was found and the frequency was low (2.2%). The type II insert with 377 bp was solely identified in one isolate in Poland. In no other country was a type II insert found. The type I insert was found in Ireland, the Netherlands, France, Germany, and Poland in frequencies ranging from 5.4 to 9.1%.

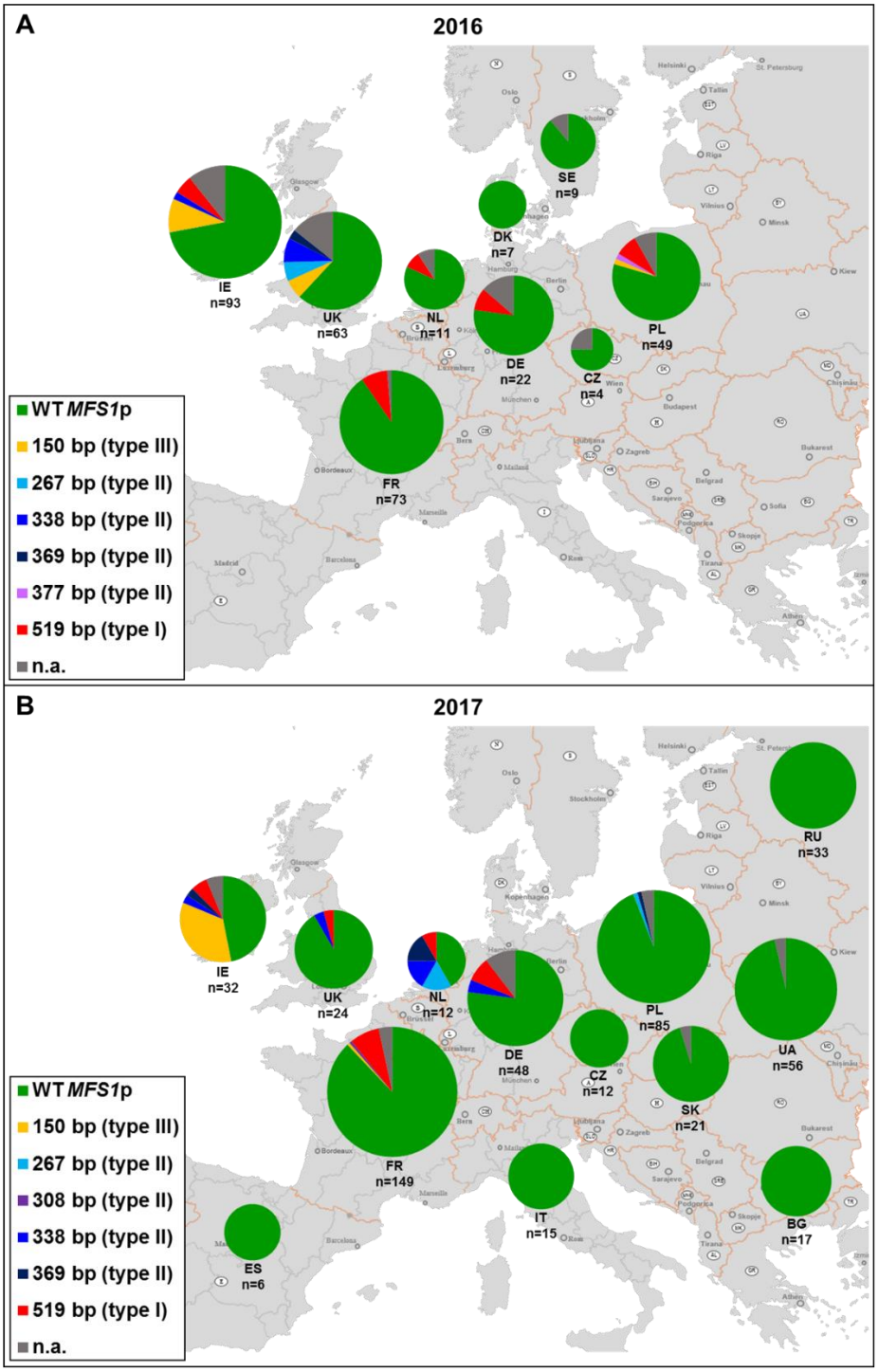


Figure 27: Frequency and distribution of inserts in *MFS1*p of *Z. tritici* across Europe. Isolates were obtained from BASF routine sensitivity monitoring. Frequency of isolates carrying a wild type *MFS1*p (WT *MFS1*p) and frequencies of isolates with an insert in *MFS1*p are shown. For some isolates no amplicon was observed after PCR (n.a.). The colour code is given in the legend in the figure. The number of tested isolates is given under the respective pie chart and the size of the pie chart is increasing with the number of tested isolates per country. The 150 bp insert was designated as type III insert, the 267, 308, 338, 369, and 377 bp inserts as type II and the 519 bp insert as type I. **A:** Distribution and frequency of isolates with WT *MFS1*p or with the 150, 267, 338, 369, 377, or 519 bp insert identified in 2016. **B:** Distribution and frequency of isolates with a WT *MFS1*p or with the 150, 267, 308, 338, 369, or 519 bp insert identified in 2017. These results have been previously published partially in Huf et al., 2020.

In 2017, the type III insert, type II inserts with a length of 267, 308, 338, and 369 bp, and the type I insert in *MFS1*p were detected in heterogenous frequencies in *Z. tritici* across Europe (Figure 27 B). In that year, the type II insert with a length of 377 bp was not detected anymore. In Russia, Ukraine, Bulgaria, Slovakia, Czech Republic, Italy, and Spain, only isolates with the WT *MFS1*p were identified. The type III insert was mainly detected in Ireland (34.4%) and only in one additional isolate in France. In no other country was the type III insert found. With the exception of the Netherlands, a low frequency of type II inserts was observed. In Ireland, respectively 3.1% of type II inserts with a length of 338 or 369 bp were detected, whereas in UK just the 338 bp type II insert was found (4.2%). Single isolates with a type II insert (267 and 369 bp) were detected in Poland. In contrast to 2016, single isolates with type II inserts were also found in Germany (338 bp) and France (308 bp). In the Netherlands, type II inserts with a length of 267, 338 and 369 bp were detected in a frequency of 16.7 %, respectively. The type I insert was found in Ireland, UK, the Netherlands, Germany, and France in frequencies ranging from 4.2 to 8.3%. In Poland, no isolate with this insert was found anymore. All in all, *MFS1*p inserts were only detected in Western Europe and in the Central European countries Germany and Poland but not in Czech Republic, Slovakia, the Eastern and Southern European countries.

Two isolates with a deletion in *MFS1*p were identified. The 148 bp deletion was detected in an isolate from Ukraine and the deletion of 165 bp in an isolate from Poland (results not shown).

Changes in the frequency of different *MFS1*p inserts between 2016 and 2017 were compared for important cereal growing countries that were tested in both years. An increase in the frequency of the type III insert (~25%) was observed in Ireland accompanied with a decrease of the WT *MFS1*p (~25%). Frequencies of type II inserts decreased in UK (in total ~20%) and increased in the Netherlands (in total ~50%). A lower number of isolates was tested in 2017 from Ireland and UK compared to 2016. In the Netherlands, the number of tested isolates was relatively small in both years. In these cases, a small sample size may contribute to strong frequency changes of *MFS1*p inserts because single isolates cause a high change in overall frequency distribution. In other countries no strong changes in the frequency of type II inserts were observed. However, as single isolates with a type II insert were also detected in Germany and France in 2017, in contrast to 2016, a wider distribution of these inserts was detected. Nevertheless, the frequency of type II inserts was low in these countries. Overall, frequencies of the type I insert stayed relatively stable in Germany, France, and Ireland across both years. In Poland, this insert was not detected anymore, and an increase was detected in UK because this insert was absent in 2016. However, the type I insert was only detected in one isolate in UK in 2017. In conclusion, these results indicate no increase in the frequency of an increased efflux activity in 2017 compared to 2016 based on the frequency of the 519 bp *MFS1*p insert. A wider spreading of isolates carrying inserts with lower impacts on tolnaftate and epoxiconazole sensitivity, thus

probably on increased efflux, was observed in 2017. However, frequencies were low, except for Ireland and the Netherlands.

4.7 Sensitivity of *Z. tritici* isolates accumulating multiple resistance mechanisms to DMIs

At the beginning of this work, the epoxiconazole sensitivity of isolates from the routine sensitivity monitoring was correlated to their *CYP51* haplotypes (shown in chapter 4.3). Across all isolates within a *CYP51* haplotype group, pronounced variation in the sensitivity was observed. This variation was assumed to be conferred by other DMI resistance mechanisms than CYP51 alteration such as *CYP51* overexpression or an increased efflux. In order to prove this hypothesis, the sensitivity distribution of *Z. tritici* isolates for all 'frequently found *CYP51* haplotypes' was visualised based on EC_{50} values of single isolates and additional DMI resistance mechanisms were highlighted. In the course of this work, it was shown that *CYP51* overexpression is mainly correlated to the 121 bp insert in *CYP51*p and increased efflux is mainly associated with the 519 bp insert in *MFS1*p. Therefore, only isolates carrying one of these two inserts were designated as isolates showing *CYP51* overexpression or an increased efflux. Other inserts in *CYP51*p and *MFS1*p will be not considered in this chapter due to their low impacts on epoxiconazole sensitivity.

Figure 28 shows that in most cases the highest adaptation to epoxiconazole within a *CYP51* haplotype could be associated with *CYP51* overexpression and/or an increased efflux activity. These results confirm an additive effect of *CYP51* overexpression and an increased efflux on epoxiconazole adaptation of *Z. tritici* isolates which already show an adaptation based on *CYP51* haplotypes. Isolates that combine all three mechanisms (only identified for haplotype F2) showed highest adaptation within the F2 haplotype group. Most of these isolates were less sensitive than isolates with a F2 haplotype and *CYP51* overexpression alone. These results indicate an additive effect of increased efflux on epoxiconazole adaptation in isolates with *CYP51* overexpression. Nevertheless, isolates combining all three mechanisms were observed in a low frequency (n=6, i.e. <1% of all investigated isolates).

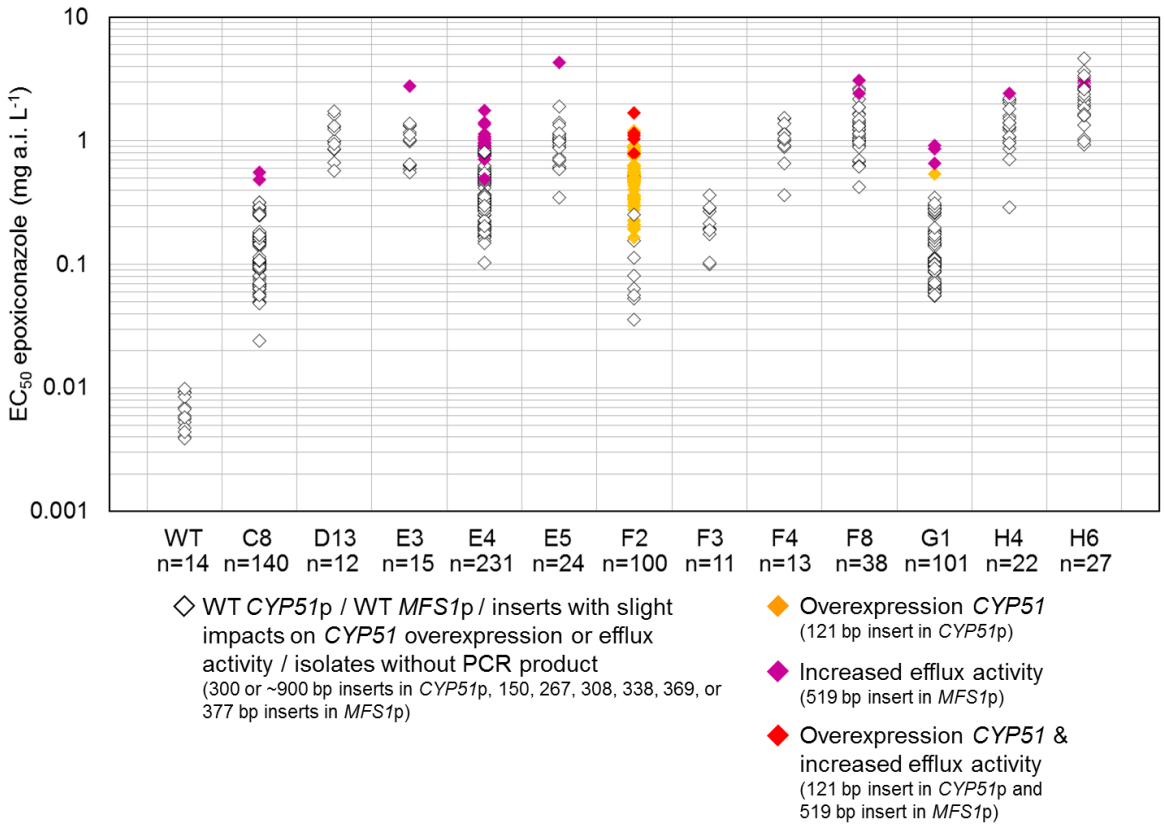


Figure 28: Sensitivity of *Z. tritici* isolates with multiple resistance mechanisms to DMIs. EC_{50} values of epoxiconazole (gained in microtiter tests from EpiLogic) for each isolate carrying a 'frequently found *CYP51* haplotype' are shown. Isolates that show additional resistance mechanisms as an increased efflux activity and/or *CYP51* overexpression are highlighted. The colour code is given in the legend. The number of tested isolates is given in the diagram.

In some cases, the highest epoxiconazole adaptation of isolates within a *CYP51* haplotype group could not be explained by *CYP51* overexpression or an increased efflux activity based on identification of the 121 bp insert in *CYP51*p and 519 bp insert in *MFS1*p. In the H6 haplotype group, highest adaptation of isolates could be correlated to other *MFS1*p inserts that confer in most cases only low effects on an increased efflux (results not shown). Despite the highest adaptation that can be explained by an increased efflux activity or *CYP51* overexpression, a broad range in sensitivity of the remaining isolates within a *CYP51* haplotype group was still observed. These results indicate that high variability in epoxiconazole sensitivity may be observed based on natural variability in epoxiconazole sensitivity of *Z. tritici* field isolates or that other mechanisms may play a role in epoxiconazole adaptation. Further studies would be required to test these assumptions in future studies.

After investigation of the epoxiconazole sensitivity of *Z. tritici* populations across Europe, cross-resistance between different DMIs was investigated in the next chapter.

4.8 Evaluation of cross-resistance between different DMIs of *Z. tritici* isolates

The correlation of the sensitivity to different DMIs resulting from microtiter tests was analysed to identify if adapted *Z. tritici* isolates show cross-resistance for different DMIs. Moreover, the composition of *CYP51* haplotypes in surviving *Z. tritici* populations after DMI treatment was examined based on isolates derived from field trials.

4.8.1 Correlation of the sensitivity of *Z. tritici* isolates to DMIs

Z. tritici isolates were tested for their sensitivity towards epoxiconazole, mefentrifluconazole, and prothioconazole-desthio in order to evaluate cross-resistance between different DMIs. EC_{50} values were determined in microtiter tests by EpiLogic with a commercial formulation of epoxiconazole and mefentrifluconazole and the technical a.i. for prothioconazole-desthio. The technical a.i. of prothioconazole-desthio was used as this metabolite is suggested to be responsible for fungicidal activity of prothioconazole (Parker et al., 2011, 2013). A subset of isolates of the routine sensitivity monitoring was tested and sensitivity of isolates carrying a 'frequently found *CYP51* haplotype' (n=376) was analysed in this chapter. EC_{50} values for tested DMIs were plotted against each other and the linear correlation was analysed. The cross-resistance pattern of different 'frequently found *CYP51* haplotypes' was investigated based on resistance factors for each tested compound.

Correlations of EC_{50} values of 'frequently found *CYP51* haplotypes' for the three tested DMIs are shown in Figure 29, Figure 30, and Figure 31. Across all tested isolates, EC_{50} values ranged from 0.004 to 4.630 mg epoxiconazole L^{-1}, 0.003 to 1.736 mg prothioconazole-desthio L^{-1}, and 0.001 to 1.609 mg mefentrifluconazole L^{-1}. Most of the EC_{50} values of a respective *CYP51* haplotype clustered in groups for all three compounds, but variation in sensitivity within *CYP51* haplotypes was observed. In this chapter, isolates with similar *CYP51* haplotypes were not separated for additional DMI resistance mechanisms (*CYP51* overexpression, increased efflux) because in most cases only a single isolate with or without an additional mechanism was detected within a haplotype group. Therefore, in some isolates, variation in sensitivity may be correlated to additional resistance mechanisms. For example, within the F2 haplotype group, one isolate showed a higher sensitivity, whereas remaining isolates showed a lower sensitivity to all three compounds. This higher adaptation could be correlated to *CYP51* overexpression in these isolates demonstrating that all tested DMIs are affected by this mechanism.

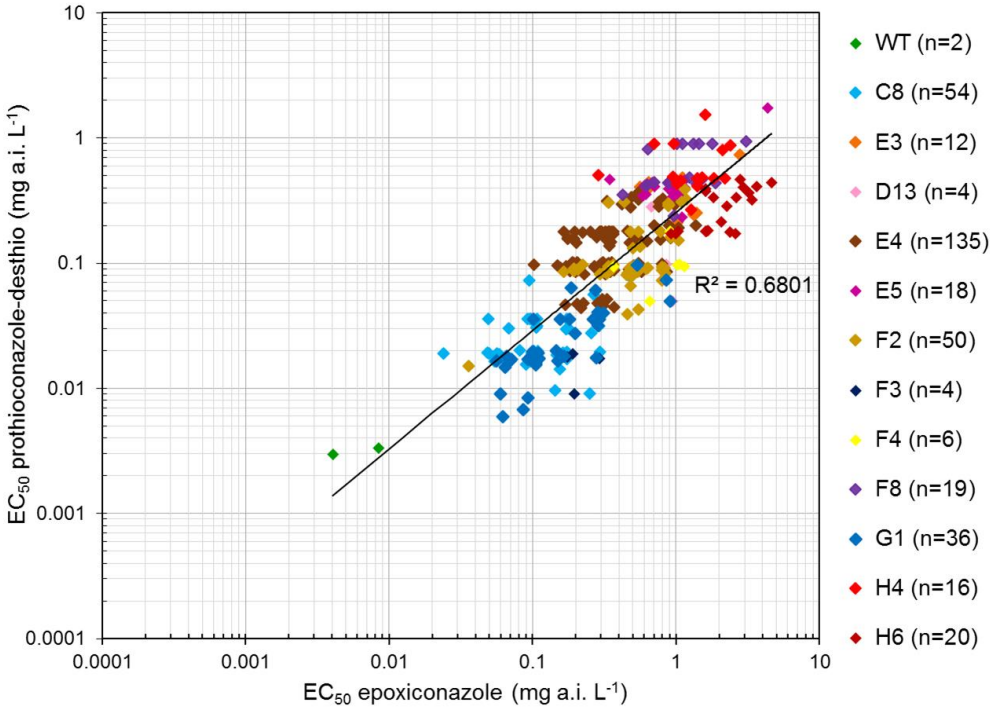


Figure 29: Correlation of EC_{50} values of *Z. tritici* isolates with 'frequently found *CYP51* haplotypes' for epoxiconazole and prothioconazole-desthio. The log-transformed EC_{50} values were used to calculate linear regression and the coefficient of determination (R^2 = 0.6801) over all isolates. Isolates for which EC_{50} values for all three tested DMIs (epoxiconazole, mefentrifluconazole and prothioconazole-desthio) were available, are plotted in the diagram and the number is shown. These results have been previously published partially in Huf et al., 2018.

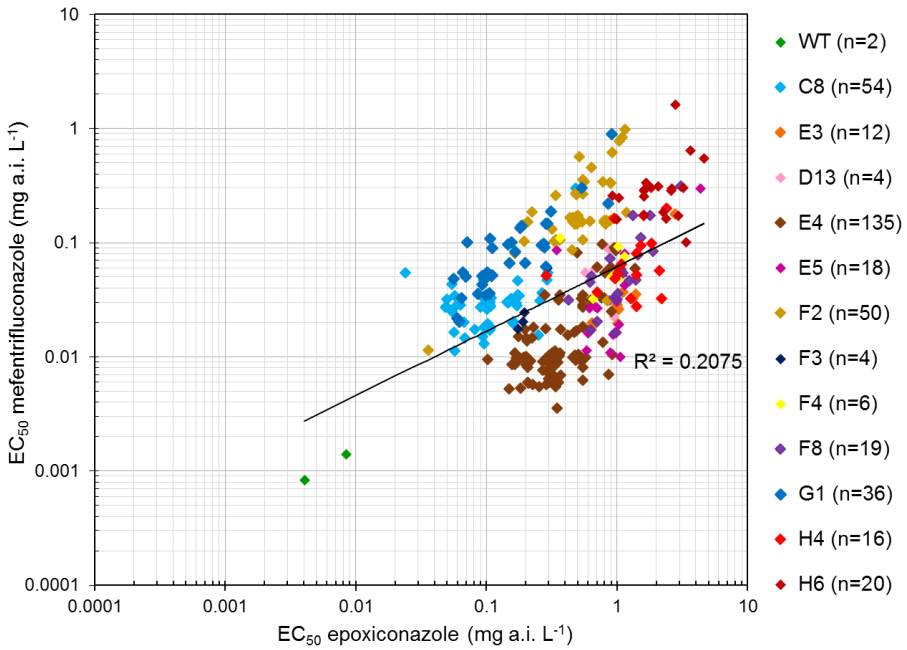


Figure 30: Correlation of EC_{50} values of *Z. tritici* isolates with 'frequently found *CYP51* haplotypes' for epoxiconazole and mefentrifluconazole. The log-transformed EC_{50} values were used to calculate linear regression and the coefficient of determination (R^2 = 0.2075) over all isolates. Isolates for which EC_{50} values for all three tested DMIs (epoxiconazole, mefentrifluconazole and prothioconazole-desthio) were available, are plotted in the diagram and the number is shown.

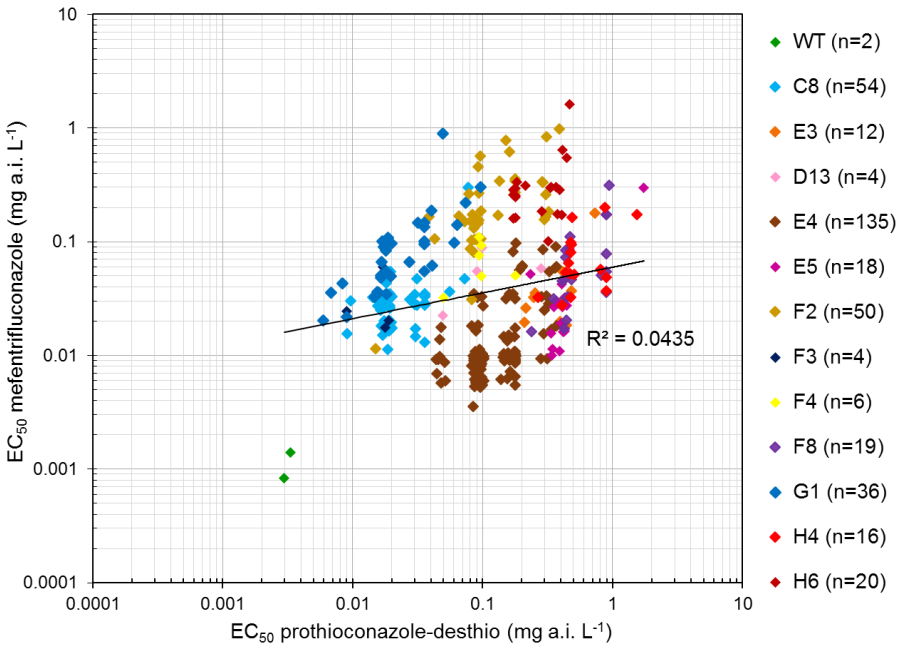


Figure 31: Correlation of EC_{50} values of *Z. tritici* isolates with 'frequently found *CYP51* haplotypes' for prothioconazole-desthio and mefentrifluconazole. The log-transformed EC_{50} values were used to calculate linear regression and the coefficient of determination (R^2 = 0.0435) over all isolates. Isolates for which EC_{50} values for all three tested DMIs (epoxiconazole, mefentrifluconazole and prothioconazole-desthio) were available, are plotted in the diagram and the number is shown.

A high correlation of the EC_{50} values (R^2=0.6801) for most of the isolates was observed for epoxiconazole and prothioconazole-desthio (Figure 29). In contrast to this, a weak correlation (R^2=0.2075) of EC_{50} values for most of the isolates was observed for epoxiconazole and mefentrifluconazole (Figure 30). Almost no correlation was observed for the EC_{50} values (R^2=0.0435) of most of the isolates for prothioconazole-desthio and mefentrifluconazole as shown in Figure 31.

The cross-resistance pattern of 'frequently found *CYP51* haplotypes' based on resistance factors for the tested DMIs is shown in Table 19. Resistance factors were calculated as the ratio of mean EC_{50} of an adapted *CYP51* haplotype to mean EC_{50} of the *CYP51* wild type. Resistance factors are dependent on the intrinsic activity of a compound for wild type isolates. Therefore, the mean EC_{50} values for wild type isolates (chosen for resistance factor calculation) are additionally depicted in Table 19. Compounds with a high intrinsic activity often lead to higher resistance factors than compounds with a lower intrinsic activity. Mefentrifluconazole showed highest activity for wild type isolates, followed by prothioconazole-desthio and epoxiconazole.

Table 19: Resistance factors (RF) of *Z. tritici* isolates with 'frequently found *CYP51* haplotypes' for different DMIs. Resistance factors (RF) were calculated for epoxiconazole, mefentrifluconazole and prothioconazole-desthio as ratio of the mean EC_{50} of an adapted *CYP51* haplotype to mean EC_{50} of sensitive isolates (*CYP51* wild type). EC_{50} values of isolates analysed for their sensitivity to epoxiconazole, mefentrifluconazole and prothioconazole-desthio from routine sensitivity monitoring were used and additionally of five reference isolates from EpiLogic. EC_{50} values were determined in microtiter tests by EpiLogic. Resistance factors are coloured according to resistance levels: low RF: < 30 = bright yellow, moderate RF: 31-65 = dark yellow and 66-100 = orange, high RF: >100 = red.

Haplotype	RF		
	Epoxiconazole	Mefentrifluconazole	Prothioconazole-desthio
C8	22	26	9
D13	132	43	49
E3	189	33	143
E4	69	13	53
E5	183	36	176
F2	97	180	49
F3	37	23	6
F4	149	53	39
F8	206	58	224
G1	34	83	11
H4	240	61	235
H6	393	266	109
Mean EC_{50} (mg a.i. L^{-1}) sensitive isolates	0.006	0.001	0.002

Resistance factors ranged from 6 to 393 across all haplotypes and DMIs. Different cross-resistance patterns for some haplotypes were observed. In Table 19, low resistance factors are coloured bright yellow, moderate resistance factors dark yellow and orange, and high resistance factors were coloured red. It was observed that cross-resistance patterns were in most cases more similar for epoxiconazole and prothioconazole-desthio compared to mefentrifluconazole. For example, high resistance factors were observed for haplotypes E3, E5, F8, and H4 for epoxiconazole and prothioconazole-desthio, whereas they were moderate for mefentrifluconazole. In contrast, haplotype F2 showed a high resistance factor for mefentrifluconazole, whereas resistance factors for epoxiconazole and prothioconazole-desthio were lower. Additionally, moderate resistance factors were observed for epoxiconazole and prothioconazole-desthio with haplotype E4, whereas mefentrifluconazole showed a low resistance factor. In contrast, haplotype G1 showed a moderate resistance factor for mefentrifluconazole, whereas resistance factors were lower for epoxiconazole and prothioconazole-desthio. Haplotype C8 showed low resistance

factors for all three compounds, whereas high resistance factors for all three DMIs were observed for haplotype H6.

In conclusion, results of the *in vitro* studies indicate that across all 'frequently found *CYP51* haplotypes' the level of cross-resistance is higher for epoxiconazole and prothioconazole-desthio compared to mefentrifluconazole. Dependent on specific *CYP51* haplotypes limited cross-resistance as well as full cross-resistance could be detected for the three tested DMIs.

4.8.2 Composition of *CYP51* haplotypes in *Z. tritici* populations following DMI treatment

Isolates generated from field samples taken after fungicide application were investigated for their *CYP51* haplotypes. The aim was to identify if different cross-resistance patterns between DMIs, that were indicated in *in vitro* studies in the previous chapter, can be associated with differences in the composition of *CYP51* haplotypes of *Z. tritici* after DMI application in the field. Four field trials were conducted across Ireland, Germany, UK and Denmark from BASF SE and samples were taken in untreated, mefentrifluconazole (Revysol®) and prothioconazole (Proline®) treated plots in each trial. EpiLogic generated 10 isolates from each sample resulting in 120 isolates. Afterwards, *CYP51* haplotypes of these isolates were identified in this work.

CYP51 haplotypes and their relative frequencies identified per sample and country are shown in Figure 32.

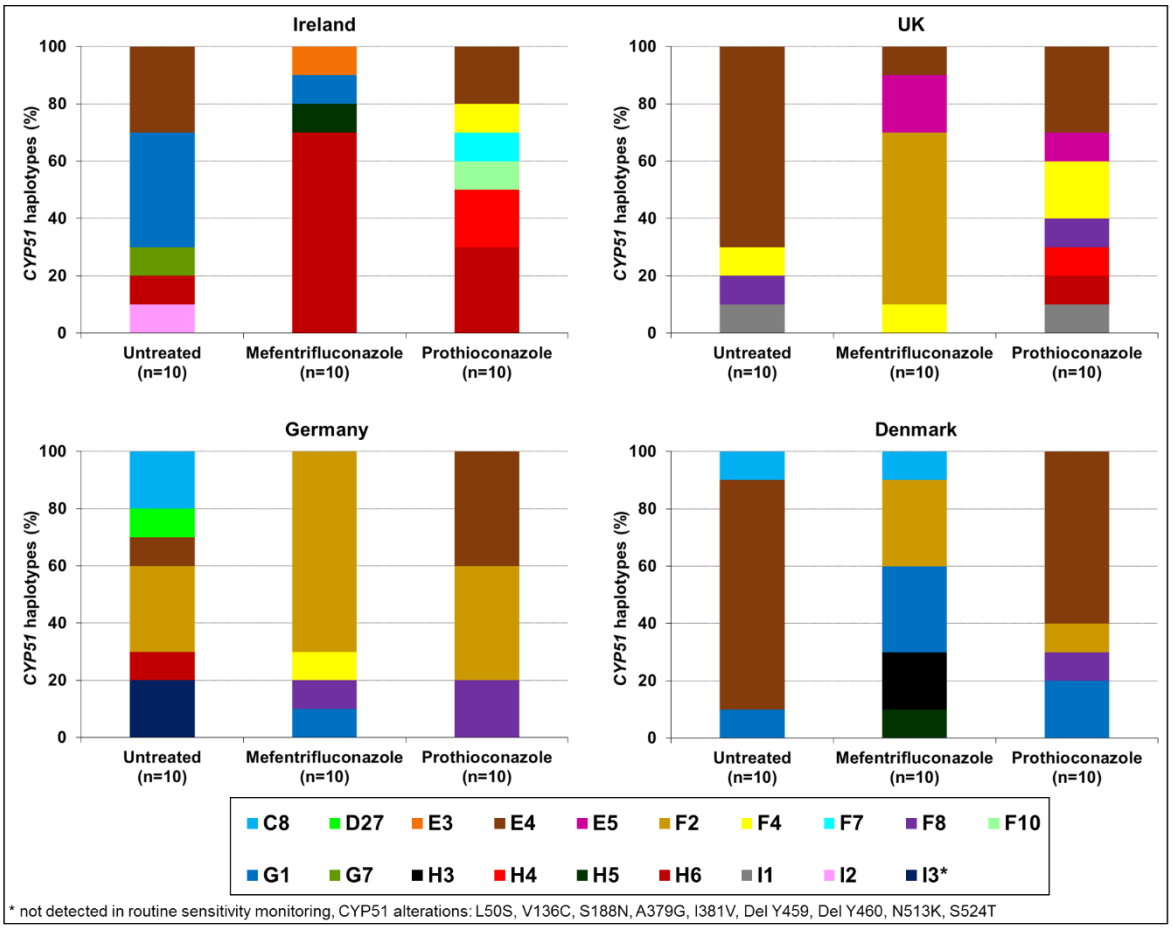


Figure 32: *CYP51* haplotypes of *Z. tritici* and their relative frequency in field trials after application of mefentrifluconazole (Revysol®) and prothioconazole (Proline®). Field trials were conducted in four different countries (Ireland, UK, Germany, Denmark). Samples were taken in untreated, mefentrifluconazole and prothioconazole treated plots and 10 isolates were generated per sample by EpiLogic. Isolates were investigated for their *CYP51* haplotypes and relative frequency of *CYP51* haplotypes per country and treatment is shown.

Untreated plots showed different compositions and varying relative frequencies of *CYP51* haplotypes across all four investigated countries. Compared to untreated plots, changes in the composition and frequencies of *CYP51* haplotypes were detected in all fungicide treated plots across all four countries. Over all trials, a different trend in the pattern of remaining *CYP51* haplotypes after treatment with mefentrifluconazole or prothioconazole was observed. In plots treated with mefentrifluconazole often haplotypes F2 and H6 were identified among others that were found in lower frequencies. Compared to this, in Ireland and UK, the pattern of *CYP51* haplotypes detected after prothioconazole treatment seems more variable. Except haplotype E4, all haplotypes found after prothioconazole treatment in UK and Ireland had in common that they carry the alteration S524T. In contrast to this, haplotypes E4 and F2 were frequently detected after prothioconazole treatment in Germany. Haplotype E4 was also frequently found in Denmark after treatment with prothioconazole. Although a different trend in the selection pattern of prothioconazole and mefentrifluconazole is indicated, it should be noted that in some cases similar haplotypes

were observed in surviving isolates such as H6 in Ireland, F2 in Germany, and G1 in Denmark.

Similarities between resistance patterns observed *in vitro* in the previous chapter (4.8.1) and *CYP51* haplotype patterns after DMI application in the field can be observed partially. High resistance factors of haplotype F2 and H6 *in vitro* correlate to frequent detection of these haplotypes after mefentrifluconazole treatment. *In vitro*, high resistance factors for prothioconazole were mainly detected for haplotypes carrying alteration S524T. These findings correlate to frequent detection of haplotypes with this alteration after prothioconazole treatment. Haplotype F2 and E4 that were frequently found in prothioconazole treated plots in Germany (F2 and E4) and Denmark (E4) showed moderate resistance factors to prothioconazole *in vitro*. An important point to mention is that the relative frequencies shown in results of this chapter does not provide information about absolute frequencies of these *CYP51* haplotypes in the field. This fact is further evaluated in the discussion in chapter 5.3.

All in all, results of this chapter indicate a different trend in the selection pattern of *CYP51* haplotypes after application of mefentrifluconazole and prothioconazole supporting the assumption of limited cross-resistance observed *in vitro*. Nevertheless, the same *CYP51* haplotypes can be detected after both treatments in some cases. Correlation of *in vitro* resistance patterns and emergence of *CYP51* haplotypes after DMI treatment was observed. It should be noted, however, that relative frequencies of *CYP51* haplotypes were analysed in this chapter and that resistance factors *in vitro* do not necessarily correlate to efficacy of fungicides *in vivo*. Therefore, efficacy of different DMIs was tested in the glasshouse (*in vivo*) in this work and results are shown in the next chapter.

4.9 Efficacy of DMIs in glasshouse studies

DMI efficacies were tested in the glasshouse to evaluate DMI efficacy for *Z. tritici* isolates under conditions that correspond more to realistic conditions in the field. *Z. tritici* isolates with different *CYP51* haplotypes and different levels of *CYP51* expression or increased efflux were used in these studies. *CYP51* haplotypes were chosen based on the frequency of haplotypes identified in the routine sensitivity monitoring from 2016 and 2017 and/or due to a high adaptation in microtiter tests (EC_{50} values of microtiter tests are shown in supplementary material Table 27). Isolates were obtained from routine sensitivity monitoring of 2017 or were provided from isolate collection of BASF SE. The DMIs epoxiconazole (Opus®), mefentrifluconazole (Revysol®) and prothioconazole (Proline®) were applied in solo formulations one day preventatively. Visual assessment of the diseased leaf area was performed 21 dpi and the efficacy (inhibition %) of fungicides was calculated. Each isolate

was tested in two independent experiments (except the isolate carrying haplotype E1 that was tested three times due to methodological split of all tested isolates).

In Figure 33, efficacy of epoxiconazole, mefentrifluconazole, and prothioconazole in the glasshouse for the tested isolates is shown. The isolate carrying haplotype E1 was used as an internal control (reference isolate). This isolate showed a low DMI adaptation in microtiter tests. In glasshouse tests it was fully controlled (99-100%) by epoxiconazole and mefentrifluconazole and a high efficacy was observed for prothioconazole (93%). Across all other isolates, a variable efficacy was observed dependent on the tested DMI and isolate. Statistical differences between the efficacy for the reference isolate and the grouped efficacies across all remaining haplotypes were calculated with the Kruskal-Wallis-Test (significance level: $p<0.05$). Compared to the reference, a significantly reduced efficacy of epoxiconazole and prothioconazole was observed across all remaining haplotypes, whereas no significantly reduced efficacy was observed for mefentrifluconazole. Efficacy of epoxiconazole was lowest for isolates with haplotype H6, which additionally exert an increased efflux activity, and haplotype I2 with mean inhibition levels of around 75%, respectively. Inhibition of remaining haplotypes was higher (> 80%). Efficacy of mefentrifluconazole was slightly reduced (85%) only for the isolate carrying haplotype G2 and additionally exerting slight *CYP51* overexpression and slight efflux. For all remaining haplotypes mean inhibition of mefentrifluconazole was high with at least 90%. Mean inhibition levels of prothioconazole ranged between 44-67%. Highest reduction in efficacy was observed for isolates carrying haplotypes H6 (with increased efflux) and I2. Methodological issues may interfere with lower and more variable efficacies of prothioconazole compared to epoxiconazole. With regard to *Z. tritici* on wheat, in BASF SE, it is experienced that prothioconazole often performs more poorly under controlled conditions in the glasshouse compared to the field.

Isolates carrying the F2 haplotype additionally showed *CYP51* overexpression. A high efficacy of epoxiconazole (89-93%) and mefentrifluconazole (93-99%) was observed for these isolates.

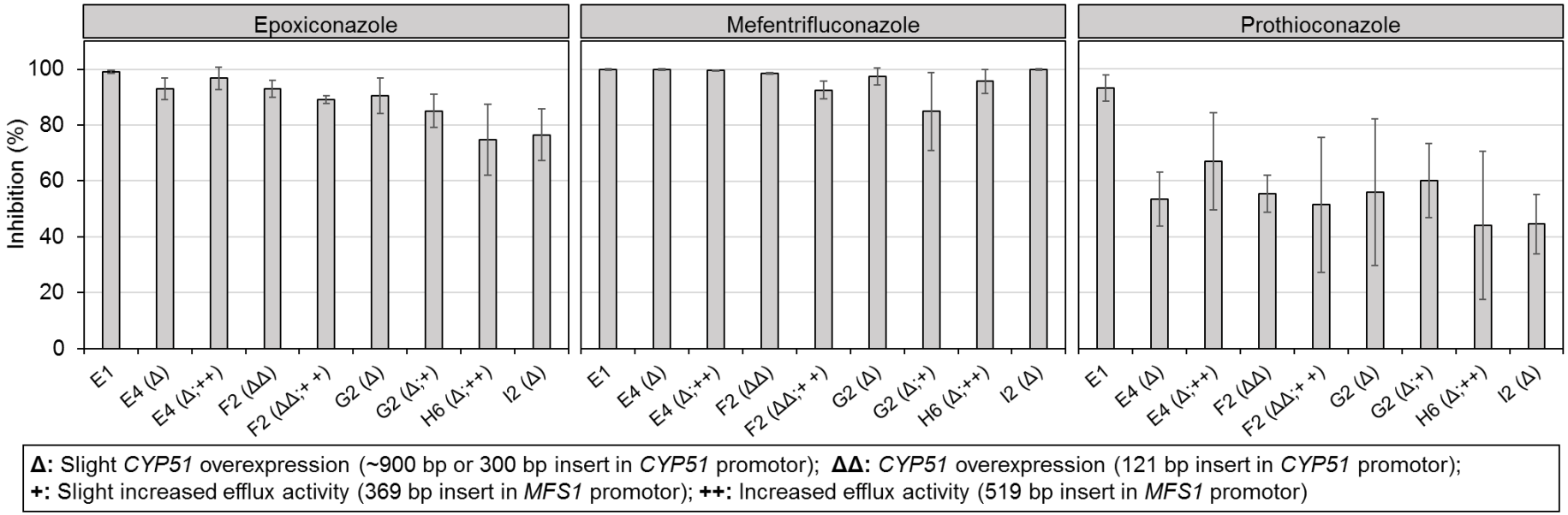


Figure 33: Efficacy of epoxiconazole (Opus®), mefentrifluconazole (Revysol®), and prothioconazole (Proline®) for *Z. tritici* isolates in glasshouse experiments. Fungicides at full registered field rate (epoxiconazole: 125 g a.i. ha^{-1}, mefentrifluconazole: 150 g a.i. ha^{-1}, prothioconazole: 200 g a.i. ha^{-1}) were applied one day preventative in three replicates. Isolates with different *CYP51* haplotypes and with or without different levels of *CYP51* expression (indicated by sign Δ) and/or an increased efflux activity (indicated by sign +) were tested (explanation of signs is given in the diagram). An isolate (n=1) with a low DMI adapted *CYP51* haplotype (E1) was used as internal control (reference isolate). Respectively one isolate (n=1) was tested for haplotype E4 (Δ), E4 (Δ;++), F2 (ΔΔ), F2 (ΔΔ;++), G2 (Δ), G2 (Δ;+) and two isolates (n=2) for H6 (Δ;++) and I2 (Δ). Each isolate was tested in two independent experiments (except for haplotype E1 that was tested three times due to methodological split of all tested isolates). Mean values of inhibition (%) of the applied DMIs and standard deviations are shown. Mean values and standard deviations were calculated across independent experiments. Statistical differences between the efficacy for the reference and grouped efficacies across all haplotypes were calculated with Kruskal-Wallis-Test (significance level: p= <0.05). In comparison to the reference isolate, statistical differences for grouped efficacies across all remaining haplotypes were observed in the efficacy of epoxiconazole (p=0.0106) and prothioconazole (p=0.0062) but not for mefentrifluconazole (p=0.0802).

In glasshouse tests, isolates with the same *CYP51* haplotype but differing in the absence or presence of an increased efflux were tested. In the case of haplotype E4 no decrease in efficacy of all DMIs was observed for the isolate with an increased efflux compared to the isolate carrying haplotype E4 and no increased efflux. In case of haplotype F2 and G2 a slight decrease (4-8%) in the efficacy of epoxiconazole and mefentrifluconazole was observed for the isolate with an increased efflux. Among all *CYP51* haplotypes, prothioconazole only showed a slight reduced efficacy (4%) for the isolate with haplotype F2 and increased efflux. As isolates with haplotype F2 additionally show *CYP51* overexpression and varying impacts associated with *CYP51* overexpression were observed (shown in 4.5.2), it cannot be clarified if lower efficacy for the isolate carrying *CYP51* overexpression and an increased efflux is driven by an increased efflux or by a stronger *CYP51* overexpression. Additionally, as no isolate carrying haplotype H6 without an additional increased efflux was tested, it cannot be clarified if an increased efflux contributes to lower efficacy of epoxiconazole and prothioconazole for these isolates compared to remaining isolates. Altogether, no clear conclusion about the impact of an increased efflux on efficacy of DMIs *in vivo* could be drawn. A contribution to a slight reduction in DMI efficacy seems possible in single cases. Nevertheless, this reduction was observed to be low and in cases in which a reduction was observed, efficacy of epoxiconazole and mefentrifluconazole was still at a high level.

Resistance factors observed in *in vitro* studies for haplotypes E4, F2, and H6 (described in chapter 4.8.1) could only be correlated partly to efficacies of different DMIs in the glasshouse. High resistance factors for haplotype H6 were observed for epoxiconazole and prothioconazole-desthio, whereas the resistance factors for the E4 and F2 haplotype were moderate. These results correlate to lowest efficacy of epoxiconazole and prothioconazole for haplotype H6 (mean inhibition) in glasshouse tests, whereas efficacy for haplotype E4 and F2 (mean inhibition) was higher. In contrast, high resistance factors were detected for mefentrifluconazole for haplotypes F2 and H6 *in vitro*. Efficacies of mefentrifluconazole in glasshouse tests for isolates carrying a F2 or H6 haplotype were on average high with at least 90% inhibition. These results indicate that high resistance factors *in vitro* do not necessarily imply a low efficacy of DMIs in the glasshouse and that the correlation between resistance factors observed *in vitro* and the efficacy of DMIs in the glasshouse (*in vivo*) may occur weak.

5 Discussion

During the last few decades, DMIs have been the most widely used fungicides and still represent an important tool in disease management of wheat, including STB (Parker et al., 2011; Cools and Fraaije, 2013; Jørgensen et al., 2018; Heick et al., 2017a; Kildea et al., 2019; Jørgensen et al., 2019). Over many years, intensive application of DMIs has resulted in the evolution of DMI adaptation and a gradual shift towards a reduced sensitivity of the *Z. tritici* population in Europe (Cools et al., 2011; Lucas et al., 2015; FRAC, 2019). Molecular mechanisms conferring DMI resistance in *Z. tritici* include alteration of the CYP51 target enzyme, *CYP51* overexpression and an increased efflux activity of membrane transporters. The most studied and reported mechanism is the alteration of CYP51, whereas contribution of *CYP51* overexpression and an increased efflux activity in *Z. tritici* across Europe is less intensively studied so far (Stammler et al., 2008b; Stammler and Semar, 2011; Leroux and Walker, 2011; Cools and Fraaije, 2013; Jørgensen et al., 2019).

In this work, an overview about the occurrence of different DMI resistance mechanisms in *Z. tritici* across Europe and their impact on DMI sensitivity has been shown. In the following chapter the emergence, distribution, and impact of these mechanisms is discussed. Combination of different DMI resistance mechanisms and further evolution of these mechanisms is considered. Furthermore, cross-resistance patterns of different DMIs are debated with regard to future DMI usage and resistance management approaches. The impact of different DMI resistance mechanisms on field performance and possible consequences for future DMI application for STB control are also discussed.

5.1 Emergence of DMI resistance in *Z. tritici*

5.1.1 Alteration of *CYP51*: Ongoing evolution after 30 years of changes?

Since the first detection of a CYP51 alteration in isolates from 1988, new alterations in *CYP51* of *Z. tritici* emerged successively over time and space and the sequential accumulation of these alterations has led to the emergence of increasingly more complex *CYP51* haplotypes (Cools and Fraaije, 2013; Lucas et al., 2015). In this work, sequencing of the *CYP51* gene of *Z. tritici* isolates from routine sensitivity monitoring programmes for 2016 and 2017 revealed the prevalence of 23 *CYP51* alterations that have already been described in literature (Stammler et al., 2008b; Cools and Fraaije, 2013). Previously, deletions in CYP51 of *Z. tritici* were shown to occur in amino acid region YGYG at positions 459-462 coded by nucleotides TAT/GGC/TAC/GGC (Stammler et al., 2008b). With the exception of two isolates, all investigated isolates that showed a deletion in CYP51 showed

missing codons for Y459 (TAT) and Y460 (GGC) with remaining codons in this region observed to be TAC/GGC (data not shown). Consequently, isolates carrying a *CYP51* haplotype with a deletion frequently showed a combination of deletions Y459 and Y460. Seldomly a single amino acid deletion was detected (only found in one isolate with haplotype E26). Additionally, only one isolate showed a remaining DNA sequence of TAT/GGC at codon positions 459-462 (data not shown). In this isolate the amino acid at position Y461 was deleted and either G460 or G462 (haplotype E21). It could not be clarified in this work if amino acid position G460 or G462 was deleted based on sequence alignments. Occurrence of such isolates was already reported before (Stammler et al., 2008b), but clarification if amino acid G460 or G462 is deleted, would need further investigations.

Besides the 23 existing alterations, 11 new CYP51 alterations that were not described so far were identified. Molecular characterisation of the location of these new alterations within the CYP51 enzyme and phenotypical assessment of the sensitivity of isolates carrying these new alterations gave information about their potential impact on DMI binding and DMI sensitivity.

Amino acid alterations in CYP51 can affect binding of triazoles in different ways. In their work, Mullins et al. (2011) and Becher and Wirsel (2012) described that an alteration may lead to conformational changes that confer a loss of interaction between the triazole and a specific residue (or residues). Another possibility is the direct substitution of such a key residue. Both can lead to a disturbed accommodation of the triazole within the binding cavity that may lead to a hindered interaction of the triazole with the haem. A third possibility is the disturbed access of the triazole to the active site (Mullins et al., 2011; Becher and Wirsel, 2012). In Figure 34, binding of the triazole triadimenol to the wild type CYP51 is shown. Amino acid positions V136, Y137, I381, Y459, G460, and Y461 known to be the subject of existing alterations associated to DMI adaptation were localised at the border of the binding pocket (Mullins et al., 2011). In contrast, amino acids L50 and A379 were not observed to be directly bordering the binding pocket but form parts of the secondary structure adjacent to the binding cavity. Amino acids S188 and N513 are located far away from the binding region (Mullins et al., 2011). This modelling is in accordance with the observation that alterations L50S, S188N, and N513K are not associated with DMI adaptation, whereas alterations of amino acids V136, Y137, I381, Y459, G460, and Y461 drive DMI adaptation or compensatory effects enabling DMI adaptation (Cools et al., 2010, 2011; Mullins et al., 2011; Cools and Fraaije, 2013; Lucas et al., 2015).

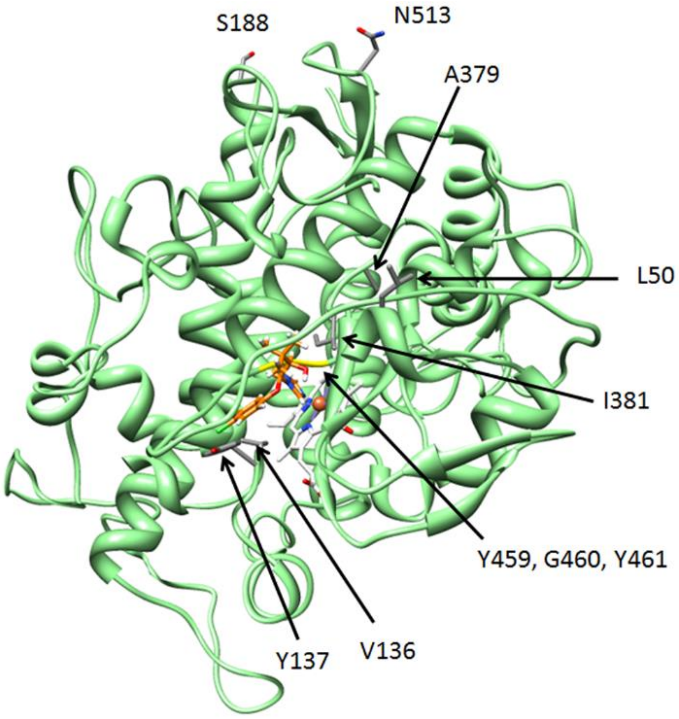


Figure 34: Protein model of CYP51 wild type with bound triadimenol (orange) and location of amino acids subject to alterations. Picture was extracted from Mullins et al., 2011. Amino acids L50, V136, Y137, S188, A379, I381, and N513 labelled in grey, region of Y459, G460, Y461 labelled in yellow, haem group labelled white. Amino acids L50 and A379 form parts of secondary structure directly adjacent to the binding cavity but do not border it. Amino acid S188 and N513 are located far away from the binding cavity at the outside of the protein. Amino acids V136 and Y137 are located at the end of the access channel of the binding pocket, whereas I381, Y459, G460, and G461 are located at the end of the binding pocket at haem site (Mullins et al., 2011).

As positions of amino acids K95, I452, and N507, that were observed to be subject of new CYP51 alterations, are located far away from the haem group, and thus from the binding pocket, the relevance of these residues in triazole binding may be considered to be minor. Mullins et al. (2011) identified 86 specific residues lining the binding pocket of the wild type protein and additionally several residues, among them V136, Y137, I381, Y459, G460, and Y461, in close proximity to different bound triazoles. Although compared to position of amino acids K95, I452, and N507, the remaining amino acids that were observed to be subject of new alterations were located in closer proximity to the haem, only positions L197, E457, D458, and Y461 represent residues lining the binding pocket according to Mullins et al. (2011). Among those four amino acid residues, E457, D458, and Y461 were observed additionally to be located in close proximity to the docked triazoles but not L197 (Mullins et al., 2011). Based on these results it may be deduced that across all new alterations probably only the deletion of E457 and D458 as well as alteration Y461N may affect DMI binding. This assumption may be supported by findings for neighbouring residues in the region Y459-Y461. Deletions or alterations in this region result in severe structural rearrangements of CYP51 and/or in the loss of residues that confer a disturbed interaction to triazoles (Mullins et al., 2011).

The effect of new *CYP51* alterations on DMI sensitivity was tested by comparing the sensitivity of *CYP51* haplotypes that differ in the presence or absence of new alterations or to the *CYP51* wild type. As *CYP51* alterations almost always occur in combinations, this is a commonly applied method to assess the effect of single alterations (Cools et al., 2011; Leroux and Walker, 2011). Depending on the alteration and test method around a 2- to 8-fold higher epoxiconazole adaptation was correlated to the individual impact of alterations V136A, I381V, and S524T, known as key alterations in DMI resistance evolution (Cools et al., 2011; Leroux and Walker, 2011; Lucas et al., 2015). Compared to this, no strong effects on epoxiconazole sensitivity could be associated with a high level of certainty to new *CYP51* alterations identified in this work. It should be clarified in future studies, whether the 2.5-fold higher adaptation in the isolate carrying alteration A318G is driven by this alteration or a slight increased efflux. However, such a study would require an isolate with alteration A318G but no additional resistance mechanism or, for example, heterologous expression studies with this alteration in *S. cerevisiae* (Cools et al., 2010) which are currently not available. Furthermore, future studies would be required to assess the individual effect of alteration L197V and deletion of amino acids K456, E457, D458, and L463. As these alterations occur together with deletion of Y459, G460, Y461, and G462, already known for their relevant role in DMI adaptation (Mullins et al., 2011; Cools and Fraaije, 2013), it may be speculated that these latter alterations contribute to the 3-fold higher epoxiconazole adaptation in isolates carrying haplotype I4 (L197V, Del K456-L463). Resistance levels of isolates carrying haplotype I4, however, were observed to be very low compared to isolates carrying more adapted *CYP51* haplotypes that are frequently found in *Z. tritici* (this work; Leroux and Walker, 2011; Stammler et al., 2008b; Cools et al., 2011; Buitrago et al., 2014). Therefore, no further studies were conducted to investigate haplotype I4. Nevertheless, it cannot be excluded that deletion of the whole amino acid region from K456 to L463 becomes relevant when combined with other *CYP51* haplotypes. As most of the new alterations were only found in a single isolate, it is recommended to confirm results of this work in future studies when new isolates with these alterations are found. In addition, molecular techniques such as CRISPR-Cas9 may be used to explore the individual effect of new target site alterations. Compared to traditional genetic approaches, CRISPR-Cas9 was shown to be a simple as well as precise system that facilitates the introduction of desired mutations (reviewed in Deng et al., 2017; reviewed in Schuster and Kahmann, 2019). Introduction of the new *CYP51* mutations into the *CYP51* wild type would enable investigation of their individual effect on DMI sensitivity without the interference of the effect of other mutations.

Alterations with no clear effect on DMI sensitivity of *Z. tritici* such as L50S, S188N, and N513K were commonly identified in modern *Z. tritici* populations (this work; Leroux et al., 2007; Cools and Fraaije, 2013). The widespread occurrence of these alterations is suggested to rely on the emergence of amino acid alterations that affect DMI sensitivity in

CYP51 haplotype backgrounds that already carry either L50S, S188N, or N513K alone or in combination (Cools and Fraaije, 2013). Additionally, it was shown that CYP51 alterations with or without an impact on DMI sensitivity may confer conformational changes in the enzyme that increase selective pressure of triazoles on individual amino acid residues or enable the emergence of other alterations that were otherwise lethal (e.g. alteration I381V) (Mullins et al., 2011; Cools et al., 2010, 2011; Lucas et al., 2015). Consequently, it cannot be excluded that new alterations observed in this work may drive such conformational changes of CYP51 and may enable emergence of new alterations in the future. This assumption, however, was not proven in this work and would require further elucidation using protein modelling studies of CYP51 proteins carrying the new alterations. Although there was no strong impact of the new alterations on epoxiconazole sensitivity in this work, their occurrence indicates that CYP51 can still accept changes in the protein structure without losing its enzymatic function. In addition, it confirms the high level of flexibility of the protein which was already suggested after the emergence of various amino acid alterations in the past (Becher and Wirsel, 2012). Lucas et al. (2015) suggested that the occurrence of alterations in CYP51 is a dynamic process in *Z. tritici* that was expected to continue at the time of publication. This assumption was confirmed in this work based on the identification of newly emerged CYP51 alterations in *Z. tritici* isolates and thus confirm the ongoing evolution of *CYP51* which is likely to continue in the future.

Most of the *CYP51* alterations were found in different combinations leading to distinct *CYP51* haplotypes. An ongoing emergence of new *CYP51* haplotypes was described for *Z. tritici* over three decades (Stammler et al., 2008b; Brunner et al., 2008; Stammler and Semar, 2011; Leroux and Walker, 2011; Lucas et al., 2013; Buitrago et al., 2014). The occurrence of new CYP51 alteration has led simultaneously to the emergence of new *CYP51* haplotypes. Additionally, so far not described *CYP51* haplotypes based on new combinations of already existing alterations were identified during the current work. New combinations of already existing alterations were shown probably to occur due to intragenic recombination (Brunner et al., 2008). The occurrence of new *CYP51* haplotypes confirms the assumption above that *CYP51* evolution is still ongoing and that new combinations of new and/or already existing amino acid alterations continue to occur. This finding was additionally confirmed by studies of Kildea et al. (2019) who also identified new *CYP51* haplotypes based on new combinations of already existing alterations. In total, 57 *CYP51* haplotypes were identified across all investigated isolates from 2016 and 2017, but only 13 haplotypes were identified more frequently (‘frequently found *CYP51* haplotypes’). The 13 ‘frequently found *CYP51* haplotypes’ represented the majority (~89%) of all investigated isolates from routine sensitivity monitoring of 2016 and 2017, whereas the remaining 44 ‘rarely found *CYP51* haplotypes’ only accounted for around 11%. Therefore, the main focus in this discussion is on these ‘frequently found *CYP51* haplotypes’. In the course of this work, a new nomenclature was proposed to describe *CYP51* haplotypes of *Z. tritici*. Due to

the complex evolution and occurrence of different evolutionary pathways for various *CYP51* haplotypes (Lucas et al., 2013) the proposed approach is not intended to reflect *CYP51* evolution. Instead, it is intended to give a more systematic and sustainable description of *CYP51* haplotypes enabling a description of haplotypes in the ongoing evolution of *CYP51* because it can be used and amended continuously in the future. An assignment of the new nomenclature to *CYP51* haplotypes that were detected in the past was published in the course of this work in Huf et al. (2018) and the nomenclature was already used and amended by different authors (Kildea et al., 2019; McDonald et al., 2019) during the time of this work.

5.1.2 Emergence and identification of *CYP51* overexpression and increased efflux activity in *Z. tritici*

CYP51 overexpression has been reported as a mechanism contributing to DMI resistance in plant and human pathogens (Ma and Michailides, 2005; Cools and Fraaije, 2013; reviewed in Ziogas and Malandrakis, 2015). Increased *CYP51* expression was shown to rely either on an increased *CYP51* copy number (Marichal et al., 1997; Coste et al., 2007) or on inserts in the *CYP51* regulatory region (Cools et al., 2013; Ziogas and Malandrakis, 2015). A possible contribution of an increased *CYP51* expression in DMI adaptation of *Z. tritici* was already suggested in 2003 (Stergiopoulos et al., 2003). In 2012, a 120 bp insert in *CYP51*p was identified and shown to be correlated to an increased *CYP51* expression of *Z. tritici* (Cools et al., 2012).

An increased efflux activity in fungal pathogens, commonly referred to as multi-drug-resistance (MDR) in literature, is based on the overexpression of membrane bound efflux transporters and known to reduce sensitivity to unrelated fungicides (reviewed in Gulshan and Moye-Rowley, 2007; Morschhäuser, 2010; Hahn and Leroch, 2015). In 2011, *Z. tritici* isolates were detected that were highly resistant to DMIs but also to other unrelated fungicides suggesting that an active efflux of membrane bound transporters contribute to DMI resistance of *Z. tritici* (Leroux and Walker, 2011). In 2015, RNA sequencing revealed that the overexpression of the MFS1 transporter is responsible for an increased efflux activity in *Z. tritici*. This overexpression was conferred by different insertions in *MFS1*p (Omrane et al., 2015, 2017).

In the current study, the *CYP51*p and *MFS1*p of all isolates was investigated for the presence of inserts in order to identify the occurrence and frequency of *CYP51* overexpression and an increased efflux activity in *Z. tritici* across Europe.

Across all isolates, four different *CYP51*p inserts and seven different *MFS1*p inserts were identified. Length of inserts in comparison to inserts described in literature are summarised in Table 20.

Table 20: Summary of inserts and insertion sites identified in *CYP51*p and *MFS1*p of *Z. tritici* in this work and comparison to inserts in the literature, when available. The sign '-' shows that this insert was identified for the first time in the current work.

Promotor region	Length of insert (bp) / insertion site (bp upstream of start codon in IPO323) in this work	Length of insert (bp) / insertion site in literature (Source)
CYP51	121 / -83	120 / -83 (1)
	863 / - 207	862 / -205 (4)
	868 / -184	866 / -184 (2)
	300 / -118	-
MFS1	150 / -214	150 / -201 (3)
	267 / -468	-
	308 / -468	-
	338 / -468	338 / -455 (3)
	369 / -468	369 / -455 (3)
	377 / -468	-
	519 / -402	519 / -401 (2)

[1]Cools et al., 2012; [2]Omrane et al., 2015; [3]Omrane et al., 2017; [4]Kildea et al., 2019

*MFS1*p inserts identified in this work showed similar sequences and length compared to *MFS1*p inserts described by Omrane et al. (2015, 2017). *CYP51*p inserts were almost identical in length and sequence compared to the 120 (Cools et al., 2012), 862 (Kildea et al., 2019), and 866 bp inserts (Omrane et al., 2015) described in literature. Nevertheless, small differences of one or two nucleobases in length were observed. Additionally, for some inserts, differences in the insertion sites between inserts of this work and inserts in literature were observed. Sequence duplications (target site duplications) were observed in all isolates carrying an insert in *MFS1*p or *CYP51*p except for the 121 bp *CYP51*p insert. In contrast to most parts of this work, the term target site is not restricted to a gene in this chapter but can also refer to a promotor. Emergence of target site duplications in association with insertions will be discussed later in this chapter. However, at this point it should be mentioned that based on the sequence alignment alone, insertion sites of the *MFS1*p or *CYP51*p inserts could not be determined with a high level of certainty from this work. It was not clarified if insertion of the inserts occurred left- or right-hand site of the duplicated sequence upon insertion in the native gene. Therefore, it is possible that insertion sites shown here have to be shifted by the number of nucleobases of the respective sequence duplication. Especially in the case of the 338 bp *MFS1*p insert, insertion sites may have to be shifted by a couple of nucleobases. However, precise assignment of these insertion sites was no issue in this work and would need further bioinformatical investigations in the future. Nevertheless, these circumstances may explain differences between insertion sites of inserts in this work and published in literature. Small differences in length or position of

insertion sites between inserts identified in this work and already published ones may also result from a high genetic variability known for *Z. tritici* field isolates (Schnieder et al., 2001; reviewed in Simón et al., 2012). Furthermore, as sequencing was performed based on PCR products, methodological issues during sequencing or afterwards during sequence alignment may contribute to these small differences, because alignment of sequences is highly dependent on the algorithm used and on the number of aligned sequences. Due to these reasons, it was assumed that small differences between inserts identified in this work and described in literature are negligible and that inserts identified in this work are equal to inserts identified in literature. Therefore, the *CYP51*p inserts with a length of 121, 863, and 868 bp identified in this work will be described hereafter in the discussion as 120, 862, and 866 bp inserts.

Compared to the WT *CYP51*p, the 120 bp insert in *CYP51*p was correlated to a 10- to 40-fold constitutive overexpression of *CYP51* that was not induced by addition of epoxiconazole (Cools et al., 2012; Kildea et al., 2019). *CYP51*p inserts associated with *CYP51* overexpression were identified also in other plant pathogens. For example, a 126 bp tandem repeated sequence or a 199 bp insert confer a higher *PdCYP51* expression in *P. digitatum* (Hamamoto et al., 2000; Sun et al., 2013). A 533 bp insert confers a 18-fold constitutive *ViCYP51* overexpression in *V. inaequalis* (Schnabel and Jones, 2001) and a 65 bp repetitive element (named *mona*) in *Monilinia fructicola* results in a 5- to 11-fold higher increased constitutive *MfCYP51* expression (Luo and Schnabel, 2008). In contrast to the 120 bp insert, it is assumed that the 862 and 866 bp inserts alone do not alter *CYP51* expression of *Z. tritici* (Omrane et al., 2015; Kildea et al., 2019). A slight increase in *CYP51* expression of isolates carrying either the 862 or 866 bp insert was observed upon addition of epoxiconazole, however, expression levels were still at a low level compared to the wild type (Kildea et al., 2019). The molecular effect of the newly identified 300 bp insert on *CYP51* expression has not been tested during this work and analysing *CYP51* mRNA levels in isolates carrying the 300 bp insert is recommended for future research.

Molecular studies revealed that the 150, 338, 369, and 519 bp inserts in *MFS1*p are correlated to an increased expression of *MFS1* with highest effect exerted by the 519 bp insert (Omrane et al., 2017). In *Z. tritici*, overexpression of *MFS1* varied between 28- to 50-times in strains showing an MDR profile compared to sensitive strains (Omrane et al., 2015). A comparison to the effect of *MFS1*p inserts on *MFS1* expression in other fungal plant pathogens is critical because increased efflux based on *MFS1* overexpression is not a widespread phenomenon so far. Besides *Z. tritici*, only in *B. cinerea*, one of the best studied fungal plant pathogens concerning increased efflux activity, overexpression of an MFS transporter was reported. In *B. cinerea*, two types of a promotor insert lead to an over 600-times increased constitutive *BcMFSM2* expression (Kretschmer et al., 2009; Mernke et al., 2011). Molecular examination of the *MFS1* expression level in *Z. tritici* isolates carrying the

newly described 267, 369, and 377 bp inserts was not performed in this work but is recommended for future studies.

As described above, target site duplications directly adjacent to all inserts in *CYP51*p (except the 120 bp insert) and *MFS1*p were identified. These target site duplications may yield information about the origin of these inserts. Insertion of transposable elements (TEs) almost always results in short target site duplications based on the process of transposition. This process includes cleavage of the target molecule that results in cohesive (sticky) ends and subsequent filling of unpaired oligonucleotides after insertion of the TE (Shapiro, 1979; Kleckner, 1981; Kempken and Kück, 1998; Wicker et al., 2007). In filamentous fungi it was observed that these target site duplications can vary between 2 to 17 bp dependent on the organism and the type of transposon (reviewed in Kempken and Kück, 1998). Therefore, target site duplications identified at insertion sites of *CYP51*p and *MFS1*p inserts may indicate that these inserts derive from transposition of transposable elements (or parts of those). This assumption is supported by the fact that BLASTn searches with the 120, 862, and 866 bp inserts of *CYP51*p and with the 150 and 519 bp insert of *MFS1*p revealed multiple regions showing high sequence similarities throughout the *Z. tritici* genome (Cools et al., 2012, Omrane et al., 2015, 2017; Kildea et al., 2019). Indeed, the 519 bp insert in *MFS1*p region was described to be a relic of a Class 1 Ty1/Copia retrotransposon that is still active in *Z. tritici* (Omrane et al., 2015). Additionally, a high number of sequence similarities between the 338 and 369 bp inserts to other sequences of IPO323 were identified and consequently it was suggested that these inserts may also derive from a repeated element or part of it (Omrane et al., 2017). As the newly identified inserts of *MFS1*p (267, 308, and 377 bp) showed a high identity to each other and to the 338 and 369 bp insert, it may be speculated that they derive from the same repeated element. Interestingly, these type II inserts from *MFS1*p showed a high sequence identity to the newly identified 300 bp insert in *CYP51*p (results not shown). This finding supports the suggestion that these inserts represent repetitive elements derived from a TE. In many other plant pathogens it was already described that inserts in *CYP51*p conferring *CYP51* overexpression often belong to TEs and contain strong promotor sequences (Schnabel and Jones, 2001; Luo and Schnabel, 2008; Cools et al., 2013; Sun et al., 2013; Ziogas and Malandrakis, 2015). In *Z. tritici*, at least 17% of the genome were estimated to be repetitive and 70% of the total repetitive content was shown to be represented by Class I TEs (amplify via RNA intermediate) (Dhillon et al., 2014). TEs contribute to genomic rearrangements, relocation of genes, generation of new genes and can alter regulation of gene expression. Therefore, TEs are suggested to play a relevant role in the evolutionary adaptation of living organisms as they are the main source of genome plasticity (reviewed in Gozukirmizi, 2019). Further clarification about the role of TEs in gene and genome evolution may help to understand genome plasticity and its effect on the adaptation of organisms, among them *Z. tritici*, to changing environments (Kempken and Kück, 1998; Dhillon et al., 2014; Gozukirmizi, 2019).

Therefore, future research about the origin of *CYP51*p and *MFS1*p inserts and analysing if they derive from transposable elements may be helpful to understand evolutionary adaptation of *Z. tritici*.

The insertion of TEs inside or outside of the promotor may introduce regulatory elements that impact gene expression (reviewed in Girard and Freeling, 1999; Rebollo et al., 2012; Krishnan et al., 2018). Different effects of inserts in *MFS1*p on gene expression were suggested to occur due to different upstream activation sequences (UAAs) that may recruit transcription activators or coactivators and thus increase transcription (Hahn and Young, 2011; Omrane et al., 2017). In fact, different regulatory elements were identified in the 519 and 338/369 bp inserts, whereas no regulatory elements were found in the 150 bp insert. Consequently, it was speculated that the 519 and 338/369 bp inserts induce overexpression on their own, whereas the 150 bp insert may function through the release of inhibition of *MFS1* transcription (Omrane et al., 2017). Research on how inserts in *MFS1*p drive overexpression, however, is just at its beginning and further studies are needed to fully understand this phenomenon as well as different levels of overexpression. Additionally, *CYP51*p inserts were not investigated for regulatory elements so far. Such studies may help to understand why the 120 bp insert is responsible for *CYP51* overexpression (Cools et al., 2012), whereas the inserts around ~900 bp do not alter *CYP51* expression (Kildea et al., 2019).

5.2 DMI resistance mechanisms of *Z. tritici*: Impact on DMI sensitivity, evolution, and prevalence across Europe

5.2.1 *CYP51* haplotypes of *Z. tritici*

In vitro sensitivity studies (microtiter tests) revealed that all isolates with ‘frequently found *CYP51* haplotypes’ showed a reduced sensitivity to epoxiconazole compared to wild type isolates. A 20- to 350-fold higher adaptation dependent on the *CYP51* haplotype was observed. Differences in epoxiconazole sensitivity between isolates with these *CYP51* haplotypes in this work confirm effects of *CYP51* alterations that have been reported before (Leroux and Walker, 2011; Cools et al., 2011, 2013; Cools and Fraaije, 2013). An additive effect of alteration D134G and V136A on adaptation to epoxiconazole was observed in this work. As alteration D134G always occurs in combination with other alterations, the individual effect of this alteration is still unclear (Cools and Fraaije, 2013). However, the effect of alteration V136A on a reduced DMI sensitivity has been proven before (Leroux and Walker, 2011; Cools et al., 2011; Cools and Fraaije, 2013) and it is likely that higher adaptation of haplotype E4 compared to haplotype C8 (only differing in alteration D134G

and V136A) is driven by alteration V136A. In this work, the highest adaptation to epoxiconazole was correlated to alteration S524T. This finding confirms previous results which demonstrate that alteration S524T further affects sensitivity to all triazoles, among them epoxiconazole and prothioconazole, the two most effective and widely used triazoles in the past years (Cools et al., 2011; Lucas et al., 2015; Kirikyali et al., 2017; Kildea et al., 2019). In general, different *CYP51* haplotypes were shown to affect DMI sensitivity to some triazoles differently (Leroux et al., 2007; Fraaije et al., 2007; Leroux and Walker, 2011; Buitrago et al., 2014) and sensitivity of 'frequently found *CYP51* haplotypes' to different triazoles will be discussed at a later point in this work (see chapter 5.3).

A large sensitivity range for epoxiconazole was identified across all isolates with 'rarely found *CYP51* haplotypes'. Their low frequency identified in 2016 and 2017 indicate a minor importance of these haplotypes in DMI adaptation of *Z. tritici* compared to 'frequently found *CYP51* haplotypes'. Nevertheless, as some of these *CYP51* haplotypes also express high adaptation towards epoxiconazole, especially those carrying alteration S524T, their frequency may increase in the future under continued DMI selection pressure.

A selective replacement in the occurrence and frequency of increasingly DMI adapted *CYP51* haplotypes was observed in *Z. tritici* over time and space during the last three decades based on widespread use of different DMI fungicides (Gisi et al., 2005; Leroux et al., 2007; Brunner et al., 2008; Stammler et al., 2008b; Stammler and Semar, 2011; Leroux and Walker, 2011; Lucas et al., 2013; Buitrago et al., 2014; Lucas et al., 2015). A heterogenous frequency and distribution of 'frequently found *CYP51* haplotypes' was observed across Europe in 2016 and 2017. Some of these haplotypes were detected in each investigated country, whereas others were only found in a few countries. Isolates carrying the wild type *CYP51* were only detected in the Eastern European countries Bulgaria, Russia, and Slovakia. In previous studies it was shown that already in 2004 and 2007 no *CYP51* wild type isolates were detected anymore in Central and Western European countries such as France, Germany, and UK and that they were only found in Eastern European countries as well as in Sweden (Chassot et al., 2008; Stammler et al., 2008b). Since 2002, an increase in the frequency of isolates carrying alterations L50S, I381V and Y461H, as found in the C8 haplotype, or isolates with L50S, S188N, A379G, I381V, DelG460 and DelY461, as observed in haplotype G1 (except positions of deletions that are DelY459 and DelG460), were found. The *Z. tritici* populations of 2006 were mainly composed of these isolates and isolates carrying alterations at position V136 and/or alterations at position Y459, G460, and Y461. From 2007 to 2010, the *Z. tritici* populations across Europe (except for Ireland) were dominated by isolates carrying alteration I381V in combination with alteration of Y461 (as found in C8) or alterations A379G, I381V, DelY459, and DelG460 (as found in G1) (Stammler et al., 2008b; Stammler and Semar, 2011; Buitrago et al., 2014). From 2009 to 2011, a slow decrease in the frequency of *CYP51* haplotype C8 was observed, whereas the frequency of isolates with haplotype E4 (L50S,

D134G, V13A, I381V, Y461H) increased (Buitrago et al., 2014). In 2015, haplotypes E4 and F2 were most frequently found in an isolate collection across UK, Germany, and France, followed by haplotype C8 (Kirikyali et al., 2017). During the present study, it was shown that haplotype E4, expressing moderate adaptation to epoxiconazole, was identified most frequently across all tested isolates and was detected in almost every country of Central (except for Czech Republic and Slovakia) and Western Europe (except for Ireland in 2017). This finding confirms the increasing importance of haplotype E4. Additionally, a high frequency of haplotype F2 and C8 was confirmed, but haplotype C8 was mainly detected in France, in Central European countries, and especially in Southern and Eastern European countries.

A west-to-east gradient in the DMI adaptation of *Z. tritici* across Europe has been reported previously in several studies (Strobel et al., 2017; Heick et al., 2017a; Jørgensen et al., 2018) and was also confirmed in this work for 2016 and 2017. The Eastern European countries were mainly composed of isolates with haplotypes C8 and G1 exerting highest sensitivity to epoxiconazole among all 'frequently found *CYP51* haplotypes'. Based on historical data about *CYP51* haplotype occurrence described above, the composition of the *Z. tritici* populations in Eastern European countries in 2016 and 2017 is comparable to the composition of the *Z. tritici* populations in Central Europe around 10 years ago. In 2016 and 2017, *Z. tritici* populations of France, Germany, and Poland were mainly composed of haplotypes C8 and G1, together with moderately adapted haplotypes E4 and F2. The frequency of isolates that carry haplotypes with alteration S524T, showing highest adaptation towards epoxiconazole, is higher in France and Germany in 2016 and 2017 compared to previous studies (Stammler et al., 2008b; Stammler and Semar, 2011). The higher frequency of haplotypes with moderate adaptation and higher frequency of haplotypes with alteration S524T in France and some countries of Central Europe demonstrates that evolution towards a reduced DMI sensitivity of *Z. tritici* is still ongoing. A further increase in the adaptation of the *Z. tritici* populations was observed in the Western European countries, Ireland, UK, and the Netherlands compared to France, Germany, and Poland. In Ireland, UK, and the Netherlands, a higher frequency of *CYP51* haplotypes with alteration S524T was found. This alteration was described for the first time in an isolate from UK from 2007 (Stammler et al., 2008b). Afterwards, a high frequency of isolates with alteration S524T was observed in Ireland in 2010, whereas it was lower in the UK (Stammler and Semar, 2011). In the current study, *Z. tritici* populations in Ireland, UK, and the Netherlands were mainly composed of haplotypes conferring moderate or high epoxiconazole adaptation indicating a further shift in DMI sensitivity in these countries compared to previous studies (Stammler et al., 2008b; Stammler and Semar, 2011).

On one side, the west-to-east gradient in DMI adaptation of *Z. tritici* may be explained based on the historical origin of DMI resistance. Brunner et al. (2008) proposed that *CYP51* alterations or new combinations of those alterations conferring DMI resistance arose locally

in North-West Europe and were then disseminated eastwards through wind-dispersed ascospores. A selective replacement of *CYP51* haplotypes by *CYP51* haplotypes with higher adaptation to DMIs followed (Brunner et al., 2008). On the other side, variability in disease prevalence and fungicide use intensity may also play a role in the west-east adaptation gradient (Stammler et al., 2008b; Strobel et al., 2017; Jørgensen et al., 2018). Intensive wheat production and a high disease prevalence is known for Northern France, Germany, and UK (Jørgensen et al., 2014; Fones and Gurr, 2015). Among these countries, fungicide input in winter wheat is highest in UK with often 3 to 4 fungicide applications (Jørgensen et al., 2014). Both together, the intensive use of fungicides and high disease prevalence most probably drives higher adaptation of *Z. tritici* populations to DMIs in countries as UK and Ireland. In contrast, agricultural production is more extensive in Eastern European countries and fungicide selection pressure is not as high as in Western European countries and some countries of Central Europe. A west-to-east gradient was already observed in the resistance emergence of *Z. tritici* to QoIs and SDHIs (Torriani et al., 2009; Jørgensen et al., 2017a; FRAC, 2019). Alteration G143A conferring QoI-resistance of *Z. tritici* was shown to have evolved independently through at least four mutational events and was subsequently distributed from West to East Europe through wind dispersal of ascospores (Torriani et al., 2009). These patterns support the assumption that increasingly adapted *CYP51* haplotypes emerge in countries with a high fungicide selection pressure and high disease prevalence and subsequently spread across Europe.

Compared to the development of QoI resistance in *Z. tritici*, evolution of DMI resistance proceeded much more slowly and gradually (reviewed in Jørgensen et al., 2019). As described previously in this chapter, a stepwise replacement of *CYP51* haplotypes was observed over many years until the distribution of *CYP51* haplotypes presently described for 2016 and 2017. In this work, to test whether changes continue to emerge slowly over a short period of time, the composition of *CYP51* haplotypes and their frequency between 2016 and 2017 was compared for chosen countries that were tested in both years. Results demonstrated that development in occurrence and frequency of *CYP51* haplotypes between 2016 and 2017 was dependent on the country and no general pattern was observed across all countries. In Ireland, a further shift towards a reduced epoxiconazole sensitivity was indicated in 2017. This finding confirms the gradual shift in the sensitivity of the Irish *Z. tritici* population reported steadily during the past years (Stammler and Semar, 2011; Kildea et al., 2019). In Germany and France, at least a slight further shift towards a reduced DMI sensitivity was indicated, whereas in Poland and UK no clear further shift was observed. An important point to mention here is the fact that in 2016 not all isolates from the European sensitivity monitoring were tested for their *CYP51* haplotypes during this work but rather a selection of isolates. In contrast to this, all isolates from the European sensitivity monitoring were investigated for their *CYP51* haplotypes in 2017. Therefore, results of 2017 may be more representative and differences in sample size between both years may

partially explain why no further shift could be detected at molecular basis for Poland and UK. Such a shift could be expected based on *CYP51* haplotype evolution in past years. Analysing a comparable number of isolates from the same locations within a country is recommended for future studies, if it is intended to compare changes in *CYP51* haplotype frequency over a short period of time between individual consecutive years.

In the past, recurring phases of shifts in sensitivity and a stabilised situation, that often lasted two to three years, were observed for *Z. tritici* (FRAC, 2019). Taking all results of this work into account compared to previous studies (Stammler et al., 2008b; Stammler and Semar, 2011; Buitrago et al., 2014), a further evolution in the frequency and distribution of increasingly adapted *CYP51* haplotypes was observed. This demonstrates an ongoing shift towards a reduced DMI sensitivity in regional populations of *Z. tritici* across Europe, especially in intensive wheat growing areas. The previously reported west-to-east gradient in DMI adaptation of *Z. tritici* was confirmed across Europe in this work. At a worldwide scale DMI adaptation in *Z. tritici* is most advanced in Europe. Compared to Europe, DMI resistance development is less advanced in other countries such as Tunisia (Taher et al., 2014) as well as USA and Australia in which beginning emergence of *CYP51* haplotypes that affect DMI sensitivity was reported (Estep et al., 2015; Milgate et al., 2016). These differences may result from different intensities in the use of DMIs (Estep et al., 2015) and different disease severities of *Z. tritici* (Goodwin, 2019).

5.2.2 *CYP51* overexpression in *Z. tritici*

The epoxiconazole sensitivity of *Z. tritici* isolates carrying the same *CYP51* haplotype but differing in the presence or absence of a *CYP51*p insert was compared in order to analyse the impact of the 120, 862, and 866 bp inserts in *CYP51*p on DMI sensitivity. On average, a 5- to 8-fold reduction in sensitivity was associated with the 120 bp insert. Compared to isolates with *CYP51* haplotype F2 and a WT *CYP51*p, isolates carrying haplotype F2 and the 120 bp insert in *CYP51*p showed on average a 7-fold higher epoxiconazole adaptation. Adaptation across all of these isolates varied between 2- to 16-fold. These results reflect the findings of Cools et al. (2012) who reported that the 120 bp insert confers *CYP51* overexpression. In their studies, isolates carrying a F2 haplotype and the 120 bp insert showed a 7- to 16-fold reduction in sensitivity to four different triazoles. Additionally, it was found that for most triazoles tested there was an increasing reduction in sensitivity with increasing levels of *CYP51* expression which varied between a 10- to 40-fold overexpression (Cools et al., 2012). Although this finding was based on only 3 isolates, the results may indicate a positive correlation of *CYP51* quantity and a lower DMI sensitivity. This in turn may account for the variation in sensitivity of isolates carrying haplotype F2 and the 120 bp insert in *CYP51*p. On the other hand, natural variability in azole sensitivity of *Z. tritici* (Gisi et al., 1997; Stergiopoulos et al., 2003) may contribute to sensitivity variation

of these isolates. Moreover, it cannot be excluded that other mechanisms not investigated in the current work, as for example an increased efflux activity due to membrane transporters other than MFS1, may play a role. Determining the quantity of *CYP51* mRNA in several F2 haplotypes expressing varying adaptation to epoxiconazole could help to further elucidate the relationship between *CYP51* quantity and DMI adaptation.

Across all investigated isolates carrying a ~900 bp insert in *CYP51*p, no clear trend in the impact on epoxiconazole sensitivity of *Z. tritici* was observed compared to isolates carrying the same *CYP51* haplotype and a WT *CYP51*p. The effect correlated to the ~900 bp insert was either negligible or low (1.5- to 3.5-fold reduction in sensitivity). A low impact on epoxiconazole adaptation (~3-fold reduction in sensitivity) was also observed for the 300 bp insert in *CYP51*p. A 2.5-fold difference in sensitivity was observed across wild type isolates investigated in this work (described in chapter 4.3). Moreover, a high variability in azole sensitivity of *Z. tritici* has been reported before (Gisi et al., 1997; Stergiopoulos et al., 2003). Therefore, it is challenging to determine whether such minor differences in sensitivity can be attributed to natural variation in triazole sensitivity or to the ~900 bp or 300 bp inserts in *CYP51*p. Based on molecular studies that showed no or only slight alteration of *CYP51* expression conferred by ~900 bp inserts (Heick Wieczorek, 2017; Kildea et al., 2019), no or only slight effects on epoxiconazole sensitivity would be expected. Quantification of *CYP51* mRNA levels in isolates with a ~900 bp insert in *CYP51*p showing slightly higher adaptation in comparison to isolates carrying the same insert but without increased adaptation could help to clarify if increased *CYP51* expression drives an adaptation in these isolates. Nevertheless, compared to the impact correlated to the 120 bp insert, the previous assumption, that the ~900 bp inserts alone only confer a low impact (if at all) on triazole sensitivity (Leroux and Walker, 2011; Lucas et al., 2015; Heick Wieczorek, 2017; Kildea et al., 2019) was confirmed. Determining *CYP51* mRNA levels in isolates carrying the 300 bp insert would help to correlate the impact of this insert to epoxiconazole sensitivity. In general, further sensitivity studies with a higher number of isolates are recommended for *CYP51* haplotypes for which only single isolates with a *CYP51*p insert were available and could be tested (e.g. the 120 bp insert combined to haplotype C2, D7, or G1, or the 300 bp insert). The prerequisite for this, however, is an increase in the frequency of these isolates in field populations of *Z. tritici*. Additionally, introduction of promotor inserts into the wild type promotor with molecular techniques such as CRISPR-Cas9 (reviewed in Deng et al., 2017; reviewed in Schuster and Kahmann, 2019) would be helpful. Based on this method, isolates with similar genetic background that only differ in the presence or absence of a promotor insert could be used to determine the individual effect of promotor inserts on *CYP51* overexpression and DMI sensitivity. Thereby, interference of other resistance mechanisms or natural variation between field isolates would be excluded.

Compared to some target site alterations, *CYP51* overexpression in several pathogens was shown to reduce sensitivity to all DMIs tested at the time of publication, thus to confer cross-

resistance (Cools et al., 2012, 2013; Ziogas and Malandrakis, 2015). Therefore, it can be assumed that results obtained for epoxiconazole in this work would most likely also account for other common DMIs. As described in chapter 4.5.2, in comparison to resistance levels of isolates carrying different *CYP51* haplotypes and no *CYP51* overexpression (9- to 166-fold higher adaptation compared to *CYP51* wild type), the impact on epoxiconazole sensitivity of the ~900 and 300 bp inserts appears of minor relevance. Additionally, as epoxiconazole adaptation associated with the 120 bp insert was shown to be on average 5- to 8-fold higher compared to isolates with the same *CYP51* haplotype and a WT *CYP51*p, the potential impact associated with inserts in *CYP51*p seems lower than the combination of *CYP51* alterations. This assumption is confirmed by previous authors who reported for several pathogens that resistance levels conferred by *CYP51* overexpression were lower compared to resistance levels correlated to target site alterations (Cools et al., 2013; Ziogas and Malandrakis, 2015). Nevertheless, as *CYP51* overexpression based on the 120 bp insert in *CYP51*p exhibits an additive effect on DMI adaptation (this work; Cools et al., 2012; Kildea et al., 2019) and confers cross-resistance between most common DMIs (Cools et al., 2012, 2013; Ziogas and Malandrakis, 2015; Kildea et al., 2019), monitoring of the occurrence and frequency of this mechanism in field populations of *Z. tritici* is required.

Occurrence and frequency of *CYP51* overexpression in *Z. tritici* was investigated in this study across Europe in 2016 and 2017. Isolates overexpressing *CYP51* associated with the 120 bp insert in *CYP51*p were identified in almost every country of Central and Western Europe in 2016 and 2017. In 2016, frequencies of isolates exhibiting *CYP51* overexpression were low to moderate (~4 to 25%) in these countries. In UK, the 120 bp insert in *CYP51*p was detected for the first time in isolates from 2009 (Cools et al., 2012). Afterwards, this insert was detected in two isolates from UK in 2010 and 2011 (Buitrago et al., 2014) as well as in isolates from the Nordic-Baltic area in 2014 (Heick et al., 2017a). The frequency of the 120 bp insert across Europe now observed in 2016 has therefore increased compared with earlier findings. A higher number of isolates carrying the 120 bp insert compared to previous studies described above was already reported for France, Germany and UK in 2015 (Kirikyali et al., 2017) and an increase in the frequency of this insert of up to 14% was reported in Ireland in 2015 compared to 2011 (Kildea et al., 2019). In addition, a further increase in the frequency of the 120 bp insert was detected in 2017 compared to 2016 in this work. An increase was observed in the UK, Poland, and Germany and slightly in France. Although most isolates carrying the 120 bp insert only express moderate DMI adaptation *in vitro* among all isolates with 'frequently found *CYP51* haplotypes', this increase supports the previous assumption of Kildea et al. (2019) that *CYP51* overexpression confers a selective advantage for *Z. tritici* isolates upon DMI treatment in the field. It may be speculated that the main driver of this selective advantage is cross-resistance for most common DMIs conferred by *CYP51* overexpression (Leroux et al., 2007; Fraaije et al., 2007; Leroux and Walker, 2011; Buitrago et al., 2014). In contrast to the 120 bp insert, the majority

of countries showed high frequencies of the ~900 bp inserts in *Z. tritici* populations from 2016 and 2017 investigated in this work. These results confirm an increase in the frequency and now widespread distribution of these inserts in Europe that was reported before for Ireland and the Nordic-Baltic area compared to previous years (Heick et al., 2017a; Kildea et al., 2019).

Interestingly, as observed for the distribution of *CYP51* haplotypes across Europe (lower adaptation in the East) (this work; Strobel et al., 2017; Heick et al., 2017a; Jørgensen et al., 2018), lower frequencies of *CYP51*p inserts were identified in Eastern European countries and Italy compared to Western and two Central European countries (Germany and Poland). In this work, as in previous studies (Cools et al., 2012; Buitrago et al., 2014; Kildea et al., 2019) the occurrence of the 120 bp insert was generally restricted to isolates carrying *CYP51* haplotype F2. In contrast, a ~900 bp insert was combined to several *CYP51* haplotypes. As described for the emergence and distribution of *CYP51* alterations from West to East Europe (Brunner et al., 2008), the fact that the 120 bp insert in *CYP51*p is mostly restricted to the F2 haplotype, may indicate that this insertion occurred once in the evolution of *Z. tritici* and then was spread within the population. It may be speculated that the same holds true for the 862 and 866 bp inserts. Although combinations of these inserts with different *CYP51* haplotypes were observed, a pattern still seems to exist. Sequencing results for promotor fragments with a ~900 bp insert in this work confirmed results from Kildea et al. (2019) who reported that the 866 bp insert is always combined to *CYP51* haplotypes with alterations Y461S/H. In contrast, the 862 bp insert is always combined to *CYP51* haplotypes with deletion of Y459 and G460 (except for one isolate). It was suggested that the evolutionary history of both groups is slightly different (Kildea et al., 2019) indicating that insertion occurred once. The pattern of *CYP51* alterations of haplotypes that have either the 862 or 866 bp *CYP51*p insert in common are more similar to each other compared to the contrasting group (results not shown). From this finding it may be deduced that new alterations evolved in *CYP51* haplotypes which were already combined to one of these *CYP51*p inserts. In contrast to the 120 bp insert, combination of a ~900 bp insert was detected with almost all 'frequently found *CYP51* haplotypes' (with exception of F3) in this work. This indicates that development of alterations in *CYP51* already combined to a *CYP51*p insert has happened more frequently for *CYP51* haplotypes previously combined with a ~900 bp insert compared to the 120 bp insert. Combinations of a ~900 bp insert with almost all 'frequently found *CYP51*' haplotypes may explain the wider distribution and higher frequencies of these inserts compared to the 120 bp insert. As ~900 bp inserts were not correlated to substantial impacts on DMI sensitivity (this work; Kildea et al., 2019), selection occurred probably in parallel with the corresponding *CYP51* haplotypes. High frequency of the WT *CYP51*p in Eastern Europe may support the assumption that combination of *CYP51*p inserts to *CYP51* haplotypes emerged in Western Europe and afterwards distribution of these genotypes occurred from West to East associated with the

distribution of the *CYP51* haplotypes. Differences in fungicide use patterns and disease severity, however, as described for the distribution of *CYP51* haplotypes (see chapter 5.2.1), may have also contributed to regional differences and distribution of *CYP51*p inserts observed between Western or Central Europe and Eastern Europe.

In this work, for the first time the 120 bp insert was identified in single isolates in combination to *CYP51* haplotypes other than F2. These *CYP51* haplotypes and their CYP51 alterations are shown in Table 21.

Table 21: *CYP51* haplotypes combined to the 120 bp insert in *CYP51*p.

***CYP51* haplotype**	**Amino acid alterations**
C2	L50S, I381V, Y459S
D7	L50S, V136A, Y461S, S524T
F2	L50S, S188N, I381V, Del Y459, Del G460, N513K
F6	L50S, V136A, S188N, Del Y459, Del G460, S524T
G1	L50S, S188N, A379G, I381V, Del Y459, Del G460, N513K
H3	L50S, S188N, N284H, A379G, I381V, Del Y459, Del G460, N513K

Only two of these *CYP51* haplotypes (G1 and H3) share an evolutionary pathway with the haplotype F2 (Lucas et al., 2013) and may have evolved through the emergence of new alterations in the F2 *CYP51* haplotype background already combined to the 120 bp insert in the promotor. The other *CYP51* haplotypes combined to the 120 bp insert in *CYP51*p (C2, D7, and F6) do not show similarities in their evolution (Lucas et al., 2013). Therefore, emergence of these genotypes through successive emergence of alterations in a *CYP51* haplotype background already combined to the promotor insert seems unlikely. The emergence of the 120 bp insert combined to these *CYP51* haplotypes may be explained due to intragenic recombination that has been previously described by Brunner et al. (2008) for the combination of alterations in CYP51.

Previous authors predicted that if frequency of *CYP51* overexpression increases in *Z. tritici* populations, probability of recombination of the overexpression trait with more evolved *CYP51* haplotypes or emergence of new alterations in the overexpression background may increase (Lucas et al., 2015; Kildea et al., 2019). This assumption can be supported based on results of the current work. It is possible that further selection of isolates with *CYP51* overexpression (based on 120 bp insert in *CYP51*p) in combination with more evolved *CYP51* haplotypes may lead to a further reduction in sensitivity of *Z. tritici* to DMIs (this work; Lucas et al., 2015; Kildea et al., 2019). Therefore, it is important that further monitoring studies should be carried out to monitor whether the frequency of the 120 bp insert combined to the F2 haplotype or to other *CYP51* haplotypes increases. The possible impact of *CYP51* overexpression on field performance of DMIs will be discussed in chapter 5.4.

5.2.3 Increased efflux activity in *Z. tritici*

In many studies (Kretschmer et al., 2009; Leroux and Walker, 2011; Heick et al., 2017a), tolnaftate, a squalene-epoxidase inhibitor used in dermatophytes (Barrett-Bee et al., 1986) has been used as a common indicator for the phenotypical identification of an increased efflux activity. Tolnaftate has no role in agriculture and therefore is not linked to specific resistances of *Z. tritici* (Omrane et al., 2017). The designation for *MFS1*p inserts of Omrane et al. (2017) was accepted and amended in order to describe *MFS1*p inserts in the course of this work. The 150 bp insert was designated as type III, the 267, 308, 338, 369, 377 bp inserts as type II and the 519 bp insert as type I. Tolnaftate sensitivity of *Z. tritici* isolates carrying different inserts in *MFS1*p was tested to identify their impact on an increased efflux activity. In comparison to isolates with a WT *MFS1*p a reduced tolnaftate sensitivity was observed for all isolates carrying an *MFS1*p insert (except for the 308 bp insert that was not tested). Based on tolnaftate adaptation, the highest increased efflux was associated with the 519 and 369 bp inserts, with a lower effect correlated to the 150, 267, 338, and 377 bp inserts. These results partially confirm molecular results from Omrane et al. (2017) who reported that all tested *MFS1*p inserts were correlated to *MFS1* overexpression. However, expression levels conferred by the 519 bp insert were significantly higher than by the 150, 338, or 369 bp inserts (Omrane et al., 2017). Based on these results, it would not be expected that isolates with a 369 bp insert would behave differentially than isolates carrying another type II insert. Unexpectedly however, across all isolates carrying a 369 bp insert in the current work, significant differences in tolnaftate sensitivity were observed compared to isolates with a WT *MFS1*p, whereas no significant differences were observed for isolates with remaining type II inserts (267, 338, 377 bp). It is possible that the low number of tested isolates with the 369 bp insert resulted in a high impact of the sensitivity of outliers which may contribute to these differences in significance. It is not always possible to obtain a representative number of isolates to investigate all effects under consideration. Analysing tolnaftate sensitivity of more isolates carrying a type II insert, especially the 369, 377, or 308 bp insert, would help to further characterise the effect of those inserts. A combination of these analyses with the molecular determination of *MFS1* expression level could help to further correlate increased *MFS1* expression to phenotypical sensitivity. Such studies would be especially valuable for characterisation of the 267, 308 and 377 bp inserts as their effect on *MFS1* expression has not been investigated via molecular studies to date.

The epoxiconazole sensitivity of isolates carrying the same *CYP51* haplotype but differing in the presence or absence of an insert in *MFS1*p was compared in order to analyse if and to which extend higher tolnaftate adaptation implying increased efflux activity is reflected in DMI sensitivity of *Z. tritici*. Impacts associated with different *MFS1*p inserts based on tolnaftate sensitivity were correlated with epoxiconazole sensitivity of isolates in most cases. Across all *MFS1*p inserts, the highest impact on epoxiconazole sensitivity (~2- to 8-fold reduction in sensitivity) was correlated to the 519 bp insert (type I). In single cases a low

impact was correlated to the 519 bp insert. As this low impact was observed for isolates carrying highly adapted *CYP51* haplotypes, it may be speculated that the impact of the 519 bp insert is “masked” by the already high adaptation driven by *CYP51* alterations. Type III (150 bp) and type II (267, 308, 338, 369, or 377 bp) inserts were associated with lower effects on epoxiconazole sensitivity (<2- to 2.5-fold reduction in sensitivity). These results support the molecular impact of different inserts on *MFS1* expression observed by Omrane et al. (2015, 2017). They reported highest *MFS1* expression driven by the 519 bp insert, whereas type III and type II inserts lead to lower levels of *MFS1* overexpression. Furthermore, results shown here are consistent with the findings of Kildea et al. (2019), who reported a minor effect on DMI sensitivity correlated to the type III (150 bp) insert. Distinct differences in the impact on epoxiconazole sensitivity correlated to type II inserts were not observed in this work. This supports the assumption that differences described previously for tolnaftate sensitivity may result from methodological issues due to a low sample size. Consequently, results for epoxiconazole sensitivity indicate that all type II inserts confer more or less similar impacts on sensitivity. This is in line with the molecular results from Omrane et al. (2017), who reported that type II inserts exert similar effect on *MFS1* expression. So far, only a few studies exist that investigate correlation of *MFS1*p inserts on increased efflux and consequently on DMI sensitivity in *Z. tritici*. For this reason, further studies on DMI sensitivity of isolates carrying *MFS1*p inserts would be helpful to confirm the results of this work. Increased efflux activity of *Z. tritici* was shown to reduce sensitivity to common DMIs tested at the time of publication and even to fungicides with unrelated MOAs (Leroux and Walker, 2011; Omrane et al., 2015, 2017). Therefore, it may be assumed that effects of an increased efflux shown here by means of epoxiconazole would most likely also account for other DMIs.

Characterisation of *MFS1*p was not achieved in all investigated isolates as several isolates produced no amplicon during PCR. Evaluation of epoxiconazole sensitivity indicated no specific mechanisms across all of these isolates. Nevertheless, single isolates with uncharacterised *MFS1*p showed slightly higher adaptation to epoxiconazole compared to isolates with a WT *MFS1*p. Therefore, further studies are recommended in order to characterise *MFS1*p in these isolates and to clarify if this sensitivity variation occurred due to natural variation in sensitivity of *Z. tritici* to azoles (Gisi et al., 1997; Stergiopoulos et al., 2003) or whether alteration of *MFS1*p and increased efflux plays a role.

Compared to resistance levels of isolates carrying different *CYP51* haplotypes but no other additional resistance mechanism (19- to 306- fold reduction in sensitivity compared to *CYP51* wild type) described in chapter 4.6.3, potential impact of an increased efflux was assumed to be lower compared to combination of *CYP51* alterations. This assumption is supported by previous studies for *Z. tritici* that reported moderate resistance factors due to an increased efflux activity (Leroux and Walker, 2011; Omrane et al., 2015). Comparison to other fungal plant pathogens is challenging. An increased efflux has only been reported in

a few fungal plant pathogens, for example, in *B. cinerea* (Kretschmer et al., 2009) and in eyespot on wheat, *O. yallundae* (Leroux et al., 2013). For *B. cinerea* comparable results were published. Resistance levels towards fungicides due to an increased efflux were observed to be low to moderate (10- to 20-fold) while high resistance factors were conferred by target site alterations (up to several hundredfold) (Hahn and Leroch, 2015). Nevertheless, as an increased efflux activity based on the 519 bp insert in *MFS1*p was associated with an additive effect on DMI adaptation of *Z. tritici* (this work; Leroux and Walker, 2011; Omrane et al., 2015, 2017) and potentially can affect sensitivity to several fungicides with unrelated MOA (Roohparvar et al., 2007, 2008; Leroux and Walker, 2011; Omrane et al., 2015, 2017), monitoring of the occurrence and frequency of this mechanism in *Z. tritici* is recommended and of value.

Across all investigated isolates from 2016 and 2017, the frequency of inserts in *MFS1*p was relatively low (around 10%). Across both years, the type I insert associated with the highest impacts on DMI adaptation (this work; Omrane et al., 2017) was only found in countries of Central Europe (Germany and Poland) and Western Europe in a relatively low frequency of around 5 to 10%, respectively. Only single isolates carrying the type I insert in *MFS1*p were detected in isolate collections from Ireland from 2006 to 2015 (Kildea et al., 2019), from Germany, France, and UK from 2015 (Kirikyali et al., 2017), and a very low frequency (1.5%) of *MFS1*p inserts was observed in isolate collections from 2006 to 2011 from Europe and New Zealand (Buitrago et al., 2014). Compared to these earlier studies (Buitrago et al., 2014; Kirikyali et al., 2017; Kildea et al., 2019), a slight increase in the frequency and distribution of the type I insert in *MFS1*p was observed across Europe. Among all *MFS1*p inserts identified in isolates of this work, the type I insert was identified most frequently. This finding is in line with results from a population survey which revealed that among all isolates with increased efflux activity, the type I insert in *MFS1*p was found most frequently and was detected since 2009. This was followed by the type II inserts (338 and 369 bp), detected since 2012 and the least frequent type III insert which was only found since 2015 (reviewed in Omrane et al., 2017). In the current work the type III and type II inserts were mainly detected in Ireland and UK and only in single isolates from other countries in 2016. In 2017, there was a wider distribution of type II inserts in the Netherlands, Germany, and France, although still at a low level (with exception of the Netherlands). In addition, there was an increase in the frequency of the type III insert in Ireland. This finding is supported by Kildea et al. (2019), who already observed that the type III insert was detected most frequently in *MFS1*p in *Z. tritici* from 2015 in Ireland. Together, these results indicate a selective advantage of isolates carrying one of the type III or type II inserts and it may be speculated that frequency of the smaller inserts may increase in the future. The frequency of the type I insert has not increased between 2016 and 2017 with exception of UK. As mentioned before, sample size in UK was limited in 2017 and single isolates gained higher impact on changes in frequency of *MFS1*p inserts. The fact, that despite the first isolates with a 519 bp

insert in *MFS1*p being detected 10 years ago (Omrane et al., 2017) and that no widespread distribution of these isolates has occurred may indicate a reduced fitness of isolates exerting increased efflux activity. Fitness aspects correlated to DMI resistance mechanisms will be discussed in the next chapter (5.2.4).

Inserts in *MFS1*p were mainly restricted to isolates found in Central European countries, Germany and Poland, and to Western European countries. *MFS1*p inserts were not detected in isolates from Slovakia, Czech Republic, the Eastern, Northern, and Southern European countries. A higher frequency of *MFS1*p inserts in Western and some Central European countries compared to Eastern European countries was observed as was already seen for inserts in *CYP51*p and increasingly adapted *CYP51* haplotypes. As already hypothesized for *CYP51* haplotypes and *CYP51*p inserts, distribution of *MFS1*p inserts is likely to be influenced by the geographic emergence of DMI resistance mechanisms, differences in fungicide use patterns and disease prevalence (Brunner et al., 2008; Stammler et al., 2008b; Strobel et al., 2017; Jørgensen et al., 2018). It may be speculated again that insertions in *MFS1*p arose under high fungicide selection pressure in Western European countries and were then distributed to the East via windborne ascospores. A contrasting argument to this is the fact that the 308 and 377 bp inserts were detected in France and Poland. These two inserts were detected for the first time during this work and only in a single isolate, respectively. These results may indicate that fungicide selection pressure in these countries is sufficient for the selection of new *MFS1*p inserts. Practical relevance of an increased efflux for DMI field performance will be discussed at later point in this discussion (chapter 5.4).

5.2.4 Combination of DMI resistance mechanisms and possibilities for future evolution

Different DMI resistance mechanisms have evolved in several human and plant pathogenic fungi. Nevertheless so far, *Z. tritici* seems to be one of the only plant pathogens which combined all three described resistance mechanisms in individual isolates (this work; Cools et al., 2013; Ziogas and Malandrakis, 2015; Kirikyali et al., 2017). Four isolates combining *CYP51* alterations, *CYP51* overexpression (based on 120 bp *CYP51*p insert) and increased efflux (based on 519 bp *MFS1*p insert) were found in 2015 (Kirikyali et al., 2017). Occurrence of such isolates was confirmed at a very low frequency (<1%) in isolates investigated from 2016 and 2017 in the current work. In the further discussion, these isolates are described as isolates with an additional resistance mechanism, that combine target site alterations, *CYP51* overexpression (based on 120 bp insert in *CYP51*p), and/or an increased efflux (based on 519 bp insert in *MFS1*p). As has already been suggested here and in previous studies (Omrane et al., 2017; Kildea et al., 2019) the remaining *CYP51*p and *MFS1*p inserts are probably of minor relevance for DMI adaptation. Therefore, isolates carrying one of these inserts were not designated as isolates carrying *CYP51*

overexpression or an increased efflux in this chapter. It should be mentioned that the impact of different inserts in *MFS1*p or *CYP51*p was only tested based on the sensitivity comparison of isolates carrying the same *CYP51* haplotype but differing in the presence or absence of an insert because all inserts were detected in combination to *CYP51* alterations. In order to investigate the individual impact of *MFS1*p and *CYP51*p inserts on DMI sensitivity and to exclude the factor of natural variation in azole sensitivity between field isolates of *Z. tritici* (this work; Gisi et al., 1997; Stergiopoulos et al., 2003), future studies are recommended that comprise the introduction of promotor inserts in sensitive parental strains via transformation, as for example performed for the 519 bp insert in *MFS1*p (Omrane et al., 2017), and subsequent analyses of DMI sensitivity.

In vitro sensitivity studies described in the current work showed that highest adaptation to epoxiconazole within a *CYP51* haplotype group could be associated with *CYP51* overexpression and/or increased efflux. Kirikyali et al. (2017) reported that isolates combining all three resistance mechanisms showed highest sensitivity reduction to triazoles. These results were confirmed within haplotype group F2 for epoxiconazole sensitivity in this work demonstrating the additive effect of an increased efflux and *CYP51* overexpression on DMI adaptation. Nevertheless, a high variability in epoxiconazole sensitivity of isolates within a *CYP51* haplotype group, which could not be explained by *CYP51* overexpression or an increased efflux, was observed (chapter 4.7). In some cases, natural variation in azole sensitivity of *Z. tritici* (Gisi et al., 1997; Stergiopoulos et al., 2003; de Waard et al., 2006) probably contributes to this variation in sensitivity. In other cases, transporters other than the MFS1 and/or other mechanisms leading to an increased efflux or *CYP51* overexpression could be responsible for this sensitivity variation although have not been elucidated. One isolate was defined to carry the WT *MFS1*p but showed an adaptation towards tolnaftate indicating an increased efflux activity (shown in chapter 4.6.2). As this isolate showed a small insertion in *MFS1*p (<50 bp, described in chapter 4.6.2), it may be useful to investigate whether individual small insertions can confer an effect on an increased efflux. Single isolates without the 519 bp insert in *MFS1*p but displaying an MDR phenotype were also identified previously by Omrane et al. (2015). They suggested that transporters other than the *MFS1* transporter may contribute to an increased efflux in *Z. tritici*. Furthermore, *Z. tritici* isolates exerting *CYP51* overexpression independently from the 120 or ~900 bp insert were detected (Omrane et al., 2015). In *C. beticola*, it was observed that *CbCYP51* overexpression was not caused by promotor alterations or by copy number variation and it was proposed that *trans*-acting factors may contribute to *CbCYP51* overexpression (Bolton et al., 2012). Further studies are needed to clarify if other transporters or *CYP51* overexpression mediated by mechanisms other than the 120 bp promotor insert play a role in variation of epoxiconazole sensitivity in isolates of this work. Omrane et al. (2015) already identified candidate transporters which may be involved in an increased efflux activity in addition to the MFS1 transporter. They claimed that studies

including mutagenesis of these transporters are necessary to define their contribution to an increased efflux activity in *Z. tritici*.

So far, combination of all three DMI resistance mechanisms have only been found in association with the F2 haplotype (this work; Kirikyali et al., 2017). *CYP51* overexpression is mainly restricted to isolates carrying the F2 *CYP51* haplotype (this work; Cools et al., 2012; Buitrago et al., 2014; Kildea et al., 2019). Therefore, combination of *CYP51* overexpression and an increased efflux to other *CYP51* haplotypes is probably mainly dependent on the emergence of *CYP51* overexpression combined to other *CYP51* haplotypes than F2. As described in chapter 5.2.2, combination of *CYP51* overexpression to *CYP51* haplotypes other than F2 was detected in isolates from 2016 and 2017. It may be speculated that if the frequency of these isolates increases in field populations of *Z. tritici*, the likelihood of combination of all three DMI resistance mechanisms including other *CYP51* haplotypes than F2 will also increase.

Predictions about further evolution, increase and spread of individual DMI resistance mechanisms or combinations of those in *Z. tritici* is challenging due to its high complexity. Factors such as fungicide use intensity, disease pressure, anti-resistance-strategies, and fitness of a resistant isolate determine the increase and spread of resistances in fungal populations (van den Bosch et al., 2011, 2014; Ma and Michailides, 2005; Mikaberidze and McDonald, 2015). Due to fungicide selection pressure, frequency of isolates carrying a resistance mechanism that confers a selective advantage increases (van den Bosch et al., 2014; Oliver and Hewitt, 2014). Mutations conferring fungicide resistance, however, may disturb efficiency of physiological and biochemical processes that lead to a lower pathogen fitness (Anderson, 2005; Mikaberidze and McDonald, 2015). The fitness of resistant isolates determines if they persist or decrease in fungal populations in the absence of fungicide selection pressure and fitness costs may delay resistance development (Ma and Michailides, 2005; Mikaberidze and McDonald, 2015). The frequency of resistant individuals may decline in the absence of fungicide selection pressure when resistant populations show a lower fitness compared to the sensitive one (Ma and Michailides, 2005; Ishii, 2015). Fitness costs associated with different DMI resistance mechanisms may therefore impact further evolution, spread, and persistence of those mechanisms in field populations of *Z. tritici*. In this work, fitness of isolates with different DMI resistance mechanisms or combination of those was not investigated. However, information about fitness costs associated with *CYP51* alterations in *Z. tritici* and with increased efflux or *CYP51* overexpression in other pathogens is available in literature.

Some alterations or combinations of alterations in CYP51 of *Z. tritici* have been correlated to negative impacts on enzymatic activity and consequently to potentially lower pathogen fitness (Bean et al., 2009; Cools et al., 2010, 2011; Mullins et al., 2011; Becher and Wirsel, 2012; Oliver and Hewitt, 2014). Yet, some alterations were shown to destroy *CYP51*

function completely, but it was observed that compensatory alterations in other parts of the gene partially restored *CYP51* function (Brunner et al., 2008; Mullins et al., 2011; Cools et al., 2010). Therefore, the sequence and frequency by which *CYP51* alterations have occurred was probably driven by differential effects on enzyme activity (Cools et al., 2013). The requirement for multiple alterations to confer DMI resistance is one factor suggested to be responsible for the slow emergence of DMI resistance in *Z. tritici* (Cools et al., 2013) compared, for example, to QoI resistance. Regarding QoI resistance, the single alteration G143A, which is claimed not to affect enzyme activity of CYTB, was enough to confer a highly resistant phenotype that rapidly predominated the pathogen population (Gisi et al., 2002; Fraaije et al., 2005; Lucas and Fraaije, 2008; Jørgensen et al., 2019). Although detection of new *CYP51* alterations in this work confirms the high level of flexibility of the CYP51 protein (Becher and Wirsel, 2012), it was predicted that the scope for changing the gross architecture of the haem pocket is limited, because native function of the enzyme has to be restored (Mullins et al., 2011). It may be speculated that the possibility for the development of new alterations with strong effects on the haem environment and consequently on DMI binding is now less likely because of the high number of already existing alterations. Nevertheless, occurrence of compensatory alterations may facilitate the ongoing evolution of new alterations and it remains to be seen if new alterations that affect DMI sensitivity will occur in the future. Due to the ongoing evolution in the frequency and distribution of existing increasingly adapted *CYP51* haplotypes described in chapter 5.2.1, a further increase of these haplotypes may be expected in the future.

Whether constitutive overexpression of *CYP51* and *MFS1* (increased efflux) reduces the fitness of *Z. tritici* is not clear to date. However, energy dependent increased efflux (MDR) is often associated with fitness penalties in plant pathogenic fungi in the absence of fungicide selection pressure (de Waard et al., 2006). It is conceivable that resource allocation for overexpression of *CYP51* or increased efflux is associated with fitness costs (Hawkins and Fraaije, 2018). Among other factors, this may contribute to the fact that widespread occurrence of these mechanisms in plant pathogens do not seem to have occurred so far (Cools et al., 2013; Leroux et al., 2013). Nevertheless, it was observed that fitness costs associated for example with MDR phenotypes can be compensated for in *C. albicans* (Cowen et al., 2001). Moreover, it was observed that MDR strains of *B. cinerea* did not show any differences in fitness parameters compared to non-MDR strains and seem to compete efficiently under field conditions (Hahn and Leroch, 2015). In addition, Cools et al. (2013) suggested that additional resistance mechanisms leading to cross-resistance between DMIs may increase in *Z. tritici* in the future despite any fitness costs associated with them. This prediction is supported in findings reported in the current work as an increase in the frequency of additional resistance mechanisms was observed compared to past years. However, as first isolates with increased *CYP51* or *MFS1* expression already appeared in 2009, the relatively long period until their increase in frequency and still low

levels of strong *MFS1* overexpression in 2016 and 2017 may support the assumption of fitness penalties associated with these mechanisms. Fitness tests such as, for example, competition studies commonly used to assess pathogen fitness (Karaoglanidis et al., 2001; Ma and Uddin, 2009; Rehfus, 2018; Graf, 2018; Hawkins and Fraaije, 2018) are recommended for future research. Such studies should incorporate *Z. tritici* isolates that carry the same *CYP51* haplotype and only differ in the presence or absence of an additional resistance mechanism or in combination of different mechanisms. Thereby, information about fitness aspects associated with *CYP51* overexpression and an increased efflux or combination of those may be gained. Knowledge about fitness costs may help to establish efficient resistance management strategies (Mikaberidze and McDonald, 2015; Hawkins and Fraaije, 2018) and may support predictions about further evolution of *CYP51* overexpression and increased efflux in *Z. tritici*.

5.3 Cross-resistance of *Z. tritici* to different DMIs

As all DMIs show the same mode of action, they are generally considered to be cross-resistant with each other (FRAC, 2019). Indeed, *CYP51* overexpression and an increased efflux activity was shown to affect sensitivity of *Z. tritici* to most common DMIs on the market at the time of publication (Cools et al., 2012, 2013; Ziogas and Malandrakis, 2015; Omrane et al., 2015, 2017; Kildea et al., 2019). Nevertheless, incomplete cross-resistance was observed between individual DMIs or a subset of DMIs for specific *CYP51* haplotypes based on specific *CYP51* alterations (this work; Cools et al., 2011; Leroux and Walker, 2011; Cools et al., 2013; Lucas et al., 2015; Strobel et al., 2017; Jørgensen et al., 2018).

Isolates obtained from field trials of 2015 and 2016 showed a high correlation with the *in vitro* sensitivity between epoxiconazole, prothioconazole, and metconazole, whereas correlation to tebuconazole was lower (Jørgensen et al., 2018). Differences in the sensitivity profile of DMIs result in different cross-resistance patterns with epoxiconazole, prothioconazole, and cyproconazole belonging to one group and difenoconazole and tebuconazole to another (Leroux and Walker, 2011; Buitrago et al., 2014). Correlations of the *in vitro* sensitivity of all ‘frequently found *CYP51* haplotypes’ during this work confirmed a high correlation between epoxiconazole and prothioconazole-desthio, while correlation to the new DMI compound mefentrifluconazole was weaker or almost absent. As expected from overall sensitivity correlations across all ‘frequently found *CYP51* haplotypes’, the cross-resistance patterns, based on resistance factors of individual *CYP51* haplotypes, between epoxiconazole and prothioconazole-desthio were more similar compared to mefentrifluconazole. These findings indicate a limited cross-resistance of epoxiconazole as well as prothioconazole-desthio to mefentrifluconazole *in vitro*. In the past, efficacy data for STB control from field trials confirmed that cross-resistance relationships observed *in vitro* could also be seen *in vivo* (Jørgensen et al., 2018). A higher correlation of epoxiconazole

and prothioconazole efficacy compared to correlation of prothioconazole and tebuconazole was observed (Jørgensen et al., 2018). In addition, different selection patterns after DMI treatment seem to occur in the field (Heick et al., 2017b). A different trend in the pattern of *CYP51* haplotypes of *Z. tritici* after solo treatment of prothioconazole or mefentrifluconazole was also observed among isolates from field trials in this work. This result supports the assumption of limited cross-resistance from *in vitro* results. Relative frequencies of *CYP51* haplotypes after DMI treatment showed that *CYP51* haplotypes expressing high resistance factors *in vitro* (in case of prothioconazole also moderate RF values) preferentially survived DMI treatment in the field. Nevertheless, an important point to mention is that results presented in chapter 4.8.2 represent relative frequencies of different *CYP51* haplotypes gained from a defined number of investigated isolates. Therefore, they do not reflect absolute number of isolates with these haplotypes in the field. For example, in Ireland, a relative frequency of 70% of all investigated isolates from the mefentrifluconazole treated plot showed haplotype H6, whereas only 30% were identified among isolates after prothioconazole treatment (Figure 32). An important aspect contributing to the absolute number of *Z. tritici* isolates in the field is the field efficacy conferred by different DMIs. Quantification of *Z. tritici* DNA in a field sample with real-time PCR would help to correlate the relative frequency of *CYP51* haplotypes to the absolute occurrence of *Z. tritici* with these *CYP51* haplotypes in the field. In this work, quantification of *Z. tritici* in field samples was not performed. Therefore, assessment data of STB infection were used here in order to give an estimation about the absolute occurrence of isolates with specific *CYP51* haplotypes in the field. In Figure 35, the relative frequency of *CYP51* haplotypes was visualised in relation to the STB infection in untreated plots and after DMI treatment. For this evaluation mean STB infection (%) on the second- and flag leaf detected at the final assessment date of the respective field trial was used.

Results shown in Figure 35 illustrate that a large difference between the relative frequency of *CYP51* haplotypes (described in Figure 32) does not result in same differences in the absolute frequency of these *CYP51* haplotypes in the field when regarded in relation to STB infection. For example, based on Figure 35, it could be assumed that the absolute frequency of isolates with haplotype H6, was more similar in mefentrifluconazole and prothioconazole treated plots in the field in Ireland than expected from differences in relative frequencies described above. A higher level of STB infection in the prothioconazole treated plot compared to the mefentrifluconazole treated plot in Ireland results in the approximation of absolute frequencies of this haplotype. Across all locations, efficacy of mefentrifluconazole was higher compared to prothioconazole resulting in a lower level of *Z. tritici* infection after mefentrifluconazole treatment. Except for Denmark, a higher proportion of the STB population was composed of a single *CYP51* haplotype following treatment with mefentrifluconazole compared to prothioconazole. A higher level of *Z. tritici* infection was

observed in the prothioconazole treated plots, but composition of *Z. tritici* in these plots tended to be more variable.

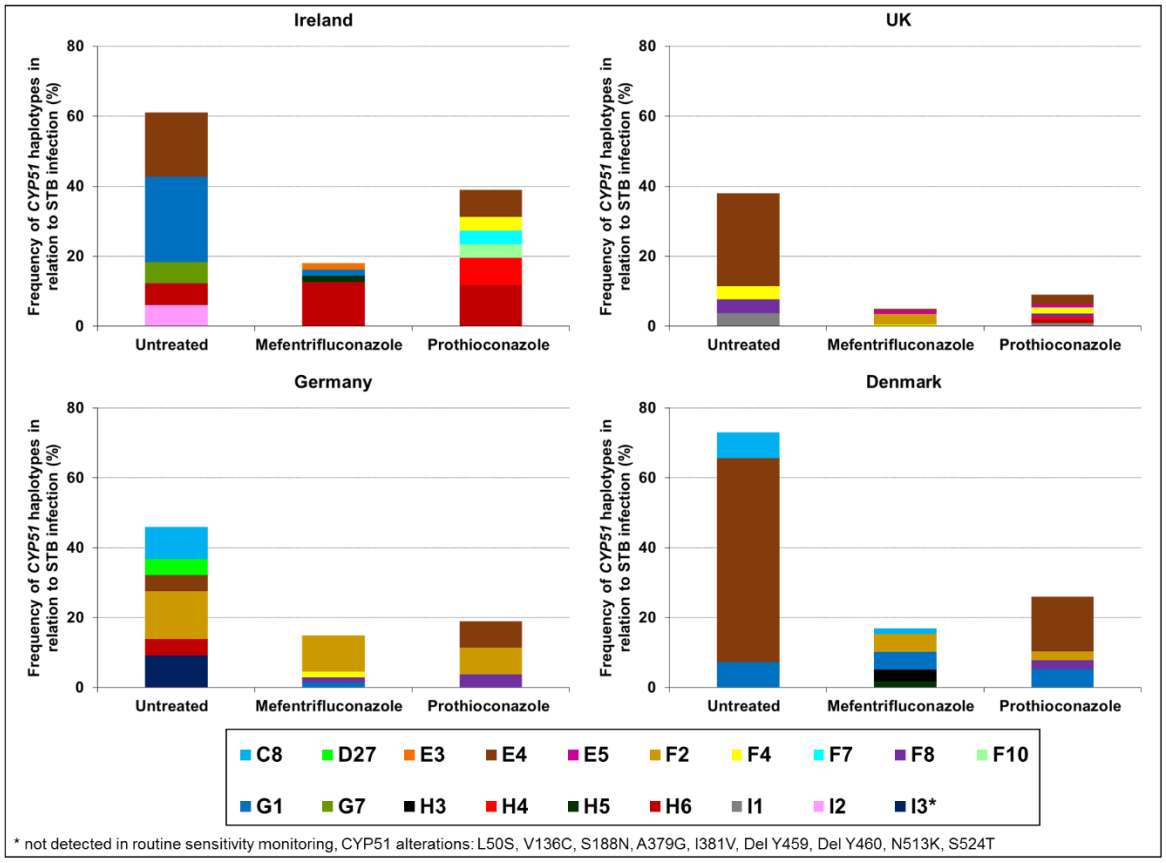


Figure 35: *CYP51* haplotypes of *Z. tritici* and their frequency in field trials after application of mefentrifluconazole (Revysol®) and prothioconazole (Proline®) in relation to *Z. tritici* infection (%). Field trials were conducted in four different countries (Ireland, UK, Germany, Denmark). Samples were taken in untreated, mefentrifluconazole and prothioconazole treated plots and 10 isolates were generated per sample by EpiLogic. Relative frequency of *CYP51* haplotypes (shown in Figure 32) in relation to *Z. tritici* infection is presented per country and treatment.

The importance of these findings for further resistance evolution can be considered in different ways. For example, in the absence of any fitness penalty associated with different *CYP51* haplotypes a low absolute frequency of STB, independently from any proportions of different *CYP51* haplotypes, may help to slow down further resistance evolution because future build-up of the STB population would start at a lower level. On the other hand, van den Bosch et al. (2014) suggested that selection for fungicide resistance can only be reduced when the increase of both, the sensitive and resistant strain is reduced or when the increase of the resistant strain is reduced relative to that of the sensitive one. It is assumed that the probability of a resistant strain to build a population is higher when the density of the sensitive population is low (van den Bosch et al., 2011). As the *CYP51* wild type can no longer be found in Western and Central Europe (described in chapter 5.2.1), this hypothesis could only be transferred to less adapted and highly adapted *CYP51* haplotypes. A higher absolute frequency of STB with varying *CYP51* haplotypes may help

to slow down resistance evolution if fitness penalties are associated with highly adapted *CYP51* haplotypes. If the higher absolute STB frequency is composed of moderately and highly adapted *CYP51* haplotypes, further increase of highly adapted *CYP51* haplotypes may be slower as they have to compete in future STB build-up with eventually more fit moderately adapted *CYP51* haplotypes. Under this assumption, in the long-run, resistance build-up may be slower compared to the scenario of population build-up after a low absolute STB frequency with high proportions of single *CYP51* haplotypes. In the end, no final conclusion can easily be drawn about these hypotheses here and further studies are recommended for clarification. Additionally, as this is the first report of selection studies for mefentrifluconazole and only a limited number of isolates in a limited number of trials was tested, results of this work need confirmation in future studies. Moreover, quantification of *Z. tritici* DNA in field samples and subsequent determination of *CYP51* haplotypes is recommended for future studies when relative frequencies *in vitro* should be correlated to absolute frequency of a haplotype in the field.

Taken all results from *in vivo* studies and from field trials into account, a limited cross-resistance between epoxiconazole as well as prothioconazole compared to mefentrifluconazole was demonstrated. Protein modelling in combination with azole docking studies revealed that limited cross-resistance between existing DMIs probably occurs due to different binding specificities of different triazoles and consequently due to different effects of alterations on triazole binding (Mullins et al., 2011). Such studies may clarify if different binding properties of mefentrifluconazole compared to other triazoles are responsible for limited cross-resistance for specific *CYP51* haplotypes and are recommended.

Application of DMIs that differ in their cross-resistance pattern and differentially select for DMI resistance (*CYP51* haplotypes) in mixtures or alternation is suggested to be a suitable strategy of anti-resistance management and has been proven in field trials (Cools and Fraaije, 2008; Heick et al., 2017b; Jørgensen et al., 2018). In field trials of 2015 and 2016, a high variability in tebuconazole sensitivity was observed (Jørgensen et al., 2018). High efficacy of this triazole was correlated to high proportions of alteration V136A that exert different effects to different triazoles (Cools et al., 2011; Leroux and Walker, 2011; Buitrago et al., 2014; Jørgensen et al., 2018). Heick et al. (2017b) demonstrated in field trials of 2015 and 2016 that selection for CYP51 alterations conferring DMI adaptation was reduced when spray strategies were applied consisting of DMIs of different cross-resistance groups compared to spray strategies consisting of DMIs of the same cross-resistance group. Therefore, maintenance of DMI diversity in the fungicide market and registration of new DMIs that show a different cross-resistance pattern to existing DMIs is considered to be an important aspect of disease management of STB. Nevertheless, in the long-run as suggested before (Heick et al., 2017b; Jørgensen et al., 2018), application of DMI mixtures may select for *CYP51* haplotypes that show cross-resistance to all common DMIs or for

additional resistance mechanisms conferring cross-resistance, such as *CYP51* overexpression or an increased efflux. Therefore, application of DMIs in mixtures with fungicides with different MOAs is also recommended in order to counteract this selection (Hobbelen et al., 2013; van den Bosch et al., 2014; Heick et al., 2017b).

5.4 Impact of *in vitro* DMI adaptation on *in vivo* DMI efficacy and implications for field performance of DMIs

Detection of resistant isolates in fungal field populations is not necessarily associated with sudden and direct disease control problems under field conditions (Brent and Hollomon, 2007a). Therefore, it is important to distinguish between 'field resistance' that describes the presence of resistant isolates in field populations and 'practical resistance' that indicates losses in disease control based on fungicide resistance (Brent and Hollomon, 2007a). 'Practical resistance' arises when the resistance levels of isolates are high enough to survive application rates in the field and the prevalence of these isolates is also high enough to dominate the population (Oliver and Hewitt, 2014). Resistance factors determined in *in vitro* studies do not necessarily correlate linearly to fungicide efficacies in the glasshouse (this work; Rehfus, 2018) and consequently probably not to field performance. Monitoring of field performance of fungicides is therefore an important issue of fungicide resistance monitoring, alongside the identification and characterisation of resistances in the laboratory (Brent and Hollomon, 2007a).

Although adapted *CYP51* haplotypes were detected in field populations of *Z. tritici* and a shift in sensitivity was observed *in vitro*, field performance of registered field rates of epoxiconazole was not significantly affected until 2008 (Stammler et al., 2008b). Across 26 field trials in 2015 and 2016, triazoles provided significant control of STB (50-70%) with highest levels of control associated with triazole mixtures, epoxiconazole+metconazole (Osiris®) and prothioconazole+tebuconazole (Prosaro®) (Jørgensen et al., 2018). Highly variable field performance across Europe was observed and correlated at least to some extend to varying DMI adaptation of the *Z. tritici* populations and different impacts on triazoles (see chapter 5.3) (Jørgensen et al., 2018). In glasshouse studies in this work, it was observed that epoxiconazole showed substantial efficacy (on average around 75% or higher) even for isolates carrying *CYP51* haplotypes that showed high adaptation *in vitro*. Additionally, mefentrifluconazole showed high efficacy for all tested *CYP51* haplotypes (on average at least around 85% and in most cases higher) including haplotype F2 and H6, which showed high resistance factors *in vitro*. Limited correlation of *in vitro* and *in vivo* results may result from the interaction of metabolic processes in plant cells with fungicides. For example, in case of mefentrifluconazole, a high CYP51 binding affinity has been

observed indicating a lower concentration required for an effective binding of CYP51 compared to currently registered DMIs. Furthermore, mefentrifluconazole showed a lower solubility and consequently lower translocation within the plant compared to reference triazoles (depot effect). Interaction of these two properties may lead to lower degradation of free mefentrifluconazole by plant metabolism over time compared to older triazoles (personal communication Dieter Strobel, technical manager BASF SE). All in all, results of the glasshouse tests imply that DMIs still contribute to STB control in the field despite the occurrence of isolates that show high adaptation *in vitro*. In glasshouse trials, a high spore density of individual highly *in vitro* adapted isolates with a specific *CYP51* haplotype was tested and conditions for fungal growth optimised. These conditions would not occur in nature. The assumption that DMIs can still contribute to the control of the tested *CYP51* haplotypes in the field is supported by these facts, because under natural conditions a lower frequency of these *CYP51* haplotypes occur (this work). Under natural conditions a high variation of different *CYP51* haplotypes is prevalent, even within a single field (Stammler et al., 2008b; Stammler and Semar, 2011). In glasshouse studies in this work, only a limited number of *CYP51* haplotypes was tested. Therefore, additional glasshouse studies with an extended number of *CYP51* haplotypes are recommended for further predictions about field efficacy of triazoles.

In field studies of 2015 and 2016, efficacy of triazoles could not always be correlated to the frequency of *CYP51* mutations and to *in vitro* adaptation of regional *Z. tritici* populations across different countries (Jørgensen et al., 2018). It was suggested that disease pressure, weather conditions, leaf emergence, and application timing may be crucial factors determining field performance of triazoles (Paveley et al., 2000; Strobel et al., 2017; Blake et al., 2018). It may be speculated that under suboptimal application conditions (e.g. high disease pressure, unfavourable application timing, lower doses) the impact of DMI adaptation measured *in vitro* on field performance of triazoles is more severe than indicated by glasshouse studies of this work. This fact may contribute to overall decline in the field performance of triazoles reported in the past (Kildea, 2016; Heick et al., 2017a; Strobel et al., 2017; Blake et al., 2018; Jørgensen et al., 2018). Although, in glasshouse studies epoxiconazole showed substantial control for isolates that showed highest adaptation *in vitro*, a reduced efficacy was observed compared to isolates that showed lower adaptation *in vitro*. Ongoing evolution of *CYP51* haplotypes and an ongoing shift in sensitivity identified in this work across Europe may consequently reduce field efficacy of individual triazoles further if no resistance management strategies would be applied. Therefore, constant monitoring of field performance of existing and new DMIs entering the market is recommended.

In vitro studies of this work and previous studies (Cools et al., 2012) revealed that *CYP51* overexpression confers an additive effect on DMI adaptation, but the decrease in sensitivity based on *CYP51* overexpression is lower compared to the potential impact of combination

of CYP51 alterations (see chapter 5.2.2). In glasshouse studies of this work, epoxiconazole and mefentrifluconazole showed a high efficacy for isolates with *CYP51* overexpression which supports findings from Cools et al. (2012). They reported that isolates with *CYP51* overexpression were completely controlled in the glasshouse when fungicides were applied at full rates. Resistance levels correlated to *CYP51* overexpression in the laboratory may not be high enough to impact efficacy at field dose rates (Oliver and Hewitt, 2014; Blake et al., 2018). Consequently, it was suggested that disease control of STB is not affected due to *CYP51* overexpression under field conditions when triazoles are used at full recommended rates, optimal application timing and uniform coverage of the crop is ensured (Cools et al., 2012). However, the increase of *CYP51* overexpression in countries of Western Europe and in Poland as well as in Germany (this work; Kildea et al., 2019) may be interpreted as a contrasting argument. It may allow the assumption that under suboptimal application conditions and reduced application rates the impact of *CYP51* overexpression on DMI sensitivity measured *in vitro* may be high enough to affect field performance of DMIs. It was reported that isolates overexpressing *CYP51* grew at higher fungicide doses in the glasshouse compared to isolates with the same *CYP51* haplotype but without *CYP51* overexpression indicating that these isolates will be selected in the field (Cools et al., 2012). Furthermore, the selective advantage of isolates with *CYP51* overexpression may rely on cross-resistance to most common DMIs associated with *CYP51* overexpression (Cools et al., 2012, 2013; Ziogas and Malandrakis, 2015; Kildea et al., 2019). Finally, however, it remains to be tested for example in the glasshouse or in field trials if these hypotheses play a role in the increase of *CYP51* overexpression. In this work, *CYP51* overexpression was identified in combination with *CYP51* haplotypes other than the F2 haplotype. Therefore, the assumption from previous authors (Lucas et al., 2015; Kildea et al., 2019) that it cannot be ruled out that combination of *CYP51* overexpression with highly evolved *CYP51* haplotypes will lead to further reduction in sensitivity and may contribute to reduced fungicide efficacy in the field is supported.

The practical relevance of an increased efflux activity for fungicide performance in the field was not investigated so far. As for *CYP51* overexpression, in this work and previous studies (Leroux and Walker, 2011; Omrane et al., 2015, 2017), it was demonstrated that an increased efflux is correlated to an additive effect on DMI adaptation, but this effect is smaller compared to the potential impact of combination of *CYP51* alterations. In this work, *in vivo* results for isolates exerting increased efflux were presented for the first time for *Z. tritici*. Overall, epoxiconazole and mefentrifluconazole showed high efficacies for isolates with an increased efflux. If at all, only a slight decrease in efficacy (4-8%) was observed for all tested compounds for isolates with increased efflux (compared to isolates with the same *CYP51* haplotype but without increased efflux). For one isolate, it could not be clarified if lower efficacy is conferred by an increased efflux or *CYP51* overexpression. Additionally, a high variability of *Z. tritici* field isolates in general, makes it hard to predict if slight differences

in efficacy are conferred by an increased efflux. Moreover, it could not be clarified if an increased efflux has contributed to the lowest efficacy of epoxiconazole and prothioconazole for isolates carrying haplotype H6 with increased efflux, because no isolates with a H6 haplotype and without increased efflux were tested. However, *in vitro* results (Figure 26) showed that *CYP51* haplotype H6 alone confers high adaptation to epoxiconazole and an increased efflux only has a slight additive effect on DMI adaptation of this haplotype (around 1.5-fold). Based on these results, it may be assumed that lower efficacy for the H6 haplotype in glasshouse is mainly driven by combination of *CYP51* alterations.

All in all, no clear conclusion could be drawn for the impact of an increased efflux on *in vivo* efficacy of different DMIs, but a contribution to a slightly lower efficacy seems possible. Nevertheless, efficacy of epoxiconazole and prothioconazole for isolates that only carry haplotype I2 and no additional resistance mechanism was lower compared to isolates carrying other haplotypes and an increased efflux and/or *CYP51* overexpression (except haplotype H6). Based on this fact, it may be speculated that combination of *CYP51* alterations has higher relevance for epoxiconazole and prothioconazole efficacy *in vivo* compared to additional resistance mechanisms. Due to the high variability of *Z. tritici* field isolates and the low number of tested isolates in glasshouse studies of this work, further studies with a higher number of isolates that carry the same *CYP51* haplotype and only differ in the presence or absence of an increased efflux are recommended. These studies would help to gain further information about the impact of an increased efflux on DMI efficacy *in vivo*. As assumed for *CYP51* overexpression and *CYP51* haplotypes, it may be speculated that the impact associated with an increased efflux on DMI sensitivity *in vitro*, may be high enough to affect field performance of DMIs under suboptimal application conditions. Additionally, it cannot be excluded that combination of an increased efflux activity with highly evolved *CYP51* haplotypes will lead to further reduction in sensitivity and field efficacy of DMIs will be affected. An increase in the frequency of these isolates, however, would still be required in order to constrain field performance of triazoles for STB. Such an example was found for another pathogen, where for *B. cinerea* an increase in the frequency of such isolates was detected (Hahn and Leroch, 2015). For *B. cinerea* in strawberries, an additive effect on adaptation to some fungicides was shown to be due to a combination of target site mutations and MDR (Grabke and Stammler, 2015). Combination of multiple target site resistances and MDR1, an MDR type that relies on overexpression of ABC transporters (Kretschmer et al., 2009), seems to have practical relevance (Hahn and Leroch, 2015). Nevertheless, it was stated that a complete failure of fungicide efficacy due to MDR is unlikely in *B. cinerea*, but combination with multiple target site resistances makes disease control increasingly difficult (Hahn and Leroch, 2015). The role of multiple target site resistances to fungicides with different MOAs combined to an increased efflux for future control of STB has not been investigated so far and seems a relevant issue based on the

example of *B. cinerea*. Nevertheless, for *B. cinerea* in strawberries a different situation exists compared to *Z. tritici* in wheat, since several fungicide treatments are applied in strawberries for *B. cinerea* control within a couple of weeks. Additionally, in this crop/pathogen system, MDR is mainly driven by fludioxonil applications for which no target site alterations occur in field isolates. MDR is the only resistance mechanism of field isolates of *B. cinerea* against fludioxonil since more than 20 years of product launch. This fungicide, however, is not registered and applied in cereals (Leroch et al., 2013; Grabke and Stammler, 2015; personal communication Dr. Gerd Stammler, fungicide resistance monitoring BASF SE).

In summary, the combination of *CYP51* alterations in *Z. tritici* and an increase of the frequency of these *CYP51* haplotypes were associated with a reduced field performance of DMIs (Heick et al., 2017a; Jørgensen et al., 2018). Nevertheless, it was shown that existing DMIs still contribute to STB control under glasshouse- or field conditions (this work; Jørgensen et al., 2018) and the new DMI probably will provide good STB control for some time (this work). Glasshouse studies indicate up to now, that the impact of *CYP51* overexpression and an increased efflux activity on field performance of DMIs is relatively low under optimal application conditions. If conditions are not ideal, then the effect of an increased efflux or *CYP51* overexpression may be high enough to contribute to a reduced field performance and selection of these mechanisms under field conditions may be possible. Additionally, due to the possible combination of additional resistance mechanisms with increasingly adapted *CYP51* haplotypes, and cross-resistance between triazoles conferred by an increased efflux and *CYP51* overexpression, these mechanisms may be selected in the future. These assumptions, however, need to be confirmed experimentally in future studies.

5.5 Prospects for future DMI application

DMI resistance in *Z. tritici* has been intensively studied since many years. Making predictions about further development of DMI efficacy in the control of STB is difficult due to a high level of complexity. Different DMI resistance mechanisms exert different effects on DMI sensitivity (this work; Leroux and Walker, 2011; Cools and Fraaije, 2013). A heterogenous distribution of these mechanisms across Europe exist (this work), meaning that resistance development is spatially variable. In addition, limited cross-resistance of *CYP51* haplotypes to different DMIs was shown at one site (this work; Cools et al., 2011; Leroux and Walker, 2011; Cools et al., 2013; Lucas et al., 2015; Strobel et al., 2017; Jørgensen et al., 2018), but cross-resistance conferred by *CYP51* overexpression as well as an increased efflux on the other site (Leroux and Walker, 2011; Cools et al., 2012, 2013; Ziogas and Malandrakis, 2015; Omrane et al., 2015; Kildea et al., 2019).

Currently, despite the emergence of DMI resistance over many years, triazoles still play a crucial role in STB control (Strobel et al., 2017; Jørgensen et al., 2018; Kildea et al., 2019). This crucial role is based on direct STB control but also on the importance to have DMIs as mixing partners for other fungicides with different MOAs as option for anti-resistance management (Jørgensen et al., 2018). A heterogenous distribution of *CYP51* haplotypes with varying resistance levels, occurrence of *CYP51* overexpression and an increased efflux across Europe demonstrated that resistance in Western and in the Central European countries, Germany and Poland, is more advanced than in Eastern Europe. As described in the previous chapter, ongoing spread and increase of increasingly adapted *CYP51* haplotypes probably will affect field performance of individual DMIs further. Nevertheless, results of this work supported the assumption made before (Cools and Fraaije, 2008; Heick et al., 2017b; Jørgensen et al., 2018; Kildea et al., 2019), that incomplete cross-resistance between existing DMIs as well as to new DMIs entering the market may be exploited for anti-resistance management by alternation or mixing of different DMIs and consequently could help to maintain STB control.

Although the impact conferred by *CYP51* overexpression and an increased efflux in *Z. tritici* seems lower than the potential impact of combinations of *CYP51* alterations, they further reduce triazole sensitivity of *Z. tritici in vitro* (this work; Cools et al., 2012; Omrane et al., 2015, 2017). As assumed in the previous chapter, a potential impact of these additional mechanisms on field performance of DMIs under suboptimal application conditions (including application timing, disease pressure, weather conditions) could not be excluded. Furthermore, *CYP51* overexpression or an increased efflux may contribute to a reduced field performance in STB control when combined with increasingly adapted *CYP51* haplotypes in the future. The same is assumed when all three mechanisms will be combined involving highly adapted *CYP51* haplotypes. Therefore, further distribution and increase of isolates with additional DMI resistance mechanisms in *Z. tritici* should be prevented. As described in chapter 5.2.4, fitness aspects correlated to additional resistance mechanisms may affect distribution and increase of these mechanisms in *Z. tritici*. Investigation of such fitness aspects is recommended in order to establish appropriate anti-resistance management strategies. As *CYP51* overexpression and an increased efflux confer cross-resistance between DMIs (Cools et al., 2012, 2013; Ziogas and Malandrakis, 2015; Omrane et al., 2015; Kildea et al., 2019), principal resistance management strategies that aim to reduce fungicide resistance build-up should be applied (Jørgensen et al., 2019). Alternation or especially mixtures of fungicides with different effective MOAs or multi-site inhibitors have been shown to delay the evolution of fungicide resistance (Hobbelen et al., 2013; van den Bosch et al., 2014; Heick et al., 2017b) and are recommended. Moreover, an optimal application timing, rather preventive than curative, can provide anti-resistance management (van den Berg et al., 2013; Jørgensen et al., 2019). Additionally, limiting the number of

fungicide applications according to their requirement is also an important tool in anti-resistance management (van den Bosch et al., 2014; Jørgensen et al., 2019).

A relevant aspect that has to be considered for anti-resistance management strategies and future disease control of STB is the occurrence of multiple resistances to different MOAs. Multiple resistances were not investigated in this work, but as frequency of QoI resistance is generally high in Western Europe (Fraaije et al., 2005; Lucas and Fraaije, 2008; Jørgensen et al., 2019), the combination of DMI adaptation and QoI resistance in the majority of isolates in Western Europe is likely. Moreover, field isolates that exhibit SDHI resistance, QoI resistance, benzimidazole resistance and that carry an evolved *CYP51* haplotype have been already detected (Rehfus, 2018). Availability of different active MOAs for effective anti-resistance management strategies has therefore been suggested to be at risk (Jørgensen et al., 2019). Moreover, a stricter regulation approach in the EU is likely to reduce the availability of different MOAs on the European fungicide market in the future (Hollomon, 2015; Bryson and Brix, 2019). Consequently, future resistance management strategies should incorporate integrated disease management approaches such as host plant resistance and the combined application of biopesticides with conventional fungicides. These strategies aim to keep disease pressure, and consequently the requirement for fungicide applications, low (reviewed in Hollomon, 2015; Jørgensen et al., 2019). Thereby, they may help to protect active ingredients entering the market as well as existing DMIs and the important role of DMIs in control of STB may be maintained.

5.6 Proposal for future research on DMI resistance

Monitoring of DMI resistance in *Z. tritici*, as performed in this work, is recommended for future studies in order to determine future evolution, spread, and frequency of *CYP51* haplotypes and additional DMI resistance mechanisms across Europe. For a better understanding of DMI resistance and for prediction about further evolution, future studies could also comprise:

- Protein modelling studies with new CYP51 alterations identified in this work could help to predict if they bear evolutionary potential for the emergence of other alterations that may affect DMI sensitivity.
- Characterisation of inserts in *CYP51*p and *MFS1*p with respect to their origin and how they drive overexpression could help to understand evolution of adaptation in *Z. tritici*.
- Introduction of *CYP51*p and *MFS1*p inserts in sensitive isolates, for example, via molecular techniques as CRISPR-Cas9 in order to investigate their individual effect on DMI sensitivity of *Z. tritici*.

- Investigation if other transporters or mechanisms play a role in increased efflux activity or *CYP51* overexpression of *Z. tritici*.
- Extension of glasshouse studies with more isolates with different *CYP51* haplotypes carrying *CYP51* overexpression and/or increased efflux or not (preventive as well as curative application) for better estimation of their impact on DMI field performance.
- Assessment of the fitness of isolates, for example, via competition studies in the glasshouse with isolates that show the same *CYP51* haplotypes but differ in the presence or absence of *CYP51* overexpression and/or increased efflux.
- Establishment of a method for absolute quantification of *Z. tritici* in field samples that are subsequently analysed for *CYP51* haplotypes after solo DMI treatment in order to assess absolute occurrence of haplotypes in a sample.

6 Literature

Abbott, W. S. (1925). A method of computing the effectiveness of an insecticide. *Journal of Economic Entomology*, 18: 265-267.

Abe, F., Usui, K., & Hiraki, T. (2009). Fluconazole modulates membrane rigidity, heterogeneity, and water penetration into the plasma membrane in *Saccharomyces cerevisiae*. *Biochemistry*, 48: 8494-8504.

Akins, R. A. (2005). An update on antifungal targets and mechanisms of resistance in *Candida albicans*. *Medical Mycology*, 43: 285-318.

Ammermann, E., Lorenz, G., Schelberger, K., Wenderoth, B., Sauter, H., & Rentzea, C. (1992). BAS 490 F – a broad-spectrum fungicide with a new mode of action. In *Proceedings Brighton Crop Protection Conference – Pests and Diseases 1992*, Vol. 1 (pp. 403-410). Farnham, UK: British Crop Protection Council.

Anderson, J. B. (2005). Evolution of antifungal-drug resistance: mechanisms and pathogen fitness. *Nature Reviews Microbiology*, 3: 547-556.

Ao, H. C., & Griffiths, E. (1976). Change in virulence of *Septoria nodorum* and *S. tritici* after passage through alternative hosts. *Transactions of the British Mycological Society*, 66: 337-340.

Arraiano, L. S., & Brown, J. K. M. (2006). Identification of isolate-specific and partial resistance to septoria tritici blotch in 238 European wheat cultivars and breeding lines. *Plant Pathology*, 55: 726-738.

Bailey, K. L., Gossen, B. D., Lafond, G. P., Watson, P. R., & Derksen, D. A. (2001). Effect of tillage and crop rotation on root and foliar diseases of wheat and pea in Saskatchewan from 1991 to 1998: univariate and multivariate analyses. *Canadian Journal of Plant Science*, 81: 789-803.

Baldwin, B. C. (1983). Fungicidal inhibitors of ergosterol biosynthesis. *Biochemical Society Transactions*, 11: 659-663.

Baldwin, B. C. (1990). Inhibitors of ergosterol biosynthesis as crop protection agents. *Biochemical Society Transactions*, 18: 61-62.

Baloch, R. I., Mercer, E. I., Wiggins, T. E., & Baldwin, B. C. (1984). Inhibition of ergosterol biosynthesis in *Saccharomyces cerevisiae* and *Ustilago maydis* by tridemorph, fenpropimorph and fenpropidin. *Phytochemistry*, 23: 2219-2226.

Barrett-Bee, K. J., Lane, A. C., & Turner, R. W. (1986). The mode of antifungal action of tolnaftate. *Journal of Medical and Veterinary Mycology*, 24: 155-160.

Bartlett, D. W., Clough, J. M., Godwin, J. R., Hall, A. A., Hamer, M., & Parr-Dobrzanski, B. (2002). The strobilurin fungicides. *Pest Management Science*, 58: 649-662.

Bean, T. P., Cools, H. J., Lucas, J. A., Hawkins, N. D., Ward, J. L., Shaw, M. W., & Fraaije, B. A. (2009). Sterol content analysis suggests altered eburicol 14α-demethylase (CYP51) activity in isolates of *Mycosphaerella graminicola* adapted to azole fungicides. *FEMS Microbiology Letters*, 296: 266-273.

Becher, R., Weihmann, F., Deising, H. B., & Wirsel, S. G. (2011). Development of a novel multiplex DNA microarray for *Fusarium graminearum* and analysis of azole fungicide responses. *BMC Genomics*, 12: 52.

Becher, R., & Wirsel, S. G. (2012). Fungal cytochrome P450 sterol 14α-demethylase (CYP51) and azole resistance in plant and human pathogens. *Applied Microbiology and Biotechnology*, 95: 825-840.

Becker, W. F., Von Jagow, G., Anke, T., & Steglich, W. (1981). Oudemansin, strobilurin A, strobilurin B and myxothiazol: new inhibitors of the bc_1 segment of the respiratory chain with an E-*β*-methoxyacrylate system as common structural element. *FEBS Letters*, 132: 329-333.

Beddington, J., Asaduzzaman, M., Fernandez, A., Clark, M., Guillou, M., Jahn, M., Erda, L., Mamo, T., Van Bo, N., Nobre, C. A., Scholes, R., Sharma, R., & Wakhungu, J. (2011). Achieving food security in the face of climate change: Summary for policy makers from the Commission on Sustainable Agriculture and Climate Change. *CGIAR Research Program on Climate Change, Agriculture and Food Security (CCAFS)*. Available online: www.ccafs.cgiar.org/commission [accessed 2019-10-25].

Berry, P. M., Griffin, J. M., Sylvester-Bradley, R., Scott, R. K., Spink, J. H., Baker, C. J., & Clare, R. W. (2000). Controlling plant form through husbandry to minimise lodging in wheat. *Field Crops Research*, 67: 59-81.

Bjerre, K., Jørgensen, L. N., & Olesen, J. E. (2006). Site-specific management of crop disease. In A. Srinivasan (Eds.), *Handbook of Precision Agriculture - Principles and Applications* (pp. 207-252). Binghamton, NY, USA: The Haworth Press Inc.

Blake, J. J., Gosling, P., Fraaije, B. A., Burnett, F. J., Knight, S. M., Kildea, S., & Paveley, N. D. (2018). Changes in field dose–response curves for demethylation inhibitor (DMI) and quinone outside inhibitor (QoI) fungicides against *Zymoseptoria tritici*, related to laboratory sensitivity phenotyping and genotyping assays. *Pest Management Science*, 74: 302-313.

Bolton, M. D., Birla, K., Rivera-Varas, V., Rudolph, K. D., & Secor, G. A. (2012). Characterization of *CbCyp51* from field isolates of *Cercospora beticola*. *Phytopathology*, 102: 298-305.

Bossche, H. V., Lauwers, W., Willemsens, G., Marichal, P., Cornelissen, F., & Cools, W. (1984). Molecular basis for the antimycotic and antibacterial activity of N-substituted imidazoles and triazoles: The inhibition of isoprenoid biosynthesis. *Pesticide Science*, 15: 188-198.

Bottalico, A., & Perrone, G. (2002). Toxigenic *Fusarium* species and mycotoxins associated with head blight in small-grain cereals in Europe. *European Journal of Plant Pathology*, 108: 611-624.

Bouma, E. (2005). Development of comparable agro-climatic zones for the international exchange of data on the efficacy and crop safety of plant protection products. *EPPO Bulletin*, 35: 233-238.

Brading, P. A., Verstappen, E. C., Kema, G. H., & Brown, J. K. (2002). A gene-for-gene relationship between wheat and *Mycosphaerella graminicola*, the Septoria tritici blotch pathogen. *Phytopathology*, 92: 439-445.

Brent, K. J., & Hollomon, D. W. (2007a). *Fungicide resistance in crop pathogens: how can it be managed?* 2nd, revised edition. Brussels, Belgium: Fungicide Resistance Action Committee. Available online: https://www.frac.info/docs/default-source/publications/monographs/monograph-1.pdf?sfvrsn=8&sfvrsn=8 [accessed 2019-11-07].

Brent, K. J., & Hollomon, D. W. (2007b). *Fungicide resistance: the assessment of risk*, 2nd revised edition. Brussels, Belgium: Fungicide Resistance Action Committee. Available online: https://www.frac.info/docs/default-source/publications/monographs/monograph-2.pdf [accessed 2019-11-20].

Brent, K. (2012). Historical Perspectives of Fungicide Resistance. In T. S. Thind (Eds.), *Fungicide Resistance in Crop Protection: Risk and Management* (pp. 3-18). Wallingford, UK: CABI.

Brokenshire, T. (1975). The Role of Graminaceous Species in the Epidemiology of *Septoria tritici* on Wheat. *Plant Pathology*, 24: 33-38.

Brown, J. S., Kellock, A. W., & Paddick, R. G. (1978). Distribution and dissemination of *Mycosphaerella graminicola* (Fuckel) Schroeter in relation to the epidemiology of speckled leaf blotch of wheat. *Australian Journal of Agricultural Research*, 29: 1139-1145.

Brown, J. K., & Hovmøller, M. S. (2002). Aerial dispersal of pathogens on the global and continental scales and its impact on plant disease. *Science*, 297: 537-541.

Brown, J. K., Chartrain, L., Lasserre-Zuber, P., & Saintenac, C. (2015). Genetics of resistance to *Zymoseptoria tritici* and applications to wheat breeding. *Fungal Genetics and Biology*, 79: 33-41.

Brunner, P. C., Stefanato, F. L., & McDonald, B. A. (2008). Evolution of the *CYP51* gene in *Mycosphaerella graminicola*: evidence for intragenic recombination and selective replacement. *Molecular Plant Pathology*, 9: 305-316.

Brunner, P. C., Torriani, S. F., Croll, D., Stukenbrock, E. H., & McDonald, B. A. (2013). Coevolution and life cycle specialization of plant cell wall degrading enzymes in a hemibiotrophic pathogen. *Molecular Biology and Evolution*, 30: 1337-1347.

Bryson, R. J., & Brix, H. D. (2019). Challenges and prospects for fungicidal control of wheat diseases. In R. Oliver (Eds.), *Integrated disease management of wheat and barley* (pp. 219-234). Cambridge, UK: Burleigh Dodds Science Publishing Limited.

Buitrago, C., Frey, R., Wullschleger, J., & Sierotzki, H. (2014). An update on the genetic changes in the *CYP51* gene of *Mycosphaerella graminicola* and their relationship to DMI fungicide sensitivity. In H. W. Dehne, H. B. Deising, B. Fraaije, U. Gisi, D. Hermann, A. Mehl, E. C. Oerke, P. E. Russel, G. Stammler, K. H. Kuck, & H. Lyr (Eds.), *Modern Fungicides and Antifungal Compounds VII* (pp. 103-110). Braunschweig, Germany: Deutsche Phytomedizinische Gesellschaft.

Cha, R. S., Zarbl, H., Keohavong, P., & Thilly, W. G. (1992). Mismatch amplification mutation assay (MAMA): Application to the c-H-*ras* gene. *Genome Research*, 2: 14-20.

Chartrain, L., Brading, P. A., Widdowson, J. P., & Brown, J. K. M. (2004). Partial resistance to Septoria tritici blotch (*Mycosphaerella graminicola*) in wheat cultivars Arina and Riband. *Phytopathology*, 94: 497-504.

Chassot, C., Hugelshofer, U., Sierotzki, H., & Gisi, U. (2008). Sensitivity of CYP51 genotypes to DMI fungicides in *Mycosphaerella graminicola*. In H. W. Dehne, H. B. Deising, U. Gisi, K. H. Kuck, P. E. Russel, & H. Lyr (Eds.), *Modern Fungicides and Antifungal Compounds V* (pp. 129-136). Braunschweig, Germany: Deutsche Phytomedizinische Gesellschaft.

Chen, R. S., & McDonald, B. A. (1996). Sexual reproduction plays a major role in the genetic structure of populations of the fungus *Mycosphaerella graminicola*. *Genetics*, 142: 1119-1127.

Clark, W. S. (2006). *Septoria tritici* and azole performance. Fungicide resistance: are we winning the battle but losing the war? *Aspects of Applied Biology*, 78: 127-132.

Coleman, J. J., & Mylonakis, E. (2009). Efflux in fungi: la pièce de résistance. *PLoS Pathogens*, 5: e1000486.

Cools, H. J., Fraaije, B. A., & Lucas, J. A. (2005). Molecular examination of *Septoria tritici* isolates with reduced sensitivities to triazoles. In H. W. Dehne, U. Gisi, K. H. Kuck, P. E. Russel, & H. Lyr (Eds.), *Modern Fungicides and Antifungal Compounds IV* (pp. 103-114). Alton, UK: British Crop Protection Council.

Cools, H. J., & Fraaije, B. A. (2008). Are azole fungicides losing ground against Septoria wheat disease? Resistance mechanisms in *Mycosphaerella graminicola*. *Pest Management Science*, 64: 681-684.

Cools, H. J., Parker, J. E., Kelly, D. E., Lucas, J. A., Fraaije, B. A., & Kelly, S. L. (2010). Heterologous expression of mutated eburicol 14α-demethylase (CYP51) proteins of *Mycosphaerella graminicola* to assess effects on azole fungicide sensitivity and intrinsic protein function. *Applied and Environmental Microbiology*, 76: 2866-2872.

Cools, H. J., Mullins, J. G., Fraaije, B. A., Parker, J. E., Kelly, D. E., Lucas, J. A., & Kelly, S. L. (2011). Impact of recently emerged sterol 14α-demethylase (CYP51) variants of *Mycosphaerella graminicola* on azole fungicide sensitivity. *Applied and Environmental Microbiology*, 77: 3830-3837.

Cools, H. J., Bayon, C., Atkins, S., Lucas, J. A., & Fraaije, B. A. (2012). Overexpression of the sterol 14α-demethylase gene (*MgCYP51*) in *Mycosphaerella graminicola* isolates confers a novel azole fungicide sensitivity phenotype. *Pest Management Science*, 68: 1034-1040.

Cools, H. J., & Fraaije, B. A. (2013). Update on mechanisms of azole resistance in *Mycosphaerella graminicola* and implications for future control. *Pest Management Science*, 69: 150-155.

Cools, H. J., Hawkins, N. J., & Fraaije, B. A. (2013). Constraints on the evolution of azole resistance in plant pathogenic fungi. *Plant Pathology*, 62: 36-42.

Coste, A., Turner, V., Ischer, F., Morschhäuser, J., Forche, A., Selmecki, A., Berman, J., Bille, J., & Sanglard, D. (2006). A mutation in Tac1p, a transcription factor regulating *CDR1* and *CDR2*, is coupled with loss of heterozygosity at chromosome 5 to mediate antifungal resistance in *Candida albicans*. *Genetics*, 172: 2139-2156.

Coste, A., Selmecki, A., Forche, A., Diogo, D., Bougnoux, M. E., d'Enfert, C., Berman, J., & Sanglard, D. (2007). Genotypic evolution of azole resistance mechanisms in sequential *Candida albicans* isolates. *Eukaryotic Cell*, 6: 1889-1904.

Cowen, L. E., Kohn, L. M., & Anderson, J. B. (2001). Divergence in fitness and evolution of drug resistance in experimental populations of *Candida albicans*. *Journal of Bacteriology*, 183: 2971-2978.

Cowger, C., Hoffer, M. E., & Mundt, C. C. (2000). Specific adaptation by *Mycosphaerella graminicola* to a resistant wheat cultivar. *Plant Pathology*, 49: 445-451.

Cowger, C., McDonald, B. A., & Mundt, C. C. (2002). Frequency of sexual reproduction by *Mycosphaerella graminicola* on partially resistant wheat cultivars. *Phytopathology*, 92: 1175-1181.

Cowger, C., & Mundt, C. C. (2002). Effects of wheat cultivar mixtures on epidemic progression of Septoria tritici blotch and pathogenicity of *Mycosphaerella graminicola*. *Phytopathology*, 92: 617-623.

Daboussi, M. J., & Capy, P. (2003). Transposable elements in filamentous fungi. *Annual Reviews in Microbiology*, 57: 275-299.

De Jonge, R., van Esse, H. P., Kombrink, A., Shinya, T., Desaki, Y., Bours, R., van der Krol, S., Shibuya, N., Joosten, M. H. A. J., & Thomma, B. P. H. J. (2010). Conserved fungal LysM effector Ecp6 prevents chitin-triggered immunity in plants. *Science*, 329: 953-955.

Delp, C. J., & Dekker, J. (1985). Fungicide resistance: definitions and use of terms. *EPPO Bulletin*, 15: 333-335.

Délye, C., Laigret, F., & Corio-Costet, M. F. (1997). A mutation in the 14 alpha-demethylase gene of *Uncinula necator* that correlates with resistance to a sterol biosynthesis inhibitor. *Applied and Environmental Microbiology*, 63: 2966-2970.

De Miccolis Angelini, R. M., Pollastro, S., & Faretra, F. (2015). Genetics of Fungicide Resistance. In H. Ishii, & D. W. Hollomon (Eds.), *Fungicide Resistance in Plant Pathogens. Principles and a Guide to Practical Management* (pp. 13-34). Tokyo, Japan: Springer.

Deng, H., Gao, R., Liao, X., & Cai, Y. (2017). CRISPR system in filamentous fungi: current achievements and future directions. *Gene*, 627: 212-221.

Desmazières, J. B. H. J. (1842). Cryptogames nouvelles. *Annales des Sciences Naturelles*, 17: 91-118.

De Waard, M. A., Andrade, A. C., Hayashi, K., Schoonbeek, H. J., Stergiopoulos, I., & Zwiers, L. H. (2006). Impact of fungal drug transporters on fungicide sensitivity, multidrug resistance and virulence. *Pest Management Science,* 62: 195-207.

Dhillon, B., Gill, N., Hamelin, R. C., & Goodwin, S. B. (2014). The landscape of transposable elements in the finished genome of the fungal wheat pathogen *Mycosphaerella graminicola*. *BMC Genomics*, 15: 1132.

EPPO (2019). European and Mediterranean Plant Protection Organization Global Database. Available online: https://gd.eppo.int/taxon/SEPTTR [accessed 2019-10-28].

Eriksen, L., & Munk, L. (2003). The occurrence of *Mycosphaerella graminicola* and its anamorph *Septoria tritici* in winter wheat during the growing season. *European Journal of Plant Pathology*, 109: 253-259.

Estep, L. K., Torriani, S. F., Zala, M., Anderson, N. P., Flowers, M. D., McDonald, B. A., Mundt, C. C., & Brunner, P. C. (2015). Emergence and early evolution of fungicide resistance in North American populations of *Zymoseptoria tritici*. *Plant Pathology*, 64: 961-971.

European Commission (2019). *Final Renewal report for the active substance chlorothalonil* [PDF file]. European Commission Directorate-General for Health and Food Safety. Available online: https://ec.europa.eu/food/plant/pesticides/eu-pesticides-database/public/?event=activesubstance.detail&language=EN&selectedID=1125 [accessed 2020-09-05].

Eyal, Z., Scharen, A. L., Prescott, J. M., & van Ginkel, M. (1987). *The Septoria Diseases of Wheat: Concepts and methods of disease management.* Mexico city, Mexico: International Maize and Wheat Improvement Center (CIMMYT).

Fan, J., Urban, M., Parker, J. E., Brewer, H. C., Kelly, S. L., Hammond-Kosack, K. E., Fraaije, B. A., Liu, X., & Cools, H. J. (2013). Characterization of the sterol 14α-demethylases of *Fusarium graminearum* identifies a novel genus-specific CYP 51 function. *New Phytologist*, 198: 821-835.

FAO (2009). *World Summit on food security. Rome, 16 – 18 November 2009. Opening Statement of FAP Director-General, Dr Jacques Diouf* [PDF file]. Food and Agriculture Organization of the United Nations. Available online: http://www.fao.org/tempref/docrep/fao/meeting/018/k6628e.pdf [accessed 2019-10-25].

FAO (2019). Food and Agriculture Organization of the United Nations. Available online: http://fenix.fao.org/faostat/internal/en/#data/QC [accessed 2019-10-25].

Finckh, M. R., & Mundt, C. C. (1992). Stripe rust, yield, and plant competition in wheat cultivar mixtures. *Phytopathology*, 82: 905-913.

Fischer, R. A., & Stapper, M. (1987). Lodging effects on high-yielding crops of irrigated semidwarf wheat. *Field Crops Research*, 17: 245-258.

Fones, H., & Gurr, S. (2015). The impact of Septoria tritici Blotch disease on wheat: An EU perspective. *Fungal Genetics and Biology*, 79: 3-7.

Fraaije, B. A., Butters, J. A., Coelho, J. M., Jones, D. R., & Hollomon, D. W. (2002). Following the dynamics of strobilurin resistance in *Blumeria graminis* f. sp. *tritici* using quantitative allele-specific real-time PCR measurements with the fluorescent dye SYBR Green I. *Plant Pathology*, 51: 45-54.

Fraaije, B. A., Burnett, F. J., Clark, W. S., Motteram, J., & Lucas, J. A. (2005). Resistance development to QoI inhibitors in populations of *Mycosphaerella graminicola* in the UK. In H. W. Dehne, U. Gisi, K. H. Kuck, P. E. Russel, & H. Lyr (Eds.), *Modern Fungicides and Antifungal Compounds IV* (pp. 63-71). Alton, UK: British Crop Protection Council.

Fraaije, B. A., Cools, H. J., Kim, S. H., Motteram, J., Clark, W. S., & Lucas, J. A. (2007). A novel substitution I381V in the sterol 14α-demethylase (CYP51) of *Mycosphaerella graminicola* is differentially selected by azole fungicides. *Molecular Plant Pathology*, 8: 245-254.

Fraaije, B. A., Bayon, C., Atkins, S., Cools, H. J., Lucas, J. A., & Fraaije, M. W. (2012). Risk assessment studies on succinate dehydrogenase inhibitors, the new weapons in the battle to control Septoria leaf blotch in wheat. *Molecular Plant Pathology*, 13: 263-275.

FRAC (2019). Fungicide Resistance Action Committee. Available online: https://www.frac.info/home [accessed 2019-11-11].

Gadher, P., Mercer, E. I., Baldwin, B. C., & Wiggins, T. E. (1983). A comparison of the potency of some fungicides as inhibitors of sterol 14-demethylation. *Pesticide Biochemistry and Physiology*, 19: 1-10.

Germer, S., Holland, M. J., & Higuchi, R. (2000). High-throughput SNP allele-frequency determination in pooled DNA samples by kinetic PCR. *Genome Research*, 10: 258-266.

Gigot, C., Saint-Jean, S., Huber, L., Maumené, C., Leconte, M., Kerhornou, B., & de Vallavieille-Pope, C. (2013). Protective effects of a wheat cultivar mixture against splash-dispersed Septoria Tritici Blotch epidemics. *Plant Pathology*, 62: 1011-1019.

Girard, L., & Freeling, M. (1999). Regulatory changes as a consequence of transposon insertion. *Developmental Genetics*, 25: 291-296.

Gisi, U., Hermann, D., Ohl, L., & Steden, C. (1997). Sensitivity profiles of *Mycosphaerella graminicola* and *Phytophthora infestans* populations to different classes of fungicides. *Pesticide Science*, 51: 290-298.

Gisi, U., Sierotzki, H., Cook, A., & McCaffery, A. (2002). Mechanisms influencing the evolution of resistance to Qo inhibitor fungicides. *Pest Management Science*, 58: 859-867.

Gisi, U., Pavic, L., Stanger, C., Hugelshofer, U., & Sierotzki, H. (2005). Dynamics of *Mycosphaerella graminicola* populations in response to selection by different fungicides. In H. W. Dehne, U. Gisi, K. H. Kuck, P. E. Russel, & H. Lyr (Eds.), *Modern Fungicides and Antifungal Compounds IV* (pp. 89-101). Alton, UK: British Crop Protection Council.

Glaab, W. E., & Skopek, T. R. (1999). A novel assay for allelic discrimination that combines the fluorogenic 5′ nuclease polymerase chain reaction (TaqMan®) and mismatch amplification mutation assay. *Mutation Research*, 430: 1-12.

Gladders, P., Paveley, N. D., Barrie, I. A., Hardwick, N. V., Hims, M. J., Langton, S., & Taylor, M. C. (2001). Agronomic and meteorological factors affecting the severity of leaf blotch caused by *Mycosphaerella graminicola* in commercial wheat crops in England. *Annals of Applied Biology*, 138: 301-311.

Glättli, A., Grote, T., & Stammler, G. (2011). SDH-inhibitors: history, biological performance and molecular mode of action. In H. W. Dehne, H. B. Deising, U. Gisi, K. H. Kuck, P. E. Russel, & H. Lyr (Eds.), *Modern Fungicides and Antifungal Compounds VI* (pp. 159-169). Braunschweig, Germany: Deutsche Phytomedizinische Gesellschaft.

Godwin, J. R., Anthony, V. M., Clough, J. M., & Godfrey, C. R. A. (1992). ICIA5504: a novel, broad spectrum, systemic β-methoxyacrylate fungicide. In *Proceedings Brighton Crop Protection Conference – Pests and Diseases 1992*, Vol. 1 (pp. 435-442). Farnham, UK: British Crop Protection Council.

Goodwin, S. B., Cavaletto, J. R., Waalwijk, C., & Kema, G. H. J. (2001). DNA fingerprint probe from *Mycosphaerella graminicola* identifies an active transposable element. *Phytopathology*, 91: 1181-1188.

Goodwin, S. B., Ben M'Barek, S., Dhillon, B., Wittenberg, A. H. J., Crane, C. F., Hane, J. K., Foster, A. J., van der Lee, T. A. J., Grimwood, J., Aerts, A., Antoniw, J., Bailey, A., Bluhm, B., Bowler, J., Bristow, J., van der Burgt, A., Canto-Canché, B., Churchill, A. C. L., Conde-Ferràez, L., … Kema, G. H. J. (2011). Finished genome of the fungal wheat pathogen *Mycosphaerella graminicola* reveals dispensome structure, chromosome plasticity, and stealth pathogenesis. *PLoS Genetics*, 7: e1002070.

Goodwin, S. B. (2019). Diseases affecting wheat: *Septoria tritici* blotch. In R. Oliver (Eds.), *Integrated disease management of wheat and barley* (pp. 47-68). Cambridge, UK: Burleigh Dodds Science Publishing Limited.

Gozukirmizi, N. (2019). Transposons continue the amaze. *International Journal of Science Letters*, 1: 1-13.

Grabke, A., & Stammler, G. (2015). A *Botrytis cinerea* population from a single strawberry field in Germany has a complex fungicide resistance pattern. *Plant Disease*, 99: 1078-1086.

Graf, S. (2018). *Characterisation of metrafenone and succinate dehydrogenase inhibitor resistant isolates of the grapevine powdery mildew* Erysiphe necator. Doctoral dissertation, Technische Universität Kaiserslautern, Kaiserslautern, Germany.

Grimmer, M. K., van den Bosch, F., Powers, S. J., & Paveley, N. D. (2015). Fungicide resistance risk assessment based on traits associated with the rate of pathogen evolution. *Pest Management Science*, 71: 207-215.

Grkovic, S., Brown, M. H., & Skurray, R. A. (2002). Regulation of bacterial drug export systems. *Microbiology and Molecular Biology Review*, 66: 671-701.

Gulshan, K., & Moye-Rowley, W. S. (2007). Multidrug resistance in fungi. *Eukaryotic Cell*, 6: 1933-1942.

Gustafson, P., Raskina, O., Ma, X., & Nevo, E. (2009). Wheat evolution, domestication, and improvement. In B. F. Carver (Eds.), *Wheat Science and Trade* (pp. 5-30). Ames, IA, USA: Wiley-Blackwell.

Hägerhäll, C. (1997). Succinate: quinone oxidoreductases variations on a conserved theme. *Biochimica et Biophysica Acta*, 1320: 107-141.

Haggag, W. M., Abouziena, H. F., Abd-El-Kreem, F., & El Habbasha, S. (2015). Agriculture biotechnology for management of multiple biotic and abiotic environmental stress in crops. *Journal of Chemical and Pharmaceutical Research*, 7: 882-889.

Hahn, S., & Young, E. T. (2011). Transcriptional regulation in *Saccharomyces cerevisiae*: transcription factor regulation and function, mechanisms of initiation, and roles of activators and coactivators. *Genetics*, 189: 705-736.

Hahn, M., & Leroch, M. (2015). Multidrug Efflux Transporters. In H. Ishii, & D. W. Hollomon (Eds.), *Fungicide Resistance in Plant Pathogens. Principles and a Guide to Practical Management* (pp. 233-248). Tokyo, Japan: Springer.

Hamamoto, H., Hasegawa, K., Nakaune, R., Lee, Y. J., Makizumi, Y., Akutsu, K., & Hibi, T. (2000). Tandem Repeat of a Transcriptional Enhancer Upstream of the Sterol 14α-Demethylase Gene (*CYP51*) in *Penicillium digitatum*. *Applied and Environmental Microbiology*, 66: 3421-3426.

Hansen, J. G., Secher, B. J., Jørgensen, L. N., & Welling, B. (1994). Thresholds for control of *Septoria* spp. in winter wheat based on precipitation and growth stage. *Plant Pathology*, 43: 183-189.

Hawkins, N. J., & Fraaije, B. A. (2018). Fitness penalties in the evolution of fungicide resistance. *Annual Review of Phytopathology*, 56: 339-360.

Heick, T. M., Justesen, A. F., & Jørgensen, L. N. (2017a). Resistance of wheat pathogen *Zymoseptoria tritici* to DMI and QoI fungicides in the Nordic-Baltic region-a status. *European Journal of Plant Pathology*, 149: 669-682.

Heick, T. M., Justesen, A. F., & Jørgensen, L. N. (2017b). Anti-resistance strategies for fungicides against wheat pathogen *Zymoseptoria tritici* with focus on DMI fungicides. *Crop Protection*, 99: 108-117.

Heick Wieczorek, T. M. (2017). *Fungicide resistance of wheat pathogen* Zymoseptoria tritici-*Ways of minimising fungicide resistance in practice.* Doctoral dissertation, Aarhus Universitet, Aarhus, Denmark.

Hilu, H. B., & Bever, W. M. (1957). Inoculation, oversummering and suscept-pathogen relationships of *Septoria tritici* on *Triticum* species. *Phytopathology*, 47: 474-480.

Hobbelen, P. H. F., Paveley, N. D., Oliver, R. P., & Van den Bosch, F. (2013). The usefulness of fungicide mixtures and alternation for delaying the selection for resistance in populations of *Mycosphaerella graminicola* on winter wheat: A modeling analysis. *Phytopathology*, 103: 690-707.

Hollomon, D. W. (1981). Genetic control of ethirimol resistance in a natural population of *Erysiphe graminis* f. sp. *hordei*. *Phytopathology*, 71: 536-540.

Hollomon, D.W. (2015). Fungicide Resistance: 40 Years on and Still a Major Problem. In H. Ishii, & D. W. Hollomon (Eds.), *Fungicide Resistance in Plant Pathogens. Principles and a Guide to Practical Management* (pp. 3-11). Tokyo, Japan: Springer.

Holmes, S. J. I., & Colhoun, J. (1974). Infection of wheat by *Septoria nodorum* and *S. tritici* in relation to plant age, air temperature and relative humidity. *Transactions of the British Mycological Society*, 63: 329-338.

Holmes, S. J. I., & Colhoun, J. (1975). Straw-borne inoculum of *Septoria nodorum* and *S. tritici* in relation to incidence of disease on wheat plants. *Plant Pathology*, 24: 63-66.

Huf, A., Rehfus, A., Lorenz, K. H., Bryson, R., Voegele, R. T., & Stammler, G. (2018). Proposal for a new nomenclature for *CYP51* haplotypes in *Zymoseptoria tritici* and analysis of their distribution in Europe. *Plant Pathology*, 67: 1706-1712.

Huf, A., Pfleger, S., Strobel, D., Bryson, R., Voegele, R. T., & Stammler, G. (2020). Distribution and changes of genotypes associated to DMI sensitivity of *Zymoseptoria tritici* in Europe. In H. B. Deising, B. Fraaije, A. Mehl, E. C. Oerke, H. Sierotzki, & G. Stammler (Eds.), *Modern Fungicides and Antifungal Compounds IX* (pp. 93-98). Braunschweig, Germany: Deutsche Phytomedizinische Gesellschaft.

Hunter, T., Coker, R., & Royle, D. (1999). The teleomorph stage, *Mycosphaerella graminicola*, in epidemics of Septoria Tritici Blotch on winter wheat in the UK. *Plant Pathology*, 48: 51-57.

Ishii, H. (2015). Stability of resistance. In H. Ishii, & D. W. Hollomon (Eds.), *Fungicide Resistance in Plant Pathogens. Principles and a Guide to Practical Management* (pp. 35-48). Tokyo, Japan: Springer.

Jabs, T., Cronshaw, K., & Freund, A. (2001). New strobilurin resistance mechanism in apple scab (*Venturia inaequalis*). *Phytomedizin*, *31*: 15-16.

Jefcoate, C. R. (1978). Measurement of substrate and inhibitor binding to microsomal cytochrome P-450 by optical-difference spectroscopy. In S. Fleischer, & L. Packer (Eds.), *Methods in Enzymology*, Vol. 52 (pp. 258-279). Cambridge, MA, USA: Academic Press.

Jlibene, M., Gustafson, J. P., & Rajaram, S. (1994). Inheritance of resistance to *Mycosphaerella graminicola* in hexaploid wheat. *Plant breeding*, 112: 301-310.

Jones, D. G., & Cooke, B. M. (1969). The Epidemiology of *Septoria tritici* and *S. nodorum*: I. A tentative key for assessing *Septoria Tritici* infection on wheat heads. *Transactions of the British Mycological Society*, 53: 39-46.

Joosten, M. H., Hendrickx, L. J., & De Wit, P. J. (1990). Carbohydrate composition of apoplastic fluids isolated from tomato leaves inoculated with virulent or avirulent races of *Cladosporium fulvum* (syn. *Fulvia fulva*). *Netherlands Journal of Plant Pathology*, 96: 103-112.

Jørgensen, L. N., Secher, B. J. M., Olesen, J. E., & Mortensen, J. (1997). Need for fungicide treatments when varying agricultural parameters. *Aspects of Applied Biology*, 50: 285-292.

Jørgensen, L. N., Nielsen, G. C., Ørum, J. E., Jensen, J. E., & Pinnschmidt, H. O. (2008). Integrating disease control in winter wheat–optimizing fungicide input. *Outlooks on Pest Management*, 19: 206-213.

Jørgensen, L. N., Hovmøller, M. S., Hansen, J. G., Lassen, P., Clark, B., Bayles, R., Rodemann, B., Flath, K., Jahn, M., Goral, T., Czembor, J. J., Cheyron, P., Maumene, C., de Pope, C., Ban, R., Nielsen, G. C., & Berg, G. (2014). IPM strategies and their dilemmas including an introduction to www. eurowheat. org. *Journal of Integrative Agriculture*, 13: 265-281.

Jørgensen, L. N., van den Bosch, F., Oliver, R. P., Heick, T. M., & Paveley, N. D. (2017a). Targeting fungicide inputs according to need. *Annual Review of Phytopathology*, 55: 181-203.

Jørgensen, L. N., Matzen, N., Semaskiene, R., Korbas, M., Danielewicz, J., Glazek, M., Maumene, C., Rodemann, B., Weigand, S., Hess, M., Blake, J., Clark, B., Kildea, S., Bataille, C., & Ban, R. (2017b). Azoles have different strengths and perform diversely across Europe. In H. B. Deising, B. Fraaije, A. Mehl, E. C. Oerke, H. Sierotzki, & G. Stammler (Eds.), *Modern Fungicides and Antifungal Compounds VIII* (pp. 129-134). Braunschweig, Germany: Deutsche Phytomedizinische Gesellschaft.

Jørgensen, L. N., Matzen, N., Hansen, J. G., Semaskiene, R., Korbas, M., Danielewicz, J., Glazek, M., Maumene, C., Rodemann, B., Weigand, S., Hess, M., Blake, J., Clark, B., Kildea, S., Batailles, C., Ban, R., Havis, N., & Treikale, O. (2018). Four azoles' profile in the control of Septoria, yellow rust and brown rust in wheat across Europe. *Crop Protection*, 105: 16-27.

Jørgensen, L. N., Oliver, P. O., & Heick, T. M. (2019). Occurrence and avoidance of fungicide resistance in cereal diseases. In R. Oliver (Eds.), *Integrated disease management of wheat and barley* (pp. 235-259). Cambridge, UK: Burleigh Dodds Science Publishing Limited.

Karaoglanidis, G. S., Thanassoulopoulos, C. C., & Ioannidis, P. M. (2001). Fitness of *Cercospora beticola* field isolates – resistant and – sensitive to demethylation inhibitor fungicides. *European Journal of Plant Pathology*, 107: 337-347.

Kellner, R., Bhattacharyya, A., Poppe, S., Hsu, T. Y., Brem, R. B., & Stukenbrock, E. H. (2014). Expression profiling of the wheat pathogen *Zymoseptoria tritici* reveals genomic patterns of transcription and host-specific regulatory programs. *Genome Biology and Evolution*, 6: 1353-1365.

Kelly, S. L., Lamb, D. C., Corran, A. J., Baldwin, B. C., & Kelly, D. E. (1995). Mode of action and resistance to azole antifungals associated with the formation of 14α–methylergosta-8,24(28)-dien-3β,6α-diol. *Biochemical and Biophysical Research Communications*, 207: 910-915.

Kelly, S. L., & Kelly, D. E. (2013). Microbial cytochromes P450: biodiversity and biotechnology. Where do cytochromes P450 come from, what do they do and what can they do for us? *Philosophical Transactions of the Royal Society B*, 368: 20120476.

Kema, G. H., Verstappen, E. C., Todorova, M., & Waalwijk, C. (1996a). Successful crosses and molecular tetrad and progeny analyses demonstrate heterothallism in *Mycosphaerella graminicola*. *Current Genetics*, 30: 251-258.

Kema, G. H., Yu, D., Rijkenberg, F. H., Shaw, M. W., & Baayen, R. P. (1996b). Histology of the pathogenesis of *Mycosphaerella graminicola* in wheat. *Phytopathology*, 86: 777-786.

Kempken, F., & Kück, U. (1998). Transposons in filamentous fungi—facts and perspectives. *BioEssays*, 20: 652-659.

Keon, J., Antoniw, J., Carzaniga, R., Deller, S., Ward, J. L., Baker, J. M., Beale, M. H., Hammond-Kosack, K., & Rudd, J. J. (2007). Transcriptional adaptation of *Mycosphaerella graminicola* to programmed cell death (PCD) of its susceptible wheat host. *Molecular Plant-Microbe Interactions*, 20: 178-193.

Khush, G. S. (1999). Green revolution: preparing for the 21st century. *Genome*, 42: 646-655.

Kildea, S. (2016). Wheat disease control and resistance issues. In *National Tillage Conference 2016* (pp. 39-46). Carlow, Ireland: Teagasc.

Kildea, S., Heick, T. M., Grant, J., Mehenni-Ciz, J., & Dooley, H. (2019). A combination of target-site alterations, overexpression and enhanced efflux activity contribute to reduced azole sensitivity present in the Irish *Zymoseptoria tritici* population. *European Journal of Plant Pathology*, 154: 529-540.

Kirikyali, N., Diez, P., Luo, J., Hawkins, N., & Fraaije, B. A. (2017). Azole and SDHI sensitivity status of *Zymoseptoria tritici* field populations sampled in France, Germany and the UK during 2015. In H. B. Deising, B. Fraaije, A. Mehl, E. C. Oerke, H. Sierotzki, & G. Stammler (Eds.), *Modern Fungicides and Antifungal Compounds VIII* (pp. 153-158). Braunschweig, Germany: Deutsche Phytomedizinische Gesellschaft.

Kleckner, N. (1981). Transposable elements in prokaryotes. *Annual Review of Genetics*, 15: 341-404.

Kretschmer, M., Leroch, M., Mosbach, A., Walker, A. S., Fillinger, S., Mernke, D., Schoonbeek, H. J., Pradier, J. M., Leroux, P., de Waard, M. A., & Hahn, M. (2009). Fungicide-driven evolution and molecular basis of multidrug resistance in field populations of the grey mould fungus *Botrytis cinerea*. *PLoS Pathogens*, 5: e1000696.

Krishnan, P., Meile, L., Plissonneau, C., Ma, X., Hartmann, F. E., Croll, D., McDonald, B. A., & Sánchez-Vallet, A. (2018). Transposable element insertions shape gene regulation and melanin production in a fungal pathogen of wheat. *BMC Biology*, 16: 78.

Kuck, K. H., Stenzel, K., & Vors, J. P. (2012). Sterol biosynthesis inhibitors. In W. Krämer, U. Schirmer, P. Jeschke, & M. Witschel (Eds.), *Modern Crop Protection Compounds*, *Second Edition* (pp. 761-805). Weinheim, Germany: Wiley-VCH Verlag GmbH & Co. KGaA.

Lamb, D. C., Kelly, D. E., Corran, A. J., Baldwin, B. C., & Kelly, S. L. (1996). Role of sterol $\Delta^{5,6}$-desaturase in azole antifungal mode of action and resistance. *Pesticide Science*, 46: 294-298.

Lamb, D., Kelly, D., & Kelly, S. (1999). Molecular aspects of azole antifungal action and resistance. *Drug Resistance Updates*, 2: 390-402.

Lamb, D. C., Ikeda, H., Nelson, D. R., Ishikawa, J., Skaug, T., Jackson, C., Omura, S., Waterman, M. R., & Kelly, S. L. (2003). Cytochrome P450 complement (CYPome) of the avermectin-producer *Streptomyces avermitilis* and comparison to that of *Streptomyces coelicolor* A3(2). *Biochemical and Biophysical Research Communications*, 307: 610-619.

Leroch, M., Plesken, C., Weber, R. W., Kauff, F., Scalliet, G., & Hahn, M. (2013). Gray mold populations in German strawberry fields are resistant to multiple fungicides and dominated by a novel clade closely related to *Botrytis cinerea*. *Applied and Environmental Microbiology*, 79: 159-167.

Leroux, P., Debieu, D., Albertini, C., Arnold, A., Bach, J., Chapeland, F., Fournier, E., Fritz, R., Gredt, M., Hugon, M., Lanen, C., Malosse, C., & Thebaud, G. (2002). The hydroxyanilide botryticide fenhexamid: mode of action and mechanism of resistance. In H. W. Dehne, U. Gisi, K. H. Kuck, P. E. Russel, & H. Lyr (Eds.), *Modern Fungicides and Antifungal Compounds III* (pp. 29-40). Bonn, Germany: AgroConcept GmbH.

Leroux, P., Albertini, C., Gautier, A., Gredt, M., & Walker, A. S. (2007). Mutations in the *CYP51* gene correlated with changes in sensitivity to sterol 14α-demethylation inhibitors in field isolates of *Mycosphaerella graminicola*. *Pest Management Science*, 63: 688-698.

Leroux, P., & Walker, A. S. (2011). Multiple mechanisms account for resistance to sterol 14α-demethylation inhibitors in field isolates of *Mycosphaerella graminicola*. *Pest Management Science*, 67: 44-59.

Leroux, P., Gredt, M., Remuson, F., Micoud, A., & Walker, A. S. (2013). Fungicide resistance status in French populations of the wheat eyespot fungi *Oculimacula acuformis* and *Oculimacula yallundae*. *Pest Management Science*, 69: 15-26.

Lesniak, K. E., Proffer, T. J., Beckerman, J. L., & Sundin, G. W. (2011). Occurrence of QoI resistance and detection of the G143A mutation in Michigan populations of *Venturia inaequalis*. *Plant Disease*, 95: 927-934.

Lev-Yadun, S., Gopher, A., & Abbo, S. (2000). The cradle of agriculture. *Science*, 288: 1602-1603.

Li, X., Schuler, M. A., & Berenbaum, M. R. (2007). Molecular mechanisms of metabolic resistance to synthetic and natural xenobiotics. *Annual Review of Entomology*, 52: 231-253.

Lucas, J. A., & Fraaije, B. A. (2008). QoI resistance in *Mycosphaerella graminicola*: what have we learned so far? In H. W. Dehne, H. B. Deising, U. Gisi, K. H. Kuck, P. E. Russel, & H. Lyr (Eds.), *Modern Fungicides and Antifungal Compounds V* (pp. 71-77). Braunschweig, Germany: Deutsche Phytomedizinische Gesellschaft.

Lucas, J., Cools, H., & Fraaije, B. (2013). *Understanding the evolution of fungicide resistance* [PDF file]. Agriculture and Horticulture Development Board. Available online: https://cereals.ahdb.org.uk/media/243910/John_Lucas.pdf [accessed 2018-03-18].

Lucas, J. A., Hawkins, N. J., & Fraaije, B. A. (2015). The evolution of fungicide resistance. In S. Sariaslani, & G. M. Gadd (Eds.), *Advances in Applied Microbiology*, Vol. 90 (pp. 29-92). Waltham, MA, USA: Academic Press.

Luo, C. X., & Schnabel, G. (2008). The cytochrome P450 lanosterol 14α-demethylase gene is a demethylation inhibitor fungicide resistance determinant in *Monilinia fructicola* field isolates from Georgia. *Applied and Environmental Microbiology*, 74: 359-366.

Ma, Z., & Michailides, T. J. (2005). Advances in understanding molecular mechanisms of fungicide resistance and molecular detection of resistant genotypes in phytopathogenic fungi. *Crop Protection*, 24: 853-863.

Ma, B., & Uddin, W. (2009). Fitness and competitive ability of an azoxystrobin-resistant G143A mutant of *Magnaporthe oryzae* from perennial ryegrass. *Plant Disease*, 93:1044-1049.

Mair, W., Lopez-Ruiz, F., Stammler, G., Clark, W., Burnett, F., Hollomon, D., Ishii, H., Thind, T. S., Brown, J. K. M., Fraaije, B., Cools, H., Shaw, M., Fillinger, S., Walker, A. S., Mellado, E., Schnabel, G., Mehl, A., & Oliver, R. P. (2016). Proposal for a unified nomenclature for target-site mutations associated with resistance to fungicides. *Pest Management Science*, 72: 1449-1459.

Marichal, P., Bossche, H. V., Odds, F. C., Nobels, G., Warnock, D. W., Timmerman, V., van Broeckhoven, C., Fay, S., & Mose-Larsen, P. (1997). Molecular biological characterization of an azole-resistant *Candida glabrata* isolate. *Antimicrobial Agents and Chemotherapy*, 41: 2229-2237.

Marshall, R., Kombrink, A., Motteram, J., Loza-Reyes, E., Lucas, J., Hammond-Kosack, K. E., Thomma, B. P. H. J., & Rudd, J. J. (2011). Analysis of two in planta expressed LysM effector homologs from the fungus *Mycosphaerella graminicola* reveals novel functional properties and varying contributions to virulence on wheat. *Plant Physiology*, 156: 756-769.

Mavroeidi, V. I., & Shaw, M. W. (2005). Sensitivity distributions and cross-resistance patterns of *Mycosphaerella graminicola* to fluquinconazole, prochloraz and azoxystrobin over a period of 9 years. *Crop Protection*, 24: 259-266.

McDonald, B. A., & Martinez, J. P. (1990). DNA restriction fragment length polymorphisms among *Mycosphaerella graminicola* (anamorph *Septoria tritici*) isolates collected from a single wheat field. *Phytopathology*, 80: 1368-1373.

McDonald, B. A., & Martinez, J. P. (1991). Chromosome length polymorphisms in a *Septoria tritici* population. *Current Genetics*, 19: 265-271.

McDonald, M. C., Renkin, M., Spackman, M., Orchard, B., Croll, D., Solomon, P. S., & Milgate, A. (2019). Rapid parallel evolution of azole fungicide resistance in Australian populations of the wheat pathogen *Zymoseptoria tritici*. *Applied and Environmental Microbiology*, 85: e01908-18.

Mercer, E. I. (1993). Inhibitors of sterol biosynthesis and their applications. *Progress in Lipid Research*, 32: 357-416.

Mernke, D., Dahm, S., Walker, A. S., Lalève, A., Fillinger, S., Leroch, M., & Hahn, M. (2011). Two promoter rearrangements in a drug efflux transporter gene are responsible for the appearance and spread of multidrug resistance phenotype MDR2 in *Botrytis cinerea* isolates in French and German vineyards. *Phytopathology*, 101: 1176-1183.

Mikaberidze, A., & McDonald, B. A. (2015). Fitness cost of resistance: impact on management. In H. Ishii, & D. W. Hollomon (Eds.), *Fungicide Resistance in Plant Pathogens. Principles and a Guide to Practical Management* (pp. 77-89). Tokyo, Japan: Springer.

Milgate, A., Adorada, D., Orchard, B., & Pattemore, J. (2016). First report of resistance to DMI fungicides in Australian populations of the wheat pathogen *Zymoseptoria tritici*. *Plant Disease*, 100: 522.

Mille, B., Fraj, M. B., Monod, H., & de Vallavieille-Pope, C. (2006). Assessing four-way mixtures of winter wheat cultivars from the performances of their two-way and individual components. *European Journal of Plant Pathology*, 114: 163-173.

Milne, A., Paveley, N., Audsley, E., & Livermore, P. (2003). A wheat canopy model for use in disease management decision support systems. *Annals of Applied Biology*, 143: 265-274.

Mirzadi Gohari, A., Ware, S. B., Wittenberg, A. H. J., Mehrabi, R., Ben M'Barek, S., Verstappen, E. C. P., van der Lee, T. A. J., Robert, O., Schouten, H. J., de Wit, P. P. J. G. M., & Kema, G. H. J. (2015). Effector discovery in the fungal wheat pathogen *Zymoseptoria tritici*. *Molecular Plant Pathology*, 16: 931-945.

Moreau, J. M., & Maraite, H. (2000). Development of an interactive decision-support system on a web site for control of *Mycosphaerella graminicola* in winter wheat. *EPPO Bulletin*, 30: 161-163.

Morschhäuser, J. (2010). Regulation of multidrug resistance in pathogenic fungi. *Fungal Genetics and Biology*, 47: 94-106.

Mullins, J. G. L., Parker, J. E., Cools, H. J., Togawa, R. C., Lucas, J. A., Fraaije, B. A., Kelly, D. E., & Kelly, S. L. (2011). Molecular modelling of the emergence of azole resistance in *Mycosphaerella graminicola*. *PLoS One*, 6: e20973.

Newe, M., Meier, H., Johnen, A., & Volk, T. (2003). ProPlant expert.com–an online consultation system on crop protection in cereals, rape, potatoes and sugarbeet. *EPPO Bulletin*, 33: 443-449.

O'Driscoll, A., Kildea, S., Doohan, F., Spink, J., & Mullins, E. (2014). The wheat–Septoria conflict: a new front opening up? *Trends in Plant Science*, 19: 602-610.

Oerke, E. C. (2006). Crop losses to pests. *The Journal of Agricultural Science*, 144: 31-43.

Oliver, R. P., & Hewitt, H. G. (2014). *Fungicides in Crop Protection*, 2nd Edition. Wallingford, UK: CABI.

Omrane, S., Sghyer, H., Audéon, C., Lanen, C., Duplaix, C., Walker, A. S., & Fillinger, S. (2015). Fungicide efflux and the MgMFS1 transporter contribute to the multidrug resistance phenotype in *Zymoseptoria tritici* field isolates. *Environmental Microbiology*, 17: 2805-2823.

Omrane, S., Audéon, C., Ignace, A., Duplaix, C., Aouini, L., Kema, G., Walker, A. S., & Fillinger, S. (2017). Plasticity of the *MFS1* promoter leads to multidrug resistance in the wheat pathogen *Zymoseptoria tritici*. *mSphere*, 2: e00393-17.

Orton, E. S., Deller, S., & Brown, J. K. (2011). *Mycosphaerella graminicola*: from genomics to disease control. *Molecular Plant Pathology*, 12: 413-424.

Parker, J. E., Warrilow, A. G. S., Cools, H. J., Martel, C. M., Nes, W. D., Fraaije, B. A., Lucas, J. A., Kelly, D. E., & Kelly, S. L. (2011). Mechanism of binding of prothioconazole to *Mycosphaerella graminicola* CYP51 differs from that of other azole antifungals. *Applied and Environmental Microbiology*, 77: 1460-1465.

Parker, J. E., Warrilow, A. G. S., Cools, H. J., Fraaije, B. A., Lucas, J. A., Rigdova, K., Griffiths, W. J., Kelly, D. E., & Kelly, S. L. (2013). Prothioconazole and prothioconazole-desthio activities against *Candida albicans* sterol 14-α-demethylase. *Applied and Environmental Microbiology*, 79: 1639-1645.

Paveley, N. D., Lockley, D., Vaughan, T. B., Thomas, J., & Schmidt, K. (2000). Predicting effective fungicide doses through observation of leaf emergence. *Plant Pathology*, 49: 748-766.

Pedersen, E. A., & Hughes, G. R. (1992). The effect of crop rotation on development of the septoria disease complex on spring wheat in Saskatchewan. *Canadian Journal of Plant Pathology*, 14: 152-158.

Peng, J. H., Sun, D., & Nevo, E. (2011). Domestication evolution, genetics and genomics in wheat. *Molecular Breeding*, 28: 281-301.

Ponomarenko, A., Goodwin, S. B., & Kema, G. H. J. (2011). Septoria tritici blotch (STB) of wheat. *Plant Health Instructor.* Available online: DOI:10.1094/PHI-I-2011-0407-01 [accessed: 2019-10-28].

PubChem (2019). National Center for Biotechnology Information. Available online: https://pubchem.ncbi.nlm.nih.gov [accessed 2020-02-13].

Quaedvlieg, W., Kema, G. H. J., Groenewald, J. Z., Verkley, G. J. M., Seifbarghi, S., Razavi, M., Mirzadi Gohari, A., Mehrabi, R., & Crous, P. W. (2011). *Zymoseptoria* gen. nov.: a new genus to accommodate *Septoria*-like species occurring on graminicolous hosts. *Persoonia*, 26: 57-69.

Rebollo, R., Romanish, M. T., & Mager, D. L. (2012). Transposable elements: an abundant and natural source of regulatory sequences for host genes. *Annual Review of Genetics*, 46: 21-42.

Rehfus, A. (2018). *Analysis of the emerging situation of resistance to succinate dehydrogenase inhibitors in* Pyrenophora teres *and* Zymoseptoria tritici *in Europe*. Doctoral dissertation, Universität Hohenheim, Stuttgart, Germany.

Rehfus, A., Matusinsky, P., Strobel, D., Bryson, R., & Stammler, G. (2019). Mutations in target genes of succinate dehydrogenase inhibitors and demethylation inhibitors in *Ramularia collo-cygni* in Europe. *Journal of Plant Diseases and Protection*, 126: 447-459.

Roohparvar, R., de Waard, M. A., Kema, G. H. J., & Zwiers, L. H. (2007). MgMfs1, a major facilitator superfamily transporter from the fungal wheat pathogen *Mycosphaerella graminicola*, is a strong protectant against natural toxic compounds and fungicides. *Fungal Genetics and Biology*, 44: 378-388.

Roohparvar, R., Mehrabi, R., van Nistelrooy, J. G., Zwiers, L. H., & de Waard, M. A. (2008). The drug transporter MgMfs1 can modulate sensitivity of field strains of the fungal wheat pathogen *Mycosphaerella graminicola* to the strobilurin fungicide trifloxystrobin. *Pest Management Science*, 64: 685-693.

Rudd, J. J., Keon, J., & Hammond-Kosack, K. E. (2008). The wheat mitogen-activated protein kinases TaMPK3 and TaMPK6 are differentially regulated at multiple levels during compatible disease interactions with *Mycosphaerella graminicola*. *Plant Physiology*, 147: 802-815.

Rudd, J. J., Kanyuka, K., Hassani-Pak, K., Derbyshire, M., Andongabo, A., Devonshire, J., Lysenko, A., Saqi, M., Desai, N. M., Powers, S. J., Hooper, J., Ambroso, L., Bharti, A., Farmer, A., Hammond-Kosack, K. E., Dietrich, R. A., & Courbot, M. (2015). Transcriptome and metabolite profiling of the infection cycle of *Zymoseptoria tritici* on wheat reveals a biphasic interaction with plant immunity involving differential pathogen chromosomal contributions and a variation on the hemibiotrophic lifestyle definition. *Plant Physiology*, 167: 1158-1185.

Sánchez-Vallet, A., Saleem-Batcha, R., Kombrink, A., Hansen, G., Valkenburg, D. J., Thomma, B. P. H. J., & Mesters, J. R. (2013). Fungal effector Ecp6 outcompetes host immune receptor for chitin binding through intrachain LysM dimerization. *eLife*, 2: e00790.

Sánchez-Vallet, A., McDonald, M. C., Solomon, P. S., & McDonald, B. A. (2015). Is *Zymoseptoria tritici* a hemibiotroph? *Fungal Genetics and Biology*, 79: 29-32.

Sanderson, F. R. (1976). *Mycosphaerella graminicola* (Fuckel) Sanderson comb. nov., the ascogenous state of *Septoria tritici* Rob. apud Desm. *New Zealand Journal of Botany*, 14: 359-360.

Sanglard, D., Ischer, F., Koymans, L., & Bille, J. (1998). Amino acid substitutions in the cytochrome P-450 lanosterol 14α-demethylase (CYP51A1) from azole-resistant *Candida albicans* clinical isolates contribute to resistance to azole antifungal agents. *Antimicrobial Agents and Chemotherapy*, 42: 241-253.

Sauter, H., Steglich, W., & Anke, T. (1999). Strobilurins: evolution of a new class of active substances. *Angewandte Chemie International Edition*, 38:1328-1349.

Schmitz, H. K., Medeiros, C. A., Craig, I. R., & Stammler, G. (2014). Sensitivity of *Phakopsora pachyrhizi* towards quinone-outside-inhibitors and demethylation-inhibitors, and corresponding resistance mechanisms. *Pest Management Science*, 70: 378-388.

Schnabel, G., & Jones, A. L. (2001). The 14α-demethylase (*CYP51A1*) gene is overexpressed in *Venturia inaequalis* strains resistant to myclobutanil. *Phytopathology*, 91: 102-110.

Schnieder, F., Koch, G., Jung, C., & Verreet, J. A. (2001). Genotypic diversity of the wheat leaf blotch pathogen *Mycosphaerella graminicola* (anamorph) *Septoria tritici* in Germany. *European Journal of Plant Pathology*, 107: 285-290.

Schuh, W. (1990). Influence of tillage systems on disease intensity and spatial pattern of Septoria leaf blotch. *Phytopathology*, 80: 1337-1340.

Schuster, M., & Kahmann, R. (2019). CRISPR-Cas9 genome editing approaches in filamentous fungi and oomycetes. *Fungal Genetics and Biology*, 130: 43-53.

Seidl, M. F., & Thomma, B. P. H. J. (2014). Sex or no sex: evolutionary adaptation occurs regardless. *BioEssays*, 36: 335-345.

Sevastos, A., Labrou, N. E., Flouri, F., & Malandrakis, A. (2017). Glutathione transferase-mediated benzimidazole-resistance in *Fusarium graminearum*. *Pesticide Biochemistry and Physiology*, 141: 23-28.

Shapiro, J. A. (1979). Molecular model for the transposition and replication of bacteriophage Mu and other transposable elements. *Proceedings of the National Academy of Sciences of the United States of America*, 76: 1933-1937.

Shaw, M. W. (1987). Assessment of upward movement of rain splash using a fluorescent tracer method and its application to the epidemiology of cereal pathogens. *Plant Pathology*, 36: 201-213.

Shaw, M. W., & Royle, D. J. (1989a). Airborne inoculum as a major source of *Septoria tritici (Mycosphaerella graminicola*) infections in winter wheat crops in the UK. *Plant Pathology*, 38: 35-43.

Shaw, M. W., & Royle, D. J. (1989b). Estimation and validation of a function describing the rate at which *Mycosphaerella graminicola* causes yield loss in winter wheat. *Annals of Applied Biology*, 115: 425-442.

Shaw, M. W., & Royle, D. J. (1993). Factors determining the severity of epidemics of *Mycosphaerella graminicola* (*Septoria tritici*) on winter wheat in the UK. *Plant Pathology*, 42: 882-899.

Siegel, M. R. (1981). Sterol-inhibiting fungicides: effects on sterol biosynthesis and sites of action. *Plant Diseases*, 65: 986-989.

Simón, M. R., Cordo, C. A., Perello, A. E., & Struik, P. C. (2003). Influence of nitrogen supply on the susceptibility of wheat to *Septoria tritici*. *Journal of Phytopathology*, 151: 283-289.

Simón, M. R., Cordo, C. A., Castillo, N. S., Struik, P. C., & Börner, A. (2012). Population structure of *Mycosphaerella graminicola* and location of genes for resistance to the pathogen: recent advances in Argentina. *International Journal of Agronomy*, 2012: 680275.

Solomon, P. S., & Oliver, R. P. (2001). The nitrogen content of the tomato leaf apoplast increases during infection by *Cladosporium fulvum*. *Planta*, 213: 241-249.

Stammler, G., Brix, H. D., Glättli, A., Semar, M., & Schoefl, U. (2007). Biological properties of the carboxamide boscalid including recent studies on its mode of action. In *Proceedings XVI International Plant Protection Congress Glasgow* (pp. 16-21). Alton, UK: British Crop Protection Council.

Stammler, G., Brix, H. D., Nave, B., Gold, R., & Schoefl, U. (2008a). Studies on the biological performance of boscalid and its mode of action. In H. W. Dehne, H. B. Deising, U. Gisi, K. H. Kuck, P. E. Russel, & H. Lyr (Eds.), *Modern Fungicides and Antifungal Compounds V* (pp. 45-51). Braunschweig, Germany: Deutsche Phytomedizinische Gesellschaft.

Stammler, G., Carstensen, M., Koch, A., Semar, M., Strobel, D., & Schlehuber, S. (2008b). Frequency of different CYP51-haplotypes of *Mycosphaerella graminicola* and their impact on epoxiconazole-sensitivity and -field efficacy. *Crop Protection*, 27: 1448-1456.

Stammler, G., Cordero, J., Koch, A., Semar, M., & Schlehuber, S. (2009). Role of the Y134F mutation in *cyp51* and overexpression of *cyp51* in the sensitivity response of *Puccinia triticina* to epoxiconazole. *Crop Protection*, 28: 891-897.

Stammler, G., & Semar, M. (2011). Sensitivity of *Mycosphaerella graminicola* (anamorph: *Septoria tritici*) to DMI fungicides across Europe and impact on field performance. *EPPO Bulletin*, 41: 149-155.

Stammler, G., Wolf, A., Glaettli, A., & Klappach, K. (2015). Respiration inhibitors: complex II. In H. Ishii, & D. W. Hollomon (Eds.), *Fungicide Resistance in Plant Pathogens. Principles and a Guide to Practical Management* (pp. 105-117). Tokyo, Japan: Springer.

Stanis, V. F., & Jones, A. L. (1985). Reduced sensitivity to sterol-inhibiting fungicides in field isolates of *Venturia inaequalis*. *Phytopathology*, 75: 1098-1101.

Stergiopoulos, I., van Nistelrooy, J. G. M., Kema, G. H. J., & de Waard, M. A. (2003). Multiple mechanisms account for variation in base-line sensitivity to azole fungicides in field isolates of *Mycosphaerella graminicola*. *Pest Management Science*, 59: 1333-1343.

Strobel, D., Bryson, R., Roth, J., & Stammler, G. (2017). Field Performance of DMI Fungicides against *Zymoseptoria tritici* across Europe-Compromized by Further Sensitivity Shift? In H. B. Deising, B. Fraaije, A. Mehl, E. C. Oerke, H. Sierotzki, & G. Stammler (Eds.), *Modern Fungicides and Antifungal Compounds VIII* (pp. 249-254). Braunschweig, Germany: Deutsche Phytomedizinische Gesellschaft.

Suffert, F., Sache, I., & Lannou, C. (2011). Early stages of septoria tritici blotch epidemics of winter wheat: build-up, overseasoning, and release of primary inoculum. *Plant Pathology*, 60: 166-177.

Sun, X., Xu, Q., Ruan, R., Zhang, T., Zhu, C., & Li, H. (2013). PdMLE1, a specific and active transposon acts as a promoter and confers *Penicillium digitatum* with DMI resistance. *Environmental Microbiology Reports*, 5: 135-142.

Taher, K., Graf, S., Fakhfakh, M. M., Salah, H. B. H., Yahyaoui, A., Rezgui, S., Nasraoui, B., & Stammler, G. (2014). Sensitivity of *Zymoseptoria tritici* Isolates from Tunisia to Pyraclostrobin, Fluxapyroxad, Epoxiconazole, Metconazole, Prochloraz and Tebuconazole. *Journal of Phytopathology*, 162: 442-448.

Thomas, M. R., Cook, R. J., & King, J. E. (1989). Factors affecting development of *Septoria tritici* in winter wheat and its effect on yield. *Plant Pathology*, 38: 246-257.

Thomma, B. P. H. J., van Esse, H. P., Crous, P. W., & de Wit, P. J. G. M. (2005). *Cladosporium fulvum* (syn. *Passalora fulva*), a highly specialized plant pathogen as a model for functional studies on plant pathogenic Mycosphaerellaceae. *Molecular Plant Pathology*, 6: 379-393.

Tillman, R. W., Siegel, M. R., & Long, J. W. (1973). Mechanism of action and fate of the fungicide chlorothalonil (2, 4, 5, 6-tetrachloroisophthalonitrile) in biological systems: I. Reactions with cells and subcellular components of *Saccharomyces pastorianus*. *Pesticide Biochemistry and Physiology*, 3: 160-167.

Torriani, S. F., Brunner, P. C., McDonald, B. A., & Sierotzki, H. (2009). QoI resistance emerged independently at least 4 times in European populations of *Mycosphaerella graminicola*. *Pest Management Science*, 65: 155-162.

Torriani, S. F., Melichar, J. P., Mills, C., Pain, N., Sierotzki, H., & Courbot, M. (2015). *Zymoseptoria tritici*: a major threat to wheat production, integrated approaches to control. *Fungal Genetics and Biology*, 79: 8-12.

Van den Berg, F., van den Bosch, F., & Paveley, N. D. (2013). Optimal fungicide application timings for disease control are also an effective anti-resistance strategy: A case study for *Zymoseptoria tritici* (*Mycosphaerella graminicola*) on wheat. *Phytopathology*, 103: 1209-1219.

Van den Bosch, F., Paveley, N., Shaw, M., Hobbelen, P., & Oliver, R. (2011). The dose rate debate: does the risk of fungicide resistance increase or decrease with dose? *Plant Pathology*, 60: 597-606.

Van den Bosch, F., Oliver, R., van den Berg, F., & Paveley, N. (2014). Governing principles can guide fungicide-resistance management tactics. *Annual Review of Phytopathology*, 52: 175-195.

Verreet, J. A., Klink, H., & Hoffmann, G. M. (2000). Regional monitoring for disease prediction and optimization of plant protection measures: The IPM wheat model. *Plant Disease*, 84: 816-826.

Vidal, T., Boixel, A. L., Durand, B., de Vallavieille-Pope, C., Huber, L., & Saint-Jean, S. (2017). Reduction of fungal disease spread in cultivar mixtures: impact of canopy architecture on rain-splash dispersal and on crop microclimate. *Agricultural and Forest Meteorology*, 246: 154-161.

Von Schmeling, B., & Kulka, M. (1966). Systemic fungicidal activity of 1,4-oxathiin derivatives. *Science*, 152: 659-660.

Wicker, T., Sabot, F., Hua-Van, A., Bennetzen, J. L., Capy, P., Chalhoub, B., Flavell, A., Leroy, P., Morgante, M., Panaud, O., Paux, E., SanMiguel, P., & Schulman, A. H. (2007). A unified classification system for eukaryotic transposable elements. *Nature Reviews Genetics*, 8: 973-982.

Wittenberg, A. H. J., van Der Lee, T. A. J., Ben M'Barek, S., Ware, S. B., Goodwin, S. B., Kilian, A., Visser, R. G. F., Kema, G. H. J., & Schouten, H. J. (2009). Meiosis drives extraordinary genome plasticity in the haploid fungal plant pathogen *Mycosphaerella graminicola*. *PLoS One*, 4: e5863.

Wu, Q., Yang, Z., Nie, Y., Shi, Y., & Fan, D. (2014). Multi-drug resistance in cancer chemotherapeutics: mechanisms and lab approaches. *Cancer Letters*, 347: 159-166.

Wyand, R. A., & Brown, J. K. M. (2005). Sequence variation in the CYP51 gene of *Blumeria graminis* associated with resistance to sterol demethylase inhibiting fungicides. *Fungal Genetics and Biology*, 42: 726-735.

Yang, F., Li, W., & Jørgensen, H. J. (2013). Transcriptional reprogramming of wheat and the hemibiotrophic pathogen *Septoria tritici* during two phases of the compatible interaction. *PLoS One*, 8: e81606.

Yoshida, Y., & Aoyama, Y. (1984). Yeast cytochrome P-450 catalyzing lanosterol 14 alpha-demethylation. I. Purification and spectral properties. *The Journal of Biological Chemistry*, 259: 1655-1660.

Yoshida, Y., & Aoyama, Y. (1987). Interaction of azole antifungal agents with cytochrome $P\text{-}450_{14DM}$ purified from *Saccharomyces cerevisiae* microsomes. *Biochemical Pharmacology*, 36: 229-235.

Ypema, H. L., & Gold, R. E. (1999). Kresoxim-methyl: modification of a naturally occurring compound to produce a new fungicide. *Plant Disease*, 83: 4-19.

Yu, Q., & Powles, S. (2014). Metabolism-based herbicide resistance and cross-resistance in crop weeds: a threat to herbicide sustainability and global crop production. *Plant Physiology*, 166: 1106-1118.

Zhang, X., Haley, S. D., & Jin, Y. (2001). Inheritance of Septoria Tritici Blotch resistance in winter wheat. *Crop Science*, 41: 323-326.

Ziogas, B. N., & Malandrakis, A. A. (2015). Sterol Biosynthesis Inhibitors: C14 Demethylation (DMIs). In H. Ishii, & D. W. Hollomon (Eds.), *Fungicide Resistance in Plant Pathogens. Principles and a Guide to Practical Management* (pp. 199-216). Tokyo, Japan: Springer.

7 Supplementary material

Table 22: *Z. tritici* isolates from European sensitivity monitoring of 2016 and 2017 used in this work and their origin. All isolates were tested for their sensitivity to epoxiconazole. Isolates marked in bold were additionally tested for their sensitivity to prothioconazole-desthio and mefentrifluconazole. Microtiter tests were performed by the company EpiLogic.

Year	Country	Region	Isolates
2016	CZ	Jihocesky kraj	**6871, 6872, 6873**
		Olomoucky kraj	**6874**
	DE	Baden-Württemberg	**6460, 6461, 6813, 6814, 6815, 6816, 6817, 6818, 6819**
		Bayern	**6439, 6446**
		Niedersachsen	**6472, 6473, 6811**
		Rheinland-Pfalz	**6466, 6468, 6812**
		Schleswig-Holstein	**6498, 6500, 6809, 6501, 6810**
	DK	Midtjylland	**6802, 6803, 6804**
		Sjæland	**6805, 6806, 6807, 6808**
	FR	Aquitaine	**6787, 6788**
		Bourgogne	**6445, 6448, 6773, 6774, 6775, 6776, 6777, 6778, 6779**
		Bretagne	**6452, 6453, 6757, 6758, 6759, 6760, 6761, 6762**
		Centre	**6467, 6769, 6770, 6771, 6772**
		Champagne-Ardenne	**6436, 6447, 6755, 6756**
		Haute Normandie	**6443, 6444, 6741, 6742, 6743**
		Île de France	**6752, 6753**
		Midi-Pyrenees	**6789, 6790, 6791, 6792**
		Nord-Pas de Calais	**6438, 6454, 6455, 6456, 6735, 6736, 6737, 6738, 6739, 6740**
		Pays de Loire	**6442, 6763, 6764, 6765, 6766, 6767, 6768**
		Picardie	**6437, 6459, 6744, 6745, 6746, 6747, 6748, 6749, 6750, 6751**
		Poitou-Charente	**6780, 6781, 6782**
		Rhône-Alpes	**6783, 6784, 6785, 6786**
	IE	Leinster	**6410, 6412, 6413, 6415, 6416, 6435, 6476, 6477, 6478, 6479, 6480, 6481, 6482, 6484, 6485, 6486, 6487, 6488, 6489, 6628, 6629, 6630, 6631, 6632, 6633, 6634, 6635, 6636, 6637, 6638, 6639, 6640, 6641, 6642, 6643, 6644, 6645, 6646, 6647, 6648, 6649, 6650, 6651, 6652, 6653, 6654, 6655, 6656, 6657, 6658, 6659, 6660, 6661, 6662, 6663, 6664, 6665, 6666, 6667, 6668**

Year	Country	Region	Isolates
2016	IE	Munster	**6417, 6418, 6419, 6420, 6421, 6440, 6474, 6475, 6490, 6492, 6493, 6494, 6669, 6670, 6671, 6672, 6673, 6674, 6675, 6676, 6677, 6678, 6679, 6680, 6681, 6682, 6683, 6684, 6685, 6686, 6687, 6688, 6689**
	NL	Flevoland	**6425, 6426, 6505**
		Groningen	**6422, 6423, 6424, 6427, 6428, 6429, 6502, 6504**
	PL	Dolnośląskie	**6853, 6854, 6855**
		Kujawsko-Pomorskie	**6846, 6847, 6848, 6849, 6850, 6851,**
		Łódzkie	**6852**
		Lubelskie	**6868**
		Lubuskie	**6827, 6828, 6829, 6830, 6831, 6832**
		Mazowieckie	**6867**
		Opolskie	**6856, 6857, 6858, 6859, 6860, 6861, 6862**
		Podkarpackie	**6869, 6870**
		Pomorskie	**6823, 6824, 6825, 6826**
		Slaskie	**6863**
		Warminsko-Mazurskie	**6864, 6865, 6866**
		Wielkopolskie	**6834, 6835, 6836, 6837, 6838, 6839, 6840, 6841, 6842, 6843, 6844, 6845**
		Zachodniopomorskie	**6821, 6822**
	SE	Blekinge	**6793**
		Östergötland	**6794, 6795, 6796, 6797**
		Skåne	**6798, 6799, 6800**
		Västra Götaland	**6801**
	UK	East Anglia	**6462, 6469, 6730, 6731, 6732, 6733**
		East Midlands	**6407, 6430, 6431, 6434, 6464, 6720, 6721, 6722, 6723, 6724, 6725, 6726, 6727, 6728, 6729**
		North West	**6470, 6471, 6716, 6717**
		Scotland	**6450, 6451, 6457, 6458, 6690, 6691, 6692, 6693, 6694, 6695, 6696, 6697, 6698, 6699, 6700, 6701, 6702, 6703, 6704, 6705, 6706, 6707, 6708, 6709, 6710, 6711, 6712, 6713, 6714, 6715**
		South West	**6432, 6433, 6465, 6734**
		West Midlands	**6409, 6496, 6719**
		Yorkshire/Humberside	**6718**
2017	BG	Severozapaden	7676, 7677, 7678
		Yuzhen tsentralen	7662, 7663, 7664, 7665, 7666, 7667, 7668, 7669, 7670, 7671, 7672, 7673, 7674, 7675
	CZ	Jihocesky kraj	7572, 7573, 7574, 7575, 7576, 7577

Year	Country	Region	Isolates
2017	CZ	Olomoucky kraj	7581, 7582, **7583**
		Pardubicky kraj	7578, **7579**, 7580
	DE	Baden-Württemberg	7458, 7459, 7460, **7461**, 7462, **7463**, 7464, 7465, 7466
		Bayern	7467, **7468**, 7469, 7470, **7471**, **7472**, **7473**, 7474, 7475, **7476**, 7477, 7478, 7479, 7480, 7481, 7482, 7483, 7484
		Mecklenburg-Vorpommern	**7436**, **7438**, 7439, 7440, 7441
		Niedersachsen	**7442**, **7443**, 7444, **7445**, **7446**, 7447, **7448**, 7449, 7450, **7451**
		Rheinland-Pfalz	**7455**, 7456, 7457
		Sachsen	7452, 7453, 7454
	ES	Andalucía	**7679**, 7680, 7681, 7682, **7683**, 7684
	FR	Aquitaine	7420, 7421, 7422, 7423, 7424, 7425, 7426, 7427, 7428, **7429**, 7430, 7431, 7432, 7433, 7434
		Basse Normandie	7306, 7307, 7308
		Bourgogne	**7388**, 7389, 7390, 7391, 7392, 7393, 7394, 7395, 7396, 7397, 7398, 7399, 7400, 7401, 7402, 7403, 7404
		Bretagne	**7341**, 7342, **7343**, 7344, 7345, 7346, 7347, 7348, 7349, 7350, 7351, 7352
		Centre	7367, 7368, 7369, 7370, 7371, 7372, 7373, 7374, 7375, 7376, 7377, 7378, 7379, 7380, 7381, **7382**, 7383, 7384, **7385**, 7386, 7387
		Champagne-Ardenne	**7332**, 7333, 7334, 7335, 7336, 7337, **7338**, 7339, 7340
		Haute Normandie	7309, 7310, 7311, 7312, 7313, 7314, 7315, 7316, 7317, **7318**, 7319, 7320
		Île de France	7329, 7330, **7331**
		Midi-Pyrenees	**7435**
		Nord-Pas de Calais	7288, 7289, 7290, 7291, **7292**, 7293, 7294, 7295, 7296, 7297, 7298, **7299**, 7300, 7301, 7302, 7303, 7304, 7305
		Pays de Loire	**7353**, **7354**, 7355, 7356, **7357**, **7358**, 7359, 7360, 7361, **7362**, **7363**, 7364, 7365, 7366, 7863
		Picardie	7321, **7322**, 7323, 7324, 7325, 7326, **7327**, 7328,
		Poitou-Charente	**7405**, 7406, **7407**, **7408**, 7409, 7410, 7411, 7412, 7413, 7414, 7415, **7416**, 7417, 7418, 7419
	IE	Leinster	6999, 7000, 7001, 7002, 7003, 7235, 7236, 7237, **7238**, 7239, 7240, 7241, 7242, **7243**, 7244, 7245, 7246, 7247, 7248, 7249, 7250, **7251**, **7252**, 7253, 7254, 7255
		Munster	**7256**, 7257, 7258, **7259**, 7260, 7261

Year	Country	Region	Isolates
2017	IT	Emilia Romagna	7694, 7695, 7696, 7697, 7698
		Lombardia	7685, 7686, 7687, **7688**, 7689, 7690, 7691, 7692
		Toscana	7699, 7700
	NL	Flevoland	7285, 7286, 7287
		Groningen	7276, 7277, **7278**, **7279**, **7280**, 7281, 7282, 7283, 7284
	PL	Dolnośląskie	7532, 7533, 7534, 7535, 7536, 7537, 7538
		Kujawsko-Pomorskie	7523, **7524**, 7525, **7526**
		Łódzkie	7527, 7528, 7529, **7530**, 7531
		Lubelskie	**7563**, 7564, 7565
		Lubuskie	7498, 7499
		Małopolskie	**7566**, **7567**, 7568, **7569**, 7570, 7571
		Opolskie	7539, 7540, 7541, **7542**, 7543, 7544, 7545, 7547, 7548, 7549, **7550**, 7551, 7552, 7553, 7554, 7555, 7556
		Pomorskie	**7488**, 7489, 7490, 7491, 7492, 7493, 7494, 7495, **7496**, **7497**
		Warminsko-Mazurskie	7557, 7558, 7559, 7560, 7561, 7562
		Wielkopolskie	7500, 7501, 7502, 7503, **7504**, 7505, **7506**, 7507, 7508, 7509, 7510, 7511, **7512**, **7513**, **7514**, 7515, 7516, 7517, 7518, 7519, **7520**, 7521, 7522
		Zachodniopomorskie	7485, **7486**, 7487
	RU	Juschny	7848, 7849, 7850, 7851, 7852, 7853, 7854
		Sewero-kavkaskyi	7855, 7856, 7857, 7858, 7859, 7860, 7861, 7862
		Sewero-sapadny	7829, 7830, 7831, 7832, 7833, 7834
		Zentralny	7835, 7837, 7838, 7839, 7840, 7841, 7842, 7843, 7844, 7845, 7846, 7847
	SK	Banskobystrický kraj	7584, 7585, 7586, 7587, 7588, 7589, 7590, 7591, 7592
		Košický kraj	**7599**, **7600**, **7601**, 7602, 7603, 7604
		Prešovský kraj	**7593**, 7594, 7595, 7596, 7597, **7598**
	UA	Chersonska	7656, 7657, 7658, **7659**, **7660**, 7661
		Chmelnyzka	7623, 7624, 7625, 7626, 7627, 7628, 7629, 7630, 7631
		Kyjiwska	7644, 7645, 7646
		Lwiwska	**7620**, 7621, 7622
		Mykolajiwska	7653, 7654, 7655
		Odeska	7638, 7639, 7640, 7641, 7642, 7643
		Riwnska	**7611**, 7612, 7613, **7614**, 7615, 7616, 7617, 7618, **7619**

Year	Country	Region	Isolates
2017	UA	Sumska	7647, 7648, 7649
		Tscherkaska	7650, 7651, **7652**
		Tschernihiw	7635, 7636, 7637
		Winnyzka	7632, 7633, 7634
		Wolynska	7605, 7606, 7607, 7608, 7609, 7610
	UK	East Anglia	7077, 7078, 7079, 7080, 7081, 7262, **7263**, **7264**
		South East	7158, 7159, 7160, 7161, 7162, 7271, 7272, 7273, 7274, 7275
		South West	7265, 7266, **7267**, 7268, 7269, **7270**

Table 23: *Z. tritici* isolates obtained from trial sites used in this work. Leave samples from trial sites were sent to EpiLogic and they generated single pycnidia isolates.

Country	Region	Treatment	Isolates
DE	Niedersachsen	Untreated	6598, 6599, 6600, 6601, 6602, 6603, 6604, 6605, 6606, 6607
		Mefentrifluconazole	6608, 6609, 6610, 6611, 6612, 6613, 6614, 6615, 6616, 6617
		Prothioconazole	6618, 6619, 6620, 6621, 6622, 6623, 6624, 6625, 6626, 6627
DK	Sjæland	Untreated	6568, 6569, 6570, 6571, 6572, 6573, 6574, 6575, 6576, 6577
		Mefentrifluconazole	6578, 6579, 6580, 6581, 6582, 6583, 6584, 6585, 6586, 6587
		Prothioconazole	6588, 6589, 6590, 6591, 6592, 6593, 6594, 6595, 6596, 6597
IE	Munster	Untreated	6508, 6509, 6510, 6511, 6512, 6513, 6514, 6515, 6516, 6517
		Mefentrifluconazole	6518, 6519, 6520, 6521, 6522, 6523, 6524, 6525, 6526, 6527
		Prothioconazole	6528, 6529, 6530, 6531, 6532, 6533, 6534, 6535, 6536, 6537
UK	East Anglia	Untreated	6538, 6539, 6540, 6541, 6542, 6543, 6544, 6545, 6546, 6547
		Mefentrifluconazole	6548, 6549, 6550, 6551, 6552, 6553, 6554, 6555, 6556, 6557
		Prothioconazole	6558, 6559, 6560, 6561, 6562, 6563, 6564, 6565, 6566, 6567

Table 24: Distances (Å) from wild type amino acids observed as position of alteration to the haem group in the CYP51 enzyme. Distances were calculated from the iron atom of the haem to the alpha carbon on the protein backbone for each alteration. Amino acids that were observed as subject of alterations already described for *Z. tritici* were designated as 'existing'. Amino acids that were observed as subject of new alterations identified in this work were designated as 'new'.

Position of wild type amino acid observed as subject of alteration	Status of occurrence	Distance to haem group (Å)
L50	Existing	37.0
D134	Existing	19.3
V136	Existing	12.3
Y137	Existing	14.1
S188	Existing	34.4
N284	Existing	19.2
A379	Existing	11.1
I381	Existing	9.8
A410	Existing	14.8
Y459	Existing	16.0
G460	Existing	16.3
Y461	Existing	13.6
G462	Existing	15.0
N513	Existing	28.3
S524	Existing	14.0
K95	New	25.4
L197	New	17.6
A318	New	12.9
I377	New	16.7
I452	New	20.1
K456	New	18.5
E457	New	19.4
D458	New	17.2
L463	New	16.2
N507	New	30.1

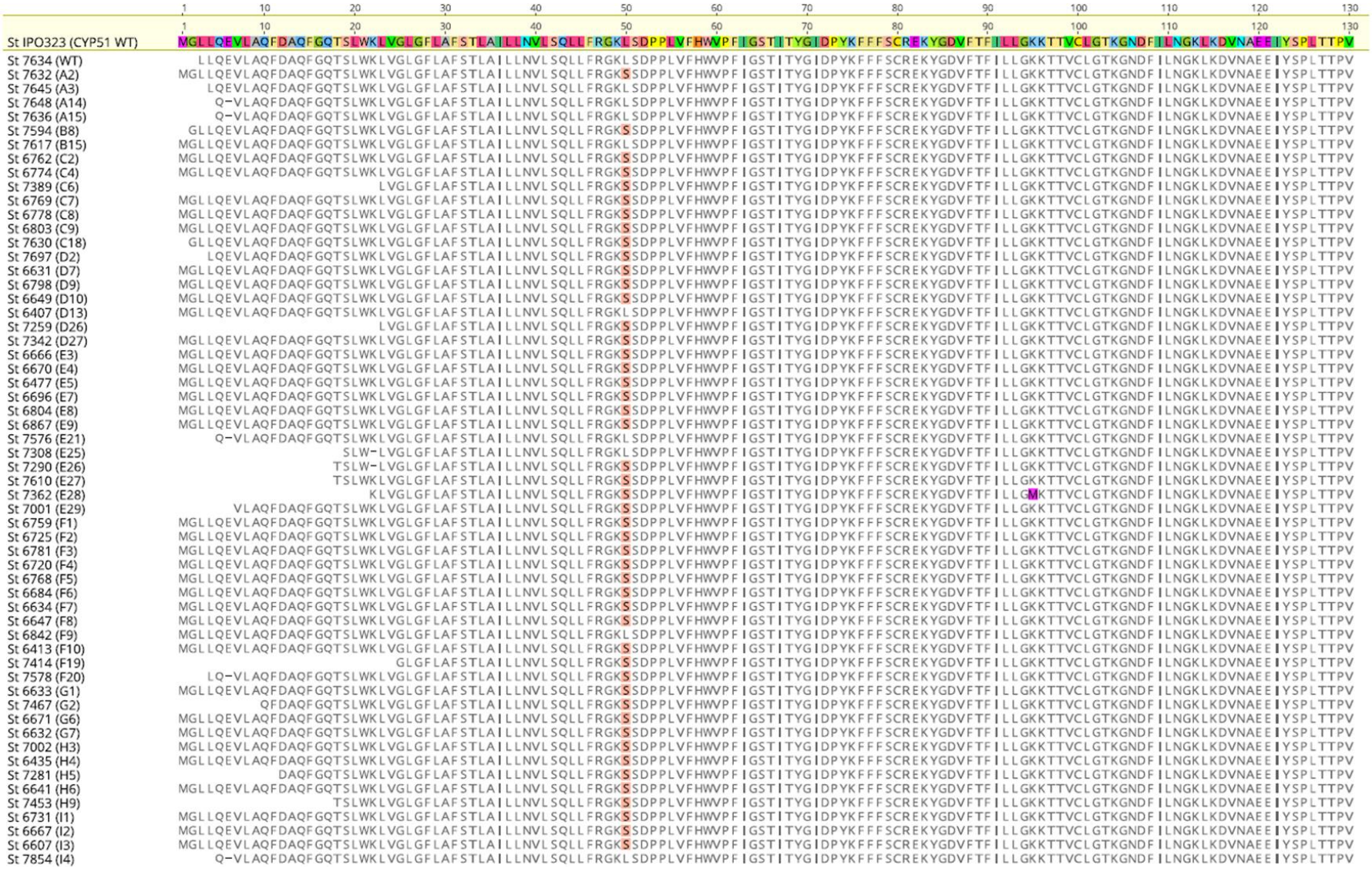


Figure 36-Part 1: Amino acid sequence of all *CYP51* haplotypes identified in *Z. tritici* isolates of this work. Further information is given on page 175.

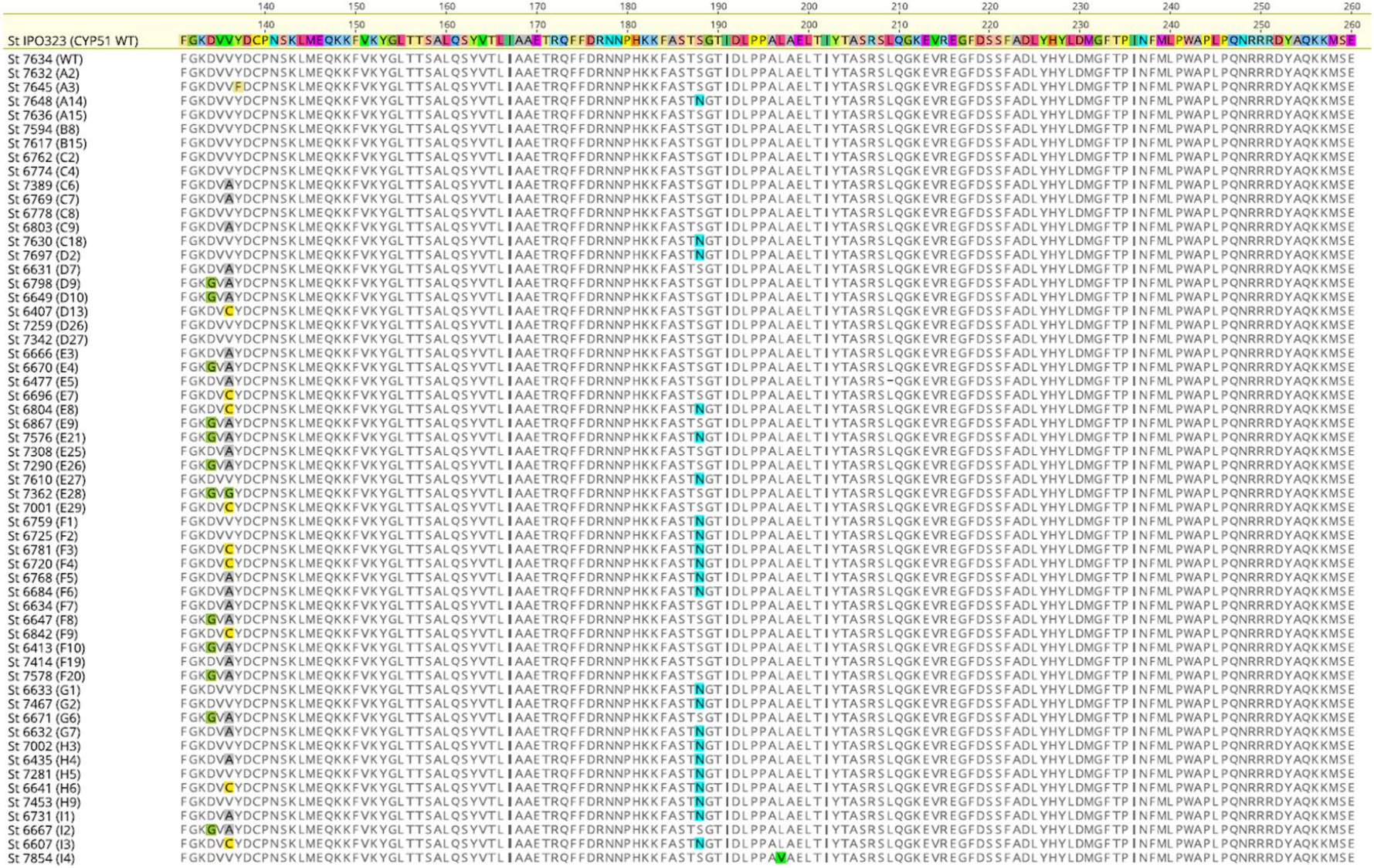


Figure 36-Part 2: Amino acid sequence of all *CYP51* haplotypes identified in *Z. tritici* isolates of this work. Further information is given on page 175.

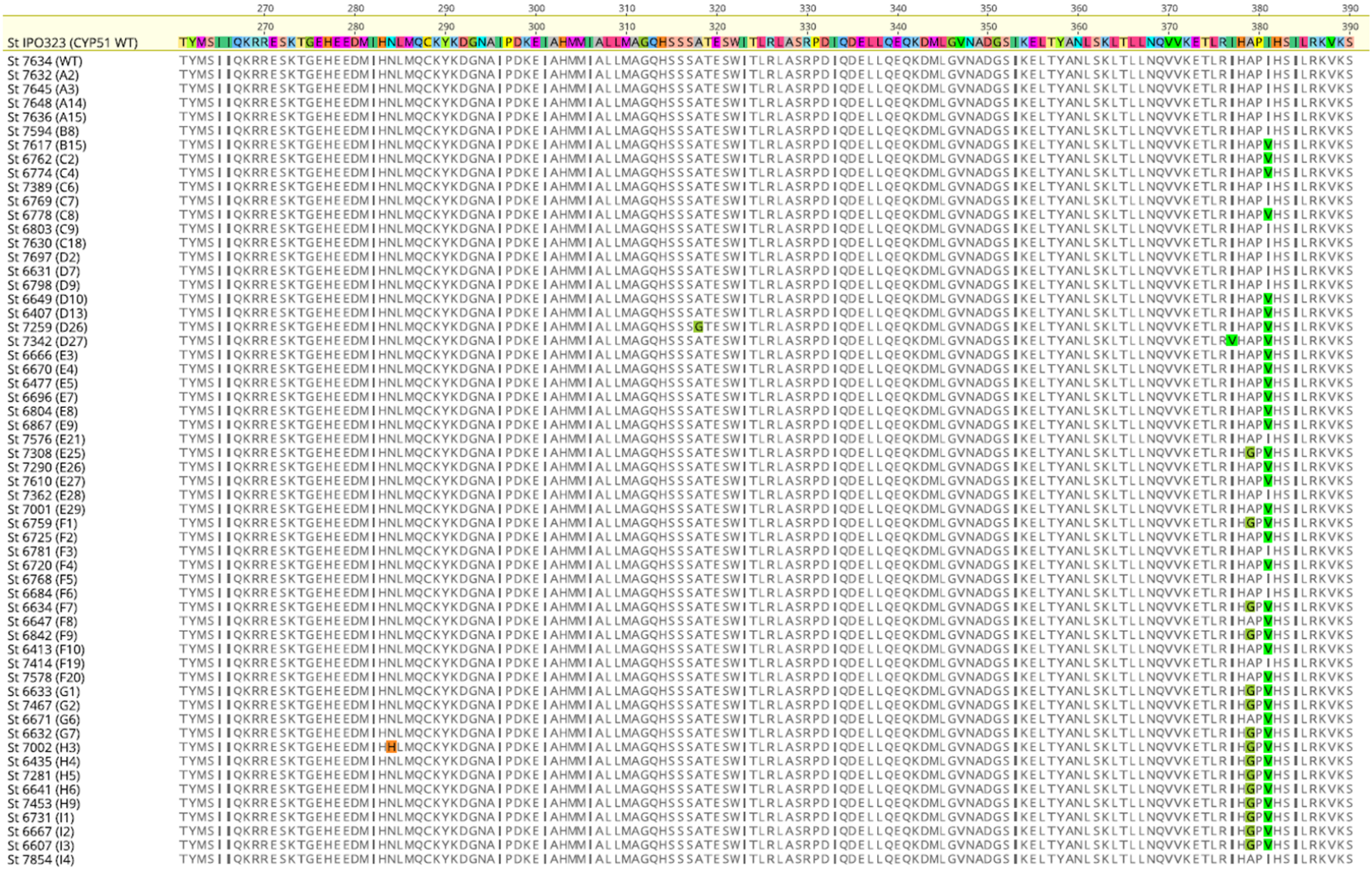


Figure 36-Part 3: Amino acid sequence of all *CYP51* haplotypes identified in *Z. tritici* isolates of this work. Further information is given on page 175.

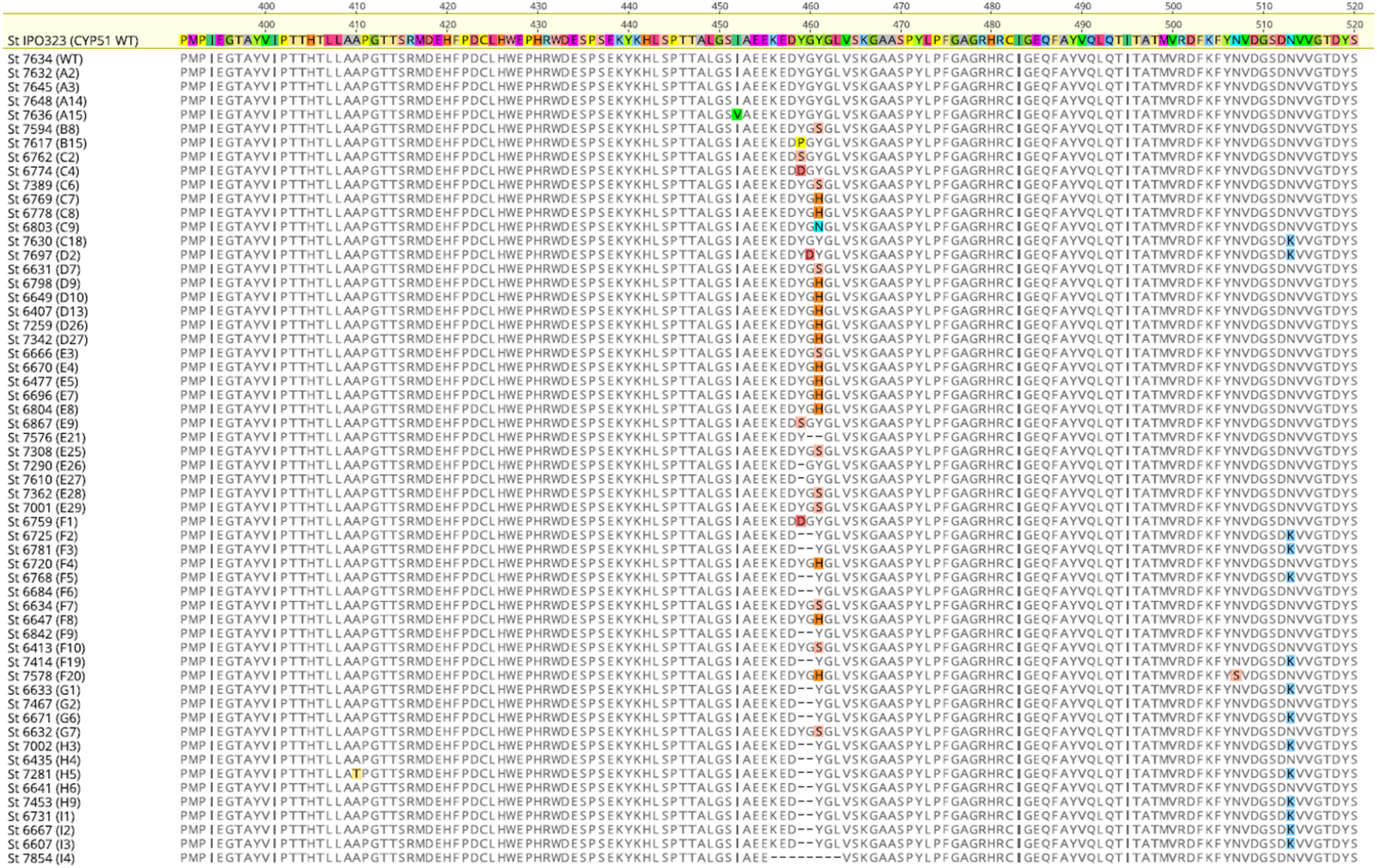


Figure 36-Part 4: Amino acid sequence of all *CYP51* haplotypes identified in *Z. tritici* isolates of this work. Further information is given on page 175.

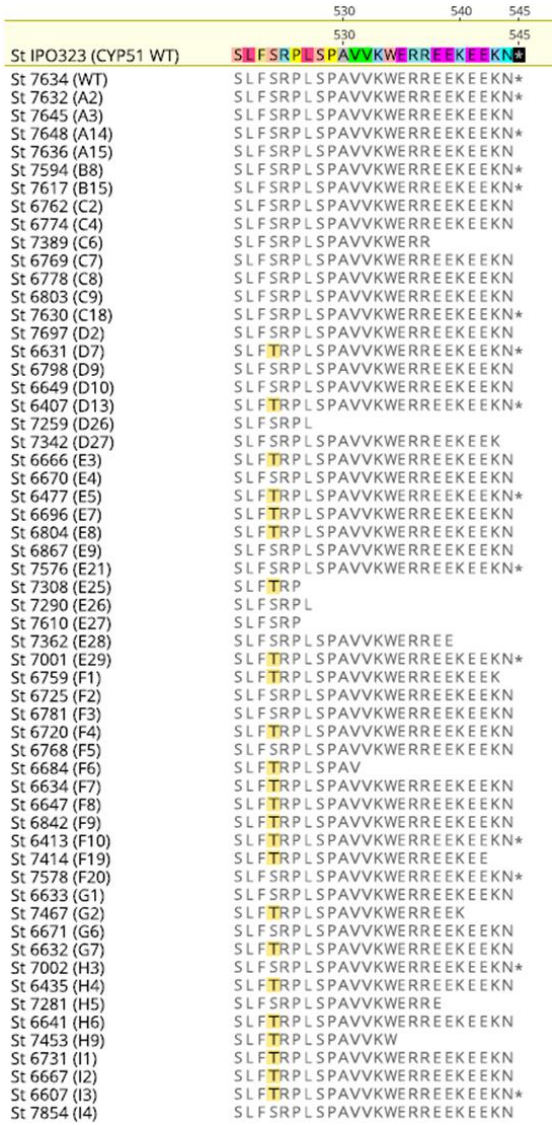


Figure 36-Part 5: Amino acid sequence of all *CYP51* haplotypes identified in *Z. tritici* isolates of this work. Alignment was performed with one *Z. tritici* isolate for each identified *CYP51* haplotype (including the WT CYP51 of St IPO323). For some *CYP51* haplotypes the beginning and end of the amino acid sequences are missing. However, it is known that in these regions resistance alterations are less likely. Amino acids of the reference sequence of St IPO323 and all disagreements to this sequence are highlighted in colour. *CYP51* haplotypes that carry new CYP51 alterations are shown. Following isolates (*CYP51* haplotypes) carry a new alteration: St 7636 (A15): I452V, St 6803 (C9): Y461N, St 7259 (D26): A318G, St 7342 (D27): I377V, St 7362 (E28): K95M, St 7578 (F20): N507S, St 7854 (I4): L197V, Del K456, Del E457, Del D458, Del L463. Alignment was performed with MUSCLE algorithm and manually corrected for positions of deletions.

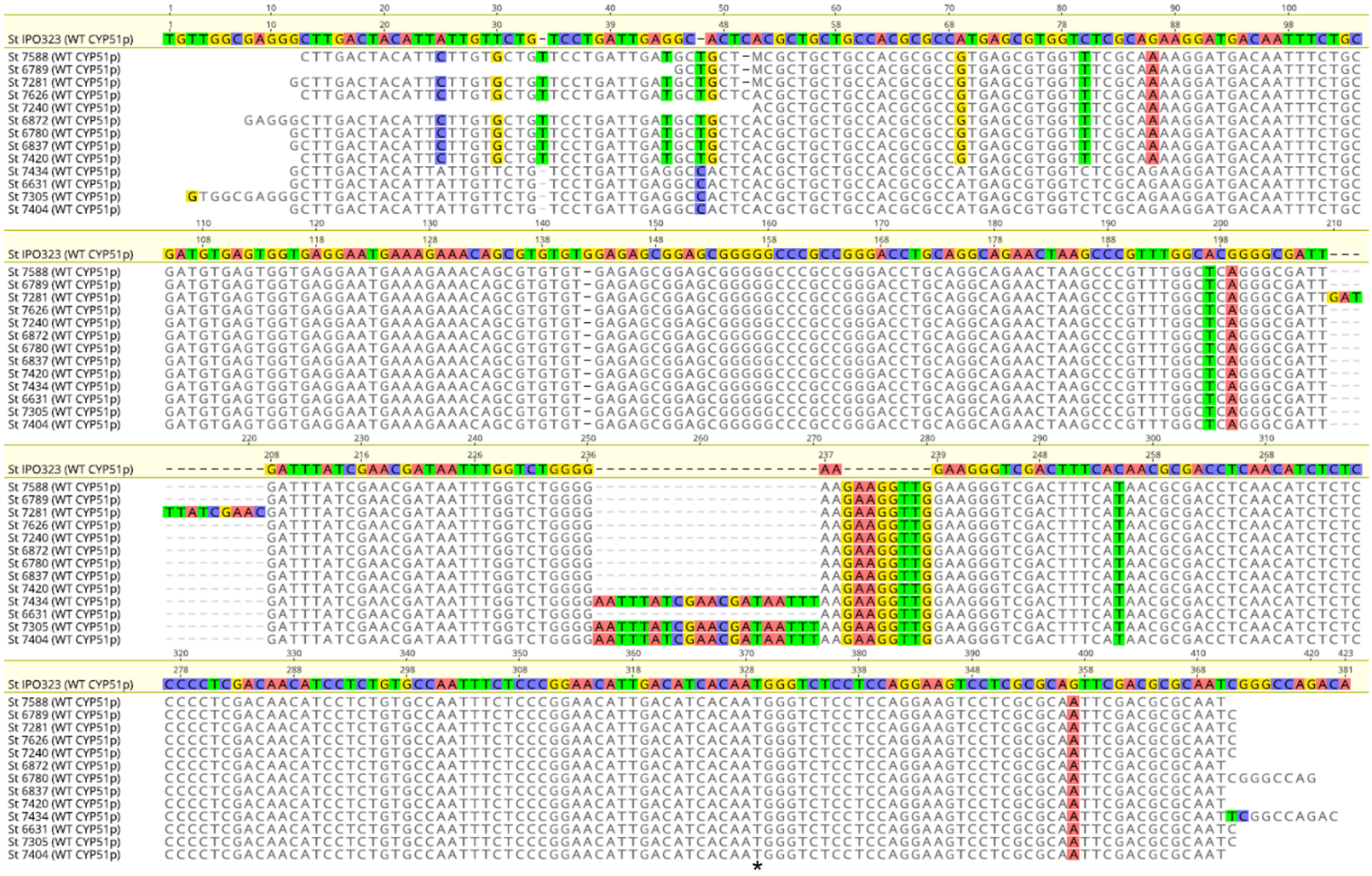


Figure 37: Alignment of *Z. tritici* isolates carrying the wild type *CY51*p (WT *CYP51*p). Nucleobases of the reference sequence of St IPO323 and all disagreements to this sequence are highlighted in colour (adenine = red, guanine = yellow, thymine = green, cytosine = blue). The *CYP51* start codon is indicated by the asterisk. Compared to reference sequence of St IPO323, all isolates showed small insertions ranging between 8 and <30 bp. Nevertheless, these isolates were regarded in this work to carry a WT *CYP51*p (described in 4.6.1). Alignment was performed with MUSCLE algorithm.

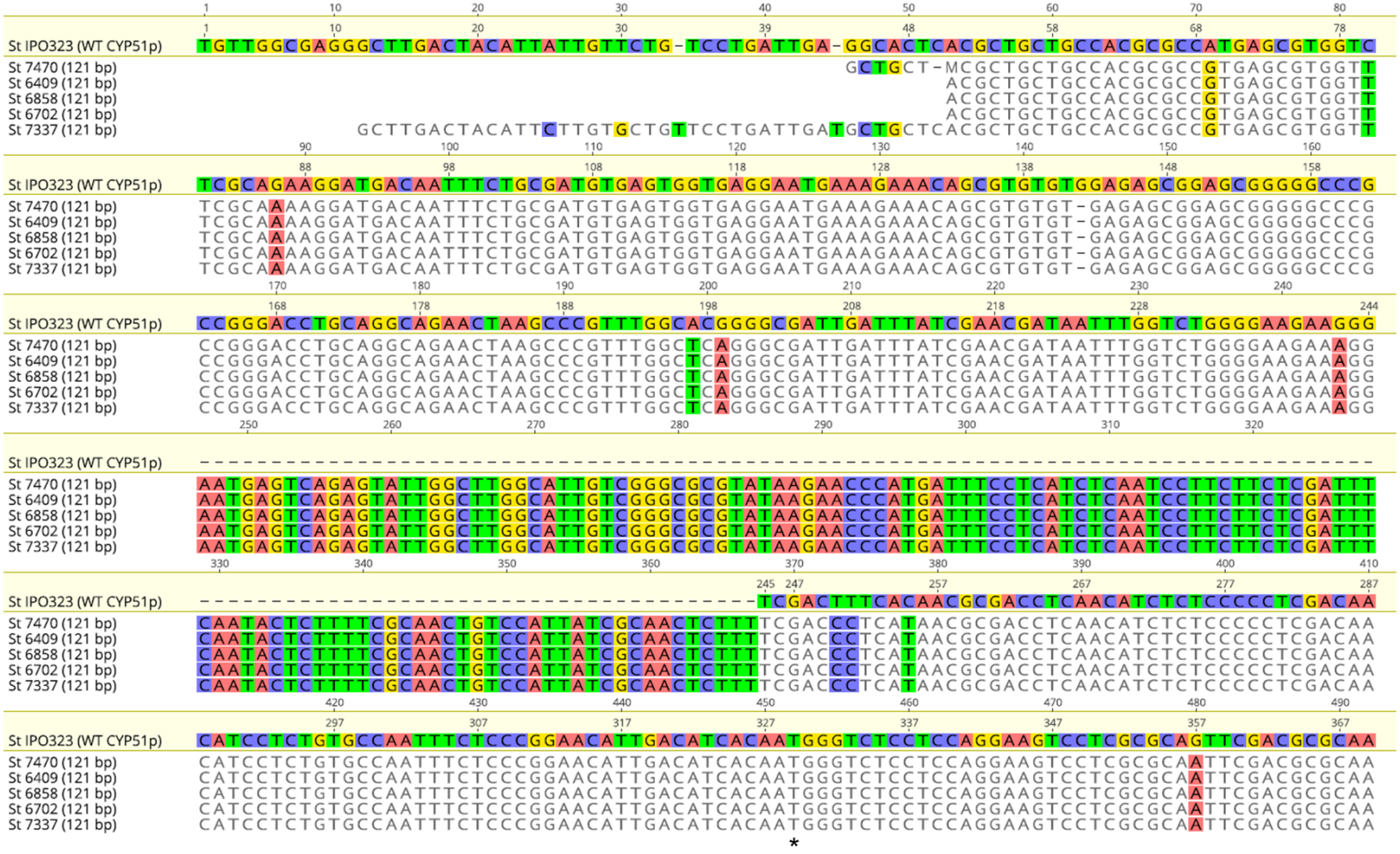


Figure 38: Alignment of *Z. tritici* isolates carrying the 121 bp insert in *CYP51*p. Isolates carrying the 121 bp insert in *CYP51*p were aligned to the WT *CYP51*p (St IPO323). Nucleobases of the reference sequence of St IPO323 and all disagreements to this sequence are highlighted in colour (adenine = red, guanine = yellow, thymine = green, cytosine = blue). The alignment is only shown until the beginning of the *CYP51* gene and the start codon is indicated by the asterisk. The insertion site of the 121 bp insert is located -83 bp upstream of the start codon (based on WT *CYP51*p of St IPO323). Alignment was performed with MUSCLE algorithm.

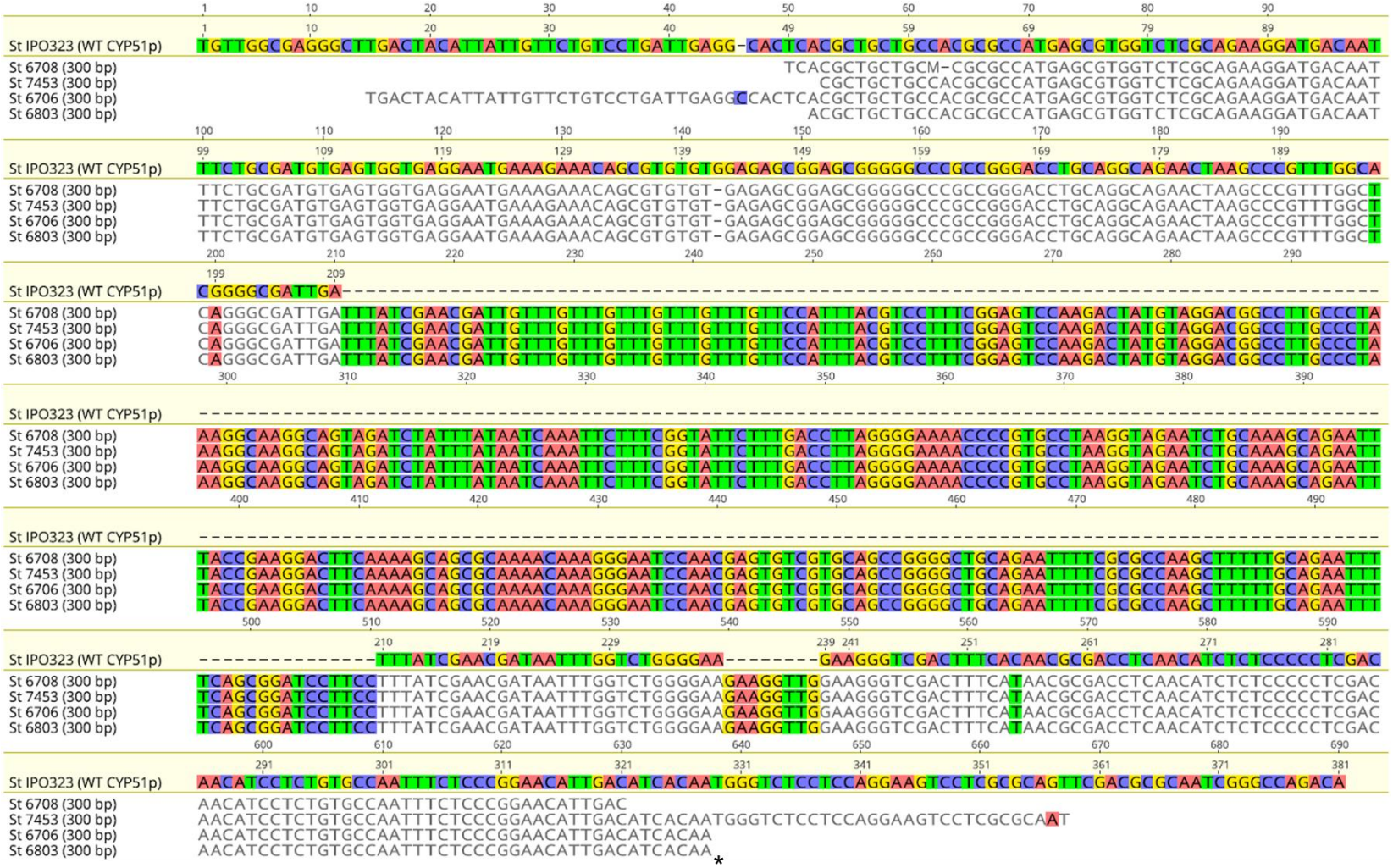


Figure 39: Alignment of *Z. tritici* isolates carrying the 300 bp insert in *CYP51*p. Isolates carrying the 300 bp insert in *CYP51*p were aligned to the WT *CYP51*p (St IPO323). Nucleobases of the reference sequence of St IPO323 and all disagreements to this sequence are highlighted in colour (adenine = red, guanine = yellow, thymine = green, cytosine = blue). The *CYP51* start codon is indicated by the asterisk. The insertion site of the 300 bp insert is located -118 bp upstream of the start codon (based on WT *CYP51*p of St IPO323). Alignment was performed with MUSCLE algorithm.

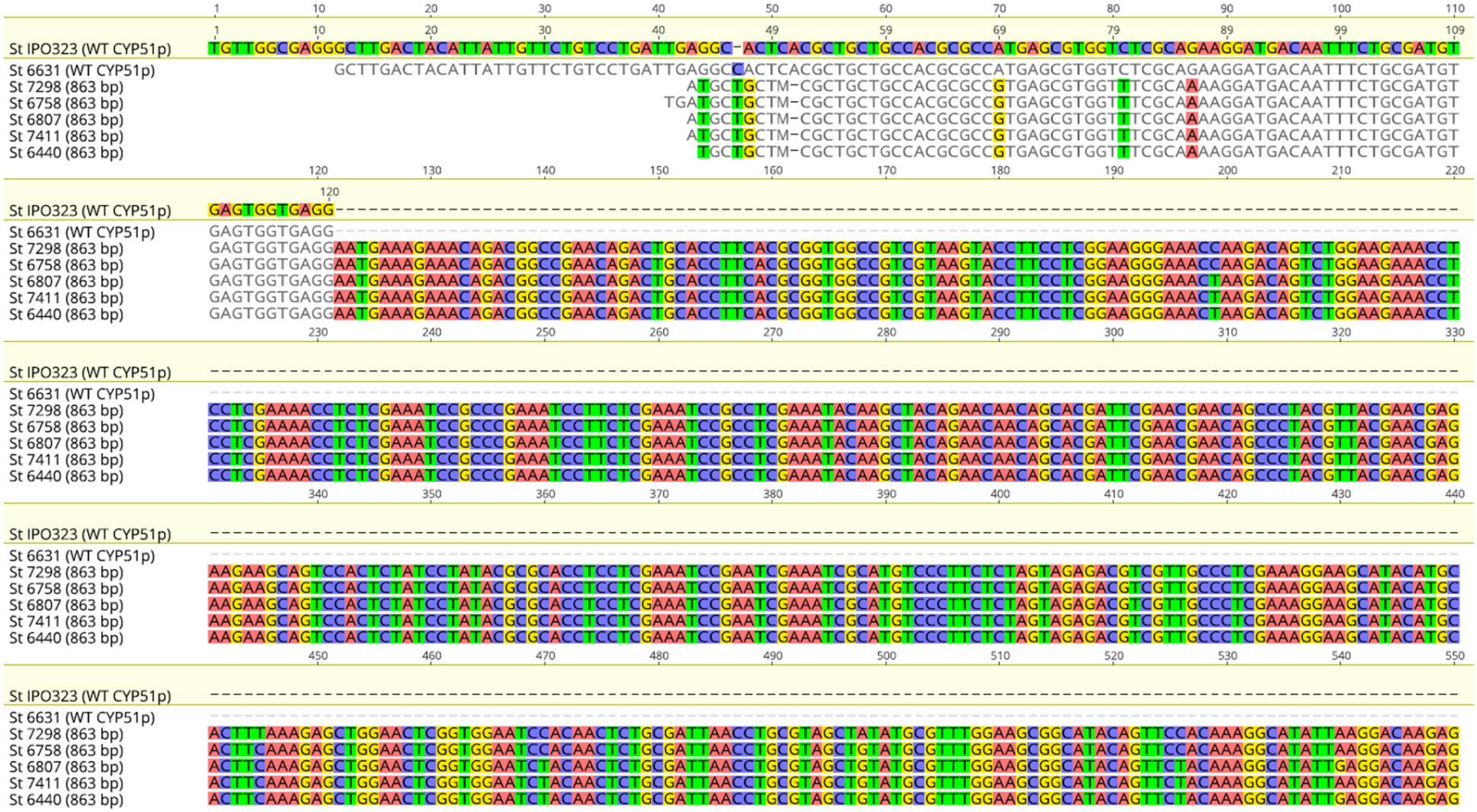


Figure 40-Part 1: Alignment of *Z. tritici* isolates carrying the 863 bp insert in *CYP51*p. Further information is given on page 181.

Figure 40-Part 2: Alignment of *Z. tritici* isolates carrying the 863 bp insert in *CYP51*p. Further information is given on page 181.

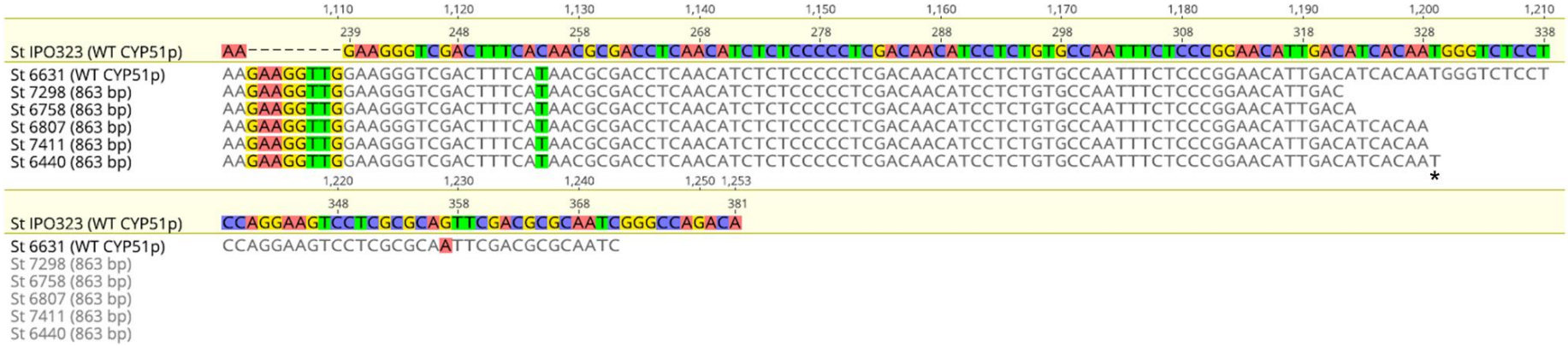


Figure 40-Part 3: Alignment of *Z. tritici* isolates carrying the 863 bp insert in *CYP51*p. Isolates carrying the 863 bp insert in *CYP51*p were aligned to the WT *CYP51*p (St IPO323, St 6631). The second WT *CYP51*p sequence was aligned for correct alignment. Nucleobases of the reference sequence of St IPO323 and all disagreements to this sequence are highlighted in colour (adenine = red, guanine = yellow, thymine = green, cytosine = blue). The *CYP51* start codon is indicated by the asterisk. The insertion site of the 863 bp insert is located -207 bp upstream of the start codon (based on WT *CYP51*p of St IPO323). Alignment was performed with MUSCLE algorithm.

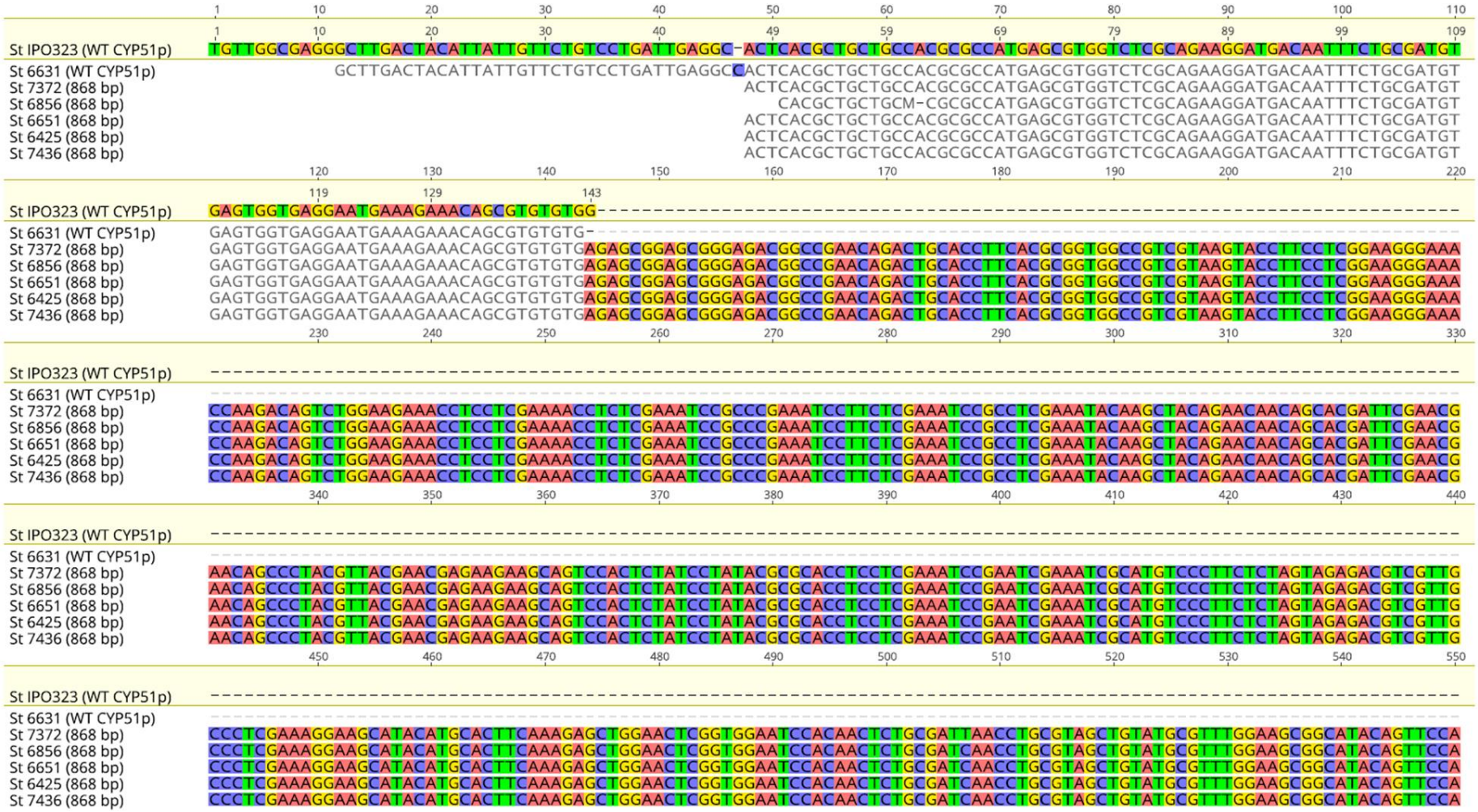


Figure 41-Part 1: Alignment of *Z. tritici* isolates carrying the 868 bp insert in *CYP51*p. Further information is given on page 184.

Figure 41-Part 2: Alignment of *Z. tritici* isolates carrying the 868 bp insert in *CYP51*p. Further information is given on page 184.

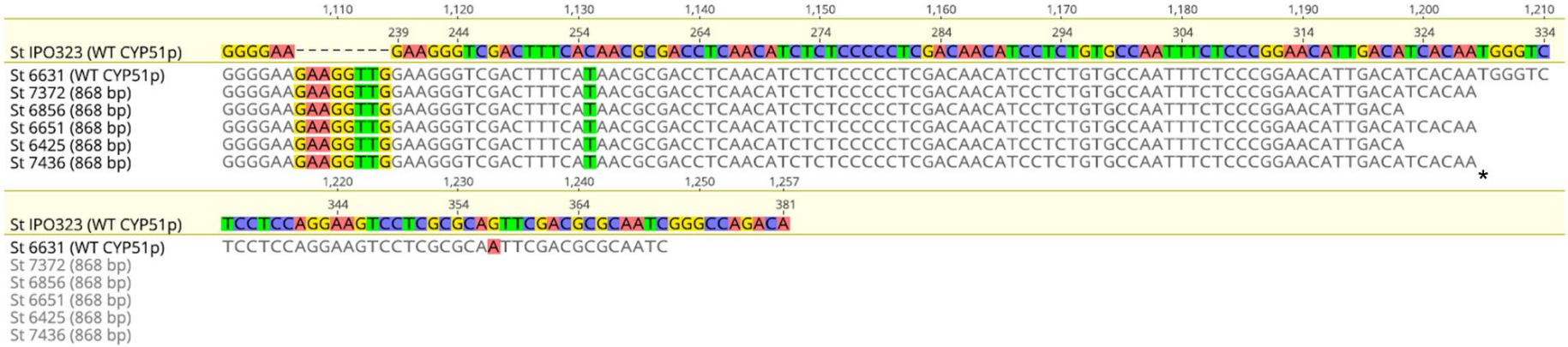


Figure 41-Part 3: Alignment of *Z. tritici* isolates carrying the 868 bp insert in *CYP51*p. Isolates carrying the 868 bp insert in *CYP51*p were aligned to the WT *CYP51*p (St IPO323, St 6631). The second WT *CYP51*p sequence was aligned for correct alignment. Nucleobases of the reference sequence of St IPO323 and all disagreements to this sequence are highlighted in colour (adenine = red, guanine = yellow, thymine = green, cytosine = blue). The *CYP51* start codon is indicated by the asterisk. The insertion site of the 868 bp insert is located -184 bp upstream of the start codon (based on WT *CYP51*p of St IPO323). Alignment was performed with MUSCLE algorithm.

Table 25: Number of *Z. tritici* isolates carrying a wild type *CYP51*p (WT *CYP51*p) or a *CYP51*p insert shown separately for 'rarely found *CYP51* haplotypes'. Isolates that did not produce a PCR product are described with n.a..

***CYP51* haplotype**	**WT *CYP51*p**	**Length of *CYP51*p insert (bp)**			**n.a.**
		121	**300**	**~900**	
A2	-	-	-	-	1
A3	1	-	-	-	-
A14	2	-	-	-	-
A15	1	-	-	-	-
B8	3	-	-	-	-
B15	1	-	-	-	-
C2	4	1	-	-	-
C4	4	-	-	-	-
C6	1	-	-	-	-
C7	1	-	-	1	-
C9	-	-	1	-	-
C18	1	-	-	-	-
D2	1	-	-	-	-
D7	3	1	-	-	-
D9	1	-	-	1	-
D10	-	-	-	1	-
D26	-	-	-	1	-
D27	-	-	-	1	-
E7	-	-	-	2	-
E8	-	-	-	1	-
E9	1	-	-	-	-
E21	1	-	-	-	-
E25	1	-	-	-	-
E26	-	-	-	1	-
E27	1	-	-	-	-
E28	1	-	-	-	-
E29	1	-	-	-	-
F1	-	-	-	1	-
F5	2	-	-	2	-
F6	-	1	-	8	-
F7	4	-	-	1	-
F9	1	-	-	-	-
F10	-	-	-	5	-

***CYP51* haplotype**	**WT *CYP51*p**	**Length of *CYP51*p insert (bp)**			**n.a.**
		121	**300**	**~900**	
F19		-	-	1	-
F20		-	-	1	-
G2	-	-	-	2	-
G6	-	-	-	2	-
G7	3	-	-	-	-
H3	1	1	-	-	-
H5	2	-	-	1	-
H9	-	-	1	-	-
I1	-	-	-	5	-
I2		-	-	2	-
I4	5	-	-	-	-

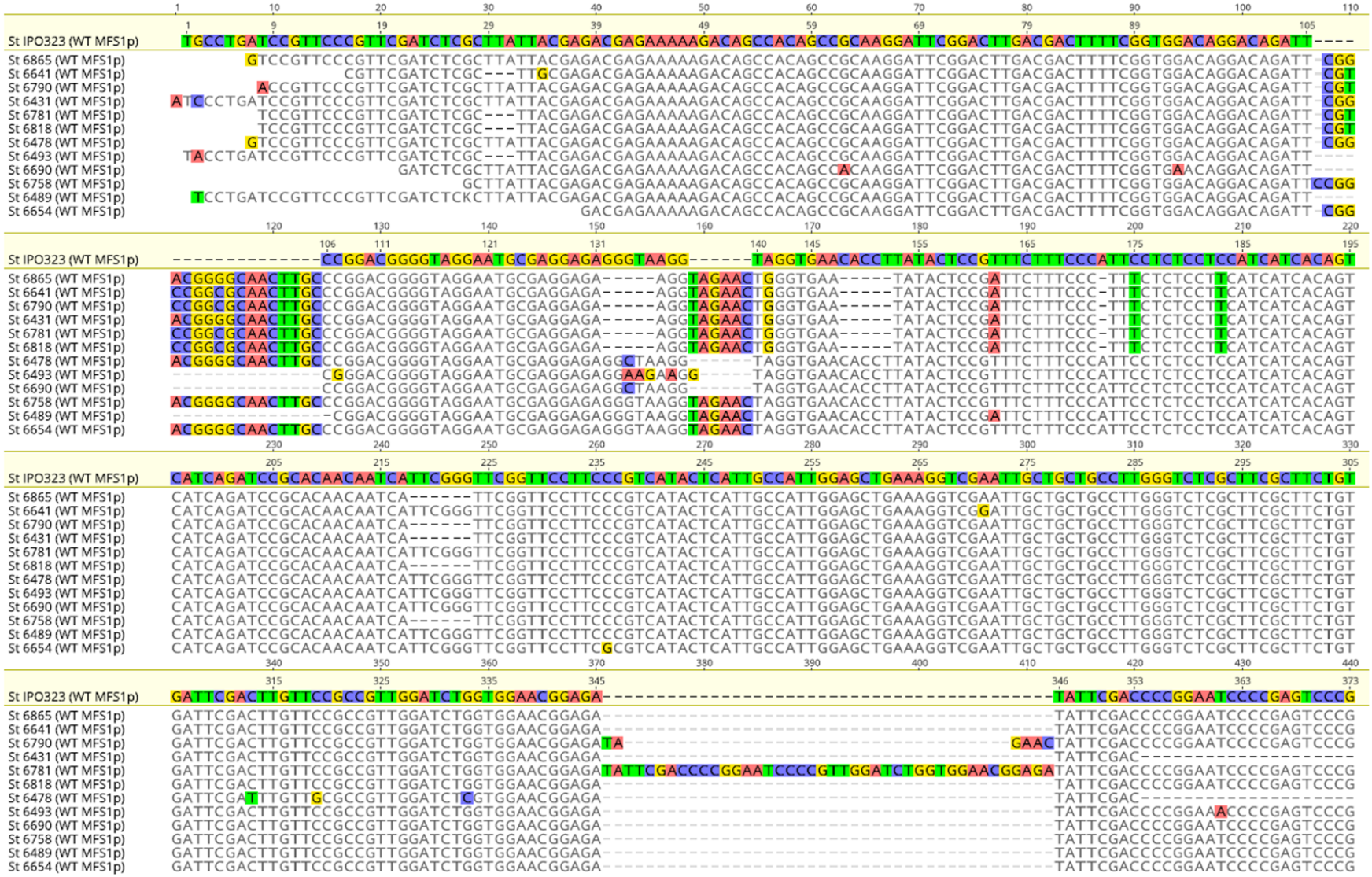


Figure 42-Part 1: Alignment of *Z. tritici* isolates carrying the wild type *MFS1*p (WT *MFS1*p). Further information is given on the next page.

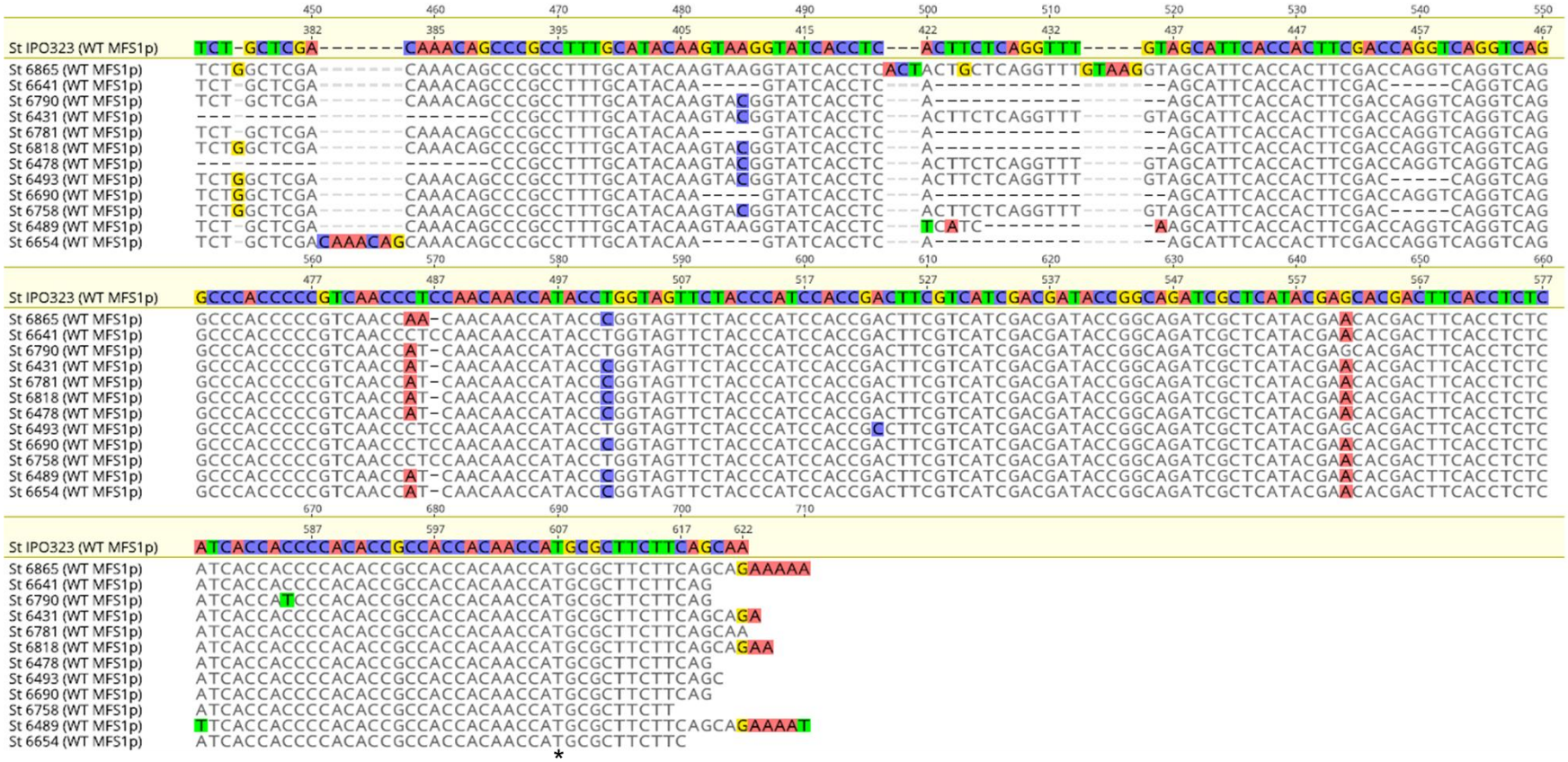


Figure 42-Part 2: Alignment of *Z. tritici* isolates carrying the wild type *MFS1*p (WT *MFS1*p). Nucleobases of the reference sequence of St IPO323 and all disagreements to this sequence are highlighted in colour (adenine = red, guanine = yellow, thymine = green, cytosine = blue). The *MFS1* start codon is indicated by the asterisk. Compared to the reference sequence of St IPO323, all isolates showed small insertions ranging between 3 and ≤42 bp. Nevertheless, these isolates were regarded in this work to carry a WT *MFS1*p (described in 4.6.1). Alignment was performed with MUSCLE algorithm.

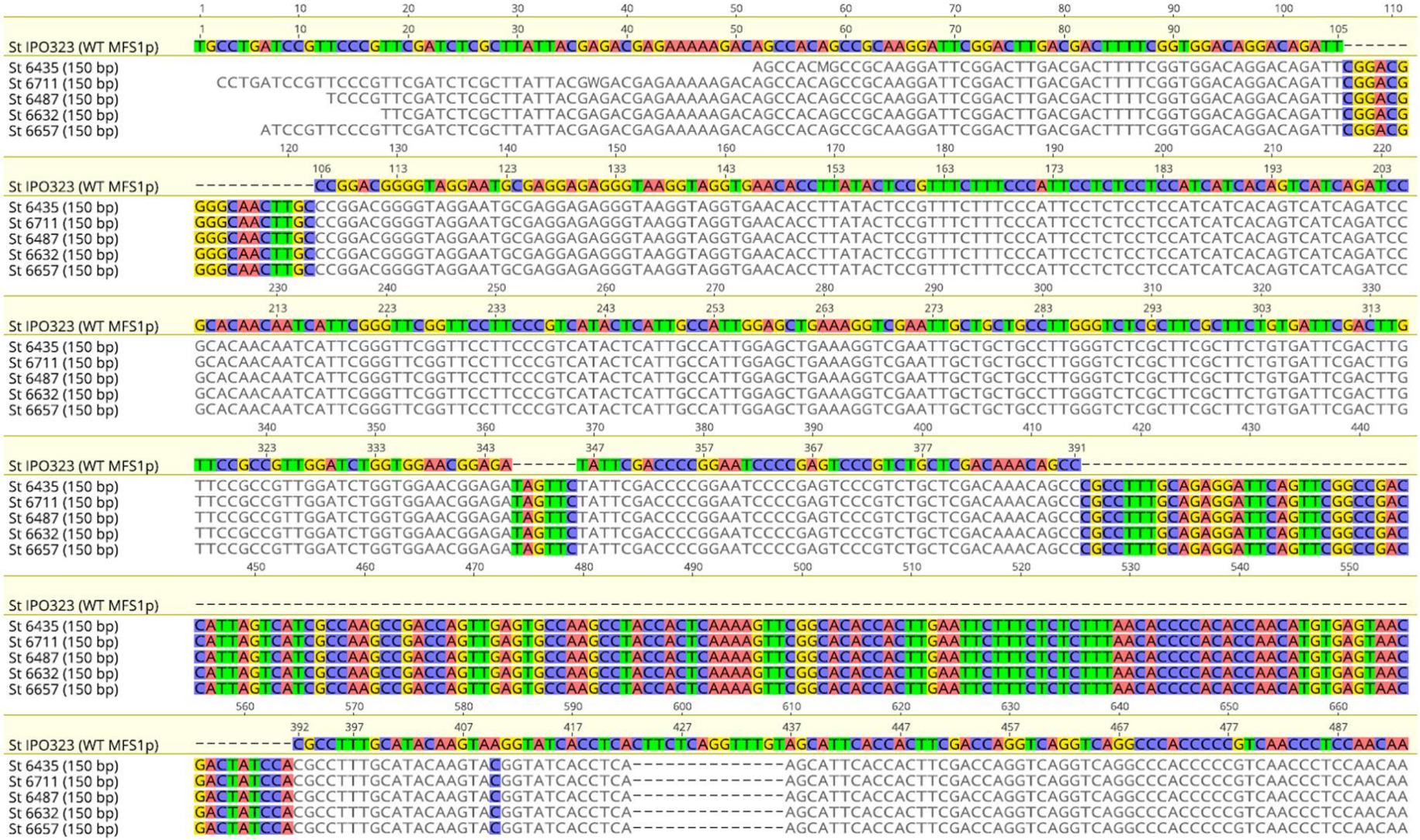


Figure 43-Part 1: Alignment of *Z. tritici* isolates carrying the 150 bp insert in *MFS1*p. Further information is given on the next page.

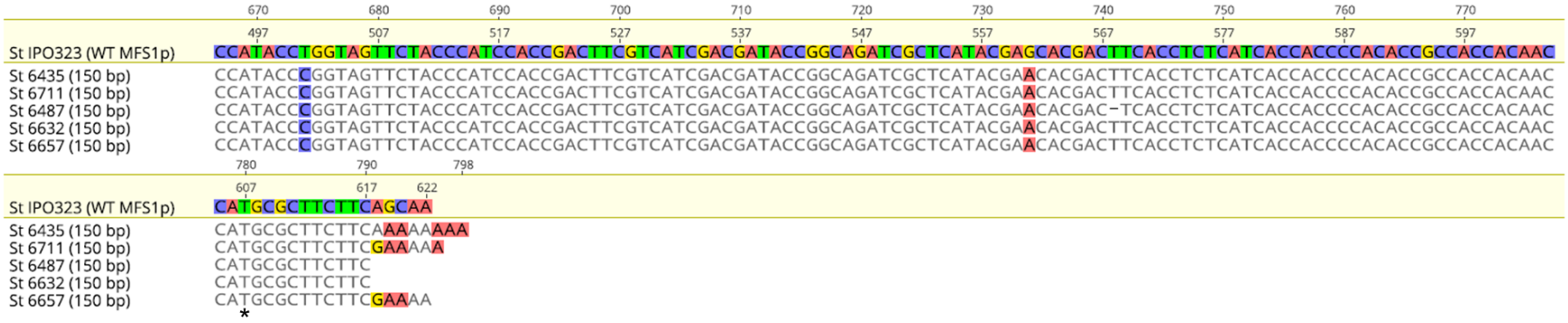


Figure 43-Part 2: Alignment of *Z. tritici* isolates carrying the 150 bp insert in *MFS1*p. Isolates carrying the 150 bp insert in *MFS1*p were aligned to the WT *MFS1*p (St IPO323). Nucleobases of the reference sequence of St IPO323 and all disagreements to this sequence are highlighted in colour (adenine = red, guanine = yellow, thymine = green, cytosine = blue). The *MFS1* start codon is indicated by the asterisk. The insertion site of the 150 bp insert is located -214 bp upstream of the *MFS1* start codon (based on WT *MFS1*p of St IPO323). Alignment was performed with MUSCLE algorithm

Figure 44-Part 1: Alignment of *Z. tritici* isolates carrying different inserts with a length of around 300 bp in *MFS1*p. Further information is given on page 194.

Figure 44-Part 2: Alignment of *Z. tritici* isolates carrying different inserts with a length of around 300 bp in *MFS1*p. Further information is given on page 194.

Figure 44-Part 3: Alignment of *Z. tritici* isolates carrying different inserts with a length of around 300 bp in *MFS1*p. Further information is given on page 194.

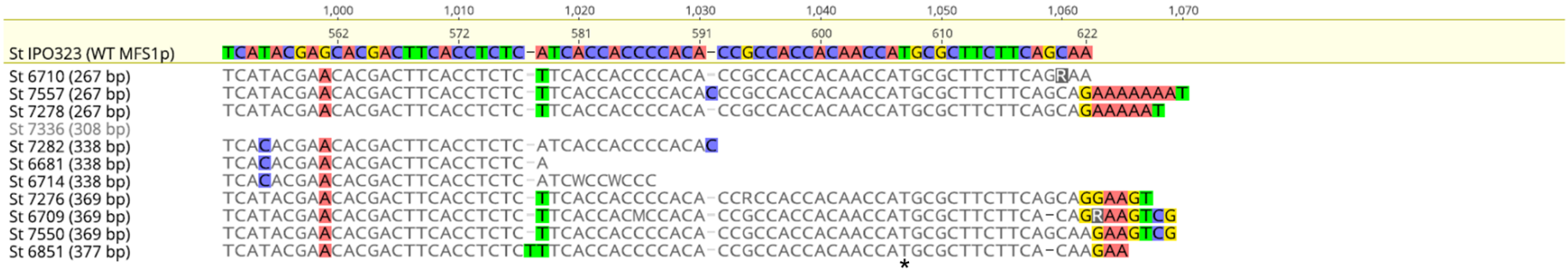


Figure 44-Part 4: Alignment of *Z. tritici* isolates carrying different inserts with a length of around 300 bp in *MFS1*p. Isolates carrying either the 267, 308, 338, 369, or 377 bp insert in *MFS1*p were aligned to the WT *MFS1*p (St IPO323). Nucleobases of the reference sequence of St IPO323 and all disagreements to this sequence are highlighted in colour (adenine = red, guanine = yellow, thymine = green, cytosine = blue). The *MFS1* start codon is indicated by the asterisk. The insertion site is located -468 bp upstream of the *MFS1* start codon (based on WT *MFS1*p of St IPO323). Alignment was performed with MUSCLE algorithm.

Figure 45-Part 1: Alignment of *Z. tritici* isolates carrying the 519 bp insert in *MFS1*p. Further information is given on page 197.

Figure 45-Part 2: Alignment of *Z. tritici* isolates carrying the 519 bp insert in *MFS1*p. Further information is given on page 197.

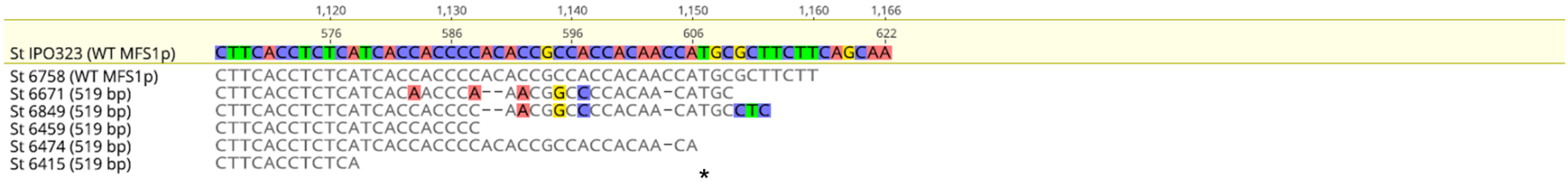


Figure 45-Part 3: Alignment of *Z. tritici* isolates carrying the 519 bp insert in *MFS1*p. Isolates carrying the 519 bp insert in *MFS1*p were aligned to the WT *MFS1*p (St IPO323, St 6758). The second WT *MFS1*p fragment was aligned for correct alignment. Nucleobases of the reference sequence of St IPO323 and all disagreements to this sequence are highlighted in colour (adenine = red, guanine = yellow, thymine = green, cytosine = blue). The *MFS1* start codon is indicated by the asterisk. The insertion site of the 519 bp insert is located -402 bp upstream of the *MFS1* start codon (based on WT *MFS1*p of St IPO323). Alignment was performed with MUSCLE algorithm.

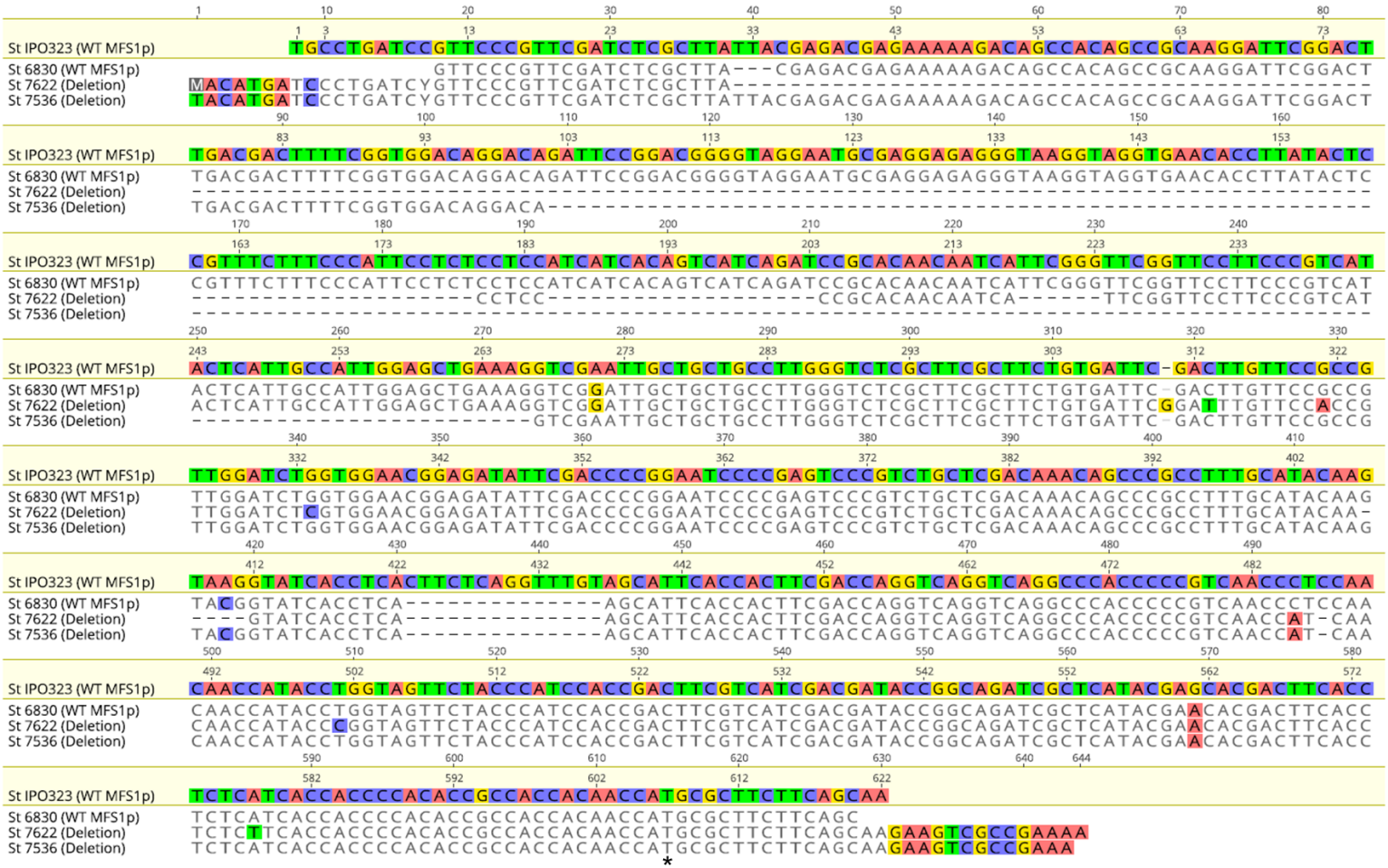


Figure 46: Alignment of *Z. tritici* isolates carrying a 148 or 165 bp deletion in *MFS1*p. Isolates carrying a WT *MFS1*p (St IPO323, St 6830) were aligned to isolate St 7622 carrying a 148 bp deletion in *MFS1*p and to isolate St 7536 with a 165 bp deletion. The second WT *MFS1*p fragment was aligned for correct alignment. Nucleobases of the reference sequence of St IPO323 and all disagreements to this sequence are highlighted in colour (adenine = red, guanine = yellow, thymine = green, cytosine = blue). The *MFS1* start codon is indicated by the asterisk. The site of the 148 bp deletion is located -426 bp upstream of the *MFS1* start codon (based on WT *MFS1*p of St IPO323), whereas the site of the 165 bp deletion is located -339 bp upstream of the *MFS1* start codon. Alignment was performed with MUSCLE algorithm.

Table 26: Number of *Z. tritici* isolates carrying a wild type *MFS1*p (WT *MFS1*p) or an *MFS1*p insert per *CYP51* haplotype. Isolates that did not produce a PCR product are described with n.a..

***CYP51* haplotype**	**WT *MFS1*p**	**Length of *MFS1*p insert (bp)**							**n.a.**
		150	**267**	**308**	**338**	**369**	**377**	**519**	
WT	14	-	-	-	-	-	-	-	-
A2	1	-	-	-	-	-	-	-	-
A3	1	-	-	-	-	-	-	-	-
A14	2	-	-	-	-	-	-	-	-
A15	1	-	-	-	-	-	-	-	-
B8	3	-	-	-	-	-	-	-	-
B15	1	-	-	-	-	-	-	-	-
C2	5	-	-	-	-	-	-	-	-
C4	4	-	-	-	-	-	-	-	-
C6	1	-	-	-	-	-	-	-	-
C7	2	-	-	-	-	-	-	-	-
C8	131	1	-	-	-	-	-	2	5
C9	1	-	-	-	-	-	-	-	-
C18	1	-	-	-	-	-	-	-	-
D2	1	-	-	-	-	-	-	-	-
D7	3	-	-	-	-	-	-	-	1
D9	2	-	-	-	-	-	-	-	-
D10	1	-	-	-	-	-	-	-	-
D13	10	1	-	-	1	-	-	-	-
D26	-	1	-	-	-	-	-	-	-
D27	1	-	-	-	-	-	-	-	-
E3	7	4	-	-	-	-	-	1	3
E4	188	4	4	1	3	1	1	15	13
E5	18	2	-	-	1	-	-	1	2
E7	1	-	1	-	-	-	-	-	-
E8	1	-	-	-	-	-	-	-	-
E9	1	-	-	-	-	-	-	-	-
E21	1	-	-	-	-	-	-	-	-
E25	1	-	-	-	-	-	-	-	-
E26	1	-	-	-	-	-	-	-	-
E27	1	-	-	-	-	-	-	-	-
E28	1	-	-	-	-	-	-	-	-
E29	1	-	-	-	-	-	-	-	-
F1	1	-	-	-	-	-	-	-	-
F2	80	1	1	-	2	1	-	6	-

***CYP51* haplotype**	**WT *MFS1*p**	**Length of *MFS1*p insert (bp)**							**n.a.**
		150	**267**	**308**	**338**	**369**	**377**	**519**	
F3	11	-	-	-	-	-	-	-	-
F4	13	-	-	-	-	-	-	-	-
F5	4	-	-	-	-	-	-	-	-
F6	7	1	-	-	-	1	-	-	-
F7	3	-	-	-	1	-	-	-	1
F8	28	1	-	-	2	3	-	2	2
F9	1	-	-	-	-	-	-	-	-
F10	3	1	-	-	-	-	-	-	1
F19	1	-	-	-	-	-	-	-	-
F20	1	-	-	-	-	-	-	-	-
G1	94	2	-	-	1	-	-	3	1
G2	1	-	-	-	-	-	-	-	1
G6	-	1	-	-	-	-	-	1	-
G7	2	1	-	-	-	-	-	-	-
H3	1	1	-	-	-	-	-	-	-
H4	14	4	-	-	-	-	-	1	3
H5	1	-	-	-	1	-	-	1	-
H6	17	-	1	-	1	-	-	3	5
H9	1	-	-	-	-	-	-	-	-
I1	4	-	-	-	-	-	-	1	-
I2	1	-	-	-	-	-	-	-	1
I4	5	-	-	-	-	-	-	-	-

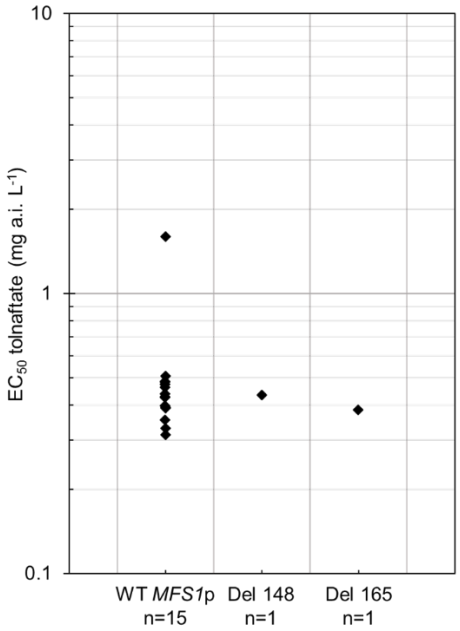


Figure 47: Sensitivity of *Z. tritici* isolates with a deletion in *MFS1*p. EC_{50} values of the isolates with respectively a 148 or 165 bp deletion for tolnaftate are shown compared to the EC_{50} values of isolates with a WT *MFS1*p. The number of tested isolates (n) is given in the figure. Tolnaftate is used as an indicator for an increased efflux activity of *Z. tritici* isolates. EC_{50} values of the isolates with a deletion were located in the sensitivity range of the isolates with a WT *MFS1*p.

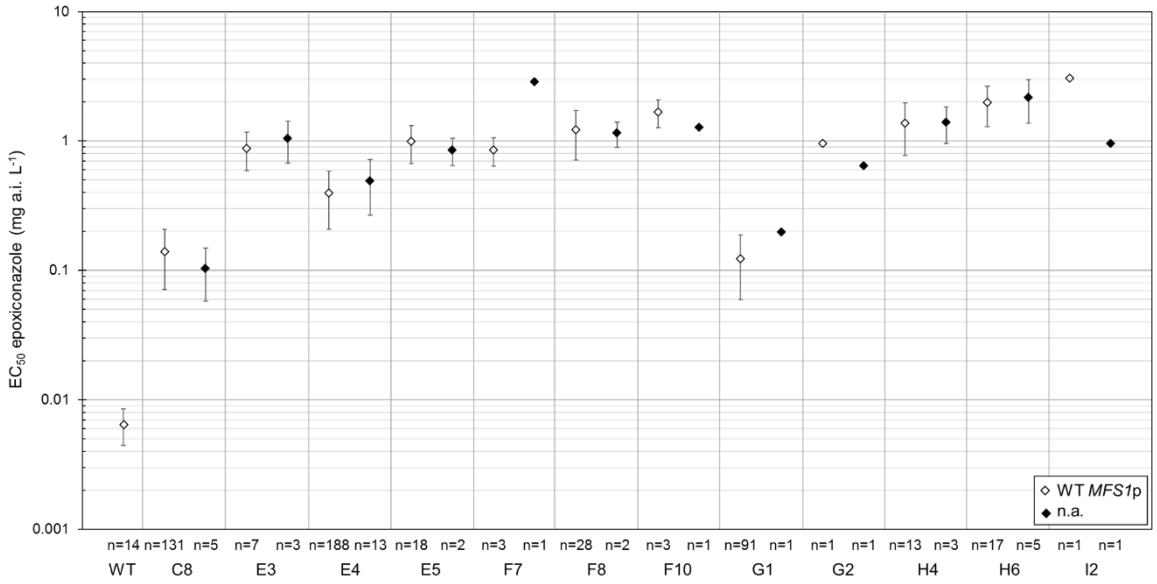


Figure 48: Sensitivity of *Z. tritici* isolates with not characterised *MFS1*p to epoxiconazole. In 2016 and 2017, *Z. tritici* isolates were observed that produced no amplicon during PCR that was used to identify *MFS1*p inserts. EC_{50} values of these isolates for epoxiconazole are shown and compared to EC_{50} of isolates carrying the same *CYP51* haplotype and a WT *MFS1*p. Single EC_{50} values are shown when only one isolate was detected and mean EC_{50} values and standard deviations were calculated when more than one isolate was identified. Mean EC_{50} and standard deviations were calculated over all isolates with the same *CYP51* haplotype and a WT *MFS1*p or with a *MFS1*p that was not characterised, respectively. The number of tested isolates is given in the diagram. Only isolates without distinct *CYP51* overexpression (121 bp insert in *CYP51*p) are shown. No clear trend in the differences between the EC_{50} or mean EC_{50} of isolates with a WT *MFS1*p and isolates for which *MFS1*p was not characterised were observed.

Table 27: EC_{50} values of different DMIs for *Z. tritici* isolates used in glasshouse studies. Epoxiconazole (ECA), mefentrifluconazole (MFA), and prothioconazole-desthio (PTH-D) were tested in microtiter tests by EpiLogic. EC_{50} values marked with an asterisk were generated in microtiter tests internally in BASF SE. *CYP51* haplotype, absence or presence and respective size of *CYP51*p and *MFS1*p inserts is shown.

Isolate	Year of detection	*CYP51* haplotype	*CYP51*p insert	*MFS1*p insert	EC_{50} (mg a.i. L^{-1})		
					ECA	MFA	PTH-D
3718	2004	E1	-	-	0.047*	0.018*	0.005*
7473	2017	E4	900	-	0,527	0,010	0,152
7332	2017	E4	900	519	1,366	0,059	0,199
7327	2017	F2	120	WT	0,540	0,153	0,085
7438	2017	F2	120	519	1,035	0,782	0,151
5302	2012	G2	900	-	1,128	0,735*	0,144*
5950	2014	G2	300	369	1,732	2,99*	0,235*
6072	2015	H6	900	519	2,517	0,431	0,181
6090	2015	H6	900	519	3,002	0,197	0,215
6101	2015	I2	900	-	1,33	0,188*	1,6672
6144	2015	I2	900	-	3,665	0,295*	1,6404

Zusammenfassung

Der pilzliche Schaderreger *Zymoseptoria tritici* verursacht die Septoria-Blattdürre (STB) an Weizen, eine der wichtigsten Krankheiten hinsichtlich der Ertragsreduktion im Weizenanbau. Neben ackerbaulichen Maßnahmen und dem Anbau von Weizensorten mit geringerer Krankheitsanfälligkeit, beruht die Kontrolle dieses Pathogens überwiegend auf der Anwendung von blattaktiven Fungiziden mit unterschiedlichen Wirkmechanismen. In den letzten Jahrzenten stellten hierbei die Demethylierungsinhibitoren (DMIs) eine der meist genutzten Fungizidklassen dar. Bis heute kommt den DMIs noch immer eine große Bedeutung in der Bekämpfung von *Z. tritici* zu. DMIs inhibieren die Sterol 14α-Demethylase, ein wichtiges Enzym im Sterolbiosyntheseweg von Pilzen, welches durch das *CYP51* Gen kodiert wird. Die langanhaltende und intensive Nutzung führte zu einer kontinuierlichen Sensitivitätsreduktion von STB gegenüber DMIs, die über einen langen Zeitraum genutzt wurden. Diese Sensitivitätsverschiebung beruht hauptsächlich auf der Akkumulation von Mutationen im *CYP51* Gen. Hieraus wurden in der Vergangenheit zahlreiche *CYP51* Haplotypen selektiert. Zusätzlich wurde in den letzten Jahren berichtet, dass eine *CYP51* Überexpression und gesteigerte Effluxaktivität, die auf der Überexpression des MFS1 Membrantransporters beruht, die DMI-Sensitivität von STB beeinflussen. Die Überexpression wird hierbei durch Insertionen in der Promotorregion des *CYP51* (*CYP51*P) und *MFS1* (*MFS1*P) Gens hervorgerufen. In der vorliegenden Arbeit wurde das Vorkommen unterschiedlicher DMI-Resistenzmechanismen in *Z. tritici* in den Jahren 2016 und 2017 über Europa untersucht. Der Effekt unterschiedlicher Mechanismen auf die DMI Anpassung von STB wurde analysiert. Um die *CYP51* Haplotypen zu bestimmen, wurde das *CYP51* Gen von allen Isolaten sequenziert. Zusätzlich wurde die *CYP51*P sowie *MFS1*P Region auf Insertionen untersucht, um eine *CYP51* Überexpression sowie gesteigerte Effluxaktivität zu identifizieren.

Im Zuge dieser Arbeit wurden neue *CYP51* Mutationen in *Z. tritici* identifiziert. Obwohl diese wahrscheinlich nicht zu einer verringerten DMI Sensitivität führen, zeigt deren Entstehung, dass die Evolution des *CYP51* Gens noch immer voranschreitet. Zusätzlich wurden neue *CYP51* Haplotypen identifiziert, welche auf neuen Kombinationen von bereits existierenden CYP51 Alterationen beruhen. Aufgrund der Identifikation zahlreicher neuer *CYP51* Haplotypen wurde im Zuge dieser Arbeit eine neue Nomenklatur etabliert. Diese ermöglichte die Beschreibung der 57 *CYP51* Haplotypen, die über alle Isolate von 2016 und 2017 gefunden wurden. Des Weiteren ermöglicht diese Nomenklatur die zukünftige Beschreibung neu entstehender *CYP51* Haplotypen. In dieser Arbeit wurde gezeigt, dass 13 der 57 *CYP51* Haplotypen ungefähr 89% aller untersuchten Isolate darstellen. Diese wurden als „häufig gefundene *CYP51* Haplotypen“ bezeichnet. Innerhalb dieser *CYP51* Haplotypen zeigten Isolate mit den Haplotypen C8, G1 und F3 die höchste Sensitivität gegenüber Epoxiconazol, gefolgt von den Haplotypen E4 und F2, welche eine moderate

Anpassung zeigten. Isolate mit den Haplotypen F4, E3, D13, E5, F8, H4 und H6, welche die CYP51 Alteration S524T gemeinsam haben, zeigten die höchste Anpassung gegenüber Epoxiconazol. *In vitro* Sensitivitätsstudien mit diesen Haplotypen und verschiedenen DMIs deuten auf eine begrenzte Kreuzresistenz zwischen Epoxiconazol sowie Prothioconazol im Vergleich zu Mefentrifluconazol hin. Diese Ergebnisse konnten durch Selektionsstudien im Feld gestützt werden, die einen unterschiedlichen Trend in der Selektion von *CYP51* Haplotypen von Prothioconazol und Mefentrifluconazol zeigten.

Über Europa wurde eine heterogene Verbreitung von den „häufig gefundenen *CYP51* Haplotypen“, mit einem Ost-West Gradient in der DMI Anpassung von *Z. tritici*, beobachtet. Die Mehrheit der *Z. tritici* Populationen in den West-Europäischen Ländern Irland, UK und den Niederlanden wies *CYP51* Haplotypen mit moderater oder höchster Epoxiconazol-Anpassung auf. In Zentral-Europa hingegen, z.B. in Deutschland und Polen wies der Großteil der Populationen *CYP51* Haplotypen mit geringster oder moderater Anpassung innerhalb der „häufig gefundenen *CYP51* Haplotypen“ auf. In Osteuropa wurde eine hohe Häufigkeit an Haplotypen mit höchster Sensitivität innerhalb der „häufig gefundenen *CYP51* Haplotypen“ gefunden. Im Vergleich zu früheren Studien wurde jedoch eine voranschreitende Sensitivitätsverschiebung in Richtung einer reduzierten DMI Sensitivität über Europa, insbesondere in Regionen mit intensivem Weizenanbau, beobachtet.

Die Untersuchung des *CYP51*P führte zur Identifizierung eines 121, 863, 868 und 300 bp Inserts. Es wird angenommen, dass diese Inserts den bereits publizierten 120, 862, und 866 bp Inserts entsprechen. Aus diesem Grund wurde das 121 bp Insert im Verlauf dieser Arbeit als 120 bp, das 863 bp als 862 bp und das 868 bp als 866 bp Insert bezeichnet. In vorherigen Studien wurde gezeigt, dass das 120 bp Insert im *CYP51*P mit einer gesteigerten *CYP51* Expression einhergeht, wohingegen das 862 und 866 bp Insert wahrscheinlich keinen Einfluss auf die *CYP51* Expression aufweisen. In dieser Arbeit korrelierte das 120 bp Insert mit einer im Durchschnitt 5- bis 8-fachen Sensitivitätsreduktion gegenüber Epoxiconazol. Im Gegensatz hierzu zeigten das 300, 862 und 866 bp Insert keine oder nur geringe Effekte. Im Jahr 2016 wurden Isolate mit einer *CYP51* Überexpression, basierend auf dem 120 bp *CYP51*P Insert, in einer geringen bis moderaten Häufigkeit in jedem untersuchten Land gefunden (mit Ausnahme von Dänemark). Im Vergleich hierzu wurde im Jahr 2017 ein Anstieg in der Häufigkeit dieser Isolate in einigen Ländern in Europa beobachtet. Das 120 bp Insert wurde fast ausschließlich in Kombination mit dem *CYP51* Haplotypen F2 beobachtet. Zum ersten Mal wurde es im Zuge dieser Arbeit auch in Kombination mit anderen *CYP51* Haplotypen gefunden. Diese Ergebnisse deuten darauf hin, dass die Evolution der *CYP51* Überexpression und des *CYP51*P von *Z. tritici* weiter voranschreiten.

Die Untersuchung des *MFS1*P führte zur Identifizierung von sieben verschiedenen Inserts mit einer Größe von 150, 267, 308, 338, 369, 377 bzw. 519 bp. Das 150, 338, 369 und

519 bp Insert wurde bereits in der Literatur beschrieben und es wurde gezeigt, dass diese Inserts zu einer gesteigerten *MFS1* Expression führen. Hierbei wurde berichtet, dass das 519 bp Insert im *MFS1*P den größten Effekt zeigt. In *in vitro* Sensitivitätsstudien dieser Arbeit korrelierte das 519 bp Insert mit einer bis zu 8-fachen Sensitivitätsreduktion gegenüber Epoxiconazol, wohingegen die kürzeren *MFS1*P Inserts zu geringeren Effekten führten. Im Jahr 2016 wurden Isolate mit einer gesteigerten Effluxaktivität, basierend auf dem 519 bp *MFS1*P Insert, nur in einer geringen Häufigkeit in Deutschland, Polen und in West-Europa gefunden. Das Vorkommen kleinerer *MFS1*P Inserts wurde hauptsächlich in UK und Irland beobachtet. Im Jahr 2017 wurde ein Anstieg in der Häufigkeit von kleineren *MFS1*P Inserts beobachtet, jedoch verblieb diese auf einem niedrigen Niveau mit Ausnahme von Irland und den Niederlanden. Die Häufigkeit des 519 bp Insert ist zwischen 2016 und 2017 relativ stabil geblieben.

Insgesamt konnte gezeigt werden, dass das Auftreten von verschiedenen *CYP51* Haplotypen noch immer den häufigsten und wichtigsten DMI-Resistenzmechanismus in *Z. tritici* in Europa darstellt. Im Vergleich zu früheren Studien wurde jedoch ein Anstieg in der Häufigkeit von Isolaten mit *CYP51* Überexpression oder mit gesteigerter Effluxaktivität beobachtet. Sensitivitätsstudien im Gewächshaus zeigten, dass DMIs noch immer zu einer STB Kontrolle beitragen oder die Krankheit in manchen Fällen komplett kontrollieren können, auch wenn Isolate moderat oder stark angepasste *CYP51* Haplotypen und/oder *CYP51* Überexpression, sowie eine gesteigerte Effluxaktivität zeigten. Um eine weitere Verbreitung von immer stärker angepassten *CYP51* Haplotypen und zusätzlichen DMI-Resistenzmechanismen in Europa zu verhindern, sollten Antiresistenz-Strategien in der Anwendung von DMIs eine große Bedeutung zukommen. Vor allem sollten ebenfalls die Prinzipien des integrierten Pflanzenschutzes, wie z.B. eine geeignete Sortenwahl beachtet werden, um den STB-Krankheitsdruck und somit die Anzahl der Fungizidapplikationen zu verringern. Des Weiteren könnten Resistenzmanagement-Strategien auf der begrenzten Kreuzresistenz zwischen verschiedener DMIs beruhen, zum Beispiel durch die Anwendung von Mischungen oder der Alternierung verschiedener DMI Fungizide. Zudem sollten Fungizide mit anderen Wirkmechanismen in die Bekämpfungsstrategie einbezogen werden. Das Ziel all dieser Maßnahmen ist es die Selektion angepasster *Z. tritici* Isolate zu vermindern und somit die Effizienz der DMIs in der STB-Bekämpfung zu verlängern.

Danksagung

Meinen ganz besonderen Dank möchte ich Prof. Dr. Vögele aussprechen für die Betreuung und die hilfreichen Gespräche während meiner Promotion. Ralf, ich möchte mich ebenfalls bei Dir dafür bedanken, dass Du mich während meiner gesamten akademischen Laufbahn begleitet hast. Deine Unterstützung half mir dabei mich von der Bachelorarbeit über die Masterarbeit bis heute zur Promotion weiterzuentwickeln.

Ganz besonders bedanken möchte ich mich auch bei Dr. Stammler für die Möglichkeit meine Doktorarbeit in einem solch kompetenten, liebenswerten und hilfsbereitem Team der BASF durchführen zu können. Danke Dir, Gerd, für die Unterstützung während meiner Masterarbeit und Doktorarbeit, die mir dabei half mich fachlich wie auch persönlich in den letzten Jahren weiterzuentwickeln.

Ich möchte mich ebenfalls ganz herzlich bei den Europäischen Kollegen der BASF bedanken, vor allem bei Rosie (Dr. Bryson) und Dieter (Herr Strobel) für die hilfreichen Gespräche zu meinem Thema und die Einblicke in die Praxis („ins Feld“). Danke Dir, Rosie, für die Korrektur meiner Doktorarbeit und die Möglichkeit an zahlreichen Meetings und Tagungen teilzunehmen.

Vielen Dank an Herrn Prof. Dr. Philipp Schlüter und Herrn Prof. Dr. Thomas Miedaner für die Übernahme der 2. und 3. Korrektur meiner Doktorarbeit.

Des Weiteren möchte ich mich bei Herrn Dr. Ulf Groeger, Herrn Dr. Horst-Dieter Brix und Herrn Dr. Karl-Heinz Lorenz für die Unterstützung während meiner Zeit bei BASF bedanken und für die Möglichkeit an zahlreichen Meetings und Tagungen teilzunehmen.

Mein herzlichster Dank gilt dem kompletten Laborteam der Fungizidresistenzforschung für die freundliche Aufnahme und die hilfsbereite sowie freundschaftliche Atmosphäre im Arbeitsalltag. Ganz besonders bedanken möchte ich mich bei Sina und Christoph für die Unterstützung bei den Laborarbeiten. Vielen Dank auch an Birgit, Angi, Kerstin, Gabi, Xenia und Sarah S. für die Unterstützung bei jeglichen Fragen oder „Problemchen“, die aufkamen. Ich habe mich in eurer Arbeitsgruppe unglaublich wohl gefühlt. Ganz besonders bedanken möchte ich mich auch bei meinen Kolleginnen (jetzt Freundinnen) Alexandra und Sarah, die mich herzlichst als Mit-Doktorandin aufgenommen haben. Ihr habt mir dabei geholfen auch die schwierigen Phasen, die bei einer Doktorarbeit schon einmal auftreten können, zu überstehen. Liebe Alex, vielen Dank auch für die Betreuung und Unterstützung bereits während meiner Bachelor- und Masterarbeit. Du hast ebenfalls maßgeblich zu meiner persönlichen und fachlichen Weiterentwicklung in den letzten Jahren beigetragen, wofür ich Dir von Herzen danke. Danke ebenfalls an Andreas Koch, seine Arbeitsgruppe, das Gewächshausteam und alle anderen Kollegen der BASF, die mir bei der Durchführung der

Doktorarbeit geholfen haben. Vielen Dank an Iain (Dr. Proctor) für die Hilfe bei der statistischen Auswertung und Ian (Dr. Craig) für die Hilfe bei den Proteinmodelling-Studien.

Zum Schluss möchte ich mich von ganzem Herzen bei meiner Familie und meinen Freunden bedanken. Ganz besonders bedanken möchte ich mich bei meinen Eltern, Birgit und Thomas und meinem Bruder, Johannes für den fortwährenden Glauben an mich und jegliche Unterstützung während meines Studiums. Ohne euch wäre all dies nicht möglich gewesen. Ebenfalls möchte ich mich insbesondere bei meinem Partner, Jan und meinen Freundinnen, Anna-Lena, Laura und Eileen für den Rückhalt und das Verständnis während meiner Doktorarbeitszeit bedanken. Danke euch Allen für die ermutigenden Worte, die mir dabei geholfen haben meine Ziele stets weiter zu verfolgen.

Eidesstattliche Erklärung

Eidesstattliche Versicherung über die eigenständig erbrachte Leistung

gemäß § 18 Absatz 3 Satz 5 der Promotionsordnung der Universität Hohenheim für die Fakultäten Agrar-, Natur- sowie Wirtschafts- und Sozialwissenschaften

1. Bei der eingereichten Dissertation zum Thema

Characterisation of the sensitivity of *Zymoseptoria tritici* to demethylation inhibitors in Europe

handelt es sich um meine eigenständig erbrachte Leistung.

2. Ich habe nur die angegebenen Quellen und Hilfsmittel benutzt und mich keiner unzulässigen Hilfe Dritter bedient. Insbesondere habe ich wörtlich oder sinngemäß aus anderen Werken übernommene Inhalte als solche kenntlich gemacht.

3. Ich habe nicht die Hilfe einer kommerziellen Promotionsvermittlung oder -beratung in Anspruch genommen.

4. Die Bedeutung der eidesstattlichen Versicherung und der strafrechtlichen Folgen einer unrichtigen oder unvollständigen eidesstattlichen Versicherung sind mir bekannt.

Die Richtigkeit der vorstehenden Erklärung bestätige ich. Ich versichere an Eides Statt, dass ich nach bestem Wissen die reine Wahrheit erklärt und nichts verschwiegen habe.

Ort, Datum	Unterschrift

Lebenslauf

Zur Person

Name:	Huf
Vorname:	Anna
Geburtsdatum:	26.09.1990 in 55543 Bad Kreuznach
Familienstand:	Ledig

Beruflicher Werdegang

Seit 09/2020	Technischer Projektmanager Fungizide, BASF SE, Deutschland
04/2020-08/2020	Koordination im Bereich Pflanzenbau am Kompetenzzentrum für Ökologischen Landbau, Dienstleistungszentrum Ländlicher Raum Rheinhessen-Nahe-Hunsrück, Deutschland

Akademischer Werdegang

07/2016-06/2019	Promotion bei BASF SE, Fungizid-Resistenzforschung, Limburgerhof, Thema: „Characterisation of the sensitivity of *Zymoseptoria tritici* to demethylation inhibitors in Europe", Universität: Universität Hohenheim, Stuttgart
10/2013-8/2016	Studium der Agrarwissenschaften, Universität Hohenheim, Stuttgart, Fachrichtung: Pflanzenproduktionssysteme, Abschluss: M.Sc. Agrarwissenschaften (Note: 1,1)
	Masterarbeit bei BASF SE, Fungizid-Resistenzforschung, Limburgerhof, Thema: „Untersuchungen zur Sensitivität von *Venturia inaequalis* gegenüber Succinat-Dehydrogenase Inhibitoren" (Note 1,0)
10/2010-11/2013	Studium der Agrarbiologie, Universität Hohenheim, Stuttgart, Profil: Pflanzenzüchtung und Saatgutkunde, Abschluss: B.Sc. Agrarbiologie (Note: 1,4)
	Forschungsprojekt „Humboldt relaoded", Universität Hohenheim, Stuttgart, Thema: „Erprobung von Varianten der Propagation von Rostpilztransformanten unter Selektionsbedingungen" (04-07/2012)
	Bachelorarbeit am Institut für Phytomedizin, Fachgebiet Phytopathologie, Universität Hohenheim, Stuttgart, Thema: „Reinigung und Analyse von Transposase 5 - Varianten zur Transformation von *Uromyces fabae"* (Note: 1,3)

Schulausbildung

2007 – 2010	Alfred-Delp-Schule Hargesheim, Abschluss: Allgemeine Hochschulreife (Note: 2,3)
2001 – 2007	Realschule Bad Münster am Stein-Ebernburg, Abschluss: Mittlere Reife

Publikationen

Huf, A., Rehfus, A., Teichmann, M., Randall Gold, R., & Stammler, G. (2017). Sensitivity of *Venturia inaequalis* to Fungicides. In: H. B. Deising, B. Fraaije, A. Mehl, E. C. Oerke, H. Sierotzki, & G. Stammler (Eds.), Modern Fungicides and Antifungal Compounds VIII (pp. 197-198). Braunschweig, Deutschland: Deutsche Phytomedizinische Gesellschaft.

Huf, A., Rehfus, A., Lorenz, K. H., Bryson, R., Voegele, R. T., & Stammler, G. (2018). Proposal for a new nomenclature for *CYP51* haplotypes in *Zymoseptoria tritici* and analysis of their distribution in Europe. *Plant Pathology*, 67: 1706-1712.

Huf, A., Pfleger, S., Strobel, D., Bryson, R., Voegele, R. T., & Stammler, G. (2020). Distribution and changes of genotypes associated to DMI sensitivity of *Zymoseptoria tritici* in Europe. In: H. B. Deising, B. Fraaije, A. Mehl, E. C. Oerke, H. Sierotzki, & G. Stammler (Eds.), Modern Fungicides and Antifungal Compounds IX (pp. 93-98). Braunschweig, Deutschland: Deutsche Phytomedizinische Gesellschaft.

Beiträge Konferenzen

04/2019	Teilnahme am 19. Internationalen Reinhardsbrunn Symposium der DPG in Friedrichroda, Deutschland mit Vortrag: „DMI sensitivity of *Zymoseptoria tritici* and studies on contributing mechanisms in Europe“
01/2019	Teilnahme am 13. Treffen des Fachausschusses Pflanzenschutzmittelresistenz-Fungizide am Julius-Kühn-Institut in Braunschweig, Deutschland mit Vortrag: „Resistenzentwicklung von *Zymoseptoria tritici* gegenüber DMIs und Vorschlag einer neuen Nomenklatur zur Differenzierung und Identifizierung der Isolate“
09/2018	Teilnahme an der 61. Deutschen Pflanzenschutztagung in Stuttgart-Hohenheim, Deutschland mit Vortrag: „Aktuelle Studien zu *CYP51* Haplotypen von *Zymoseptoria tritici* in Europa“
09/2018	Teilnahme am Septoria tritici blotch Meeting in Zürich, Schweiz mit Vortrag: „Studies on DMI sensitivity of *Zymoseptoria tritici*: *CYP51* haplotypes and other mechanisms“

03/2018	Teilnahme am 10. NorBaRAG Meeting in Oslo, Norwegen mit Vortrag: „Update on *CYP51* haplotype frequency and distribution of *Zymoseptoria tritici* in Europe”
03/2018	Teilnahme an der Jahrestagung der DPG-Arbeitskreise Mykologie und Wirt-Parasit-Interaktion in Köln, Deutschland mit Vortrag: „Distribution and frequency of *CYP51* haplotypes in the European population of *Zymoseptoria tritici* in 2016“
03/2017	Teilnahme an der Jahrestagung der DPG-Arbeitskreise Mykologie und Wirt-Parasit-Interaktion in Rostock, Deutschland mit Vortrag: „Sensitivity and fitness studies of fungicide resistant *Venturia inaequalis* strains“
03/2016	Teilnahme an der Jahrestagung der DPG-Arbeitskreise Mykologie und Wirt-Parasit-Interaktion in Gießen, Deutschland mit Posterbeitrag: „Sensitivity status of the causal agent of apple scab (*Venturia inaequalis*) in Europe“

Ort, Datum

Unterschrift