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Genome-wide transcriptional response to multi-site inhibitors and resistance mechanisms to SDHIs in *Zymoseptoria tritici*

Thesis submitted for the degree of Doctor of Philosophy

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Declaration

I confirm that this is my own work and the use of all material from other sources has been properly and fully acknowledge.

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Abstract

Zymoseptoria tritici causes Septoria leaf blotch (SLB), the most important foliar disease in winter wheat in Northern Europe and the UK. Chemical control of *Z. tritici* has seen a continuous introduction and substitution of fungicides with distinct mode of actions, due to the development of fungicide resistance. Emergence of diverse resistance mechanisms and their fixation in field populations of *Z. tritici* represents a constant threat to the control of SLB by fungicides. The aim of this research was to determine the biological potential of *Z. tritici* to adapt to the multi-site inhibitors chlorothalonil and folpet, and the single-site succinate dehydrogenase inhibitor (SDHI) fluxapyroxad. *In vitro* microtitre plate based fungicide sensitivity assays indicated that there was evidence for reduced sensitivity to chlorothalonil or folpet in the *Z. tritici* field isolates tested. Field isolates obtained from plots treated with solo applications of chlorothalonil or folpet were less sensitive to the fungicides compared with isolates sampled from non-treated plots. No evidence was found for reduced sensitivity to fluxapyroxad in the same set of field isolates. RNA sequencing analysis of the genome-wide transcriptional response of the reference *Z. tritici* isolate IPO323 after exposure to chlorothalonil or folpet in the lag and log phase of growth revealed a compound-specific "functional gene expression signature". In addition, several genes encoding glutathione S-transferase (GST), ATP-binding cassette (ABC) or major facilitator superfamily (MFS) efflux pumps were significantly overexpressed in response to chlorothalonil or folpet exposure. *In vitro* evolutionary studies determined the course of evolution of resistance to the succinate dehydrogenase inhibitor (SDHI) fluxapyroxad in replicate populations of *Z. tritici* derived from the sensitive isolate IPO323. Resistance to fluxapyroxad arose mainly through alterations in the target protein that also often conferred cross-resistance to other SDHIs (e.g. fluopyram and carboxin). Additionally, overexpression of an ABC transporter or a GST gene was associated with resistance to fluxapyroxad and lower sensitivity to fluopyram or carboxin in a mutant without target-site alteration. The frequency of six amino acid substitutions in the target protein subunits sdhB, sdhC or sdhD – determined by SNP pyrosequencing assays – indicated that evolution of resistance was driven by a successive substitution of fitter mutants carrying distinct amino acid substitutions as selection at increasing concentrations of fluxapyroxad continued.

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Abbreviations

ABC:	ATP-Binding Cassette
AOX:	Alternative Oxidase
ATP:	Adenosine TriPhosphate
DEGs:	Differentially Expressed Genes
DMI:	sterol-Demethylation Inhibitor
DNA:	Deoxyribonucleic Acid
DMSO:	Dimethyl sulfoxide
EC_{50}:	50% Effective Concentration
EC_{80}:	80% Effective Concentration
EDTA:	Ethylenediaminetetraacetic acid
FRAC:	Fungicide Resistance Action Committee
GST:	Glutathione S-Transferase
MBC:	Methyl Benzimidazole Carbamate
MFS:	Major Facilitator Superfamily
MDR:	Multi Drug Resistance
ORF:	Open Reading Frame
PCR:	Polymerase Chain Reaction
QoI:	Quinone Outside Inhibitor
qRT-PCR:	Quantitative Real-Time PCR
RNA:	Ribonucleic acid
SDB:	Sabouraud Dextrose Broth
SDHI:	Succinate Dehydrogenase Inhibitor
SDW:	Sterile Distillate Water
SLB:	Septoria Leaf Blotch
YPD:	Yeast extract Peptone and Dextrose agar

Contents

Chapter 3: Sensitivity of field populations of *Zymoseptoria tritici* to multi- and single-site inhibitors

Chapter 4: Insights into the transcriptional response of *Zymoseptoria tritici* to multi-site inhibitors

Chapter 6: General discussion

List of Tables

List of Figures

Chapter 1: Introduction

1.1 *Zymoseptoria tritici*

Zymoseptoria tritici (Desm.) Quaedvlieg & Crous (anamorph: *Septoria tritici* Roberge in Desmaz.) is a hemibiotrophic fungus that causes the septoria leaf blotch (SLB) disease in both bread (*Triticum aestivum* L.) and durum (*Triticum turgidum* ssp. *durum* L.) wheat (see Palmer and Skinner, 2002; Quaedvlieg et al., 2011). The SLB symptoms are characterised by development of chlorotic blotches on wheat leaves. Subsequently, the chlorotic spots become necrotic leaf lesions where asexual reproductive structures (i.e. pycnidia) and, at much later stage, sexual structures (pseudothecia) can be found (Fig. 1.1; Kema et al., 1996b).

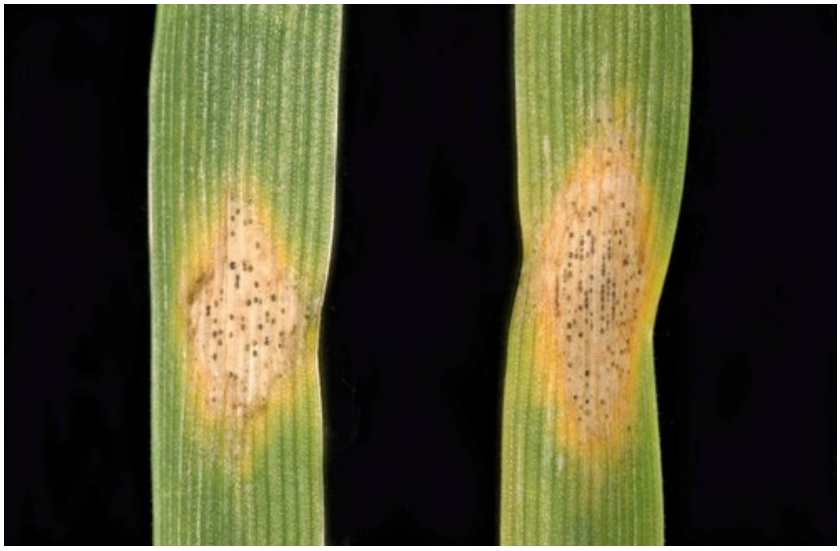

Figure 1.1 Septoria leaf blotch (SLB) symptoms on wheat leaves caused by *Zymoseptoria tritici*. Black dots within lesions are pycnidia which each can release hundreds of asexual pycnidiospores. (Source: Rothamsted visual communication unit).

1.1.1 *Zymoseptoria tritici* infection process

Zymoseptoria tritici infects wheat leaves mainly through stomata, and has a long temperate dependent latent period before symptoms are evident (Fig. 1.2). Typically, the primary inoculum source of SLB is airborne ascospores released from the sexual reproductive structures pseudothecia, which can develop in stubble from previous cropping during autumn and winter (Shaw and Royle, 1989a). Subsequent disease progress within the crop during spring and summer is mainly caused by the asexual stage of the fungus (*Septoria tritici*) (Hunter et al., 1999). Once

sexual ascospores or asexual pycnidiospores have landed on wheat leaves, germination occurs under high humidity conditions (Shaw, 1991). Light microscopy, scanning and transmission electron microscopy studies demonstrated that after spore germination, germ tubes grow randomly from the spore until they penetrate stomata without development of an appressorium within 48 hours post inoculation (Kema et al., 1996b). After stomatal penetration, the fungus can complete its asexual reproductive cycle within two or three weeks through intracellular growth without host cell penetration (see Deller et al., 2011; Kema et al., 1996b). Early in the colonisation phase there is a latency period of exceptionally slow growth that last approximately 7 days, depending on the strain-cultivar combination. During the latency period, the fungus colonises the sub-stomatal cavity and between the mesophyll cell layer of the leaf without a dramatic increase in biomass or visible symptoms development (Kema et al., 1996b; Keon et al., 2007; Pnini-Cohen et al., 2000). This initial symptomless phase has been linked with the secretion of an effector protein (i.e. Mg3LysM) by *Z. tritici* that suppress chitin (PAMP) recognition by the wheat chitin elicitor binding protein (TaCEBiP) and chitin elicitor receptor kinase1 (TaCERK1; Lee et al., 2014; Marshall et al., 2011). Under ideal conditions, after 7 to 10 days post infection fungal biomass increase dramatically and pycnidia formation begins. This increase in biomass coincides with the sudden death of wheat cells (Keon et al., 2007). During this phase the leaf becomes chlorotic and pycnidia become visible (Fig. 1.1). Keon et al. (2007) reported that the appearance of disease symptoms was restricted to inoculated leaf area in a susceptible wheat cultivar. This response displays biochemical features of programmed cell death (Rudd et al., 2008). This massive cell death may provide nutrients – to the fungus – as a consequence of the loss of host cell membrane integrity. Lastly, *Z. tritici* enters in the reproductive phase in the death tissue, forming pycnidia full of asexual spores in the substomatal cavities (Kema et al., 1996b). Under high humidity and rainfall conditions, pycnidiospores are extruded through the stomatal aperture as a spore-rich hydrophobic extracellular matrix known as cirrus (Duncan and Howard, 2000). These pycnidiospores are propagated via rain splash to stablish further cycles of infection. Under optimal experimental conditions in the Netherlands, the sexual cycle of *Z. tritici* can be completed in approximately 35 days (Kema et al., 1996a). In the UK, *Z. tritici* completes its cycle from pycnidium formation to pseudothecia in 62 up to 95 days under field conditions (Hunter et al., 1999).

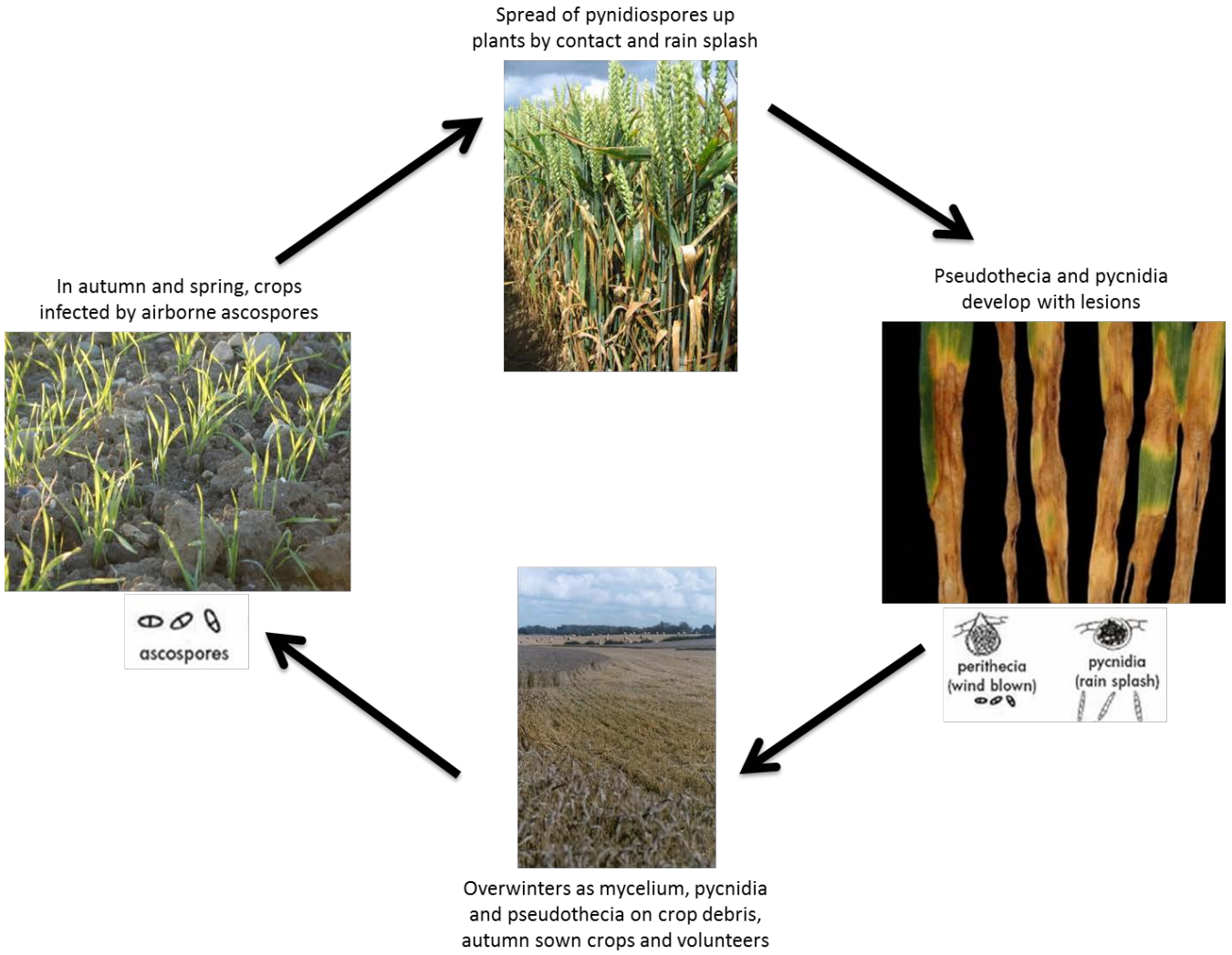

Figure 1.2 Infection cycle of *Zymoseptoria tritici* on winter wheat in the UK. (Source: Rothamsted visual communication unit).

1.1.2 Epidemiology and distribution

Population structure studies have indicated high levels of genetic variability in *Z. tritici* populations. Zhan et al. (2003) reported that approximately 90 % of the global genetic variation of the fungus can be found in a single infected wheat field. This genetic variability can be explained by the high degree of sexual recombination (Cowger et al., 2008), generation of mutations during asexual reproduction (Hunter et al., 1999), and a large population size which reduces genetic drift (Szendro et al., 2013). For plant pathogens, asexual reproduction allows a rapid clonal multiplication of fitter phenotypes under specific conditions (McDonald and Linde, 2002). Sexual reproduction generates new genotypes through recombination, which increases the probability of adaptation to new threats, including fungicide exposure (Brown and Hovmoller, 2002; Wittenberg et al., 2009). Genes or mutated genes encoding resistance to fungicides may recombine with better fitness traits to originate new phenotypes that can spread under field conditions. Moreover, sexual ascospores of the new phenotypes can be wind-dispersed long distances, whereas dispersion of asexual spores can be limited to rain-splash events (Fraaije et al., 2005). Thus, the sexual stage of *Z. tritici* increases population size and speed the spread of fungicide resistance (Brent and Hollomon, 2007b).

Septoria leaf blotch is the most important foliar disease of winter wheat in the UK (Fig. 1.3) and Northern Europe. Since the early 1980s, *Z. tritici* has been the most abundant foliar plant pathogen in wheat crops grown in the UK (Bearchell et al., 2005). Severe epidemics of *Z. tritici* can cause up to 50 % wheat yield losses (see Eyal et al., 1987; Royle et al., 1986). This reduction in wheat yield occurs when the upper three leaves in the crop (i.e. Leaf flag, Leaf 1 and Leaf 2) are infected by *Z. tritici* (Shaw and Royle, 1989b; Thomas et al., 1989). National surveys recorded that approximately 60 % of wheat crops was affected by *Z. tritici* in 2013 (CropMonitor, 2013). Despite of use of fungicides, the disease levels recorded for the leaf flag and leaf 2 were 0.2 and 0.7 % respectively. In 2012, a year with high disease pressure, the average severity of SLB in the flag leaf and leaf 2 was 5.0 and 9.6 %, respectively, and approximately 90 % of the wheat crops were affected by *Z. tritici* (Fig. 1.4; CropMonitor, 2012).

Management of SLB can be achieved by the protection of the three upper wheat leaves from *Z. tritici* infection using fungicide applications and resistant wheat varieties (HGCA, 2014). Although less susceptible varieties to SLB are available, under north European conditions which often leads to high disease pressure, chemical control is the most reliable crop protection strategy against *Z. tritici*. Currently, the control of SLB involves two or three sprays with high rates of azoles (e.g. prochloraz, tebuconazole, metconazole, epoxiconazole or prothioconazole) mixed with succinate

dehydrogenase inhibitors (SDHIs; e.g. penthiopyrad, bixafen, isopyrazam, fluxapyroxad or boscalid) and/or preventive applications of multi-site inhibitor (i.e. chlorothalonil or folpet; HGCA, 2014).

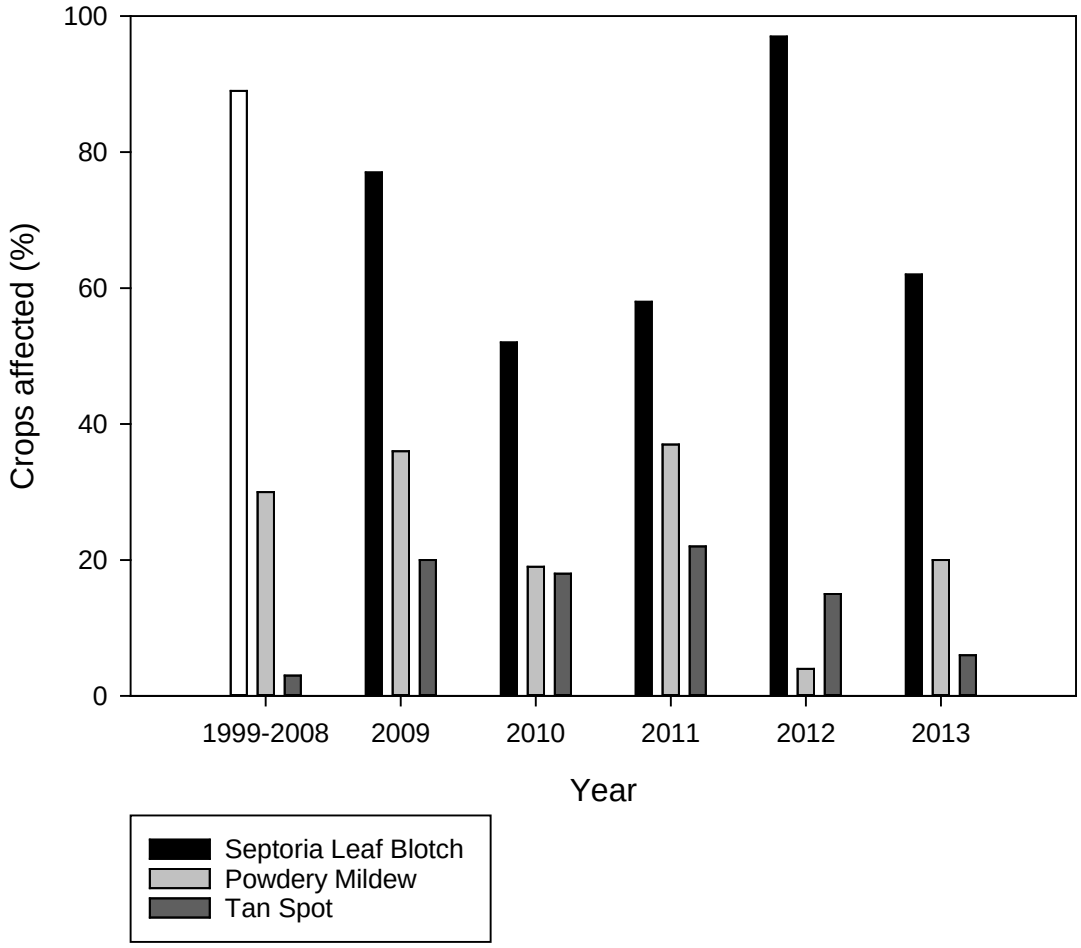

Figure 1.3 National foliar disease incidence of septoria leaf blotch *(Zymoseptoria tritici*), powdery mildew (*Blumeria graminis*) and tan spot (*Pyrenophora tritici-repentis*). Approximately 300 winter wheat crops across England were sampled every year. Disease incidence data was based on disease recorded for the flag and Leaf 2 (Adapted from CropMonitor, 2013).

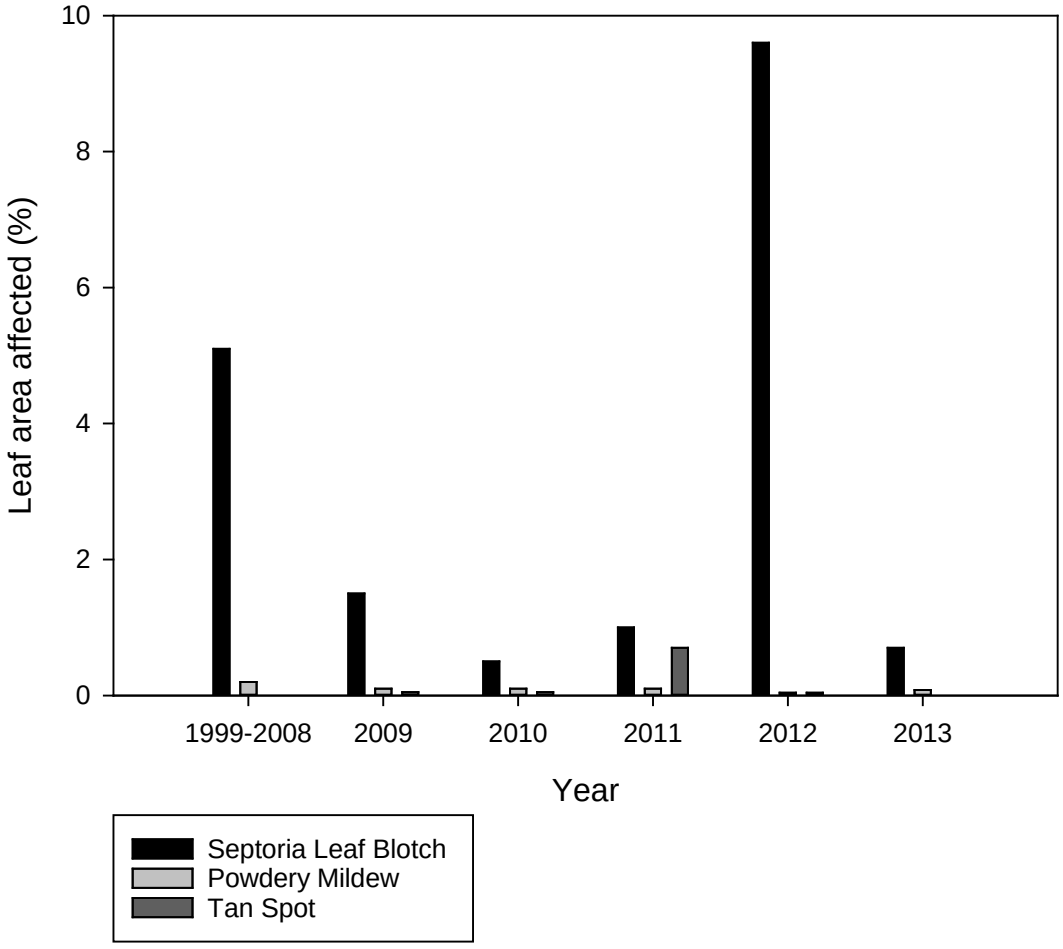

Figure 1.4 National average foliar disease severity on Leaf 2 for septoria leaf blotch (*Zymoseptoria tritici*), powdery mildew (*Blumeria graminis*) and tan spot (*Pyrenophora tritici-repentis*). Approximately 300 winter wheat crops across England were sampled each year (Adapted from CropMonitor, 2013).

1.2 Fungicide resistance

Global crop production has been boosted in part by the use of fungicides to control crop diseases, improve quality and ensure crop production stability. The intense use of fungicides became more common since late 1960's and increased selection pressure in fungal plant pathogen populations (Russell, 2005). Fungal populations under this selection pressure, perhaps, inevitably adapted to fungicides, therefore fungicide resistance became evident. Reduce sensitivity to aromatic hydrocarbons or dodine in *Penicillium* species or *Venturia inaequalis*, respectively, was one of the first reports of fungicide resistance (see Brent, 2012). Intriguingly, resistance to organomercurial fungicides in *Pyrenophora avenae* has been also reported (Noble et al., 1966). Cases of fungicide resistance became more common since 1970's when new classes of fungicide were available and widely used in crop protection (Brent, 2012). Most of the novel selective fungicides impair particular metabolic pathways and bind to specific proteins. These specific site fungicides are considered as single-site fungicides whereas fungicides that affect diverse metabolic pathways are considered as multi-site fungicides (FRAC, 2014). Reduce sensitivity to single-site fungicides is commonly linked with changes in the target protein, whereas for multi-site fungicides many changes might be required. Typically, single-site fungicides can be absorbed and translocate in plant tissue, feature that enables a better disease control at low dose rate. However, this high fungicide efficacy in combination with fungal pathogens with short generation times and large production of propagules can lead to selection of less sensitive individuals carrying alterations in the target protein conferring resistance. Therefore, the emergence and development of resistance to single-site fungicides can be driven by diverse factor such as fungicide mode of action, usage and efficacy, and pathogen biology (see Lucas et al., 2015).

Typically, the control of fungal plant pathogens has been characterised by sequential introduction and replacement of fungicides with distinct mode of action due to the emergence and development of fungicide resistance (Lucas et al., 2015; Van den Bosch et al., 2011). To achieve a long lasting disease control is necessary to understand what mechanisms underlay reduced sensitivity or resistance to fungicides. Diverse studies have reported a number of mechanisms conferring reduced sensitivity to fungicides, including alterations in the target site, increase production of the target protein, metabolic breakdown of the fungicide, and exclusion of the fungicide through ATP-ase dependent or other transporters (Fig. 1.5; see Brent and Hollomon, 2007a; see Lucas et al., 2015; see Thind, 2011).

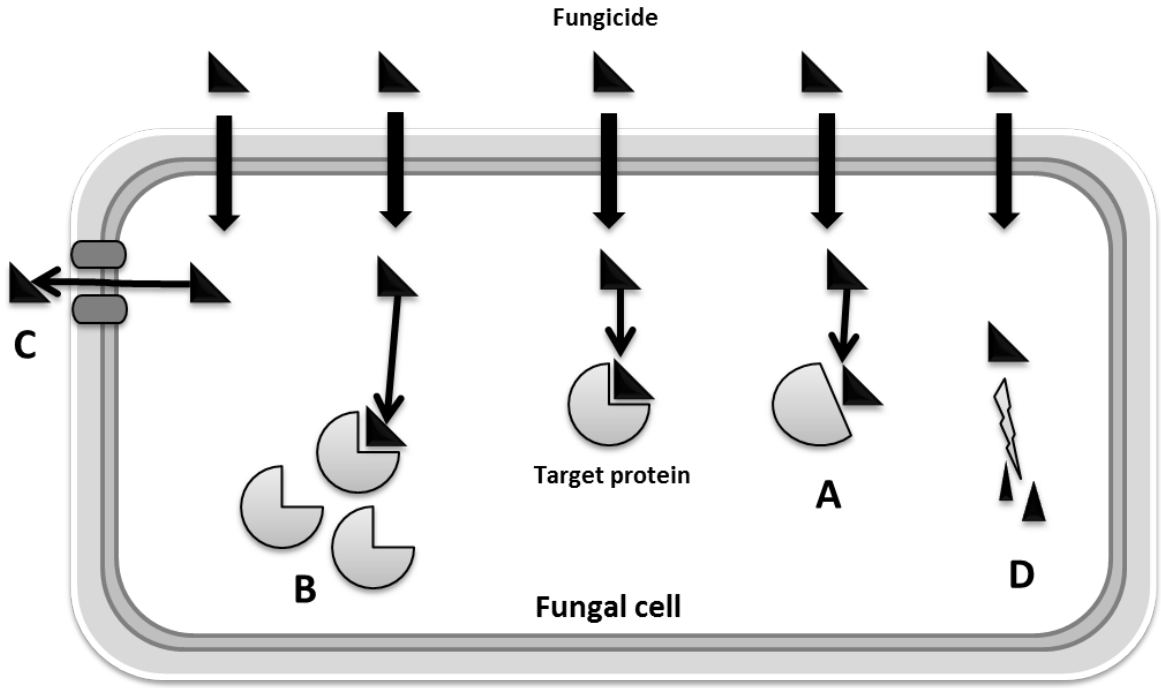

Figure 1.5 Representation of resistance mechanisms to single-site fungicides. Alteration in the target protein (A), overexpression of target protein (B), efflux pump detoxification, and degradation of fungicide (D). (Adapted from Lucas et al., 2015)

1.2.1 Alteration of the target protein

Perhaps the most common resistance mechanism is alteration in the target protein due to mutations in the encoding gene. Point mutations leading to amino acid substitution can confer resistance to most of the single-site fungicides, including methyl benzimidazole carbamates (MBC), quinone outside inhibitors (QoI), sterol-demethylation inhibitors (DMI), and succinate dehydrogenase inhibitors (SDHI; see Brent and Hollomon, 2007a; FRAC, 2013a). Distinct amino acid substitution in the target protein can confer different levels of resistance. Sierotzki et al. (2005) reported two distinct amino acid substitutions in the mitochondrial respiratory chain at the cytochrome b (complex III) that separately can confer different levels of resistance to QoIs in *Plasmopara viticola*. The amino acid substitution at codon 143 from glycine to alanine (G143A) confers higher levels of resistance than the amino acid substitution at codon 129 from phenylalanine to leucine (F129L). Moreover, high levels of resistance can be conferred by a single amino acid substitution. The amino acid substitution from glutamic acid to alanine at codon 198

(E198A) in the target protein β-tubulin confers high levels of resistance to most of the MBC fungicides in *Z. tritici* (Fraaije et al., 2005; Lucas and Fraaije, 2008).

1.2.2 Overexpression of target protein

Overexpression of the target protein can increase resistance to toxicants (Palmer and Kishony, 2014). White (1997) reported an increase of mRNA levels of ERG16 encoding gene - the target protein of azole fungicides – in less sensitive strains of *Candida albicans*. Overexpression of the cyp51A gene – encoding the target protein of azole fungicides – in less sensitive *Aspergillus fumigatus* isolates was correlated with resistance to triazole fungicides (Mellado et al., 2007). Similarly, increased expression of the cyp51 gene in *Penicillium digitatum* can confer high levels of resistance to azole fungicides (Hamamoto et al., 2000). They found five replications of a 126bp tandem repeat – acting as an upstream transcriptional enhancer – in the promoter region in a resistant *P. digitatum* isolate. This increase in gene expression has been associated with changes in the cis- or trans-acting regulatory loci (see Li et al., 2007).

1.2.3 Efflux pump detoxification

Reduce fungicide accumulation due to increased activity of efflux pumps is a common resistance mechanism in both human and plant pathogens (de Waard et al., 2006; White, 1997). ATP-binding cassette transporters (ABC) and major facilitator superfamily transporters (MFS) are transporter proteins with low substrate specificity able to export toxic compounds. Typically, mutations leading to overexpression of ABC or MFS transporters lead to increase detoxification and reduced sensitivity to a number of toxicants (see Gulshan and Moye-Rowley, 2007; see Nikaido, 2009). Overexpression of two ABC transporters (CDR1 and CDR2) or a MFS transporter was observed in multidrug resistance (MDR) phenotypes of *Candida* spp. isolated from human patients treated with fluconazole fungicide (Morschhauser et al., 2007). Similarly, MDR field isolates of *Botrytis cinerea* showed overexpression of efflux pumps encoding genes (Kretschmer et al., 2009). They found mutations in the transcription factor of the ABC transporter AtrB gene or a rearrangement in the promoter region of the MFS transporter mfsM2 gene in MDR isolates with increased efflux activity. Recently, overexpression of the MgMFS1 gene in a field isolate of *Z. tritici* was associated with resistance to fungicides with different mode of action (Omrane et al., 2015).

1.2.4 Degradation of fungicide by metabolic enzymes

An important group of metabolic enzymes involved in toxicant resistance are glutathione S-transferases (GST; see Li et al., 2007). The GST is a phase II metabolic enzyme able of conjugating reduced glutathione to the electrophilic centres of exogenous or endogenous toxic compounds, resulting in detoxification (see Salinas and Wong, 1999). Shin et al. (2003) suggested that glutathione content and GST activity may be important factors in resistance to chlorothalonil in *Saccharomyces cerevisiae*. Additionally, fungal cytochrome P450 monooxigenases are enzymes involved in primary and secondary metabolism pathways (Brase et al., 2009) and able to detoxify a range of xenobiotics, including phenolic plant defence compounds (Lah et al., 2011).

1.3 Septoria leaf blotch management and fungicide resistance

Despite the efforts of wheat breeding programmes, disease management of SLB in Northern Europe and the UK is largely achieved by fungicide applications. However, field populations of *Z. tritici* have developed resistance to most of the systemic single-site fungicide used for its control, including methyl benzimidazole carbamates (MBC), quinone outside inhibitors (QoI) and sterol-demethylation inhibitors (DMI).

1.3.1 Methyl benzimidazole carbamate fungicides

Resistance to MBC fungicides in UK *Z. tritici* field isolates was reported in 1984 (Griffin and Fisher, 1985). Similarly, Leroux et al. (2007) reported shifts in sensitivity to MBC fungicides in *Z. tritici* field isolates collected in France between 1988 and 2005. Resistance to MBCs is associated with a mutation in the gene encoding β-tubulin, leading to an amino acid substitution from glutamic acid to alanine at codon 198 (E198A) of the target protein (Fraaije et al., 2005). Evolution of resistance to MBC fungicides was marked by a sudden shift in sensitivity in *Z. tritici* field populations. Lucas and Fraaije (2008), using ancient archived SLB infected straw samples of wheat from the long-term experiment “Broadbalk” at Rothamsted and an allele-specific PCR assay, detected a rapid increase in frequency of *Z. tritici* MBC-resistant variants (E198A) within populations between 1984 and 1985. They suggested that the E198A variant was present in field populations at low frequencies before 1985, and applications of MBC fungicides in the Broadbalk plots increased its frequency dramatically in 1985. Currently, *Z. tritici* strains within UK field populations mainly carry

the A198 allele without any apparent penalty in fitness, even in absence of MBC fungicide selection pressure.

1.3.2 Quinone outside inhibitor fungicides

After the development of MBC resistance, control of SLB was mainly achieved through the use of azoles and, later, QoI fungicide applications. The QoI fungicides inhibit complex III, cytochrome bc1 (ubiquinol oxidase) at the Qo site (quinone oxidising pocket) affecting the mitochondrial respiration chain (Becker et al., 1981). After the introduction of the QoIs in the crop protection market in the UK in 1997, shifts in sensitivity were detected in *Z. tritici* field populations sampled in 2002 (Fraaije et al., 2003). Resistance to QoIs is caused by a point mutation in the cytochrome *b* target encoding gene, leading to the substitution of glycine to alanine at codon 143 (G143A) (Fraaije et al., 2003). Another substitution from phenylalanine to leucine at codon 129 (F129L) was also reported in a strain originating from Ireland but only confers low levels of resistance to QoIs (Fraaije et al., 2003). Once resistance to QoIs was detected in field populations, the frequency of *Z. tritici* strains carrying the G143A mutation increased rapidly, outcompeting "wild-type" and F129L isolates under QoI fungicide selection pressure. Fraaije et al. (2005) detected an increase from 35 to 90 % of isolates carrying mutation G143A after just two field applications of QoIs in the same season. To date, *Z. tritici* G143A variants are distributed in many regions of wheat production around the world with no apparent fitness penalty (see Lucas et al., 2015). Torriani et al. (2009) suggested that resistance to QoIs arose through mutation G143A independently on at least in four occasions in Europe. Once resistance arose in *Z. tritici* populations, further long distance spread of resistance occurred through ascospores blown by wind mainly from west to east.

Alternative respiration can also confer low levels of insensitivity to QoIs. Ziogas et al. (1997) reported reduced sensitivity to azoxystrobin in *Z. tritici* laboratory mutants. Lower sensitivity to QoIs was associated with increased activity of the alternative oxidase (AOX). However, this alternative resistance mechanism does not have a significant impact *in planta*. As a result of QoI resistance development in *Z. tritici* field populations, the control of SLB disease now relies heavily on the use of DMI fungicides, especially azoles, and the recently introduced new generation of succinate dehydrogenase inhibitors.

1.3.3 Azole fungicides

Azoles fungicides have been a key component in SLB management since the 1980s. Although variation in sensitivities against azole fungicides was initially observed in *Z. tritici* field populations, no shifts in sensitivity were detected *in vitro* (Gisi et al., 1997). Eventually, a subtle reduction in sensitivity – approximately a resistance factor of 10 – to the triazole fluquinconazole was observed in *Z. tritici* field populations collected between 1993 and 2005 (Mavroeidi and Shaw, 2005). Cools et al. (2005) also reported reduced sensitivity – up to 40-fold less sensitivity – to epoxiconazole and flusilazole in *Z. tritici* field isolates sampled in the UK in 2003. Finally, Clark (2006) reported shifts in field efficacy and/or resistance to some older azole fungicides (e.g. tebuconazole, cyproconazole or propiconazole) after fungicide treatments.

Resistance to some DMI – azole – fungicides has developed gradually. Azole fungicides bind to the sterol 14α-demethylase (CYP51) enzyme which catalyses the oxidative removal of the 14α-methyl group from the sterol core (Lamb et al., 1998). Inhibition of the CYP51 enzyme blocks ergosterol production and can cause accumulation of toxic 14α-methylated sterols which lead to pathogen growth inhibition and cell death. The reduced effectiveness of azole fungicides has been mainly linked to accumulation of different amino acid alterations – substitutions (e.g. D134G, V136A/C/G, Y137F, A379G, I381V, Y459D/S/N, G460D, Y461H/S and S524T) and a 6 bp deletion (DEL) resulting in the removal of two amino acids (codons 459 and 460) – and promoter changes in the target encoding gene, sterol 14α-demethylase (see Cools and Fraaije, 2013). The impact of most target site changes on azole binding was confirmed by CYP51 protein modelling (Mullins et al., 2011) and yeast complementation studies (Cools et al., 2010). Azole docking studies with the CYP51 model of *Z. tritici* confirmed the role of Y137F in triadimenol resistance, whereas variants with I381V and V136A are generally less sensitive to tebuconazole and prochloraz. Additionally, the deletion of codons 459 and 460 confers resistance to tebuconazole and epoxiconazole but sensitivity to prochloraz when variants also have mutations A379G and I381V. Another resistance mechanism based on CYP51 overexpression, due to a 120 bp insertion in the predicted *MgCYP51* promoter has also recently been reported (Cools et al., 2012). Additionally, laboratory studies using heterologous expression of *Z. tritici* genes encoding efflux pumps (e.g. MgArt1, MgArt5 or Mgmfs1) in yeast showed that these genes can confer protection against a range of unrelated natural and synthetic toxic compounds, including azole and QoI fungicides (Roohparvar et al., 2007a; Roohparvar et al., 2007b; Zwiers et al., 2002).

1.3.4 Succinate dehydrogenase inhibitor fungicides

Carboxamide fungicides inhibit the succinate dehydrogenase (SDH) enzyme (Georgopoulos et al., 1972). The SDH enzyme (complex II) transfers electrons from succinate to the ubiquinone pool as part of the mitochondrial respiration chain (Saraste, 1999). SDH is a membrane-bound enzyme that consists of a flavoprotein (SDHA) and an iron-sulphur protein (SDHB), which are anchored to the inner membrane of the mitochondria by two hydrophobic sub-units (SDHC and SDHD) (Horsefield et al., 2006). The fungicide carboxin was the first SDH inhibitor (SDHI) fungicide launched on to the market. It was sold to control basidiomycete pathogens (Schmeling and Kulka, 1966; Snel et al., 1970). Modifications of the carboxin molecule led later to the development of diverse groups of SDHIs that were able to control a number of ascomycete fungi (see Glattli et al., 2010). Boscalid – introduced in the UK in 2005 – was the first of these "new" generation of carboxamides, with fungicidal activity against a wide range of plant pathogens affecting fruits, vegetables, canola and cereals (Stammler et al., 2008). Other members, including penthiopyrad, bixafen, isopyrazam and fluxapyroxad, were introduced later and provided excellent control of SLB (HGCA, 2013, 2014). However, laboratory studies have shown a high risk of resistance development against SDHIs in *Z. tritici*. Skinner et al. (1998) reported that resistance to carboxin in UV exposed mutants was linked to different mutations in the SDH target protein. Resistance to carboxin was conferred by a substitution from histidine to leucine or tyrosine at codon 267 (H267L/Y) in the *SDH* gene encoding sub-unit B (*SDHB*). Other amino acid substitutions in *SDHB* (e.g. S221P/T, N225H/I, R265P and I269V/P), *SDHC* (e.g. T79I, S83G, L85P, N86K and H152R), and *SDHD* (e.g. D129E/G/S/T) have been reported to confer different levels of sensitivity to a range of SDHIs in *Z. tritici* laboratory mutants (Fraaije et al., 2012; Scalliet et al., 2012). Field monitoring of *Z. tritici* populations detected two isolates carrying amino acid substitutions in the SDH target protein (FRAC, 2013b). One isolate carrying an amino acid substitution from threonine to asparagine at codon 79 (T79N) in the *SDHC* encoding gene was found in France in 2012. The other isolate, carrying an amino acid substitution from tryptophan to serine at codon 80 (W80S) in SDHC encoding gene, was found in the UK in the same season. *In vitro* sensitivity assays indicated that both variants had low levels of insensitivity to SDHIs (FRAC, 2013b). The new generation of carboxamides are always applied in mixtures with other fungicides with different modes of action in UK cereal crop, which should reduce the chance for resistance emerging and slow down spread of resistance (FRAC, 2013b; HGCA, 2014).

1.3.5 Multi-site inhibitor fungicides

Chlorothalonil and folpet are protective multi-site inhibitors with low risk for fungicide resistance development (FRAC, 2014). However, multi-site inhibitors offer lower disease control in field applications. Applications of folpet in a protectant situation at full label recommendation were able to reduce SLB symptoms from 25 to 17 %, whereas systemic single-site fungicides reduced disease levels from 25 to 5 % disease severity (HGCA, 2013). Although multi-site fungicides provide relatively poor SLB disease control, they are believed to reduce or delay fungicide resistance development to single-site inhibitors in *Z. tritici* when used in mixtures (HGCA, 2014).

Chlorothalonil and folpet interact with thiol-containing proteins in the cell. It has been suggested that chlorothalonil exerts its fungicidal activity through inactivation of sulfhydryl groups of thiol-containing proteins (e.g. cysteine, glutathione or CoA) leading to depletion in the cell (Vincent and Sisler, 1968). Vincent and Sisler (1968) also suggested that chlorothalonil might be able to inhibit thiol-containing enzymes involved in vital cellular process such glycolysis or respiration. Similarly, folpet is able to react with thiol-containing (e.g. glyceraldehyde 3-phosphate dehydrogenase; Siegel (1971b)) and non-thiol-containing proteins (i.e. lysozyme; Siegel, 1971a).

Lower sensitivity to multi-site inhibitors has been reported in field isolates of plant pathogens. Holm et al. (2003) and more recently Fairchild et al. (2013) reported field isolates of *Alternaria solani* less sensitive to chlorothalonil fungicide. Similarly, less sensitive field isolates of *Botrytis cinerea* to folpet were reported after continuous applications of carboxamides and folpet in vineyards (Fourie and Holz, 2001). However, no genomic changes underlying lower sensitivity to multi-site inhibitors in field populations of plant pathogens have been reported. It has been suggested that resistance mechanisms such as metabolisation of fungicide or increased efflux pump activity might underlie lower sensitivity to multi-site inhibitors (Sisler, 1988). Fungicide metabolism through glutathione and glutathione S-transferase activity has been suggested to confer resistance to chlorothalonil in yeast (Shin et al., 2003). Resistance to diverse single-site fungicides with distinct modes of action in *B. cinerea* was conferred through alterations in the promoter region of genes encoding an ATP binding cassette (ABC) transporter or a major facilitator superfamily (MFS; Kretschmer et al., 2009). Leroux and Walker (2011) suggested, based on cross resistance studies, that overexpression of genes encoding ABC or MFS efflux pumps might confer resistance to a range of unrelated fungicides. Laboratory studies have reported that efflux via ABC (Zwiers et al., 2003; Zwiers et al., 2002) or MFS (Roohparvar et al., 2007a) transporters can protect *Z. tritici* against diverse natural metabolites and single-site fungicides.

However, to date, fungicide metabolism has not been associated with fungicide resistance in *Z. tritici* field populations.

1.4 Genomics in fungicide research

As a model microorganism, *Zymoseptoria tritic*i has been subjected to a range of genetic studies. The reference *Z. tritici* isolate IPO323, originating from the Netherlands (Kema and van Silfhout, 1997) was sequenced by Goodwin et al. (2011). The entire genome comprises approximately 40 Mb. It is organised as 21 chromosomes ranging from approximately 0.4 to 6 Mb in size. Karyotyping studies (Mehrabi et al., 2007), and sexual crosses and genetic analysis of the progeny (Wittenberg et al., 2009) found a large variation in chromosome numbers in field isolates, including the reference isolate IPO323. These studies reported 13 core and up to eight dispensable chromosomes (Fig. 1.6). The core or essential chromosomes are always present in all field isolates and progeny; whereas the dispensable chromosomes can be absent in field isolates and recombinants. The dispensable chromosomes range in size from 0.3 to 0.7 Mb and represent approximately 12 % of the genome size. They contain repetitive genomic DNA with approximately 650 genes, most of them with unknown function (Goodwin et al., 2011; Mehrabi et al., 2007). Currently, the function of these dispensable chromosomes in the lifestyle of the Z. tritici is unknown. However, it has been suggested that they may facilitate the evolutionary process of the fungus (Croll and McDonald, 2012; Croll et al., 2013). Stukenbrock et al. (2010) suggested that dispensable chromosomes may be involved in host specialisation in *Z. tritici*. Kellner et al. (2014) looking at genome-wide expression profiles of *Z. tritici* under axenic culture or plant infection, observed that genes residing on the dispensable chromosomes were expressed at a relative low level compared with genes on the essential chromosomes. Similar results were reported by Rudd et al. (2015). They observed a low expression of genes residing on the dispensable chromosomes compared to those genes on the essential chromosomes. This suggests that genes residing on the dispensable chromosomes may not play a large role in the pathogen's life cycle in host tissue. Lopez-Leon et al. (1994) reported that the supernumerary, dispensable or B chromosome – in the grasshopper *Eyprepocnemis plorans* – contains mainly a 180-bp tandem repeat and ribosomal DNA. Genetic studies indicated no recombination of the dispensable chromosomes, suggesting that distributive disjunction – segregation in the absence of homologous recombination – may drive the meiotic process of these chromosomes in *Z. tritici* (Wittenberg et al., 2009). Thus, dispensable chromosomes can be lost with no apparent penalty in fitness (Mehrabi et al., 2007; Wittenberg et al., 2009). Dispensable chromosomes have also

been reported in other fungal plant pathogens. Miao et al. (1991), using electrophoretic karyotype mapping identified a gene (*pda6*) in a dispensable chromosome of *Nectria haematococca* conferring protection against the phytoalexin pisatin. In addition to *pda6*, Han et al. (2001) identified other genes in the same dispensable chromosome of *N. heamatococca* that were expressed during the infection process of pea tissue. Shaw and Hewitt (1991) based on genetic studies in *Myrmeleotettix maculatus* (Shaw, 1984; Shaw and Hewitt, 1985) suggested that dispensable or B chromosomes might not have a function.

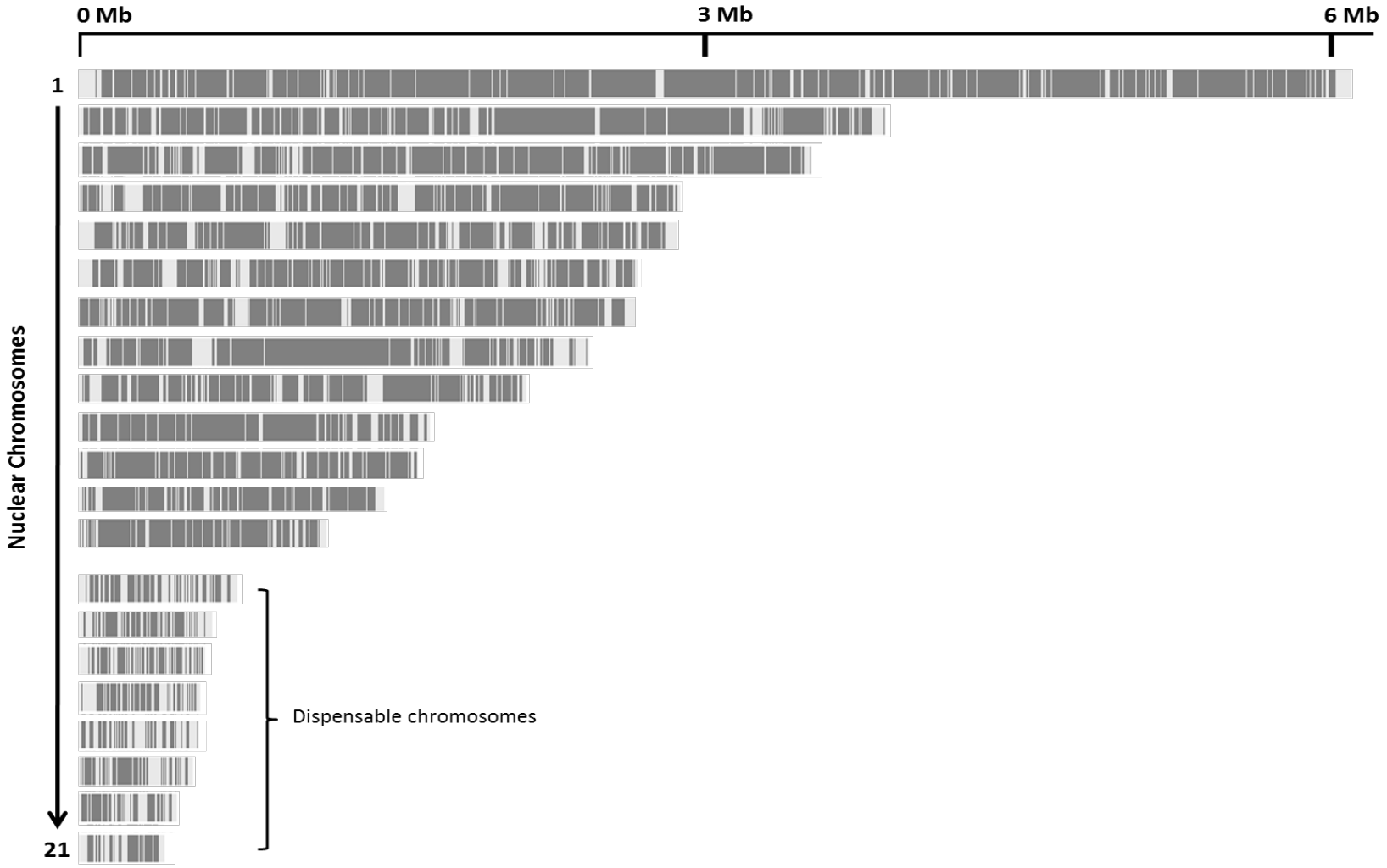

Figure 1.6 Representation of the 10,933 predicted genes and their distribution on the chromosomes of *Zymoseptoria tritici*. Location of genes is indicated by dark grey lines on each chromosome denoted in light grey.

1.4.1 Genome-wide transcriptomic studies using microarray technology

The development of cDNA microarray technology allowed us to assess the transcriptional response associated with toxicant exposure (Amin et al., 2002). Moreover, classification of differentially expressed sequences or genes into functional categories can provide insights into the putative metabolic pathways involved in the response to a given stress (Mewes et al., 1997). Jia et al. (2000) using an array comprising 1,529 open reading frames (ORFs) of *Saccharomyces cerevisiae* genes determined the transcriptional response of yeast after exposure to sulfometuron methyl (SM) – an inhibitor of amino acid biosynthesis. This array allowed detection of many altered metabolic pathways in yeast exposed to SM, such as amino acid and nucleotide biosynthesis, carbohydrate metabolism, sulphur assimilation and stress responses. Additionally, a link between SM exposure and impaired ergosterol metabolism was revealed in yeast (Jia et al., 2000). Caba et al. (2005) using an Affymetrix probe array were able to identify gene expression signatures associated with genotoxic or cytotoxic stress in yeast. For example, genes encoding the glucosidase SUN4 – involved in cell wall organisation and biogenesis – or the IMP cyclohydrolase ADE17 were expressed in yeast only in response to bleomycin and cisplatin (Caba et al., 2005). Similarly, Liu et al. (2005), using microarray technology determined antifungal-specific changes in gene expression in *Candida albicans* after exposure to ketoconazole, amphotericin B, caspofungin or flucytosine. Genes involved in lipid, fatty acid and sterol metabolism were up-regulated in cells exposed to ketoconazole – an ergosterol inhibitor. Amphotericin B – another type of ergosterol inhibitor – induced expression of genes involved in transport of small molecules, and general cell stress. Caspofungin induced overexpression of genes encoding cell wall maintenance proteins, including the target protein β-1,3-glucan synthase. Exposure to flucytosine – a thymidylate synthase inhibitor – induced expression of genes involved in protein synthesis, purine and pyrimidine biosynthesis. Becher et al. (2011) determined the transcriptional response of *Fusarium graminearum* to tebuconazole. Genes involved in the ergosterol biosynthesis pathway were overexpressed in response to tebuconazole, in agreement with the mode of action of azole fungicides, as well as twelve ABC transporter encoding genes. Likewise, Cools *et al*. (2007), using *Z. tritici,* reported differential expression of genes involved in ergosterol biosynthesis, mitochondrial respiration and cell transport mechanisms upon exposure to an azole fungicide. They also reported overexpression of a vacuolar glutathione S-conjugated ABC transporter encoding gene (EST: *mga1012f*) in resistant and sensitive *Z. tritici* after epoxiconazole exposure, but expression of this gene did not appear to be linked to azole resistance. Genome-wide transcriptomic studies have also revealed expression of genes encoding mechanism conferring protection against toxicants. Cowen et al. (2002b) determined that resistance to the DMI

fungicide fluconazole in laboratory populations of *C. albicans* grown over 300 generations in the presence of the fungicide was conferred by overexpression of the efflux pumps ABC CDR2 or the MFS MDR1. Similarly, resistance to fluconazole through overexpression of the ABC transporter CaCDR1 in resistant-strains *C. albicans* obtained from patients (Marr et al., 1998) was confirmed by a cDNA microarray containing approximately 3,000 unigenes of the fungus (Xu et al., 2006). They also reported overexpression of other genes encoding ABC transporters (i.e. IPF7530, CaYOR1 and CaPXA1), oxidative stress response (i.e. IPF10565, CaALD5, CaGRP1, CaSOD2 and CaCTA1), and copper transport (i.e. CaCRD2) after fluconazole exposure in fluconazole-resistant *C. albicans* strains. Rogers and Barker (2002) also found differentially expressed genes in association with azole resistance in *C. albicans*. These genes were implicated to play a role in amino acid and carbohydrate metabolism; cell stress, cell wall maintenance; lipid, fatty acid and sterol metabolism; and small molecule transport. Liu et al. (2005) observed overexpression of the ABC transporter *cdr1* in a sensitive *C. albicans* strain after ketoconazole, flucytosine, amphotericin B or caspofungin exposure. Interestingly, ketoconazole also induced overexpression of the ABC transporter *cdr2* gene.

1.4.2 Genome-wide transcriptomic studies through RNA sequencing technology

Advances in DNA sequencing technologies – high-throughput sequencing, also called next-generation sequencing (NGS) – have allowed to determine gene expression levels through a sequencing-based approach (Mardis, 2008; Schuster, 2008). Analysis of RNA by sequencing cDNA – also called RNA sequencing – provides a more sensitive method than microarray technology to characterise the transcriptional response of diverse organisms, tissues or cells under contrasting conditions (Wang et al., 2009). RNA sequencing technology has proved to be a reliable method to measure changes in gene expression (Marioni et al., 2008), discover novel transcribed regions (Bruno et al., 2010; Emrich et al., 2007) and detect mechanisms conferring protection against toxicants (Wacker et al., 2012). Sun et al. (2013) using RNA sequencing analysis reported reduced expression of genes encoding ABC transporters (i.e. CDR1 and CDR2) in the *C. albicans* laboratory mutant goa1Δ and ndh51Δ after fluconazole exposure. The gene *Goa1p* is required for electron transport in the respiratory chain complex I (Bambach et al., 2009), and Hdh51 encodes the 51-kDa sub-unit of the NADH dehydrogenase in complex I (McDonough et al., 2002). It was suggested that disruption of the electron transport chain at complex I in mutants of *C. albicans* caused the down-regulation of the genes *cdr1* and *cdr2* (Sun et al., 2013) which are associated with resistance to azole fungicides (Cowen et al., 2002b; Marr et al., 1998). Interestingly, mutant

ndh51Δ showed down-regulation of genes involved in the ergosterol metabolic pathway which is affected by azole fungicides (Sun et al., 2013). Wacker et al. (2012) were able to determine single nucleotide polymorphisms (SNPs) in target genes (i.e. *plk1* and *psmb5*), and detoxification mechanisms (i.e. ABC transporters) conferring resistance to drugs (i.e. BI 2536 and bortezomib) in less sensitive laboratory mutant human cell lines through RNA sequencing.

Studying whole-genome transcriptional responses to toxicants in different microorganisms has revealed reliable toxicant-specific responses, as predicted from their mode of action in some cases where this is known. In addition, transcriptional responses have also shown other metabolic pathways that may be affected in the target microorganisms. Furthermore, this knowledge has enabled identification of mechanisms conferring protection against toxicant compounds. However, to date, studies of the transcriptional responses of plant pathogens to fungicides through RNA sequencing are scarce. The coincidence of completion of *Z. tritici* genome sequence, availability of high-throughput sequencing techniques, and improved computational biology skills provide new opportunities to study the transcriptional response of *Z. tritici* to fungicides used in the control of SLB as outlined in this thesis.

1.5 Evolutionary studies of fungicide resistance

Fungal human (Anderson, 2005) or plant (Lucas et al., 2015) pathogen populations frequently evolve resistance to fungicides due to selection pressure imposed by continuous use of antifungal compounds. Development of fungicide resistance in plant pathogen populations, including *Z. tritici*, remains a major concern in agriculture (Lucas et al., 2015). As described in section 1.2, due to development of resistance to fungicides, the management of this fungal diseases relies on continuous substitution of fungicides with distinct mode of action (Van den Bosch et al., 2011). The main and long-term concern is the limited number of fungicides available to current and future management crop diseases.

Studies of the evolution of fungicide resistance in plant pathogens populations are relatively rare. Typically, studies of the dynamics of fungicide resistance in plant pathogens populations, including *Z. tritici*, are based on field experiments (see Van den Bosch et al., 2011) or mathematical model approaches (see van den Bosch and Gilligan, 2008). Field studies have looked at the evolution of resistance after resistant strains of *Z. tritici* are present in field populations and how fungicide usage drives the further spread of resistance (Fraaije et al., 2006; Mavroeidi and Shaw, 2006; Metcalfe et al., 2000). Mathematical models have tried to infer how fungicide usage

drives the dynamics of evolution (see van den Bosch and Gilligan, 2008). Understanding the evolution of resistance may aid long-term management of fungicide resistance (Anderson, 2005). Thus, the evolutionary processes that may determine the fate of resistance in fungal plant pathogen populations, such the emergence of resistance mechanisms, their impact on pathogen fitness, and interaction with other resistance mechanisms need to be studied in more detail.

Experimental evolution is an approach to study evolutionary processes in model microbial populations in response to determined conditions or threats (Kawecki et al., 2012). Selection exerted during evolutionary experiments can act on any or all traits of the microorganism, including genes related with fitness associated to the selection regimen (Lang et al., 2013). A long-term experiment with *Escherichia coli* conducted over 60,000 generations since 1988 was undertaken to study the dynamics of adaptation and divergence in 12 replicate populations (Lenski et al., 1991). Detailed studies on these 12 replicate populations have found links between phenotypic and genetic evolution (Barrick et al., 2009) and correlated responses to new threats (Travisano and Lenski, 1996). Cooper and Lenski (2000) reported the role of antagonistic pleiotropy and mutation accumulation in specialisation to new environments after 20,000 generations in the *E. coli*-derived populations. Moreover, other studies on these 12 replicated populations reported an increase in mutation rate (Sniegowski et al., 1997), forces maintaining genetic variability (Barrick and Lenski, 2009) and the role of epistasis in adaptation (Khan et al., 2011). In addition, it was possible to study the influence of historical contingency on new morphological (Travisano et al., 1995) and physiological (Blount et al., 2008) traits.

In vivo or *in vitro* studies have provided evidence for the evolution of fungicide resistance in fungal human pathogens or other model microorganisms. White (1997) provided a well-documented example of evolution of fungicide resistance in a population of *C. albicans* obtained from a single patient. Over a period of two years, 17 *C. albicans* strains were isolated from a single patient undergoing fluconazole treatment in that study. Resistance to fluconazole developed gradually through accumulation of distinct resistance mechanisms (i.e. overexpression of the genes encoding a major facilitator superfamily efflux pump, lanosterol 14 α-demethylase target and ABC transporters) in an evolutionary lineage of *C. albicans*. Similar resistance mechanisms were found in replicate populations *of C. albicans* after more than 300 generations at increasing concentrations of fluconazole *in vitro* (Cowen et al., 2002a). They found that resistance to fluconazole was conferred by overexpression of genes encoding ABC or MFS efflux pumps. Similarly, Anderson et al. (2003) determined the evolution of resistance to fluconazole in replicate populations of *S. cerevisiae* for 400 generations at increasing concentrations of the fungicide.

After 100 generations, some populations developed resistance to fluconazole through overexpression of two distinct genes encoding ABC transporters. Then, after 400 generations some population accumulated an additional mutation in an unidentified gene (*unk1*) which seems to be linked to improved fitness at the highest concentration of fluconazole tested (Anderson et al., 2003). Therefore, experimental evolution studies can provide insights into how fungi adapt to new environments.

1.6 Project aims

The evolution of fungicide resistance in *Z. tritici* remains as constant threat to wheat production in Northern Europe and the UK. Due to resistance development to the MBC, QoI, and some DMIs and recent detection of less sensitive field strains to SDHI fungicides in *Z. tritici* field populations, chemical options for the management of SLB are becoming even more limited. Multi-site inhibitors such chlorothalonil or folpet are also included in programmes for the chemical control of SLB to reduce or delay further fungicide resistance development. However, multi-site inhibitors offer lower disease control than single-site fungicides, particularly when *Z. tritici* hyphae have penetrated leaves. Identification of the biological potential for development of resistance mechanisms (i.e. target- or non-target-site) could inform new anti-resistance strategies. Therefore, the overall aim of this study was to investigate how *Z. tritici* is able to adapt to multi- and single-site fungicides.

The following objectives were addressed:

1. To determine the baseline sensitivities to chlorothalonil, folpet and fluxapyroxad in recent *Z. tritici* field isolates (Chapter 3).
2. To characterise the genome-wide transcriptional response of *Z. tritici* after exposure to chlorothalonil or folpet using RNA sequencing technology (Chapter 4).
3. To determine the course of evolution of resistance *in vitro* to the single-site fungicide fluxapyroxad in replicate populations of *Z. tritici* (Chapter 5).

Chapter 2: General Materials and Methods

2.1. Media, sterilisation and antibiotics

Solid culture medium Yeast extract Peptone and Dextrose agar (YPD) was purchased from ForMedium™ (Norwich, UK), and Sabouraud Dextrose broth (SDB) from Oxoid (Basingstoke, UK). All media were prepared according to the manufacturer's instructions and autoclaved at 121 °C for 15 minutes. When required, the antibiotics penicillin G sodium and streptomycin sulphate (Sigma-Aldrich, Gillingham, UK) were added at a concentration of 100 µg/mL after cooling down the media below 50 ^{o}C.

2.2. Isolation, maintenance and storage of *Z. tritici* from wheat leaves

Wheat leaves of cultivar Consort with Septoria leaf blotch (SLB) symptoms were sampled from untreated plots and plots treated with fungicide at T1 (i.e. first and second node detectable) and T2 (i.e. flag leaf blade visible Tottman et al., 1979) from a field trial at Rothamsted in 2012 (2012/R/WW/1208, Long Hoos 5). Symptoms of SLB in 2012 accounted for an average of 5 and 9.6 % of the flag leaf and leaf 2, respectively (CropMonitor, 2012). Ten leaves, representative for Leaf 3, were collected from each plot after no treatments or after two applications of chlorothalonil (Bravo 500 1.0 l/ha) or folpet (Phoenix 1.5 l/ha). (Fig. 2.1). SLB lesions were cut from the sampled leaves, attached to Whatman filter paper with staples and incubated under damp conditions (filter paper wetted by adding 500 µL of sterile distilled water (SDW)) in petri dishes sealed with parafilm. Incubation took place overnight at 20 °C in the dark to induce cirrhi production from pycnidia. Cirrhi from single pycnidia were collected using watchmaker's forceps, dispersed in 30 µL of SDW and plated out onto antibiotica amended YPD plates. Single spore strains of typical *Z. tritici* morphology (yeast like small pink/brown colonies) were sub-cultured by transferring single colonies to new YPD plates. Spores were retrieved with a loop after seven days incubation at 15 °C in the dark and stored in 80 % glycerol at – 80 °C as a stock for further studies. Approximately 18 isolates per plot were obtained in total. Yeast extract peptone and dextrose agar was used to grow *Z. tritici* because the media induces better yeast-like growth. This makes easier to harvest and recover *Z. tritici* spores (Bart Fraaije 2015, personal communication).

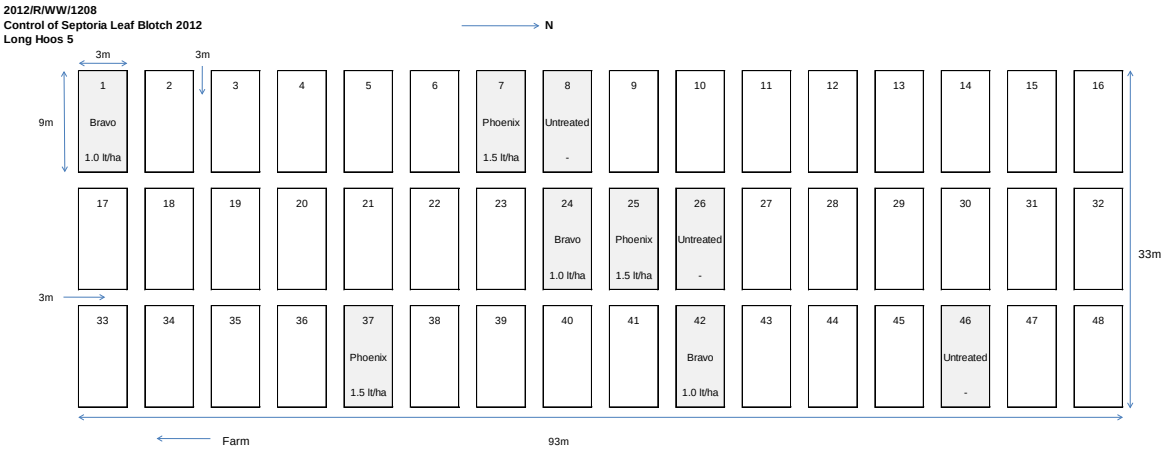

Figure 2.1 Experimental design of fungicide field trial at Rothamsted. Samples of *Zymoseptoria tritici* were collected from untreated plots (plots 8, 26 and 46), and plots treated twice with chlorothalonil (Bravo 500, 40.4 % i.a. Zeneca; treated plots 1, 24 and 42) or folpet (Phoenix 89.5 % i.a. ADAMA; treated plots 7, 25 and 37).

2.3. Production of conidia suspensions

Spores of *Z. tritici* isolates from 80 % glycerol stock suspension were plated out onto YPD plates for seven days at 15 °C in the dark, harvested with a loop and suspended in SDW. Spore concentration was determined under a microscope using a haemocytometer (Webber Scientific International Middlesex, UK).

2.4. Standard growth curves in liquid media

Spores from seven-day-old cultures of *Z. tritici* grown on YPD plates were harvested and suspended in 100 mL of fungicide amended SDB or SDB liquid media at a final concentration of 5×10^5 spore/mL. Cultures were incubated at 21 °C in the dark on an orbital shaker at 200 rpm for 96 hours. Mycelium growth was determined by measuring optical density at 600 nm in a spectrophotometer (BioPhotometer: Eppendorf, Hamburg, Germany) every 24 h according to the manufacturer's instructions. Fungal growth phases (lag, log and stationary) were identified by plotting the optical density against time.

2.5. Fungicide *in vitro* sensitivity assays using microtitre plates

Spore suspensions of *Z. tritici* strains, grown on YPD plates for seven days at 15 °C in the dark, were harvested and adjusted at 2.5×10^4 spore/mL concentration in SDW after counting spore suspensions under a microscope. Aliquots of 100 µL spore suspension were added to 100 µL double strength SDB amended with increasing fungicide concentrations in clear, flat-bottomed, 96 well cell culture plates (Greiner Bio-One, Frickenhausen, Germany) (Fig. 2.2). Plates were incubated at 21 °C in the dark for 96 hours, and growth measured using absorbance readings at 630 nm (A_{630nm}) with a FLUOstar OPTIMA microplate reader (BMG Labtech GmbH, Offenberg, Germany). Absorbance was measured in a well-scanning mode with a 2×2 matrix of scanning points set at 3 mm diameter. Fungicide sensitivity was determined as the concentration which inhibited growth by 50% (EC_{50} in µg/mL). The EC_{50} values were calculated with the OPTIMA software v2.20OR2 which fits a dose-response curve.

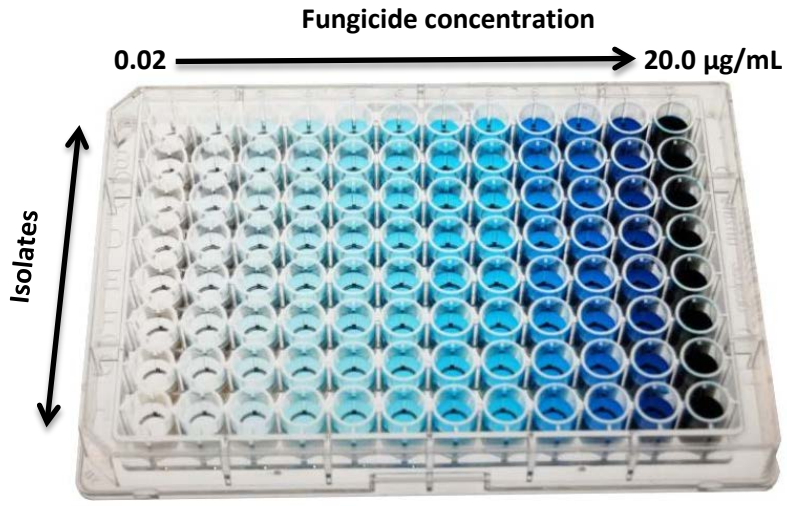

Figure 2.2 *In vitro* microtitre plate sensitivity testing. Every row corresponds to a single *Z. tritici* isolate and column to a specific fungicide concentration (e.g. chlorothalonil or folpet fungicide concentration shown in the figure). Wells were filled up with 100 µL of fungicide-amended Sabouraud dextrose liquid medium (SDB) and 100 µL of spore suspension ($2.5X10^4$ spore/mL). The first column was filled up with SDB as a control with no added fungicide.

2.6. Total RNA extraction and quantification

The referenced and sequenced *Z. tritici* isolate IPO323 (Goodwin et al., 2011; Kema and van Silfhout, 1997) was exposed to chlorothalonil or folpet in the lag or log phase growth. Spores of the IPO323 were harvested from seven-day-old cultures grown on YPD plates at 15 °C in the dark. For treatments in the lag phase growth, *Z.tritici* spores (5×10^5 spore/mL) were added to flasks

containing 100 mL of SDB amended with chlorothalonil or folpet at 0.1 or 0.5 µg/mL final concentrations, respectively. After 24 h growth at 21 °C in the dark at 200 r.p.m, mycelia were harvested by vacuum filtration through 8 µm pore nitrocellulose filters (Millipore, Ireland) and snap frozen in liquid nitrogen. For treatments applied in the log phase of growth, IPO323 spores were suspended in 100 mL of SDB at a final concentration of 5×10^5 spore/mL. After 24 h incubation at 21 °C in the dark at 200 r.p.m, cultures were adjusted to 0.1 µg/mL of chlorothalonil or 0.5 µg/mL of folpet (Fig. 4.1B). Mycelia were harvested after further 24 h growth in the same conditions by vacuum filtration and snap frozen. IPO323 cultures grown for 24 h or 48 h in the absence of fungicide were used as untreated controls for treatments applied in the lag or log phase growth, respectively. Technical degree fungicide was dissolved in acetone then diluted in SDW before adding to the SDB or inoculated-SDB

Total RNA was extracted from overnight freeze-dried samples with TRIzol reagent (Invitrogen Carlsbad, USA) according to the manufacturer's protocol with 1-bromo-3-chloropropane (Molecular Research Center, Cincinnati, USA) as the phase separating agent. RNA was purified by precipitating overnight in 4 M lithium chloride (Sigma-Aldrich, Steinheim, Germany) at -20 °C. Purified RNA was quantified with a Nanodrop spectrophotometer (Nano Technologies, Delaware, USA) according to manufacturer's instruction. Quality was determined by the ratios of absorption at 260/280 nm and 260/230 nm. Only samples with both ratios above 2.0 were used for further studies.

2.7. First strand cDNA synthesis

Ten micrograms of total purified RNA was reversed transcribed with random primers using the High-Capacity cDNA reverse transcription kit (Applied Biosystem, California, USA) according to the manufacturer's protocol. Products of this reaction were diluted one in ten and stored at -20 °C for further use as a template in quantitative real-time PCR.

2.8. Oligonucleotide primers design

Primers for measuring gene expression in quantitative real-time PCR were designed in Geneious R6 v6.1.4 (Biomatters Ltd., Auckland, New Zealand) using Primer3 tool (Untergasser et al., 2012) and custom synthesised by Sigma-Aldrich (Haverhill, UK). Primer design parameters were set at: length 20 – 24 bp; melting temperature (Tm) 58 – 62 °C; product size 80 – 150 bp; and GC content

40 – 60 %. Only pairs of primers with similar GC content and Tm, and amplicon size around 120 bp were selected for qRT-PCR.

2.9. Quantitative real-time PCR

Gene expression was determined by quantitative real-time PCR (qRT-PCR) reactions using SYBR® Green JumpStart Taq ReadyMix (Sigma-Aldrich, Missouri, USA). Five microliters of diluted cDNA was used as template in a 20 µL reaction with 5 µM of each primer. Thermal cycling conditions were 95 °C for 2 min, 40 cycles at 95 °C for 15 seconds, 58 °C for 30 seconds and 72 °C for 40 seconds, and SYBR Green fluorescent emission data collection was carried out at each 72 °C elongation step with the default settings. Following thermocycling, melt curves were run for all primers pairs to check for dimerization. Reactions were carried out on an ABI 7500 Real Time PCR System (Applied Bioscience, California, USA) or a Stratagene Mx300P QPCR System (Agilent Technologies, USA). Relative transcript abundance (RQ) of target genes was calculated by the $2^{-[\Delta][\Delta]Ct}$ method (Pfaffl, 2001), using β-tubulin as the endogenous control and samples from untreated IPO 323 isolate as calibrator:

$$RQ = 2^{-\Delta\Delta Ct}$$

where:

ΔΔCt = ΔCt gene – ΔCt calibrator

ΔCt = Ct gene – Ct endogenous control

Ct = cycle at which threshold level of amplification is reached

2.10. Genomic DNA extraction and quantification

Seven-day-old spores of *Z. tritici* isolates grown on YPD plates at 15 °C in the dark were harvested, snap frozen and freeze dried overnight. Fungal biomass was placed into 2-mL screw top tubes with a 3.2 mm chrome steel bead (BioSpec Products Inc.) and homogenised using a FastPrep shaker (FP120, Bio101/Savant, MPBiomedicals, California, USA) at 4.5 m/s for 25 s. Samples were incubated with 900 µL of DNA extraction buffer [TEN (500mM NaCl, 400mM Tris-HCl, 50mM EDTA, pH 8.0), 2 % sodium dodecyl sulphate, 5mM Phenanthroline, 2 % polyvinylpyrrolidone K30 and 1 % β-mercaptoethanol] at 65 °C for 20 min. Then 350 µL of cold 7.5 M ammonium acetate was added to each sample. The samples were kept on ice for 20 min and centrifuged at 13200

rpm for 15 min. Nine hundred microliter of supernatant was transferred into 2-mL micro-tubes prefilled with 900 µL of isopropanol, incubated at room temperature for 15 min and centrifuged at 13200 rpm for another 15 min. Finally, the DNA pellet was washed with 400 µL of 70 % ethanol, centrifuged at 13200 rpm for 5 min, dried and suspended into 200 µL of sterile distilled water.

DNA was quantified with a Nanodrop spectrophotometer (Nano Techonologies, Delaware, USA) according to manufacturer's instruction and diluted to 20 or 100 ng/µL for further studies.

2.11. Polymerase chain reactions

PCR reactions were carried out in a Biometra T3000/T3 thermocyclers (Biometra GmbH, Göttingen, Germany) using Phusion High Fidelity DNA polymerase (Finnymes, Espoo, Finland), Easy-A High Fidelity PCR cloning enzyme (Stratagene, La Jolla, California, USA) or Red Hot DNA polymerase (ABgene, Epsom, Surrey, UK). Reactions contained 20 or 100 ng of template DNA, 200 µM of each deoxynucleotide triphosphate (dNTP), and 0.5 µM of each primer in 1× buffer reaction. For reactions using Red Hot DNA polymerase, $MgCl_2$ was added at a final concentration of 1.5 mM. Sterile distilled water was included as negative control. Amplification conditions are given in table 2.1.

PCR products were mixed with 20 % (v/v) DNA loading dye (Bioline, UK) and separated in ethidium bromide-stained 1 – 3 % (w/v) agarose gels in 1×TBE buffer (0.8 M Tris Borate, 20 mM Na_2EDTA). Five microliters of PCR product or 4 µL of 25 or 100 bp Gene ruler ladder (HyperLadder, Bioline) were loaded into gels. Gels were electrophoresed at 120 V for 30 min and exposed to 320 nm UV light in a transilluminator (Syngene, Marylan, USA) to visualise DNA fragments.

Table 2.1 DNA polymerases used in PCR reactions.

DNA polymerase	Application	Units of polymerase (50 µL reaction)	Thermocycling parameters
Phusion High Fidelity DNA polymerase	Gene amplification for sequencing the *sdh* subunits *B* and *D* encoding gene.	1.0	95 °C – 1 min 30 cycles at 95 °C – 15 s 70 °C – 30 s 72 °C – 1 min 72 °C – 5 min
Easy-A High Fidelity PCR cloning enzyme	Gene amplification for sequencing the *sdh* subunit *C* encoding gene.	2.5	95 °C – 1 min 30 cycles at 95 °C – 40 s 65 °C – 30 s 72 °C – 2.5 min 72 °C – 7 min
Red Hot DNA polymerase	Routine PCR	1.25	94 °C – 2 min 40 cycles at 94 °C – 10 s 58 °C – 30 s 72 °C – 30 s 72 °C – 4.50 min

2.12. Statistical analysis

Statistical analysis of general data was carried out in GenStat 16th edition v16.1.0.10916, Microsoft Excel 2010 using the Analysis TookPak add-ins or SigmaPlot v12.3. Specific statistical analysis techniques used for specific data sets are explained in detail in the relevant sections.

Chapter 3: Sensitivity of field populations of *Zymoseptoria tritici* to multi- and single-site inhibitors

3.1 Introduction

Management of fungal plant pathogens relies extensively on fungicide usage when other alternatives such as host resistance or cultural approaches are unable to provide adequate disease control. With current varieties and intensity of growing, fungicides are the most important tool for control of Septoria leaf blotch (SLB) (HGCA, 2014), the most important foliar disease in winter wheat in the UK since the 1980s (Bearchell et al., 2005). *Zymoseptoria tritici* (aka *Mycosphaerella graminicola* Desm.) Quaedvlieg & Crous, the causal agent of SLB, is able to overcome host resistance and can cause up to 50 % wheat yield loss in severe epidemics (Royle et al., 1986). Currently, under north European conditions, chemical control is the most reliable crop protection strategy against *Z. tritici*. However, *Z. tritici* has developed resistant to several systemic single-site fungicides with diverse modes of action including the methyl-benzimidazole carbamates (MBCs) (Griffin and Fisher, 1985), the quinone-outside inhibitors (QoIs) (Fraaije et al., 2005) and some sterol-demethylation inhibitors (DMIs) (Clark, 2006). Resistance development against MBC and QoI fungicides has been associated with selection for less sensitive strains carrying a single amino acid substitution in the target proteins. An amino acid substitution from glutamic acid to alanine at codon 198 (E198A) in the β-tubulin encoding gene, and a substitution from glycine to alanine at codon 143 (G143A) in the cytochrome *b* encoding gene have conferred high levels of resistant to benzimidazole and strobilurin/Quinone outside Inhibitor (QoI) fungicides, respectively (Fraaije et al., 2005). The mutation E198A remains at high frequency in *Z. tritici* UK field populations and therefore presumably imposes no fitness penalty in the absence of MBC fungicides. Similarly, mutation G143A can be found at high frequency in *Z. tritici* field population in the UK and Northern Europe with no apparent fitness penalty (see Lucas et al., 2015). Although, the combination of QoIs and *Z. tritici* can have a high risk of fungicide resistance development, low frequency or no evidence of mutation G143A can be found in South Europe (Siah et al., 2010) or North Africa (Boukef et al., 2012; Stammler et al., 2012). Thus, strubirulin fungicides are still used to control SLB and exerting selection pressure on *Z. tritici* populations (Sierotzki, 2015 personal communication). In contrast, resistance to other some other DMI fungicides has developed gradually; selection for less sensitive isolates has led to accumulation of different amino acid alterations and promoter changes in the target encoding gene, sterol 14α-

demethylase (see Cools and Fraaije (2013)). Although some azole fungicides still provide acceptable control of *Z. tritici* at full rate, resistance evolution in this plant pathogen remains a constant threat.

Currently, the management of SLB relies on mixtures of fungicides with different modes of action. The SLB fungicide programme relies on application of high doses of azoles in mixture with the new-generation of succinate dehydrogenase inhibitors (SDHIs) and/or the multi-site inhibitor chlorothalonil (HGCA, 2014). It has been suggested that chlorothalonil inhibits many thiol dependent reactions in fungal and yeast cells (Shin et al., 2003; Vincent and Sisler, 1968), and folpet reacts with thiol compounds in fungal cells (see Lukens (1966)). The new-generation of carboxamides affect succinate dehydrogenase (*Sdh*) of the mitochondrial respiration chain (complex II) (Fraaije et al., 2012; Scalliet et al., 2012). Using a range of carboxamide fungicides, laboratory mutational studies have shown a high risk of fungicide resistance development in *Z. tritici* (Fraaije et al., 2012; Scalliet et al., 2012; Skinner et al., 1998). In contrast, the risk of fungicide resistance development to multi-site fungicides (e.g. chlorothalonil and folpet) is considered low as they interfere directly with many cell processes (FRAC, 2014). However, multi-site inhibitors give poorer SLB disease control than systemic single-site fungicides, particularly when applied after infection of the host plant. Applications of folpet or chlorothalonil in a protectant situation at full label recommendation are able to reduce from 25 to 17 or 9 % of SLB symptoms, respectively, whereas some systemic single-site fungicides can reduce from 25 to 5 % disease severity (HGCA, 2013). Although multi-site fungicides give a relative poor SLB disease control, they aim to reduce or delay fungicide resistance development in *Z. tritici* when used in mixtures with systemic single-site inhibitors (HGCA, 2015).

Monitoring of resistance to a specific fungicide is based on comparison of the sensitivity profile of the target fungus population before market introduction (i.e. baseline sensitivity), with that after exposure to fungicides. Shifts in sensitivity can be detected by comparing sensitivity profiles to the baselines, preferably using stored reference strains to allow comparison under the same experimental conditions (Bernhard et al., 2002). Evolution of resistance in *Z. tritici* to single-site fungicides has been extensively studied, using both sensitivity baselines to compare changes in phenotypic distribution, and directly detecting resistance mechanisms with molecular techniques when genetic markers linked to fungicide resistance are available (e.g. cytochrome *b* G143A and QoI resistance). Although resistance of *Z. tritici* to single-site inhibitors is well documented, little is known about its adaptation to multi-site inhibitors. In this chapter, I present the chlorothalonil, folpet and fluxapyroxad sensitivity status using *Z. tritici* field isolates sampled from a fungicide

field trial at Rothamsted in 2012. Outcomes from this study indicate selection of *Z. tritici* for less sensitive field isolates to the multi-site inhibitors chlorothalonil (Bravo 500) and folpet (Phoenix) in a year of high disease pressure. In contrast, no shifts in sensitivity to the single-site fungicide fluxapyroxad were found in the same trial.

3.2 Materials and methods

3.2.1 Isolate collection

Isolates for this study were obtained from an annual fungicide field trial at Rothamsted (see chapter 2). This fungicide field trial aims to detect shifts in sensitivity in *Z. tritici* field populations against a range of fungicides with different modes of action. It uses a susceptible wheat variety, Consort, to promote high natural infection levels (HGCA, 2007). A total of 165 *Zymoseptoria tritici* field isolates were obtained, as described in chapter 2, section 2.2. Fifty five isolates from plots treated with formulated chlorothalonil (Bravo 500), 56 isolates from plots treated with formulated folpet (Phoenix), and 54 isolates from untreated plots were included in the *in vitro* fungicide sensitivity assays against chlorothalonil or folpet. A total of 135 isolates randomly sampling from these 165 isolates were tested against fluxapyroxad. Nine isolates did not grow enough for the sensitivity test. The reference *Z. tritici* isolate IPO323 (Goodwin et al., 2011; Kema and van Silfhout, 1997) was also included as a control in all tests.

3.2.2 *In vitro* fungicide sensitivity assays

Sensitivity tests were carried out as described in chapter 2, section 2.5. Double strength SDB medium was amended with increasing concentrations of chlorothalonil or folpet (0.019, 0.039, 0.078, 0.156, 0.313, 0.625, 1.25, 2.5, 5, 10 and 20 µg/mL) or fluxapyroxad (0.002, 0.005, 0.015, 0.046, 0.14, 0.4, 1.2, 3.7, 11.1, 33.3 and 100 µg/mL). Technical grade chlorothalonil and folpet (10 mg/mL) were dissolved in dimethylsulphoxide (DMSO) before dilution in the liquid media. Formulated fluxapyroxad as emulsifiable concentrate (62.5 g/L EC) (BASF, Ludwigshafen, Germany) was dissolved in SDW at 10 mg/mL as a stock concentration before dilution. Microtitre wells were filled with 100 µL of fungicide-amended SDB and 100 µL of spore suspension (2.5×10^4 spore/mL). After four days incubation, absorbance A_{630nm} was recorded using a FLUOstar OPTIMA microplate reader. Fungicide sensitivity was determined as the 50 % effective concentration to inhibit growth using a dose-response relationship according to OPTIMA software v2.20OR2. Results are given as EC_{50} values in microgram of fungicide per millilitre (µg/mL) to each isolate. The presented chlorothalonil and folpet EC_{50} values are the averages of four independent experiments. Fluxapyroxad sensitivity testing was only done once. For every *in vitro* sensitivity test, *Z. tritici* field isolates were re-grown from the glycerol stock suspension stored at -80 °C.

3.2.3. Statistical analysis

Calculated EC_{50} values by treatment were analysed by Kolmogorov-Smirnov two-sample test (KS-test) in GenStat v16. The KS-test looks for the greatest difference between two cumulative distributions. The test statistic is the largest distance between the distributions, D, found (at any point along the x-axis).

Pearson's correlation coefficient between log_{10}-EC_{50} values of all pairs of fungicide was estimated in SigmaPlot v12.3.

3.3 Results

3.3.1 Baseline sensitivity of *Z. tritici* field isolates to chlorothalonil

Chlorothalonil sensitivity (EC_{50}) values were determined for 165 *Z. tritici* field isolates obtained in the annual fungicide field trial at Rothamsted in 2012. On average, these '2012' isolates were less sensitive to chlorothalonil than the reference *Z. tritici* isolate IPO323, which had an average EC_{50} of 0.09 µg/mL (Table 3.1). Chlorothalonil EC_{50} values in field isolates taken from untreated plots or after treatment with chlorothalonil or folpet ranged from 0.08 to 0.23, 0.11 to 0.36 and 0.09 to 0.31 µg/mL, respectively. Isolates taken from plots treated with chlorothalonil were skewed to greater EC_{50} values compared with isolates from plots left untreated or treated with folpet (Fig. 3.1). These isolates sampled after two field applications of chlorothalonil were less sensitive to chlorothalonil (Kolmogorov-Smirnov two-sample test, D=0.5, χ^2 approximation = 21.9, 2 df, p<0.001) than isolates collected from plots left untreated (Fig. 3.3A). Similarly, isolates from plots treated with two applications of folpet tended to be less sensitive to chlorothalonil (Kolmogorov-Smirnov two-sample test, D=0.3, χ^2 approximation = 11.9, 2 df, p = 0.003) than isolates sampled from untreated plots (Fig. 3.3A).

3.3.2 Baseline sensitivity of *Z. tritici* field isolates to folpet

In general, the recent '2012' *Z. tritici* field isolates sampled at Rothamsted were less sensitive to folpet than the reference isolate IPO323, which had an average EC_{50} of 0.53 µg/mL (Table 3.1). Folpet EC_{50} values in field isolates taken from untreated plots or plots treated with chlorothalonil or folpet ranged from 0.35 to 0.87, 0.43 to 1.58 and 0.43 to 1.65 µg/mL, respectively. Although no positive skew distribution of folpet EC_{50} values was observed, the frequency of isolates with greater folpet EC_{50} values was higher in isolates taken from plots treated with two sprays of formulated chlorothalonil or folpet (Fig. 3.2; folpet: Kolmogorov-Smirnov two-sample test, D=0.6, χ^2 approximation = 40.4, 2 df, p<0.001; chlorothalonil: D=0.5, χ^2 approximation = 22.3, 2 df, p<0.001) than isolates collected from untreated plots (Fig. 3.3B).

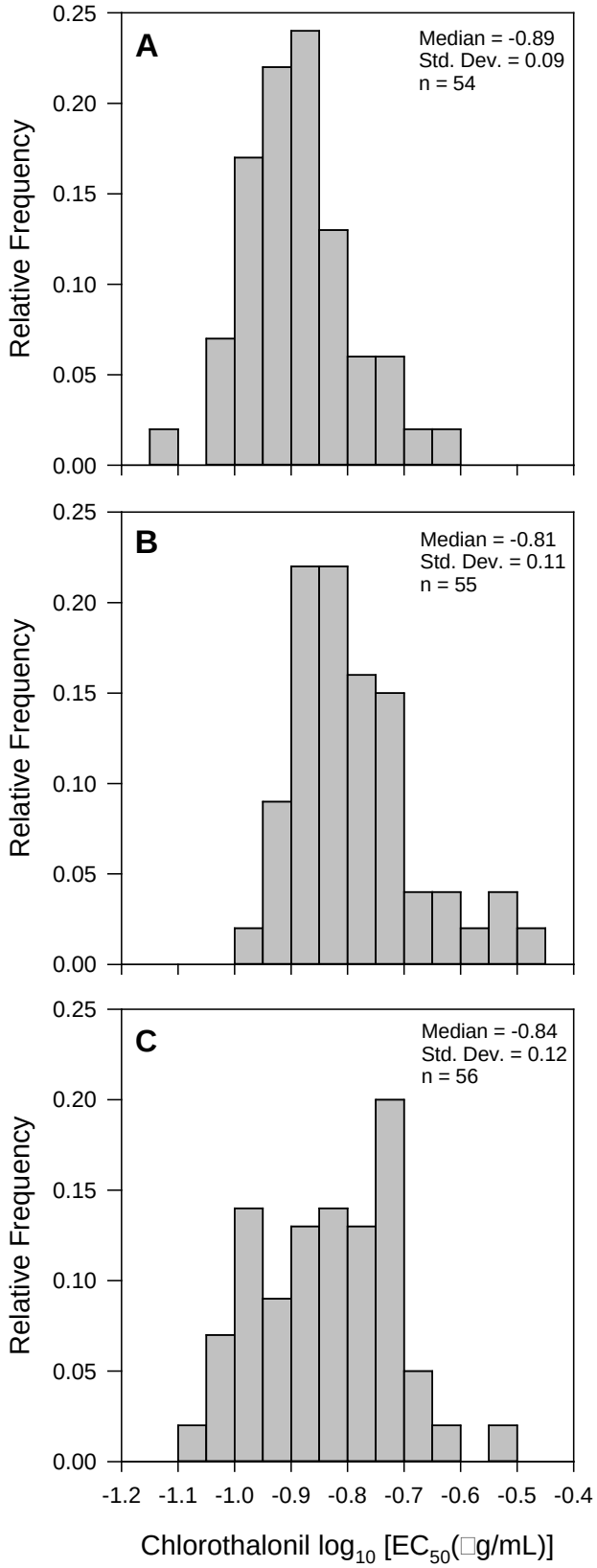

Figure 3.1 Frequency distribution of chlorothalonil EC_{50} values of *Z. tritici* isolates recovered from untreated plots (A), plots treated with chlorothalonil (B) or folpet (C).

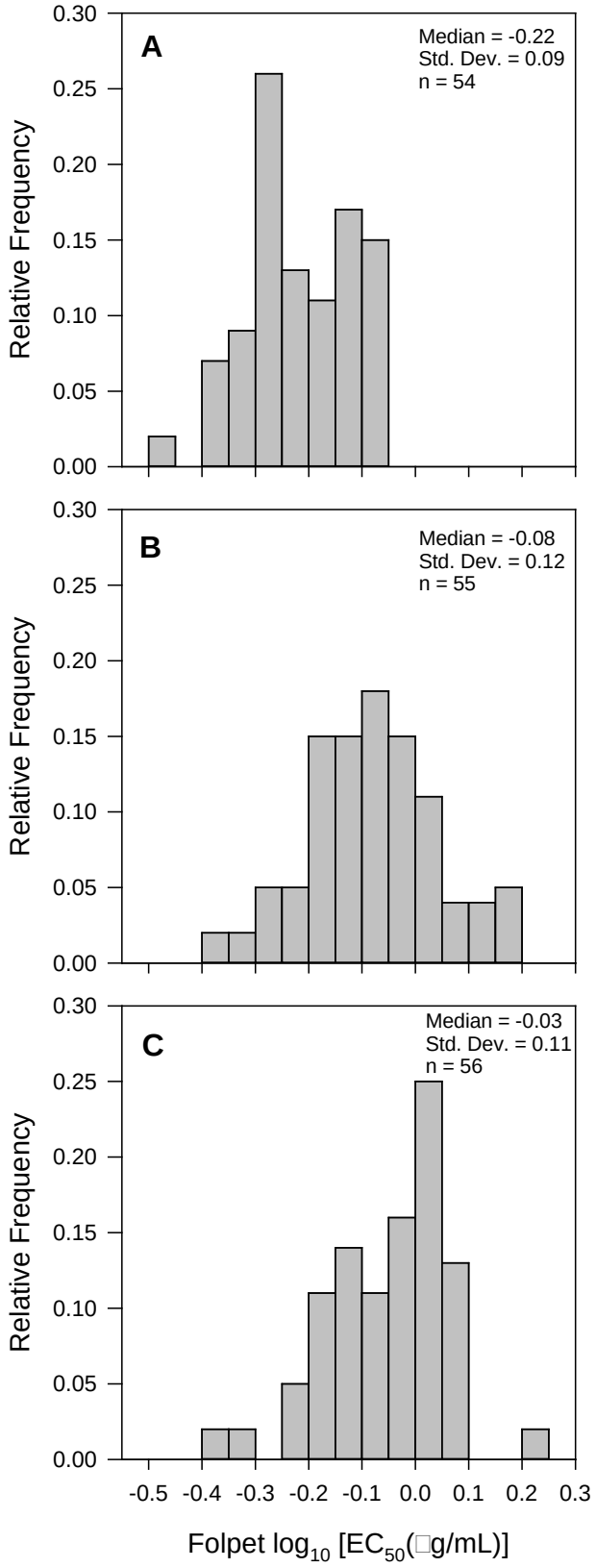

Figure 3.2 Frequency distribution of folpet EC_{50} values of *Z. tritici* isolates recovered from untreated plots (A), plots treated with chlorothalonil (B) or folpet (C).

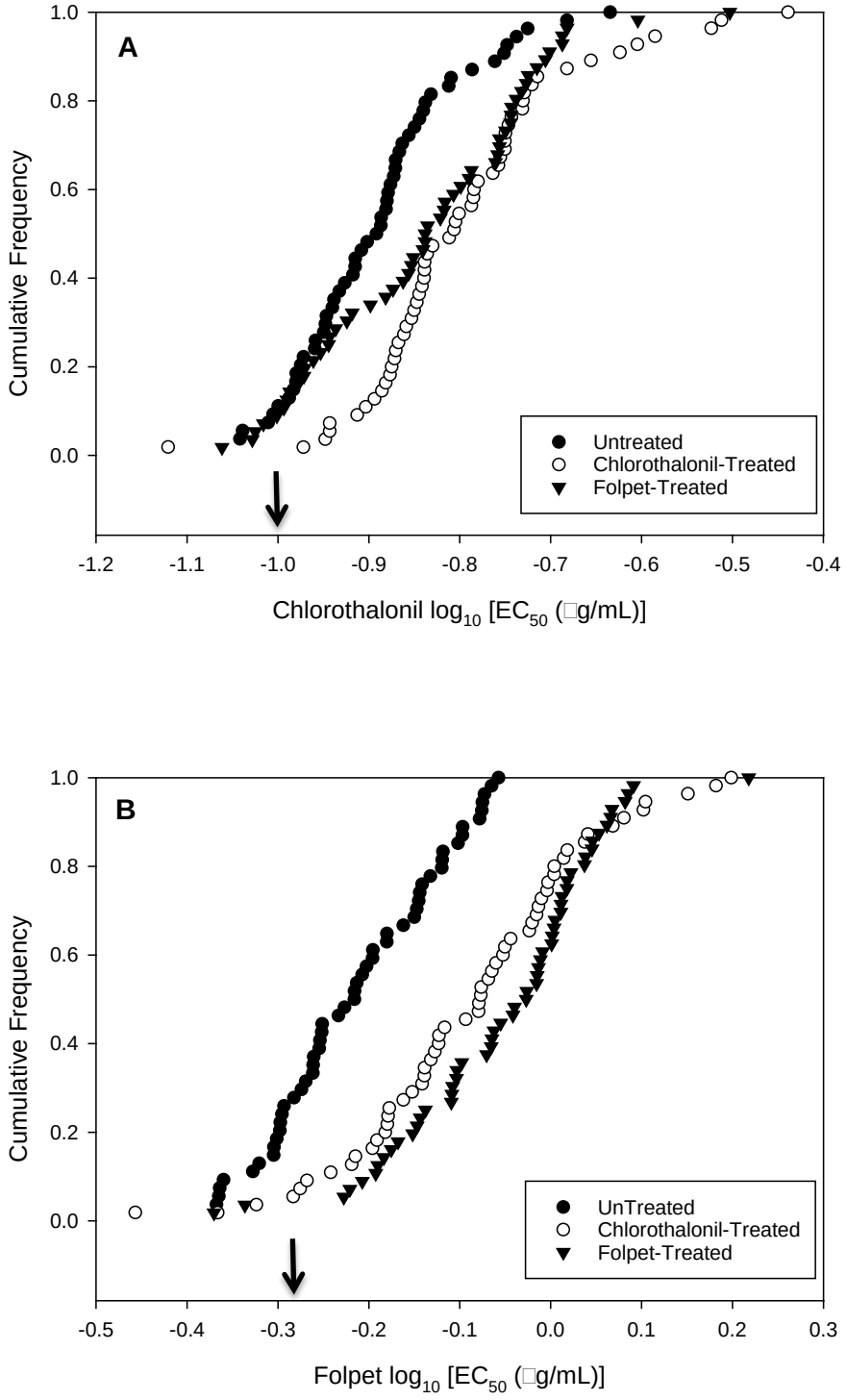

Figure 3.3 Cumulative frequency distribution of chlorothalonil (A) and folpet (B) EC_{50} values of *Z. tritici* isolates sampled from untreated plots or from plots treated with chlorothalonil or folpet. Sensitivity values are the mean of four independent experiments. Sensitivity of the reference IPO323 isolate is indicated by the arrow.

Table 3.1. Sensitivities of *Zymoseptoria tritici* field isolates sampled from untreated plots or plots treated with chlorothalonil or folpet.

Treatment	*n*	Chlorothalonil (µg/mL)		Folpet (µg/mL)	
		EC_{50}±SE[1] (mean)	SD	EC_{50}±SE[1] (mean)	SD
Untreated	54	0.131±0.004	0.03	0.623±0.018	0.13
Chlorothalonil[2]	55	0.168±0.007	0.05	0.863±0.034	0.25
Folpet[3]	56	0.151±0.006	0.04	0.911±0.029	0.23

[1]EC_{50} are the mean of four independent experiments base on the physical concentration scale.
[2]Two applications of chlorothalonil (Bravo 500 1.0 L/ha)
[3]Two applications of folpet (Phoenix 1.5 L/ha)

3.3.3 Baseline sensitivity of *Z. tritici* field isolates to fluxapyroxad

The fluxapyroxad sensitivities (EC_{50} values) were determined for 126 *Z. tritici* field isolates sampled at Rothamsted in 2012. These field isolates had similar EC_{50} values to the reference isolate IPO323 (0.04 µg/mL) regardless of the treatment given to the plot from which they came (Table 3.2). Calculated fluxapyroxad EC_{50} values in isolates sampled from untreated plots or plots treated twice with chlorothalonil or folpet ranged from 0.02 to 0.11, 0.01 to 0.11, and 0.01 to 0.14 µg/mL, respectively (Fig. 3.4). Isolates from plots treated with chlorothalonil, folpet or water had similar distributions of sensitivity to fluxapyroxad (Fig. 3.5; Kolmogorov-Smirnov two-sample tests: chlorothalanil-water: D=0.2, χ^2 approximation = 3.4, 2 df, p = 0.18; folpet-water: D=0.2, χ^2 approximation = 3.1, 2 df, p = 0.22).

Table 3.2. Sensitivities of *Z. tritici* field isolates taken from untreated plots or plots treated with chlorothalonil or folpet.

Treatment	*n*	Fluxapyroxad (µg/mL)	
		EC_{50}±SE[1] (mean)	SD
Untreated	45	0.046±0.003	0.02
Chlorothalonil[2]	37	0.044±0.005	0.03
Folpet[3]	44	0.049±0.004	0.03

[1]EC_{50} are the mean of four independent experiments base on the physical concentration scale.
[2]Two applications of chlorothalonil (Bravo 500 1.0 L/ha)
[3]Two applications of folpet (Phoenix 1.5 L/ha)

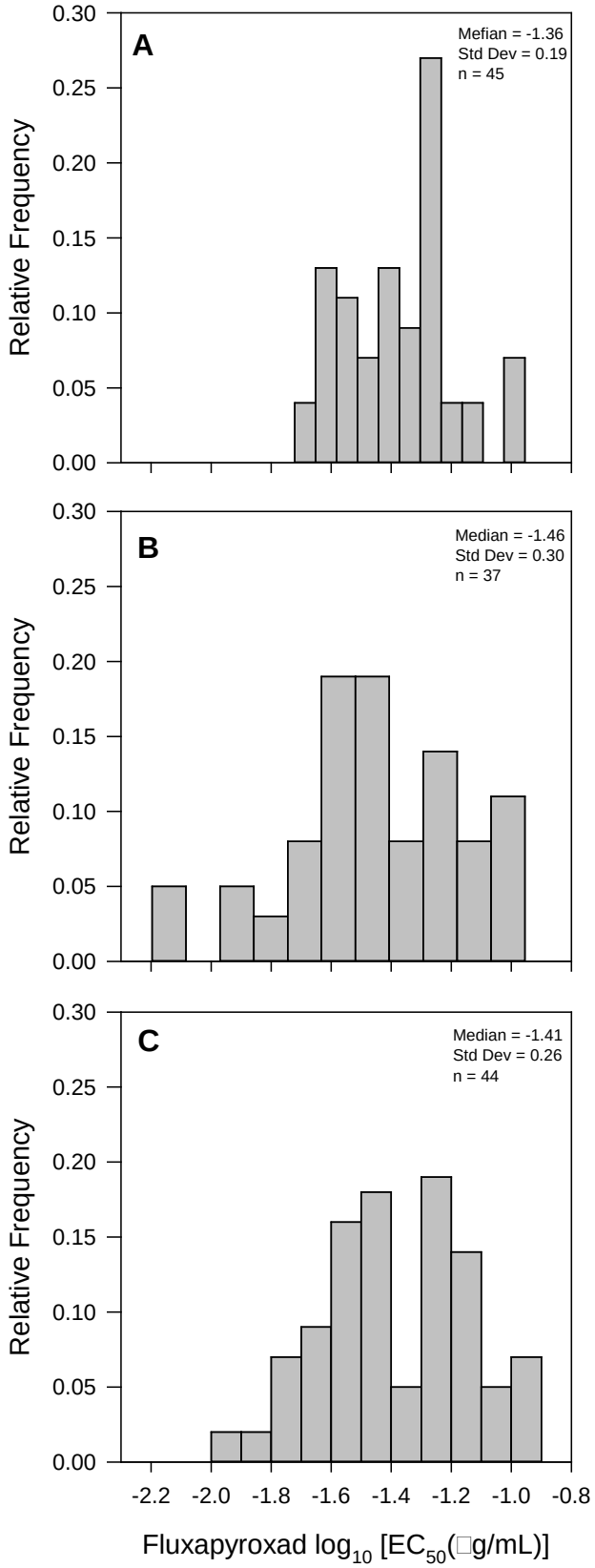

Figure 3.4 Frequency distribution of fluxapyroxad EC_{50} values of *Z. tritici* isolates sampled from untreated plots (A), plots treated with chlorothalonil(B) or folpet (C).

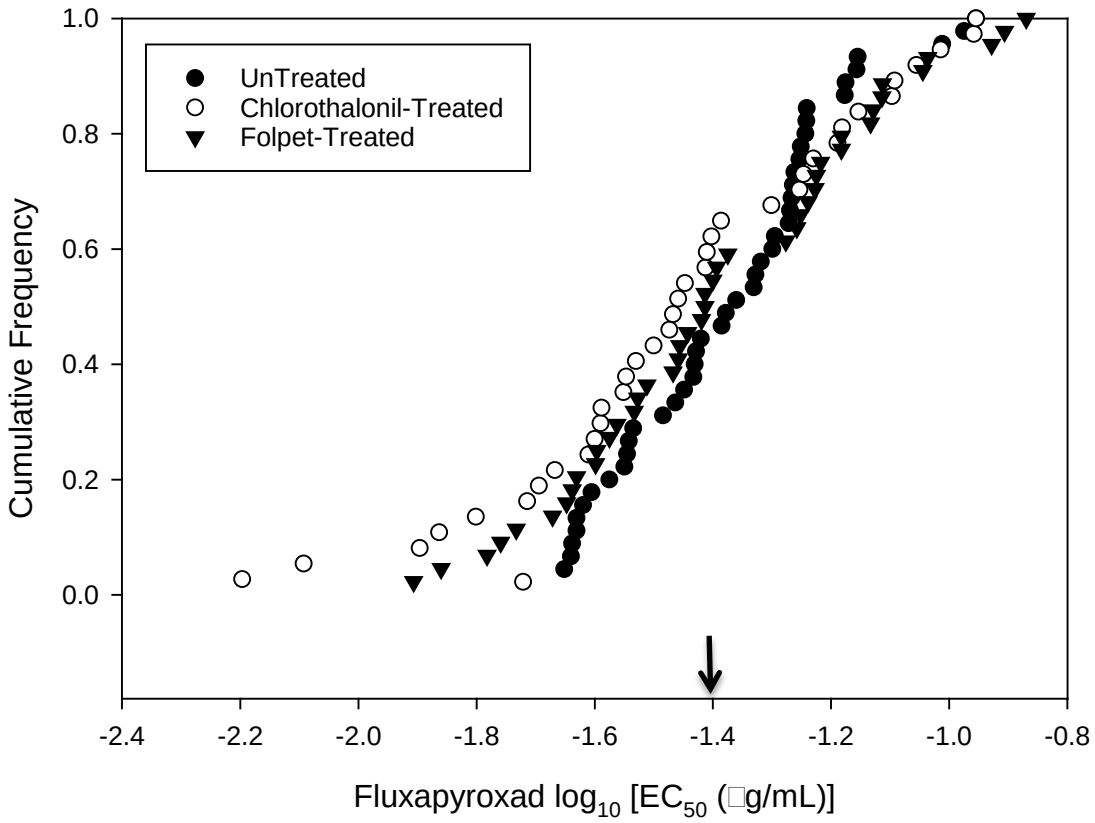

Figure 3.5 Cumulative frequency distribution of fluxapyroxad EC_{50} values of *Z. tritici* isolates sampled from untreated plots or plots treated with chlorothalonil or folpet. Sensitivity of the reference non-adapted IPO323 isolate is indicated by the arrow.

3.3.4 Cross resistance

Chlorothalonil and folpet sensitivity values of the 2012 field isolates were significantly correlated regardless of the treatment applied to the plot from which they came (Fig. 3.6; r = 0.60, p<0.001, n = 165). No evidence was found of separate slopes (p = 0.4, F-test) in the relation between folpet and chlorothalonil EC_{50} of isolates taken from plots sprayed with chlorothalonil, folpet or left untreated, but the intercepts differed (Fig. 3.6D). Correlations between folpet and chlorothalonil sensitivity of isolates from plots treated with two applications of chlorothalonil, folpet or left untreated were 0.62, 0.55 and 0.51 respectively (p<0.001 in all cases, n = 55, 56, 54).

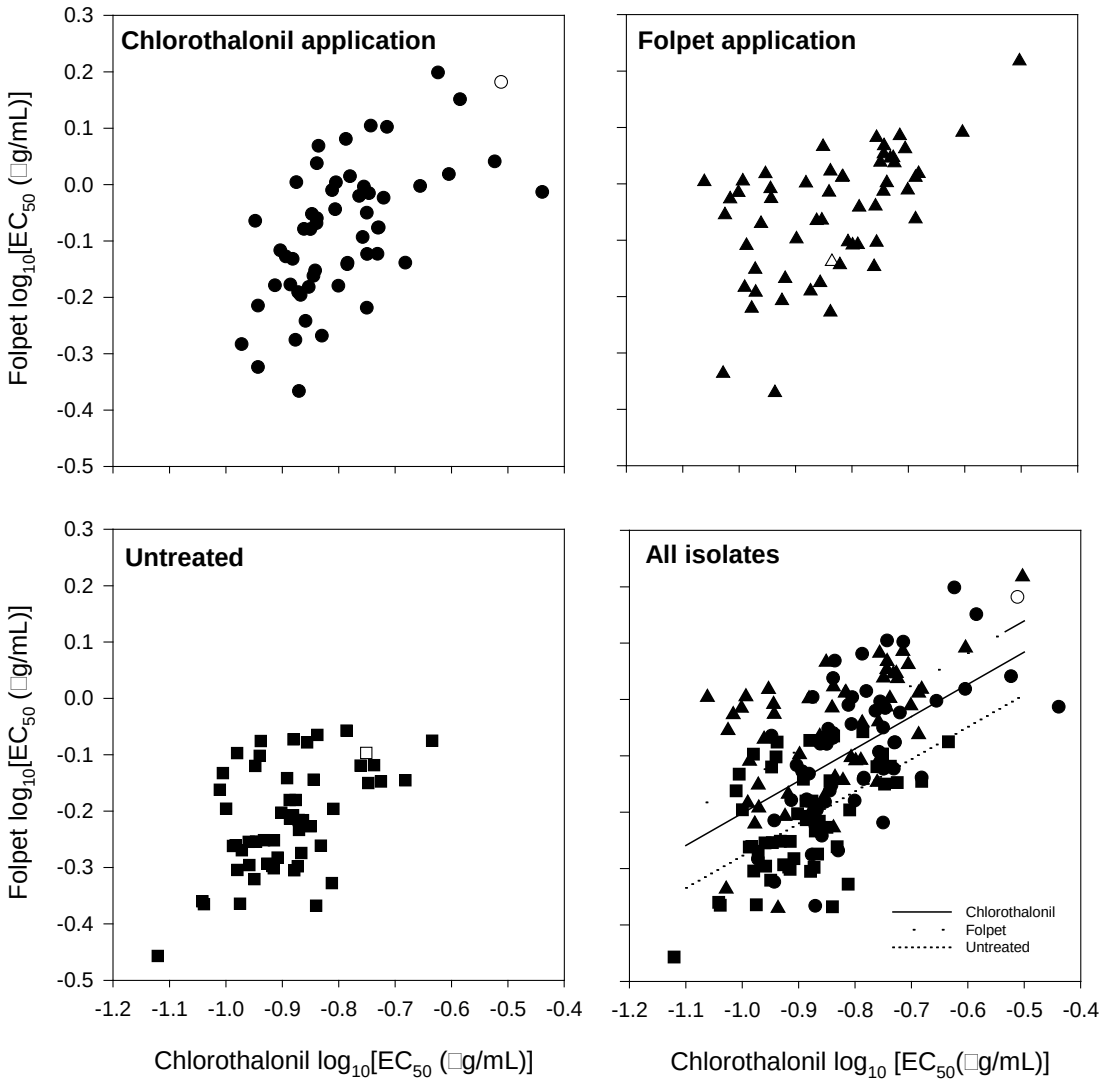

Figure 3.6 Correlation between chlorothalonil and folpet $log_{10}EC_{50}$ values of *Z. tritici* isolates sampled from plots treated with chlorothalonil, folpet or left untreated. Parallel curve analysis (all isolates): isolates from chlorothalonil treated plots: log_{10} folpetEC_{50} = 0.37+0.57*Log_chlorothalonil (SEs 0.05, 0.07); isolates from folpet treated plots: log_{10} folpetEC_{50} = 0.42+0.57* Log_chlorothalonil (SEs 0.06, 0.07); and isolates from untreated plots: log_{10} folpetEC_{50} = 0.29+0.57*Log_chlorothalonil (SEs 0.06, 0.07). R^2 = 0.51, S^2 = 0.008, 161 df

Taking all populations together, sensitivity to chlorothalonil and fluxapyroxad were uncorrelated (Fig. 3.7; r = 0.15, p = 0.105, n = 126). Sensitivity to folpet and fluxapyroxad were weakly but significantly correlated (Fig. 3.7; r = 0.23, p = 0.009, n = 126)

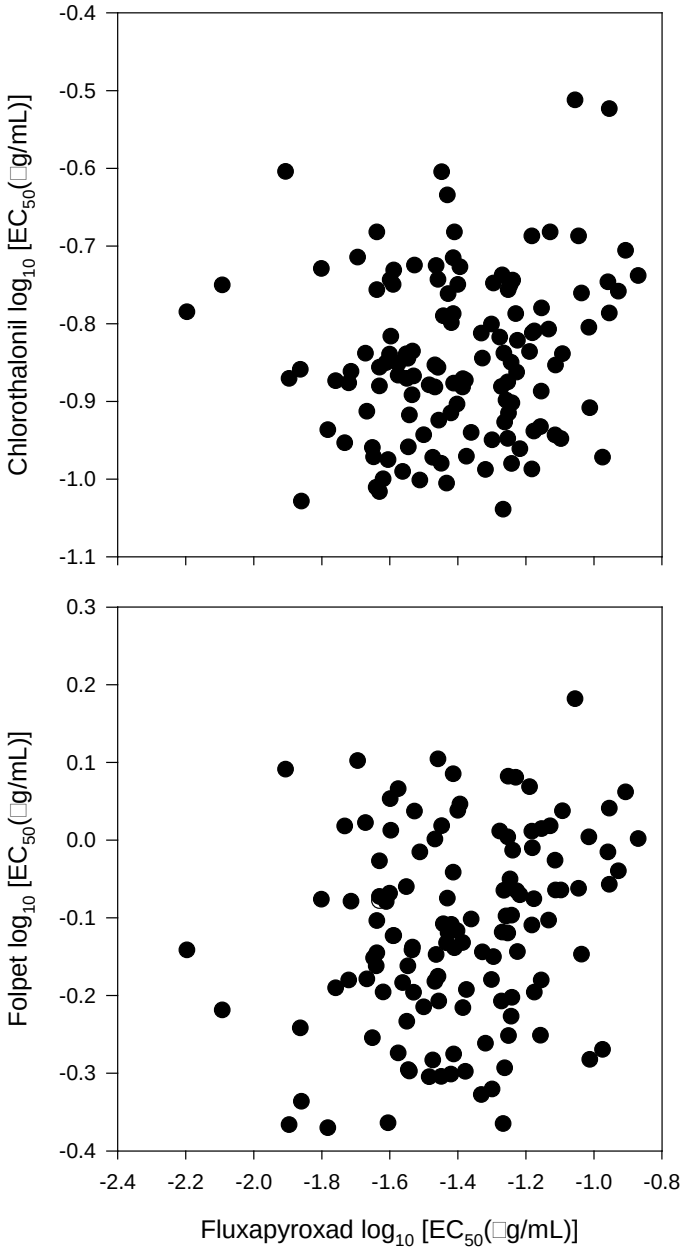

Figure 3.7 Correlation between fluxapyroxad and chlorothalonil or folpet $log_{10}EC_{50}$ values of *Z. tritici* field isolates.

3.4 Discussion

3.4.1 Multi-site inhibitors chlorothalonil and folpet

Although multi-site inhibitors (i.e. chlorothalonil) have been used for many years for SLB control in wheat, no shifts in sensitivity in *Z. tritici* have been reported before. In this study, selection for *Z. tritici* isolates less sensitive to chlorothalonil (from 0.09 to 0.36 µg/mL) or folpet (from 0.43 to 1.65 µg/mL) was observed after two solo field applications of formulated chlorothalonil (Bravo 1.0 L/ha) or folpet (Phoenix 1.5 L/ha). These selection differentials may have been greater than average because the environmental conditions during 2012 resulted in a high disease pressure (CropMonitor, 2012) and a susceptible cultivar Consort (HGCA, 2007) was used. This environment may have allowed the fittest *Z. tritici* isolates to multiply more rapidly in presence of the fungicide (Gisi et al., 1997) than in an average season or on a more resistant cultivar.

Selection for less sensitive strains has been reported in other plant pathogens after continuous applications of multi-site inhibitors. Holm et al. (2003) reported significantly reduced sensitivity in *Alternaria solani* field isolates after several applications of chlorothalonil in potato. Recently, Fairchild et al. (2013) reported *A. solani* field isolates resistant to chlorothalonil. Reduced sensitivity to folpet has been reported in *Botrytris cinerea* in vineyards under intense fungicide programmes of carboxamides and folpet (Fourie and Holz, 2001). Nevertheless, shifts in sensitivity to multi-site inhibitors can be temporary, once the fungicide applications stop the population can become sensitive again (Holm et al., 2003). This is because sensitive strains are fitter in the absence or in the presence of a low dose of the fungicide (Van den Bosch et al., 2011) and sexual recombination breaks down beneficial combinations of genes which have to be re-selected each year (Linde et al., 2002).

Sisler (1988) suggested that low sensitivity to multi-site inhibitors might arise from resistance mechanisms such as detoxification, reduced uptake or increased efflux of fungicide. Multiple drug resistance caused by increased fungicide efflux activity after overexpression of efflux transporter genes has been reported in *B. cinerea* (Kretschmer et al., 2009). Detoxification by glutathione S-transferase activity has been suggested to confer resistance to chlorothalonil in *Saccharomyces cerevisiae* (Shin et al., 2003). To date, there has been no report of mechanisms that reduce sensitivity to multi-site inhibitors in *Z. tritici*. However, low levels of insensitivity to chlorothalonil and folpet was measured for *Z. tritici* field populations studied here, suggesting that detoxification mechanisms might be operating. Further studies are needed to identify the resistance mechanisms that are linked to insensitivity to chlorothalonil or folpet. In chapter 5, expression of

genes encoding related-detoxification mechanisms in *Z. tritici* isolate IPO323 after exposure sub-lethal concentrations of chlorothalonil or folpet is presented.

3.4.2 Single-site inhibitor fluxapyroxad

No shift in sensitivity to fluxapyroxad was found in the sampled *Z. tritici* populations during the season. The *Z. tritici* field isolates had not been previously exposed to fluxapyroxad, as this fungicide was launched into the UK cereal market in 2012. Thus, the sensitivity distributions presented here can be considered as the reference baseline sensitivity for further studies.

A low but significant correlation in sensitivity between fluxapyroxad and folpet was observed in this study. This correlation could be due to expression of genes encoding general detoxification mechanisms (e.g. ATP-binding cassette transporters that are able to reduce fungicide concentration in the fungal cell: Zwiers et al. (2003)). Interestingly, constitutive overexpression of a putative ABC transporter (*abct*-2) encoding gene was identified in a lab mutant of *Z. tritici* strain with reduced sensitivity to fluxapyroxad; but no mutations were detected in the sdh sub-unit B, C and D encoding genes (Chapter 6). The same ABC transporter (*abct*-2) encoding gene was also overexpressed in the *Z. tritici* isolate IPO323 after exposure to chlorothalonil or folpet (Chapter 5). Although, shifts in sensitivity to multi-site inhibitors are linked to general detoxification mechanisms, they generally do not confer high levels of resistance.

However, *in vitro* evolutionary studies determined target site mutations in the sdh subunits B, C and D encoding genes conferring resistance to fluxapyroxad and other new-generation carboxamide fungicides (Chapter 6). This indicates that the risk of resistance development based on target-site mutations to fluxapyroxad in *Z. tritici* is high. In addition, extensive field monitoring across Europe and the UK have identified two *Z. tritici* isolates with reduced sensitivity to the new-generation of SDHI fungicides. These less sensitive isolates carried target site mutations in the sdh subunit C encoding gene at codon 79 (T79N) or 80 (W80S) (FRAC, 2013) but due to low resistance factors and low frequencies of these strains field performance has not been affected. It is particularly interesting that a mutation at codon 79 in the subunit C (T79I) was also found in the evolutionary studies (Chapter 6). Shifts in sensitivity to single-site inhibitors caused by single amino acid substitutions often remain permanent in field populations, even in absence of the fungicide where there is no fitness cost associated with mutations (Fraaije et al., 2003; Griffin and Fisher, 1985). Thus, further monitoring of specific mutations (especially T79N) is required.

3.5 Conclusions

Field applications of formulated chlorothalonil or folpet selected for less sensitive *Z. tritici* field isolates at Rothamsted. Further monitoring of chlorothalonil and folpet sensitivities across the UK and Northern Europe would determine the sensitivity landscape in *Z. tritici* to multi-site inhibitors at large scales and improve our understanding of how pathogens can adapt to anti-fungal compounds. Correlation of sensitivities between chlorothalonil and folpet, and fluxapyroxad and folpet suggests that similar detoxification mechanisms might underlie lower sensitivity to these fungicides in *Z. tritici*. Little is known about the mode of action of multi-site inhibitors. Therefore, it is important to understand multi-site inhibitors' modes of action. Insights into this are presented in chapter 4.

Chapter 4: Insights into the transcriptional response of *Zymoseptoria tritici* to multi-site inhibitors

4.1 Introduction

Fungicide resistance to single-site fungicides is widespread in *Zymoseptoria tritici* populations and remains a constant threat for wheat production (see chapter 3). Currently, the management program of Septoria leaf blotch (SLB) involves applications of multi-site inhibitors (e.g. chlorothalonil or folpet) as straights or in mixture with azoles and/or SDHIs to reduce and/or delay fungicide resistance development (FRAC, 2014; HGCA, 2014). Although chlorothalonil and folpet have a low risk for resistance development (FRAC, 2014), they can select for less sensitive *Z. tritici* field isolates in solo applications (Chapter 3). Typically, some mechanisms underlying lower sensitivity to fungicides can be inferred from their mode of action (see Cools and Hammond-Kosack (2013)). However, the mode of action of chlorothalonil and folpet remains unclear. Therefore, it is important to study the mode of action of these two fungicides to understand the process of adaptation to multi-site inhibitors.

Current understanding of chlorothalonil and folpet indicates that these fungicides are fungitoxic by depletion of thiol-containing proteins in the pathogen. Chlorothalonil (2,4,5,6-tetrachloroisophthlonitrile) is a halogenated benzonitrile fungicide with a broad spectrum of action against plant pathogens (FRAC, 2014). Vincent and Sisler (1968) suggested that chlorothalonil exerts its fungicidal action by alkylation of sulfhydryl groups of thiol-containing proteins (e.g. cysteine, glutathione, and CoA) and their subsequent depletion in the cell. Additionally, It has been suggested that chlorothalonil might inhibit physiological processes depending on thiol-containing enzymes, like glucose oxidation and possibly respiration (Vincent and Sisler, 1968). On the other hand, folpet [N-(trichloromethylthio)phthalimide] is a phthalimide fungicide (FRAC, 2014). Typically, folpet reacts with both thiol-containing (e.g. glutathione, alcohol dehydrogenase and glyceraldehyde 3-phosphate dehydrogenase) and non-thiol-containing proteins (i.e. lysozyme) in the cell (Siegel, 1971a, b; Siegel and Sisler, 1968b). Reactions with thiols produce trichloromethylthio ($SCCl_3$) then thiophosgene ($S=CCl_2$), and further reactions of thiophosgene with thiols can produce thiazolidine-2-thione-4-carboxylic acid (TTCA) (Lukens, 1966). Some evidence indicates that reactions of $SCCl_3$ and $S=CCl_2$ with thiol-containing proteins are the basis of folpet's fungicidal action in plant pathogens (Siegel, 1971b; Siegel and Sisler, 1968a, b). Although glutathione (GSH) depletion by chlorothalonil or folpet is associated with

fungitoxicity, GSH along with glutathione S-transferase (GST) is also related with cell defence against free radicals and xenobiotics (Cummins et al., 2013; Pastore et al., 2003; Shin et al., 2003). This picture lacks detail and none of the work cited used *Z. tritici* as target pathogen. Thus, the metabolic pathways and/or cell process that chlorothalonil or folpet can affect in *Z. tritici* need confirmation.

Recent progress in nucleic acid manipulation and sequencing has enabled the transcriptional response associated with chemical exposure to be determined. Genome-wide expression studies are used to determine changes in response to xenobiotics in human and plant pathogens to gain insights into the molecular pathways or target-site proteins affected by the toxicant (Becher et al., 2011; Cools et al., 2007; Cowen et al., 2002; Lindsey et al., 2011; Liu et al., 2005; Rogers et al., 2007; Xu et al., 2006). Cools et al. (2007) reported differential expression of genes involved in ergosterol biosynthesis, mitochondrial respiration and cell transport mechanisms upon exposure to an azole fungicide. They used a cDNA microarray representing around 25 % of the *Z. tritici* genome as expressed sequence tags (EST). However, high-throughput ("next-generation") DNA sequencing has emerged as alternative to microarrays for genotyping, analysis of methylation patterns, identification of transcripts binding sites and gene expression (Cokus et al., 2008; Korbel et al., 2007; Mikkelsen et al., 2007; Nagalakshmi et al., 2008). Analysis of RNA by sequencing cDNA at large scale has proved to be a reliable method to study changes in gene expression between organisms, tissues, conditions or treatments (An et al., 2014; Marioni et al., 2008; Mortazavi et al., 2008; Nagalakshmi et al., 2010).

Typically, genome-wide expression studies generate large datasets of gene expression profiles. The challenge now relies on the interpretation of these gene expression profiles to gain insights into their biological function (Subramanian et al., 2005). Functional annotation of DNA sequences is an approach to facilitate genomic data mining (see Curtis et al., 2005). The Gen Ontology (GO) is the most extensive database that classifies genes or sequences products with similar function into a hierarchy structure (Ashburner et al., 2000). Other databases such as InterPro (Labarga et al., 2007), Enzyme Encode (Schomburg et al., 2004), KEGG pathways (Kanehisa et al., 2014) or FunCat (Ruepp et al., 2004) also classify expressed sequences as product functions and can be used in genomic data mining. Bioinformatics methods using the Gene Ontology or other databases as framework to analysis large gene sets have allowed to gain biological interpretation of gene expression profiles (see Huang da et al., 2009). Enrichment analysis, a variant of gene set enrichment analysis (Subramanian et al., 2005), determines differences in functional classes between two sets of genes or sequences, based on their available functional annotation (Conesa

et al., 2005). Becher et al. (2011) using enrichment analysis detected differentially expressed genes encoding the ergosterol-biosynthesis pathway in *Fusarium graminearum* upon tebuconazole exposure. Moreover, enrichment analysis of genome-wide expression profiles in diverse organisms has revealed detoxification pathways in response to iron, copper (Jo et al., 2008), insecticides (Yang et al., 2013), or herbicides (An et al., 2014) exposure.

In the first phase of the work reported in this chapter, I used a custom design Affymetrix GeneChip expression array to characterise the genome-wide transcriptional response of the sequenced *Z. tritici* isolate IPO323 (Goodwin et al., 2007) to chlorothalonil or folpet *in vitro* during log phase growth. However, there was no correlation between microarray data and quantitative RT-PCR results, possible due to mis-hybridisation among probes on the microarray. Therefore, these results were not reliable. In the second approach, the sequenced *Z. tritici* isolate IPO323 was exposed *in vitro* to chlorothalonil or folpet during the lag and log phase of growth, and the gene expression response was determined with RNA-seq technology using an Illumina platform. Comparing treated and control samples, small but consistent changes in gene expression were detected. Functional annotation and enrichment analysis of the significantly differentially expressed genes (DEGs) determined that transcripts from several distinct functional classes of genes were particularly enriched in reaction to chlorothalonil or folpet. These findings provide insights into the metabolic pathways that might be affected by chlorothalonil or folpet in *Z. tritici*. In addition, a detailed analysis of chlorothalonil- and folpet-DEGs identified several genes encoding detoxification mechanisms, such glutathione S-transferase, ATP-binding cassette (ABC) transport, and major facilitator superfamily (MFS) drug efflux transport.

4.2 Materials and methods

4.2.1 Transcriptional response of *Z. tritici* to chlorothalonil or folpet using an Affymetrix GeneChip expression array

4.2.1.1 Growth of fungal cultures, and RNA extraction for Affymetrix GeneChip expression array and quantitative RT-PCR

The sequenced *Z. tritici* isolate IPO323 originally from the Netherlands (Goodwin et al., 2011; Kema and van Silfhout, 1997) was grown on YPD plates for seven days at 15° C in the dark. Spores were suspended in 100 mL of sabouraud dextrose broth (SDB) at final concentration 5×10^5 spore/mL and incubated at 21° C in the dark at 200 r.p.m. After 24 h incubation, cultures were adjusted to 0.1 µg/mL of chlorothalonil or 0.5 µg/mL of folpet - approximate EC_{50} concentration previously determined – (Chapter 3). Fungal biomass was harvested by filtration after 24 h further fungicide exposure. Filtered biomass was snap frozen in liquid nitrogen and stored at -80° C for further total RNA extraction from untreated and treated samples of three replicate experiments as described in chapter 2, section 2.6. Technical grade fungicides were dissolved in acetone then diluted in SDW before adding to the cultures.

Aliquots of 25 µL of purified total RNA from 200 ng/µL stocks were sent to SourceBioScience imaGenes (Berlin, Germany) for synthesis, hybridisation and scan of a custom Affymetrix GeneChip expression array according to the manufacture's protocol (GeneChip® 3' IVT Express Kit, Affymetrix). Quality control of the samples was carried out using nanodrop ND-1000 UV-VIS spectrophotometer (Thermo Fisher Scientific, Wilmingtong, DE, U.S.A.) and Agilent 2100 Bioanalyzer G2938A (Santa Clara, CA, U.S.A.) according to manufacturer's instruction.

The "Affymetrix GeneChip *Mycosphaerella graminicola* expression array" (code MgramEXPs520703; P/N 520703) has a density of 11µm 100-3660 array format GeneChip containing 49079 probe sets – 11 probe sets (perfect match/mismatch) per gene. Approximately, 69 % of the probe sets correspond to nuclear and mitochondrial *Z. tritici* genes, 21 % to wheat (*Triticum aestivum*) genes and 10 % to *Pyrenophora tritici-repentis* genes.

4.2.1.2 Transcriptomic analysis of Affymetrix GeneChip expression array.

Statistical analysis was carried out by SourceBioScience imaGenes as part of their provided service using the Expression console™ software v1.4 according to manufacturer's manual (Affymetrix, Santa Clara, CA, U.S.A.). Raw data were annotated manually as the annotation file was not

available. The SourceBioScience imaGenes analysis pipeline is as follow: raw data are read in, and normalised by quantile normalisation method. After normalisation, all gene expression values lower than 0 % quantile were set to the value of this 0 % quantile to eliminate low level signals. After normalisation, M versus A analysis (i.e. log ratios versus mean average intensity) was carried out to identify dependent bias in the microarray data. Comparisons were performed between two chips, datapoint by datapoint (gene by gene), after log-transformation. Significant (p<0.05; q<0.05) differentially expressed genes were identified by comparing fold-changes (ratios) and log fold-changes of treated versus control samples, and applying t-test statistics on quantile normalised signal intensities for each gene.

4.2.2 Transcriptional response of *Z. tritici* to chlorothalonil and folpet using Next Generation RNA sequencing (RNA-seq)

Based on the results obtained with the Affymetrix GeneChip expression array, a different approach was undertaken for the RNA-seq technology. This time, chlorothalonil or folpet was added in the lag and log phase growth of *Z. tritici* (Fig. 4.1). This is because typically, EC_{50} values are determined by adding the fungicide in the lag phase (Chapter 3; FRAC, 2012).

4.2.2.1 Growth of fungal cultures, and RNA extraction for sequencing and quantitative RT-PCR

The sequenced *Z. tritici* isolate IPO323 (Goodwin et al., 2011) was grown on YPD plates for seven days at 15° C in the dark. For treatments in the lag phase growth (Fig. 4.1A), *Z.tritici* spores (5×10^5 spore/mL) were added to flasks containing 100 mL of SDB amended with chlorothalonil or folpet at 0.1 or 0.5 µg/mL final concentrations, respectively. These concentrations were chosen to generate a gene expression profile "baseline" in response to the approximate EC_{50} values previously determined (Chapter 3). After 24 h growth at 21 °C in the dark at 200 r.p.m, mycelia were harvested by vacuum filtration and snap frozen in liquid nitrogen. For treatments applied in the log phase of growth, IPO323 spores were suspended in 100 mL of SDB at a final concentration of 5×10^5 spore/mL. After 24 h incubation at 21 °C in the dark at 200 r.p.m, cultures were adjusted to 0.1 µg/mL of chlorothalonil or 0.5 µg/mL of folpet (Fig. 4.1B). Mycelia were harvested after further 24 h growth in the same conditions and snap frozen. IPO323 cultures grown for 24 h or 48 h in the absence of fungicide were used as untreated controls for treatments applied in the lag or log phase growth, respectively. Technical grade fungicide was dissolved in acetone then diluted in SDW before adding to the SDB or inoculated-SDB containing flasks.

Total RNA extraction from triplicate untreated and treated samples was carried out as described in chapter 2, section 2.6. Aliquots of 20 µL of purified total RNA at a concentration of 100 ng/µL were sent to the TGAC, Norwich, UK for sequencing. The sequencing was carried out in 9 samples/lane on the Illumina HiSeq 2000 using 100bp single-end reads, with an average yield of 21,614,254 reads per sample.

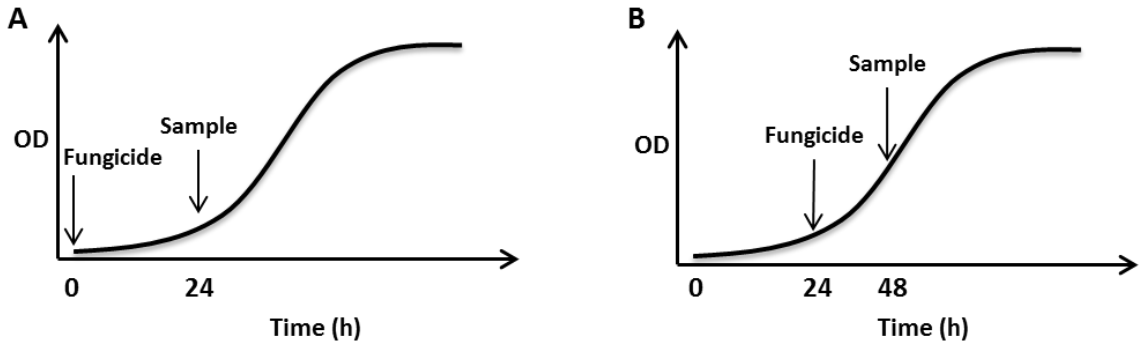

Figure 4.1 Application of fungicides in the lag (A) and log (B) phase growth of the reference *Z. tritici* isolate IPO323. Fungicide application and sample collection are indicated by the arrows.

4.2.2.2 Transcriptomic analysis of RNA sequencing.

Raw data generated from RNA sequencing and contained in libraries were checked as follows. The Phred quality score (Q) of each library was determined with the FastQC option on the Galaxy platform v0.5 (Cock et al., 2013) with the default settings. Differential analysis of RNA-seq data was carried out with "Tuxedo tools" as described in Trapnell et al. (2012) on Galaxy with the default settings (Fig 4.2). The reference *Z. tritici* isolate IPO323 genome (Goodwin et al., 2011) was used as reference in the analysis. Reads were mapped against the reference genome with TopHat (v2.0.6). Reads per kilobase of exon model per million mapped reads (RPKM; Mortazavi et al., 2008) were calculated with Cufflinks (v2.1.1) for reference annotations but excluding genes annotated as rRNA. Significantly (p<0.05; q<0.05) differentially expressed genes (DEGs) between untreated control and fungicide treated samples were determined with Cuffdiff. Analysis was performed on the three biological replicates of each fungicide. Chlorothalonil-DEGs or folpet-DEGs refer to transcripts mapped to specific gene induced by chlorothalonil or folpet exposure, respectively. Gene expression ratios (fold-changes) of significant DEGs were transformed to binary logarithms (log_2).

The significantly DEGs were mapped onto the *Z. tritici* reference genome using OmmniMapFree software v2008.07.17 (Antoniw et al., 2011). Log_2-transformed gene expression ratios of significantly DEGs were plotted as heatmaps. Heatmaps were done in R software v1.15.3 using gplots package. This function uses a euclidean measure to arrange genes according to similarity in patter of gene expression and complete agglomeration method for clustering analysis (Eisen et al., 1998; Frigui and Krishnapuram, 1997).

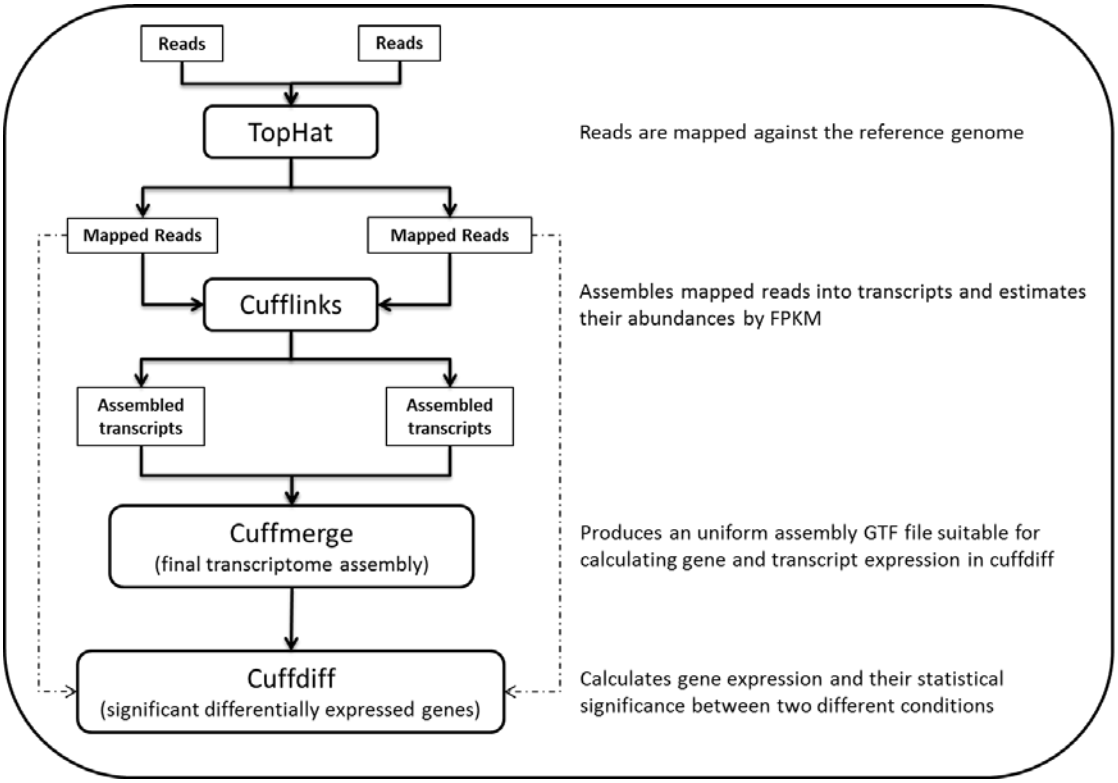

Figure 4.2 Diagram of RNA-seq data analysis workflow on Galaxy platform using "Tuxedo tools". Adapted from Trapnell et al. (2012).

4.2.2.3 Enrichment analysis of significantly differentially expressed genes

The reference *Z. tritici* isolate IPO323 genome was functionally annotated with gene ontology (GO), protein sequence analysis and classification (InterPro), Enzyme Code, and Kyoto encyclopaedia of genes and genomes (KEGG) database using Blast2Go v2.7.0 software (Gotz et al., 2008). Firstly, the *Z. tritici* whole-genome sequence was downloaded from the Joint Genome Institute (JGI) (Grigoriev et al., 2011) and blasted against the database of the National Center for Biotechnology Information (NCBI) using the Blastp option in Blast2GO. Blastp was carried out with the default parameters except for the "number of Blast hits" that was set to 20. The GO annotation was carried out using the "Run annotation step" option with the default settings. Annotation expander (ANNEX) option was used to improve the annotation (see Myhre et al., 2006). InterPro terms were obtained from InterProScan at EBI (Labarga et al., 2007) by the "InterPro search function" in Blast2GO. Finally, Enzyme Code and KEGG pathway map annotations were retrieved from the KEGG database (Kanehisa et al., 2014) through the "mapping engine" option in Blast2GO.

Enrichment analysis was carried out in Blast2GO with the "enrichment analysis (Fisher's Exact Test)" option. The enrichment analysis determines statistical differences in functional classes - denoted by GO terms – between two groups of sequences (Conesa et al., 2005). The list of significantly (p<0.05) DEGs of each treatment or any particular set of genes was loaded in Blast2GO and compared against the whole-genome of *Z. tritici* previously annotated. The cutoff for FDR-corrected p values was set to 0.05. The enrichment analysis in Blast2Go uses Fisher's exact test, corrects for multiple testing and tests if GO terms are significantly associated with a group of selected genes (Bluthgen et al., 2005). The results is a list of significantly (p<0.05) enriched GO terms with their adjusted p value. Enriched graphs or GO DAGs (i.e. direct acyclic graphs) were done in Blast2GO with the option "Make Enriched Graph".

4.2.3 Quantitative Real-Time PCR

Transcriptional levels of selected genes were measured with quantitative RT-PCR to validate the microarray and RNA-seq results, using purified total RNA from independent growth experiments. Total RNA extraction from triplicate untreated and treated samples was carried out as described in chapter 2, section 2.6. Ten micrograms of total purified RNA was reversed transcribed and diluted one in ten (see chapter 2, section 2.7). The top five significantly (p<0.05) up- or down-regulated DEGs from each treatment in the microarray or RNA-seq experiments were selected for

validation. The selected genes were targeted with primers (Table 4.1 and 4.2) designed as described in chapter 2, section 2.8.

Quantitative RT-PCR reactions were carried out using the SYBR® Green JumpStart Taq ReadyMix (Sigma-Aldrich, Missouri, USA) in a final volume of 20 µL containing 5 µL of diluted cDNA and 0.25 µM of each primer (Table 4.1 and 4.2). Thermal cycling conditions were as described in chapter 2, section 2.9. Reactions were carried out on the ABI 7500 Real-Time PCR System (Applied Biosystems). Relative transcript abundance (RQ) of target genes was calculated by the $2^{-[\Delta][\Delta]Ct}$ method (Pfaffl, 2001), using β-tubulin as endogenous control (Cools et al., 2007) and samples from the untreated controls as calibrators (see chapter 2, section 2.9). Relative transcript abundance of selected genes was compared to transformed gene expression ratios obtained in the microarray or RNA-seq experiments on a $\log_2$ scale. Pearson's correlation coefficients between $\log_2$-transformed gene expression ratios obtained with microarray, RNA-seq and qRT-PCR were calculated in SigmaPlot v12.3 (Systat Software, Erkath, Germany).

Table 4.1 Selected top most up- or down-regulated genes in the microarray experiment and primers used for qualitative RT-PCR

Gene ID	JGI target gene/EST ID[1]	Gene expression (Log_2 change)	Sequence (5' – 3')	
			Forward	Reverse
β-tubulin	e_gw1.1.861.1		AGAGAGCCTCGTTGTCAATGC	CGGTATGGGAACACTTCTCATCAG
Chlo-U1	fgenesh1_pg.C_chr_13000088	+1.8	TCCTCTCTCACCTTCAGCCGCA	TCAGCAGCACCATCACCCTCCT
Chlo-U5	fgenesh1_pg.C_chr_17000025	+1.7	TTCGCCTACCCTGCCTTACTCG	TGCTTCATCTGCCCATCCATTTGC
Chlo-U6	e_gw1.17.1.1	+1.6	ACGTCAGGGAGACCAAGGACCA	TTCGCGGTGGTGTTCAGTGC
Chlo-U7	estExt_Genewise1.C_chr_170015	+1.5	CGCCATCAACCTCAACGCCAT	CGCCCACTTCTTCGCCTACTCT
Chlo-D1	mgb0512f	-1.9	AGGGCACAATAGCGGCGACA	TGTCTGAGCAGCGCCCATCT
Chlo-D2	mg0007f	-1.8	AGAAGCAACGACGACGGCCT	TGGCATTGTCTACGCCCAGGAC
Chlo-D3	e_gw1.1.628.1	-1.8	ACCTTGCCAGTGCTGCGAGT	TCCGTAAGGCTGCGACCGTT
Chlo-D4	fgenesh1_pg.C_chr_3000553	-1.8	CAGCATTCCACCTCGCCCATCT	AAAGAGAGTCGTGCCTGTGCCG
Chlo-D6	mgc06h12f	-1.7	AGGCTTCTTCGCCTATGCTGCT	CCGGGTTCTCGACCTTCTCGAA
Fol-U1	estExt_fgenesh1_pg.C_chr_11419	+0.7	TCGGTGGGCTTCACAGGCAT	CCGCTCGCATCAACCTCAAACA
Fol-U2	e_gw1.3.952.1	+0.6	ACTACTACGCCAACGGCCAACA	AGCTTGTGCCCGAACACCCA
Fol-U3	fgenesh1_kg.C_chr_12000213	+0.6	AGAGGCGGAGACGATGTGATGG	AGCGATGGAGAGTGTGGAGTGC
Fol-U4	estExt_fgenesh1_pg.C_chr_110320	+0.6	TTGGGCGAAGAAGGCGCTGT	AACCGCAAACACAATCCCGCC
Fol-U5	e_gw1.8.1046.1	+0.6	GGTGCGGTGGTCTGCATAGGAA	ATGGTGGACGTGCCTGCGAA
Fol-D1	mgc06h12f	-1.3	AGGCTTCTTCGCCTATGCTGCT	CCGGGTTCTCGACCTTCTCGAA
Fol-D3	fgenesh1_pg.C_chr_4000634	-0.9	GGTCCAGTCACCGATTCCACCA	AAAGCCAAGGGTCTCGCCGT
Fol-D4	fgenesh1_pg.C_chr_2000103	-0.9	AAACGCTGGCTGGTCCGTGT	TTGGCTTGCTCGGCTTCGGT
Fol-D5	fgenesh1_pg.C_chr_8000511	-0.8	TGCGAGGGTTCGTTGCTGTGT	CGGTGGTTCGGGCTTCGTATCA
Fol-D6	fgenesh1_pg.C_chr_7000261	-0.8	GCGGCCATTGAACGATTGACCC	CGCATCCACCGCAACGAACA

[1]The Join Genome Institute (JGI) fungal program (Grigoriev et al., 2011)

Table 4.2 Selected top most up- or down-regulated genes in the RNA-seq experiment and primers used for qualitative RT-PCR

Gene ID	JGI target gene ID[1]	Gene expression (Log_2 change)	Sequence (5' – 3')	
			Forward	Reverse
β-tubulin	e_gw1.1.861.1		CGCATGATGGCCACCTTCTC	GCAGAAGGTCTCGTCGGAATT
LagChlo-U1	estExt_fgenesh1_pg.C_chr_20058	+1.8	GATCGTCTCCACCATCGTCTT	ATCCGCTGCTATGAGAAGAACC
LagChlo-U2	estExt_fgenesh1_kg.C_chr_60209	+1.4	CGTCAAGGAGAACAAGGAGAACTA	GGTGTTCAGTGCGACCCTTAAT
LagChlo-U3	estExt_Genewise1Plus.C_chr_80251	+1.3	CGTGGGTGGGAAACTGAGAAT	GCTCTGTCGATTACTCCGAAAG
LagChlo-U4	gw1.2.2194.1	+1.2	GGACGAGAGGAAAGTTGGAGTA	CAACCCTCAGCACACCATCTT
LagChlo-U5	fgenesh1_pg.C_chr_4000436	+1.2	CGTCGATATCAACACCGAACAT	CATGGTAGAAGTCGGAGTGATC
LagChlo-D1	e_gw1.8.1362.1	-2.9	ATGTCCGCTGCAACACAATC	TCTGGTACGAATCTCTGGCTTC
LagChlo-D2	estExt_fgenesh1_pg.C_chr_11227	-2.2	GTAGGATTGAGCAAGGACGAGAA	CTCGCTCCCAGGAATCTTGTAAA
LagChlo-D3	estExt_Genewise1Plus.C_chr_10184	-1.8	GCAACATCAGAATTACGGCATGAG	TGGCAGCTTGATTCCGATTGT
LagChlo-D4	estExt_fgenesh1_kg.C_chr_140021	-1.6	CTCCAGGACTCCTCTCGGTAA	GTCCTCCTCGTCGTTGATCTC
LagChlo-D5	gw1.1.1940.1	-1.6	GACTTGAGGTTGTTGAGGCATATG	CATCCCAACTCCACTCCCAATC
LagFol-U1	gw1.2.2368.1	+2.3	GTGGTTTCGGAGGCGGATTT	GCCTGAACCTGAGCCCTTTC
LagFol-U2	gw1.11.380.1	+2.0	CCAGATGCAACACCACCGATAG	AGGCGACCATCAGAATCGATAC
LagFol-U3	fgenesh1_pg.C_chr_4000212	+1.9	ACCAAACGCACCCACTGATAC	GAAAGGCCAGAGGTCCGAGTA
LagFol-U4	estExt_fgenesh1_pg.C_chr_20058	+1.8	CGGTCTGTTGCACAAGGTTCTA	GCTGTAAGACGATGGTGGAGAA
LagFol-U5	fgenesh1_pg.C_chr_4000425	+1.7	CTGCCGCTCCCAATTGACAAA	GTAGTGCGGTTCTCCTCCTGTA
LagFol-D1	e_gw1.1.3942.1	-1.1	ACCCGTCCCGAGTCGTAAAG	CAGCTTGCACGTCACTACCATA
LagFol-D2	estExt_Genewise1Plus.C_chr_10891	-0.9	GAAGAAGGACGAGGCCATGAA	CGGTTGTGCTGATGTCGTAAC
LagFol-D3	fgenesh1_pm.C_chr_1000920	-0.9	GCAGAAAGATGGAGACGGCAATT	GCTTCAAACAACCCGATGACAAA
LagFol-D4	fgenesh1_pg.C_chr_5000064	-0.8	ACCAGTACACCGTCTCATCTG	TATTGATCGCTTGCCACGATTG
LagFol-D5	gw1.1.1706.1	-0.8	GCCATACTCGGATGCAACTTTC	GAATCACCTCCGCTTCGATCAA
LogChlo-U1	estExt_Genewise1Plus.C_chr_40627	+2.3	GTCCTCACGCTTGCCTTCAA	TGTCCAGGAACCAGCTCAACAT
LogChlo-U2	estExt_fgenesh1_pm.C_chr_120122	+1.9	GAACAGGGTCCAATCTCTATCG	ACCATTGCTTCGGGACTAGAG
LogChlo-U3	estExt_Genewise1Plus.C_chr_100358	+1.4	CTCAACACTCAGACCGCAAAC	CCTATCACCACCACATCATACTC
LogChlo-U4	estExt_Genewise1Plus.C_chr_13116	+1.4	GGATACGTGGAAGCCGAAAGT	CGTGGATGTTGGTGTCGTTTC
LogChlo-U5	estExt_fgenesh1_pg.C_chr_60151	+1.3	TCCAATACGACTCACCTCCAATAC	AGGTTGGATGGGCAGGTAGTT
LogChlo-D1	fgenesh1_pg.C_chr_16000068	-1.3	GAGCAGAGCGAGGAAGTTGTAT	GCTTGCGAATTGTTGCTTTCTTC
LogChlo-D2	fgenesh1_pg.C_chr_3000370	-1.3	GTAGTCGTAGTGTCGGTAATTTGT	GAACATTCCCGGTACCACATCT
LogChlo-D3	fgenesh1_pg.C_chr_7000254	-0.9	GATTGATCAGGATGCTGGGAGAT	CCACATCTTCCGACTCGTACT
LogChlo-D4	fgenesh1_pg.C_chr_12000014	-0.9	GTCTGTCGGAGGGCCAAGAA	GCAGCAGAGAACGCAAAGAG
LogChlo-D5	gw1.10.406.1	-0.9	CATGTTGCCTGTTACCAAGAATCT	CACATATCGCATCGGTCCAGAAG
LogFol-U1	e_gw1.2.536.1	+2.1	CCGAAGACATTGATTCTGCTCTT	CAGGCGTTGTCTCCAGAATG
LogFol-U2	fgenesh1_pg.C_chr_10000003	+2.0	TCCTCCATTCGGTACAAGATCA	GACGTTGAGATCGGTCATACTC
LogFol-U3	e_gw1.4.1123.1	+1.9	CGGAATGGAATGGTCGTGATTG	CGTTCCCAGCCCAAACAAAGT
LogFol-U4	fgenesh1_pg.C_chr_3000638	+1.8	CTCACGGCATTCCCATCATTC	GGCTCATCATTTGACCTTCGATA
LogFol-U5	fgenesh1_pg.C_chr_19000003	+1.8	ACAGAAGAAGAGGATCAGGTCATA	GTCTTCGAGTTGTTTCTCCTGAT
LogFol-D1	estExt_Genewise1Plus.C_chr_120164	-1.6	CTGGCGGTTATGACGATGAATGT	CAATGACCGTCGCAGTGATC
LogFol-D2	e_gw1.6.1406.1	-1.5	GCTGCCCGAGATCTTCATTCT	GCGTTCAAAGTGATGCCGTATG
LogFol-D3	fgenesh1_pg.C_chr_1001542	-1.2	CAGACTCAGACGACGCCTTAC	CTATCCCGACCCTTGACGATTT
LogFol-D4	estExt_Genewise1Plus.C_chr_13616	-1.2	GCTCCTTTGGGTACTGCTCTT	GGATTTCTTCGGCATCCATGAA
LogFol-D5	estExt_fgenesh1_pg.C_chr_40586	-1.2	GCACGCTTGGAGTCTCATATG	GGGAGTGCATCGGAGTAGTC

[1]The Join Genome Institute (JGI) fungal program (Grigoriev et al., 2011)

4.3 Results

4.3.1 Transcriptional response of *Z. tritici* to chlorothalonil or folpet using an Affymetrix GeneChip expression array.

4.3.1.1 Genome-wide expression response of *Z. tritici* to chlorothalonil or folpet

There were 6,317 probe sets significantly (p<0.05; q<0.05) differentially expressed on the microarray after exposure of *Z. tritici* isolate IPO323 to 0.1 µg/mL of chlorothalonil. Approximately 75 % of these probe sets corresponded to *Z. tritici* genes, 20 % to wheat genes and 5 % to *P. tritici-repentis* genes. The change in expression level of probe sets annotated as *Z. tritici* genes ranged from -1.9 to +2.0 $\log_2$ units. Fewer probe sets (685) were significantly (p<0.05; q<0.05) differentially expressed on the microarray after exposure to folpet. Approximately, 45 % of the significantly differentially expressed probe sets corresponded to *Z. tritici* genes, 40 % to wheat genes and 15 % to *P. tritici-repentis* genes. The change in expression of folpet probe sets annotated as *Z. tritici* genes ranged from -1.3 to +0.72 $\log_2$ units.

4.3.1.2 Validation of Affymetrix GeneChip expression array results by quantitative RT-PCR

The four genes in the expression array data most up-regulated in the presence of chlorothalonil (Chlo-U1, Chlo-U5, Chlo-U6 and Chlo-U7) and the five genes most down-regulated (Chlo-D1, Chlo-D2, Chlo-D3, Chlo-D4 and Chlo-D6) were validated by qRT-PCR, using the same RNA samples as those used in the microarray. Small changes in gene expression (from -1.6 to +0.7 $\log_2$ units) were found with the qRT-PCR, similar to those obtained with the microarray. However, changes measured by qRT-PCR and microarray were not significantly correlated (Fig. 4.3; r=0.48, n=9, p=0.19). Similarly, changes in gene expression in response to folpet of the five most up-regulated (Fol-U1, Fol-U2, Fol-U3, Fol-U4 and Fol-U5) and most down-regulated (Fol-D-1, Fol-D3, Fol-D4, Fol-D5 and Fol-D6) measured by qRT-PCR were not significantly correlated (Fig. 4.4; r=0.23, n=10, p=0.52) with changes measured by the microarray. In the presence of folpet, changes in relative transcript abundance measured by qRT-PCR ranged from -1.8 to +0.6 $\log_2$ units, which was similar to the changes determined with the microarray.

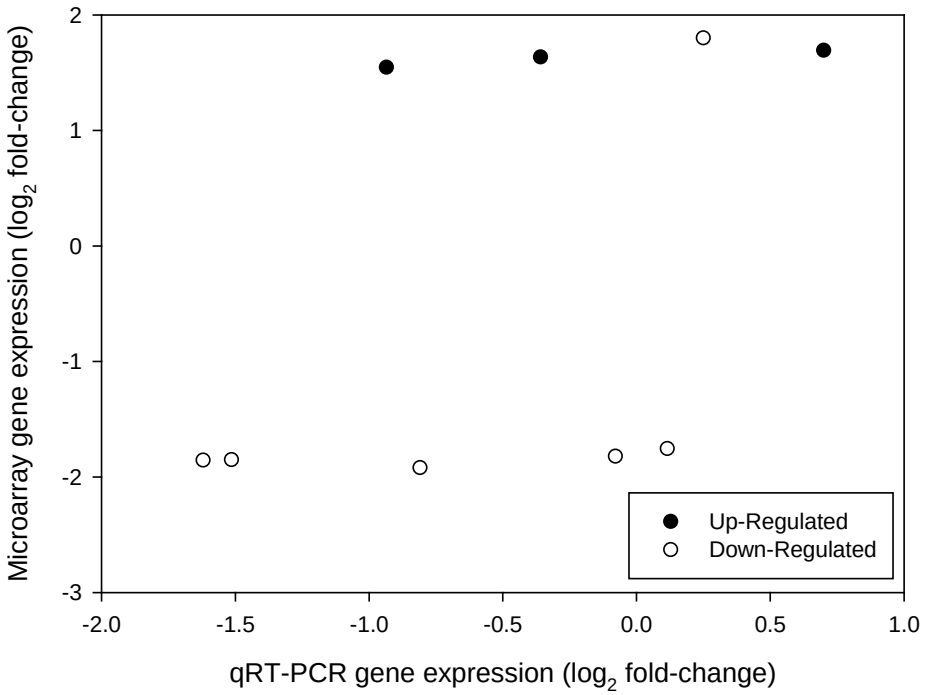

Figure 4.3 Correlation of gene expression of selected *Z. tritici* genes as determined by Affymetrix GeneChip expression array and qRT-PCR after 24 h exposure to 0.1 µg/mL of chlorothalonil in the log phase growth.

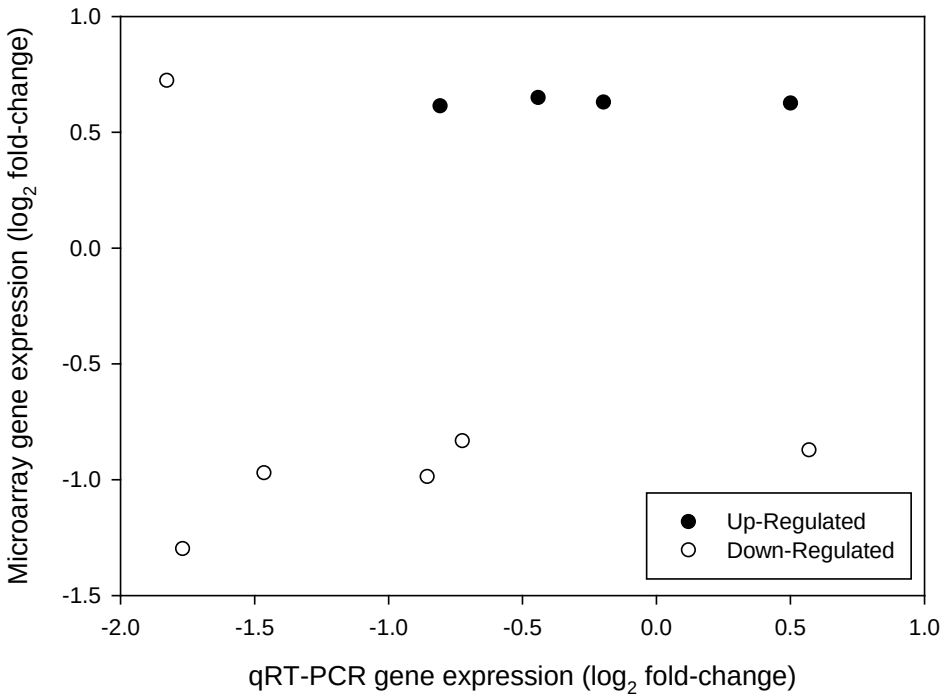

Figure 4.4 Correlation of gene expression of selected *Z. tritici* genes as determined by Affymetrix GeneChip expression array and qRT-PCR after 24 h exposure to 0.5 µg/mL of folpet in the log phase growth.

4.3.2 Transcriptional response of *Z. tritici* to chlorothalonil or folpet using RNA sequencing.

A total of 18 libraries were generated from RNA-sequencing with at least a Phred quality score Q of 30 till base-pair 100. Nine libraries of treatments applied in the lag phase of growth had an average of sequencing depth of 20 million reads. Approximately 94 % of the reads were mapped on the *Z. tritici* isolate IPO323 reference genome (Table 4.3). The other nine libraries, with treatments applied in the log phase of growth, had an average of 23 million reads. Approximately 93 % of the reads were mapped on the *Z. tritici* reference genome (Table 4.3).

Table 4.3 Summary of the *Z. tritici* isolate IPO323 transcriptome sequencing.

Growth Phase	Treatment	Sequencing Depth (reads)	Phred Quality Score (Q)	Mapped Read to the Reference Genome (%)
Lag Phase	Untreated 24 h	23,323,271	30	93.53
	Untreated 24 h	14,708,750	30	92.91
	Untreated 24 h	12,397,510	30	93.08
	Chlorothalonil	23,772,611	30	93.37
	Chlorothalonil	23,767,042	30	93.37
	Chlorothalonil	21,185,856	30	93.43
	Folpet	26,131,290	30	93.61
	Folpet	21,889,997	30	91.97
	Folpet	40,420,854	30	92.85
Log Phase	Untreated 48 h	21,739,748	30	94.09
	Untreated 48 h	22,526,784	30	94.56
	Untreated 48 h	19,588,972	30	93.79
	Chlorothalonil	20,788,327	30	94.20
	Chlorothalonil	21,269,962	30	94.18
	Chlorothalonil	15,635,753	30	94.08
	Folpet	19,280,171	30	93.76
	Folpet	20,386,665	30	93.90
	Folpet	20,243,005	30	94.66

Based on the number of predicted genes (10,933) in the *Z. tritici* isolate IPO323 reference genome (Goodwin et al., 2011), approximately 6 and 11 % of the IPO323 genes were significantly differentially expressed after exposure to chlorothalonil in the lag or log phase growth, respectively. Approximately 4 and 28 % of *Z. tritici* genes were differentially expressed after exposure to folpet in the lag or log phase growth, respectively.

The expression of 27 genes was consistently modulated in presence of either fungicide in either phase of growth (Fig. 4.5). Cluster analysis of these genes identified groups of genes with similar expression patterns in presence of chlorothalonil or folpet in the lag or log phases of growth (Fig. 4.6). Based on the available functional annotation, these 27 genes were mainly associated with carbohydrate metabolism, signal and transcriptional regulation, cell wall glycoproteins, phosphate transport, stress response, and MFS drug efflux pump activity (Fig. 4.6).

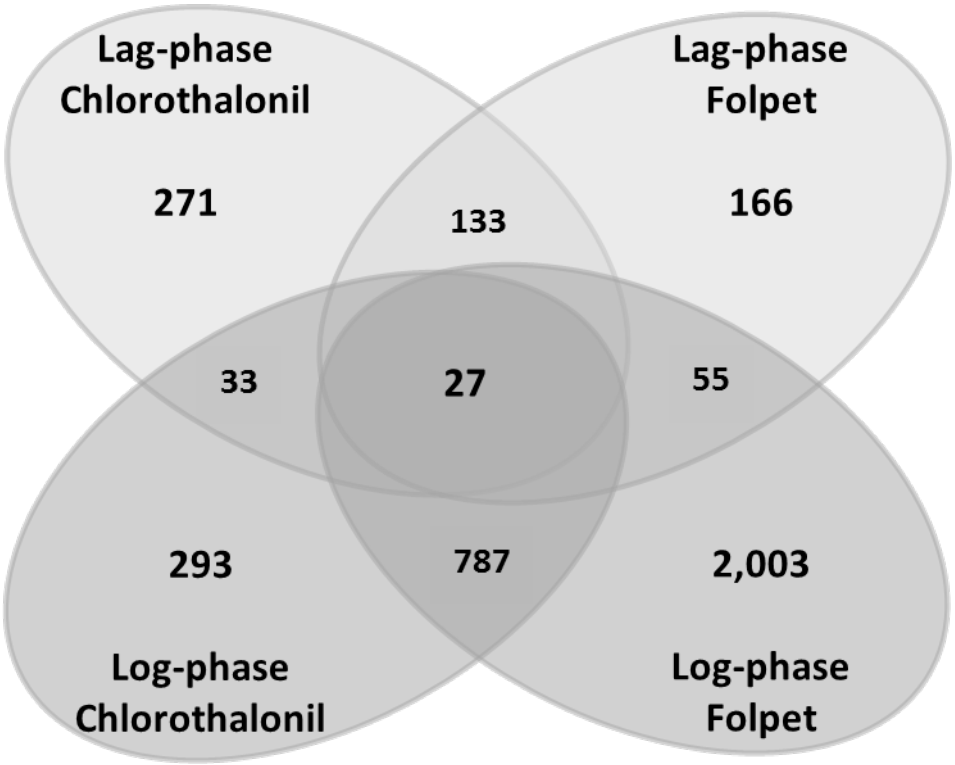

Figure 4.5 Comparison of the significantly (p<0.05; q<0.05) differentially expressed genes in *Z. tritici* after 24 h exposure to either 0.1 µg/mL of chlorothalonil or 0.5 µg/mL of folpet in either the lag or log phase growth.

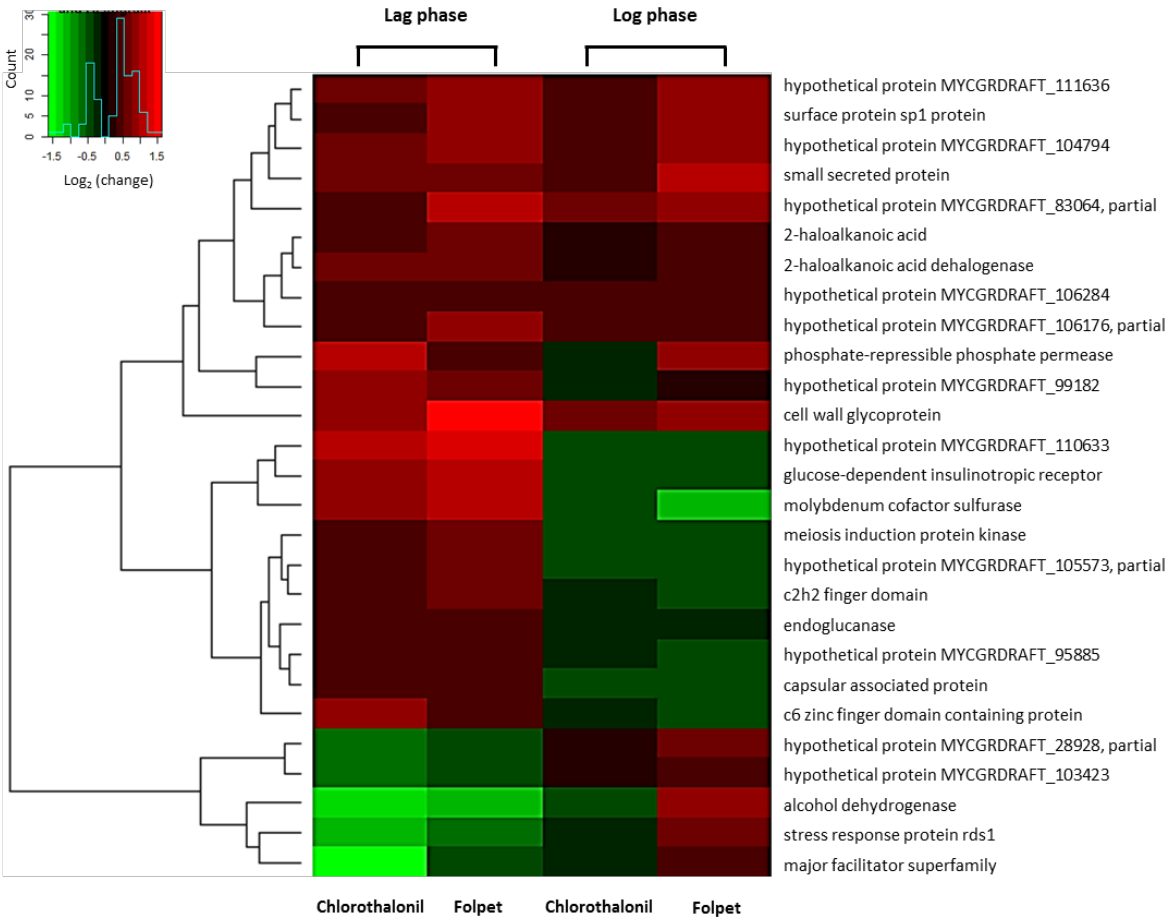

Figure 4.6 Expression pattern and putative function of the 27 genes consistently modulated in *Z. tritici* after 24 h exposure to either 0.1 µg/mL of chlorothalonil or 0.5 µg/mL folpet in either the lag or log phase of growth. Functional annotation of genes was carried out using Blast2GO software.

4.3.2.1 Genome-wide expression response of *Z. tritici* to chlorothalonil.

RNA-seq analysis found 668 genes which were significantly (p<0.05, q<0.05; Supplementary Table 4.1s) differentially expressed after 24 h exposure to chlorothalonil in the lag phase. Approximately 44 % of the DEGs showed increased expression (from +0.31 to +1.84 $\log_2$ change) while 66 % showed decreased expression (from -2.93 to -0.30 $\log_2$ change). Most of the significantly DEGs (approximately 98 %) were mapped to the 13 essential chromosomes of the *Z. tritici* isolate IPO323 genome (Fig 4.7). No GO-terms were significantly (p<0.05) commoner in the genes with significant changes in expression in response to chlorothalonil (Fisher's exact test with multiple testing correction of FRD<0.05). The top five up- or down-regulated genes were related mainly to protein folding or degradation (e.g. heat shock protein Hsp20, protein kinase and cyclin-like F-box), or glucose metabolism (i.e glycoside hydrolase and glycosyltransferase) (Table 4.4).

Comparison of chlorothalonil-DEGs in the lag or log phase treatment (Fig. 4.5) detected a set of 271 genes that were modulated only if chlorothalonil was added in the lag phase of growth (Supplementary Table 4.2s). Functional annotation and enrichment analysis of these genes revealed GO-terms that were significantly enriched (p<0.001; Fisher's exact test with multiple testing correction of FRD<0.05; Supplementary Table 4.3s). Enriched GO-terms in the biological process category were related to phosphorelay signal transduction system and signal transduction by phosphorylation (Fig. 4.8). Enriched GO-terms in the molecular function category included phosphorelay sensor kinase activity as the most specific GO-term (Fig. 4.9). Only protein histidine kinase complex GO-term was enriched in the cellular component category (Fig. 4.10).

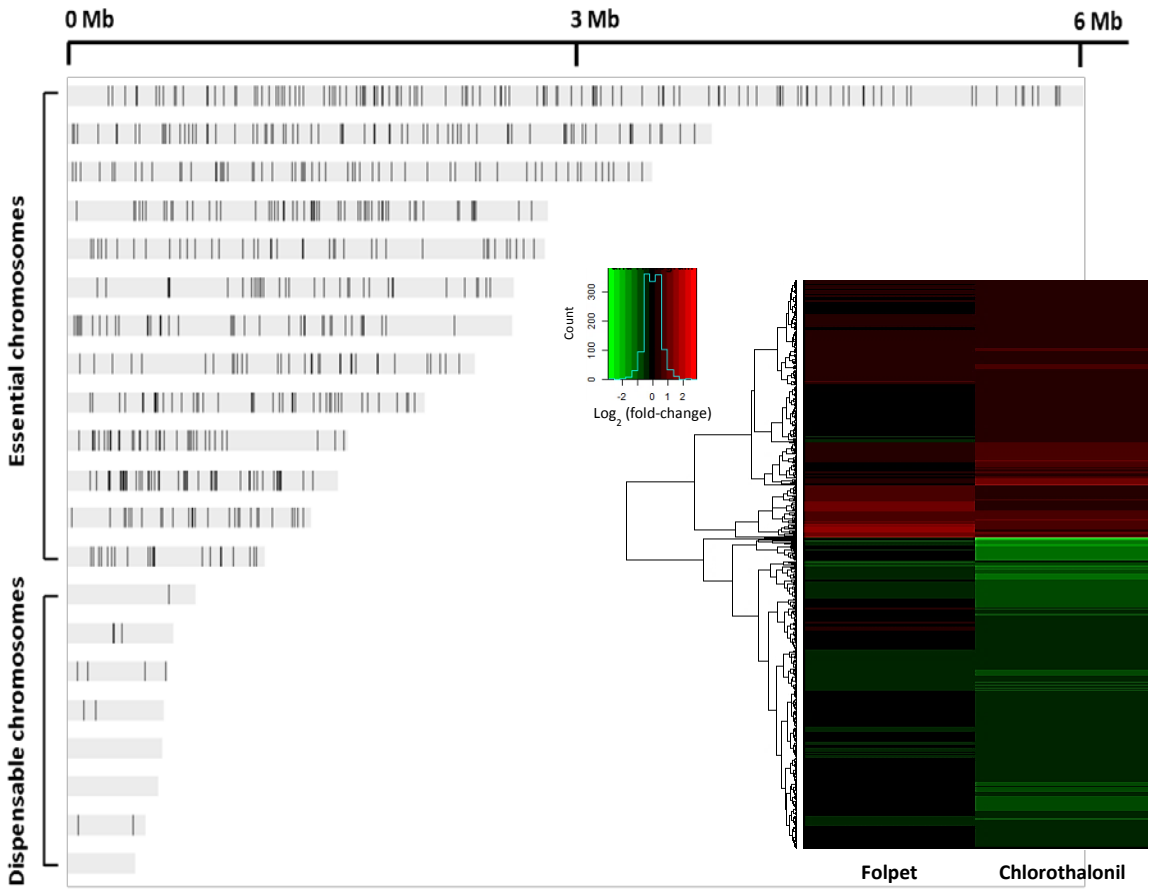

Figure 4.7 Distribution of the 668 significant (p<0.05; q<0.05) differentially expressed genes in the reference *Z. tritici* isolate IPO323 genome and their gene expression ratio (log_2 fold-change) after 24 h fungicide to 0.1µg/mL of chlorothalonil in the lag phase growth. Location of significant differentially expressed genes is indicated by black lines on each chromosome denoted in grey.

Table 4.4 The most significantly (p<0.05; q<0.05) differentially expressed genes in the *Z. tritici* after 24 h exposure to 0.1 µg/mL chlorothalonil in the lag phase growth.

Gene ID	JGI gene ID[1]	Description[2]	Log_2 (change)
LagChlo-U1	estExt_fgenesh1_pg.C_chr_20058	Hypothetical protein	+1.8
LagChlo-U2	estExt_fgenesh1_kg.C_chr_60209	Heat shock protein Hsp20	+1.4
LagChlo-U3	estExt_Genewise1Plus.C_chr_80251	Glycoside hydrolase, family 16	+1.3
LagChlo-U4	gw1.2.2194.1	Heat shock protein Hsp20	+1.2
LagChlo-U5	fgenesh1_pg.C_chr_4000436	H+-transporting two-sector ATPase, A subunit	+1.2
LagChlo-D1	e_gw1.8.1362.1	Hypothetical protein	-2.9
LagChlo-D2	estExt_fgenesh1_pg.C_chr_11227	Cyclin-like F-box (protein binding)	-2.2
LagChlo-D3	estExt_Genewise1Plus.C_chr_10184	Protein kinase, core	-1.8
LagChlo-D4	estExt_fgenesh1_kg.C_chr_140021	Hypothetical protein	-1.6
LagChlo-D5	gw1.1.1940.1	Glycosyltransferase, family 28	-1.6

[1] The Join Genome Institute (JGI) fungal program (Grigoriev et al., 2011)
[2] Description based on Blast2Go results

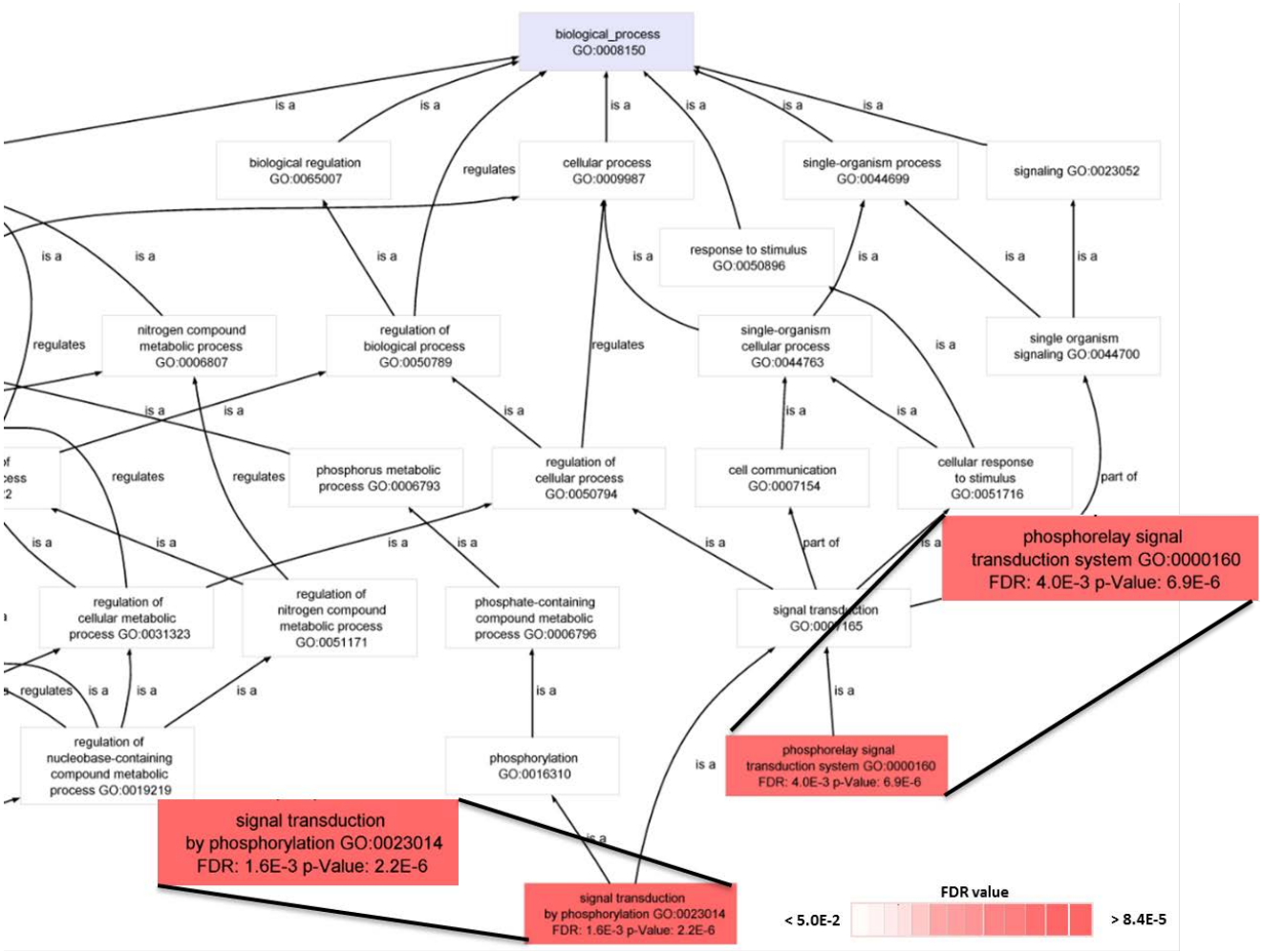

Figure 4.8 Biological process enriched graph of genes exclusively expressed in the lag phase of growth of *Z. tritici* after 24 h exposure to 0.1 µg/mL of chlorothalonil. Fisher's exact test with multiple testing correction of FDR<0.05. Nodes are coloured according to their FDR value in Fisher's exact test against the whole *Z. tritici* genome.

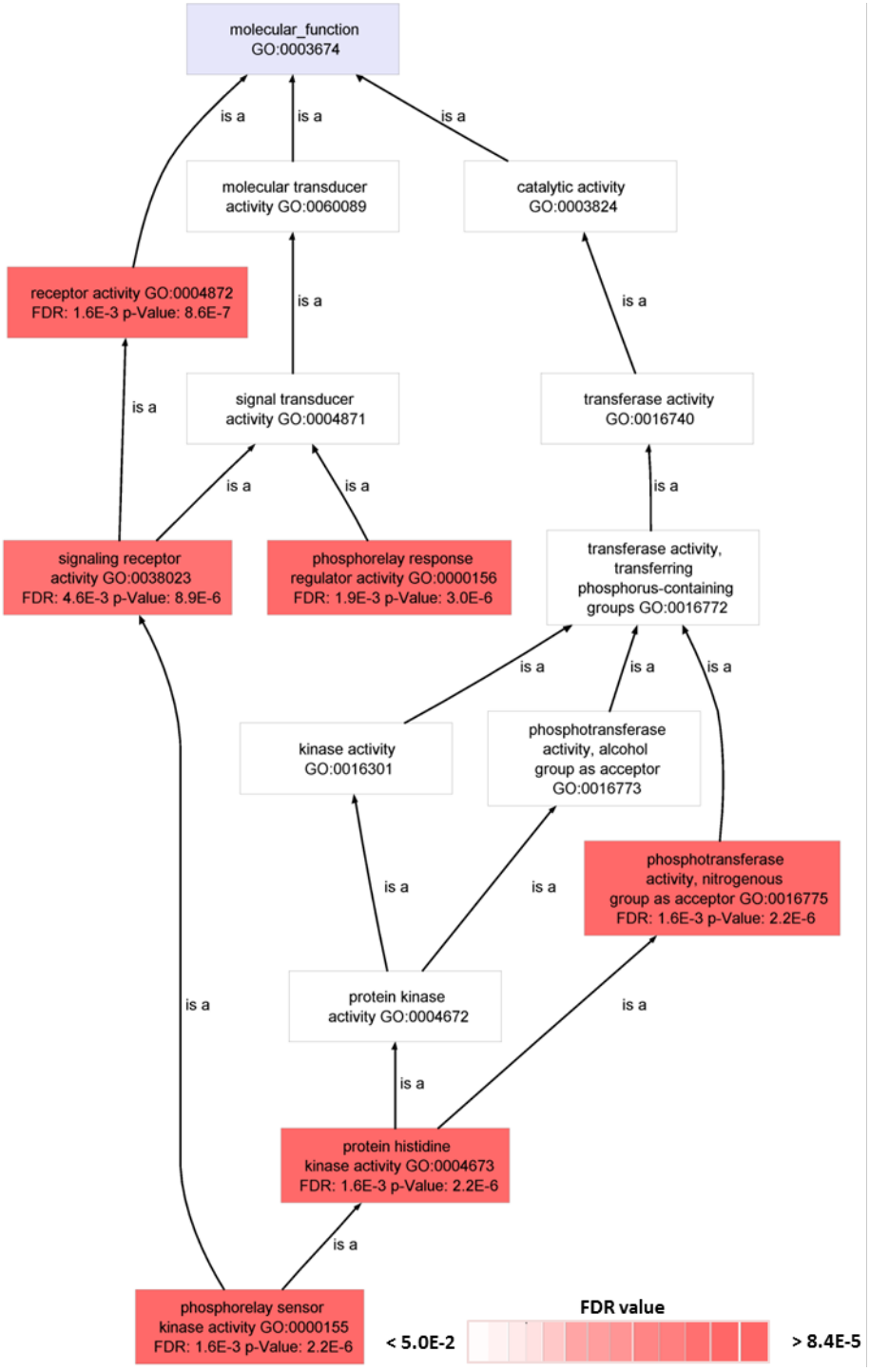

Figure 4.9 Molecular function enriched graph of genes exclusively expressed in the lag phase of growth of *Z. tritici* after 24 h exposure to 0.1 µg/mL of chlorothalonil. Fisher's exact test with multiple testing correction of FDR<0.05. Nodes are coloured according to their FDR value in Fisher's exact test against the whole *Z. tritici* genome.

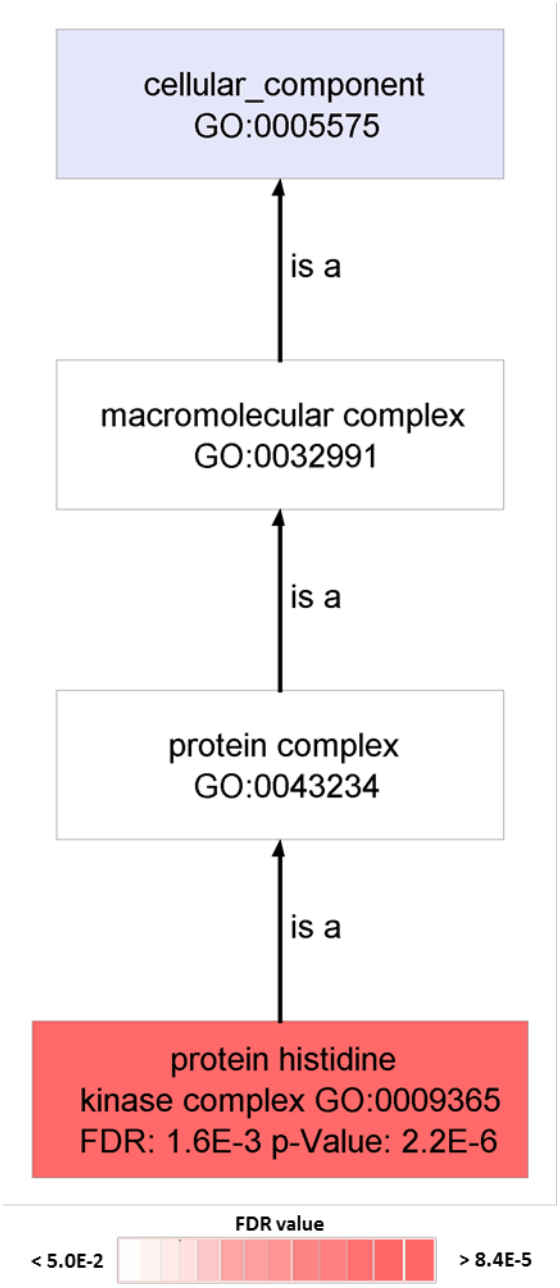

Figure 4.10 Cellular component enriched graph of genes exclusively expressed in the lag phase of growth of *Z. tritici* after 24 h exposure to 0.1 µg/mL of chlorothalonil. Fisher's exact test with multiple testing correction of FDR<0.05. Nodes are coloured according to their FDR value in Fisher's exact test against the whole *Z. tritici* genome.

More genes - a total of 1,151 - were differentially expressed (p<0.05, q<0.05; Supplementary Table 4.4s) in *Z. tritici* after exposure to chlorothalonil in the log phase of growth. Approximately 54 % of the chlorothalonil-DEGs were up-regulated (from +0.23 to +2.26 log_2 change) and the rest 46 % were down-regulated (from -1.57 to -0.23 log_2 change). According to functional annotation, top DEGs in the log phase of growth were related to transport of nitrogen or sodium, and heterocaryon incompatibility (Table 4.5). Most of the chlorothalonil-DEGs (approximately 98%) were located on the essential chromosomes of the genome (Fig. 4.11). Functional annotation and enrichment analysis of the significant chlorothalonil-DEGs revealed diverse GO-terms that were significantly enriched (p<0.001; Fisher's exact test with multiple testing correction of FRD<0.05; Supplementary Table 4.5s). Enriched GO-terms in the biological process category were related to oxidative phosphorylation, cellular respiration, alpha-amino acid catabolic process, glucose catabolic process and proton transport (Fig. 4.12). Enriched GO-terms in the molecular function category included cofactor binding, oxidoreductase activity, and oxidoreductase activity actin on NAD(P)H (Fig. 4.13). Proton-transporting V-type ATPase V_1 Domain and mitochondrion GO-terms were enriched in the cellular component category (Fig. 4.14).

Table 4.5 The most significant (p<0.05, q<0.05) differentially expressed genes in the *Z. tritici* after 24 h exposure to 0.1 µg/mL chlorothalonil in the log phase growth.

Gene ID	JGI gene ID	Description[2]	Log_2 (change)
LogChlo-U1	estExt_Genewise1Plus.C_chr_40627	Na+/solute symporter	+2.3
LogChlo-U2	estExt_fgenesh1_pm.C_chr_120122	Formate/nitrite transporter	+1.9
LogChlo-U3	estExt_Genewise1Plus.C_chr_100358	Pyridine nucleotide-disulphide oxidoreductase, class-II	+1.5
LogChlo-U4	estExt_Genewise1Plus.C_chr_13116	Arginase	+1.4
LogChlo-U5	estExt_fgenesh1_pg.C_chr_60151	"BPD_TRANSP_INN_MEMBR"	+1.3
LogChlo-D1	fgenesh1_pg.C_chr_16000068	Hypothetical protein	-1.3
LogChlo-D2	fgenesh1_pg.C_chr_3000370	Hypothetical protein	-1.3
LogChlo-D3	fgenesh1_pg.C_chr_7000254	Zinc finger, RING-type	-0.9
LogChlo-D4	fgenesh1_pg.C_chr_12000014	Hypothetical protein	-0.9
LogChlo-D5	gw1.10.406.1	Heterokaryon incompatibility	-0.9

[1] The Join Genome Institute (JGI) fungal program (Grigoriev et al., 2011)
[2] Description based on Blast2Go results

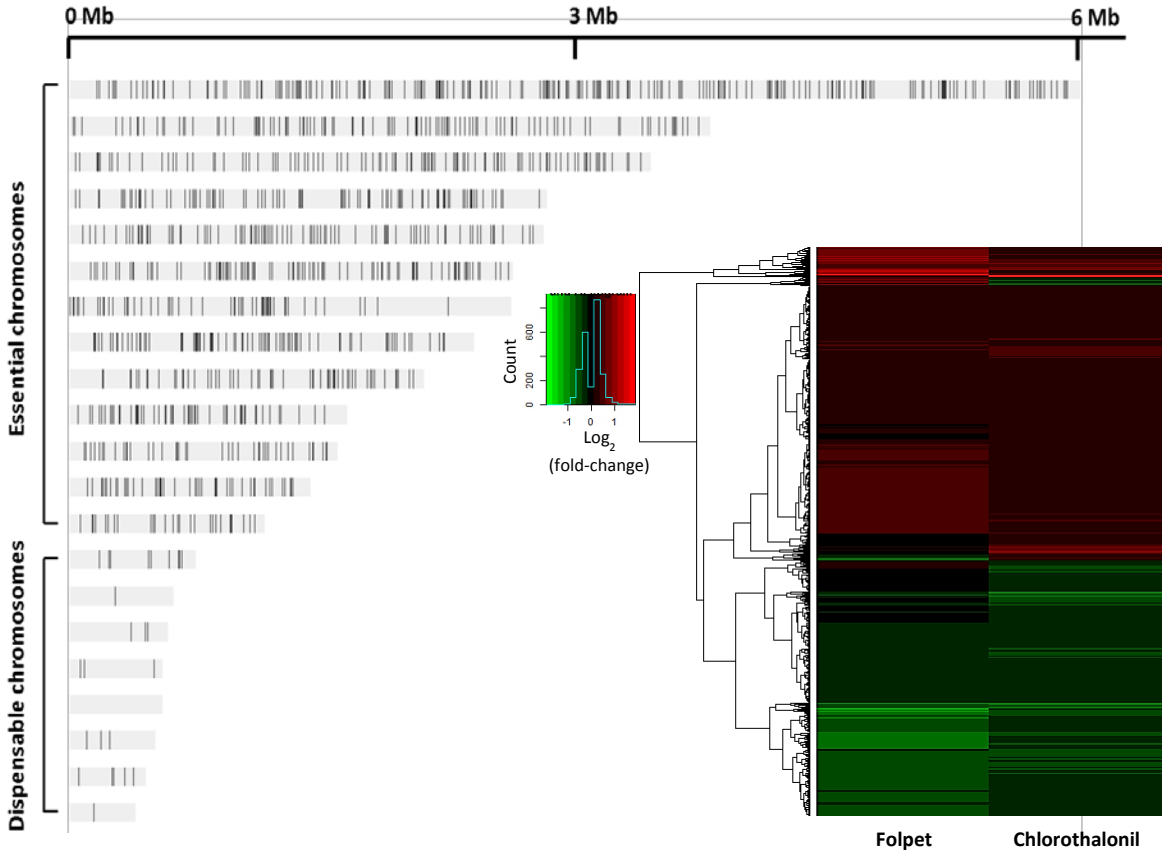

Figure 4.11 Distribution of the 1,151 significantly (p<0.05; q<0.05) differentially expressed genes in the reference *Z. tritici* isolate IPO323 genome and their gene expression ratio (log_2 fold-change) after 24 h exposure to 0.1 µg/mL of chlorothalonil in the log phase growth. Location of significant differentially expressed genes is indicated by black lines on each chromosome denoted in grey.

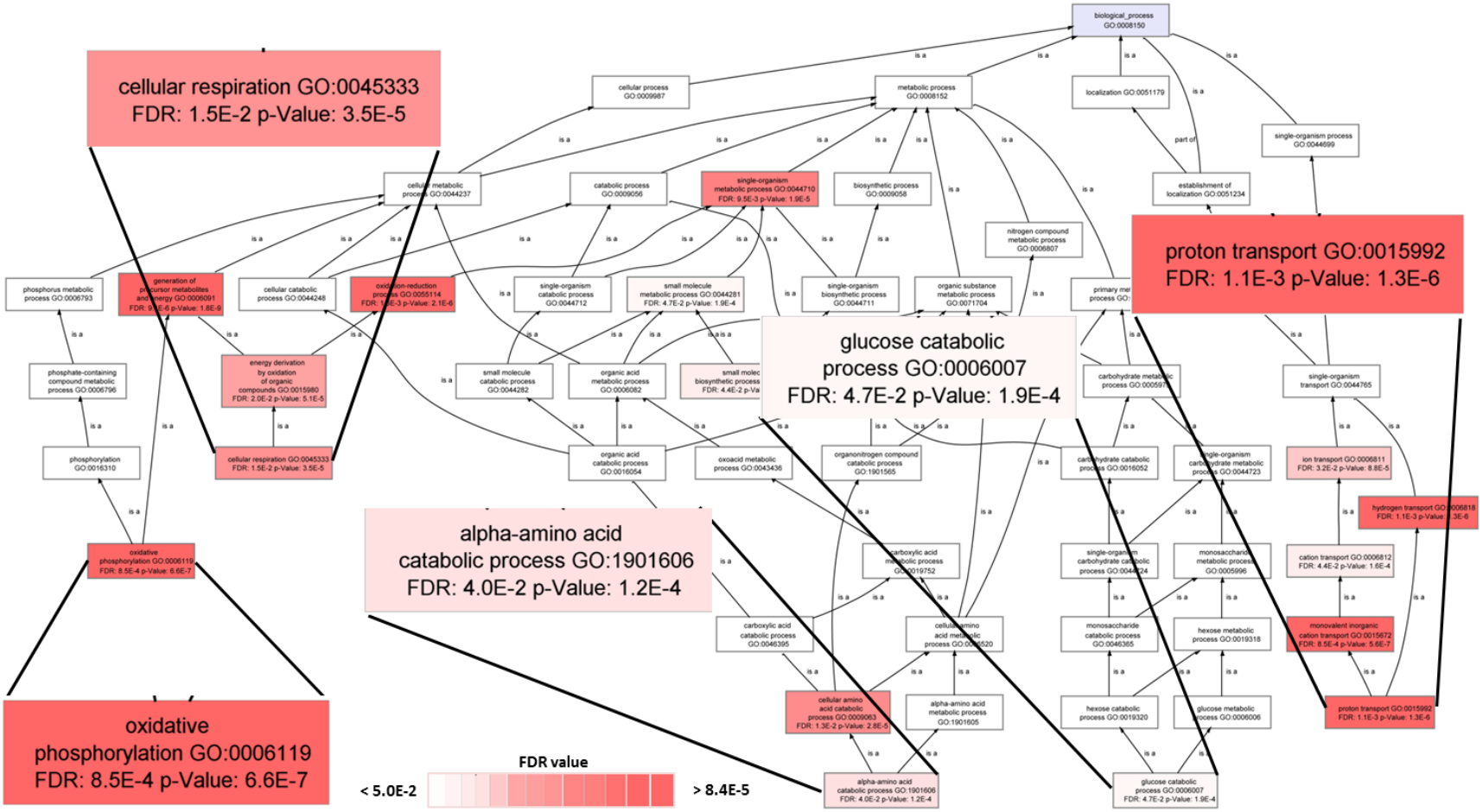

Figure 4.12 Biological process enriched graph of the significantly (*p*<0.05; *q*<0.05) differentially expressed genes in *Z. tritici* after 24 h exposure to 0.1 µg/mL of chlorothalonil in the log phase growth. Fisher's exact test with multiple testing correction of FDR<0.05. Nodes are coloured according to their FDR value in the Fisher's exact test against the whole *Z. tritici* genome.

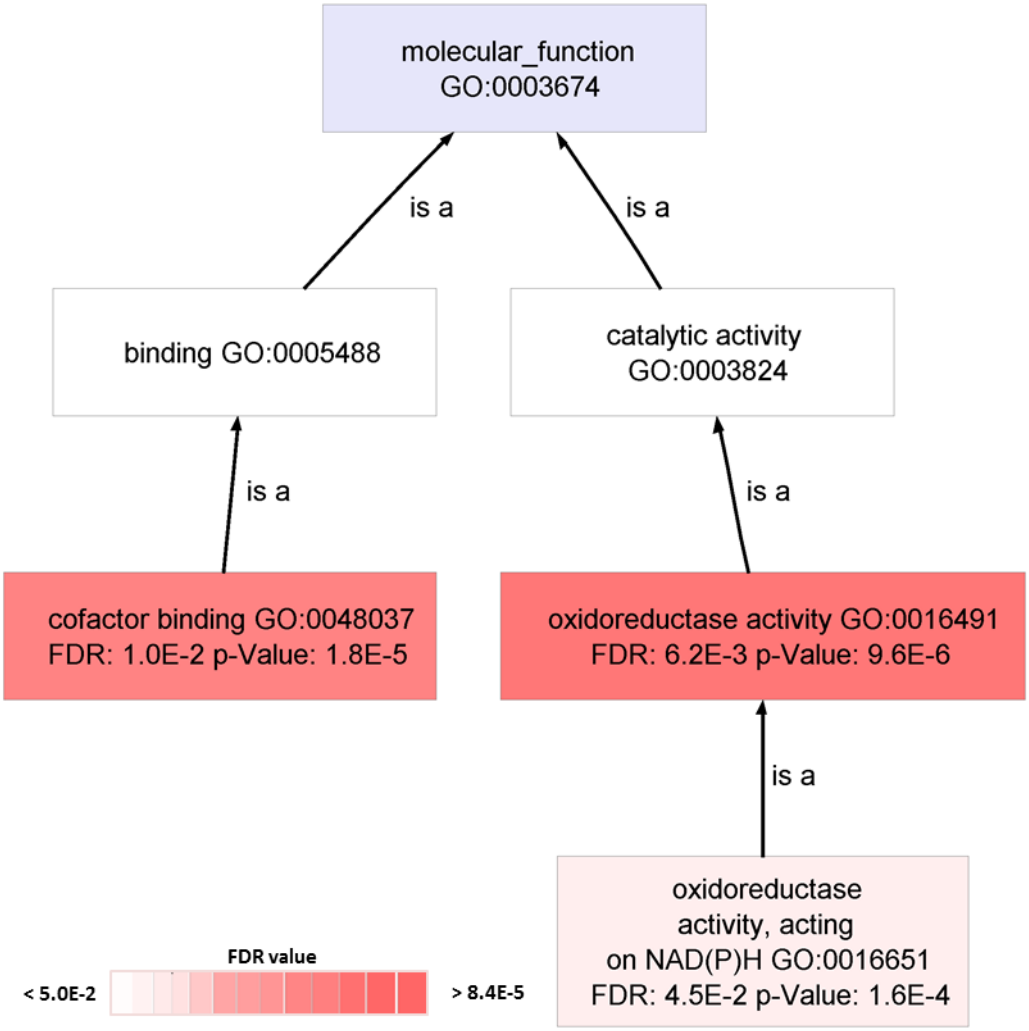

Figure 4.13 Molecular function enriched graphs of the significantly (*p*<0.05; *q*<0.05) differentially expressed genes in *Z. tritici* after 24 h exposure to 0.1 µg/mL of chlorothalonil in the log phase growth. Fisher's exact test with multiple testing correction of FDR<0.05. Nodes are coloured according to their FDR value in the Fisher's exact test against the whole *Z. tritici* genome.

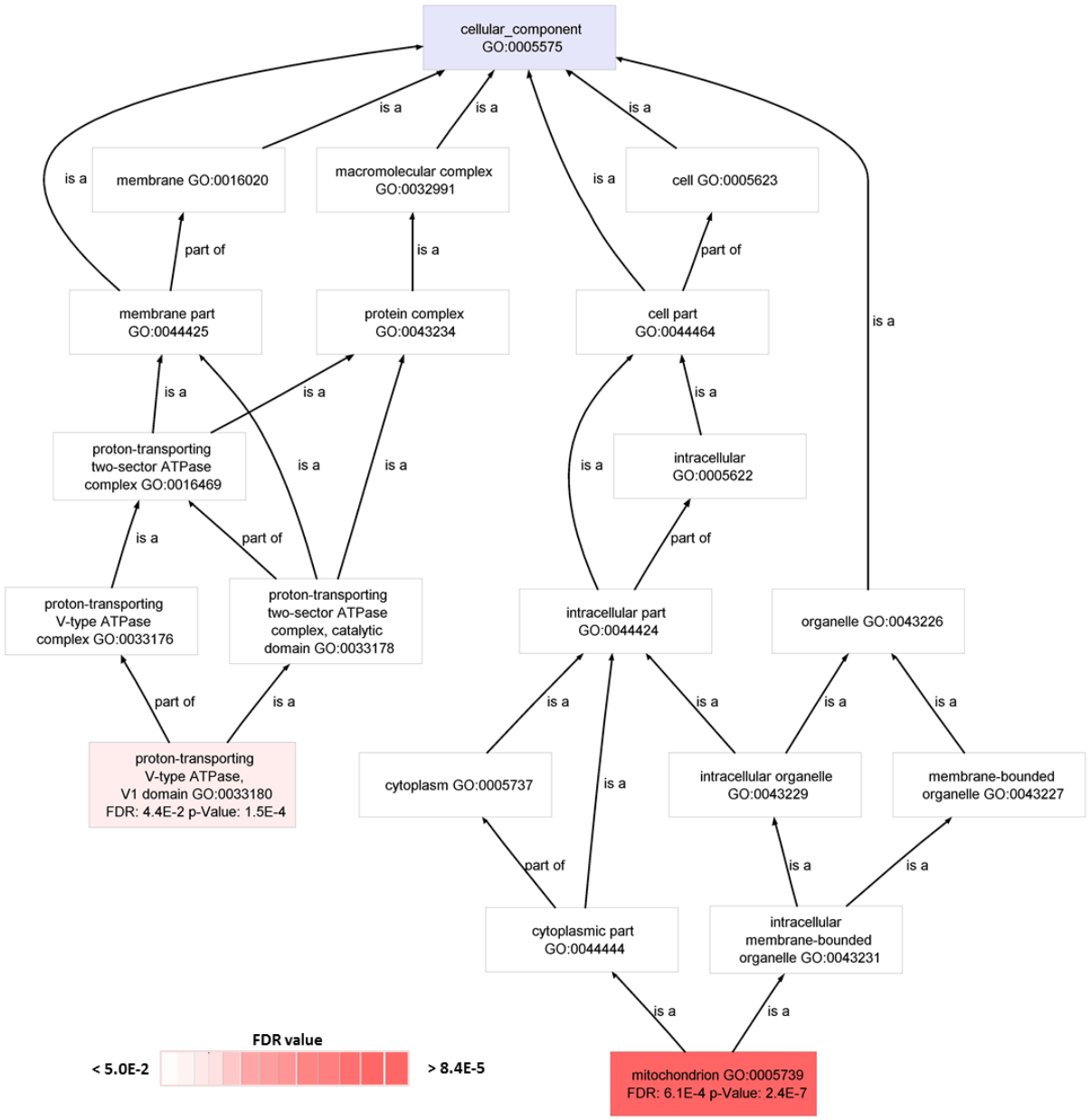

Figure 4.14 Cellular component enriched graphs of the significantly (*p*<0.05; *q*<0.05) differentially expressed genes in *Z. tritici* after 24 h exposure to 0.1 µg/mL of chlorothalonil in the log phase growth. Fisher's exact test with multiple testing correction of FDR<0.05. Nodes are coloured according to their FDR value in the Fisher's exact test against the whole *Z. tritici* genome.

In addition, comparing DEGs from each treatment (Fig. 4.5), a set of 293 genes was detected to be modulated only if chlorothalonil was added in the log phase growth (Supplementary Table 4.6s). No GO-terms were significantly (p<0.05) commoner in this set of genes (Fisher's exact test with multiple testing correction of FRD<0.05). The top five up- or down-regulated genes were related to transport and metabolism of nitrogen, and heterocaryon incompatibility (Table 4.6)

Table 4.6 Probable functions of genes exclusively modulated in *Z. tritici* after 24 h exposure to 0.1 µg/mL of chlorothalonil in the log phase of growth.

JGI Gene ID[1]	Hits	min. eValue	Similarity (mean %)	Description[2]	log_2 (change)
estExt_fgenesh1_pg.C_chr_110320	20	0	74.6	nitrate transporter	+1.2
estExt_Genewise1Plus.C_chr_40795	20	0	84.05	mfs sugar transporter	+1.1
estExt_Genewise1Plus.C_chr_20574	20	0	61.65	pq loop repeat protein	+1.1
fgenesh1_pg.C_chr_7000585	1	0	100	t-type voltage-gated ca2+ alpha1i subunit	+0.9
estExt_fgenesh1_pg.C_chr_100170	20	0	78.75	nitrate reductase	+0.8
fgenesh1_pg.C_chr_16000068	1	9.69E-144	100	hypothetical protein	-1.3
fgenesh1_pg.C_chr_3000370	1	9.40E-97	100	hypothetical protein	-1.3
fgenesh1_pg.C_chr_7000254	3	0	69.33	hypothetical protein	-1.0
fgenesh1_pg.C_chr_12000014	1	5.91E-39	100	hypothetical protein	-0.9
gw1.10.406.1	20	6.81E-110	59.7	heterokaryon incompatibility protein	-0.9

[1] The Join Genome Institute (JGI) fungal program (Grigoriev et al., 2011)
[2] Description based on Blast2Go results

A total of 33 genes were modulated in both the lag and log phase after chlorothalonil exposure (Fig. 4.5 and 4.15; Supplementary Table 4.7s). No GO-terms were significantly (p<0.05) commoner in this set of genes (Fisher's exact test with multiple testing correction of FRD<0.05). Approximately, 75 % of these genes have functional annotation. Genes related to transport of carbohydrate or proteins across the cell membrane, and protein binding were identified (Fig. 4.15). Interestingly, genes involved in carbon catabolite or nitrogen metabolite repression were down- or up-regulated after chlorothalonil exposure in the lag or log phase, respectively. Carbon catabolite or nitrogen metabolism repression can be regulated by environmental and internally changes of glucose or nitrogen availability (see Kim et al., 2013; see Marzluf, 1993).

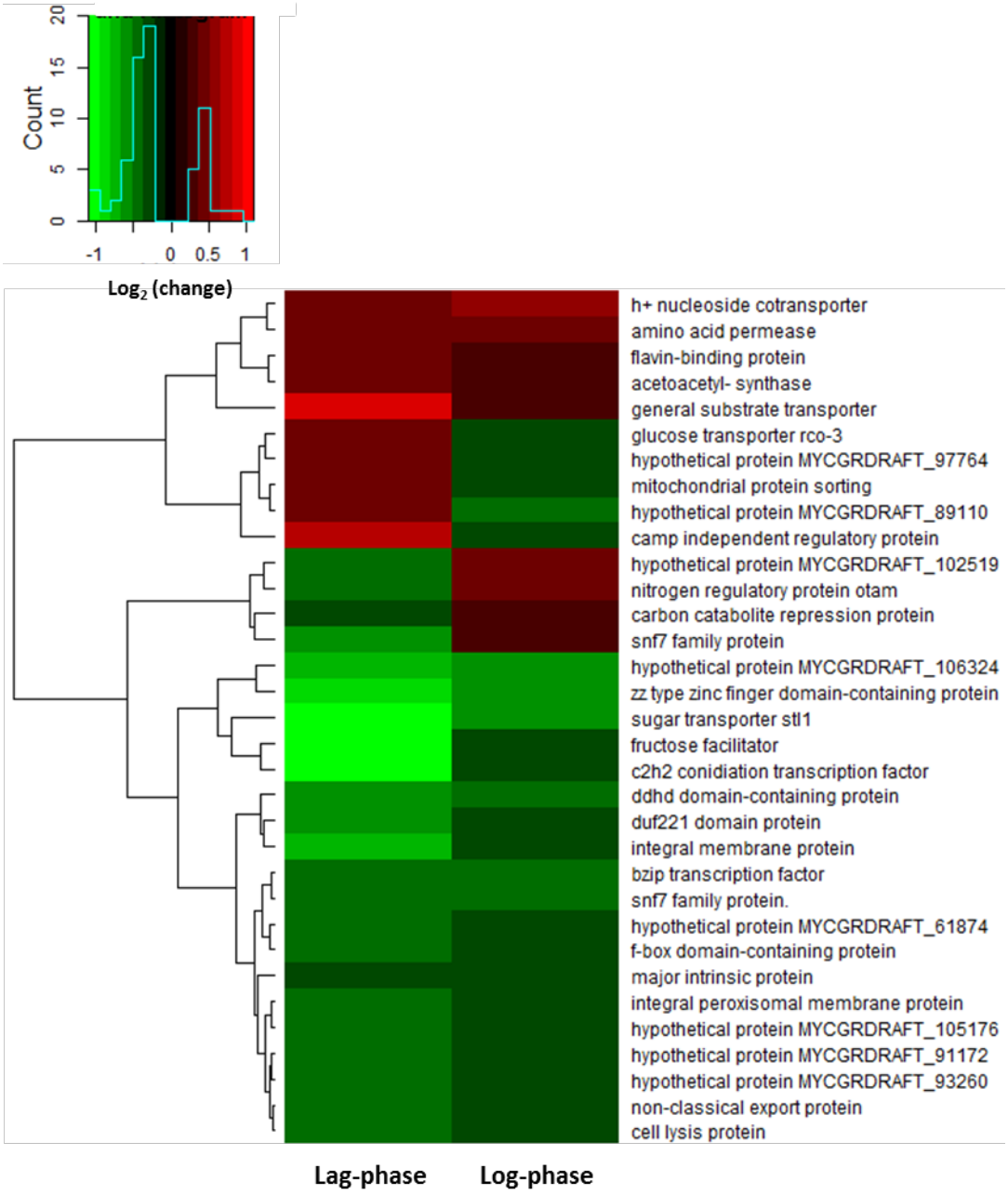

Figure 4.15 Expression patter and putative function of 33 genes consistently modulated in either the lag or log phase of growth of *Z. tritici* after 24 h exposure to 0.1 µg/mL of chlorothalonil, but not statistically significant modulated in response to folpet. Functional annotation of genes was carried out using Blast2Go software.

4.3.2.2 Genome-wide expression response of *Z. tritici* to folpet.

A total of 419 genes were significantly (p<0.05, q<0.05; Supplementary Table 4.8s) differentially expressed in response to folpet. Approximate 78 % of these showed increased gene expression (from +0.31 to +2.33 log_2 change) and around 22 % showed reduced expression (from +1.07 to -0.31 log_2 change). Approximate 97 % of folpet-DEGs were located on essential chromosomes (Fig. 4.16).

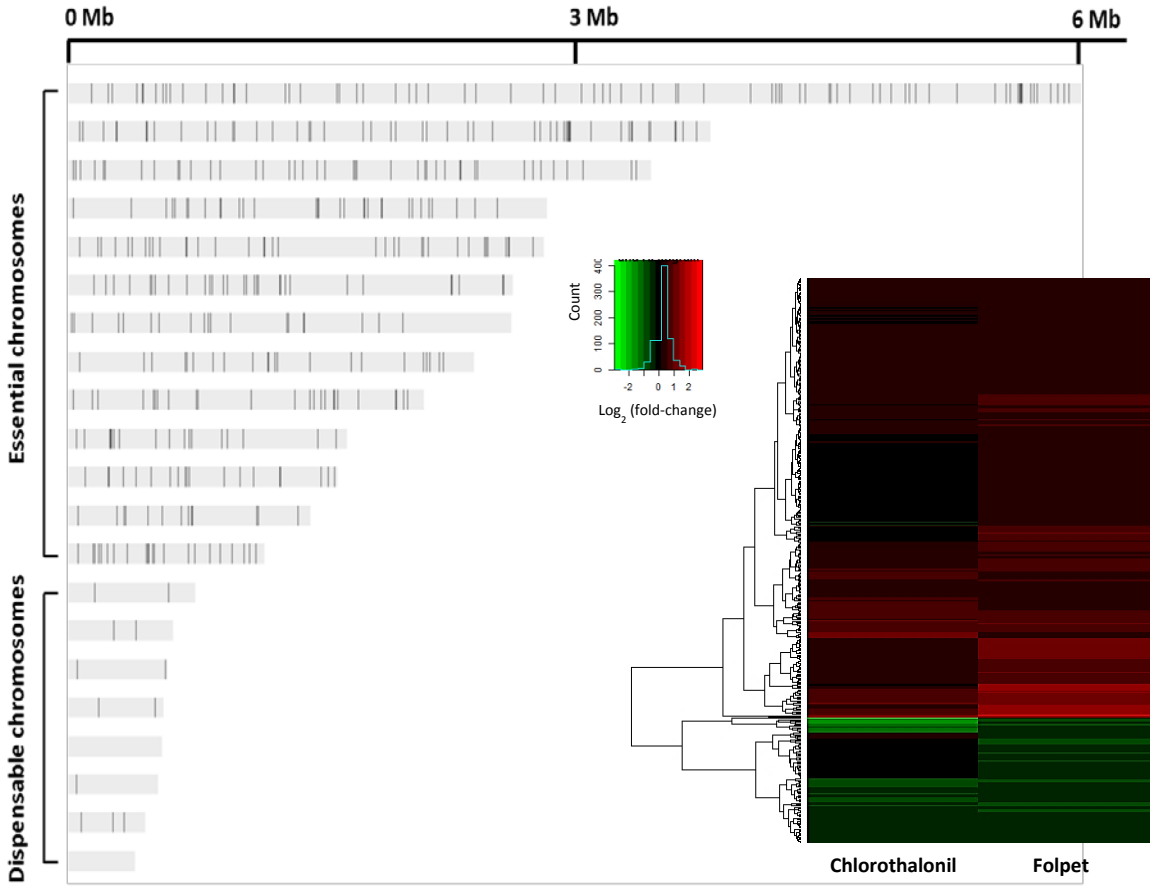

Figure 4.16 Distribution of the 419 significant (p<0.05; q<0.05) differentially expressed genes in the reference *Z. tritici* isolate IPO323 genome and gene expression (log_2 fold-change) after 24 h exposure to 0.5µg/mL of folpet in the lag phase growth. Location of significant differentially expressed genes is indicated by black lines on each chromosome denoted in grey.

The 419 significant folpet-DEGs lie disproportionately (p<0.05) in any functional groups. However, some GO-terms were enriched (p<0.001; Fisher's exact test with multiple testing correction of FDR<0.05; Supplementary Table 4.9s) when the analysis was restricted to significant DEGs with expression ratio > +1.0 and < -1.0 $\log_2$ change (53 genes; Supplementary Table 4.10s). In particular, GO-terms connected with proteolysis, peptidase activity (serine-type carboxypeptidase and aspartic-type endopeptidase activity), and gene products acting in extracellular region were significantly commoner than expected from the distribution of terms in the whole dataset (Fig. 4.17). The top ten DEGs in response to folpet were mainly genes connected with catabolic activity, such as lipase, aspartic peptidase, cutinase, alcohol dehydrogenase zinc-type and thioesterase (Table 4.7).

Table 4.7 The most significant (p<0.05; q<0.05) differentially expressed genes in the *Z. tritici* after 24 h exposure to 0.5 µg/mL folpet in the lag phase growth.

Gene ID	JGI gene ID[1]	Description[2]	Log_2 (change)
LagFol-1U	gw1.2.2368.1	Hypothetical protein	+2.3
LagFol-2U	gw1.11.380.1	Lipase, GDSL	+2.0
LagFol-3U	fgenesh1_pg.C_chr_4000212	Hypothetical protein	+1.9
LagFol-4U	estExt_fgenesh1_pg.C_chr_20058	Unique hypothetical protein Z. tritici	+1.8
LagFol-5U	fgenesh1_pg.C_chr_4000425	Aspartic Peptidase A1	+1.7
LagFol-1D	e_gw1.1.3942.1	Cutinase	-1.1
LagFol-2D	estExt_Genewise1Plus.C_chr_10891	Zinc-containing alcohol dehydrogenase superfamily (ADH)	-0.9
LagFol-3D	fgenesh1_pm.C_chr_1000920	family transcriptional	-0.9
LagFol-4D	fgenesh1_pg.C_chr_5000064	Hypothetical protein	-0.8
LagFol-5D	gw1.1.1706.1	Thioesterase superfamily	-0.8

[1] The Join Genome Institute (JGI) fungal program (Grigoriev et al., 2011)
[2] Description based on Blast2Go results

Additionally, comparing DEGs from each treatment identified 166 genes that were modulated only in the lag phase after folpet exposure (Supplementary Table 4.11s). This set of genes lies disproportionately in any functional groups (Fisher's exact test with multiple testing correction of FRD<0.05). Interestingly, top five up- or down-regulated genes with functional annotation were involved mainly in catabolic activity (e.g. lipase, aspartic peptidase and thioesterase) (Table 4.8).

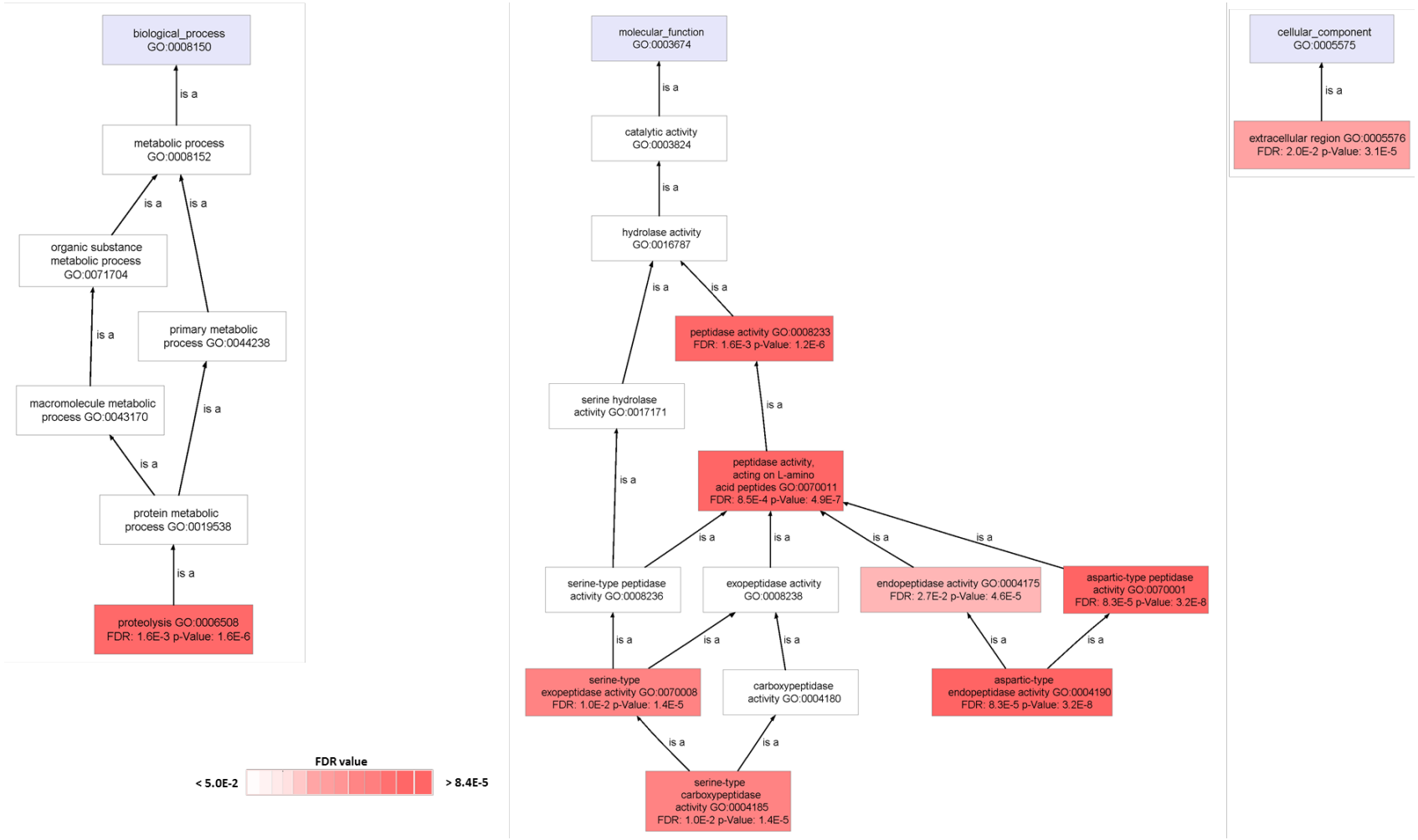

Figure 4.17 Biological process, molecular function and cellular component enriched graph of the significantly (p<0.05; q<0.05) differentially expressed genes in *Z. tritici* after 24 h exposure to 0.5 µg/mL of folpet in the lag phase growth. Fisher's exact test with multiple testing correction of FDR<0.05. Nodes are coloured according to their FDR value in Fisher's exact test against the whole *Z. tritici* genome.

Table 4.8 Probable functions of genes exclusively modulated in *Z. tritici* after 24 h exposure to 0.5 µg/mL of folpet in the lag phase of growth.

JGI Gene ID[1]	Hits	min. eValue	Similarity (mean %)	Description[2]	log_2 (change)
gw1.11.380.1	20	0	63.5	Lipase, GDSL	+2.0
fgenesh1_pg.C_chr_4000425	20	0	48.5	Aspartic Peptidase A1	+1.7
fgenesh1_pg.C_chr_8000551	1	1.13E-75	100	hypothetical protein	+1.6
estExt_fgenesh1_kg.C_chr_30061	1	1.99E-40	100	hypothetical protein	+1.5
fgenesh1_pg.C_chr_9000039	1	1.81E-65	100	hypothetical protein	+1.5
e_gw1.1.3942.1	20	3.95E-71	62.4	cutinase	-1.1
fgenesh1_pm.C_chr_1000920	20	0	63.7	family transcriptional	-0.9
fgenesh1_pg.C_chr_5000064	2	3.7E-107	85.0	hypothetical protein	-0.8
gw1.1.1706.1	20	7.82E-67	68.5	thioesterase superfamily protein	-0.8
fgenesh1_pg.C_chr_20000061	4	0	57.8	hypothetical protein	-0.8

[1] The Join Genome Institute (JGI) fungal program (Grigoriev et al., 2011)
[2] Description based on Blast2Go results

Exposing *Z. tritici* to folpet in the log phase modulated more genes (3,091; $p<0.05$, $q<0.05$; Supplementary Table 4.12s) than in the lag phase. Approximately, 52 % of the folpet-DEGs were up-regulated (from +0.23 to +2.13 log_2 change) and the rest, 48 %, were down-regulated ranging from -1.57 to -0.23 log_2 change. Around 98 % of the folpet-DEGs were located on the essential chromosomes *Z. tritici* genome (Fig. 4.18). Functional annotation and enrichment analysis revealed more diverse GO-terms enriched ($p<0.001$; Fisher's exact test with multiple testing correction of FRD<0.05; Supplementary Table 4.13s) than the chlorothalonil treatment - also in the log phase -. In the biological process category, GO-terms connected with cellular respiration, oxidative phosphorylation, carboxylic acid metabolic process (e.g. synthesis and metabolism of sulphur-, branched- and α-amino acids), translation, glycolysis, proton transport and ribosome biogenesis were significantly enriched (Fig. 4.19). In the molecular function category, cofactor binding, FMN binding, structural constituents of ribosomes, oxidoreductase, and "oxidoreductase activity acting on NAD(P)H, quinone or similar compound as acceptor" were significantly enriched (Fig. 4.20). In the cellular component category, GO-terms related to structure of ribosome, mitochondrion (i.e. mitochondrial membrane and inner mitochondrial membrane), and gene products localised in the cytosol were significantly enriched (Fig. 4.21). Additionally, genes involved in glycosylation were the most up- and copper transport the most down-regulated (Table 4.9).

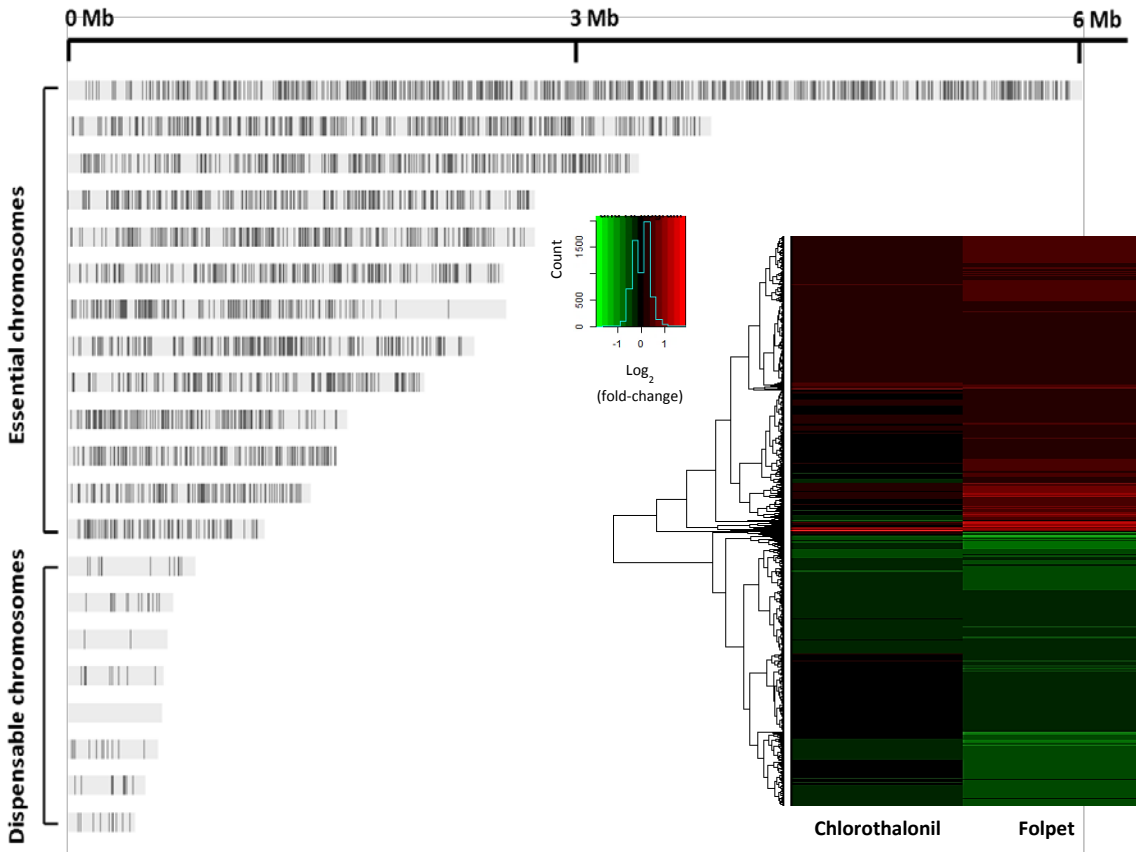

Figure 4.18 Distribution of the 3,091 significantly (p<0.05; q<0.05) differentially expressed genes in the reference *Z. tritici* isolate IPO323 genome and gene expression (log_2 fold-change) after 24 h exposure to 0.5 µg/mL of folpet in the log phase growth. Location of significant differentially expressed genes is indicated by black lines on each chromosome denoted in grey.

Table 4.9 The most significant (p<0.05, q<0.05) differentially expressed genes in the *Z. tritici* after 24 h exposure to 0.5 µg/mL folpet in the log phase growth.

Gene ID	JGI gene ID[1]	Description[2]	Log2 (change)
LogFol-U1	e_gw1.2.536.1	Glycosyltransferase Family 2 protein	+2.1
LogFol-U2	fgenesh1_pg.C_chr_10000003	Hypothetical protein	+2.0
LogFol-U3	e_gw1.4.1123.1	Hypothetical protein	+1.9
LogFol-U4	fgenesh1_pg.C_chr_3000638	Hypothetical protein	+1.8
LogFol-U5	fgenesh1_pg.C_chr_19000003	Myb, DNA-binding	+1.8
LogFol-D1	estExt_Genewise1Plus.C_chr_120164	copper transporter (Ctr)	-1.6
LogFol-D2	e_gw1.6.1406.1	Hypothetical protein	-1.5
LogFol-D3	fgenesh1_pg.C_chr_1001542	Voltage-gated potassium channels	-1.2
LogFol-D4	estExt_Genewise1Plus.C_chr_13616	Flavoprotein monooxygenase (oxidoreductase activity)	-1.2
LogFol-D5	estExt_fgenesh1_pg.C_chr_40586	Hypothetical protein	-1.2

[1] The Join Genome Institute (JGI) fungal program (Grigoriev et al., 2011)
[2] Description based on Blast2Go results

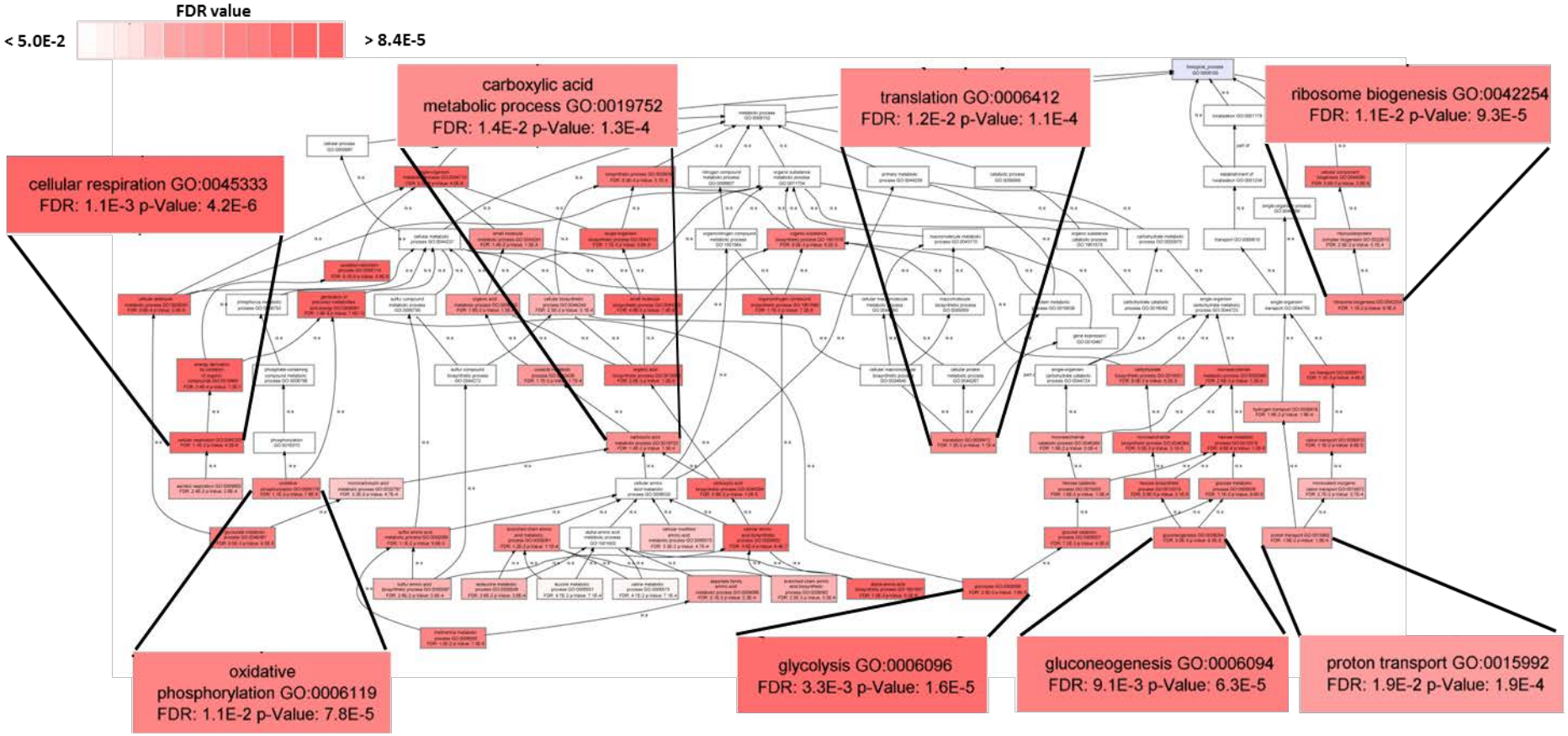

Figure 4.19 Biological process enriched graph of the significant (p<0.05; q<0.05) differentially expressed genes in *Z. tritici* after 24 h exposure to 0.5 µg/mL of folpet in the log phase growth. Fisher's exact test with multiple testing correction of FDR<0.05. Nodes are coloured according to their FDR value in Fisher's exact test against the whole *Z. tritici* genome.

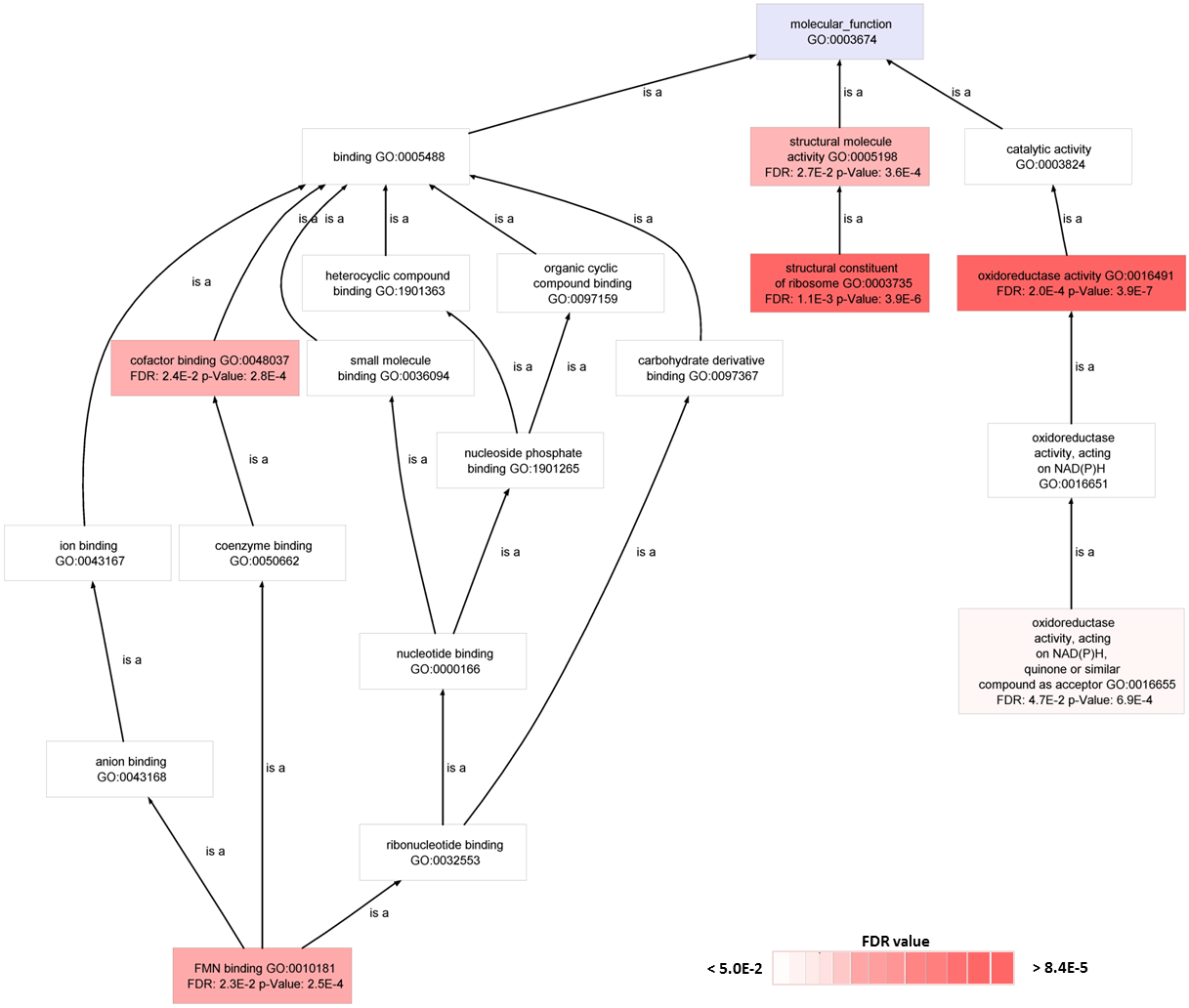

Figure 4.20 Molecular function enriched graph of the significant (*p*<0.05; *q*<0.05) differentially expressed genes in *Z. tritici* after 24 h exposure to 0.5 µg/mL of folpet in the log phase growth. Fisher's exact test with multiple testing correction of FDR<0.05. Nodes are coloured according to their FDR value in the Fisher's exact test against the whole *Z. tritici* genome.

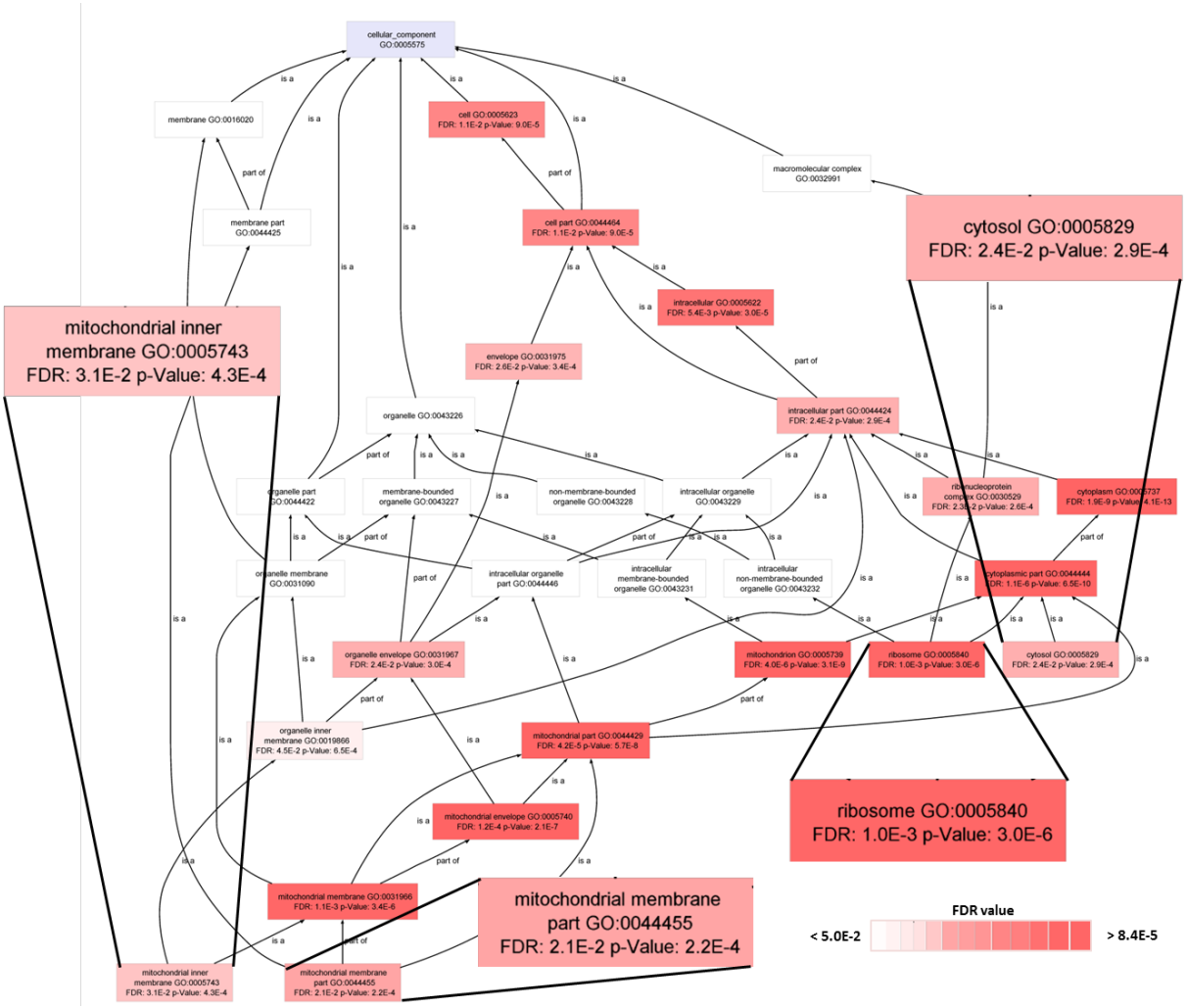

Figure 4.21 Cellular component enriched graph of the significantly (p<0.05; q<0.05) differential expressed genes in *Z. tritici* after 24 h exposure to 0.5 µg/mL of folpet in the log phase growth. Fisher's exact test with multiple testing correction of FDR<0.05. Nodes are coloured according to their FDR value in the Fisher's exact test against the whole *Z. tritici* genome.

Comparing DEGs from each treatment, a total of 2,003 genes of the 3,091 folpet-DEGs were modulated only in the log phase after folpet exposure (Supplementary Table 4.14s). Enrichment analysis of these genes found GO-terms enriched (p<0.001; Fisher's exact test with multiple testing correction of FRD<0.05; Supplementary Table 4.15s) with genes involved in translation, cellular amino acid biosynthesis and metabolism, glycolipid metabolism, membrane lipid biosynthesis, and assembly of cellular macromolecules complex in the biological process category (Fig. 4.22). In the cellular component category, a GO-term related to structure of the ribosomes was significantly enriched (Fig. 4.23; p<0.001; Fisher's exact test with multiple testing correction of FRD<0.05). No GO-terms in the molecular function category were significantly (p<0.05) enriched (Fisher's exact test with multiple testing correction of FRD<0.05) with genes only modulated in presence of folpet in the log phase of growth.

Comparison between genes modulate in the lag and log phase of growth - in presence of folpet - identified 55 genes commonly expressed (Fig. 4.5 and 4.24; Supplementary Table 4.16s). No GO-terms were significantly (p<0.05) commoner in the genes with significant changes in expression in response to folpet in either lag or log phase of growth (Fisher's exact test with multiple testing correction of FRD<0.05). Based on the functional annotation available, 44 genes are related to structure and transport across the cell membrane, oxidoreductase or hydrolase activity, and protein, zinc ion, RNA or DNA binding (Fig. 4.24).

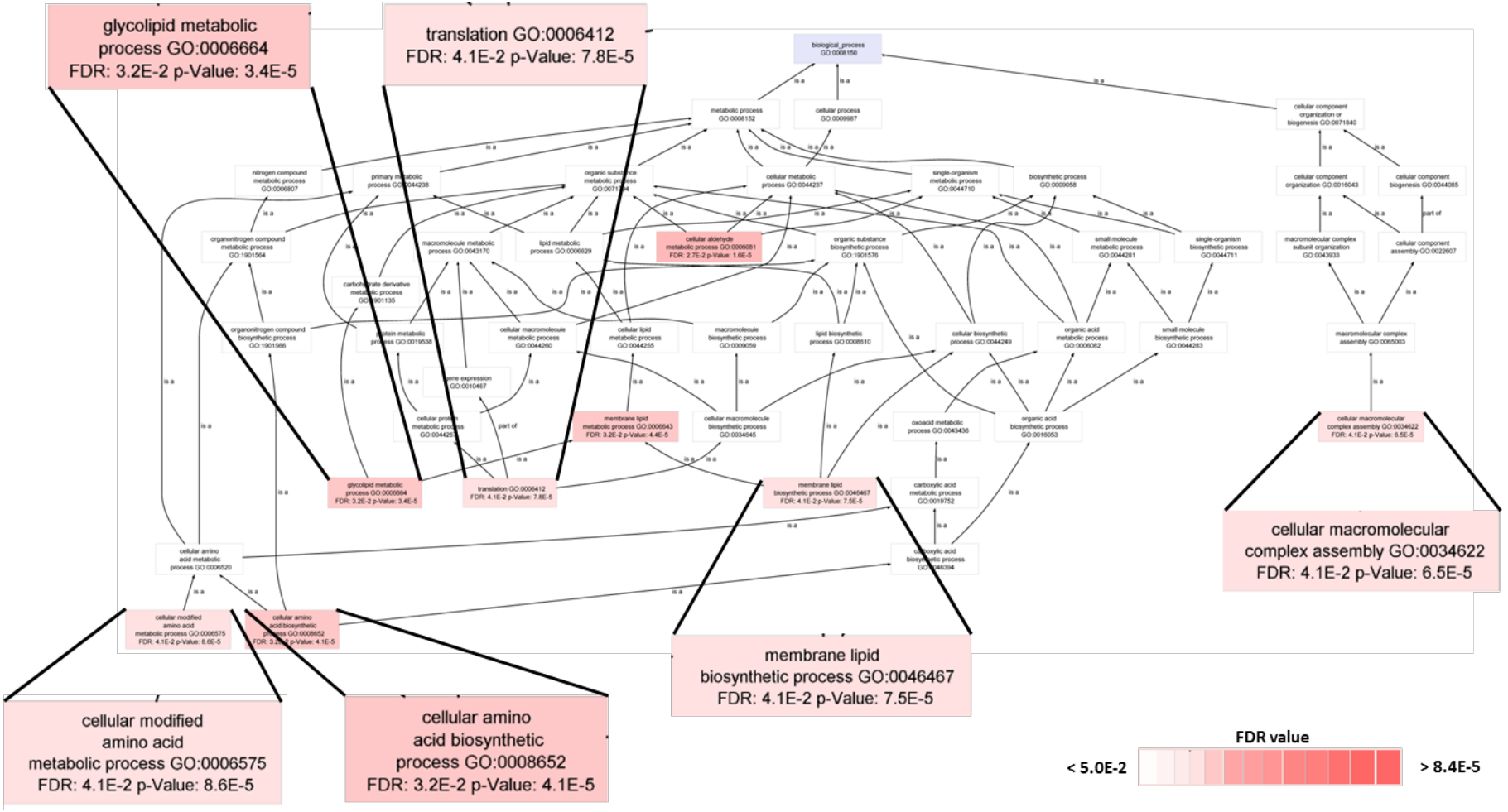

Figure 4.22 Biological process enriched graph of genes exclusively modulated in the lag phase of growth of *Z. tritici* after 24 h exposure to 0.5 µg/mL of folpet. Fisher's exact test with multiple testing correction of FDR<0.05. Nodes are coloured according to their FDR value in Fisher's exact test against the whole *Z. tritici* genome.

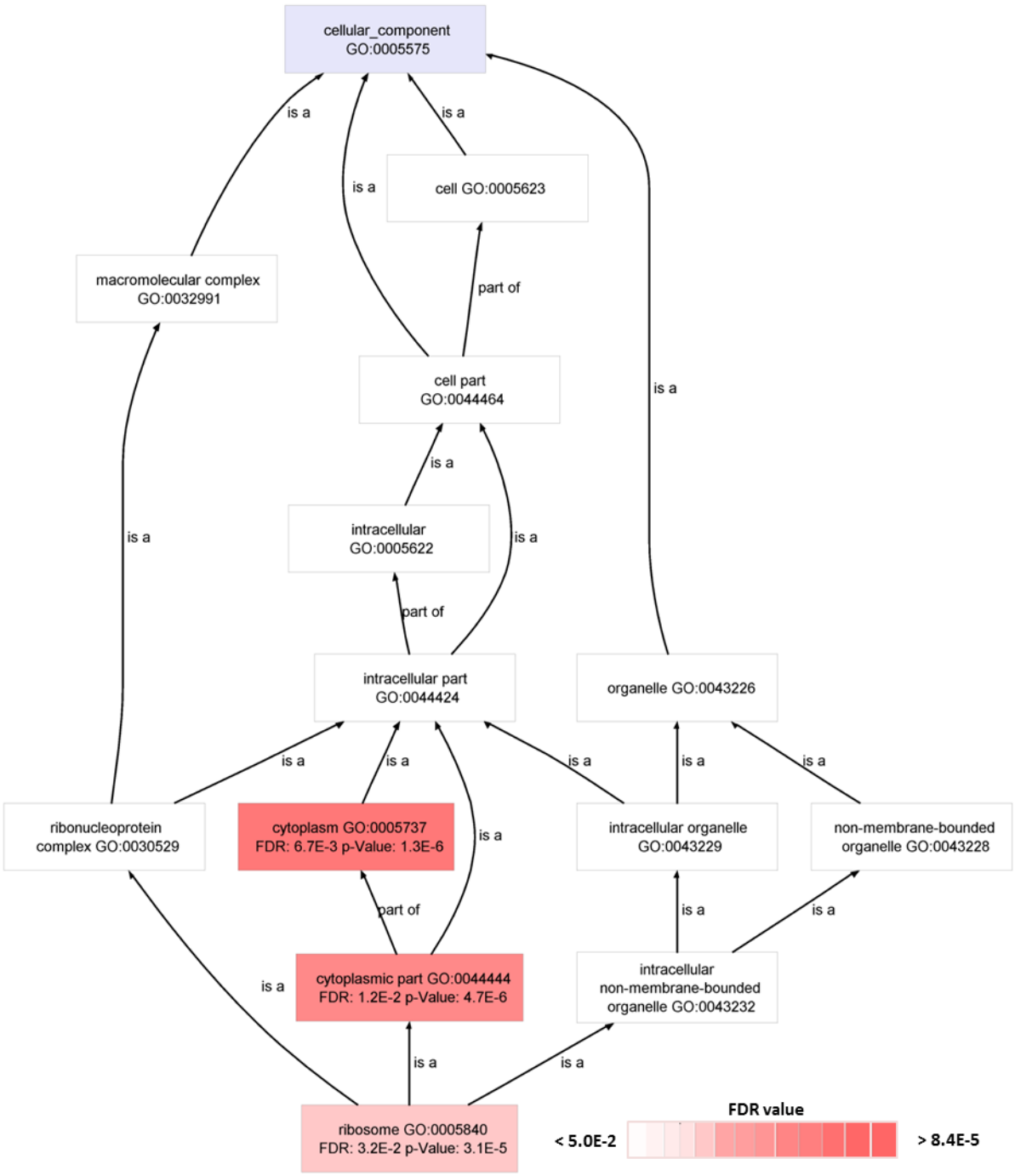

Figure 4.23 Cellular component enriched graph of genes exclusively modulated in the lag phase of growth of *Z. tritici* after 24 h exposure to 0.5 µg/mL of folpet. Fisher's exact test with multiple testing correction of FDR<0.05. Nodes are coloured according to their FDR value in Fisher's exact test against the whole *Z. tritici* genome.

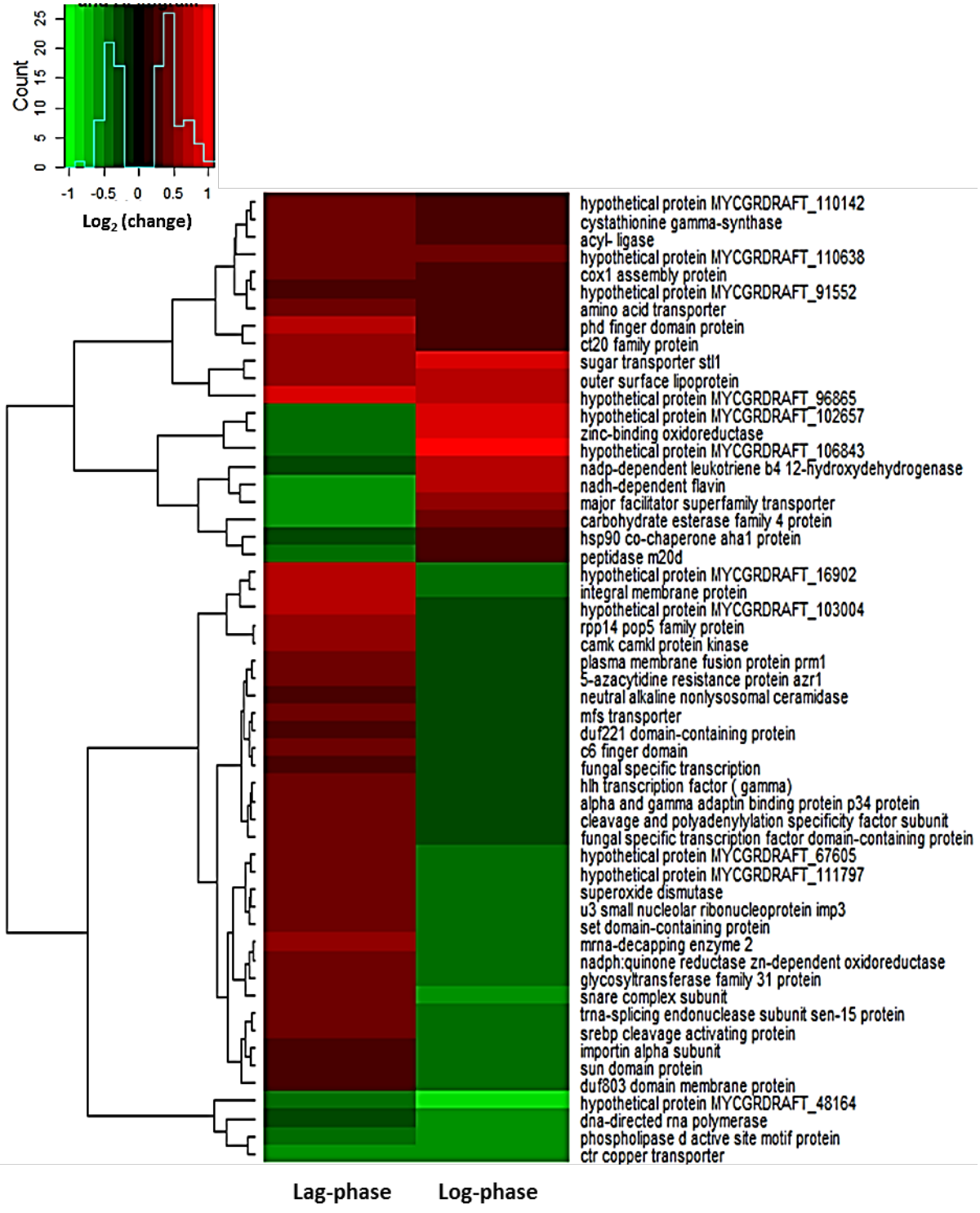

Figure 4.24 Expression pattern and putative function of 55 genes consistently modulated in either the lag or log phase of growth of *Z. tritici* after 24 h exposure to 0.5 µg/mL of folpet, but not statistically significant modulated in response to chlorothalonil. Functional annotation of genes was carried out using Blast2GO software.

4.3.2.3 Comparison between chlorothalonil and folpet genome-wide expression response in *Z. tritici*

A total of 133 genes were consistently modulated in the lag phase after either chlorothalonil or folpet exposure (Fig. 4.5; Supplementary Table 4.17s). Enrichment analysis of these genes found GO-terms enriched (p<0.001; Fisher's exact test with multiple testing correction of FRD<0.05; Supplementary Table 4.18s) with genes involved in aspartic-type endopeptidase in the molecular function category (Fig. 4.25). GO-terms in the biological process or cellular component category were not significantly enriched (p<0.05; Fisher's exact test with multiple testing correction of FRD<0.05). Based on these results and previous enrichment analysis, it was possible to identified specific functional gene expression profiles in *Z. tritici* in response to either chlorothalonil or folpet in the lag phase of growth. Annotated genes related to proteolysis (i.e. aspartic-type endopeptidase activity) were up-regulated by both fungicides, with exception of genes products acting in the carboxy-terminal end of proteins or peptides (i.e. serine-type carboxypeptidase activity) – which were only induced by folpet (Fig. 4.26). Additionally, most of the genes involved in cell wall products acting in the external region of the cell were also up-regulated only in response to folpet (Fig. 4.27). In contrast, genes related with structure and function of transmembrane proteins in the cell – histidine kinase complex - were only up-regulated by chlorothalonil in the lag phase of growth (Fig. 4.27).

On the other hand, a total of 787 genes were modulated in the log phase after either chlorothalonil or folpet (Supplementary Table 4.19s). Enrichment analysis of these genes found GO-terms enriched (p<0.001; Fisher's exact test with multiple testing correction of FRD<0.05; Supplementary Table 4.20s) with genes related to glucose and cellular amino acid catabolism, ornithine metabolism, transport of electrons in the mitochondrial membrane (i.e. NADH dehydrogenase activity), and active transport by ATP hydrolysis in the biological process category (Fig. 4.28). In the molecular function category GO-terms related to NADH dehydrogenase activity, and proton-transporting ATPase activity were significantly enriched (Fig. 4.29; p<0.001; Fisher's exact test with multiple testing correction of FRD<0.05). GO-terms related to proton-transporting V-type ATPase, proteasome, and mitochondrial membrane were significantly enriched in the cellular component category (Fig. 4.30; p<0.001; Fisher's exact test with multiple testing correction of FRD<0.05). Taking into account these results and previous enrichment analysis results, functional gene expression profiles in *Z. tritici* in response to either chlorothalonil or folpet exposure in the log phase were identified. Genes related to respiration in the mitochondrion were communally modulated by either chlorothalonil or folpet (Fig. 4.31). Genes

related to protein degradation were also modulated by both fungicides, but genes related to protein synthesis were modulated only by folpet (Fig. 4.32). Similarly, genes related to catabolism of glucose or cellular amino acids, and metabolism of ornithine were modulated by both fungicides, whereas genes related to metabolism of cellular amino acids and glycolipids were modulated only by folpet (Fig. 4.33) Genes related to catabolism of α-amino acids were associated with chlorothalonil exposure (Fig. 4.33). Genes related to transport across the cell membrane - mediated by proton-transporting V-type ATPase – were modulate by both fungicides, whereas genes related to structure of the cell membrane were modulated only by folpet in the log phase of growth of *Z. tritici* (Fig. 4.34).

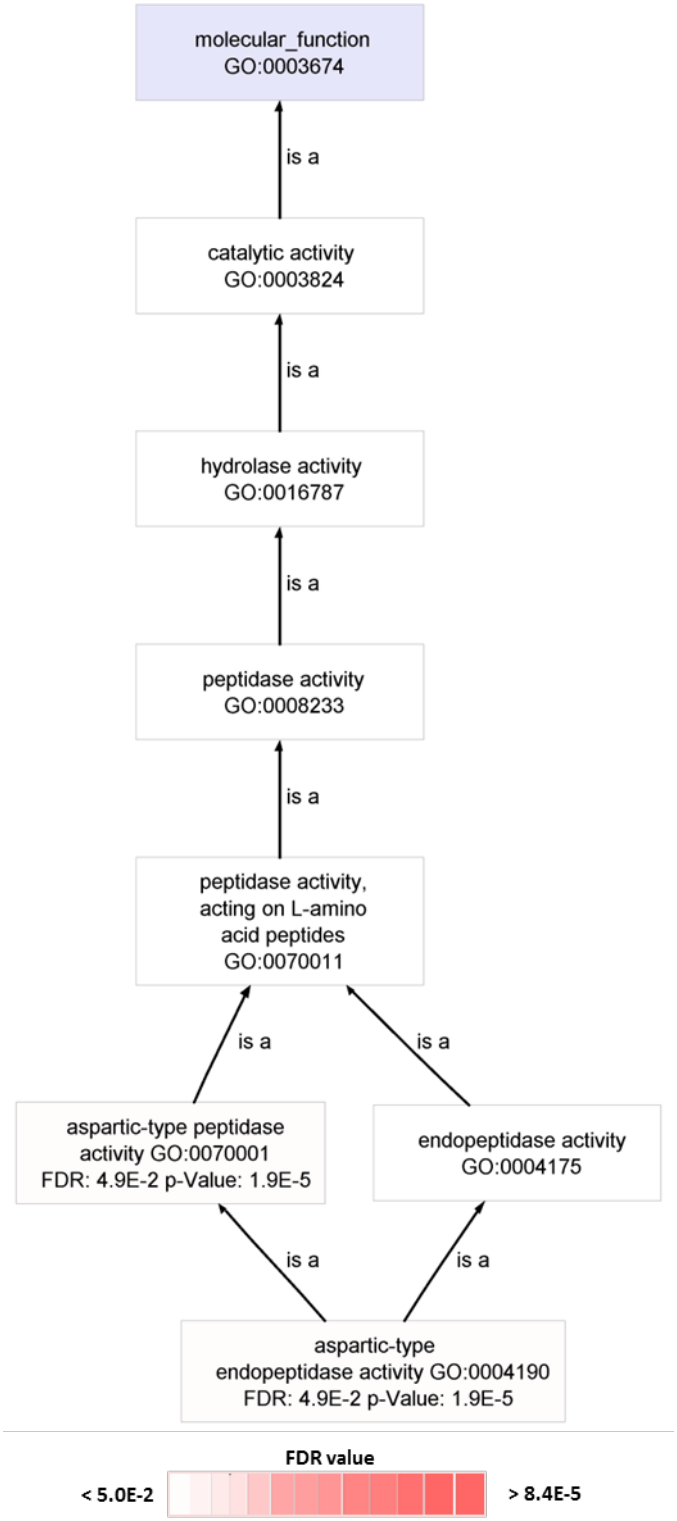

Figure 4.25 Molecular function enriched graph of genes modulated in the lag phase of growth of *Z. tritici* after 24 h exposure to either 0.1 of chlorothalonil or 0.5 µg/mL of folpet. Fisher's exact test with multiple testing correction of FDR<0.05. Nodes are coloured according to their FDR value in the Fisher's exact test against the whole *Z. tritici* genome.

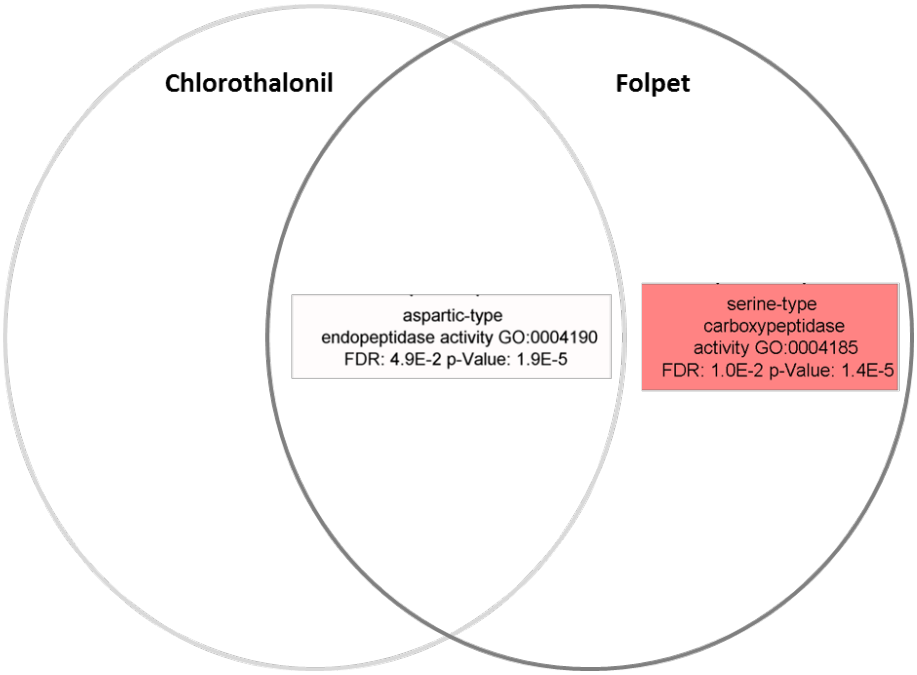

Figure 4.26 Functional groups of genes related to proteolysis - denoted by their GO-term - modulated in the lag phase of growth of *Z. tritici* after 24 h exposure to either chlorothalonil or folpet. Selected functional groups of genes shown were the most specific GO-terms with a statistically significant (p>0.05) FDR value in figures 4.17 and 4.25.

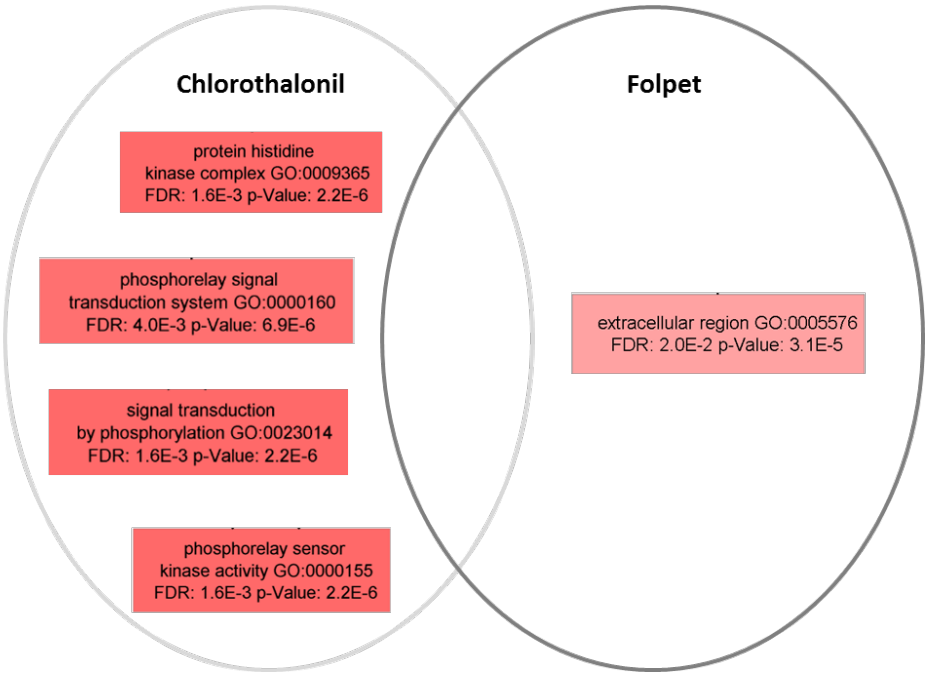

Figure 4.27 Functional groups of genes related to signaling in the cell membrane or gene products acting externally - denoted by their GO-term – modulated only in the lag phase of growth of *Z. tritici* after 24 h exposure to either chlorothalonil or folpet. Selected functional groups of genes shown were the most specific GO-terms with a statistically significant (p>0.05) FDR value in figures 4.8, 4.9, 4.10 and 4.17.

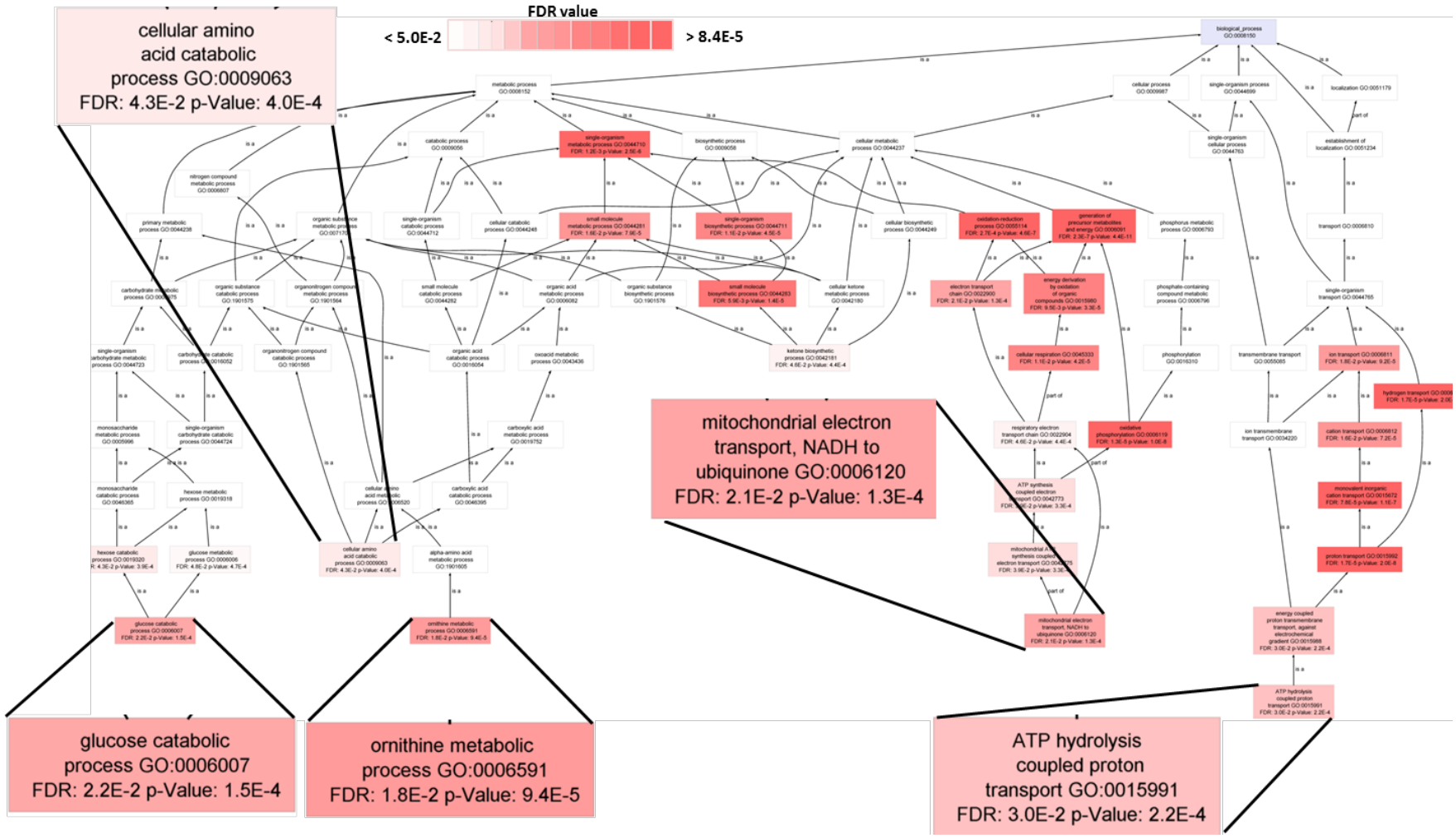

Figure 4.28 Biological process enriched graph of genes modulated in the log phase of growth of *Z. tritici* after 24 h exposure to either 0.1 µg/mL of chlorothalonil or 0.5 µg/mL of folpet. Fisher's exact test with multiple testing correction of FDR<0.05. Nodes are coloured according to their FDR value in the Fisher's exact test against the whole *Z. tritici* genome.

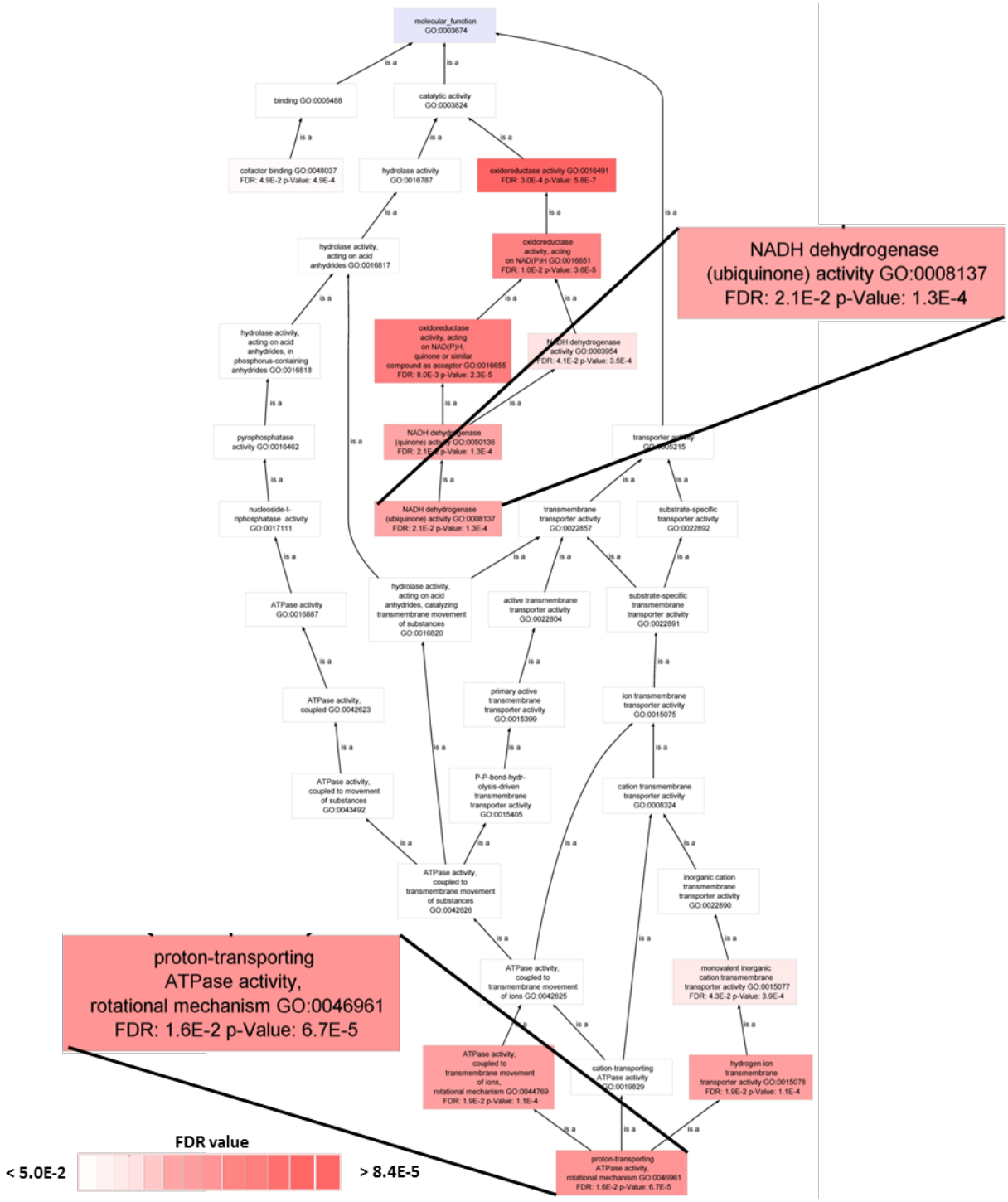

Figure 4.29 Molecular function enriched graph of genes modulated in the log phase of growth of *Z. tritici* after 24 h exposure to either 0.1 µg/mL of chlorothalonil or 0.5 µg/mL of folpet. Fisher's exact test with multiple testing correction of FDR<0.05. Nodes are coloured according to their FDR value in the Fisher's exact test against the whole *Z. tritici* genome.

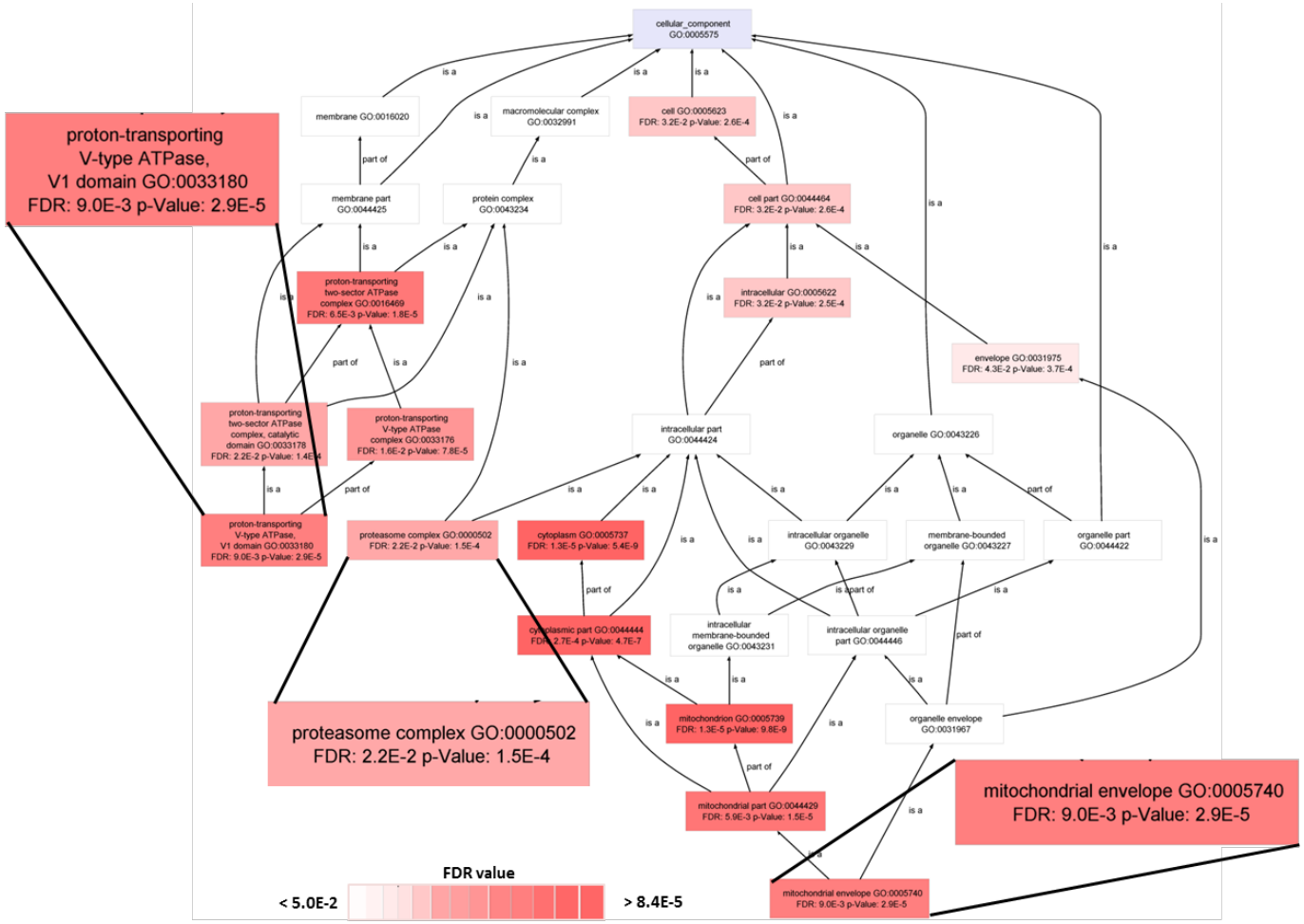

Figure 4.30 Cellular component enriched graph of genes modulated in the log phase of growth of *Z. tritici* after 24 h exposure to either 0.1 µg/mL of chlorothalonil or 0.5 µg/mL of folpet. Fisher's exact test with multiple testing correction of FDR<0.05. Nodes are coloured according to their FDR value in the Fisher's exact test against the whole *Z. tritici* genome.

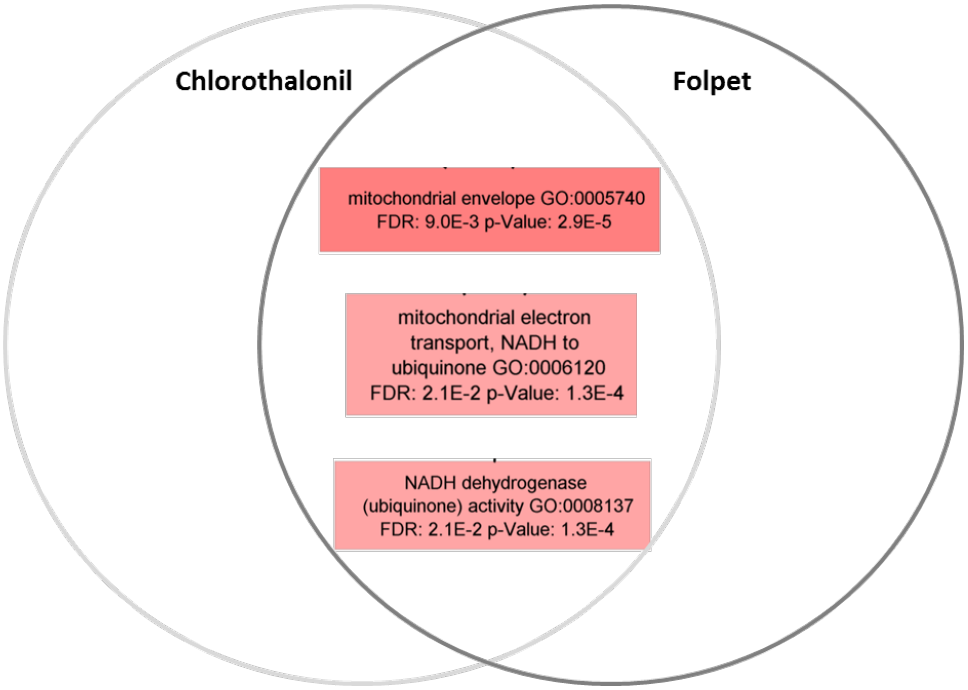

Figure 4.31 Functional groups of genes related to respiration in the mitochondria - denoted by their GO-term - modulated in the log phase of growth of *Z. tritici* after 24 h exposure to either chlorothalonil or folpet. Selected functional groups of genes shown were the most specific GO-terms with a statistically significant (p>0.05) FDR value in figures 4.28, 4.29 and 4.30.

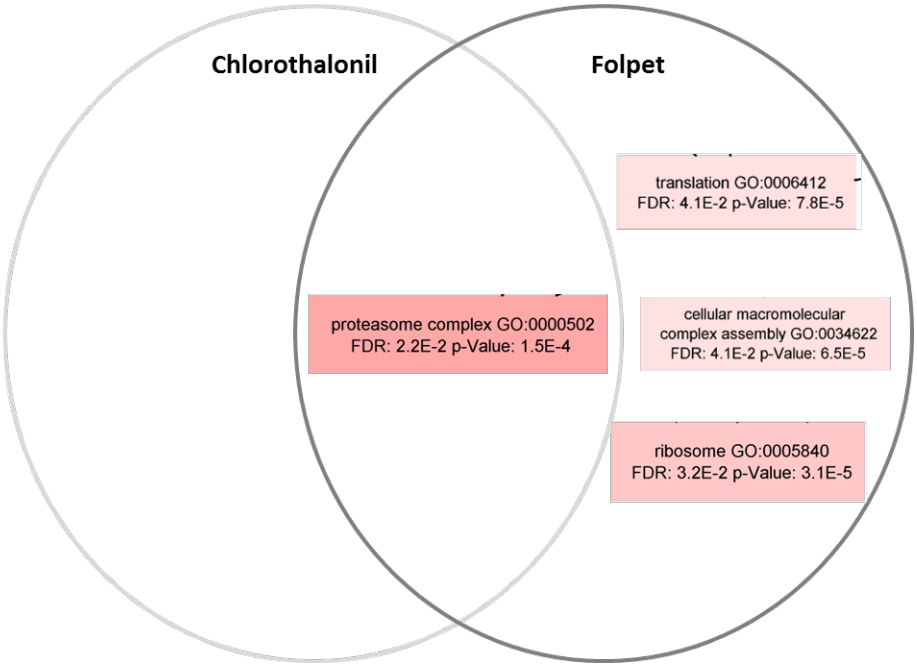

Figure 4.32 Functional groups of genes related to protein synthesis or degradation - denoted by their GO-term - modulated in the log phase of growth of *Z. tritici* after 24 h exposure to either chlorothalonil or folpet. Selected functional groups of genes shown were the most specific GO-terms with a statistically significant (p>0.05) FDR value in figures 4.28, 4.29 and 4.30.

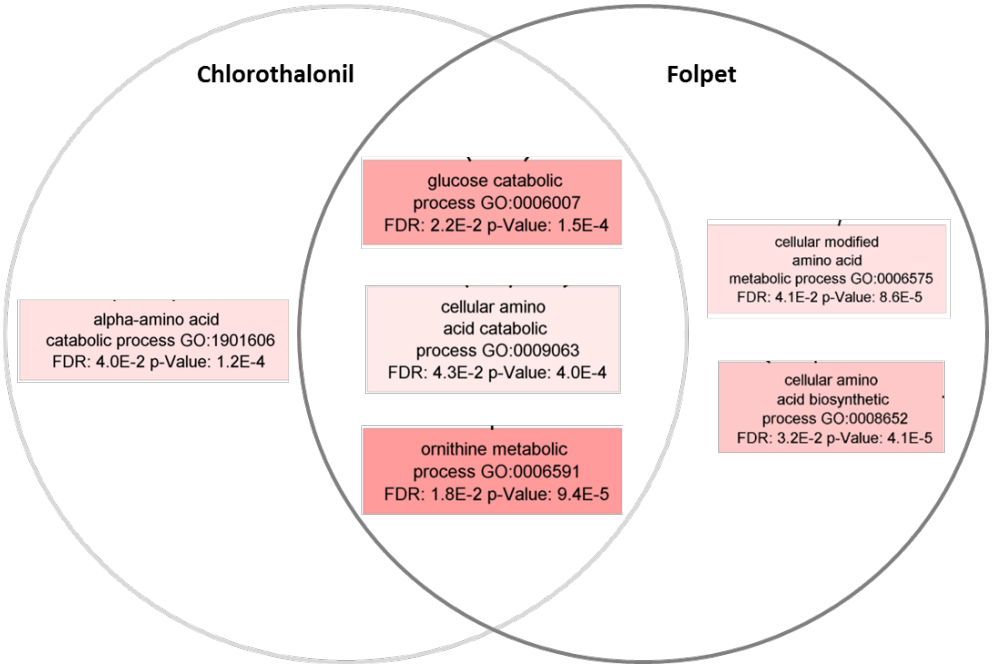

Figure 4.33 Functional groups of genes related to glucose or amino acids synthesis catabolism - denoted by their GO-term - modulated in the log phase of growth of *Z. tritici* after 24 h exposure to either chlorothalonil or folpet. Selected functional groups of genes shown were the most specific GO-terms with a statistically significant (p>0.05) FDR value in figures 4.28, 4.29 and 4.30.

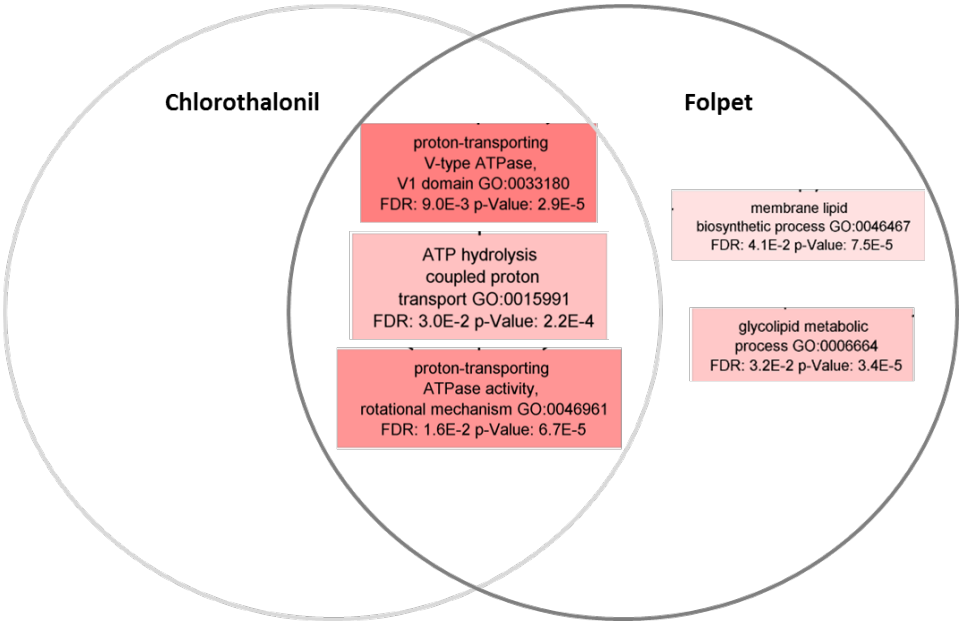

Figure 4.34 Functional groups of genes related to structure or function of the cell membrane - denoted by their GO-term - modulated in the log phase of growth of *Z. tritici* after 24 h exposure to either chlorothalonil or folpet. Selected functional groups of genes shown were the most specific GO-terms with a statistically significant (p>0.05) FDR value in figures 4.28, 4.29 and 4.30.

4.3.2.4 Genes encoding putative detoxification mechanisms in *Z. tritici* in response to either chlorothalonil or folpet

A detailed inspection of chlorothalonil- and folpet-DEGs detected several up-regulated genes encoding detoxification mechanisms during the lag or log phase of growth. Twenty genes encoding putative glutathione S-tranferase (GST) were overexpressed in response to folpet only in the log phase; nine of the 20 genes were also overexpressed in presence of chlorothalonil in the same phase of growth (Fig. 4.35).

Additional, genes encoding putative ATP-binding cassette (ABC) transport components in *Z. tritici* (Zwiers et al., 2003) were modulated by both fungicides. The expression of four putative ABC transporters encoding genes was induced by chlorothalonil exposure; the expression of one of them was only induced in the log phase (Fig. 4.36). Folpet exposure induced expression of three or two putative ABC transporters in the lag or log phase exposure, respectively (Fig. 4.36). The expression of two ABC transporters was up-regulated by chlorothalonil or folpet exposure in the lag phase.

The expression of genes encoding putative major facilitator superfamily (MFS) drug efflux transporters (Roohparvar et al., 2007) were also modulated by chlorothalonil or folpet exposure. Four genes encoding MFS transporters were up-regulated when chlorothalonil was added in the lag phase; whereas one gene with different sequence was up-regulated in presence of folpet in the same phase growth. On the other hand, six genes were up-regulated in the log phase of growth after exposure to folpet. No genes were overexpressed after chlorothalonil exposure in the log phase (Fig. 4.37).

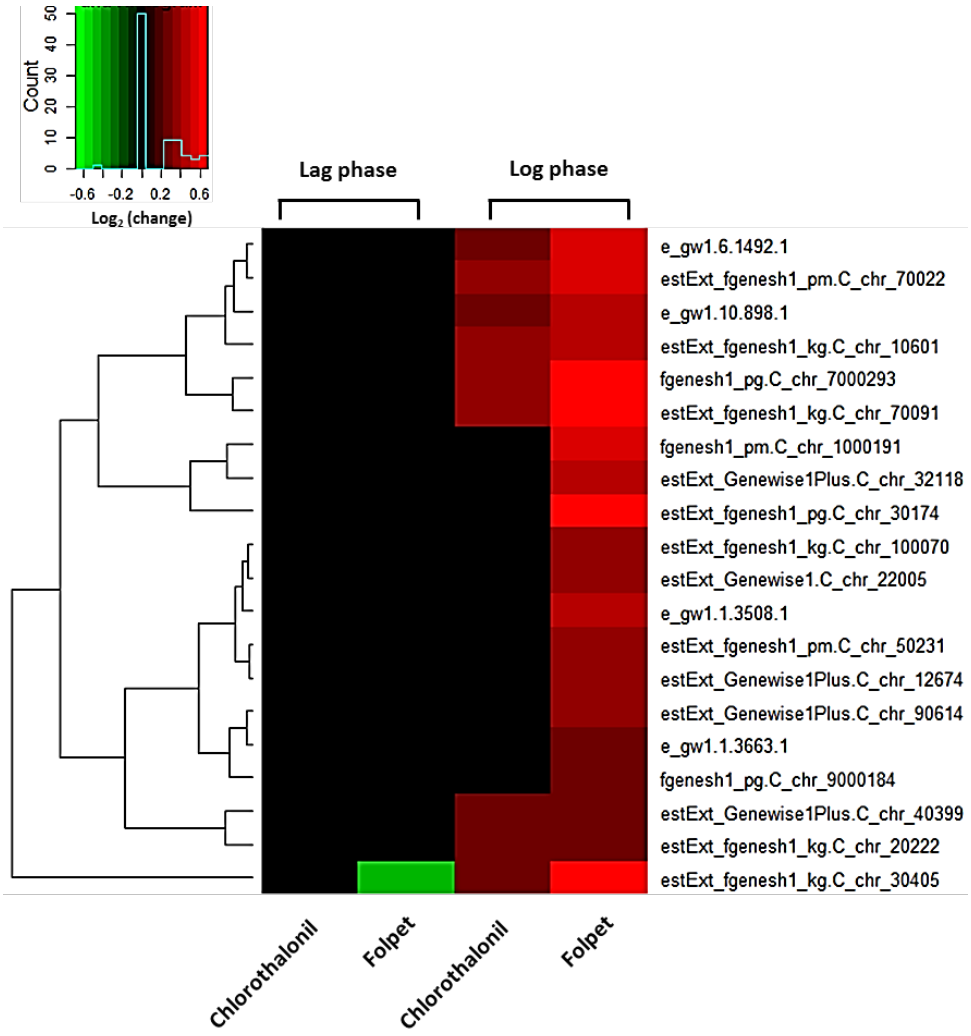

Figure 4.35 Expression of genes encoding putative glutathione S-tranferase (GST) in *Z. tritici* after 24 h exposure to 0.1 µg/mL of chlorothalonil (A) or 0.5 µg/mL of folpet (B) in the lag or log phase growth.

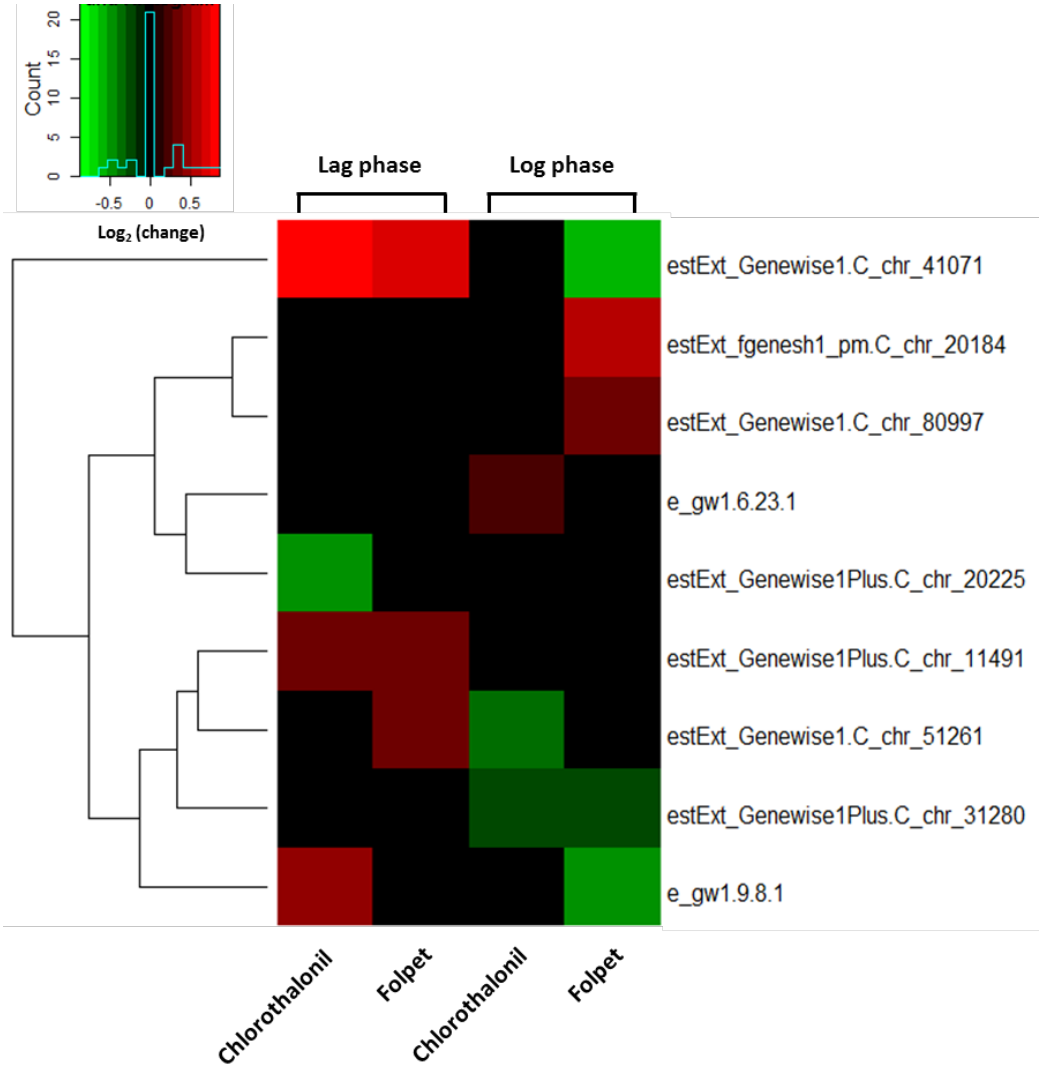

Figure 4.36 Expression of genes encoding putative ATP-binding cassette (ABC) transport components in *Z. tritici* after 24 h exposure to 0.1 µg/mL of chlorothalonil (A) or 0.5 µg/mL of folpet (B) in the lag or log phase growth. Gene sequence estExt_Genewise1.C_chr51261 is also called MgAtr5 (Zwiers et al., 2002) and e_gw1.9.8.1 is also called MgAtr7 (Zwiers et al., 2007).

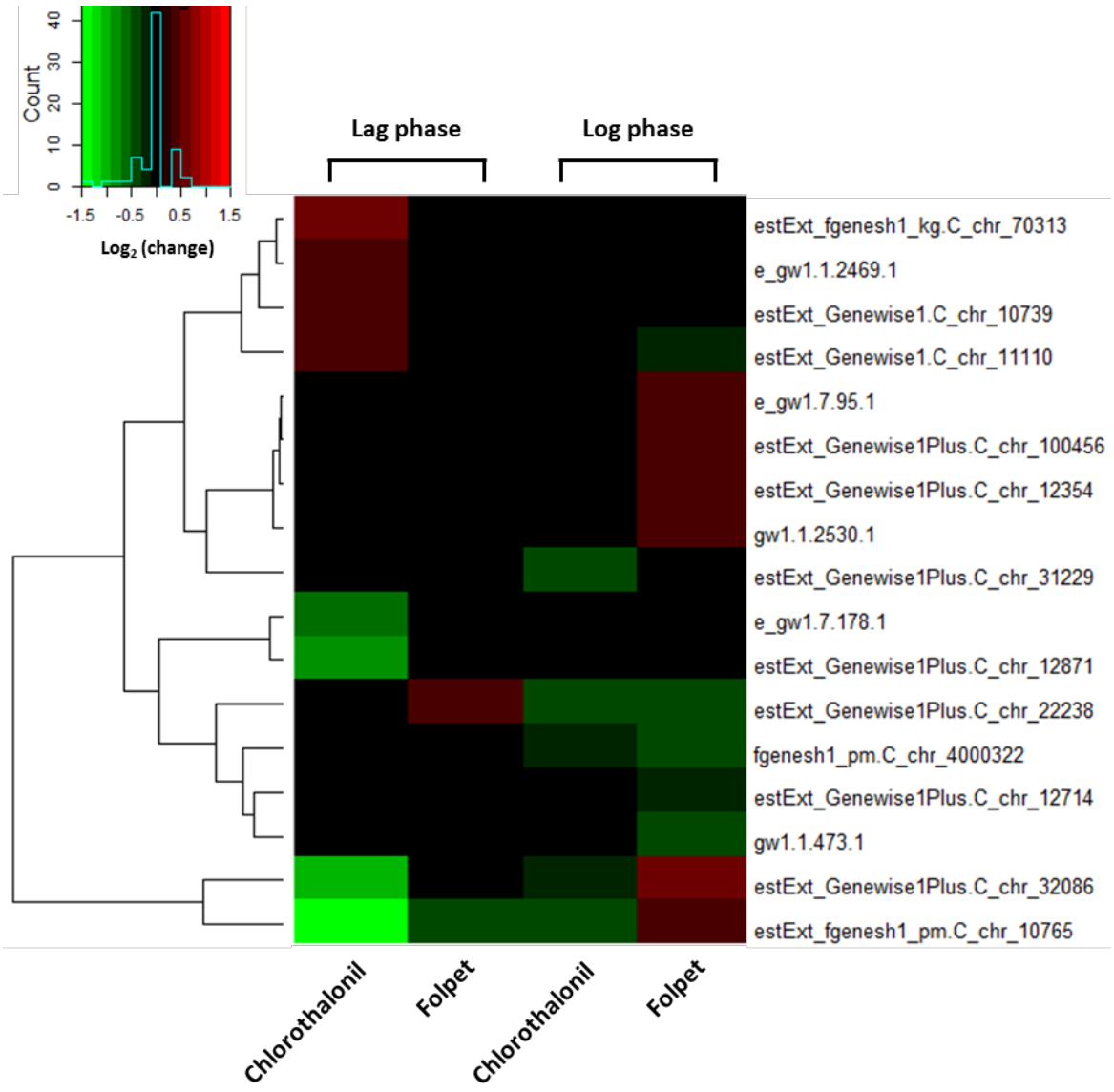

Figure 4.37 Expression of genes encoding putative major facilitator superfamily (MFS) drug efflux transport components in *Z. tritici* after 24 h exposure to 0.1 µg/mL of chlorothalonil (A) or 0.5 µg/mL of folpet (B) in the lag or log phase growth.

4.3.2.5 Validation of RNA sequencing results by quantitative RT-PCR

Changes in transcription after exposure to chlorothalonil or folpet were estimated by quantitative RT-PCR for 40 selected genes to validate the RNA-seq analyses (Table 4.2). Genes for qRT-PCR were selected based on their gene expression ratio in RNA-seq. The top five up- or down-regulated genes in presence of chlorothalonil in the lag phase (LagChlo-U1, LagChlo-U2 LagChlo-U3, LagChlo-U4, LagChlo-U5, LagChlo-D1, LagChlo-D2, LagChlo-D3, LagChlo-D4 and LagChlo-D5) or log phase (LogChlo-U1, LogChlo-U2 LogChlo-U3, LogChlo-U4, LogChlo-U5, LogChlo-D1, LogChlo-D2, LogChlo-D3, LogChlo-D4, LogChlo-D5) were selected. An equal number of genes was selected in the folpet treatments applied in the lag (LagFol-U1, LagFol-U2, LagFol-U3, LagFol-U4, LagFol-U5, LagFol-D1, LagFol-D2, LagFol-D3, LagFol-D4 and LagFol-D5) or log phase of growth (LogFol-U1, LogFol-U2, LogFol-U3, LogFol-U4, LogFol-U5, LogFol-D1, LogFol-D2, LogFol-D3, LogFol-D4 and LogFol-D5).

Transformed gene expression ratios obtained with qRT-PCR and RNA-seq analysis were significantly correlated in three of the four treatments (Fig. 4.38B chlorothalonil in the log phase, $r=0.92$, $n=10$, $p<0.001$; Fig. 4.39A folpet in the lag phase, $r=0.91$, $n=9$, $p<0.001$; Fig. 4.39B folpet in the log phase, $r=0.96$, $n=10$, $p<0.001$), with the exception of chlorothalonil in the lag phase (Fig,. 4.38A; $r=0.22$, $n=10$, $p=0.54$).

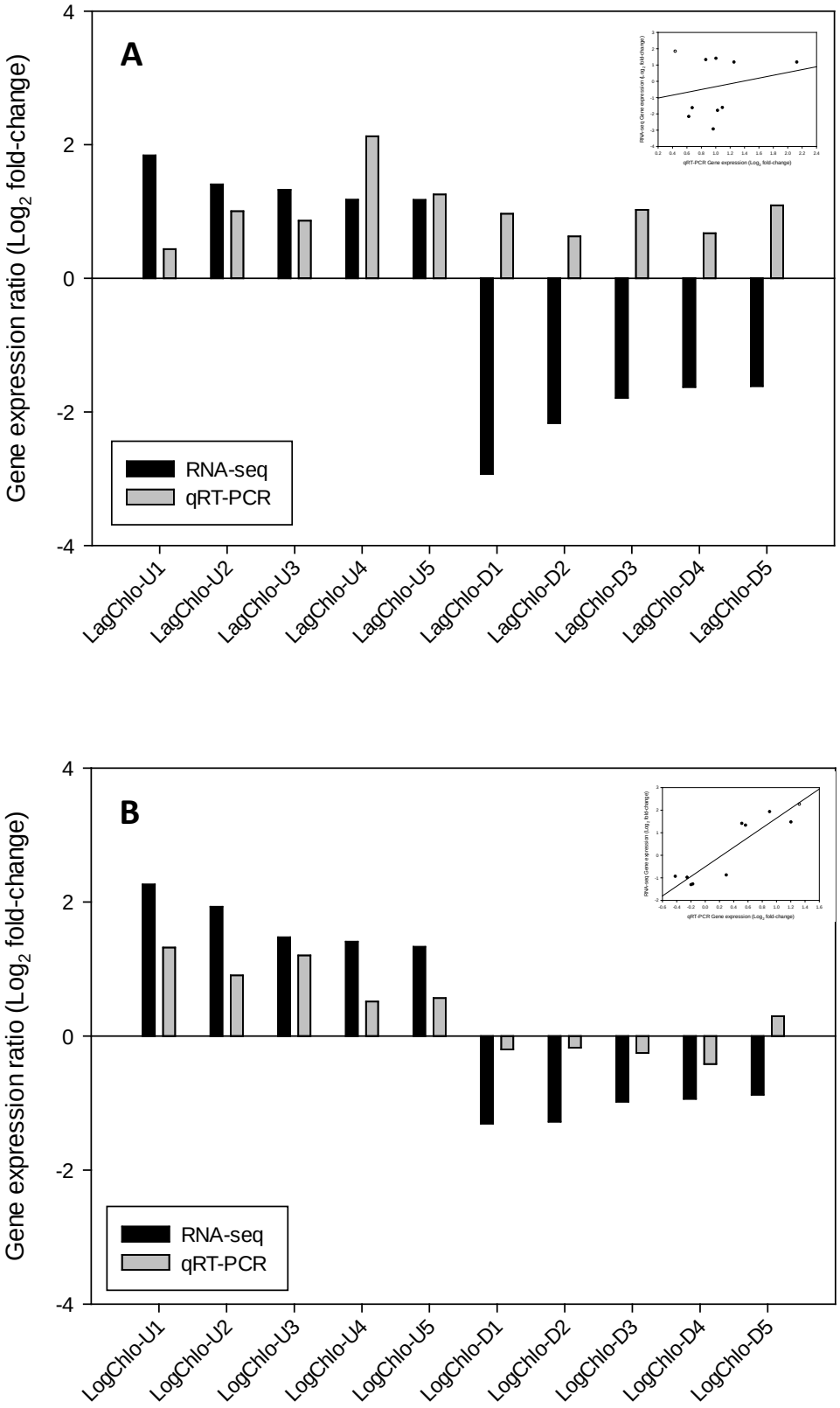

Figure 4.38 Gene expression comparison of selected *Z. tritici* genes using RNA-seq and qRT-PCR. RNA samples taken from cultures after 24 h exposure to 0.1 µg/mL of chlorothalonil in the lag (A) or log (B) phase growth.

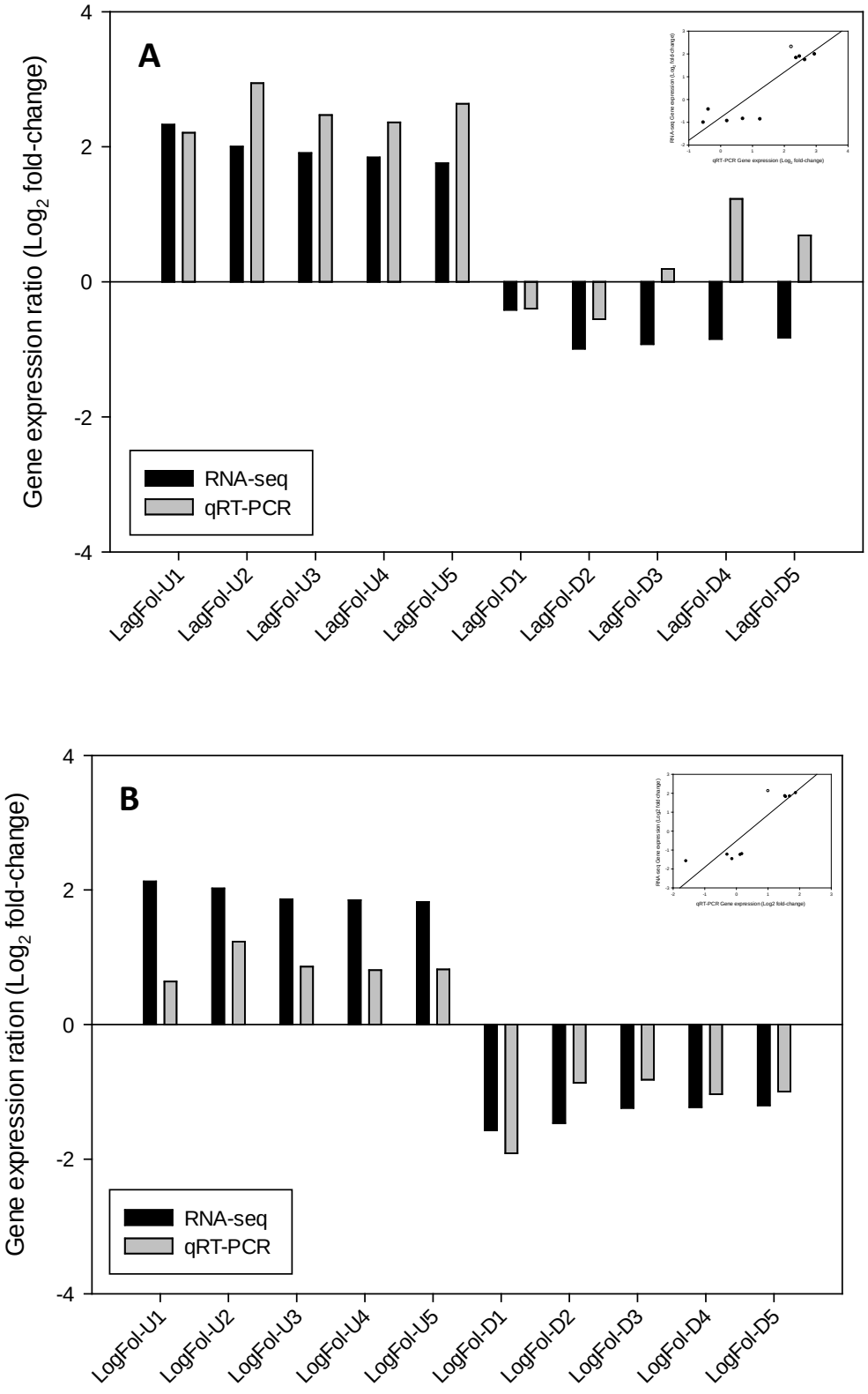

Figure 4.39 Gene expression comparison of selected *Z. tritici* genes using RNA-seq and qRT-PCR. RNA samples taken from cultures after 24 h exposure to 0.5 µg/mL of folpet in the lag (A) or log (B) phase growth.

4.4 Discussion

Profiling genome-wide expression after exposure to an antifungal compound has in other cases allowed getting insights into the mode of action and/or detoxification mechanisms (Amin et al., 2002). Using an Affymetrix GeneChip expression array, I intended to characterise the genome-wide transcriptional response of the reference *Z. tritici* isolate IPO323 to chlorothalonil and folpet. However, there was no correlation of gene expression changes between microarray measurements and quantitative RT-PCR. Furthermore, the large proportion of folpet-DEPSs annotated as wheat genes suggested possible mis-hybridisation on the microarray. This mis-hybridisation might be due to differences in hybridisation properties among probes on the microarray (Gautier et al., 2004). Therefore, results from the Affymetrix GeneChip expression array are not reliable to characterise the transcriptional response of *Z. tritici* to chlorothalonil and folpet.

As an alternative approach, changes in gene expression were measured using RNA-seq technology when *Z. tritici* isolate IPO323 was exposed to chlorothalonil or folpet in the lag or log phase of growth. Gene expression of selected genes obtained with the RNA sequencing was significantly correlated with quantitative RT-PCR in most of the treatments, with the exception of chlorothalonil in the lag phase of growth. Marioni et al. (2008) found some discrepancies in gene expression between quantitative RT-PCR and RNA-seq or microarray technology. They selected five or six genes called differentially expressed from the RNA-seq or microarray technology, respectively, and measured gene expression through quantitative RT-PCR. Results from the quantitative RT-PCR confirmed four of five genes as differentially expressed from RNA-seq, and only two of six genes from the microarray technology. Marioni et al. (2008) argued that discrepancies in gene expression between technologies may be due to either false positive discovery or differences in the genomic region that both quantitative RT-PCR and RNA-seq targeted. In my study, top up- or down-regulated DEGs – in response to chlorothalonil exposure in the lag phase – had a q-value of 0.001831 (Supplementary Table 4.1s) which indicates a low false discovery rate. The q-value is the proportion of false discoveries incurred from p-values as or more extreme (Storey, 2002; Storey and Tibshirani, 2003). Thus, discrepancies in gene expression between quantitative RT-PCR and RNA-seq might be due to differences in the genomic region that both technologies target. Nevertheless, the detection of differentially expressed genes with Illumina platform is highly replicable even for genes with low expression (Marioni et al., 2008).

Overall, small changes in gene expression level between treated and untreated samples were observed. Interestingly, diverse studies looking at effect of nutrient starvation (e.g. glucose,

nitrogen or phosphate) on gene expression also reported small changes in gene expression level in yeast (Conway et al., 2012; Kresnowati et al., 2006; Wu et al., 2004). Although chlorothalonil or folpet induced small changes in gene expression in *Z. tritici*, these changes were statistically significant. Functional annotation and enrichment analysis of significant differentially expressed genes found specific functional gene expression profiles for chlorothalonil and folpet. Moreover, most of the chlorothalonil- or folpet-DEGs were located on the core essential chromosomes of *Z. tritici,* indicating that dispensable chromosomes (Mehrabi et al., 2007; Wittenberg et al., 2009) may not play a large role in the response to chlorothalonil or folpet.

The results discuss here provide a snapshot of the genome-wide expression profile of *Z. tritici* after 24 h exposure to chlorothalonil or folpet under *in vitro* conditions. Earlier *in vitro* studies showed that chlorothalonil is able to reduce 21 or 99 % growth of *S. pastorianus* after eight hours exposure to 0.25 or 2 µg/mL, respectively, and thiols content in fungal cells of *Neurospora crassa* after two hours exposure to 2 or 4 µg/mL of the fungicide (Vincent and Sisler, 1968). Similarly, folpet is able to reduce approximately 50 % dehydrogenase activity after one hour exposure (Siegel, 1971b). Although chlorothalonil or folpet can impair metabolic pathways within few hours after exposure, the transcriptional response of *Z. tritici* measured in my study after 24 hours fungicide exposure can provide insights into the putative affected metabolic pathways. Cools et al. (2007) or Becher et al. (2011) were able to detect changes in gene expression in *Z. tritici* or *F. graminearum*, respectively, after 24 h exposure to an azole fungicide. They found significant differentially expressed genes related with ergosterol biosynthesis – the target site of azole fungicides – and transport mechanisms across the cell membrane. However, the sampling of only a single time point makes difficult to determine whether the transcriptional response was conserved during the fungicide exposure time course. In addition, in planta studies might be needed to elucidate the mode of action of chlorothalonil or folpet taking into account the plant-pathogen interaction. Comparing transcriptional response in both axenic culture and in planta may help to identify fungal genes specifically expressed during fungicide exposure (Kellner et al., 2014; Rudd et al., 2015). This can be particularly challenging due to extremely slow grow of *Z. tritici* during the early phase of colonization in wheat leaf (Kema et al., 1996; Keon et al., 2007; Pnini-Cohen et al., 2000). The slow rate growth can make difficult or impossible to get enough fungal biomass to extract RNA and detect changes in gene expression. Nevertheless, the transcriptional response presented here can be considered as baseline for further studies looking at transcriptional response of *Z. tritici* during fungicide exposure.

4.4.1 Genome-wide expression response of *Z. tritici* to chlorothalonil

Chlorothalonil is a multi-site fungicide with a broad spectrum of action, controlling a wide range of plant pathogens (FRAC, 2014). Earlier studies suggested that chlorothalonil exerts its fungicidal action by inactivation of thiol-containing compounds like cysteine, glutathione and Coenzyme A (Vincent and Sisler, 1968). Glutathione and glutathione S-transferase are involved in protecting the cell against oxidative stress and diverse xenobiotics (see Penninckx (2000); (Shin et al., 2003)). Nine genes encoding glutathione S-tranferase (GST) were up-regulated in *Z. tritici* after exposure to chlorothalonil in the log phase growth. However, no genes related to glutathione metabolism were significantly differentially expressed in the lag phase. This suggests a possible low content or depletion of glutathione in spores of *Z. tritici* during the lag phase due to chlorothalonil exposure. Shin et al. (2003) suggested that glutathione content and GST activity in yeast cells can be linked to chlorothalonil resistance. They reported reduction of glutathione content in yeast cells in presence of chlorothalonil.

Although gene expression after chlorothalonil exposure in the lag phase between RNA-seq and quantitative RT-PCR was no correlated, Marioni et al. (2008) indicated that RNA-seq data are reliable. Enrichment analysis of genes expressed only in the lag phase after chlorothalonil exposure found functional groups of genes related to structure and function of protein histidine kinase complex. The protein histidine kinase complex (PHK) is a transmembrane protein receptor dimer (see Stock et al., 2000; Yamada et al., 2009). The PHK along with its associated response regulator (RR) (Yamada et al., 2009) allow cells to sense and mediate signaling pathways in response to extracellular stimulus - including nutrients, changes in osmolarity or antibiotics - (Skerker et al., 2005; see Wolanin et al., 2002). It has been suggested that dicarboximide and phenylpyrrole fungicides exert their fungicidal action by interfering with the osmotic signal transduction pathway in the histidine kinase and MAP kinase cascade (El-Mowafy et al., 2013; Fillinger et al., 2012; Yoshimi et al., 2003). Moreover, antibiotics like vancomycin or penicillin affect the PHK function, which in turn induces cell death and lysis in bacteria (Moreillon et al., 1990; Novak et al., 1999). Thus, it is possible that chlorothalonil might exert its fungicidal activity in *Z. tritici* spores by interacting with PHK in the cell membrane that in turn modulates other signal transduction pathways, possible nutrients signaling.

Interestingly, genes related to carbon catabolite repression or nitrogen regulatory proteins were down- or up-regulated in the lag or log phase only after chlorothalonil exposure, respectively. Nitrogen regulatory proteins regulate expression of genes encoding permeases and catabolic enzymes in response to nitrogen availability as part of the nitrogen metabolite repression

pathway (Haas and Marzluf, 1995; see Marzluf, 1993). Similarly, carbon catabolite can be modulated by a glucose signaling pathway, which regulates gene transcription through the Ras/protein kinase A signal transduction pathway (New et al., 2014; Ozcan et al., 1996). Although no relationship between PHK and carbon catabolite or nitrogen metabolite repression has been reported, these signaling transduction pathways rely largely on external stimulus. This indicates that chlorothalonil may interact with signal sensors in the cell membrane and induce a limited-nutrient stress response in *Z. tritici*. Further studies are needed to determine to what extent chlorothalonil interacts with *Z. tritici* cell membrane sensors and to elucidate which metabolic pathways might be associated with alterations in the PHK.

In contrast to the lag phase, most of the enriched functional groups of genes in response of chlorothalonil in the log phase were similar to those found in response to folpet in the same phase of growth. The exception seems to be a functional group of genes related to catabolism of α-amino acids, which are categorised as cellular amino acids. However, genes related to catabolism of cellular amino acids were modulated by folpet exposure in the log phase. This large similarity in functional gene expression indicates that chlorothalonil may induce similar stress response in *Z. tritici* as folpet upon exposure in the log phase. To this end, the possible metabolic pathways affected by chlorothalonil remains elusive due to no specific respond was found in *Z. tritici* when chlorothalonil was added in the log phase of growth. A discussion of functional groups of genes detected in response to chlorothalonil and folpet exposure is presented in section 4.4.3.

4.4.2 Genome-wide expression response of *Z. tritici* to folpet

Folpet exerts its fungicidal action by reacting primarily with either thiol-containing proteins with sulfhydryl groups (e.g. glutathione, glyceraldehyde 3-phospahate dehydrogenase or alcohol dehydrogenase) or non-thiol-containing proteins (e.g. α-chymotrypsin or lysozyme) in the cell (Lukens, 1966; Siegel and Sisler, 1968b). GO-terms associated with glutathione metabolism were not preferentially associated with folpet-DEGs. However, a detailed inspection of the folpet-DEGs identified 20 genes up-regulated, which were involved in glutathione catabolism (i.e. glutathione S-transferase (GST)) in the log phase. In the lag phase a single GST encoding gene was down-regulated and none up-regulated. This difference might be related to cellular glutathione content in *Z. tritici* cell in the lag or log phase. Siegel (1971b) indicated that folpet toxicity is reached when approximately 90 % of the glutathione has reacted with the fungicide. Although glutathione content was not determined in *Z. tritici*, the large number of up-regulated genes encoding GTS in

the log phase suggests a higher concentration of glutathione than in the lag phase. However, GST activity is also related to detoxification of xenobiotics in the cell (Lukens, 1966; Siegel, 1971b). The large activity of GST in the log phase of *Z. tritici* indicates a possible detoxification mechanism protecting against folpet.

Enrichment analysis of the most differentially expressed genes in the lag phase after folpet exposure found functional groups of genes with peptidase activity related to proteolysis. Genes related to serine-type carboxypeptidases were particularly overexpressed after folpet exposure only in the lag phase. Overexpression of these proteolytic enzymes in *Z. tritici* may be due to direct interaction with folpet. The serine carboxypeptidases are glycoproteins with a free sulfhydryl group (Cooper and Bussey, 1989; Dal Degan et al., 1992) to which folpet have affinity (Lukens and Sisler, 1958). Additionally, the serine-type carboxypeptidase is an exopeptidase that releases single amino acids from the C-terminal of extracellular peptide or proteins (see Rao et al., 1998). These free amino acids can be further absorbed into the cell to overcome nutrient requirements (see Breddam, 1986). Interestingly, an amino acid transporter encoding gene was overexpressed in either the lag or log phase only in response to folpet. This suggests internal demand of nutrients in *Z. tritici*, possibly of nitrogen. To this end, folpet may exert its fungicidal action during the lag phase of growth of *Z. tritici* by an initial interaction with serine-type carboxypeptidases in the cell membrane.

In contrast, enrichment analysis of genes expressed only in the log phase after folpet exposure found more diverse functional groups significantly enriched. GO-terms associated with protein synthesis (e.g. translation or ribosome structure), biosynthesis or catabolism of cellular amino acids, glycolipid metabolism, and membrane lipid biosynthesis were preferentially associated with folpet exposure in the log phase. Synthesis of proteins is carried out by ribosomes, which consist of two subunits. The small ribosomal subunit (40S) initiates protein synthesis through binding initiation factors that help to translate the information contained in mRNA (Rabl et al., 2011). On the other hand, the large ribosomal subunit (60S) synthetises peptide bonds formation and contains the polypeptide exit tunnel (Klinge et al., 2011). Cycloheximide – a glutarimide antibiotic – inhibits protein synthesis by binding on the ribosomal protein subunit 60S, possible in the polypeptide exit tunnel (Fried and Warner, 1982; see Klinge et al., 2011; Schneider-Poetsch et al., 2010). Interestingly, a total of 15 or 21 genes encoding the ribosomal proteins subunits 40S or 60S, respectively, were up-regulated in *Z. tritici* only when folpet was added in the log phase. This large response of genes involved in the structure of ribosomes suggests that folpet might interact directly with ribosomal proteins. Siegel and Sisler (1968b) reported that folpet is able to bind to

purified proteins of *Saccharomyces pastorianus*. However, they found that folpet-protein binding is weak and folpet is released as gas. Santos et al. (2009) reported increased activity of protein translation in yeast cells exposed to mancozeb which is another multi-site inhibitor. Mancozeb – an ethylene-bis-dithiocarbamate complex with manganese and zinc – has affinity with sulfhydryl (-SH) containing proteins (Vaccari et al., 1999). Further studies are needed to determine to what extent folpet binds to ribosomal proteins and to affect protein synthesis.

Additionally, functional groups of genes related to cell membrane structure (i.e. glycolipid metabolism or membrane lipid biosynthesis) were particularly down-regulated in *Z. tritici* when folpet was added in the log phase. It has been suggested that aromatic hydrocarbons fungicides affect the structure of lipid in the cell membrane (see FRAC, 2014). Radzuhn and Lyr (1984) reported that etridiazole - an aromatic hydrocarbon – is able to lyse cell membrane phospholipids into free fatty acids and lysophosphatides. Genes related to integral membrane protein, plasma membrane fusion protein or glycosyltransferase proteins were modulated in either the lag or log phase only in folpet exposure. These genes were typically down-regulated when folpet was added in the log phase of growth. It is not surprising that folpet may interact and possible bind to the *Z. tritici* cell membrane since it is one of the external structures of the fungal cell. Siegel and Sisler (1968b) reported lower affinity of folpet to cell wall fragments of *S. pastorianus*. They found that approximately 15 % of the total folpet was bound to cell walls fragments.

4.4.3 Common genome-wide expression response in *Z. tritici* to chlorothalonil or folpet.

Functional groups of genes related to aspartic-type endopeptidase were preferable associated with chlorothalonil or folpet exposure only in the lag phase. Endopeptidases are proteolytic enzymes that cleave peptide bonds in the inner regions of polypeptides away from the N- or C- ends (see Davies, 1990). These proteolytic enzymes can be found in many cell compartments, including cytosol, vacuole or cell membrane (Teichert et al., 1989; ten Have et al., 2004; ten Have et al., 2010). Functions of endopeptidases range from degradation of proteins and peptides under nitrogen or carbon starvations (Takeshige et al., 1992; Zubenko and Jones, 1981) to pathogenicity (see Monod et al., 2002). *Zymoseptoria tritici* was not grown under starvation conditions *in vitro*. The overexpression of genes encoding aspartic-type endopeptidase in response to either chlorothalonil or folpet exposure suggests that *Z. tritici* was undergoing nutrient starvation-like stress. This nutrient starvation-like stress might be induced by alterations in the carbon catabolite or nitrogen metabolite repression pathway – exerted by chlorothalonil

exposure – and/or activity of the serine-type carboxypeptidase – exerted by folpet exposure. Exopeptidases as serine-type carboxypeptidase work together with endopeptidases during the catabolism of proteins. The endopeptidases breakdown long polypeptide chains producing large number of free ends, and the exopeptidases release single amino acids from the free ends (Monod et al., 2002). Thus, genes related to proteolytic enzymes – aspartic-type endopeptidase – may be associated to an indirect stress response through perturbations in the cell membrane exerted by either chlorothalonil or folpet.

In contrast to lag phase, enrichment analysis determined that when chlorothalonil or folpet was added in the log phase, transcription of genes associate with respiration, glucose or cellular amino acid catabolism, ornithine metabolism, structure and function of proton-transporting V-type ATPase V_1 domain, and proteasome structure was increased more than others. Several fungicides with diverse mode action are known to impair respiration by inhibiting directly complex I, II or III in the mitochondrial respiratory chain (see FRAC, 2014). Interestingly, chlorothalonil or folpet induced overexpression of genes putative involved in the structure of the complex I (NADH:ubiquinone oxidoreductase) of the respiratory chain in *Z. tritici*. The complex I – also referred as NADH dehydrogenase - is the largest enzyme of the respiratory chain (see Brandt, 2006). It has been suggested that activation of the NADH dehydrogenase may be induced through a signal transduction pathway led by alkylated sulfhydryl groups on G proteins in the cell membrane (Marques and Bicho, 1997). Chlorothalonil or folpet interact with compounds containing sulfhydryl groups (Lukens and Sisler, 1958; Vincent and Sisler, 1968). However, a gene encoding a putative G-protein-couple receptor (i.e. estExt_fgenesh1_pg.C_chr_90429) was down-regulated after chlorothalonil or folpet exposure in the log phase. Thus, chlorothalonil or folpet impair respiration possible through direct interaction with the NADH:ubiquinone oxidoreductase in *Z. tritici*. Although there is no report that folpet affects mitochondrial respiration, it is known that folpet reduces dehydrogenase activity in the cell (Siegel, 1971b). In addition, changes in respiration by chlorothalonil exposure in *S. pastorianus* and *Neurospora crassa* have been reported previously (Vincent and Sisler, 1968).

Alterations in glucose metabolism by chlorothalonil or folpet have been reported previously. These two fungicides bind to dehydrogenase enzymes, particularly folpet that has affinity for the glyceraldehyde 3-phosphate dehydrogenase, which plays a large role in glycolysis (Siegel, 1971b; Siegel and Sisler, 1968b; Vincent and Sisler, 1968). In agreement, transcription of genes related to glucose catabolism was increased in *Z. tritici* when chlorothalonil or folpet was added in the log

phase. Thus, both fungicides are able to impair glucose metabolism in the log phase of growth of *Z. tritici*.

Transcription of genes putative involved in the structure of V-type H^+ ATPase V_1 domain was increased if chlorothalonil or folpet was added in the log phase of growth of *Z. tritici*. The V-type H^+ ATPase is a multi-subunit protein complex enzyme that hydrolyses ATP to generate a gradient of protons across diverse biological membranes *via* active transport of H^+ (see Beyenbach and Wieczorek, 2006). The protein complex comprises two major ring structures a cytoplasmic V_1 complex that interacts with ATP and an integral membrane V_0 complex that mediates H^+ or Na^+ transport (see Murata et al., 2005; Nelson, 2003). Thus, V-type ATPase is responsible for intracellular acidification (see Nishi and Forgac, 2002). Expression of genes encoding the V-type H^+ ATPase complex under diverse stress conditions has been observed before. Cells undergoing stress conditions such salinity, drought, excess of heavy metals tend to modulate V-type H^+ ATPase activity to overcome the stress (see Dietz et al., 2001). Dias et al. (2010) reported that genes involved in the V-type H^+ ATPase pump structure or function were needed in yeast cells to overcome mancozeb exposure. They argued that H^+ pumps ATPase encoding genes are needed to regulate intracellular pH due to mancozeb reduced internal pH in yeast cells. Golldack and Dietz (2001) suggested that expression of genes encoding structure of the V-type ATPase under salinity stress might be due to signal transduction pathway led by G-proteins in the cell membrane. However, a gene encoding a putative G-protein-couple receptor (i.e. estExt_fgenesh1_pg.C_chr_90429) was down-regulated in *Z. tritici* if chlorothalonil or folpet was added in the log phase. To this end, the direct effect of these two fungicides on V-type H^+ ATPase V_1 domain remains elusive. Further studies are needed to determine to what extent chlorothalonil or folpet alter physiological pH or bind directly to V-type ATPase components in *Z. tritici* cells.

Proteasomes are protein complex (26 S proteasome) involve in degradation of mis-folded or particular proteins by the ubiquitin-dependent protein signaling pathway (Peters et al., 1994). The ubiquitin-proteasome system (UPS) degrades targeted proteins into small oligopeptides at particular time or in response to specific events such mutations or oxidative stress (see Baumeister et al., 1998). Oligopeptides can be degraded further into amino acids, which can be recycled for synthesis of new proteins (see Glickman and Ciechanover, 2002), glucose via gluconeogenesis (see Hers and Hue, 1983) or as nitrogen source (Shaibe et al., 1985). Up-regulation of genes related to proteasome structure indicates a controlled proteolytic activity in *Z. tritici* exerted by chlorothalonil or folpet exposure in the log phase. Previously in this chapter, transcription of genes involved in protein synthesis was increased in response to folpet exposure

in the log phase. It is possible that mis-folded proteins due to folpet exposure might be targeted for degradation through the proteolytic activity of proteasomes. Santos et al. (2009) through expression proteomics reported an increased in proteasome activity in yeast cells exposed to mancozeb. Similarly, Dias et al. (2010) reported that genes related with proteosomal function are needed to overcome mancozeb exposure in yeast cells. Moreover, Chondrogianni et al. (2005) reported overexpression of genes encoding proteasome sub-units to degrade oxidised proteins in human fibroblast cells.

Moreover, up-regulation of genes related to catabolism of cellular amino acids after chlorothalonil or folpet exposure suggest that hydrolysed proteins might undergo further reduction. Overexpression of genes involved in ornithine metabolism indicates that cellular amino acids might be used as nitrogen source when chlorothalonil or folpet is added in the log phase. Ornithine and urea are intermediated products in the catabolism of arginine – a α-amino acid – (see Racke and Warnken, 2010). Further degradation of ornithine – to glutamate – can be used as nitrogen source in yeast (Shaibe et al., 1985; Whitney and Magasanik, 1973). Although expression of genes related to protein synthesis was not linked to chlorothalonil exposure, the overexpression of genes related to catabolism of cellular- or α-amino acids suggests that these amino acids might be used to overcome nitrogen requirements in *Z. tritici* fungal cells. However, *Z. tritici* did not grow under limited-nitrogen conditions in this study. Therefore, increase in transcription of genes involved in proteasome components, and protein or amino acid catabolism in *Z. tritici* indicates indirect stress response against alterations in other metabolic pathways (e.g. synthesis of proteins) induced mainly by folpet and to less extent by chlorothalonil.

4.4.4 Detoxification mechanisms in response to chlorothalonil or folpet exposure

To gain insights into the detoxification mechanisms associated to chlorothalonil or folpet exposure, *Z. tritici* was exposed to sub-lethal concentrations of these two fungicides in either lag or log phase of growth. However, enrichment analysis of the DEGs from each treatment did not find functional groups of genes – denoted by their GO-term – associated with non-target-site detoxification mechanisms (e.g. GST, ABC transporter or MFS drug efflux pump). A detail inspection of chlorothalonil- or folpet-DEGs in the lag or log phase detected small changes in gene expression of genes encoding putative GST, and ABC transport or MFS drug efflux pump components.

Nine genes encoding glutathione S-tranferase (GST) were up-regulated when chlorothalonil was added only in the log phase of growth. These genes were also overexpressed in response to folpet. It is well known that GST metabolises diverse xenobiotics by catalysing glutathione-conjugates (Morrow et al., 1998; Pastore et al., 2003). Additionally, three genes encoding ABC transport and four genes encoding MFS drug efflux transport were up-regulated in the lag phase exposure; whereas only one gene encoding ABC transport components was up-regulated in the log phase. A MSF encoding gene in *Z. tritici* (MgMFS1) was reported to reduce sensitivity to a range of natural toxic compounds in heterologous expression in yeast (Roohparvar et al., 2007). Similarly, Zwiers et al. (2003) reported overexpression of genes encoding ABC transporters of *Z. tritici* - in heterologous expression in yeast - to a range of fungicides or plant metabolites.

Similarly, a large number of genes encoding GST (20) were overexpressed in *Z. tritici* when folpet was added only in the log phase. Additionally, three or two genes encoding ABC transporters were up-regulated in the lag or log phase, respectively, in respond to folpet. Interestingly, two of those genes encoding ABC transporters (estExt_Genewise1Plus.C_chr_11491 or estExt_Genewise1.C_chr_41071) were also expressed in response to chlorothalonil in the lag phase. Other six genes encoding MFS efflux /drug transport were up-regulated after folpet exposure in the log phase; whereas one MFS efflux/drug encoding gene was expressed only in the lag phase. It is possible that genes encoding GST or ABC transport components might underlie lower sensitivity to both chlorothalonil and folpet in *Z. tritici*, as sensitivity values of these two fungicides were significantly correlated (Chapter 3). Interestingly, the gene estExt_Genewise1.C_chr_41071 – a putative ABC transporter – was overexpressed in a *Z. tritici* laboratory mutant strain, which is resistant to fluxapyroxad (Chapter 5).

4.5 Conclusions

Using next generation RNA sequencing, I identified subtle but consistent changes in gene expression in *Z. tritici* in response to chlorothalonil or folpet. Functional annotation and enrichment analysis of differentially expressed genes in response to chlorothalonil or folpet found specific functional groups of genes associated to each fungicide. Transcription of genes related to structure or function of the protein histidine kinase complex was increased when chlorothalonil was added in the lag phase; whereas no specific group of genes was detected when chlorothalonil was added in the log phase of growth. Transcription of genes related to serine-type carboxypeptidase with activity in the extracellular region was increased when folpet was added in the lag phase; whereas transcription of genes related to protein synthesis or cellular amino acid metabolism was increased when folpet was added in the log phase. Additionally, transcription of genes related to membrane lipid synthesis was decreased only when folpet was added in the log phase. On the other hand, several functional groups of genes were modulated by chlorothalonil or folpet exposure in the lag or log phase of growth. Transcription of genes related to aspartic-type endopeptidase activity was increased when chlorothalonil or folpet was added in the lag phase. Transcription of genes related to respiration, proteasome complex, glucose or cellular amino acids catabolism, ornithine metabolism, or proton-transporting V-type ATPase V_1 domain was increased in response to either chlorothalonil or folpet exposure in the log phase. In addition, transcription of genes encoding detoxification mechanisms such ABC transporter or MFS drug efflux was slightly increased in *Z. tritici* after exposure to either chlorothalonil or folpet in either lag or log phase. Likewise genes encoding GST were slightly up-regulated only in the log phase after chlorothalonil or folpet exposure. Further studies are needed to confirm if these compound-specific responses are conserved in other strains of *Z. tritici* and can serve as valid "functional gene expression signatures" for these two fungicides.

Chapter 5: Evolution *in vitro* of fungicide resistance of *Zymoseptoria tritici* to fluxapyroxad

5.1 Introduction

Evolution of resistance to fungicides in human or plant pathogens arises from the interaction of mutation rate, selection and genetic drift (Cowen et al., 2000; Huang et al., 2012; Taylor and Feyereisen, 1996). Populations require genetic variability to adapt to new environments or toxicants. Despite natural standing variation (see Barrett and Schluter, 2008; Hawkins et al., 2014), genetic variability can arise from de novo mutations (Lenski, 2004; Torriani et al., 2009). The mutation frequency and the order in which beneficial mutations accumulate can drive the evolutionary adaptation of pathogens to toxicants (Cowen et al., 2000; Weinreich et al., 2006). Laboratory evolution studies give the opportunity to investigate the process of adaptation to new threats including toxicants. Lenski et al. (1991) founded a long-term evolution experiment from a single *Escherichia coli* bacterium in 1988 to characterise the dynamics of adaptive evolution over long-term periods under a constant environment. Since then, 12 replicate *E. coli*-derived populations have been evolving for over 60,000 generations. Diverse studies on these 12 replicated populations have found parallel phenotypic and genetic evolution. Increase and subsequent decline in fitness have been observed over time (Lenski and Travisano, 1994). Populations that evolved modifications in DNA supercoiling show parallel changes in gene expression pattern (Cooper et al., 2003). Moreover, three genes have been identified carrying distinct mutations in all 12 populations (Cooper et al., 2001; Woods et al., 2006). Interestingly, after approximately 31,500 generations only one population evolved the capability to use citrate (Ci^+) as carbon source (Blount et al., 2008). The Ci^+ phenotype reached high frequency in the population but coexisted with phenotypes that are unable to use citrate (Ci^-).

Studies looking at development of resistance to toxicants have also found a parallel increase of resistance in replicate populations. Cowen et al. (2002) reported parallel development of resistance to fluconazole after 330 generations in four replicate populations of *Candida albicans*. Resistance was conferred by overexpression of either an ATP-binding cassette transporter or a multidrug major facilitator transporter gene. Similarly, resistance to fluconazole in *Saccharomyces cerevisiae* was also found to be conferred by overexpression of two ABC transport component encoding genes (*pdr5* or *snq2*) after 400 generations in replicate populations subjected to increasing concentrations of the fungicide (Anderson et al., 2003). Toprak et al. (2012) also found

parallel development of resistance in 12 replicate populations of *E. coli* after approximately 20 days under an increasing concentration of antibiotics. Resistance was conferred by combination of mutations in three distinct genes or by accumulation of mutations in one single gene. Although *in vitro* experimental evolutionary studies using diverse microorganisms have provided insights into the evolutionary adaptation to toxicants, little is known in plant pathogen populations about the evolutionary paths toward resistance and its fixation.

In this study, I determined the course of evolution of resistance to a new succinate dehydrogenase inhibitor in replicate populations of *Zymoseptoria tritici* starting from the sensitive isolate IPO323 (Goodwin et al., 2011). *Zymoseptoria tritici* is a highly adaptable plant pathogen (Zhan and McDonald, 2004). Due to resistance development to the methyl-benzimidazole carbamates (MBCs) (Griffin and Fisher, 1985), the quinone-outside inhibitors (QoIs) (Amand et al., 2003; Fraaije et al., 2005) and some sterol-demethylation inhibitors (DMIs) (Clark, 2006), chemical control of Septoria leaf blotch (SLB) has been marked by a continuous succession of fungicides with diverse modes of action. Options for the chemical control of SLB are currently limited.

Recently, a new generation of carboxamide fungicides that inhibit the succinate dehydrogenase (SDH) enzyme has been launched in the crop protection market (FRAC, 2014). The SDH enzyme also known as succinate:quinone oxidoreductase (SQR) transfers electrons from succinate in the citrate acid cycle to the ubiquinone pool in the mitochondrial respiration chain (Saraste, 1999). The SDH is a membrane-bound enzyme (complex II) that consists of a flavoprotein (SDHA) and an iron-sulphur protein (SDHB), which are anchored to the inner membrane of the mitochondria by two hydrophobic sub-units (SDHC and SDHD) (Horsefield et al., 2006). Carboxamides exert their fungicidal action by physically blocking the transfer of succinate-derived electrons after binding in the ubiquinone-binding pocket (Qp), formed by the sub-units SDHB, SDHC and SDHD (Fraaije et al., 2012; Huang et al., 2006; Scalliet et al., 2012).

Currently, the new-generation of carboxamides (SDHIs) (e.g. penthiopyrad, bixafen, isopyrazam, fluxapyroxad or boscalid) can be sold mixed with azoles to reduce or delay fungicide resistance development in *Z. tritici* (FRAC, 2013; HGCA, 2014). However, mutational laboratory studies and field surveys have reported a number of target-site (Qp) mutations in *Z. tritici* conferring resistance or lower sensitivity to SDHIs. Skinner et al. (1998) reported a target-site mutation in the *sdh* sub-unit B encoding gene which conferred a high level of resistance to carboxin. It was an amino acid substitution from histidine to leucine or tyrosine at codon 267 (H267L/Y). Mutation H267L/Y has been reported in other mutational studies in *Z. tritici* to confer resistance to a range of SDHIs. In particular, the H267L variant confers resistance to all SDHIs, including fluopyram

(Sierotzki and Scalliet, 2013). Other amino acid substitutions in the SDH sub-unit B (e.g. S221P/T, N225H/I, R265P or I269V/P), sub-unit C (e.g. T79I, S83G, L85P, N86K or H152R), and sub-unit D (e.g. D129E/G/S/T) have been reported to confer lower sensitivity to a range of SDHIs in *Z. tritici* laboratory mutants (Fraaije et al., 2012; Scalliet et al., 2012). Field surveys detected two *Z. tritici* field isolates with reduced sensitivity to SDHIs, one carrying an amino acid substitution from threonine to asparagine at codon 79 (T79N), the other a substitution of tryptophan by serine at codon 80 (W80S) in the SDH sub-unit C (FRAC, 2013). Therefore, variation in resistance to SDHIs exists in *Z. tritici* field populations as predicted in mutational experiments. However, two questions still remain unanswered: i) Is there any fitness penalty associated to amino acid substitutions in the target protein (Qp) conferring lower sensitivity to SDHIs?, and ii) Will field selection drive these mutations to fixation in *Z. tritici* field populations, as in the case of resistance to MBCs or QoIs?.

In the present study, the emergence and subsequent dynamics of genomic adaptive changes conferring resistance to the SDHI fluxapyroxad in *Z. tritici* was investigated *in vitro*. The sensitive isolate IPO323 was exposed to increasing concentrations of fluxapyroxad in replicate populations at three different starting concentrations each with or without exposure to UV light. The experimental evolution study presented here was carried out with a controlled and known population size (10^7 spores) to reduce genetic drift (Szendro et al., 2013). Additionally, since the sensitive isolate *Z. tritici* IPO323 was used as progenitor of all populations, no external genotypes entered in the mutant populations and no genetic exchange among populations occurred, mutation was the only source of genetic variability. Therefore, alterations in the target-site (Qp) or overexpression of genes encoding detoxification mechanisms conferred adaptive advantages in the presence of fluxapyroxad and were selected to high frequencies, but genotypes requiring large numbers of simultaneous changes to confer resistance could not be generated.

5.2 Material and Methods

5.2.1 Generation of *Z. tritici* fluxapyroxad-resistant mutants

The reference *Z. tritici* isolate IPO323 (Goodwin et al., 2011; Kema and van Silfhout, 1997) was used to generate laboratory resistant mutants to fluxapyroxad. Aliquots of 100 µL of IPO323 spore suspension at 10^7 spores/mL concentration were plated out onto yeast extract peptone and dextrose agar (YPD) plates amended with 0.4 % (v/v) DMSO, and 0.04, 0.06 or 0.08 µg/mL of fluxapyroxad formulated as emulsifiable concentrate (62.5 g/L EC) (BASF, Ludwigshafen, Germany). Two YPD plates per each fluxapyroxad concentration were inoculated. An equal number of plates were inoculated and exposed to 300 J/m^2 of UV light using an UV Crosslinker (model: XLE-1000/FB, Spectroline, New York, NY, USA). UV-exposed and non-UV-exposed cultures were incubated at 21 °C in the dark for seven days.

After seven days incubation, spores were harvested from the cultures and quantified as described in chapter 2, section 2.3. Cultures with at least 10^7 spores/mL were exposed to another round of fungicide selection. An aliquot of 100 µL of spore suspension at 10^7 spores/mL from each culture was transferred to new YPD plates amended with 0.4 % (v/v) DMSO and a two-fold increased concentration of fluxapyroxad and left untreated or exposed to 300 J/m^2 of UV light before further incubation (Table 5.1). The remaining spore suspension of each culture was suspended in 80 % (v/v) glycerol and stored at -80 °C. Cultures with less than 10^7 spores/mL concentration were kept on the same fluxapyroxad concentration for another seven days at 21 °C in the dark or till they reached the spore concentration needed to be plated out on a doubled fungicide concentration. Every series of fungal cultures was kept separately, as an independent population, designated from IPOFluxa9 to IPOFlux20.

Putative fluxapyroxad-resistant mutants were isolated after ten rounds of selection (Table 5.1). Twenty single colonies of putative fluxapyroxad-resistant mutants grown on YPD were isolated from each culture/population, plated out on new YPD plates for another seven days at 21 °C in the dark, harvested, and stored in 80 % glycerol (v/v) at -80 °C for further use. Putative fluxapyroxad-resistant mutants were named in progressive number order according to the population from which they came (e.g. from IPOFluxa9-1 to IPOFluxa9-20).

Table 5.1 Treatments used in the generation of *Z. tritici* IPO323-derived laboratory mutants resistant to fluxapyroxad.

Population ID	UV light (J/m^2)	Fluxapyroxad concentration at each round of selection (µg/mL)									
		1	2	3	4	5	6	7	8	9	10[1]
IPOFluxa9	0	0.04	0.08	0.16	0.32	0.64	1.28	2.56	5.12	10.24	20.48
IPOFluxa10	0										
IPOFluxa11	300										
IPOFluxa12	300										
IPOFluxa13	0	0.06	0.12	0.24	0.48	0.96	1.92	3.84	7.68	15.36	30.72
IPOFluxa14	0										
IPOFluxa15	300										
IPOFluxa16	300										
IPOFluxa17	0	0.08	0.16	0.32	0.64	1.28	2.56	5.12	10.24	20.48	40.96
IPOFluxa18	0										
IPOFluxa19	300										
IPOFluxa20	300										

[1]Fluxapyroxad concentration was increased two-fold in every round of selection.

5.2.2 *In vitro* fungicide sensitivity testing of putative fluxapyroxad-resistant mutants

Sensitivity tests were carried out as described in chapter 2, section 2.5. Double strength SDB medium was amended with increasing concentrations of fluxapyroxad, fluopyram (0.002, 0.005, 0.015, 0.046, 0.14, 0.4, 1.2, 3.7, 11.1, 33.3 and 100 µg/mL) or carboxin (0.06, 0.05, 0.14, 0.41, 1.23, 3.70, 11.11, 33.33, 100, 300 and 900 µg/mL). Technical grade fluopyram (10 mg/mL; 32462 Fluka, Sigma-Aldrich, UK) or carboxin (50 mg/mL; 45371 Fluka, Sigma-Aldrich, UK) was dissolved in dimethylsulphoxide (DMSO) before dilution in the liquid media. Formulated fluxapyroxad as emulsifiable concentrate (62.5 g/L EC) (BASF, Ludwigshafen, Germany) was dissolved in SDW at 10 mg/mL as a stock concentration before dilution. Microtitre wells were filled with 100 µL of fungicide-amended SDB and 100 µL of spore suspension (2.5×10^4 spore/mL) of IPO323-derive putative fluxapyroxad-resistant mutants. After four days incubation, absorbance (A_{630nm}) was recorded using a FLUOstar OPTIMA microplate reader. Fungicide sensitivity was determined as the concentration which inhibited growth by 50%, using a dose-response relationship estimated with OPTIMA software v2.20OR2. Results are given as EC_{50} values in microgram of fungicide per millilitre (µg/mL). Fluxapyroxad, fluopyram or carboxin EC_{50} values are the average of two independent experiments. For every *in vitro* sensitivity test, IPO323-derived fluxapyroxad-resistant mutants were re-grown from the glycerol stock suspension stored at -80 °C. The progenitor/reference isolate IPO323 was included in the sensitivity test as control.

5.2.3 Detection of SDH sub-unit B, C or D amino acid substitutions in fluxapyroxad-resistant mutants.

Based on the results of the fungicide sensitivity test, two fluxapyroxad-resistant mutants with each of the highest, medium or lowest fluxapyroxad EC_{50} values were selected after ten rounds of selection from each population for sequencing of the succinate dehydrogenase (*sdh*) genes encoding sub-units B, C and D.

Genomic DNA for sequencing was extracted, as described in chapter 2 section 2.10, from frozen and overnight freeze dried seven-day-old spores of selected fluxapyroxad-resistant mutants grown on YPD plates at 15 °C in the dark. The *sdh* gene encoding sub-unit B, C or D were PCR amplified and sequenced using specific primers (Table 5.2).

Polymerase chain reactions were carried out on a Biometra T3000/T3 thermocycler (Applied Biosystems California, USA) in a final volume of 50 µL containing 50 ng of fungal template DNA. Polymerase chain reactions for amplification of the *sdh*B or *sdh*D encoding genes contained 0.5 µM of each primer (Table 5.2), 200 µM of dNTP, 1x Phusion HF buffer, and 1.0 units of Phusion High Fidelity DNA polymerase (Thermo Fisher Scientific, New England Biolabs, UK). Polymerase chain reactions for amplification of *sdh*C encoding gene contained 0.2 µM of each primer (Table 5.2), 200 µM of dNTP, 1x of Easy-A reaction buffer and 2.5 units of Easy-A High Fidelity PCR cloning enzyme (Stratagene Corporation). Amplification conditions were as described in chapter 2, section 2.10 (Table 2.1).

Purified PCR products were sequenced by MWG Eurofins Genomics (Wolverhampton, UK) using specific primers for each *sdh* sub-unit B, C or D encoding genes (Table 5.2). Sequences were assembled and aligned with Geneious v.6.1.4 software, and amino acid substitutions determined after sequence analysis.

Table 5.2 Primers used to amplify and sequence the succinate dehydrogenase (*sdh*) sub-unit B, C or D genes in *Z. tritici* IPO323-derived mutants.

Primer ID/Source	Target gene	Sequence (5' – 3')	Application
sdhB 752F[1]	*sdh*B	TAAACACTCCACGCCTCACG	Amplification of *sdh* sub-unit B gene
SDH2_ST1R[2]		GTCTTCCGTCGATTTCGAGAC	Amplification of *sdh* sub-unit B gene
Mgsdhbf1[3]		ACTCTTCTCACATACCACACA	Sequencing of *sdh* sub-unit B gene
Mgsdhbr1[3]		CTTTCCAATCATCTCGTTCCAT	Sequencing of *sdh* sub-unit B gene
sdhC 888F[1]	*sdh*C	TCCTGTCCTGTGATCCTGGA	Amplification of *sdh* sub-unit C gene
sdhC 1768R[1]		TCCCTTGGGTCCTGATGTAC	Amplification of *sdh* sub-unit C gene
Mgsdhcf1[3]		GGCACATCGCGTCTCACG	Sequencing of *sdh* sub-unit C gene
SDHD_NEW1RMG[2]	*sdh*D	GGCATCATCGTCAAGCAAG	Amplification of *sdh* sub-unit D gene
sdhD 1826R[1]		CAATTCTTCTTGGCAGCAACA	Amplification of *sdh* sub-unit D gene
Mgsdhdf1[3]		CTCACCCTCACCGTCGCC	Sequencing of *sdh* sub-unit D gene

[1]Hawkins, N.J. (2013) (unpublished)
[2]Dubos et al. (2013)
[3]Fraaije et al. (2012)

5.2.4 Quantification of SDHI-resistance conferring alleles in *Z. tritici* IPO323-derived mutant populations using SNP pyrosequencing assays

A pyrosequencing SNP detection assay was developed to determine the frequency of key DNA mutations linked with SDHI resistance in populations IPOFluxa9, IPOFluxa18, IPOFluxa19 and IPOFluxa20 selected after ten rounds of selection. Fluxapyroxad-resistant mutants carrying DNA mutations causing specific amino acid substitutions and the reference isolate IPO323 were included as positive and negative control, respectively. DNA was extracted, as described above, from mutant populations grown on YPD plates at 15 °C in the dark for seven days. Primers targeting regions in the *sdh* sub-unit B, C or D encoding genes conferring amino acid substitutions related to resistance to succinate dehydrogenase inhibitors were designed with the Pyrosequencing Assay Design software (Table 5.3) (version 1.0.6; Biotage, Uppsala, Sweden).

Amplification of target regions was carried out on a Biometra T3000/T3 thermocyclers (Applied Biosystems California, USA) in a final volume of 50 µL PCR reaction containing 50 ng of fungal template DNA, 0.2 units of One*Tag* DNA polymerase (New England BioLabs, Massachusetts, USA), 1x One*Taq* standard reaction buffer, 200 µM of dNTP, and 0.2 µM of each primer (Table 5.3). Thermocycling conditions were 94 °C initial denaturation for 30 seconds, 40 cycles at 94 °C for 15 seconds, 56 °C for 15 seconds and 72 C for 15 seconds, and a final DNA extension at 68 °C for 5

minutes. Fifteen microliters of biotin labelled amplified products from the previous PCR were captured in a mastermix reaction containing three microliters of Streptavidin Sepharose HP beads (GE Healthcare, Uppsala, Sweden), 37 µL of binding buffer (10 mN of Tris-HCl at pH 7.6, 2M of NaCl, 1 mM of EDTA, 0.1 % Tween 20), and 25 µL of sterile distilled water. The reaction was incubated for 10 minutes at room temperate in an Orbis plate shaker (Mikura, West Sussex, UK). Bound PCR products on the beads were aspired, purified and made single-stranded using the Pyrosequencing Vacuum Prep Tool (Biotage) as follows. Beads were aspired onto filters, washed with 70 % (v/v) ethanol for 10 seconds, incubated in denaturing buffer (0.2 M NaOH) for 10 seconds, and washed in washing buffer (10 mM of Tris-acetate, pH 7.6) for 10 seconds.

Beads with single-stranded PCR products were transferred into a PSQ 96-well (Biotage) containing 0.5 µM of sequencing primer (Table 5.3) and 45 µL of annealing buffer (20 mM of Tris-acetate, 2 mM magnesium acetate, pH 7.6). Pyrosequencing reactions were carried out on a PyroMark Q96ID (Biotage, Uppsala, Sweden) according to the manufacturer's instructions using a PyroMark GoldQ96 QSA Reagent kit (Biotage). Dispensation orders were TGCTGACAC to detect H276Y, CGACGTCGA to detect N225T, CAGCTATGC to detect T79I, TACTGAGTA to detect S83G, ACTAGCTCA to detect H152R, and CGTCAGTCT to detect I50L.

Allele frequencies in IPO323-derived fluxapyroxad-resistant mutant populations carrying specific amino acid substitution were estimated with the PyroMark ID SNIP v. 23.2 (Biotage) software. Frequency values are the mean of two technical replicate pyrosequencing reactions.

Table 5.3 Primers used to determine frequency of amino acid substitution in the succinate dehydrogenase (*sdh*) genes in *Z. tritici* IPO323-derived population mutants.

Primer ID	Amino acid substitution	Sequence (5' - 3')	Application
267S	*sdh*B - H267Y	CATGAGCTTGTACCGAT	Sequencing primer
267BioR[1]		CAGGTCCTTGAGCAATTCAGA	PCR amplification
267bF		CGCACTCAACAACAGCATGA	PCR amplification
225S	*sdh*B - N225T	GCCCATCCTACTGGT	Sequencing primer
225F		ATCTTGCCCATCCTACTGGTG	PCR amplification
225BioR		CCATCGGTATGACTGGAGAAG	PCR amplification
79S	*sdh*C - T79I	GCCGAGAGGTACCAG	Sequencing primer
79BioF		GCCCCACCTCGCAATCTA	PCR amplification
79R		TGAGGGCCGAGAGGTACCA	PCR amplification
83S	*sdh*C - S83G	CGCGGTTGAGGGCCS	Sequencing primer
83BioF		CCCCACCTCGCAATCTACAAAC	PCR amplification
83R		CCGTGACGCGGTTGAGGG	PCR amplification
152S	*sdh*C - H152R	CGTATCCCACACCAAA	Sequencing primer
152BioF		CCGGTGACGTTTCATTCGTT	PCR amplification
152R		TAATCATACTCGCCGTATCCCACA	PCR amplification
50S	*sdh*D - I50L	GGAAGAGGAGGGAGAA	Sequencing primer
50BioF		TCCGGCTTCCAGACCACTG	PCR amplification
50R		TTGCGGAAGAGGAGGGAGA	PCR amplification

[1]Bio means 5’ biotin labelled primer.

5.2.5 Gene expression studies through quantitative RT-PCR.

To examine changes in mRNA levels following the introduction of fungicide, the reference *Z. tritici* isolate IPO323 and the IPO323-derived fluxapyroxad-resistant mutant IPOFluxa9-7, with no target-site mutation in the Qp, were exposed to their respective approximate EC_{50} or EC_{80} concentrations of fluxapyroxad *in vitro* as follows. Spores of the IPO323 or IPOFluxa9-7 were grown on YPD plates at 15 °C in the dark for seven days. Flasks containing 100 mL of SDB amended with formulated fluxapyroxad to give a final concentration of 0.04 or 0.19 and 0.39 or 1.56 µg/mL were inoculated with spores from seven-day old cultures of IPO323 or IPOFluxa9-7, respectively, at final concentration of 5×10^5 spore/mL. After 24 h growth at 21 °C in the dark at 200 r.p.m, fungal biomass was harvested by vacuum filtration and snap frozen in liquid nitrogen. IPO323 or IPOFluxa9-7 grown for 24 h in absence of fungicide was used as the untreated control. Formulated fluxapyroxad (0.1 mg/mL) (EC 62.5 g/L EC, BASF) was dissolved in SDW before adding to the SDB.

Total RNA extraction from triplicate untreated and treated samples was carried out as described in chapter 2, section 2.6. Ten micrograms of total purified RNA was reverse transcribed and diluted one in ten (see chapter 2, section 2.7). Gene expression of genes encoding *sdh* sub-unit B, C or D, alternative oxidase (AOX), seven ATP-binding cassette (ABC) transporters, seven major facilitator superfamily (MFS) drug efflux transporters and seven glutathione S-transferase (GST) (Chapter 4) was determined with quantitative RT-PCR using specific oligonucleotide primers (Table 5.4) designed as described in chapter 2, section 2.8. The selected genes encoding transporters or GSTs were the seven most up-regulated genes in the *Z. tritici* isolate IPO323 after exposure to chlorothalonil or folpet in the lag or log phase of growth (Chapter 4). The *aox* encoding gene was included because overexpression can confer lower sensitivity to other fungicides that affect respiration (QoI) through non-target site mutations (Miguez et al., 2004).

Quantitative real-time PCR reactions were carried out using SYBR® Green JumpStart Taq ReadyMix (Sigma-Aldrich, Missouri, USA) in a final volume of 20 µL containing 5 µL of diluted cDNA and 0.25 µM of each primer (Table 5.3). Thermal cycling conditions were 95 °C for 2 min, 40 cycles at 95 °C for 15 seconds, 58 °C for 30 seconds and 72 °C for 40 seconds. Fluorescence was measured at each 72 °C elongation step. Reactions were carried out on a Stratagene Mx300P QPCR System (Agilent Technologies, USA). Normalised relative quantities (NRQ) of target genes were calculated by the $2^{-[\Delta][\Delta]Ct}$ method (Pfaffl, 2001), using β-tubulin as the endogenous control and samples from the untreated IPO323 isolate as calibrator. Results are presented as ratios to the control treatment for data on the $\log_2$ (NRQ) scale.

Table 5.4 Selected genes in the RNA-seq experiment and primers used for qualitative RT-PCR

Gene ID	JGI gene ID[1]	Sequence (5' – 3')	
		Forward	Reverse
sdhB	estExt_Genewise1Plus.C_chr_70864	CGAAGACAAAGACCTTCCACATCT	TCAGAGCATCCAACATCATAGGA
sdhC	fgenesh1_kg.C_chr_8000081	CTTCGGACTCCTCTACCTCG	CACACCAAATGCCTCACTCC
sdhD	estExt_Genewise1.C_chr_40018	TCCGCCATCACCGACTACTT	CCCGCAGTCAAACCAATATCG
AOX	estExt_Genewise1Plus.C_chr_60388	GCACAAGGCGTCTTCTTCAA	GGCGATTTCACGGGTGTAAGT
ABCT1	e_gw1.9.8.1	GCTCCCAGATAAGGCCGAAGA	TTGTACACCAGCCCATCCGATA
ABCT2	estExt_Genewise1.C_chr_41071	GCCATCCCTATCGCTCTCATC	GAAGCCAGACAGTTCCCATCAT
ABCT3	estExt_Genewise1Plus.C_chr_11491	GGTGACAAGGTGGATGATGATC	CAGTCGGAGCAAAGCAGAATC
ABCT4	estExt_Genewise1.C_chr_51261	AGACTGGGCTATGCGGAAATC	GGTGAGCGTTGGAAAGAAGAG
ABCT5	e_gw1.6.23.1	GGGACAACAACACAGACTTCAA	CGGTATGCAGTGAAGTGATGAT
ABCT6	estExt_fgenesh1_pm.C_chr_20184	GACGTGTGCAGTTGTGTATGG	GGGTCTCGGTCTCCTGTTTAAG
ABCT7	estExt_Genewise1.C_chr_80997	AAGCCGAGAGTGAGATGAAGAG	TAGACGGATCCATGCCATCGATA
MFS1	estExt_fgenesh1_kg.C_chr_70313	GTGGATGGTTATTGGATACCTCAT	CCCACAATGAGAGCGATTTGA
MFS2	e_gw1.1.2469.1	GGACGAATTGGGTTGTGGTGATT	CCTCCTCCTTCCTCTTCCTCTT
MFS3	estExt_Genewise1.C_chr_11110	GCCCATTGAGCGAGGTGTAT	GCGAGGCTATCAAGAGACCTTTG
MFS4	e_gw1.7.95.1	GCCAGACCCATGTACTCTAAGT	CTTCCTTATTCTCGACCCGTAGA
MFS5	estExt_Genewise1Plus.C_chr_22238	GGCGACGTTCACACATACATAC	GAGGTCAGAAATCCGGCTTGAA
MFS6	gw1.1.2530.1	GGAGTTCATGCCGAGGAAGAG	GCCATTCCCAATCGCCACAAT
MFS7	estExt_Genewise1Plus.C_chr_12354	GCAACTCATTCTCTCTGCATTCT	ACTTCCAATCGCCAGTTTCTTTG
GST1	estExt_Genewise1Plus.C_chr_32118	CATCATGAGACCATACCCGAAAG	GTACTTGCTGCCGAAGAGTTT
GST2	estExt_fgenesh1_kg.C_chr_30405	CATCAGCCAGAACATCCAGAAG	ACGGGTCACATAGGACAGAATG
GST3	estExt_fgenesh1_pg.C_chr_30174	CTCGGATCGCATTCTTCCATCAG	ATCTGCACCGTTTCCTTCGATTT
GST4	fgenesh1_pm.C_chr_1000191	GCCGAGAATGAGGGACCTTA	ATCCTTCATGATCCTTCCATACAC
GST5	fgenesh1_pg.C_chr_7000293	TTGTCAATCGAAGCGTCCACTA	CCTCCACCATCTCAGCGAATG
GST6	estExt_fgenesh1_kg.C_chr_70091	CGGGTAATGGAAAGGGACGATAT	ACCCACTTTAACAAATGCGGAAAC
GST8	estExt_fgenesh1_kg.C_chr_10601	GATACATTCACGGACGGCAAAC	GATACATTCACGGACGGCAAAC

[1]The Join Genome Institute (JGI) fungal program (Grigoriev et al., 2011)

5.2.6 *In vitro* fitness study on fluxapyroxad-resistant mutants

Fungal growth of the 54 sequenced IPO323-derived fluxapyroxad-resistant mutants was determined *in vitro* in liquid medium. Microtitre wells were filled with 100 µL of double strength SDB and 100 µL of spore suspension (2.5×10^4 spore/mL) of the fluxapyroxad-resistant mutants grown on YPD plates at 15 °C in the dark for seven days. The parental isolate IPO323 was also included in every plate used. Inoculated microtitre wells were incubated at 21 °C in the dark. Absorbance (A_{630nm}) was recorded every 24 h using a FLUOstar OPTIMA microplate reader. There were eight technical replicates (wells) for each biological replicate of a variant, where a variant is formed from a number of fluxapyroxad-resistant mutants carrying similar amino acid substitution in the *sdh* gene sub-unit B, C or D (i.e. SDHB_H267L/Y, SDHB_N225T and SDHD_I50L, SDHC_T79I, SDHC_S83G, or SDHC_H152R) or non-mutation (i.e. NM) in the encoding gene. The average absorbance was calculated over technical replicates at each time point.

5.2.7 Statistical analysis

Calculated EC_{50} values were analysed using ANOVA to compare the populations, taking account of concentration used for their generation and UV light treatment. The model was:

$$\text{Log}_{10}(EC_{50})_{ijkl} = constant + UV_i + Conc_j + (UV.Conc)_{ij} + (UV.Conc.Population)_{ijk} + E_{ijkl}$$

where *constant* is the grand mean; UV_i, i=1,2 (UV, non-UV) is the light status; $Conc_j$, j = 1, 2, 3 (0.04, 0.06, 0.08 µg/mL) is the concentration of fluxapyroxad used; $Population_k$, k = 1,…,9 (IPOFluxa9, 11,12,13,15,16,18,19,20) are the populations; and l = 1,….,20 (isolates) are the biological replicates. The standard error of the difference (SED) on the degrees of freedom from the ANOVA was used to construct a least significant difference (LSD) at the 5% level of significance with which to compare relevant means. Additionally, population resistance distributions were compared using the Kolmogorov-Smirnov two-sample test (KS-test) in GenStat (2014, 17th edition, ©VSN International, Hemel Hempstead, UK). The KS-test looks for the greatest difference between two cumulative distributions. The test statistic is the largest distance between the distributions, *D*, found (at any point along the x-axis).

The average absorbance values at each time point from each fluxapyroxad-resistant mutant was analysed in two stages. For stage one, a linear regression line was fitted to the profile for each fluxapyroxad-resistant mutant to estimate the intercept (i.e. absorbance at time zero) and slope (i.e. growth rate). In stage two, a linear mixed model was fitted to the sets of intercepts and

slopes taking account of the plates used and testing for differences between seven genotypes formed from the 54 fluxapyroxad-resistant mutants, and the parental isolate IPO323. The model was:

$$b_{ijk} = Constant + Variant_i + Plate_j + (Plate.Plate\_Plot)_{jk} + E_{ijk}$$

where *constant* is the grand mean; $Variant_i$, i = 1,…, 8 (NM, SDHB_H267L/Y, SDHB_N225T, SDHD_I50L, SDHC_T79I, SDHC_S83G, SDHC_H152R and the parent isolate) indicates the eight variants; $Plate_j$, j = 1,…,5 are the five plates used; $Plate\_Plot_k$, k = 1,…,11 are the set of eight wells per fluxapyroxad-resistant mutant – including the parent isolate – per plate used; and E_{ijk} is the error term. The *constant* and the *Variant* terms in the model are fixed effect (treatment) terms and all other terms are random (variance component) terms.

Normalised relative quantity (NRQ) values of selected target genes were transformed to the logarithmic (log_2) scale and analysed using ANOVA in GenStat. The ANOVA model was:

$$Log_2(NRQ)_{ij} = Constant + Conc_i + E_{ij}$$

with *constant* being the grand mean and where $Conc_i$, i=1,2,3 (control, EC_{50}, EC_{80}) is the concentration and j=1,2,3 is for the three biological replicates. The log_2(NRQ) values were calculated assuming an efficiency of 2 for the target and reference gene (Pfaffl, 2001). Results are presented as ratios to the control treatment, using the log_2 (NRQ) means.

5.3 Results

5.3.1 Generation of fluxapyroxad-resistant *Z. tritici* mutants

Exposing the *Z. tritici* isolate IPO323 to 300 J/m^2 of UV light resulted in approximately 45 % survival (Fig. 5.1). Six fluxapyroxad-resistant mutant populations (IPOFluxa11, IPOFluxa12, IPOFluxa15, IPOFluxa16, IPOFluxa19 and IPOFluxa20) derived from the sensitive *Z. tritici* isolate IPO323 were obtained after ten rounds of selection at increasing fungicide concentrations and 300 J/m^2 UV light exposure (Fig. 5.2). Three fluxapyroxad-resistant mutant populations (IPOFluxa9, IPOFluxa13 and IPOFluxa18) were obtained using the same series of fungicide concentrations but without UV light exposure (Fig 5.2; Table 5.1).

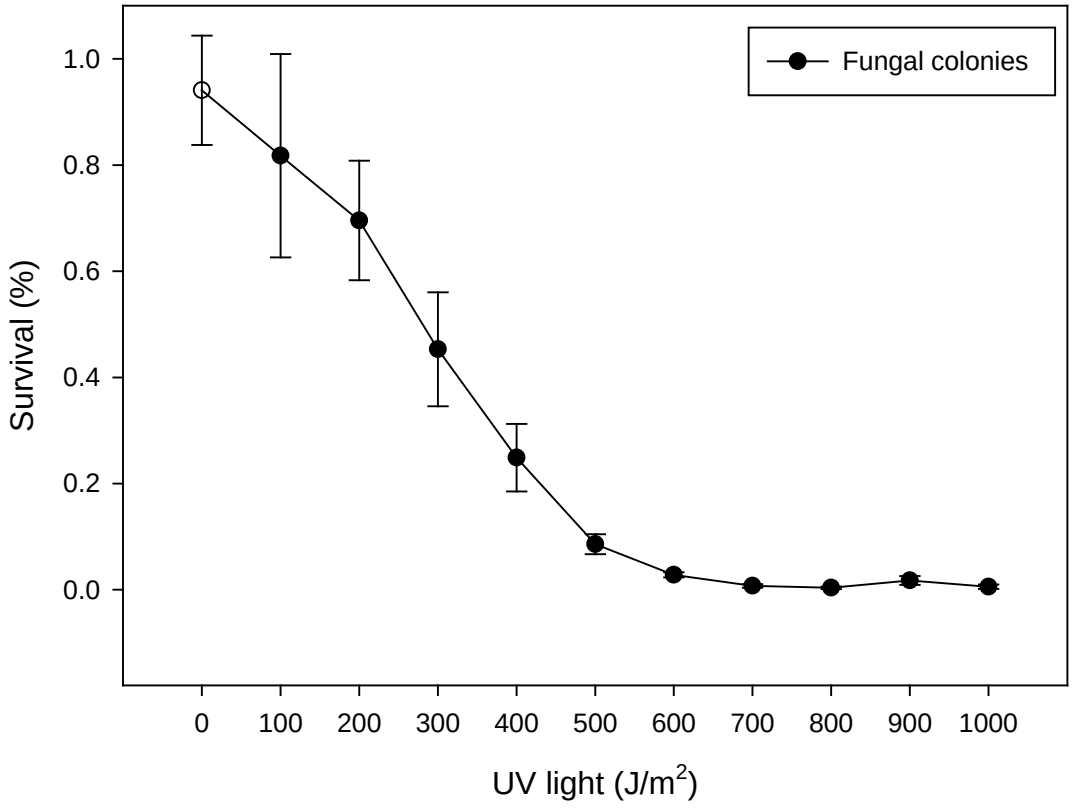

Figure 5.1 Effect of UV light exposure on the reference *Z. tritici* isolate IPO323 colonies survival. Survival values are the mean of three biological replicates. Standard error is denoted by bars.

Population IPOFluxa9 was obtained after 17 weeks, and populations IPOFluxa11 and IPOFluxa12 were obtained after 12 weeks, using a fluxapyroxad series rising from 0.04 to 20.48 µg/mL (Fig. 5.2A). Populations IPOFluxa13 was obtained after 18 weeks, and populations IPOFluxa15 and IPOFluxa16 were obtained after 12 weeks, using a fluxapyroxad, series rising from 0.06 to 30.72 µg/mL (Fig. 5.2B). Populations IPOFluxa18, IPOFluxa19 and IPOFluxa20 were obtained after 12 weeks exposure, using a fluxapyroxad series starting at 0.08 g/mL and ending at 40.96 µg/mL (Fig. 5.2C). A total of 180 fluxapyroxad-resistant mutants, 20 strains from each population, were isolated from the final populations.

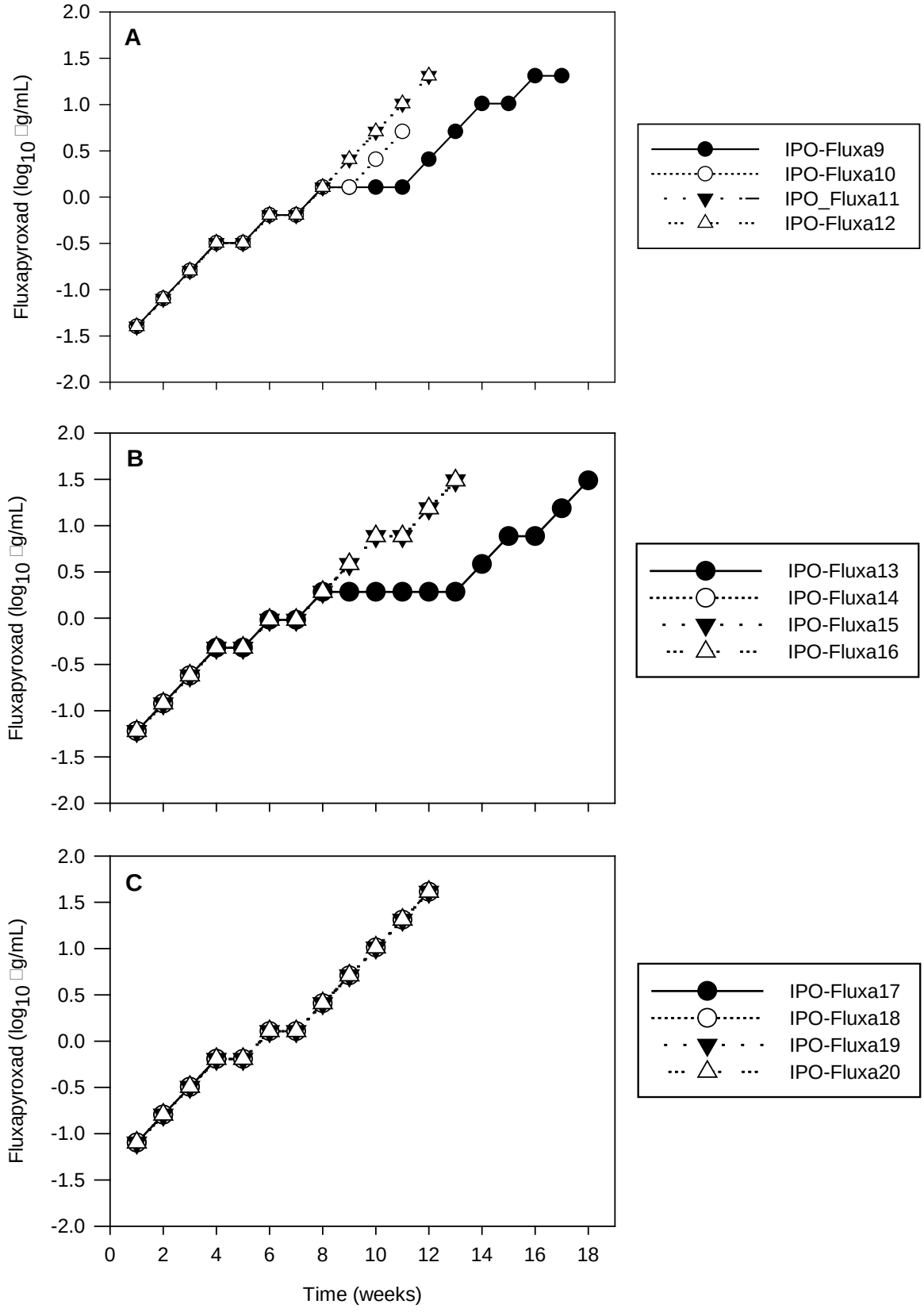

Figure 5.2 Progression of *Z. tritici* IPO323-derived populations through growth on YPD plates amended ten successively doubled concentrations of fluxapyroxad starting from 0.04 µg/mL (A), 0.06 µg/mL (B) or 0.08 µg/mL (C) of fluxapyroxad. Cultures IPOFluxa11, 12, 15, 16, 19 and 20 were also exposed to 300 J/m^2 of UV light.

5.3.2 Sensitivity of fluxapyroxad-resistant mutants to fluxapyroxad

On average, mutant isolates from the selected populations were less sensitive to fluxapyroxad than the reference *Z. tritici* isolate IPO323, which had an average EC_{50} value of 0.02 µg/mL in this study (Fig. 5.3; Table 5.5). No significant differences (p = 0.190, F-test) were detected between EC_{50} values of the nine populations studied. Fluxapyroxad sensitivity values ranged from 0.19 to 51.84 µg/mL (Fig. 5.3).There was no strong association between exposure to UV or the initial fluxapyroxad concentration. However, Kolmogorov-Smirnov test – a nonparametric test – detected significant differences between IPOFluxa9 and IPOFluxa11 or IPOFluxa12 (Kolmogorov-Smirnov two-sample tests: IPOFluxa11 vs IPOFluxa9: D=1, χ^2 approximation = 40, 2 df, $p < 0.001$; IPOFluxa12 vs IPOFluxa9: D=1, χ^2 approximation = 40, 2 df, $p < 0.001$). The IPOFluxa9 had lower fluxapyroxad EC50 values, from 0.19 to 0.47 µg/mL (Fig. 5.3).

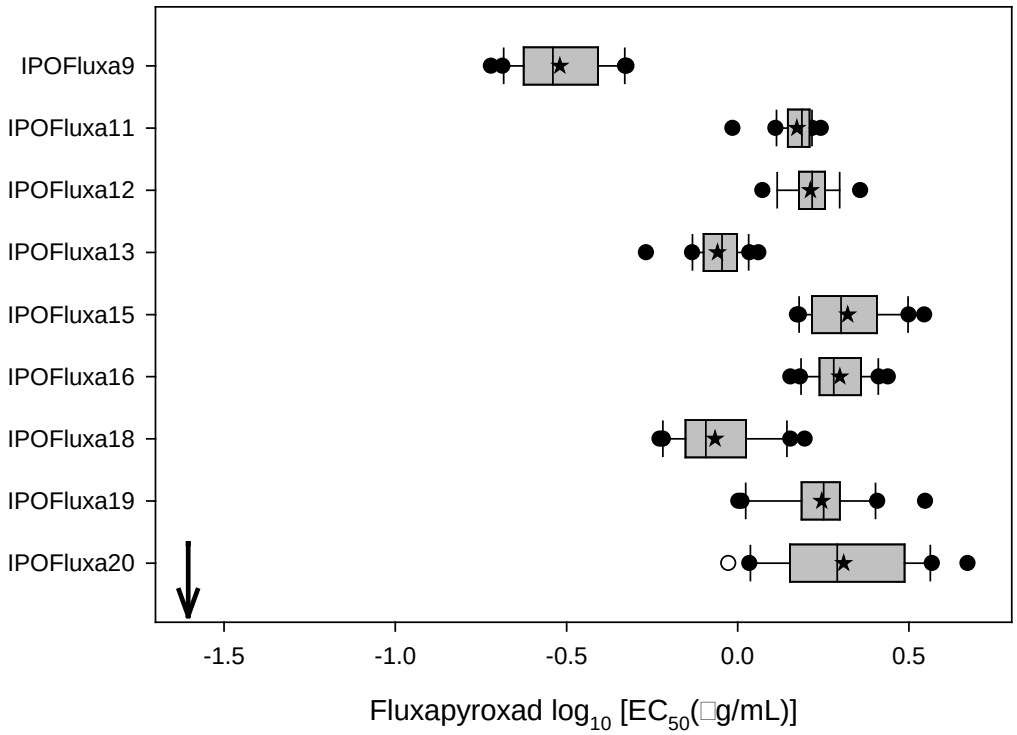

Figure 5.3 Sensitivities to fluxapyroxad of *Z. tritici* IPO323-derived mutant populations after ten rounds of selection on YPD amended with 0.04 (IPOFluxa9, 11, 12), 0.06 (IPOFluxa13, 15, 16) or 0.08 (IPOFluxa18, 19, 20) µg/mL of fluxapyroxad as starting concentration. Fungicide concentration was increased two fold every round of selection. Populations IPOFluxa11, 12, 15, 16, 19 and 20 were also exposed to 300 J/m^2 of UV light. Sensitivity values are the mean of two independent experiments. Star indicates the mean of each population (SED = 0.03567 on 170 df; LSD (5%) = 0.07042). Closed circles indicate values beyond the 90th and 10th percentiles. Sensitivity of the parent IPO323 isolate is indicated by the arrow.

Table 5.5 Succinate dehydrogenase inhibitor (SDHI) sensitivities of *Z. tritici* IPO323-derived laboratory mutant populations obtained after ten rounds of *in vitro* selection on YPD plates amended with 0.04 (IPOFluxa9, 11, 12), 0.06 (IPOFluxa13, 15, 16) or 0.08 (IPOFluxa18, 19, 20) µg/mL of fluxapyroxad as starting point or with 300 J/m^2 of UV light exposure.

Population	UV exposure	*n*	Fungicide					
			Fluxapyroxad		Fluopyram		Carboxin	
			EC_{50} ± SE (µg/mL)[1]	SD	EC_{50} ± SE (µg/mL)[1]	SD	EC_{50} ± SE (µg/mL)[1]	SD
IPOFluxa9	-	20	0.32 ± 0.02	0.09	0.56 ± 0.05	0.22	7.69 ± 0.46	2.03
IPOFluxa11	+	20	1.5 ± 0.04	0.17	3.54 ± 0.11	0.50	75.93 ± 2.9	12.97
IPOFluxa12	+	20	1.60 ± 0.07	0.33	3.10 ± 0.43	1.92	26.42 ± 1.77	7.91
IPOFluxa13	-	20	0.89 ± 0.03	0.14	0.06 ± 0.004	0.02	69.30 ± 9.68	43.31
IPOFluxa15	+	20	2.16 ± 0.13	0.58	4.63 ± 0.27	1.21	338.6 ± 45.17	202.00
IPOFluxa16	+	20	2.02 ± 0.08	0.37	89.1 ± 6.11	27.32	85.60 ± 35.70	159.83
IPOFluxa18	-	20	0.90 ± 0.06	0.27	0.09 ± 0.01	0.04	47.55 ± 1.81	8.08
IPOFluxa19	+	20	1.83 ± 0.12	0.55	32.15 ± 10.19	45.58	20.01 ± 1.16	5.19
IPOFluxa20	+	20	2.23 ± 0.22	0.99	2.59 ± 0.52	2.33	24.48 ± 4.04	18.08

[1] EC_{50} values are the mean of two independent experiments base on the physical concentration scale.

5.3.3 Sensitivity of fluxapyroxad-resistant mutants to fluopyram

Most of the fluxapyroxad-resistant mutants were also less sensitive to fluopyram than isolate IPO323, which has an average EC_{50} value of 0.10 µg/mL (Fig. 5.4). There were significant (p<0.001, F-test) differences between populations. This response depended strongly on population, with a clear decrease in sensitivity in seven populations (Fig. 5.4; IPOFluxa9, 11, 12, 15, 16, 19 and 20), but no change in two non-UV exposure populations (Fig. 5.4; IPOFluxa13 and IPOFluxa18). Populations IPOFluxa13 or IPOFluxa18 had the lowest fluopyram EC_{50} values, ranging from 0.03 to 0.20 µg/mL (Fig. 5.4). The other populations ranged from 0.28 to >100.0 µg/mL (Fig. 5.4). Differences in fluopyram sensitivities between IPO323-derived mutant populations indicate that diverse target-site (Qp) mutations may underlie lower sensitivity or resistance to the fungicide.

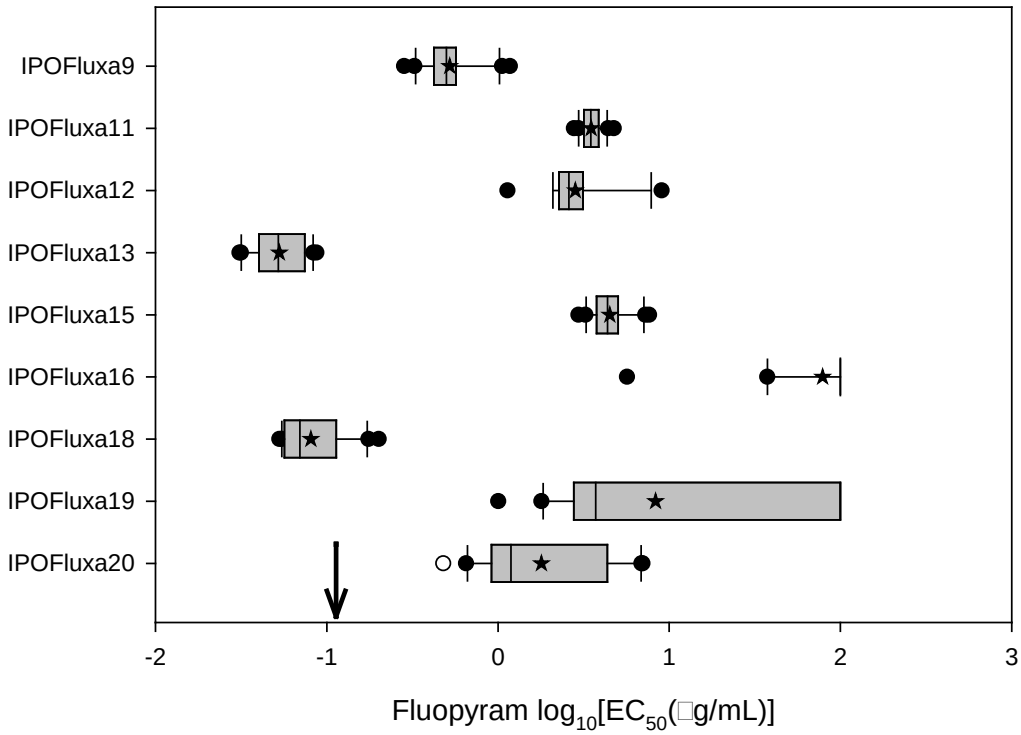

Figure 5.4 Sensitivities to fluopyram of *Z. tritici* IPO323-derived mutant populations after ten rounds of selection on YPD amended with 0.04 (IPOFluxa9, 11, 12), 0.06 (IPOFluxa13, 15, 16) or 0.08 (IPOFluxa18, 19, 20) µg/mL of fluxapyroxad as starting concentration. Fungicide concentration was increased two fold every round of selection. Populations IPOFluxa11, 12, 15, 16, 19 and 20 were also exposed to 300 J/m^2 of UV light. Sensitivity values are the mean of two independent experiments. Star indicates the mean of each population (SED = 0.10 on 170 df; LSD (5%) = 0.20). Closed circles indicate values beyond the 90th and 10th percentiles. Sensitivity of the parent IPO323 isolate is indicated by the arrow.

5.3.4 Sensitivity of fluxapyroxad-resistant mutants to carboxin

The IPO323-derived fluxapyroxad-resistant mutants with carboxin EC_{50} values ranging from 4.56 to 491 µg/mL (Fig. 5.5) were less sensitive to carboxin than the reference *Z. tritici* isolate IPO323 (average EC_{50} value of 1.7 µg/mL) regardless of the treatment given to the population from which they came (Fig. 5.5, Table 5.5). This lower sensitivity to carboxin was expected, since fluxapyroxad and carboxin are SDHI carboxamide fungicides (FRAC, 2014). However, there were significant differences between populations (Fig. 5.5; $p<0.001$, F-test).

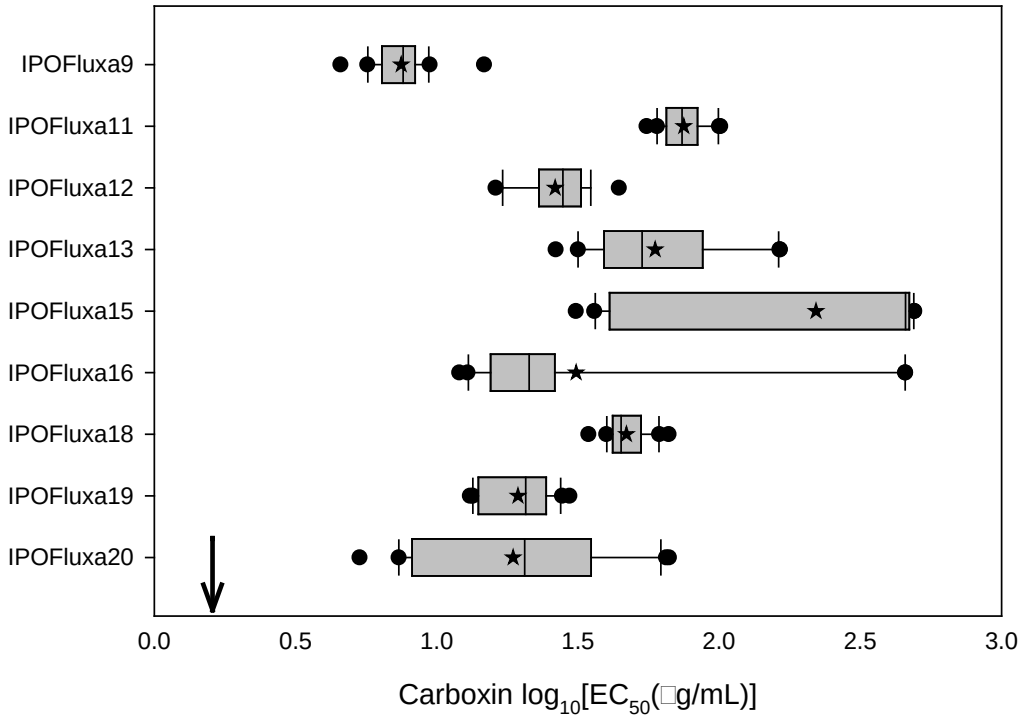

Figure 5.5 Sensitivities to carboxin of *Z. tritici* IPO323-derived mutant populations after ten rounds of selection on YPD amended with 0.04 (IPOFluxa9, 11, 12), 0.06 (IPOFluxa13, 15, 16) or 0.08 (IPOFluxa18, 19, 20) µg/mL of fluxapyroxad as starting concentration. Fungicide concentration was increased two fold every round of selection. Populations IPOFluxa11, 12, 15, 16, 19 and 20 were also exposed to 300 J/m^2 of UV light. Sensitivity values are the mean of two independent experiments. Star indicates the mean of each population (SED = 0.0912 on 170 df; LSD (5%) = 0.180). Closed circles indicate values beyond the 90th and 10th percentiles. Sensitivity of the parent IPO323 isolate is indicated by the arrow.

5.3.5 Key amino acids substitutions in the SDH sub-unit B, C or D in fluxapyroxad-resistant mutants

Based on the results of the fluxapyroxad sensitivity test, 54 fluxapyroxad-resistant mutants were selected and the genes encoding *sdh* sub-unit B, C and D sequenced. Mutations encoding seven different amino acid substitutions in the Qp were detected in 48 of these. Three amino acid substitutions were detected in the SDH sub-unit B; exchange from histidine to tyrosine or leucine at codon 267 (H267Y/L) or from asparagine to threonine at codon 225 (N225T). Three other amino acid substitutions were detected in the SDH sub-unit C; exchange from threonine to isoleucine at codon 79 (T79I), from serine to glycine at codon 83 (S83G) or from histidine to arginine at codon 152 (H152R). One amino acid substitution was detected in the SDH sub-unit D; an exchange from isoleucine to leucine at codon 50 (I50L) (Fig. 5.6). Only one fluxapyroxad-resistant mutant carrying two amino acid substitutions (SDHB_N225T, SDHD_I50L) was detected. Six fluxapyroxad-resistant mutants, all from the IPOFluxa9 population, did not carry amino acid substitutions in the target-site Qp. Pyrosequencing assays of IPOFluxa9 confirmed the absence of target-site (Qp) mutations (see section 5.3.6).

The distribution of amino acid substitution-associated mutations indicated that selection of mutations occurred at random across populations (Fig. 5.7). Most of the amino acid substitutions conferred cross-resistance to fluopyram and/or carboxin, with the exception of amino acid substitution SDHB_H267Y, which was more sensitive to fluopyram (Fig. 5.7E and H; Table 5.6). Fluxapyroxad-resistant mutants without change in the target-site Qp showed also lower sensitivity to both fluopyram and carboxin (Fig. 5.7B and C; Table 5.6).

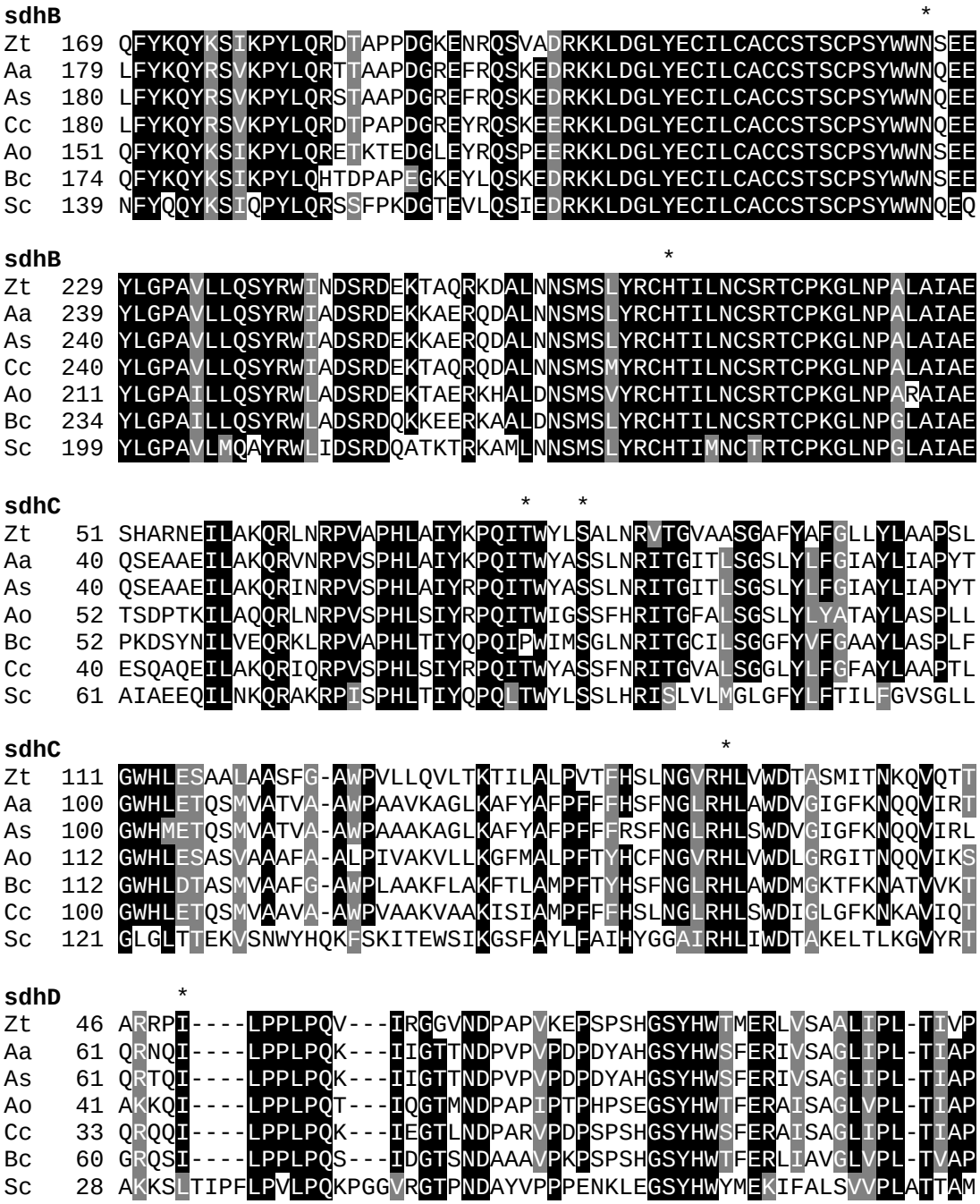

Figure 5.6 Location of key amino acid substitutions in the *sdh* sub-units in IPO323-derivated fluxapyroxad-resistant mutants. Partial *sdh* sub-units sequences of *Zymoseptoria tritici* (Zt), *Alternaria alternata* (Aa), *Alternaria solani* (As), *Aspergillus oryzae* (Ao), *Corynespora casiicola* (Cc), *Botrytis cinerea* (Bc), and *Saccharomyces cerevisiae* (Sc). Stars indicate positions where amino acid substitutions were found. Conserved residues are shaded in black or grey corresponding to 100 or 80 % conservation, respectively.

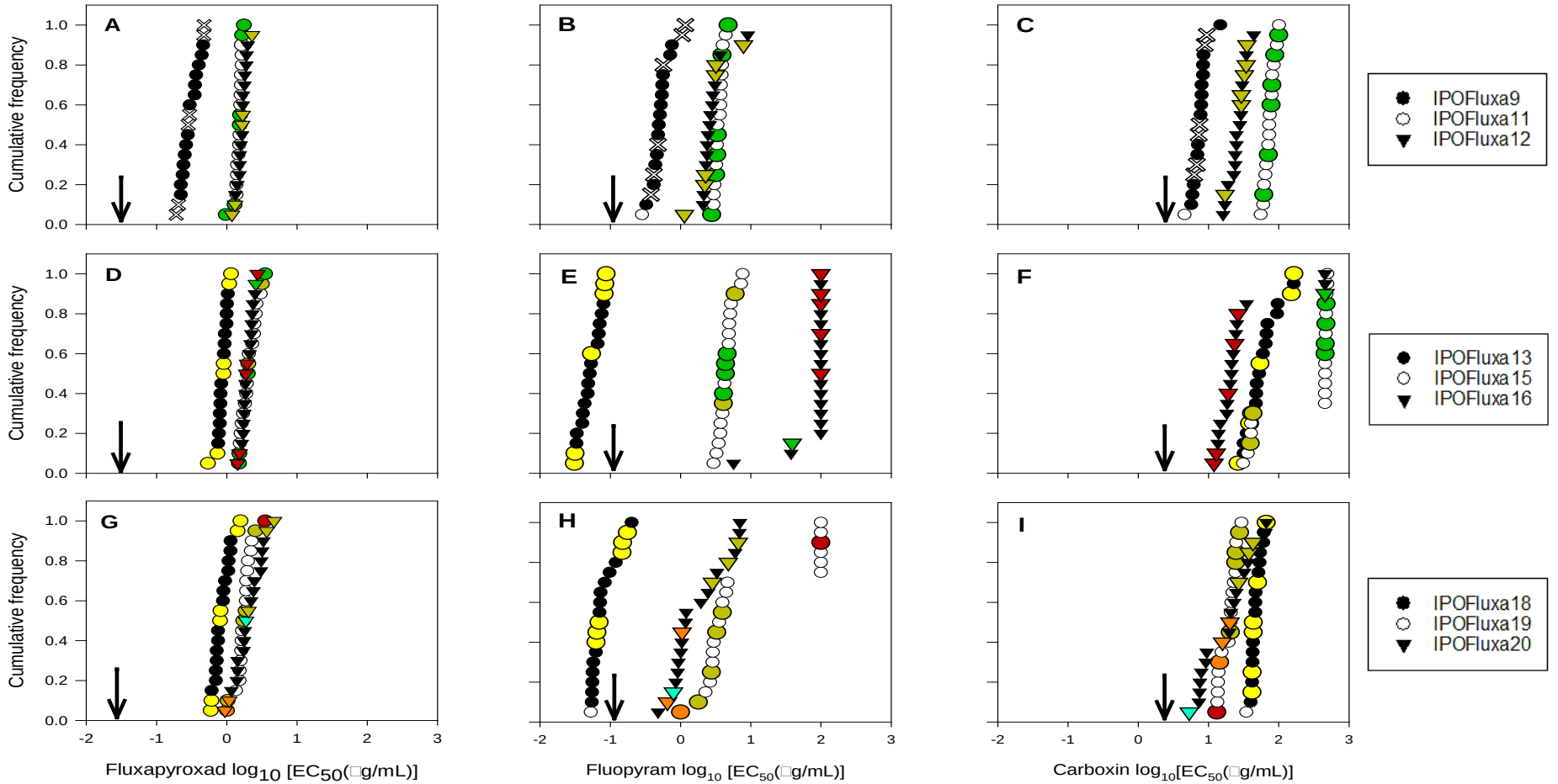

Figure 5.7 Mapping of associated amino acid substitutions in the succinate dehydrogenase (*sdh*) complex on IPO323-derived mutant populations of *Z. tritici* after ten rounds of selection *in vitro* on YPD amended with 0.04 (A, B, C), 0.06 (D, E, F) or 0.08 (G, H, I) µg/mL of fluxapyroxad as starting point. *sdh*B: H267Y, H267L and N225T; *sdh*C: T79I, S83G and H152R; *sdh*D: I50L; Non-mutation. Black filled or empty dots and black filled triangles are IPO323-derived mutants not sequenced. Fungicide concentration was increased two fold every round of selection. Populations IPOFluxa11, 12, 15, 16, 19 and 20 were also exposed to 300 J/m^2 of UV light. Sensitivity of the parent isolate IPO323 is indicated by the arrow.

Table 5.6 Succinate dehydrogenase inhibitors (SDHIs) sensitivities of IPO323-derived fluxapyroxad-resistant mutants carrying different mutations in the sdhB, C or D genes.

Strain/mutant *sdh* variants	Corresponding codon changes		Fungicide								
			Fluxapyroxad			Fluopyram			Carboxin		
		n	EC_{50} ± SE (µg/mL)[1]	SD	RF[2]	EC_{50} ± SE (µg/mL)	SD	RF	EC_{50} ± SE (µg/mL)	SD	RF
IPO323	None	1	0.02 ± 0.004	-	-	0.10 ± 0.01	-	-	1.71 ± 0.29	-	-
Non-mutation	None	6	0.32 ± 0.05	0.12	14	0.67 ± 0.15	0.36	7	7.65 ± 0.50	1.22	5
B-H267L	CAC > CTC/CTT	11	2.14 ± 0.52	1.74	94	7.04 ± 3.19	10.57	70	254.68 ± 61.39	203.62	149
B-H267Y	CAC > TAC	12	0.93 ± 0.09	0.33	40	0.09 ± 0.01	0.05	0.9	82.77 ± 29.16	101.03	48
B-N225T, D-I50L	AAC > ACC, ATT > CTT	1	1.88 ± 1.10	-	51	0.79 ± 0.08	-	8	5.33 ± 0.81	-	3
C-T79I	ACC > ATC	3	1.01 ± 0.04	0.07	44	0.90 ± 0.12	0.21	9	16.82 ± 1.74	3.02	10
C-S83G	TCG > GGG	6	2.17 ± 0.33	0.81	94	>100.00	0.00	>990	17.83 ± 2.51	6.15	10
C-H152R	CAT > CGT	15	2.12 ± 0.21	0.80	92	3.72 ± 0.48	1.88	37	30.99 ± 2.01	7.79	18

[1] EC_{50} values are the mean of two independent experiments base on the physical concentration scale.

[2] Resistance factors (RFs) of laboratory-mutants carrying the same *sdh* variant were calculated as the fold-change in mean EC_{50} compared with the mean EC_{50} value of the reference *Z. tritici* isolate IPO323.

5.3.6 Allele frequency of SDHI-resistance conferring alleles in *Z. tritici* IPO323-derived mutant populations using SNP pyrosequencing assays

The frequency of six amino-acid substitutions in Qp was determined, by pyrosequencing, at each transfer to a higher fungicide concentration in four selection lines. Population IPOFluxa9 was selected because no mutations in the Qp were detected in sequenced isolates (Fig. 5.7A, B, and C). Populations IPOFluxa18, 19 and 20 were included because fluxapyroxad-resistant isolates from these populations carried a range of amino acid substitutions in Qp (Fig. 5.7G, H and I).

Amino acid substitution SDHB_H267Y was only detected in IPOFluxa18. It was present from round of selection (RS) four to ten with an approximate allele frequency of 100% (Fig. 5.8A). The SDHB_N225T and SDHD_I50L amino acid substitutions were detected only in IPOFluxa20. The SDHB_N225T amino acid substitution was detected between RS-4 and RS-9 at low frequency (< 5 %), and at RS-10 with an approximate allele frequency of 7 % (Fig 5.8C). SDHD_I50L was detected at low frequency (< 2 %) between RS-3 and RS-6, and at RS-10 with an approximate allele frequency of 14 % (Fig. 5.8C).

The amino acid substitution SDHC_T79I was detected in IPOFluxa19 or IPOFluxa20 at RS-3 with an approximate allele frequency of 6 and 8 %, respectively, with an increase in frequency (to around 80%) at RS-5. The SDHC_T79I allele frequency decreased from approximately 80% to 6% at RS-8; it was undetectable after RS-8 in IPOFluxa19 (Fig. 5.8B). A similar pattern of increase and then decrease in allele frequency of SDHC_T79I (to approximately 15 % at RS-9) was observed in IPOFluxa20 (Fig. 5.8C).

SDHC_H152R was detected from RS-5 in IPOFluxa19 (approximately 9 % frequency) and its frequency increased to around 95 % in RS-9 and RS-10 (Fig. 5.8B). A similar pattern was observed in IPOFluxa20 for the SDHC_H152R amino acid substitution. SDHC_H152R was detected at RS-6 (approximately 5 % frequency) with an increase in allele frequency at RS-9 (to approximately 70 %), but the frequency fell to approximately 37 % at RS-10 (Fig. 5.8C).

The amino acid substitution SDHC_S83G was detected only in IPOFluxa19 at RS-10 with an approximate allele frequency of 14 % (Fig. 5.8B).

The SNP pyrosequencing assay did not detect alleles encoding target amino acid substitutions in the Qp in IPOFluxa9, confirming the result of section 5.3.5.

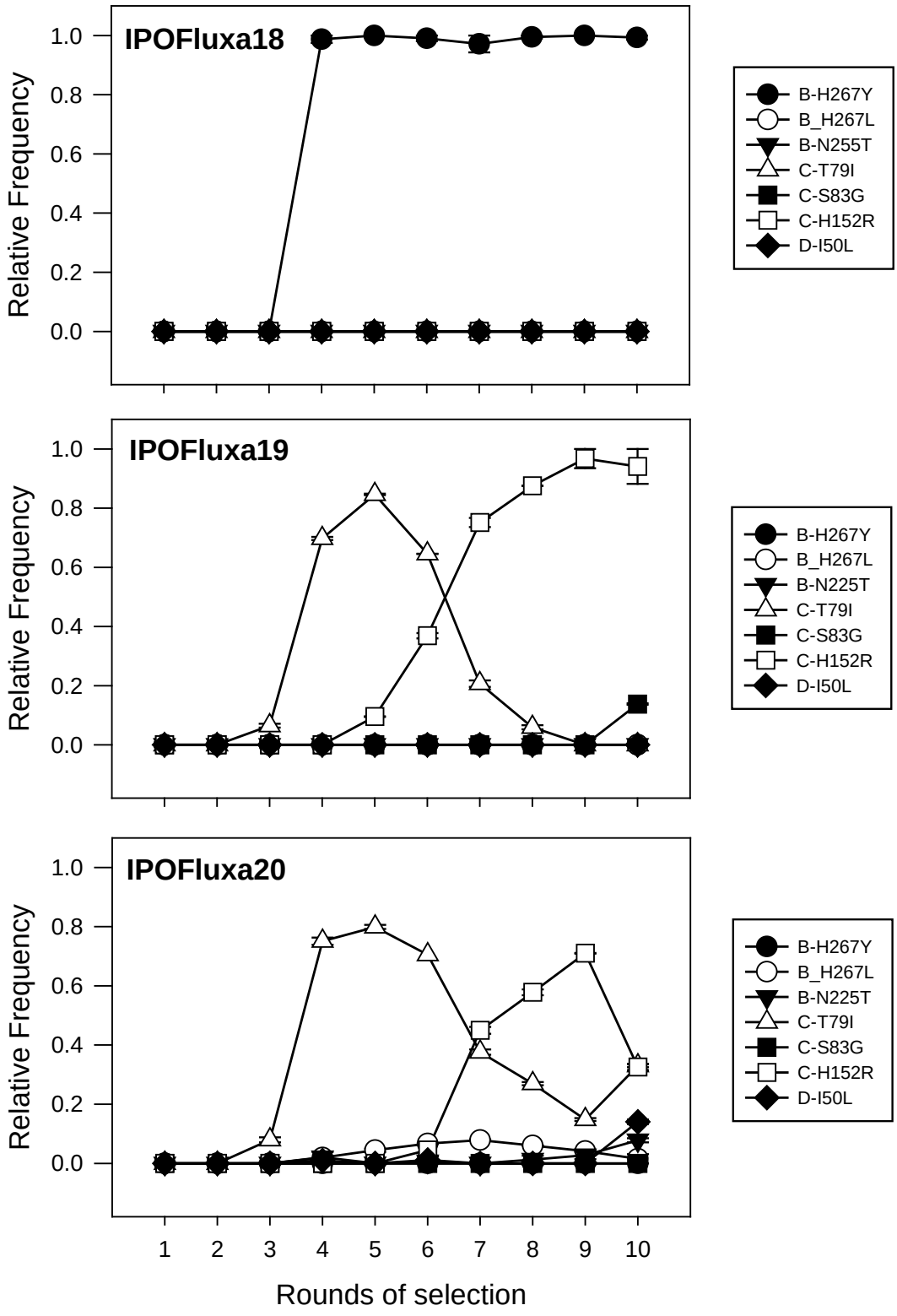

Figure 5.8 Allele frequencies of key amino acid substitutions in IPO323-derived fluxapyroxad-resistant mutant populations during ten rounds of selection *in vitro* on YPD amended with 0.08 µg/mL as starting concentration of fluxapyroxad. Fungicide concentration was increased two-fold every round of selection. Populations IPOFluxa19 and IPOFluxa20 were also exposed to 300 J/m2 of UV light. Relative allele frequency of positive control of *sdh*B: H267Y=1, N225T=0.83; *sdh*C: T79I=1, S83G=1, H152R=1; *sdh*D: I50L=1. Frequency values are the mean of two technical replicates between replicate pyrosequencing reactions.

5.3.7 Gene expression of the target- or non-target-site detoxification mechanisms in fluxapyroxad-resistant mutants

Although no target-site (Qp) alterations were detected in IPOFluxa9, this population is less sensitive to fluxapyroxad than a *Z. tritici* field population (Fig. 5.9; chapter 3 section 3.3.3). However, it is more sensitive to fluxapyroxad than IPOFluxa13 or IPOFluxa18 (Fig. 5.9), in which most or all isolates carry an amino acid substitution at codon 267 in the SDH sub-unit B conferring resistance to fluxapyroxad (Fig. 5.7D and G) and carboxin (Fig. 5.7F and I). Resistance or lower sensitivity to single-site fungicides can be conferred by overexpression of the target protein encoding gene (Cools et al., 2012) or genes encoding non-target-site resistance mechanisms (Kretschmer et al., 2009). It is possible, that lower sensitivity to SDHIs in IPOFluxa9 might be due either to overexpression of the target protein (Qp) or to expression of non-target-site resistance mechanisms. To explore these possibilities, the reference isolate IPO323 and a fluxapyroxad-resistant mutant IPOFluxa9-7 – with the highest fluxapyroxad EC_{50} value – were exposed to their approximate fluxapyroxad EC_{50} (0.04 or 0.40 µg/mL) or EC_{80} (0.19 or 1.6 µg/mL) concentrations *in vitro,* and the expression of selected genes was determined in RNA samples after 24 h incubation in fluxapyroxad-amended SDB medium at 21 °C. The 24 h exposure allowed to have enough fungal biomass to RNA extraction. Moreover, Cools et al. (2012) were able to detect changes in gene expression in *Z. tritici* after 24 h exposure to the single-site fungicide epoxiconazole. Similarly, Becher et al. (2011) detected expression of genes encoding ABC transporters in *F. graminearum* after 24 h exposure to tebuconazole.

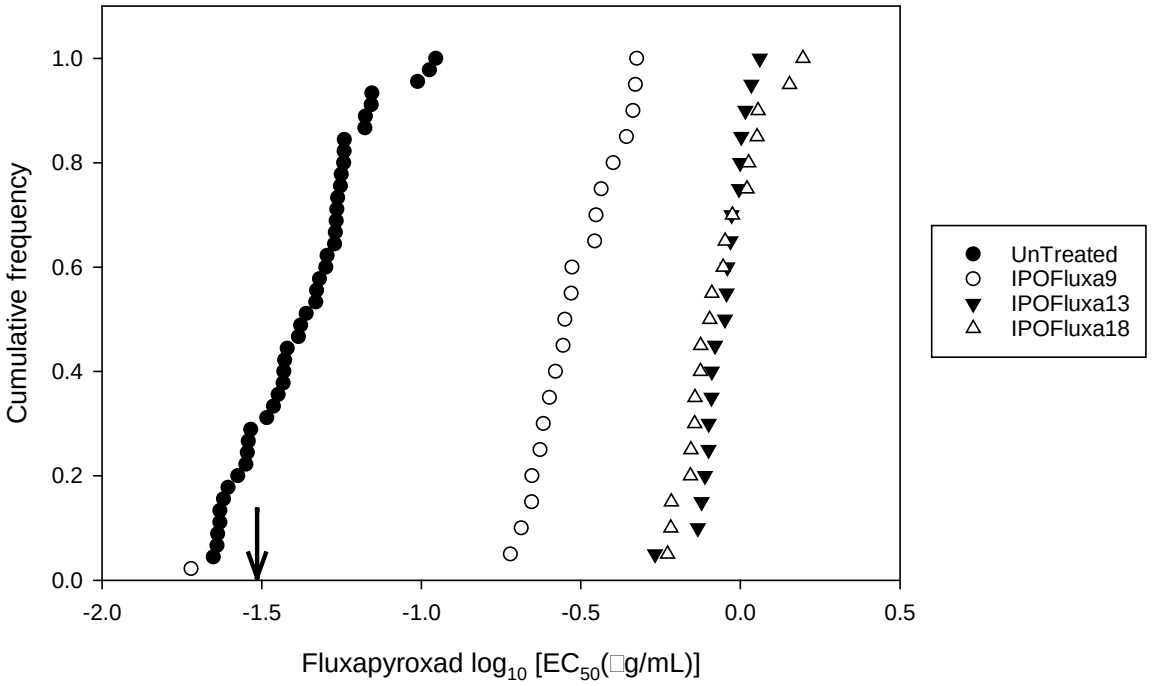

Figure 5.9 Cumulative frequency distribution of fluxapyroxad EC_{50} values of isolates taken from a *Z. tritici* field population (45 isolates: UnTreated; data taken from chapter 3 section 3.3.3), and IPO323-derived mutant populations (20 isolates) after ten rounds of selection *in vitro* on YPD amended with 0.04 (IPOFluxa9), 0.06 (IPOFluxa13) or 0.08 (IPOFluxa18) µg/mL as starting point of fluxapyroxad. Fungicide concentration was increased two fold every round of selection. Mutants tested from populations IPOFluxa13 and IPOFluxa18 carry an amino acid substitution at codon 267 in the sdh sub-unit B; IPOFluxa9 has no changes in sdh loci. Sensitivity of the reference IPO323 isolate is indicated by the arrow.

Quantitative RT-PCR detected a low but significant (p<0.05) up-regulation of genes encoding the *sdh* sub-unit B, C or D in the reference isolate IPO323. In the mutant IPOFluxa9-7 only genes encoding the *sdh* sub-unit B or D were significantly (p<0.001) slightly up-regulated (Fig 5.10). The reference isolate IPO323 and the variant IPOFluxa9-7 exhibited similar *aox* gene expression pattern. However, overexpression of *aox* encoding gene was significant only in IPO323 at its approximate fluxapyroxad EC_{50} or EC_{80} concentration (Fig 5.10).

The *abc*t-2 encoding gene, a putative ATP-binding cassette transporter (Goodwin et al., 2011), was constitutively overexpressed (p<0.05) in IPOFluxa9-7 (Fig. 5.11B). Genes *abc*t-5 and *abc*t-6 were up-regulated (p<0.05) only in IPO323 at its fluxapyroxad EC_{50} or EC_{80} concentration, respectively (Fig. 5.11A).

Similarly, a glutathione S-tranferase (*gst*-4) encoding gene was slightly but significantly (p<0.05) up-regulated in IPOFluxa9-7 exposed to its approximate fluxapyroxad EC_{50} or EC_{80} (Fig. 5.12B). *gst*-4 was also significantly (p<0.05) up-regulated, along with *gst*-1, in the reference isolate IPO323 in presence of fluxapyroxad (Fig. 4.12A).

Gene expression of seven major facilitator superfamily (MFS) efflux drug transporters was determined in the reference isolate IPO323 and the mutant IPOFluxa9-7 in presence of fluxapyroxad. *msf*-2 and *msf*-6 were significantly (p<0.05) up-regulated in the reference isolate IPO323 (Fig. 5.13A). In the fluxapyroxad-resistant mutant IPOFluxa9-7 no *msf* encoding genes was significantly (p<0.05) up- or down-regulated (Fig. 5.13B).

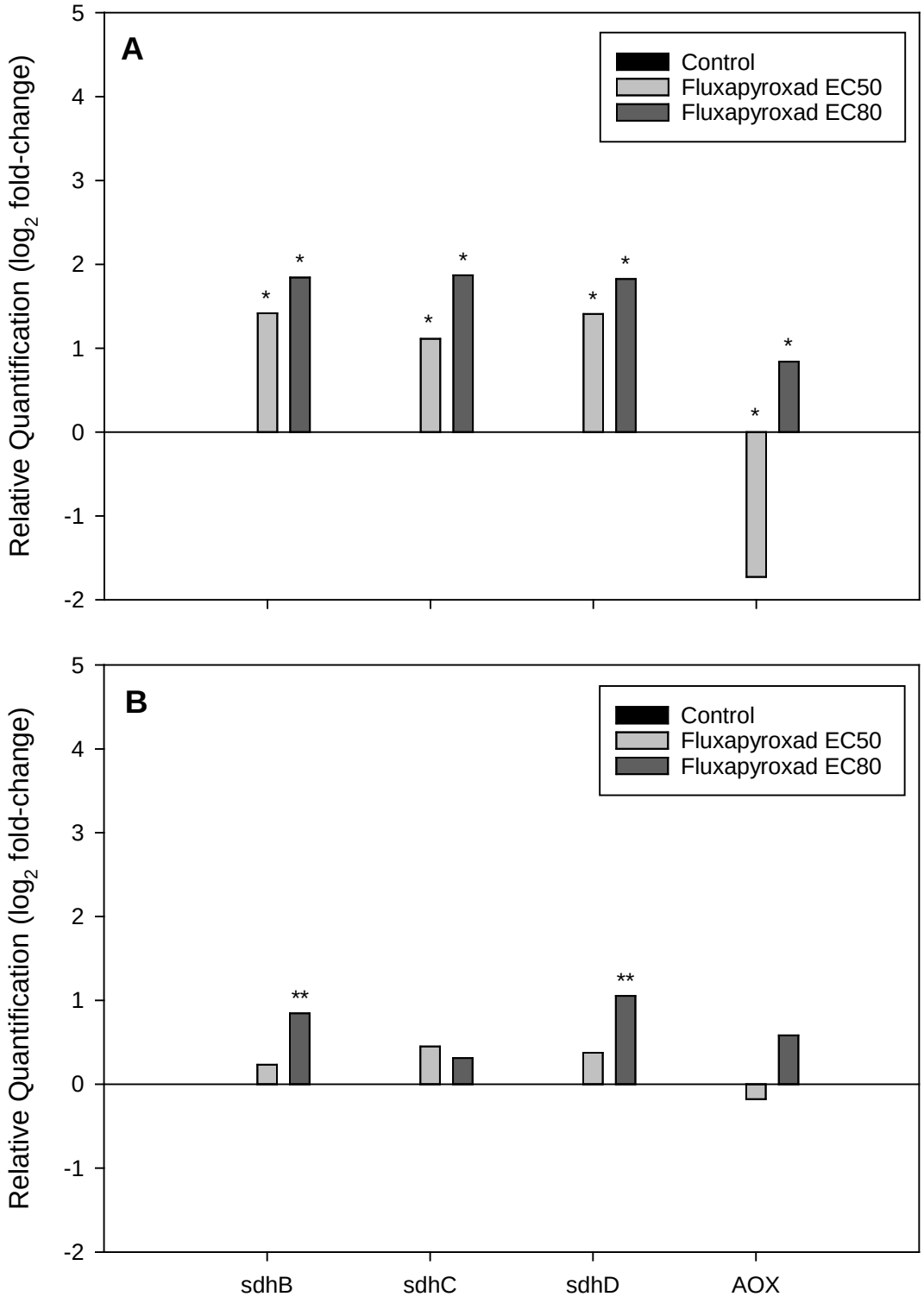

Figure 5.10 Quantitative RT-PCR of genes encoding succinate dehydrogenase (*sdh*) sub-unit B, C and D, and alternative oxidase (AOX) in the reference *Z. tritici* isolate IPO323 (A) and the laboratory mutant IPOFluxa9-7(B) after 24 h exposure to their respective fluxapyroxad EC_{50} or EC_{80} concentration. Mean of three biological replicates. Statistical significance for each gene is marked by asterisks (*=p<0.05 or **=p<0.01). See Appendix 5.1 and 5.2 for the means of log_2 fold-change expression data for the three treatments with standard error of the difference for the comparisons made.

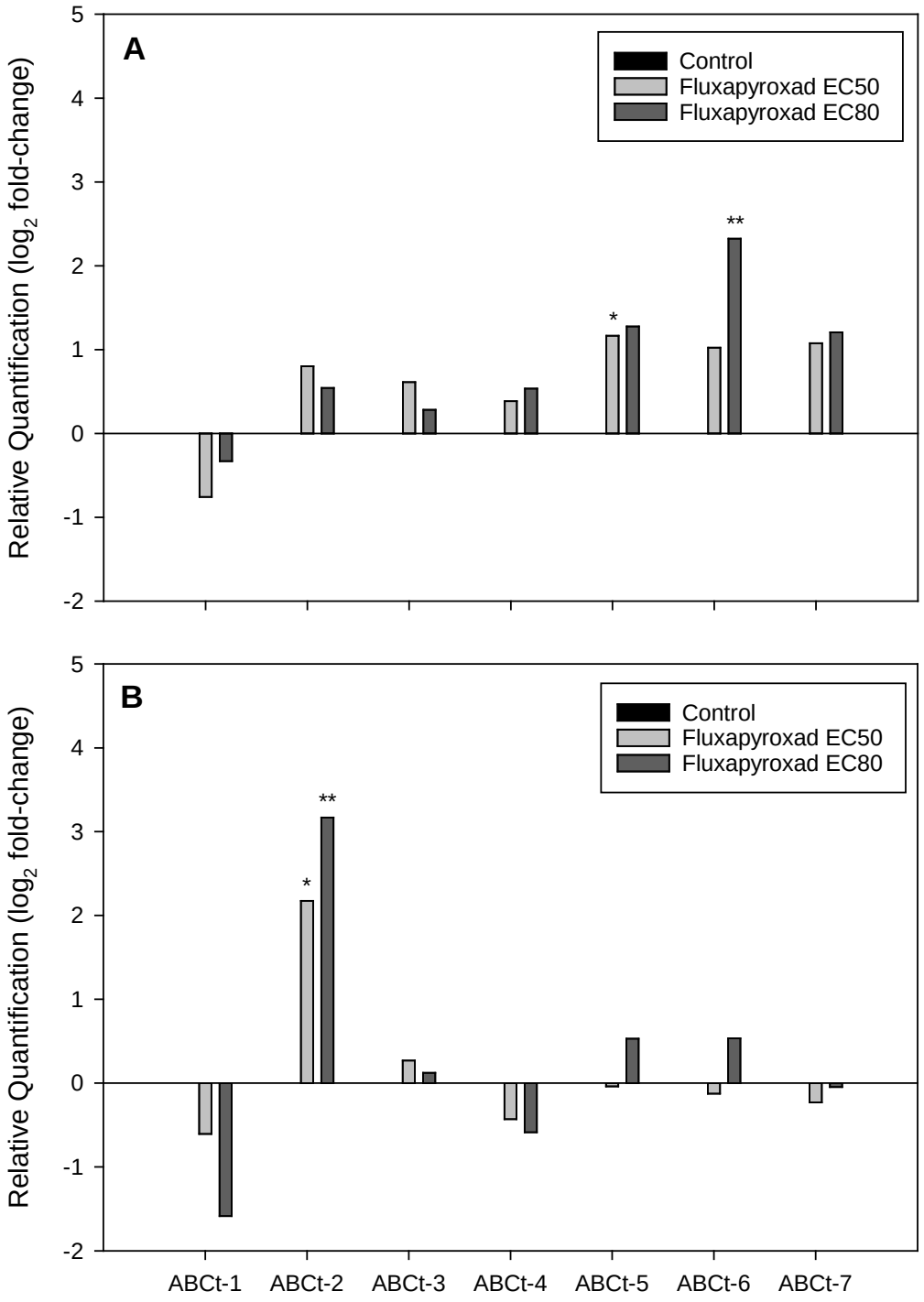

Figure 5.11 Quantitative RT-PCR of genes encoding putative ATP-binding cassette (ABC) transporters in the reference *Z. tritici* isolate IPO323(A) and the laboratory mutant IPOFluxa9-7 (B) after 24 h exposure to their respective fluxapyroxad EC_{50} or EC_{80} concentration. Mean of three biological replicates. Statistical significance for each gene is marked by asterisks (*=p<0.05 or **=p<0.01). See Appendix 5.1 and 5.2 for the means of log_2 fold-change data for the three treatments with standard error of the difference for the comparisons made.

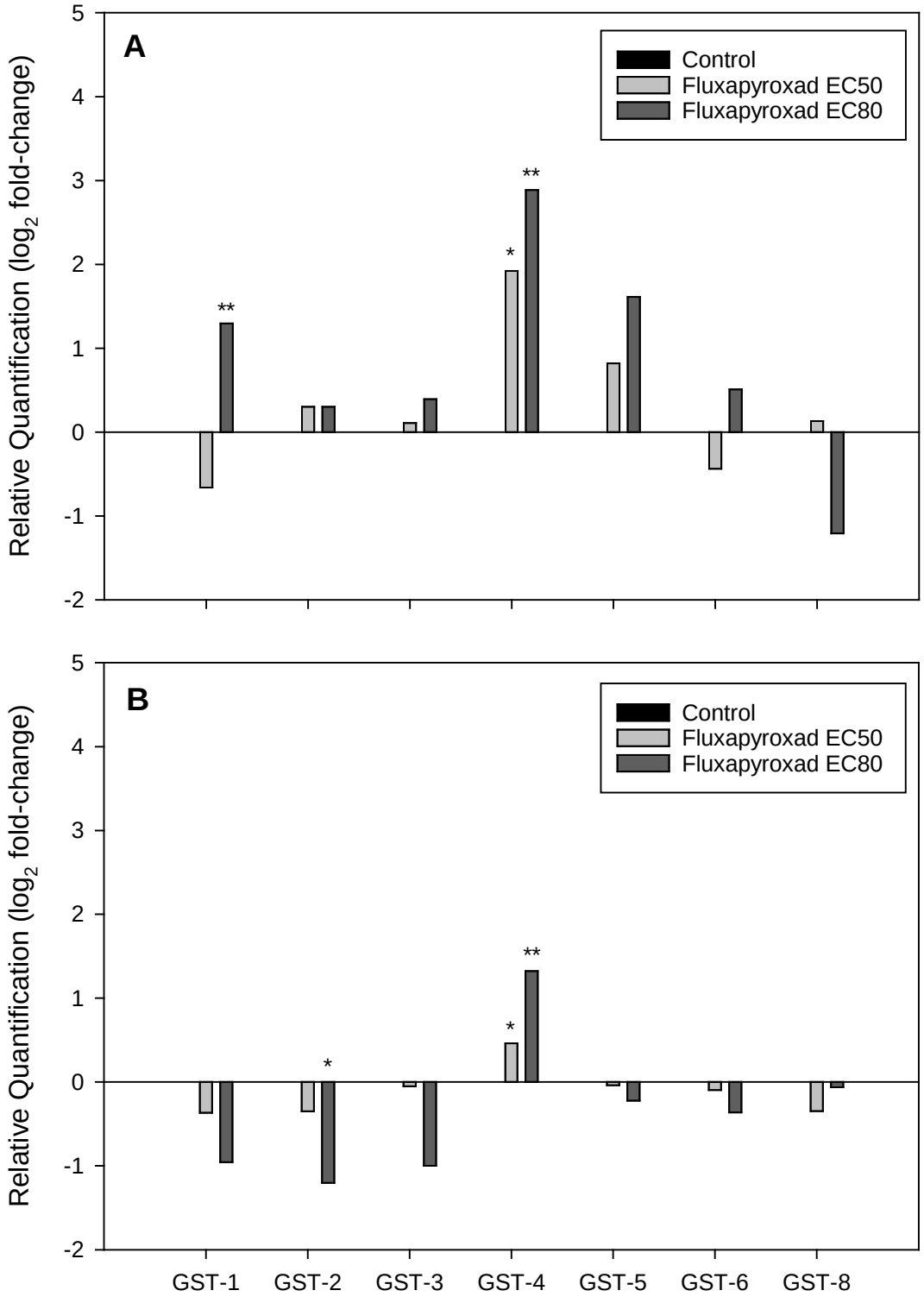

Figure 5.12 Quantitative RT-PCR of genes encoding putative glutathione S-transferase in the reference *Z. tritici* isolate IPO323 (A) and the laboratory mutant IPOFluxa9-7 (B) after 24 h exposure to their respective fluxapyroxad EC_{50} or EC_{80} concentration. Mean of three biological replicates. Statistical significance for each gene is marked by asterisks (*=p<0.05 or **=p<0.01). See Appendix 5.1 and 5.2 for the means of log_2 fold-change data for the three treatments with standard error of the difference for the comparisons made.

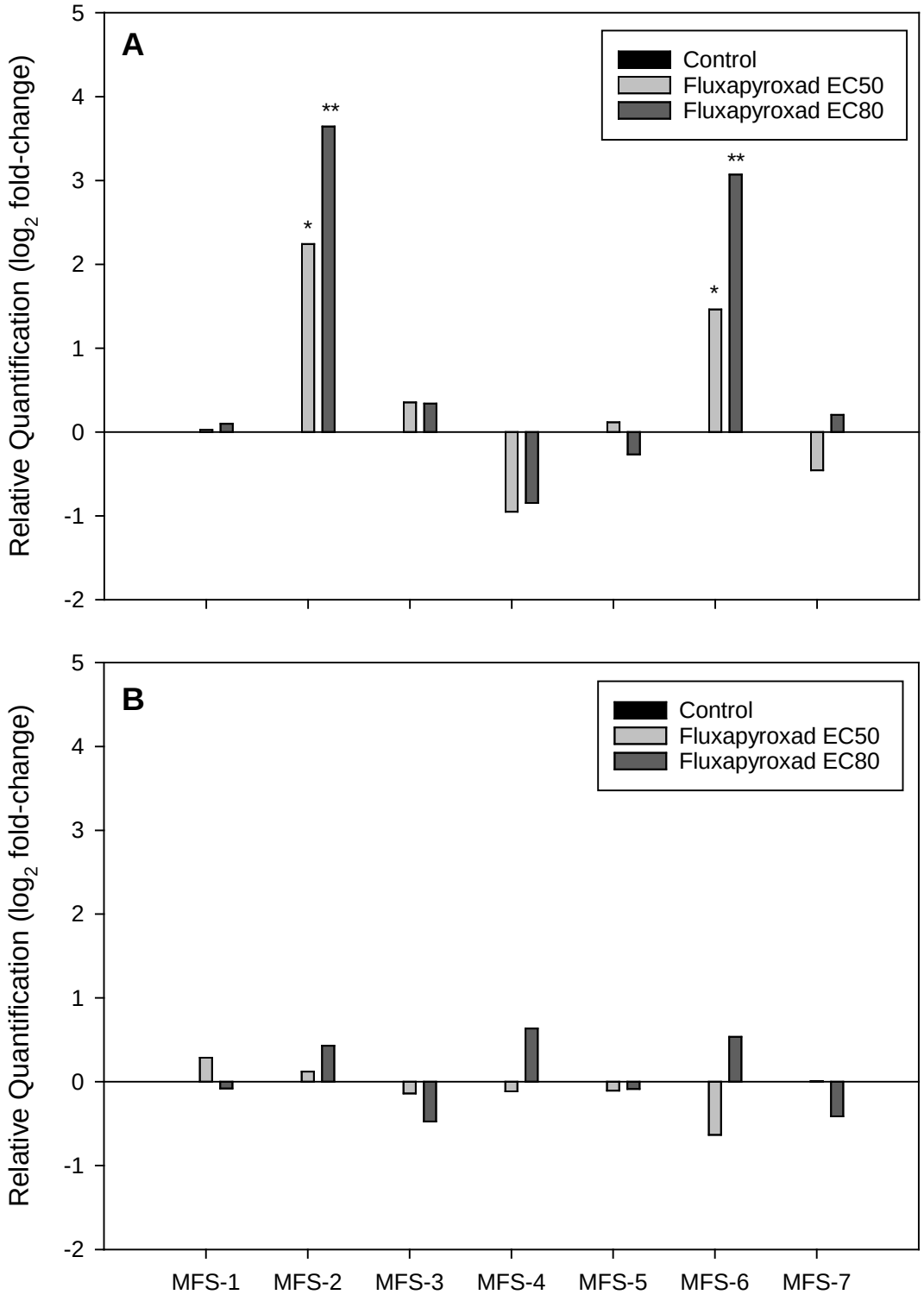

Figure 5.13 Quantitative RT-PCR of genes encoding major facilitator superfamily (MFS) drug efflux transport in the reference *Z. tritici* isolate (A) IPO323 and the laboratory mutant (B) IPOFluxa9-7 after 24 h exposure to their respective fluxapyroxad EC_{50} or EC_{80} concentration. Mean of three biological replicates. Statistical significance for each gene is marked by asterisks (*=p<0.05 or **=p<0.01). See Appendix 5.1 and 5.2 for the means of log_2 fold-change data for the three treatments with standard error of the difference for the comparisons made.

5.3.8 *In vitro* fitness study on fluxapyroxad-resistant mutants

There were no significant (post-hoc t-test, linear mixed model, $p>0.10$) differences in growth rate between the parental isolateIPO323 and the variants formed by fluxapyroxad-resistant mutants carrying similar amino acid substitutions in the *sdh* complex or with no mutation (Fig. 5.14B and 5.15). The parental isolate IPO323 and the variant carrying two amino acid substitutions (B_N225T, D_I50L) have only one biological replicate. There were significant differences between variants C_S83G (Fig. 5.14B) and B_H267L ($p<0.01$ t-test), B_H152R ($p<0.01$ t-test) or mutants with no mutation ($p<0.05$ t-test). The variant C_S83G has the highest growth rate, approximately 0.0047 OD/hours.

There were no significantly (post-hoc t-test, linear mixed model, $p>0.05$) differences in the estimated intercept at time zero between the parent isolate IPO323 and the variants formed by fluxapyroxad-resistant mutants carrying similar amino acid substitution in the *sdh* complex or with no mutation (Fig. 5.14A and 5.15). However, mutants with non-target-site mutation had significantly greater intercepts than B_H267L ($p<0.01$ t-test), B_H267Y ($p<0.01$ t-test), C_H152R ($p<0.01$ t-test) or C_S83G ($p<0.01$ t-test) variants (Fig. 5.14A) with an approximate average absorbance of 0.15 OD.

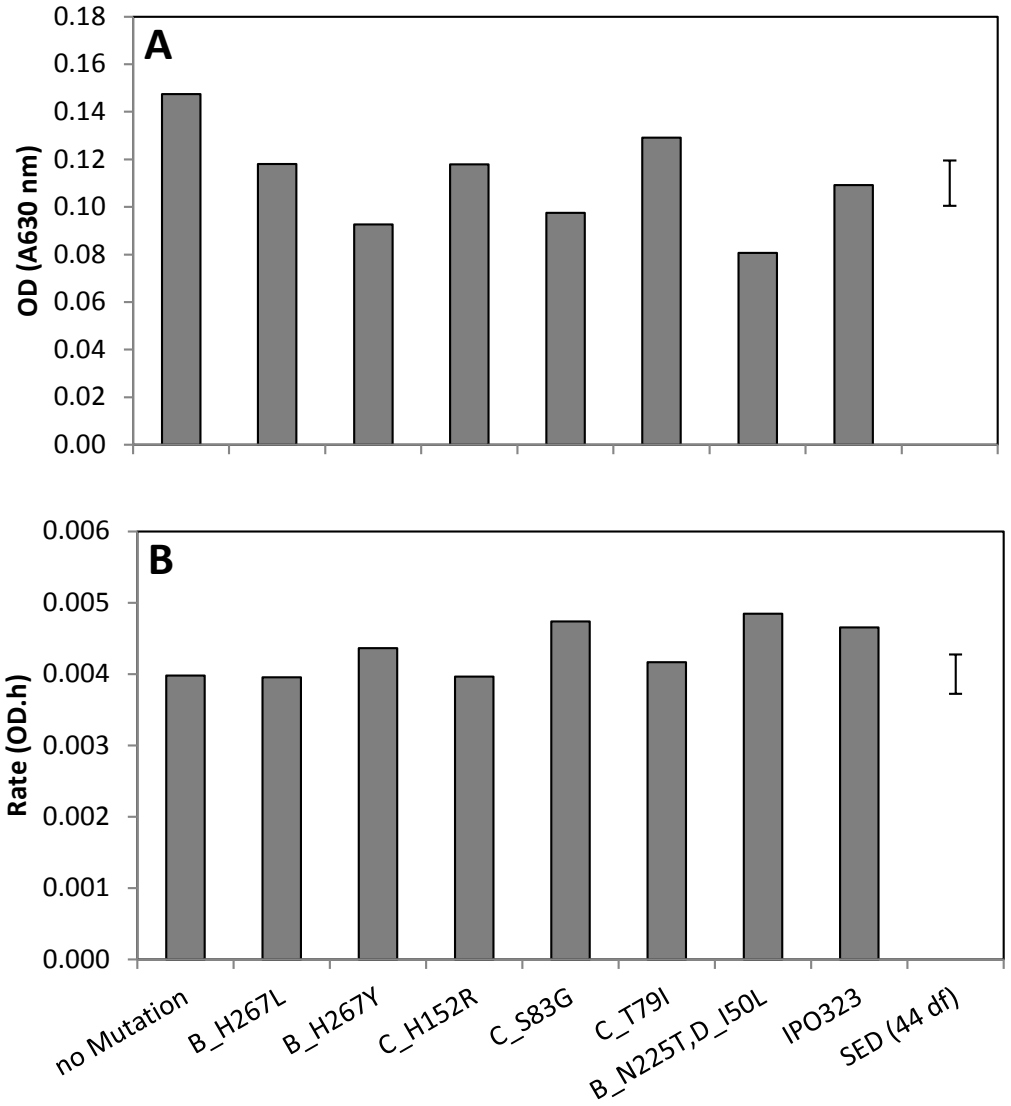

Figure 5.14 Estimated intercept at time zero (A) and growth rate (B) of seven IPO323-derived mutant genotypes and the parental isolate IPO323. There were eight technical replicates for each biological replicate of a variant, where a variant is formed from a number (*n*) of mutants carrying similar amino acid substitution in the *sdh* complex. No mutation, *n* = 6; B_H267L, *n* = 11; B_H267Y, *n* = 12; C_H152R, *n* = 15; C_S83G, *n* = 6; C_T79I, *n* = 3; B_N225T, D_I50L *n* = 1; IPO323, *n* = 1.

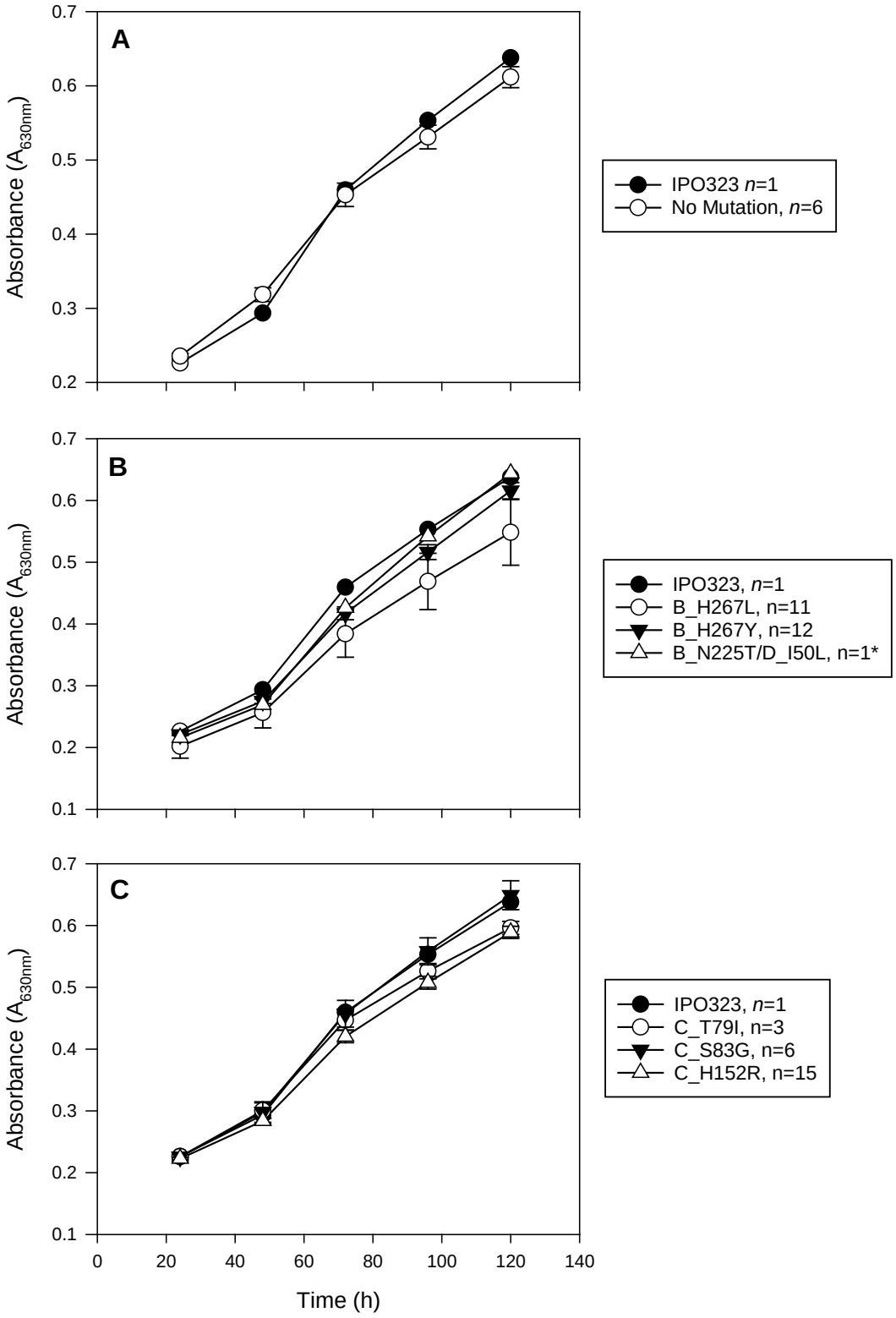

Figure 5.15 *In vitro* growth of 54 *Z. tritici* IPO323-derived mutants and the parental isolate IPO323. (A) Mutants with no mutation in the *sdh* complex encoding gene, and mutants carrying associate amino acid substitutions in the (B) *sdh*B or (C) *sdh*C encoding gene. *Mutant carrying also an amino acid substitution in the *sdh*D. There were eight technical replicates for each biological replicate of a variant, where a variant is formed from a number (*n*) of mutants carrying similar amino acid substitution in the *sdh* complex. Standard error is denoted by bars based on the number of mutants carrying similar amino acid substitution in the sdh complex.

5.4 Discussion

All nine populations of *Zymoseptoria tritici* evolved reduced sensitivity to fluxapyroxad when grown in a series of ten successive transfer onto agar amended with increasing concentrations of fluxapyroxad. Seven of nine populations were also less sensitive to fluopyram or carboxin; two were more sensitive to fluopyram.

5.4.1 Resistance mechanisms in IPO323-derived fluxapyroxad-resistant mutants

Distinct molecular mechanisms conferred resistance to fluxapyroxad, fluopyram or carboxin. Mutation encoding amino acid substitutions in the target protein (Qp) was the most common. Changes in the *sdh* sub-unit B gene were either at codon 225 (N225T) or 267 (H267Y/L). The variant sdhB_H267L conferred resistant to fluxapyroxad, carboxin and fluopyram; whereas sdhB_H267Y variant increased sensitivity to fluopyram. This negative cross-resistance of sdhB_H267Y variant was reported before for *Z. tritici* by Fraaije et al. (2012) and Scalliet et al. (2012) but has also been reported for other plant pathogens (see Sierotzki and Scalliet, 2013). The sdhB_N225T variant was found in combination with another amino acid substitution in the *sdh* gene encoding the sub-unit D at codon 50 (I50L). This double amino acid substitution (sdhB_N225T and sdhD_I50L) conferred resistance to fluxapyroxad and lower sensitivity to carboxin and fluopyram. Scalliet et al. (2012) reported changes at codon 225 in *sdh* sub-unit B gene from asparagine to histidine or isoleucine (N225H/I), which confer lower sensitivity to carboxin, isopyrazam, fluopyram and boscalid. Moreover, the amino acid substitution at codon 225 (SDHIB_N225T) in *Z. tritici* is equivalent to codon 230 (N230I) in the SDH sub-unit B in *Botrytis cinerea* (Leroux et al., 2010). They reported that the variant sdhB_N230I conferred resistance to diverse SDHIs in *B. cinerea*. However, the sdhD mutation resulting in I50L has not been reported before (see Sierotzki and Scalliet, 2013). Amino acid substitutions in the SDH sub-unit D conferring resistance to SDHIs have occurred at codon 129 in *Z. tritici* laboratory mutants (Fraaije et al., 2012; Scalliet et al., 2012). Thus, it is possible that levels of resistance in the double mutant reported here (sdhB_N225T and sdhD_I50L) might be due mainly to sdhB_N225T. Further studies with *Z. tritici* homologous recombinants carrying single amino acid substitution (Scalliet et al., 2012) or protein models (Fraaije et al., 2012; Scalliet et al., 2012) would test this hypothesis.

Amino acid substitutions in the *sdh* sub-unit C gene from threonine to isoleucine at codon 79, serine to glycine at codon 83 or histidine to arginine at codon 152 conferred resistance to fluxapyroxad, fluopyram and carboxin. Scalliet et al. (2012) reported that sdhB_T79I, sdhB_H152R

or sdhB_S83G variants can confer resistance to a range of SDHIs (i.e. carboxin, izopyrazam, fluopyram and boscalid). Interestingly, they reported that variant sdhB_S83G has the highest levels of resistance to fluopyram than variant sdhB_T79I or sdhB_H152R. Similar results were found in my study.

In vitro selection under the highest starting concentration of fluxapyroxad (0.08 µg/mL) triggered more diverse amino acid substitution in the target protein than the lowest starting concentration (0.04 µg/mL) in UV light exposed IPO323-derived mutant populations. Indeed, the number of mutations found in the target protein conferring resistance has been related to selection strength before (Oz et al., 2014). However, mutations conferring amino acid substitutions in the Qp conferred resistance to other SDH fungicides regardless of the strength of selection pressure. Similar cross-resistant patterns among SDHIs have been reported before in *Z. tritici* laboratory mutants obtained under carboxin, boscalid, isopyram or fluopyram exposure (Fraaije et al., 2012; Scalliet et al., 2012; Skinner et al., 1998).

Fluxapyroxad-resistant mutants with no target-site (Qp) alterations were also less sensitive to fluopyram or carboxin. Further studies indicated that overexpression of an ATP-binding cassette transporter and/or glutathione S-transferase may contribute to the phenotype. The *abc*t-2 gene, a putative *Z. tritici* ABC transporter (Goodwin et al., 2011), was constitutively overexpressed up to 9.5-fold in the fluxapyroxad-resistant mutant IPOFluxa9-7 at increasing concentrations of fluxapyroxad. Similarly, Cowen et al. (2002) reported overexpression of an ABC transporter in a mutant population of *Candida albicans* with no mutation in the target protein grown for 330 generations in presence of fluconazole. Overexpression of ABC transporters in *Saccharomyces cerevisiae* was also detected after 400 generations at increasing concentrations of fluconazole (Anderson et al., 2003). The overexpression of the *abc*t-2 gene indicates the ability of *Z. tritici* to develop resistance mechanisms to single-site fungicides through non-target-site mutations. Further molecular studies looking at characterising the *Z. tritici abc*t-2 gene would contribute to determine the exact role in resistance and what is causing the observed overexpression.

The up-regulation of *gst*-4 – a glutathione S-transferase – in both the reference isolate IPO323 and the mutant IPOFluxa9-7 indicates a possible protection mechanism against fluxapyroxad exposure. Glutathione S-transferase (GST) enzymes are able to detoxify many xenobiotic compounds (see Sheehan et al., 2001; Shin et al., 2003) and to protect cells from oxidative stress (Burns et al., 2005; Hayes and McLellan, 1999). The GST enzymes catalyse conjugated glutathione (GSH-xenobiotic) into more soluble and less reactive compounds, which can be removed from the cell by transmembrane transporters (Morrow et al., 1998a). Morrow et al. (1998b) reported that

resistance to chlorambucil in breast carcinoma cells is mediated by both GST catalytic activity and a multidrug resistance protein 1 (MRP1) transmembrane transporter. Similarly, da Silva et al. (2006) observed overexpression of a *gst* and an *abc* transporter encoding genes in *Aspergillus fumigatus* in presence of voriconazole. Although there is no report of carboxamide fungicides react with cellular GSH, the up-regulation of the *gst*-4 gene indicates that fluxapyroxad may bind to cellular GSH.

5.4.2 Emergence and evolution of resistance to fluxapyroxad

Emergence of amino acid substitutions in the Qp occurred in parallel in both UV light exposed mutant populations IPOFluxa19 and IPOFluxa20 at similar rate. Early in the experiment the amino acid substitution sdhC_T79I emerged in both populations at selection round three (RS-3). However, T79I was overtaken by another amino acid substitution (sdhC_H152R) that emerged at RS-5 and RS-6 in populations IPOFluxa19 and IPOFluxa20 respectively. The variants sdhC_T79I and sdhC_H152R have similar growth rate, suggesting a similar fitness. Subsequently, the sdhC_S83G variant and the double mutant (sdhB_N225T, sdhD_I50L) started to emerge at the end of the experiment.

Although variants sdhC_S83G and sdhC_T79I have similar growth rates, the sdhC_S83G grows faster than the sdhC_H152R variant. This indicates that sdhC_S83G is fitter than sdhC_H152. Scalliet et al. (2012) detected differences in pathogenicity in *in planta* studies using *Z. tritici* homologous recombinant strains carrying distinct amino acid substitutions in the Qp site including the sdhC_S83G or sdhC_H152R. The recombinant strains carrying the amino acid substitution sdhC_S83G induced more necrosis in wheat plants than those carrying the sdhC_H152R. This indicates that variant sdhC_S83G may be fitter than sdhC_H152R. Apparently, the sdhC_S83G variant has a lower growth rate than the double mutant (sdhB_N225T, sdhD_I50L), but unfortunately there was only one biological replicate of the double mutant. Additionally, the SDHB_N225T variant was detected early in the experiment, but at lower frequency (< 5 %). Allele frequencies < 5 % are not reliable because of limitations in pyrosequencing technology (Lavebratt et al., 2004; Wasson et al., 2002). Nevertheless, the sequence of mutants carrying distinct amino acid substitutions in the *sdh* sub-unit C gene – T79I > H152R > S83G – indicates a 'clonal replacement' in the evolution of resistance to fluxapyroxad. Clonal replacement takes place in asexual populations where mutations conferring beneficial advantages have to arise independently and to compete with others for fixation leading to a succession of genotypes each

one fitter than its immediate predecessor till the best adapted remains under specific conditions or threats (Muller, 1932). Atwood et al. (1951)reported a periodic replacement of strains or clonal replacement in *E. coli*-derived populations evolving *in vitro* for 1,000 generations under histidine-limited conditions. They observed a repeated cycle of clonal strains substitutions between histidine-required (h-) and histidine-independent (h+) strains in mixed *E. coli* populations consisting of various ratios of h+/h-. Additionally, Atwood et al. inferred that clonal replacement took place approximately 4.5 times during the experiment. Recently, Albrecht et al. (2011) reported clonal replacement of methicillin-resistant *Staphylococcus aureus* (MRSA) strains in human patients during a period of 11 years in German hospitals. They detected four distinct MRSA strains which their frequency fluctuated during the studied. For example, a strain named CC22-MRSA-IV was detected in 2001 and increased in frequency up to approximately 58 % in 2010; other strains such CC45-MRSA-IV decreased approximately 58 % frequency between 2002 and 2010.

In contrast, populations exposed to greater concentrations of fluxapyroxad (0.06 or 0.08 µg/mL) without UV light only the amino acid substitution SDHB_H267Y was detected. The SDHB_H267Y variant was fixed by RS-4 at approximately 100% frequency in population IPOFluxa18. This could be because IPOFluxa18 and the other IPO323-derived populations were exposed to two seven-day periods of the fluxapyroxad concentration used at RS-4. This may have allowed the increase in frequency of mutants carrying amino acid substitutions advantageous in the presence of fluxapyroxad. Although mutant populations IPOFluxa18 and IPOFluxa9 were exposed to similar fluxapyroxad concentrations (0.64 µg/mL) at RS-4 or RS-5, respectively, no mutant carrying any *sdh* variant was detected in population IPOFluxa9. Instead, resistance to fluxapyroxad in a mutant from population IPOFluxa9 was associated with overexpression of an *abc* transporter.

Adaptation to fungicides by target-site or non-target-site alterations has occurred in field populations of *Z. tritici* (Clark, 2006; see Cools and Fraaije, 2013; Fraaije et al., 2005; Leroux and Walker, 2011; Torriani et al., 2009). Recently, amino acid substitutions conferring low levels of insensitivity to SDHIs were detected in separate *Z. tritici* field isolates (FRAC, 2013). The substitutions were in the *sdh* sub-unit C gene: from threonine to asparagine at codon 79 (T79N), or from tryptophan to serine at codon 80 (W80S). In this evolutionary study, resistance to fluxapyroxad emerged first by an amino acid substitution at codon 79 (sdhC_T79I), which is the codon where the sdhC_T79N amino acid substitution was found in field isolates (FRAC, 2012). They reported that sdhC_T79N amino acid substitution induced a “low” resistant factor (RF). *In vitro* sensitivity assays indicated that the sdhC_T79I mutant had a RF of 44. Findings from this

study suggest that other amino acid substitutions (e.g. sdhD_H152R) may be found in *Z. tritici* field isolates in the future. Therefore, it is important to monitor populations of *Z. tritici* for a variety of changes at the sdh locus and elsewhere in case SDHI resistance arises. The pyrosequencing assay developed here would enable a rapid detection of *Z. tritici* isolates carrying amino acid substitutions sdhB_H267Y or _N225T, sdhC_H152R or _S83G, and sdhD_I50L and their further spread in populations can be measured.

5.5 Conclusions

Resistance to fluxapyroxad arose by distinct diverged adaptive trajectories from a sensitive *Z. tritici* isolate IPO323 after ten rounds of selection at increasing concentrations of fungicide *in vitro*. Alterations in the target protein were the most common mechanism which conferred resistance to fluxapyroxad and cross-resistance to fluopyram or carboxin. Additionally, overexpression of ABC transporter or GST genes was associated with resistance to fluxapyroxad and lower sensitivity to fluopyram or carboxin in a mutant strain with no target-site mutation. The presence of key amino acid substitutions in replicated populations indicated that resistance arose through a small number of possible pathways. There was successive substitution of fitter fluxapyroxad-resistant mutants carrying distinct amino acid substitutions as selection continued. However, in populations not exposed to UV light a single mutation arose and reached high frequency without further evolution during the experiment.

Chapter 6: General Discussion

6.1 Main findings

The aim of this PhD project was to improve our understanding of how *Z. tritici* adapts to multi- and single-site fungicides currently used for the control of Septoria leaf blotch (SLB). The multi-site inhibitors chlorothalonil and folpet were included in the study because they are key components used to reduce or delay the development of fungicide resistance to single-site fungicides. The single-site fungicide fluxapyroxad – representing a new-generation of carboxamides, which are succinate dehydrogenase inhibitors (SDHIs) – was chosen because SDHI fungicides are applied in mixture with sterol 14α-DeMethylation Inhibitor (DMI) fungicides and play a big part of the chemical control of SLB. Furthermore, fluxapyroxad is relatively new in the cereal market for crop protection and like other SDHIs, it has a high risk of resistance development. In this chapter the main findings of this PhD project are discussed in relation to future research directions.

6.1.1 *Zymoseptoria tritici* can respond to selection for reduced sensitivity to chlorothalonil or folpet exposure in the field.

Shifts in sensitivity in plant pathogen populations can be produced by recurrent use of fungicides (see van den Bosch and Gilligan, 2008). Typically, less sensitive strains – fitter in the presence of the fungicide – invade the population and cause failures in disease control (Van den Bosch et al., 2011). These shifts in sensitivity evolve at different rates, depending mainly on the fungicide mode of action and genomic changes in the pathogen conferring decreased susceptibility to the fungicide (Hollomon, 1981; Sanglard et al., 1998). Frequently, a single alteration in the target-protein encoding gene can confer a large change in phenotype with little fitness penalty in the absence of fungicide, leading to a fast shift in sensitivity to single-site fungicides. For example, the mutation G143A in the cytochrome *b* or E198A in the β-tubulin gene confers high levels of resistance to QoIs or MBCs in *Z. tritici*, respectively (Fraaije et al., 2005). In contrast, multiple alterations in either single or multiple genes often lead to a gradual shift in sensitivity, because their individual effect on phenotype is small. Accumulation of different point mutations leading to multiple amino acid alterations and overexpression of the target sterol 14α-demethylase encoding gene due to 120 bp insertion in the promoter region have contributed to a gradual shift in sensitivity to DMIs in *Z. tritici* (see Cools and Fraaije, 2013). Moreover, steady accumulation of

alterations in regulatory regions of efflux pumps encoding genes (e.g. ABC or MFS transporters) can confer shifts in sensitivity to a range of single-site fungicides in *B. cinerea* (Kretschmer et al., 2009).

Changes in the distribution of EC_{50} were observed in *Z. tritici* field populations under solo applications of chlorothalonil or folpet. These shifts in sensitivity have not been detected before. This might be due to the fact that chlorothalonil and folpet are usually sprayed in mixture with single-site fungicides (e.g. DMIs and SDHIs) to delay fungicide resistance development (HGCA, 2014) and/or assays being used were not able to detect small changes in fungicide sensitivity. Single-site fungicide applications may reduce the frequency of less sensitive strains carrying favourable combinations of genes conferring shifts in sensitivity to chlorothalonil and/or folpet. In addition, shifts in sensitivity to multi-site inhibitors might require many genomic changes in the target microorganism as these fungicides may affect diverse metabolic pathways (Chapter 4; FRAC, 2014). Nevertheless, shifts in sensitivity to multi-site inhibitors suggest that genetic variation in *Z. tritici* might allow adaption to chlorothalonil or folpet. Barak and Edgington (1984) reported shifts in sensitivity to a range of multi-site inhibitors including chlorothalonil and folpet in *B. cinerea* field isolates. Similarly, Fourie and Holz (2001) also reported shifts in folpet sensitivity in the same pathogen after intensive field applications of dicarboxamides or folpet.

Although shifts in sensitivity to multi-site inhibitors have been reported before, little is known about genomic changes that may confer decreased sensitivity to these fungicides. Genes encoding diverse detoxification mechanisms (i.e. GST, ABC or MFS efflux pumps) in response to chlorothalonil or folpet exposure were identified using genome-wide transcriptional response studies in a sensitive *Z. tritici* strain (Chapter 4). Interestingly, two genes encoding putative ABC transporters or nine GST in *Z. tritici* were overexpressed in response to both fungicides (Chapter 4, section 4.3.2.4). Changes in expression of ABC transporters or GST encoding genes might explain the correlation between chlorothalonil and folpet sensitivity values detected in *Z. tritici* field isolates (Chapter 3). Further genome-wide transcriptional studies in response to chlorothalonil or folpet using less sensitive *Z. tritici* field strains are needed to corroborate the role of detoxification mechanisms conferring lower sensitivity to multi-site inhibitors as this study and Sisler (1988) suggested.

6.1.2 Chlorothalonil or folpet modulates expression of particular functional groups of genes in *Z. tritici* according to the growth phase at which it is exposed.

Studies on changes in gene expression levels after toxicant exposure have contributed to the identification of metabolic pathways that are relevant to a toxicological response (Dias et al., 2010; Hamadeh et al., 2002b; see North and Vulpe, 2010). Typically, yeast gene expression is reprogrammed after toxicant exposure as a response to the new stress, which triggers a distinctive gene expression profile or 'signature' to the toxicant (Jia et al., 2000; Kuo et al., 2010; Nishida et al., 2013; Simmons and Portier, 2002). These gene expression signatures are tend to be similar among toxicants with similar mode of action and can be used to infer toxicological mechanisms of new or uncharacterised compounds (see Iorio et al., 2013; see Qu and Rajpal, 2012).

Chlorothalonil or folpet triggered distinct functional gene expression signatures in *Z. tritici*. Chlorothalonil exposure induced overexpression of genes related to the histidine kinase complex when the fungicide was added in the lag phase but no particular set of genes was detected when the chlorothalonil was added in the log phase. By contrast, folpet exposure induced distinct gene expression signature in the lag or log phase of growth. Expression of genes related to serine-type carboxypeptidases with activity in the extracellular region increased when folpet was added in the lag phase; whereas expression of genes related to protein synthesis or cellular amino acid metabolism was increased when folpet was added in the log phase. Therefore, functional gene expression signatures were depended on the fungal growth phase at which chlorothalonil or folpet was added. Differences in functional gene expression between lag and log phase of growth may be related with level of absorption and accumulation of the fungicide by the fungal spore or mycelium. The lag phase of growth of *Z. tritici* may be characterised by an adjustment period from growth via secondary conidial production on solid media to growth into liquid culture conditions; whereas in the log phase or active growth the fungus has already adjusted to grow into liquid culture conditions. Although chlorothalonil or folpet absorption and accumulation was not determined, it is possible that accumulation of these fungicides was higher in the lag than in the log phase of growth. Fungal spores (e.g. *Neurospora sitophila*, *Monilinia fruticola*, *Aspergillus niger*, *Alternaria oleracea*, *Glomerella cingulata*, *Venturia pyrina* or *Rhizopus nigricans*) including *S. cerevisiae* can absorb and accumulate approximately 1 % of their own weight of toxicants such as 2-heptadecyl-2-imidazoline, 2,3-dichloro-1,4-napthoquinone, silver, mercury, cerium, cadmium, zinc or copper (McCallan and Miller, 1958; see Miller, 1959). Miller (1957) reported that fungal spores are able to absorb fungicide faster than mycelia. Moreover, *in vitro* sensitivity

studies of some fungicides using spores have found lower sensitivity values than when mycelium is used. Nevertheless, sensitivity values obtained through use of spores or mycelium are often correlated (Barak and Edgington, 1984).

The transcriptional response and the functional gene expression signatures determined in the lag phase in response to chlorothalonil or folpet can provide a better picture of the putative metabolic pathways affected by these fungicides than during the log phase of growth. The fungicide concentrations – able to reduce approximately 50 % growth – used to determine the genome-wide transcriptional response (i.e. 0.1 µg/mL of chlorothalonil or 0.5 µg/mL of folpet) were estimated by adding the fungicide in the lag phase of growth of *Z. tritici* (see Chapter 2; Chapter 3). In addition, diverse studies have detected shifts in sensitivities by exposing microorganisms to the fungicides in the lag phase of growth. Pijls and Shaw (1994) developed a method to estimate flutriafol sensitivity values in *Z. tritici* through measuring fungal growth in microtitre plates based on light absorbance. They were able to estimate precise fungicide sensitivity values by adding the fungicide in the lag phase of growth of *Z. tritici*. Currently, FRAC (2012) recommends the use of the microtitre plate test to determine sensitivity baselines or shifts in sensitivity in diverse fungi – including *Z. tritici* – such as *B. cinerea* against anilinopyrimidines (APs), amines or sterol biosynthesis inhibitors (SBIs), SDHIs or QoIs; *F. graminearum* against SBIs; *Phytophthora infestans* against carboxylic acid amides (CAAs) or QoIs; *Pyrenophora teres* against SDHIs; *Rhynchosporium secalis* against APs, SBIs or QoIs.

The genome-wide transcriptional response and functional gene expression signature reported here may also depend on the concentration of fungicide used. Cools et al. (2007) reported no changes in gene expression in the reference *Z. tritici* isolate IPO323 after exposure to its approximate EC_{50} concentration during the log phase of growth. Instead, a lethal concentration of epoxiconazole was used to determine changes in gene expression in IPO323. In my study, changes in transcriptional response of target genes – determined through quantitative RT-PCR – were observed at increasing concentrations of fluxapyroxad (i.e. approximated EC_{50} or EC_{80} concentrations) in the lag phase of growth of *Z. tritici*. Genes involved in detoxification mechanisms (e.g. *abc*t-2 or *gst*-4) or genes encoding the target protein (Qp) showed a trend to increased expression as the concentration of the fungicide increased (see chapter 5). Other genes showed a different trend. For example, a gene encoding AOX was down-regulated at the lower concentration (EC_{50}) of fluxapyroxad but was up-regulated at the higher concentration (EC_{80}). Although genome-wide transcriptional response in presence of fungicides does not provide a decisive description of their mode of action, it does give insights into the putative metabolic

pathways affected by the fungicide and possible detoxification mechanisms. Quantitative proteomics – analytical chemistry approach to quantify and identify proteins in a sample – studies can identify protein alteration due to toxicant exposure (Santos et al., 2009). The combination of transcriptomic and quantitative proteomic studies could provide a more complete picture of the metabolic pathways affected by the fungicides and help to elucidate their mode of action using *Z. tritici* as a model.

Integrated genome-wide transcriptomic and proteomic expression studies after chemical exposure are scarce. Foss et al. (2007) compared the genome-wide transcriptional profile that underlies proteome abundance in progeny of *S. cerevisiae* crosses. They found a small but significant correlation between transcript levels and protein abundance, and also detected specific loci that affect protein abundance or transcript levels. Similar results reported Ghazalpour et al. (2011) by investigating genetic regulation of the transcriptome and proteome in mice. The correlation between transcript levels and protein abundance depended on the cellular component and biological function of the corresponding gene. Moreover, diverse studies in *Escherichia coli*, *Streptomyces coelicolor*, *Schizosaccharomyces pombe* or *S. cerevisiae* reported Pearson's correlation coefficients between transcript levels and protein abundance ranging from 0.3 to 0.8 (see de Sousa Abreu et al., 2009). Although protein abundance is mainly related with gene expression level, it is also affected by post-transcriptional, translation and degradation regulation (Lu et al., 2007). Nevertheless, integrated proteomic and transcriptomic expression studies in plant pathogens such *Z. tritici* may help to identify direct and/or indirect effects of fungicide exposure and provide data and/or clues on mode of action, novel target sites for fungicides, identification of fungicide stress responsive genes and biomarkers in impaired metabolic pathways, and resistance mechanisms (see Dos Santos et al., 2012).

6.1.3 Resistance to SDHIs can be conferred by non-target-site mutations in *Z. tritici* laboratory mutants.

Resistance to fluxapyroxad emerged in parallel in replicate populations of *Z. tritici* through mutations in the target protein Qp (chapter 5). Once resistance emerged *in vitro*, the evolution of resistance was driven by clonal replacement, where fitter mutants carrying beneficial amino acid substitutions in the target protein substituted less fit mutants under increasing concentrations of fluxapyroxad. It is possible that the mutations SDHC_T79N or SDHC_W80S – detected in field isolates in 2013 (FRAC, 2013), but not apparently increasing subsequently – emerged and

increased in frequency to a detectable level during the asexual field stage of *Z. tritici* but not in the following season possible due to impaired fitness during overwinter. It is likely that other amino acid substitutions in the target protein Qp might emerge in field populations of *Z. tritici* in the near future. Besides target-site mutations, resistance to fluxapyroxad was associated with overexpression of an ABC transporter encoding gene in a lab strain mutant with non-target-site alterations (chapter 3). The transmembrane protein efflux pumps ABC and MFS can play a major role in protecting the fungal cell against natural or synthetic toxicant compounds (see de Waard et al., 2006). Approximately 56 putative ABC transporters have been identified in *Zymoseptoria tritici* (see Bean, 2008). Laboratory studies have demonstrated that some ABC transporters (MgAtr1, 2, 4 or 5) can help protect *Z. tritici* against some fungicides and plant, bacteria or fungal metabolites (see Roohparvar, 2007). In heterologous expression in *S. cerevisiae*, the ABC transporter MgAtr1 conferred lower sensitivity to azole fungicides or the antibiotic cycloheximide (Zwiers et al., 2002). Similarly, Zwiers et al. (2003) reported that the ABC transporters MgAtr2 and MgAtr4 conferred protection against azole fungicides in yeast complementation studies. They also found that MgAtr1, MgAtr2 or MgAtr4 reduced sensitivity to azoles, whereas the ABC transporter MgAtr5 or MgAtr1 also provided protection against plant alkaloids. The ABC transporter encoding gene *abct*-2 – a distinct efflux pump to those reported previously – was constitutively overexpressed in the fluxapyroxad-resistant mutant IPOFluxa9-7. This mutant also showed lower sensitivity to other SDHIs (i.e. fluopyram and carboxin). Additional *in vitro* sensitivity assays testing the IPOFluxa9-7 mutant against other SDHIs (e.g. bixafen, isopyram, boscalid), DMIs (e.g. tebuconazole, epoxiconazole, prothioconazole-desthio, prochloraz), tolnaphtate, and antibiotics (e.g. cycloheximide, antimicinA or berberine) indicated shifts in sensitivity only to SDHI fungicides but not to DMIs, tolnaphatate or antibiotics (data not shown, Bart Fraaije 2014, personal communication). This indicates that overexpression of *abct*-2 gene may only confer protection against SDHI fungicides.

Overexpression of ABC transporter encoding genes in some multidrug resistant (MDR) *Candida albicans* phenotypes is due to mutations in transcription factors (Morschhauser et al., 2007). Kretschmer et al. (2009) reported mutations in the transcription factor of the ABC transporter encoding gene *AtrB* in field populations of *B. cinerea*, conferring resistance to diverse unrelated fungicides. It is possible that overexpression of the ABC transporter encoding gene *abct*-2 in the mutant IPOFluxa9-7 is due to mutations in the transcription factor of the gene. Further molecular studies characterising the transcription factors of *abct*-2 would test this hypothesis.

It is known that glutathione S-transferase enzyme detoxifies xenobiotic compounds in the cell (see Sheehan et al., 2001). The combined up-regulation of the gene encoding glutathione S-transferase (*gst-4*) and the overexpression of the ABC transporter gene *abct-2* as protective mechanism in *Z. tritici* indicates that resistance or adaptation could develop through active efflux, alone or in combination with enhanced fungicide metabolism.

6.2 Future work to study the development of fungicide resistance and perspectives for disease management of Septoria leaf blotch

Data from this study indicated that *Z. tritici* has the biological potential to adapt to the multi-site inhibitors chlorothalonil or folpet, and to develop resistance to SDHIs fungicides. However, adaptation to multi-site inhibitors is likely to evolve slowly as these fungicides affect diverse metabolic pathways (chapter 4). Therefore, although *Z. tritici* may be able to adapt to multi-site inhibitors, these fungicides will remain important for SLB disease management in wheat in the immediate future. As previously discussed, further monitoring of *Z. tritici* populations in the UK and Northern Europe will be needed to determine if subtle shifts toward sensitivity are common elsewhere and whether the rate of evolution is accelerating.

The emergence and development of resistance to the single-site SDHIs in *Z. tritici* field population is likely to occur in the near future. Two strains carrying amino acid substitutions in the target protein (SDHC_T79N or SDHC_W80S) conferring lower sensitivity to SDHIs have been detected in *Z. tritici* field populations (FRAC, 2013). Additionally, the evolutionary study presented here indicated that resistance to the SDHI fluxapyroxad can easily emerge (chapter 5). The study also suggests that once resistance emerges through point mutations, further development of resistance may be driven by clonal replacement until the fittest mutant carrying a beneficial mutation reaches high frequency in the population. Therefore, chemical options for SLB disease management in wheat will be even more limited as most, if not all SDHIs will be affected to a certain extent (Chapter 5). This is particularly worrying, as resistance to azole fungicides is likely to continue evolving in *Z. tritici* field populations (see Cools and Fraaije, 2013).

Interestingly, the evolution of fungicide resistance in *Botrytis cinerea* – causal agent of grey mould in grapevine – has been similar to *Z. tritici*. Control of grey mould is mainly achieved through fungicide applications in European vineyards (Leroch et al., 2011; Leroux et al., 2002). The chemical control programme of *B. cinerea* typically involves alternation of fungicides with distinct mode of action applied at the end of flowering, bunch closure and the beginning of berry ripening (Broome et al., 1995; Petit et al., 2010). However, as in the case of *Z. tritici*, *B. cinerea* has developed resistance to diverse single-site fungicides with distinct mode of action. Studies have reported resistance to benzimidazoles (Leroux and Clerjeau, 1985), dicarboximides (Leroux et al., 1982; Ma et al., 2007), anilinopyrimidines (Foster and Staub, 1996), QoIs (De Miccolis Angelini et al., 2014), and boscalid (SDHI; Walker et al., 2011) in field isolates of *B. cinerea*. Although resistance to single-site fungicides has mainly been linked with target-site alterations (Fillinger et al., 2008; Leroux and Clerjeau, 1985; Ma et al., 2007), non-target-site alterations can also confer

resistance to a range of unrelated fungicides in *B. cinerea* (Kretschmer et al., 2009). They reported increased drug efflux activity and overexpression of genes encoding efflux transporters in multi-drug resistant (i.e. MDR1, MDR2 and MDR3) phenotypes of *B. cinerea*. Mutations in the transcription factor Mrr1 lead overexpression of the ABC transporter ArtB which confers resistance to fludioxonil, cyprodinil and tolnaftate in MDR1 strains. In MDR2 phenotypes, a promoter rearrangement leads to overexpression of the MFS transporter MfsM2 gene that confers resistance to fenhexamid, tonlaftate, cycloheximide and cyprodinil. Sexual recombination of MDR1 and MDR2 originated MDR3 strains with higher and broader spectrum of fungicide resistance than the progenitors. Before the detection of MDR phenotypes in field population of *B. cinerea*, mixtures of cyprodinil with fludioxonil (Foster and Staub, 1996) or iprodione with captan (Northover and Matteoni, 1986) were able to control grey mould and decreased selection for fungicide resistance. However, mixtures of QoIs (i.e. pyraclostrobin, picoxystrobin or azoxystrobin) with epoxiconazole or chlorothalonil did not reduce frequency of QoI-resistant field strains of *Z. tritici* (McCartney et al., 2007). Studies suggest that mixtures of fungicides with distinct mode of action may reduce selection for fungicide resistance only if the pathogen is sensitive to one of the mixture (van den Bosch et al., 2014). It is possible that current mixtures of DMI with SDHI fungicides (HGCA, 2014) might not be able to reduce selection for fungicide resistance in the long term due to variation in sensitivity in field populations of *Z. tritici* against these two groups of fungicide. Preventive application of mixtures of DMIs or SDHIs with chlorothalonil might confer good SLB control and reduce selection for fungicide resistance. Additionally, further monitoring of *Z. tritici* field populations is needed to detect changes in frequency of strains with lower sensitivity to SDHIs.

The SNP pyrosequencing assay developed in this study to quantify fungicide resistance alleles in IPO323-derived mutant populations can be used to detect SDHC_T79N or SDHC_W80S variants in field populations. However, this pyrosequencing assay can only detect known SNPs. Other SNPs encoding amino acid substitutions conferring resistance to SDHIs that might arise in field populations will not be detected. To overcome this situation, Carter (2013) suggested a resistance diagnostic based on long read sequencing rather than targeting specific SNPs. Cloning population samples and sequencing, separately, different regions of PbCYP51 it was possible to confirm the frequency of amino acid substitutions previously detected with a pyrosequencing assay. However, cloning and sequencing is laborious and expensive, and phenotyping of strains is still needed to detect non-target site resistance mechanisms (Bell et al., 2014). High-throughput strategy using next-generation sequencing (NGS) of whole genomes may help to detect alterations in target and non-target encoding DNA sequences. Wacker et al. (2012) using RNA sequencing were able to

determine SNPs in target genes or drug efflux transporters conferring resistance to drugs in less sensitive laboratory mutant human cell lines. Harris et al. (2010) using high-throughput DNA sequencing in combination with unique-index tagged sequences were able to genotype, and to investigate the evolution and spread of methicillin-resistant *Staphylococcus aureus* (MRSA) strains at world-wide scale. Based on approximately 4,000 high-quality SNPs, they found evidence for intercontinental spread and hospital transmission of *S. aureus* isolates. Additionally, many SNPs were mapped on genes related with drug resistance. Thus, screening pool-samples of *Z. tritici* spores or wheat leaves with SLB symptoms using NGS technology may allow identify SNPs and insertions and/or deletions in target DNA sequences (e.g. *sdh* gene encoding sub-unit B, C or D; CYP51 encoding gene). This approach could be developed and validated using the IPO323-derived mutant populations obtained in this study.

The functional gene expression signature of chlorothalonil or folpet suggests hypotheses about the metabolic pathways that these fungicides may affect in *Z. tritici*. Additionally, functional annotation of the differentially expressed genes allowed me to identified genes encoding detoxification mechanisms associated with chlorothalonil or folpet exposure. However, an integrated approach using transcriptomic, proteomic and metabolomic analysis simultaneously could provide a better understanding of the metabolic pathways affected by a toxicant and its mode of action. “Toxicogenomics” involves mRNA, protein and metabolite analysis to study and elucidate the effect of toxicants on diverse organisms (see Hamadeh et al., 2002a). However, to date, studies of cell response to a specific toxicant using a full toxicogenomic approach are scarce. Kresnowati et al. (2006) reported a correlation between metabolome and transcriptome responses in energy requirement and nucleotide metabolism after a glucose pulse in yeast grown in chemical static cultures. Independent studies looking at response of yeast to mancozeb exposure through proteomics (Santos et al., 2009) or “disruptome” (Dias et al., 2010) – a collection of yeast strains with individually deleted genes – found similar results. Both studies indicated alterations in oxidative stress, protein synthesis and degradation of proteins by proteasomes and carbohydrate metabolism after mancozeb exposure. Therefore, toxicogenomic studies using *Z. tritici* as a plant pathogen model may improve the understanding of the direct and/or side effects in metabolic pathways induced by fungicides exposure.

6.3 Conclusions

Outcomes from this study indicate that *Z. tritici* has the biological potential to adapt to chlorothalonil or folpet. The genome-wide transcriptional response study also provided insights into alternative adaptation and resistance mechanisms (i.e. ABC, MFS efflux pumps or GST) that may confer lower sensitivity to chlorothalonil or folpet in *Z. tritici*. Moreover, the evolutionary study indicated that generation of *de novo* mutations in the SDH target protein was the most common resistance mechanism conferring resistance to the single-site fluxapyroxad and other SDHIs. Additionally, overexpression of an ABC transporter, alone or in combination with up-regulation of a GST gene may also confer resistance to fluxapyroxad and lower sensitivity to other SDHIs. Once resistance emerged through *de novo* mutations in the target site, the evolution of resistance was driven by clonal replacement under increasing concentrations of fluxapyroxad. Lastly, the genome-wide transcriptional response and enrichment analysis determined compound-specific functional gene expression signatures in response to chlorothalonil or folpet. The functional gene expression signatures indicated a range of metabolic pathways possibly affected in *Z. tritici* that can be further explored to elucidate the mode of action of these fungicides.

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Appendix

Appendix 4.1 Supplementary information: the following additional excel files data are available with this thesis.

Supplementary Table 4.1s. All significantly differentially expressed *Z. tritici* genes after chlorothalonil exposure in the lag phase. The spread sheet contains gene codes retrieve from the JGI genome website (http://genome.jgi-psf.org/Mycgr3/Mycgr3.home.html), expression values and available functional annotation.

Supplementary Table 4.2s. List of 271 significantly differentially expressed *Z. tritici* genes after chlorothalonil exposure only in the lag phase. The spread sheet contains gene codes retrieve from the JGI genome website (http://genome.jgi-psf.org/Mycgr3/Mycgr3.home.html), expression values and available functional annotation.

Supplementary Table 4.3s. Enriched gene ontology terms in the 271 differentially expressed genes in *Z. tritici* after chlorothalonil exposure in the lag phase of growth compared to the reference annotated genome using Fisher's exact test with multiple testing correction of FDR.

Supplementary Table 4.4s. All significantly differentially expressed *Z. tritici* genes after chlorothalonil exposure in the log phase. The spread sheet contains gene codes retrieve from the JGI genome website (http://genome.jgi-psf.org/Mycgr3/Mycgr3.home.html), expression values and available functional annotation.

Supplementary Table 4.5s. Enriched gene ontology terms in all significantly differentially expressed genes in *Z. tritici* after chlorothalonil exposure in the log phase of growth compared to the reference annotated genome using Fisher's exact test with multiple testing correction of FDR.

Supplementary Table 4.6s. List of 293 significantly differentially expressed *Z. tritici* genes after chlorothalonil exposure only in the log phase. The spread sheet contains gene codes retrieve from the JGI genome website (http://genome.jgi-psf.org/Mycgr3/Mycgr3.home.html), expression values and available functional annotation.

Supplementary Table 4.7s. List of significantly differentially expressed *Z. tritici* genes in response to chlorothalonil exposure in the lag and log phase. The spread sheet contains gene codes retrieve from the JGI genome website (http://genome.jgi-psf.org/Mycgr3/Mycgr3.home.html), expression values and available functional annotation.

Supplementary Table 4.8s. All significantly differentially expressed *Z. tritici* genes after folpet exposure in the lag phase. The spread sheet contains gene codes retrieve from the JGI genome website (http://genome.jgi-psf.org/Mycgr3/Mycgr3.home.html), expression values and available functional annotation.

Supplementary Table 4.9s. Enriched gene ontology terms in the significantly most differentially expressed genes in *Z. tritici* after folpet exposure in the lag phase of growth compared to the reference annotated genome using Fisher's exact test with multiple testing correction of FDR.

Supplementary Table 4.10s. List of significantly most differentially expressed *Z. tritici* genes in response to folpet exposure in the lag phase. The spread sheet contains gene codes retrieve from the JGI genome website (http://genome.jgi-psf.org/Mycgr3/Mycgr3.home.html), expression values and available functional annotation.

Supplementary Table 4.11s. List of 166 significantly differentially expressed *Z. tritici* genes in response to folpet exposure only in the lag phase. The spread sheet contains gene codes retrieve from the JGI genome website (http://genome.jgi-psf.org/Mycgr3/Mycgr3.home.html), expression values and available functional annotation.

Supplementary Table 4.12s. All significantly differentially expressed *Z. tritici* genes after folpet exposure in the log phase. The spread sheet contains gene codes retrieve from the JGI genome website (http://genome.jgi-psf.org/Mycgr3/Mycgr3.home.html), expression values and available functional annotation.

Supplementary Table 4.13s. Enriched gene ontology terms in all significantly differentially expressed genes in *Z. tritici* after folpet exposure in the log phase of growth compared to the reference annotated genome using Fisher's exact test with multiple testing correction of FDR.

Supplementary Table 4.14s. List of 2,003 significantly differentially expressed *Z. tritici* genes in response to folpet exposure only in the log phase. The spread sheet contains gene codes retrieve from the JGI genome website (http://genome.jgi-psf.org/Mycgr3/Mycgr3.home.html), expression values and available functional annotation.

Supplementary Table 4.15s. Enriched gene ontology terms in the 2,003 significantly differentially expressed genes in *Z. tritici* after folpet exposure only in the log phase of growth compared to the reference annotated genome using Fisher's exact test with multiple testing correction of FDR.

Supplementary Table 4.16s. List significantly differentially expressed *Z. tritici* genes in response to folpet exposure in the lag and log phase. The spread sheet contains gene codes retrieve from the JGI genome website (http://genome.jgi-psf.org/Mycgr3/Mycgr3.home.html), expression values and available functional annotation.

Supplementary Table 4.17s. List significantly differentially expressed *Z. tritici* genes in the lag phase in response to chlorothalonil or folpet exposure.

Supplementary Table 4.18s. Enriched gene ontology terms in the significantly differentially expressed genes in *Z. tritici* in the lag phase in response to chlorothalonil or folpet exposure compared to the reference annotated genome using Fisher's exact test with multiple testing correction of FDR.

Supplementary Table 4.19s. List significantly differentially expressed *Z. tritici* genes in the log phase in response to chlorothalonil or folpet exposure.

Supplementary Table 4.20s. Enriched gene ontology terms in the significantly differentially expressed genes in *Z. tritici* in the log phase in response to chlorothalonil or folpet exposure compared to the reference annotated genome using Fisher's exact test with multiple testing correction of FDR.

Appendix 5.1. ANOVA of log_2 fold-change for various target genes in *Z. tritici* isolate IPO323, showing standard errors of difference (SED) between means of 5 degrees of freedom.

Target gene ID	Control	EC50	EC80	SED
SDHB	0.0	+1.42	+1.84	0.458
SDHC	0.0	+1.11	+1.87	0.441
SDHD	0.0	+1.41	+1.83	0.488
AOX	0.0	-1.73	+0.84	0.537
ABCt-1	0.0	-0.76	-0.33	0.461
ABCt-2	0.0	+0.80	+0.54	0.595
ABCt-3	0.0	+0.61	+0.28	0.503
ABCt-4	0.0	+0.39	+0.54	0.517
ABCt-5	0.0	+1.17	+1.28	0.477
ABCt-6	0.0	+1.02	+2.32	0.448
ABCt-7	0.0	+1.08	+1.21	0.564
GST-1	0.0	-0.66	+1.30	0.436
GST-2	0.0	-0.24	+0.30	0.477
GST-3	0.0	+0.11	+0.39	0.638
GST-4	0.0	+1.92	+2.89	0.616
GST-5	0.0	+0.82	+1.61	0.643
GST-6	0.0	-0.44	+0.51	0.426
GST-7	0.0	+0.13	-1.21	1.456
MFS-1	0.0	+0.03	+0.10	0.631
MFS-2	0.0	+2.24	+3.68	0.777
MFS-3	0.0	+0.35	+0.34	0.671
MFS-4	0.0	-0.95	-0.84	0.500
MFS-5	0.0	+0.12	-0.27	0.598
MFS-6	0.0	+1.46	+3.07	0.610
MFS-7	0.0	-0.46	+0.21	0.495

Appendix 5.2 ANOVA of log_2 fold-change for various target genes in *Z. tritici* laboratory mutant IPOFluxa9-7, showing standard errors of difference (SED) between means of 5 degrees of freedom.

Target gene ID	Control	EC_{50}	EC_{80}	SED
SDHB	0.0	+0.23	+0.84	0.175
SDHC	0.0	+0.45	+0.31	0.502
SDHD	0.0	+0.37	+1.05	0.195
AOX	0.0	-0.18	+0.58	1.647
ABCt-1	0.0	-0.61	-1.59	0.745
ABCt-2	0.0	+2.17	+3.17	0.622
ABCt-3	0.0	+0.27	+0.12	0.424
ABCt-4	0.0	-0.43	-0.60	0.469
ABCt-5	0.0	-0.04	+0.53	0.683
ABCt-6	0.0	-0.13	+0.53	0.361
ABCt-7	0.0	-0.23	-0.05	0.361
GST-1	0.0	-0.37	-0.96	0.514
GST-2	0.0	-0.35	-1.20	0.403
GST-3	0.0	-0.05	-1.00	0.574
GST-4	0.0	+0.460	+1.32	0.158
GST-5	0.0	-0.04	-0.22	0.433
GST-6	0.0	-0.10	-0.36	0.381
GST-7	0.0	-0.35	-0.06	0.331
MFS-1	0.0	+0.29	-0.08	0.489
MFS-2	0.0	+0.12	+0.43	0.989
MFS-3	0.0	-0.14	-0.47	0.847
MFS-4	0.0	-0.11	+0.69	0.567
MFS-5	0.0	-0.11	-0.09	0.642
MFS-6	0.0	-0.63	+0.54	1.272
MFS-7	0.0	+0.01	-0.41	1.277