PhD Summary Report No. 7

November 2008

Project No. RD-2004-3007

Characterisation of *Mycosphaerella graminicola* isolates with reduced azole sensitivities

by

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October 2004- May 2008

Abstract

Fungicide activity against *Mycosphaerella graminicola,* the causal agent of Septoria leaf blotch (SLB) on wheat has declined over recent years. The overall aim of this study was to characterise UK *M. graminicola* field isolates and to identify the molecular mechanisms that result in reduced azole (triazole and imidazole) sensitivity.

M. graminicola isolates from treated and untreated UK field populations in 2004 had significantly lower epoxiconazole sensitivities compared to isolates from 1993. The prevalence of alterations in eburicol 14α-demethylase (CYP51), the azole target site, was assessed within the1993 and 2004 (?) isolates. This confirmed that substitution I381V correlates with slightly reduced epoxiconazole sensitivity. Cross-resistance occurred within the triazole and the imidazole groups, but not between groups. Fourteen compounds were assessed for their ability to increase epoxiconazole efficacy against isolates with reduced azole sensitivity. A formula, developed to calculate synergistic interactions, identified synergism between trifluoperazine or fluphenazine and epoxiconazole. In future this could be used to identify synergistic compounds to aid fungicide longevity.

Expression of 16 *M. graminicola* transporter genes (including 11 novel genes) was studied in several isolates, both *in planta* and *in vitro.* None of these genes were over-expressed to the extent expected to cause a reduction in epoxiconazole sensitivity. However, expression of a selection of these genes was induced by epoxiconazole in a resistant isolate. *In planta*, it was possible to identify genes involved in infection of the leaf but not those involved in reducing fungicide sensitivity.

Biochemical techniques were used to analyse differences in sterol content between sensitive isolates and isolates with reduced epoxiconazole sensitivities. Using sterol content data, it was possible to assess overall CYP51 activity, and identify the impact of individual mutations on activity. Some alterations clearly reduce CYP51 activity, whereas others probably compensate for a loss of activity. These data suggest that adaptation of *M. graminicola* to azole fungicide use, particularly evolution of the CYP51 target enzyme, is an ongoing dynamic process in modern populations of the fungus.

Introduction

Mycosphaerella graminicola is the cause of Septoria tritici blotch (STB) in winter wheat. Complete varietal resistance to STB is not available and during seasons of high disease pressure, control relies heavily on treatment with fungicides. Epidemics of STB are common in intensively farmed wheat worldwide, and in 1997 total economic losses in the UK were estimated at £35.5 million (Hardwick *et al.*, 2001).

Due to the yield reducing effects of *M. graminicola* epidemics in Northern Europe, it is economically beneficial to control the disease at key points during the season. The top three leaves of a wheat crop contribute 80% of the eventual yield, with the flag and second leaf being the most important. Protection of just these three leaves from disease ensures high yields. *M. graminicola* has been present in UK wheat production since the mid-nineteenth century, however it has only become a major problem since the 1980's, when prevalence increased and it became the most important disease of winter wheat (Bearchell *et al.*, 2005). Since then there have been sustained efforts to control this disease through both breeding genetic resistance into wheat varieties and treatment with both protectant and systemic fungicides. Initial efforts to control STB were based on the use of Methyl Benzimidazole Carbamate (MBC) fungicides, before development of resistance in 1984 (Griffin *et al.*, 1985). Since then control has consistently relied on the azoles, together with the QoI, or strobilurin, fungicides introduced in the late 1990's, before development of resistance to these compounds in 2002.

Currently it is popular for older chemistry, such as the protectants chlorothalonil and mancozeb, to be used as first sprays or mixture partners to boost control and aid resistance management. Total cereal fungicide applications in the UK are shown in Figure 1.

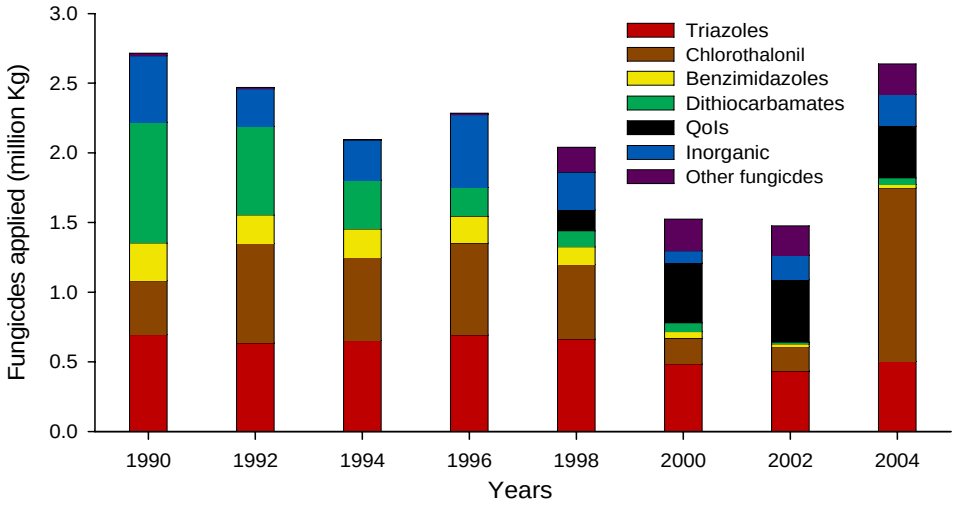

Figure 1. Total fungicides used in UK cereals, excluding seed treatments. Adapted from CSL pesticide usage statistics (http://pusstats.csl.gov.uk, accessed October 2007).

Resistance to azole antifungal agents has been observed in both medically and agriculturally important fungi. The first report of field resistance was in the cucumber pathogen *Sphaerotheca fuliginea* (Schepers, 1985) but since then resistance has been reported in several plant pathogens including *Penicillium digitatum* (Eckert, 1987), and *Erysiphe graminis* (Heaney, 1988). Resistance in medicine is now common and several literature reviews exist on the subject (Lupetti *et al.*, 2002; Sanglard *et al.*, 2002). Common human pathogens that have been investigated include *Candida albicans* and to a lesser extent *C. dublinensis, C. glabrata, C. tropicalis, C. krusei* and *Aspergillus fumigatus*. Four common mechanisms and two atypical mechanisms that cause resistance to azoles have been discovered and are discussed in detail below. The key mechanisms are: mutations in the target encoding gene; increased production of the target enzyme; reduced fungicide accumulation; and alterations in sterol biosynthesis. These mechanisms are often found in combination in human and plant pathogenic fungi (Hartman *et al.*, 1997; Stergiopoulos *et al.*, 2003a).

Much research has been done in *Candida spp.* to understand which alterations in the target enzyme, CYP51, are important in reducing affinity for azoles. It should be noted that an amino acid alteration that reduces affinity to one azole does not necessarily cause a reduced affinity to the whole family, largely due to variation in azole side chains. One substitution commonly identified in resistant *C. albicans* strains is G464S (glycine to serine change at codon 464), close to the haem binding site. G464S alters

confirmation of protein and perturbs the haem environment thus changes azole binding, reducing affinity of the CYP51 for fluconazole (Kelly *et al.*, 1999; Podust *et al.*, 2001). A further alteration, the presence of which correlates with resistance is R467K, isolated from a fluconazole treated patient (White, 1997b). One of the systems used to study the effect of point mutations in *CYP51* is heterologous expression in yeast (Lamb *et al.*, 1997; Sanglard *et al.*, 1998; Marichal *et al.*, 1999). Mutations in *C. albicans* that have been found to contribute to a significantly reduced affinity for azoles and consequently a resistant phenotype are Y132H, P230L, S405F, G464S, G465S, R467K and I471T. These mutations are often found in combination, leading to different levels of resistance, and it is thought that stepwise shifts in resistance can occur through the emergence and accumulation of new mutations.

The first example of reduced sensitivity to azoles through increased expression of *CYP51* was a correlation between resistance of a *C. albicans* strain and increased levels of *CYP51* mRNA alongside increased levels of efflux pump mRNA (*CDR1* and *MDR1* see later, (White, 1997a). The only case of increased expression of *CYP51* acting as a lone resistance mechanism is in a resistant *Penicillium digitatum* isolate with constitutively 100-fold higher levels of wild type *CYP51* than a sensitive isolate (Hamamoto *et al.*, 2000). The promoter region of *P. digitatum CYP51* in this isolate contained five replications of a 126bp tandem repeat, which was acting as an upstream transcriptional enhancer. Strains with increased levels of CYP51 have also been reported in *C. glabrata* (Pinjon *et al.*, 2003), and *Venturia inaequalis* (Schnabel *et al.*, 2001) and in an isolate of *M. graminicola* with reduced sensitivity to cyproconazole, in combination with other mechanisms (Stergiopoulos *et al.*, 2003a).

Reduced fungicide accumulation through increased active efflux is a common resistance mechanism found in many different fungi and the most commonly identified mechanism of azole resistance in *C. albicans* (Perea *et al.*, 2001). Transporter proteins that export xenobiotics are from two protein families, ATP-binding cassette transporters (ABC transporters) or major facilitator transporters (MFS). ABC transporter genes discovered in *C. albicans* do not all transport fungicides. Only two of the overexpressed ABC transporters reduce drug accumulation, CDR1 (Prasad *et al.*, 1995; Sanglard *et al.*, 1995) and CDR2 (Sanglard *et al.*, 1997), whereas only one major facilitator, MDR1, exports azole (Fling *et al.*, 1991; Ben-Yaacov *et al.*, 1994).

Reduced sensitivity to xenobiotics through decreased accumulation has been reported in *M. graminicola* (Stergiopoulos *et al.*, 2002a; Stergiopoulos *et al.*, 2002b; Stergiopoulos *et al.*, 2003a). Five *M. graminicola* ABC-transporters, *MgAtr1-5,* have been functionally characterised by targeted knockout studies and heterologous overexpression in yeast. Overexpression shows that *MgAtr1, 2* and *4* play roles in azole resistance. However, basal expression of *MgAtr1-5* in field isolates varies and has not been correlated with triazole sensitivity (Zwiers *et al.*, 2000; Stergiopoulos *et al.*, 2002b). *MgAtr* genes have distinct but overlapping substrate activity and these particular ABC-transporters play roles in protection against plant-related defence compounds as well as transporting synthetic compounds. There are undoubtedly more *M. graminicola* ABC-transporter and major facilitators and therefore the possibility of uncharacterised resistance mechanisms. It is currently possible to identify further transporter genes using the Consortium for the Functional Genomics of Microbial Eukaryotes (COGEME) Expressed Sequence Tag (EST) database and the *M. graminicola* genome.

In vivo azole resistance through altered sterol biosynthesis was first noted in *C. albicans* strains isolated from HIV positive patients. The strains, which were cross resistant to fluconazole and amphotericin B, showed reduced levels of ergosterol and accumulation of sterols lacking a $C^{5(6)}$ double bond (Kelly *et al.*, 1995; Kelly *et al.*, 1996). Similar resistance mechanisms had previously been identified in azole resistant lab generated mutants of *S.cerevisiae* and *Ustilago maydis* (Watson *et al.*, 1989; Joseph-Horne *et al.*, 1995). *U. maydis* exhibited reduced levels of ergosterol and accumulation of sterols lacking a $C^{5(6)}$ double bond and when azole treated there was an accumulation of functional 14α-methylated sterols rather than toxic 14α-methyl-3,6-diol, thereby enabling growth. Resistance in *U. maydis* and *C. albicans* was found to be due to leaky activity or null-mutation of *ERG3*, encoding the sterol Δ5,6-desaturase. This resistance mechanism appears to be uncommon among plant pathogens and has never been found in field isolates, possibly due to a fitness penalty associated with growth utilising 14α-methylated sterols rather than ergosterol. Further changes in the ergosterol biosynthesis pathway (such as mutations in *ERG4*) are not thought to affect azole sensitivities (Zweytick *et al.*, 2000). However, *C. glabrata* is able to overcome azole fungicides, not by altering sterol biosynthesis but by circumventing it through utilisation of exogenous sterols (Brun *et al.*, 2004).

Fungicide resistance management strategies are designed to delay the onset or spread of resistance. Development of resistance costs not only the fungicide producing companies but also growers and consumers and thus it is in everyone's best interest to try and slow the evolution of resistance. In order to develop fungicide resistance management strategies it is first necessary to understand the risk of resistance associated with each fungicide group and each fungal pathogen, and the options available in a given growing system. The Fungicide Resistance Action Committee (FRAC), an industrial consortium designed to promote action and awareness of resistance issues, have produced a resistance risk assessment model. Multi-site inhibitor fungicides generally act upon multiple metabolic pathways and are often only active at the initial stages of fungal growth, providing a protectant barrier between the plant and the fungus. These compounds, such as chlorothalonil, generally carry very little risk of resistance. Single-site fungicides are generally more specific, more selective and more prone to the development of resistance. Polycyclic pathogens, with multiple sexual and/or asexual stages during a season, are generally considered high risk as resistant alleles can be selected or recombine and spread rapidly within a single season. For example powdery mildews produce millions of wind-dispersed spores throughout the season, having multiple generations where phases of selection can occur and with many opportunities for resistance alleles to spread long distances. A monocyclic pathogen, such as *Leptosphaeria maculans* has a lower risk potential as it only has one sexual generation within a season. The extent to which fungicide resistant management strategies are employed depends on the risk assessment.

The only way to completely prevent the development of resistance is not to use the fungicide. Continuation of this theory states that minimisation of exposure to fungicides will allow minimum selection of resistant alleles, but this is a contentious issue. Many argue that higher doses prevent selection by killing more isolates with reduced sensitivities. It is almost certainly the case that one whole dose is better than two half doses due to exponential decay of fungicide efficacy, but the general rule is still that more fungicide causes more selection. Many resistance management strategies have been employed in the past with varying degrees of success. It is widely agreed that to slow the spread of resistance, strategies must be in place before resistance emerges. It is now the Pesticide Safety Directorate, UK policy that resistance studies based on efficacy are undertaken before pesticide registration. The key aspect of resistance management to date has been the use of tank mixtures when spraying. If fungicides of two or more modes of action are used simultaneously, lower

quantities of each active can be used, and thus it is less likely that resistance alleles will be selected. Furthermore, if isolates resistant to one fungicide are present, the other fungicide(s) should control them, unless individuals are resistant to both. As discussed earlier it is now common to spray the protectant chlorothalonil at T0, T1 and T2 for both additional protection and increasingly for the purpose of resistance management. Since the demise of the QoI fungicides against STB, the use of chlorothalonil has increase dramatically (Figure 1), perhaps reflecting a need for more actives to reduce the current reliance on azoles. This is especially important in the light of declining efficacy recently observed in many azole fungicides (Clark, 2006). A resistance management strategy, employed either when tank-mixing is not possible or when crops require many fungicide applications, is to alternate active ingredients in sprays (Brent *et al.*, 2008). Alternation of spraying is common on crops where many sprays are required within a season, simply for ease of use.

Aims

The primary aim of this project is to characterise UK field isolates of *M. graminicola* with reduced azole sensitivities and thereby understand the molecular mechanisms responsible for the recent decline in azole efficacy. Potential azole resistance mechanisms, analogous to those conferring a highly resistant phenotype in *Candida albicans*, have been studied in detail in *M. graminicola* isolates using a variety of molecular and biochemical approaches. Molecular characterisation of these mechanisms will allow for development of high-throughput assays to detect and monitor spread of azole resistant isolates in the field.

Materials and Methods

Obtaining single spore *Mycosphaerella graminicola* isolates

STB lesions were cut from wheat leaves, attached to moist filter paper and incubated in a damp environment at 20 °C for 24 h to induce extrusion of cirri from pycnidia. Individual cirri were picked off with watchmakers forceps and diluted in 50 µL water before being plated out onto penicillin G and streptomycin (100 $\mu g\ mL^{-1}$) amended yeast potato dextrose agar (YPD) (ForMedium, Norwich, UK). Spores were retrieved after seven days growth at 15 °C and stored in at -80 °C in 80% glycerol. When required, this stock was re-cultured on YPD.

Sensitivity Testing: Optical Density Assay

Each isolate was grown on YPD agar at 15 °C, for seven days, harvested, and diluted to a concentration of $2.5x10^5$ spores/mL. 100 µL of this suspension was added to 100 µL double strength liquid Czapek Dox medium (Oxoid, Basingstoke, UK) in a 96 well microtitre plate amended with 12 final epoxiconazole concentrations (50, 25, 12.5, 6.25, 3.125, 1.56, 0.78, 0.39, 0.195, 0.098, 0.049 and 0 µg/mL). Plates were incubated in the dark at 23 °C for 96 hours, after which optical densities at 630 nm were measured with a Dynex MRX plate reader (Chantilly, VA, USA). Replicate readings were used to fit dose response regression curves (with a coefficient of determination >95%) and the EC_{50} value of each isolate calculated. Isolates for which an accurate regression curve could not be found were re-tested, but discarded if this did not lead to a result. Fifteen historical isolates from a 1993 field trial in Reading, UK and four isolates of interest (Table 2) were tested alongside Terrington isolates as a reference.

An ADAS experimental field plot in Terrington, Norfolk with eight winter wheat plots (*Triticum aestivum* cv. Consort), four treated with four quarter doses of epoxiconazole, and four untreated, was sampled for *M. graminicola*. A total of 256 isolates were taken from individual lesions on leaf two (thirty-two from each plot, sampled a few metres apart) and stored in glycerol at -80°C. These isolates were tested for sensitivity to epoxiconazole with the optical density assay method previously described.

CYP51 sequencing

DNA was extracted from yeast-like fungal material and adjusted to 10 ng/µL. PCR was performed with Phusion polymerase according to manufacturers protocol (New England Biolabs, MA, USA) using *CYP51* primers CYPSEQF and CYPSEQR (Table 1). PCR fragments were purified with SV Wizard PCR purification kit (Promega, WI, USA) ligated into pGem-T plasmid (Promega, WI, USA) and transformed into *Escherichia coli* strain JM109. Transformed colonies were screened for the correct insert with CYP51 primers as above. Successful transformants were grown for 18 h at 37 °C 220 rpm. Plasmids were purified according to Sigma GenElute plasmid mini prep kit (Sigma-Aldrich, Gillingham, Dorset) and sequenced with M13 primers by MWG Biotech (Ebersberg, Germany).

Prevalence of substitutions in the target site

PCR based assays (primers shown in Table 1) were developed to identify a point mutation encoding a substitution (I381V) and a six base pair deletion encoding a two amino acid deletion (ΔY459/G460). Spore suspensions of isolates for which an EC_{50} value had been determined were boiled at 110 °C for 10 min and used as template DNA. A region of *CYP51* encompassing both alterations was amplified from each isolate using primers F3BF and CYSTR under the following conditions: 94°C for 2.30 min, followed by 40 cycles of 94°C for 30 s, 55°C for 1 min, 72°C for 1.30 min and a final extension at 72°C for 9 min. A 1 in 200 dilution of this primary PCR product was used as a DNA template for both the following assays. To identify I381V, a PCR reaction with primers CYSTF and BF381REV was carried out with cycles 94°C for 2.30 min, followed by 40 cycles of 94°C for 30 s, 55°C for 45 s and 72°C for 1 min, with a final extension at 72°C for 9 min. An additional BsrI recognition site in CYP51 is introduced by a nucleotide change encoding substitution I381V. This was detected by cutting 2 µl of PCR product with 1 unit of BsrI at 65°C for two hours. To identify ΔY459/G460 an allele-specific PCR assay was carried out with forward primer DEL-T and the reverse primer UNIREV (at 0.2µM) with cycling at 94°C for 2.30 min, followed by 40 cycles of 94°C for 30 s, 52°C for 30 s and 72°C for 30s, with a final extension at 72°C for 4.30 min. Primers are specified in Table 1.

Table 1. Primer sequences used to identify mutations in CYP51

Primer name	Sequence
F3BF	5′-GTCACAAGCAGAAGGCGTGCAGAT-3′
CYSTF	5′-GTTGCCACGCGTGTCCCAC-3′
BF381REV	5′-CAATGGAGGCAGTCGGGAAAGTGC-3′
CYSTR	5′-CCACTTYACTACTGCCGGCGA-3′
DEL-T	5′-GAAAGAAGACTACGGCCTT$_a$-3′
UNIREV	5′-CTCCCTCCTCTCCCACT-3′
CYPSEQF	5′-ATGCGTCTCCTCCAGGAAGTCCTC-3′
CYPSEQR	5′-TCAGTTCTTCTCCTCCTTCTCCTC-3′

[a] - allele specific nucleotide

Growth of Fungal Cultures for RNA extraction

Four isolates, IPO323, G303, SMR1 and R6-17 (Table 2), were grown on Yeast Potato Dextrose YPD agar (ForMedium, Norwich, UK), at 15 °C, for seven days. Spores were harvested and diluted to a concentration of 4.5 x 10^5 spores/mL in 100 mL YPD broth (ForMedium, Norwich, UK). Cultures were incubated at 21 °C, 220 rpm for a total of 72 hours. Epoxiconazole was added to treated samples at a concentration of 1 µg/mL after 48 h incubation. Fungal material was collected by vacuum filtration through 8 µM pore nitrocellulose filters (Millipore, Billerica, USA). Material was snap frozen and freeze dried overnight prior to RNA extraction.

Table 2. Isolates used in this study, their sensitivities to epoxiconazole, tebuconazole and known CYP51 alterations.

Isolate designation	Origin	EC_{50} (µg/mL)		CYP51 alterations
		Epoxiconazole	Tebuconazole	
IPO323	Netherlands - 1981	0.041	0.11	None
G303	Kent - 2003	1.41	18.98	L50S, S188N, A379G, I381V, ΔY459/ G460, N513K
SMR1	Rothamsted - 2005	3.22	nd[a]	L50S, I381V, Y461H
R6-17	Rothamsted - 2006	0.72	1.511	L50S, V136A, Y461H

[a] - not determined

RNA extraction

Roughly 30 mg of powdered freeze dried fungal material was placed in a 2 mL screw top tube with a 3 mm stainless steel ball and run through a FastPrep shaker at 4.5 ms^{-1} for 30 s (FP120, Bio101/Savant, MPBiomedicals). One mL of TRIZOL reagent (Invitrogen, Paisley, UK) was added and the samples run through the FastPrep once

again. TRIZOL was then used according to the manufacturer's protocol with 1-bromo-3-chloropropane (Helena Biosciences, Sunderland) as the phase separating agent. Further purification of the total RNA was achieved with an overnight 4 M lithium chloride precipitation (Sigma-Aldrich, Gillingham, Dorset) at -20 °C. RNA was quantified with the NanoDrop ND-1000 system according to manufacturer's protocol (NanoDrop, Thermo Fisher Scientific, DE, USA)

RNA extraction from infected leaf samples

The central five centimetres of each inoculated leaf area were removed from the plant and snap frozen. Three leaves were pooled in a 2 mL tube and freeze dried. A five milimeter stainless steel ball bearing was added to each tube, which was then run through a FastPrep shaker at 4.5 ms^{-1} for 30 s (FP120, Bio101/Savant, MPBiomedicals). One millilitre of TRIZOL reagent (Invitrogen, Paisley, UK) was then added and the tube was run through the FastPrep again. TRIZOL was used according to the manufacturer's protocol with 1-bromo-3-chloropropane (Helena Biosciences, Sunderland) as the phase separating agent. Further purification of the total RNA was achieved with an overnight precipitation in 4 M lithium chloride (Sigma-Aldrich, Gillingham, Dorset) at -20 °C.

Reverse Transcription

Reverse transcription of first strand cDNA was carried out with the Invitrogen Superscript III RT-PCR kit (Invitrogen, Paisley, UK) following the supplier's protocol using oligo d(T) primers and 5 µg total RNA per reaction. A one in ten dilution of this reaction was used as the template for the real-time PCR analysis. cDNA quality was checked by PCR, with intron spanning *tub-1* (β-tubulin accession: AJ310917) primers (Table 3) and PCR conditions 94°C for 2 min then 40 cycles of 94°C for 30 s, 56°C for 30 seconds, 72°C for 30 seconds and a final elongation at 72°C for 4 min.

Real-time PCR

Real-time PCR reactions were carried out with Invitrogen Platinum SYBR green PCR mix with ROX (Invitrogen, Paisley, UK) with a modified version of the supplier's protocol, using 5 µl cDNA solution in a 25 µl reaction. The cycling conditions used were 50 °C for 2 min, 95°C for 2 min then 40 replicates of 95°C for 15 seconds, 58°C for 30 seconds and 72°C for 36 seconds with fluorescence being recorded at each 72°C elongation step. Each reaction was carried out in triplicate, using the ABI PRISM 7500 sequence detection system (Applied Biosystems, Foster City, CA, USA). Relative

Quantification (RQ) values were calculated with the $2^{-(\Delta\Delta Ct)}$ method (Figure 2) using the constitutively expressed housekeeping β-tubulin gene as an endogenous control and gene expression levels in the IPO323 untreated sample as calibrators. Primers were designed in Vector NTI for each of the new sequences and for the five previously described PDR-ABC transporters from *M. graminicola* (Zwiers *et al.*, 2000; Stergiopoulos *et al.*, 2002b) with parameters set at Tm 58-59 °C, Length 20-24 bp, amplicon length 300-400 bp and a GC content between 45-55% (Table 3-4).

$RQ = 2^{-\Delta\Delta Ct}$ where:
$\Delta\Delta Ct = \Delta Ct$ gene - ΔCt calibrator
ΔCt = Ct gene - Ct endogenous control
Ct = Cycle at which threshold level of amplification is reached

Figure 2. The $2^{-(\Delta\Delta Ct)}$ method to calculate relative quantification (RQ) of transcript levels

Hierarchical clustering of *in planta* expression data

Expression data was logged to base two and stored in tab-delimited form. The program Cluster 3.0 (Eisen *et al.*, 1998) was used to centre and normalise the data before if was hierarchically clustered for each isolate, using a euclidean similarity matrix and the average-linkage clustering algorithm. TreeView 1.0.13 was used to view the output files from the clustering process.

Fungal culturing and epoxiconazole treatment for sterol content analysis

Each isolate was grown on YPD agar (ForMedium, Norwich, UK) at 15 °C for seven days (isolates used are shown in Tables 4 and 5). Spores were harvested and diluted to a concentration of 4.5×10^5 spores mL^{-1} in 100 mL YPD broth (ForMedium, Norwich, UK). This was incubated for 72 hours at 21 °C, with shaking at 220 rpm, and treated samples were amended with epoxiconazole at a concentration of 1 µg/mL 48 h into this incubation. Fungal material was collected by vacuum filtration through 8 µM pore nitrocellulose filters (Millipore, Billerica, USA). Collected fungal material was snap frozen and freeze dried overnight in borosilicate vials prior to free sterol and RNA extraction.

Table 4. Isolates for which sterol content was analysed in the presence and absence of epoxiconazole.

Isolate designation	Origin	EC_{50} (µg/mL) Epoxiconazole	CYP51 alterations
IPO323	Netherlands - 1981	0.041	None
G303	Kent - 2003	1.41	L50S, S188N, A379G, I381V, ΔY459/ G460, N513K
SMR1	Rothamsted - 2005	3.22	L50S, I381V, Y461H
R6-17	Rothamsted - 2006	0.72	L50S, V136A, Y461H

Table 5. Isolates for which sterol content was analysed in the absence of fungicide

Isolate designation	Origin	*T. aestivum* cv.	CYP51 alterations
IPO323	Netherlands - 1981	Arminda	None
Berdun 17	Pyrenees - 2006	Unknown	None
R6-31	Rothamsted - 2006	Robigus	Y137F
Berdun 13	Pyrenees - 2006	Unknown	Y137F
Berdun 10	Pyrenees - 2006	Unknown	L50S
Berdun 4	Pyrenees - 2006	Unknown	G460D
103.9	Kent - 2006	Consort	L50S, V136A, Y461H
115.3	Kent - 2006	Consort	L50S, I381V, Y461H
209.10	Kent - 2006	Consort	L50S, I381V, Y461H
209.9	Kent - 2006	Consort	V136A, S188N, ΔY459/G460
212.2	Kent - 2006	Consort	V136A, S188N, ΔY459/G460
214.7	Kent - 2006	Consort	L50S, S188N, A379G, I381V, ΔY459/G460, N513K
202.1	Kent - 2006	Consort	L50S, S188N, A379G, I381V, ΔY459/G460, N513K

Table 3. Sequences of oligonucleotides used for real-time PCR expression analysis and cDNA analysis.

Gene		Forward Primer		Reverse Primer
MgAtr1	F3	5'- CCAGAAAAACAAGGACGACGAGAA-3'	R3	5'- GTGCGGTGACAAAGGCGAAGTA-3'
MgAtr2	F2	5'- ACGCTTTCGCAGGCCTACTGTT-3'	R2	5'- CAGCCCACCCGTAATGTACGTAAA-3'
MgAtr3	F3	5'- GGCCGTGTTTCTGGTGCAGAT-3'	R3	5'- TGGATCGTGCAAGGTGGAGAGTA-3'
MgAtr4	F2	5'- CTTCCTCTTCTTCATGGCCCTCAT-3'	R2	5'- CCGCAACTCGAATGGAGCAGTA-3'
MgAtr5	F1	5'- CGACTGGGGACTGACAGAAGAATT-3'	R1	5'- GGTCCATCGATCCGTATTTGACAT-3'
Mgb0618f	F3	5'- CTCCGACTGGTCCGCCAAGTA-3'	R3	5'- CGGCCAGAAAACCCTCCAAAT-3'
Mga1012f	F2	5'- CGCAAAGTCCTTGGGTCATGAA-3'	R2	5'- ATGCGTGTCTTGCCGGATAGAA-3'
Mga0007r	F1	5'- ATTGGACCCGCGCTGATCAT-3'	R1	5'- TTCCAGGCGAAGTTGACCAGAA-3'
Mgc08g07f	F1	5'- GTGCTACCCATTGCCATCATCAT-3'	R1	5'- GATCGGAAGGATTGGGATCTTCTT-3'
Mgc14f08f	F1	5'- CACACCTGGACCATCTCGTAACAA-3'	R1	5'- CATCGTTGTGACACTGGCCTTGTA-3'
Mga1230f	F1	5'- GCAAGAGGTCTGGCGGTTGTT-3'	R1	5'- CGCGATTTGCGTCGAAGAATT-3'
Mgc17c03f	F1	5'- GGCACCTTTGCATCTGCTGTGTA-3'	R1	5'- AGATGTCACCCAGCACTCCTCAA-3'
Mg[0925]	F1	5'- GCCTTTTCACAGTCCGTCGAATT-3'	R1	5'- GCCTGGCTCGATCTTGATGTCTA-3'
Mgb0260f	F2	5'- CCACGCGAATCTCCATCCTCTA-3'	R2	5'- CGGCGGTAGGGAAGAAGTTCTT-3'
Mga0755f	F2	5'- TCTCTGCCTACGTCGAGCAAGAA-3'	R2	5'- GCAGCGGAGTCGAGACCAGAT-3'
Mg[0212]	F2	5'- CCGCAGTTTGATGCCTTGGATAT-3'	R2	5'- CAAGCATGCGTTTGGCCATAAT-3'
β-tubulin	F	5'- CGGTATGGGAACACTTCTCATCAG-3'	R	5'-AGAGAGCCTCGTTGTCAATGC-3'
β-tubulin intron spanning	F	5'-GCTCGAGCGCATGAACGTCTACTT-3'	R	5'-GGTGATCTGGAAACCCTGGAGGCA-3'

Total sterol extraction and derivitization

Fungal material was filtered and rinsed with water before freeze drying. Sterol extraction was adapted from Holmberg *et al.* (2002). Dihydrocholesterol (50µg) was used as internal surrogate standard. Sterols were extracted in 5 mL of 2:1 (v/v) chloroform:methanol for 30 min at 60 °C. The mixture was allowed to cool, filtered and reduced to dryness under nitrogen (BOC, Guildford, UK). The extract was transmethylated by addition of 1 mL toluene and 2 mL sodium methoxide (0.5 M) in methanol and incubation at 60 °C for 30 min. Two millilitres of 14 % boron trifluoride in methanol was added and the suspension heated at 60 °C for a further 10 min. Once cool, sterols were isolated with 2-3 mL diethyl ether. The ether was backwashed with 5 mL deionised water and isolated again. A further ether extraction was performed and both extracts combined. The ether phase was then dried with anhydrous sodium sulfate and remaining solvent was removed under nitrogen gas. Extracts were resuspended in 300 µl toluene and silylated with 200 µl *N*-methyl-*N*-(trimethylsilyl)trifluoroacetamide (MSTFA) for 10 min at 50 °C. The entire process (from culturing of fungi to GC-MS) was carried out in triplicate for each isolate and treatment. All reagents were obtained from Sigma-Aldrich (Gillingham, Dorset, UK) unless otherwise stated.

GC-MS

GC-MS analysis was done on a Hewlett Packard (Palo Alto, CA) 5890 Series GC fitted with a 5970 Series MSD fitted with automatic liquid sampler and Zebron ZB5 column with 5m Guardian (Phenomenex, Macclesfield, UK) 30 m × 0.25 mm internal diameter × 0.25 µm film. One microlitre splitless injections were made at an inlet temperature of 280 °C and carrier gas (Helium) head pressure of 105kPa. The oven was ramped from an initial temperature of 100 °C (initial hold time 2 min) at 10 °C/min to 320 °C and held for 6 min. The GC interface was 290 °C and EI+ (electron ionization) mass spectra, 41–750 amu, were acquired from 13 to 30 min, with the threshold set to 150 and a 1.15 scan/s scan rate. Data analysis was done in the QuanLynx module of MassLynx (Waters, Manchester, UK), following conversion of the data files from their native (HP) format to Masslynx files using the Mass Transit file conversion utility program (Palisade, Newfield, New York, USA).

Data were semi-quantified by integration of extracted ion chromatograms for individual sterols, against dihydrocholesterol (100%) as the internal standard. To

allow direct comparison of the absolute amounts of individual sterols, reconstituted (TIC) peak areas dividing the EIC peak area by its contribution to the TIC.

Peaks were identified by several techniques. Chemical standards were used to identify ergosterol, lanosterol, cholesterol and dihydrocholesterol (also used as the surrogate standard). Further sterols were identified by comparison with relative retention time and mass spectra in literature and with the National Institute of Standards and Technology (NIST) Mass spectrometry database. All sterols identified without the use of an authentic standard are putative designations.

CYP51 expression analysis

Levels of *CYP51* transcript were measured with real time RT-PCR following the previously described protocol. *CYP51* specific primers were from Cools *et al* (2007) (Table 6).

Table 6. Primers used for *CYP51* expression analysis

Gene	Forward Primer	Reverse Primer
CYP51	5'-ACAACGAGCCCCATGGACGA-3'	5'-CGCCGATACATCTGCGTCGT-3'
β-tubulin	5'- CGGTATGGGAACACTTCTCATCAG-3'	5'-AGAGAGCCTCGTTGTCAATGC-3'

Results

Changes in sensitivity over time

Epoxiconazole EC_{50}'s of 15 isolates taken from an untreated 1993 field trial in Reading UK, were calculated and their *CYP51* genes sequenced and translated (Table 7). The mean EC_{50} was 0.228 µg/mL with most isolates being highly sensitive to epoxiconazole and only one (Reading 17) having a relatively high EC_{50} of 2.52 µg/mL. This isolate has three CYP51 alterations, at G460D, L5a0S and S188N. Nine of the 15 isolates harbour alteration Y137F. Other alterations include Y461H and D224E. None of the isolates had alterations I381V or ΔY459/G460.

Table 7. Epoxiconazole sensitivities and CYP51 alterations in untreated isolates, Reading 1993.

Isolate designation	CYP51 alteration(s)	EC_{50} epoxiconazole (µg/mL)
Reading 1	Y137F	0.0259
Reading 4	G460D	0.0284
Reading 6	[a]	0.0995
Reading 11	Y461H	0.0346
Reading 12	[a]	0.0365
Reading 13	Y137F	0.0157
Reading 14	Y137F	0.1463
Reading 15	Y137F	0.0207
Reading 16	Y137F	0.0347
Reading 17	G460D, L50S, S188N	2.5248
Reading 20	Y137F	0.0081
Reading 22	Y137F	0.1778
Reading 23	[a]	0.0145
Reading 24	Y137F	0.0225

[a]wild type

Epoxiconazole sensitivities of 165 isolates obtained from ADAS field plots, near Terrington, UK, in 2004 were calculated. Sixty-eight isolates came from untreated plots, and 97 from epoxiconazole treated plots (Figure 3). There were no significant differences between mean epoxiconazole sensitivities of isolates in treated compared to untreated, with a mean EC_{50} of 0.262 µg/mL. The 2004 population was significantly less sensitive than the 1993 population (ANOVA $\ln EC_{50}$, $p<0.001$). The 2004 population contained several outlying isolates with reduced sensitivities but the highest of these, with an epoxiconazole EC_{50} of 1.89 µg/mL, was not as resistant as Reading 17.

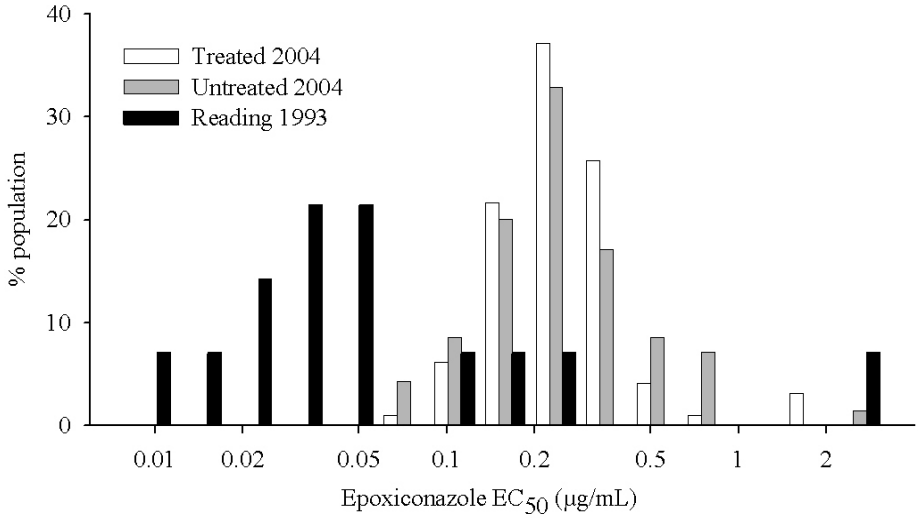

Figure 3. Distribution of EC_{50} values of isolates obtained from Reading 1993 and Terrington 2004 (untreated and epoxiconazole treated).

Fifty randomly selected Terrington isolates were further assessed using PCR-based assays (Table 8). There was no evidence that treatment selected for isolates with I381V or ΔY459/G460 over the 2004 season (t-test, p=0.131 for I381V and p=0.127 for ΔY459/G460). The majority of isolates with ΔY459/G460 also carried substitution I381V (26 out of 29). The presence of I381V was significantly associated with a change in EC_{50}, with an increase of 0.085 µg/mL in the EC_{50} of the geometric mean (anova, F=0.036). The presence of ΔY459/G460 was not significantly associated with any change in EC_{50} (ANOVA, F= 0.9).

Table 8. EC_{50} of Terrington isolates to epoxiconazole (µg/mL) and alterations in CYP51

EC50 (µg/mL)	Resistance Factor (compared to IPO323)	Number of Isolates	Isolates with ΔY459/G460	Isolates with I381V	Isolates with I381V and ΔY459/G460
<0.1	<2	2	1	1	1
0.1-0.3	2-5	39	23	25	21
>0.3	>5 (max=30)	9	5	9	5

Cross-resistance to other members of the azole family

EC_{50} of eight isolates to epoxiconazole, prothioconazole, prochloraz and ketoconazole were tested. Results demonstrate cross resistance occurs within the triazole group and within the imidazole group but not between members of the two groups. Scattergrams of results with corresponding R^2 values are shown in Figure 4.

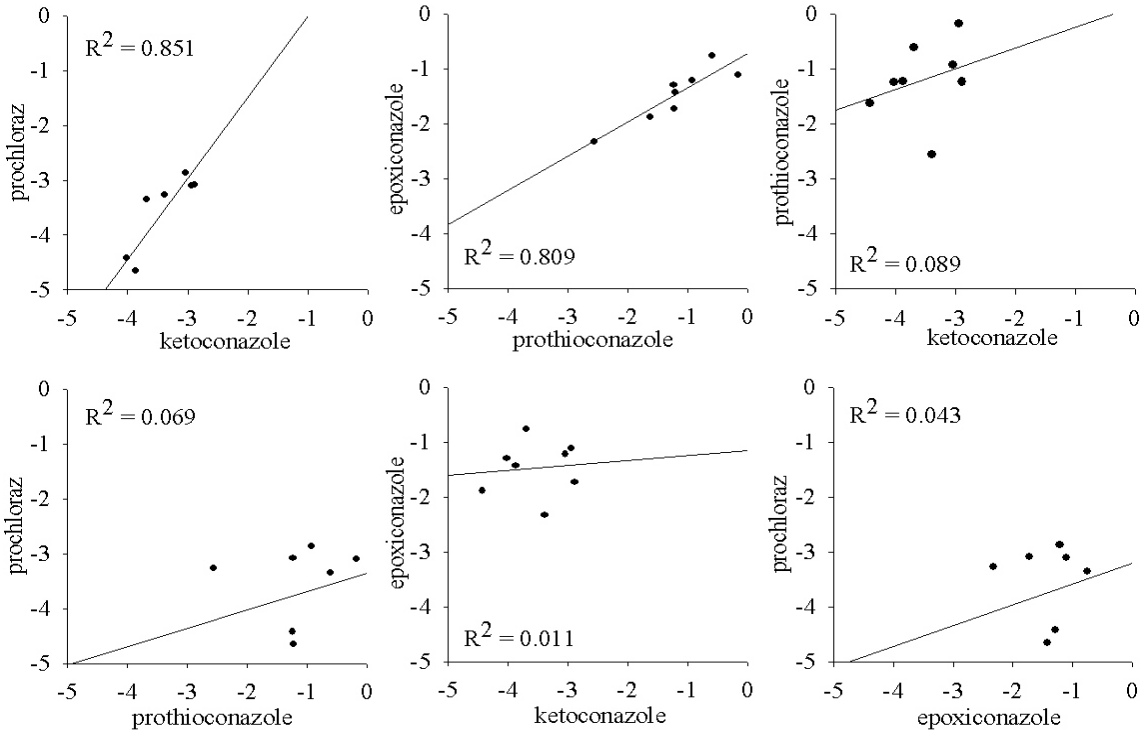

Figure 4. Scattergram showing correlations among the $\log_{10}EC_{50}$ values for *M. graminicola*. N = 8.

Relative Quantifications

Transcript levels of all 16 transporter genes, from cultures untreated or treated with epoxiconazole, are outlined in Figures 5 and 6. Levels in untreated samples differed between isolates, but it was not possible to identify a single highly, constitutively overexpressed gene in any of the isolates. Untreated transcript levels of *Mg[0212], Mga0007r, Mga1012f, MgAtr3*, and *MgAtr5* were significantly higher expressed in G303, R6-17 and SMR1 than IPO323. *MgAtr2* expression was the same in all isolates. All other genes had altered expression in at least one isolate, for example, *Mgc08g07f* was expressed 16 fold less in isolate SMR1 (significance $p<0.05$). Levels of gene expression were also measured after isolates were treated with 1 µg/mL epoxiconazole (Figure 6). The clearest differences in expression after treatment were detected for isolate G303, with significant induction of *Mga0007r, Mga1012f, Mga1230f, MgAtr2, MgAtr3, MgAtr4* and *Mgb0260f* expression. Expression levels of the MFS gene *Mga0007r* dropped significantly in IPO323 after treatment, with all other genes studied expressed at low levels irrespective of treatment in this isolate. Expression levels of transporter genes in isolates SMR1 and R6-17 varied little between treated and untreated samples.

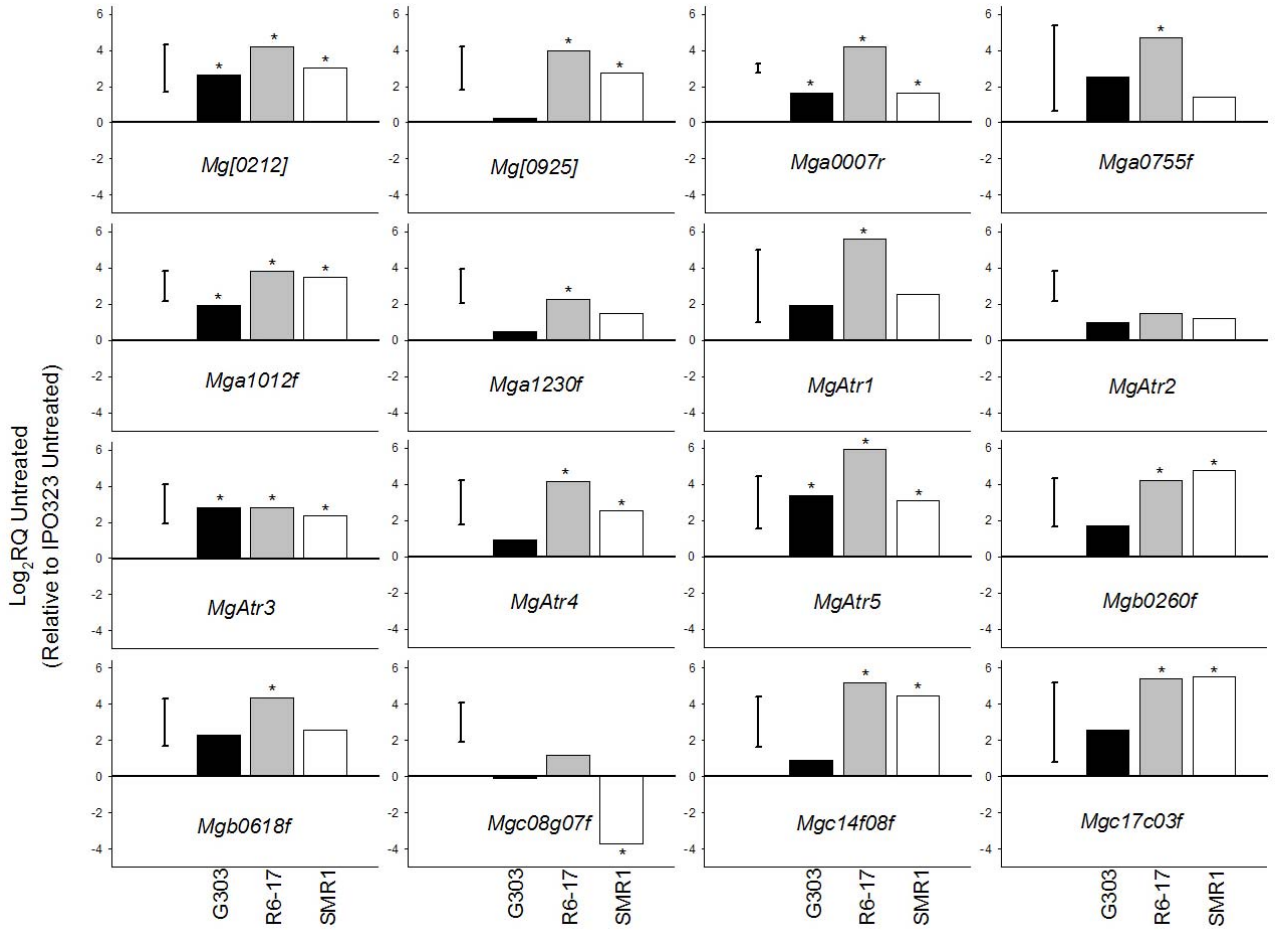

Figure 5. Expression of 16 transporter genes in untreated isolates G303, R6-17 and SMR1, relative to IPO323 untreated. Error bars represent least significant differences at p=0.05. *denotes significant (p=0.05) difference in expression from untreated IPO323.

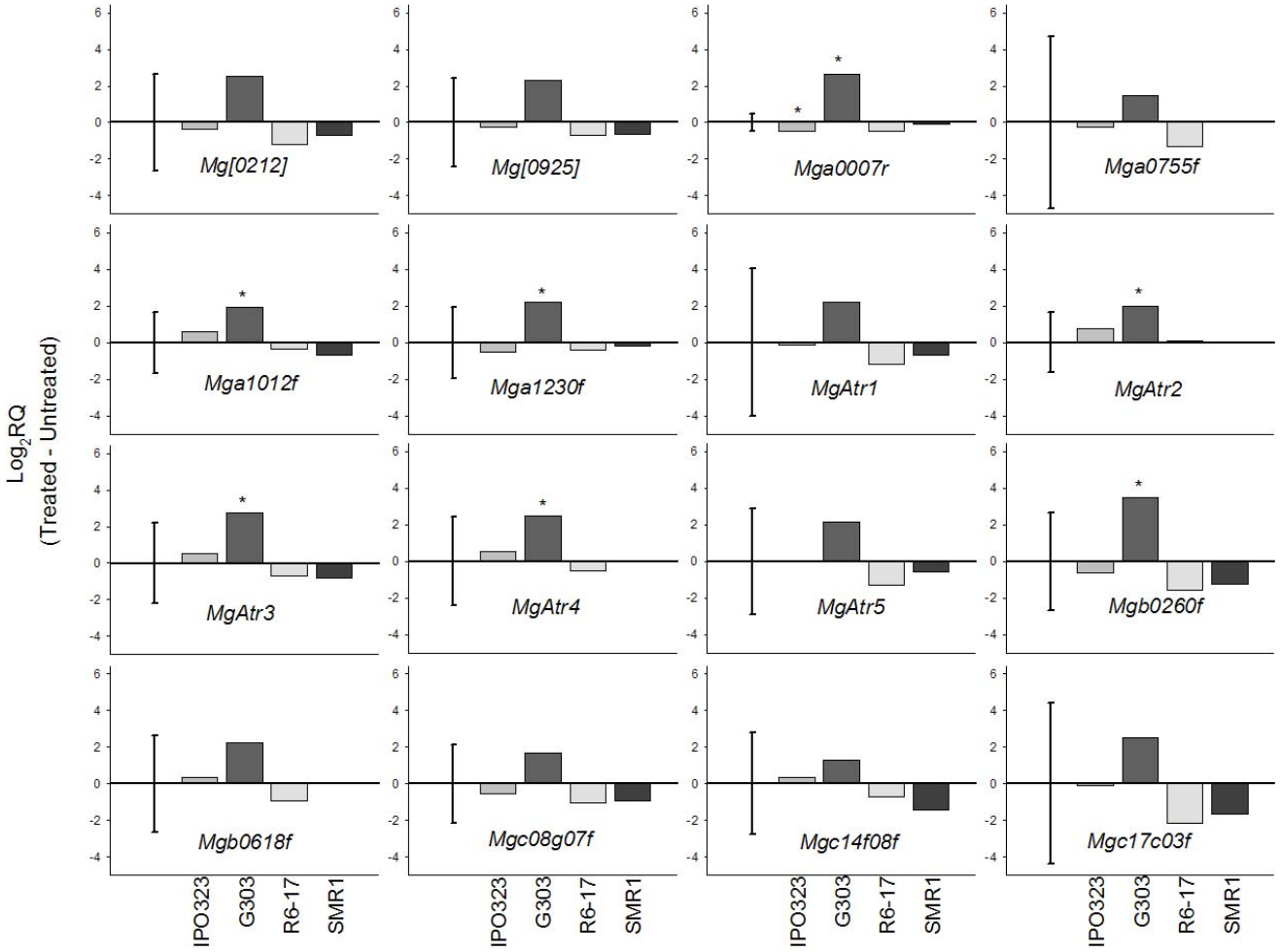

Figure 6. Expression of all 16 transporter genes in IPO323, G303, SMR1 and R6-17 when treated with 1 µg/mL epoxiconazole. Error bars represent least significant differences at p=0.05.

In planta infection assay

The infection time-course occurred at the same rate in both isolates when inoculated onto *T. aestivum* cv. Riband. Levels of β-tubulin gene transcript (a constitutively expressed housekeeping gene) generally increase over time as would be expected with increasing fungal biomass (Figure 7), with no large differences between isolates. IPO323 has reduced levels of β-tubulin transcript at the highest fungicide concentration throughout infection.

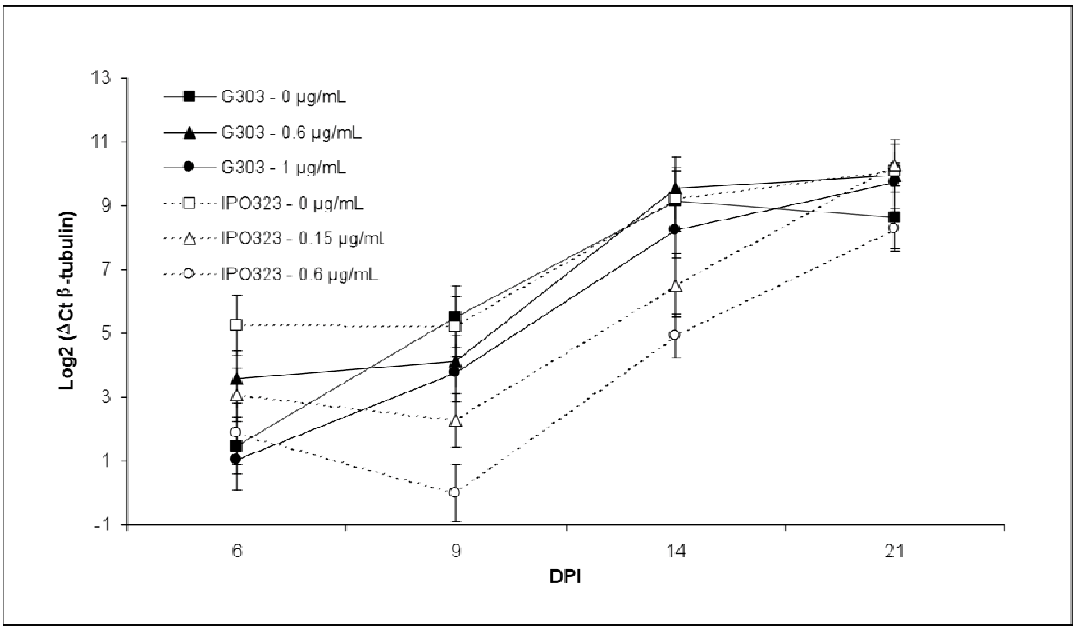

Figure 7. β-tubulin transcript levels throughout at 6, 9, 14 and 21 days post inoculation (standard errors shown).

Gene expression

Analysis of *Mgc14f08f* expression demonstrated an steady increase in relative Expression (RE) over the infection cycle in all conditions in both isolates, a pattern which is largely unaffected by fungicide treatment (Figure 8). Mgc14f08f has close homology (61% identity) to the *Zygosaccharomyces bailii* protein Ffz1*,* a hexose transporter specifically involved in the facilitation of fructose (Pina *et al.*, 2004). Relative expression of *Mga1230f* in IPO323 is increased in the presence of the fungicide (Figure 9). Blast homology searches reveal that Mga1230f has close homology to the *Aspergillus fumigatus* protein AfuMdr2, the expression of which is induced in the presence of itraconazole and has an impact on fungicide sensitivity (Ferreira *et al.*, 2004). *MgAtr1* was previously shown to increase tolerance to cyproconazole when overexpressed in *S. cerevisiae* (Stergiopoulus *et al.*, 2002b).

However this gene, along with the four other previously characterised ABC transporters (*MgAtr2-5*) showed no significant changes in relative expression that could be correlated with previous reports of function. For example, *MgAtr4* is known to be involved in colonisation of the sub-stomatal cavity but relative expression of *MgAtr4* does not reflect this function. No individual gene was constitutively overexpressed to the extent that has been observed *in vitro* in triazole resistant human pathogenic fungi, for example fluconazole resistant *C. albicans* isolates which commonly have 100-fold increases in relative expression of *CDR1* when compared to wild type isolates (Prasad *et al.*, 1995; Gaur *et al.*, 2005; De Waard *et al.*, 2006).

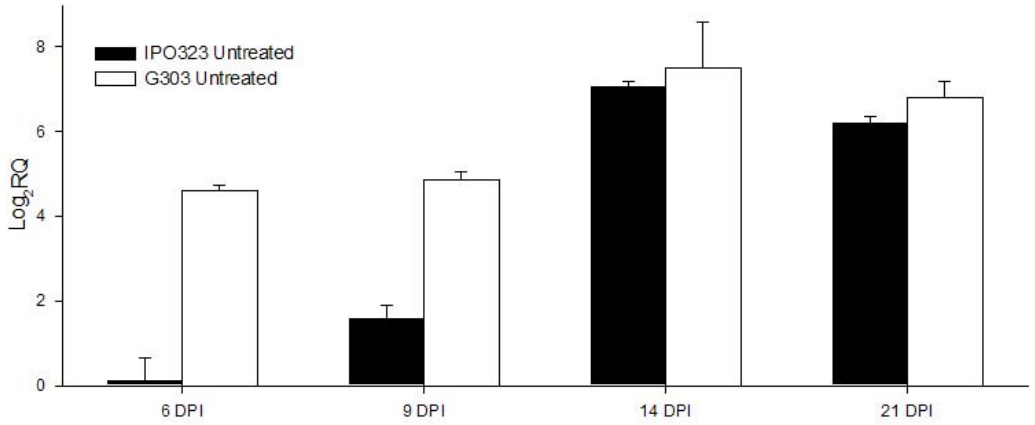

Figure 8. *Mgc14f08f* gene expression in both isolates in the absence of epoxiconazole. There is a clear pattern of increasing relative expression toward later stages of infection.

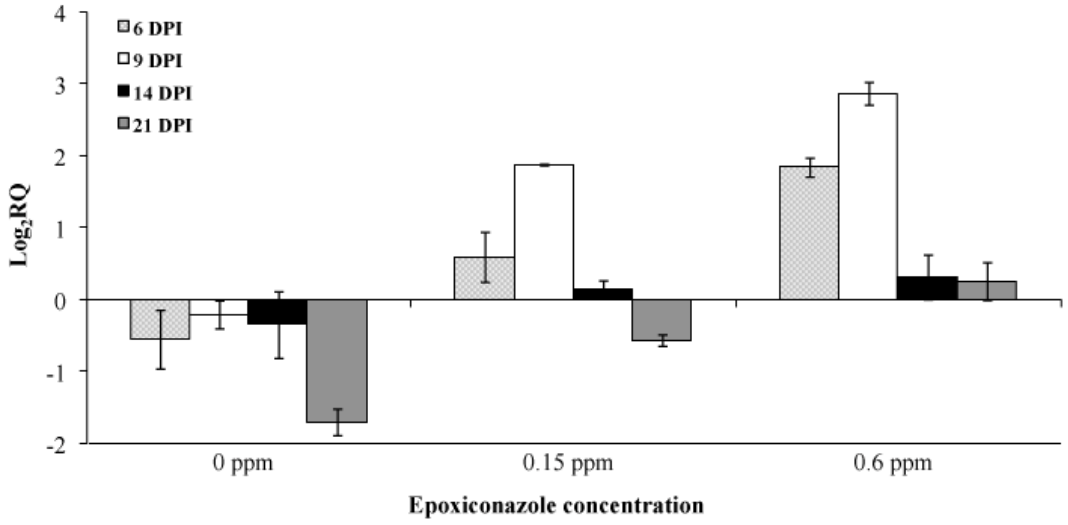

Figure 9. *Mga1230f* gene expression in IPO323 over the course of infection on treated and untreated leaves. Relative expression is up in the presence of epoxiconazole in IPO323.

Hierarchical clustering

Clustering shows that *Mg[0925]* and *Mga1230f* have similar expression patterns in all situations, in both IPO323 and G303 (Figure 10). Mg[0925] and Mga1230f are both MDR type ABC-transporters and this relationship in expression demonstrates the only significant link between transporter sub-families defined by structure and patterns of expression. The PDR family of ABC-transporters, the largest sub-family studied, demonstrated a large range of relative expression values that did not correlate significantly. IPO323 has one large cluster of genes and smaller groups according to expression. G303 has thee clear clusters but these clusters do not appear to relate to transporter structure (as derived through homology searches) except for the previously mentioned Mg[0925] and Mga1230f. Expression of many IPO323 genes is clearly more affected by the epoxiconazole than the G303 equivalent, and expression varies as much between treatments as it does between time-points. The clustering in G303 gives only three clusters of more than three genes that are related in expression pattern. Overall expression appears to be affected less by the fungicide and more by the point in the infection cycle. Groupings focus around genes that are expressed at specific times during infection, for example group containing *Mga0755f* in G303 shows genes expressed at 6 dpi, the group containing *MgAtr2* contains genes expressed 9-14 dpi and group containing *Mg[0212]* contains genes expressed at 14-21 dpi These groupings may indicate genes in G303 that are involved in the various stages of infection.

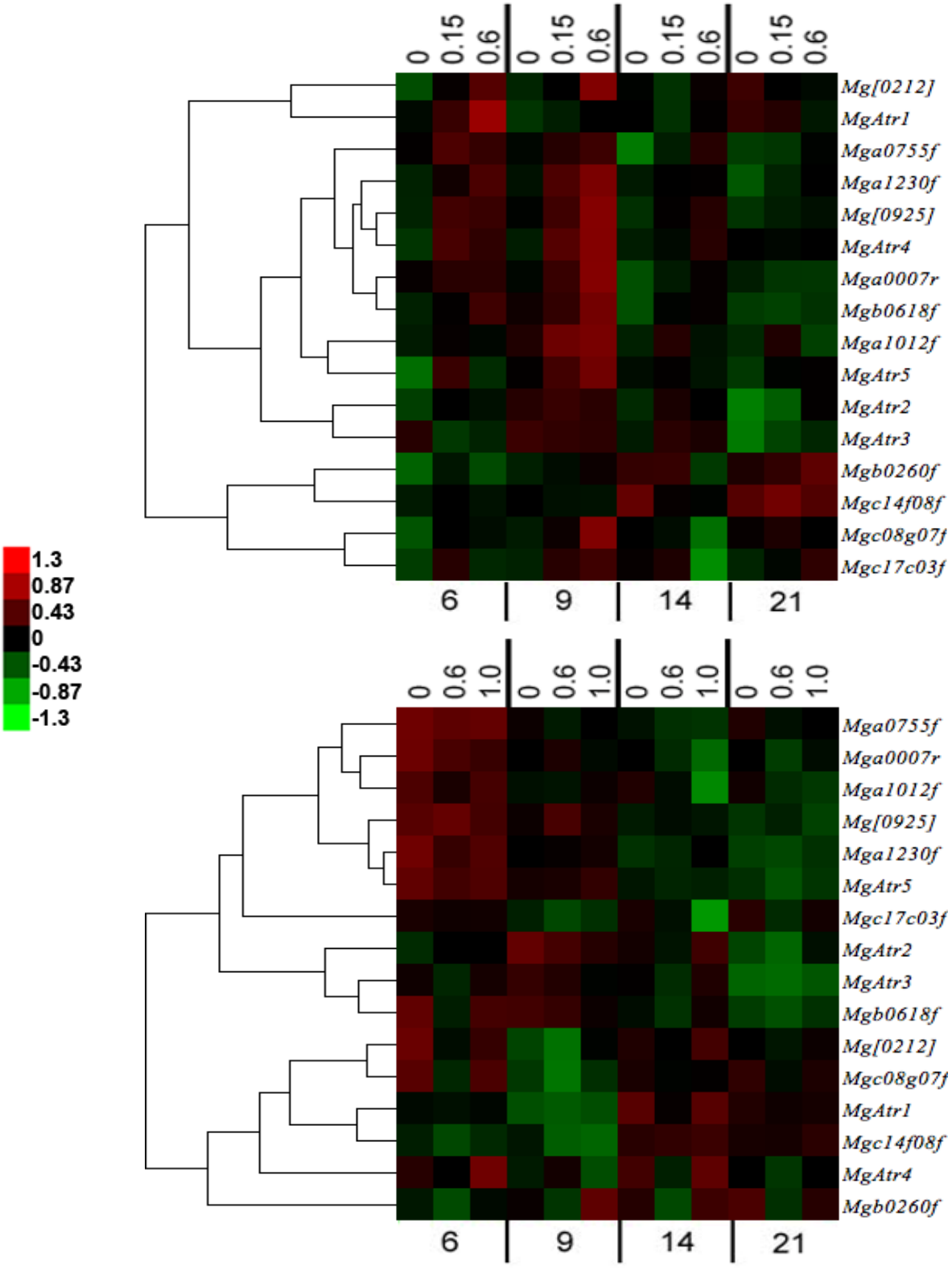

Figure 10. Hierarchically clustered IPO323 (top) and G303 gene expression, arranged by days post inoculation and treatment structure. Colour bar represents normalized expression levels (log_2)

Sterol content analysis

GC-MS was successfully performed with undiluted total lipid extract. Ergosterol, lanosterol, cholesterol and dihydrocholesterol were identified with authentic standards. Ergosta-7,22-dienol, fungisterol, epiergosterol, 4,4-dimethylfecosterol and eburicol were identifiable with retention time data and mass spectra published or present in the NIST mass spectrometry database. However, due to lack of comparisons, some sterols could not be identified with certainty. These are designated as unknown sterols 1- 5. Unknowns 3, 4 and 5 are likely to be methylated sterols, with a characteristically stronger retention than desmethyl equivalents (Table 8, Joseph-Horne *et al.*, 1996)

Table 8. Putative designation of sterols, through relative retention times and authentic samples.

GC Peak	Retention Time	RRt[a] Moretti *et al*. (2003)	RRt[a] Loeffler *et al*. (1990)	RRt[a] Barrero *et al.* (1998)	Designation
1	24.89	-	-	-	Unknown 1
2	25.21	-	-	1	Cholesterol[b]
3	25.32	1	1	-	Dihydrocholesterol[b,c]
4	25.68	-	-	-	Ergosterol isomer
5	25.91	-	-	-	Non-Sterol
6	25.98	1.19	1.096	1.12	Ergosterol[b]
7	26.12	1.23	1.117	1.155	Ergosta-7,22-dienol
8	26.49	1.31	-	-	Unknown 2
9	26.61	-	1.184	1.25	Episterol
10	26.68	1.38	-	1.259	Fungisterol
11	26.92	-	-	1.298	Lanosterol[b]
12	27.57	1.62	1.313	1.422	Eburicol
13	27.84	1.7	1.344	1.495	4,4-dimethylfecosterol
14	28.23	-	-	-	Unknown 3
15	29.40	-	-	-	Unknown 4
16	30.20	-	-	-	Unknown 5
	R^2	0.9991	0.9993	0.9962	

a - Relative retention time of the TMS derivative with respect to the TMS-ether of dihycrocholesterol or cholesterol
b - Identified with authentic sample
c - Dihydrocholesterol (containing low levels of cholesterol) used as surrogate standard

Sterol content of epoxiconazole treated isolates

Total sterol content was measured in four isolates (Table 9), untreated and treated with 1 µg/mL epoxiconazole for 24 hours. ANOVA revealed significant differences in the levels of individual sterols between treatments and between isolates (Table 9). Significantly less ergosterol was detected in treated cells of G303, SMR1 and R6-17 but not IPO323. Significantly less epiergosterol was present in G303 and R6-17. There was also a significant increase in the quantity of eburicol in treated isolates and reduced levels of 14α-desmethyl sterol intermediates ergosta-7,22-dienol, fungisterol and 4,4-dimethylfecosterol. Increased levels of unknown sterols 4, 5 and 6 in epoxiconazole treated samples add further evidence to the hypotheses that these are 14α-methylated sterols.

In untreated samples, the sterol profile of R6-17 differed most from IPO323, with increased levels of 14α-methylated sterols, including 25-fold more eburicol, and lower levels of desmethyl sterols. G303 also had more eburicol and less desmethyl sterols than IPO323. Sterol content of isolate SMR1 differed least to IPO323.

Sterol content according to *CYP51* sequence

Previous experiments suggested alterations in CYP51 structure (predicted through sequence variation) may alter enzyme activity, as indicated by higher eburicol levels in isolates with CYP51 alterations. Sterol content of isolates carrying eight different CYP51 variants was analysed for qualitative and quantitative variation (Table 7-2).

Quantitative differences in the level of different sterols were detected, although these could not be directly related to CYP51 sequence. For example eburicol content differed slightly between isolates, but it was not possible to associate levels with specific CYP51 variants. There were no significant differences in ergosterol content between isolates, suggesting all isolates are able to produce functional levels of ergosterol *in vitro* (Table 10).

There were no differences in the type of sterols present, suggesting that sterol biosynthesis occurs in the same way in each of these isolates (Table 7-7). As in earlier studies by Joseph-Horne *et al*, (1996), 14α-methylfecosterol or 14α-methyl-3,6-diol were not detected in any isolate.

Table 9. Sterol content of isolates treated and untreated.

		Sterol content (mg/g)[b]							
	GC	Untreated				Treated (1µg/mL epoxiconazole)			
Sterol	Peak	IPO323	G303	SMR1	R6-17	IPO323	G303	SMR1	R6-17
Unknown sterol 1	1	0.077	0.063	0.064	0.07	0.058[d]	0.036[d]	0.053[d]	0.032
Cholesterol[c]	3	0.017	0.017	0.014	0.015	0.017	0.015	0.015	0.015
Ergosterol isomer	4	1.678	1.807	1.564	1.416	1.452	1.089[d]	1.285	0.816[d]
Ergosterol	6	2.004	2.014	1.834	1.716	1.804	1.32[d]	1.624[d]	0.949[d]
Ergosta-7,22-dienol	7	0.361	0.129	0.251	0.11	0.194	0.063[d]	0.157	0.027[d]
Unknown sterol 2	8	0.123	0.078	0.126	0.063[e]	0.013[d]	0.018[d]	0.019[d]	0.005
Episterol	9	0.456	0.143[e]	0.33[e]	0.069[e]	0.118	0.016	0.114	0.004
Fungisterol	10	0.31	0.07[e]	0.181[e]	0.042[e]	0.083[d]	0.008[d]	0.065[d]	0.001
Lanosterol	11	0.179	0.439[e]	0.27	0.261	0.165	0.403	0.189	0.355
Eburicol	12	0.037	0.543[e]	0.202	0.921[e]	1.576[d]	2.466[d]	1.6[d]	2.261[d]
4,4-dimethylfecosterol	13	1.13E-03	3.74E-04[e]	3.65E-04[e]	1.38E-04[e]	[a]	[a]	[a]	[a]
Unknown sterol 3	14	[a]	0.001	[a]	0.001[e]	0.002[d]	0.004[d]	0.002[d]	0.004[d]
Unknown sterol 4	15	[a]	0.004	[a]	0.012	0.032[d]	0.11[d]	0.045[d]	0.118[d]
Unknown sterol 5	16	[a]	0.094	0.034	0.181[e]	0.272[d]	0.445[d]	0.283[d]	0.447[d]

[a] - sterol below limits of detection
[b] - milligrams sterol per gram fungal biomass
[c] - dihydrocholesterol used as surrogate standard, in which cholesterol occurs in low quantities
[d] - significant difference between treatments ($p<0.05$)
[e] - significantly different to IPO323 untreated ($p<0.05$)

Table 10. Isolate sterol content

	Sterol content (mg/g)[b]												
Sterol	Berdun 17	IPO323	Berdun 10	Berdun 4	Berdun 13	R6-31	103.9	115.3	209.10	209.9	212.2	202.1	214.7
Unknown sterol 1	0.018	0.042 [d]	0.029	0.016	0.046[d]	0.019	0.019	0.023	0.017	0.018	0.019	0.018	0.015
Cholesterol[c]	0.015	0.016	0.013	0.016	0.005[d]	0.017	0.014	0.015	0.016	0.015	0.015	0.015	0.016
Ergosterol isomer	1.152 [d]	0.883	0.919	1.205[d]	0.997	0.819	0.667[d]	0.893	0.785	0.844	0.778	0.856	0.930
Ergosterol	1.105	1.007	0.929	1.056	1.012	0.955	0.784	1.047	0.819	0.934	1.010	1.107	0.881
Ergosta-7,22-dienol	0.096	0.090	0.078	0.084	0.066	0.116[d]	0.049	0.074	0.109	0.048	0.053	0.049	0.089
Unknown sterol 2	0.047 [d]	0.041	0.029	0.050[d]	0.032	0.020[d]	0.020	0.029	0.024	0.018[d]	0.023	0.051[d]	0.033
Episterol	0.288 [d]	0.172	0.223[d]	0.264[d]	0.168	0.094	0.011[d]	0.264[d]	0.210	0.038[d]	0.050[d]	0.074[d]	0.155
Fungisterol	0.117	0.111	0.120	0.100	0.094	0.060	0.014[d]	0.121[d]	0.104	0.027[d]	0.026[d]	0.049	0.093
Lanosterol	4.22E-03	0.116	1.50E-03	0.003	0.157[d]	0.006	0.028	0.148[d]	0.139[d]	0.028	0.033	0.020	0.003
Eburicol	3.50E-03	0.034	0.004	0.042	0.256	0.067	0.079	0.195	0.036	0.149	0.278	0.060	0.017
4,4-dimethylfecosterol	[a]	9.58E-04 [d]	[a]	[a]	[a]	[a]	[a]	7.10E-04[d]	[a]	[a]	[a]	[a]	1.74E-04
Unknown sterol 3	[a]	[a]	[a]	[a]	4.34E-04	[a]	[a]	[a]	[a]	1.68E-04[d]	5.60E-04[d]	[a]	[a]
Unknown sterol 4	[a]	[a]	[a]	[a]	[a]	[a]	[a]	[a]	[a]	0.011	8.67E-03	[a]	[a]
Unknown sterol 5	[a]	[a]	[a]	[a]	0.039	0.015	[a]	0.041	[a]	0.014	0.060	[a]	[a]

[a] - sterol below limits of detection

[b] - milligrams sterol per gram fungal biomass

[c] - dihydrocholesterol used as surrogate standard, in which cholesterol occurs in low quantities

[d] - significantly different from the mean ($p<0.05$)

CYP51 expression

CYP51 gene expression was analysed in each untreated isolate at the same time as sterol content. *CYP51* transcript was detected in all isolates, with the lowest level detected in IPO323 to which relative expression in all other isolates was calibrated. Isolates 103.9 and 209.9 had the most *CYP51* transcript (Table 11). *CYP51* expression was not correlated with levels of the substrate eburicol (data not shown). An indicator of CYP51 activity was defined as 1/CxE (C = CYP51 expression, E = Eburicol content). Thus if CYP51 expression is low but eburicol is also low, the small amount of CYP51 must be converting the eburicol efficiently so 1/CxE is high. Table 11 suggests that isolates may group according CYP51 sequence. Wild type CYP51 alleles have the greater mean efficiency, followed by variants with only L50S, then G460D. Other CYP51 proteins have lower levels of efficiency. The lowest CYP51 efficiency was in predicted proteins harbouring A136. Isolates 202.1 and 214.7 have more CYP51 alterations than other isolates but CYP51 has greater predicted activity in these isolates than others. This may be because the changes are less damaging or because some of these alterations increase function to compensate for reductions by others (Table 12)

Table 11. Grouping of isolates by 1/(C x E).

Isolate	CYP51 alterations	Eburicol content (mg/g)[a]	*CYP51* expression[b]	1/(C x E)
Berdun 17	None	3.5E-03	2.59	110.23
IPO323	None	0.034	1	29.02
Berdun 10	S50	4.3E-03	3.54	65.61
Berdun 4	D460	0.042	1.48	16.21
Berdun 13	Y137F	0.256	1.34	2.91
R6-31	Y137F	0.067	5.12	2.93
115.3	S50, V381, H461	0.195	4.37	1.17
209.10	S50, V381, H461	0.036	15.71	1.77
103.9	S50, A136, H461	0.079	23.07	0.55
209.9	A136, N188, ΔY459/G460	0.149	26.2	0.26
212.2	A136, N188, ΔY459/G460	0.278	5.84	0.62
202.1	S50, N188, G379, V381, ΔY459/G460, K513	0.06	6.78	2.46
214.7	S50, N188, G379, V381, ΔY459/G460, K513	0.017	15.08	3.91

[a] - milligrams sterol per gram fungal biomass
[b] - relative to IPO323 untreated
[c] - C denotes CYP51 expression, E denotes eburicol content

Table 12. Impact of individual CYP51 alterations on 1/(C x E).

CYP51Alteration	Impact on activity	Reasoning
S50	None	Occurs in isolate with wild type activity
A136	Severe	Occurs in all isolates with severe disruption of activity
F137	Moderate	Occurs in all isolates with moderate disruption of activity
N188	Little or none	-
G379	Compensator	Only alteration which could be a compensatory mutation
V381	Moderate	Occurs in all isolates with moderate disruption of activity
ΔY459/G460	Little or none	-
H461	Little or none	-

Discussion

Mycosphaerella graminicola is the most economically important fungal pathogen of wheat in the UK and North West Europe. Since the escalation of disease levels in the 1980's (Bearchell *et al.*, 2005), scientific and agronomic interest in *M. graminicola* has grown. Over the same period multiple methods of disease control, including host based resistance, protectant and systemic fungicides have been developed, and many of these have been used against *M. graminicola* (Russell, 2005). This fungus however, has developed resistance to several major fungicide groups and continues to be a problem in wheat production. It is therefore surprising that in 2008, more than two decades on from the introduction of triadimefon, azole fungicides are still the primary method of controlling *M. graminicola*. Recently, examples where azole based spray programmes are failing to provide effective control against *M. graminicola* are increasing (Clark, 2006; Defra, 2007). It is crucial that the mechanisms responsible for azole resistance in *M. graminicola* are studied, to reveal not only reasons why azole efficacy is deteriorating but perhaps also to understand why the chemistry has, in the main, remained effective for so long.

Azole fungicides prevent ergosterol biosynthesis through inhibition of the eburicol-14α-demethylase (CYP51). In *S. cerevisiae*, *C. albicans* and other fungi this inhibition results in reduced ergosterol biosynthesis alongside accumulation of non-functional sterols and the toxic sterol derivative 14α-methyl-3,6-diol (Kelly *et al.*, 1995). Sterol content of *M. graminicola* confirmed the results of Joseph-Horne *et al* (1996) that treatment with epoxiconazole prevents ergosterol biosynthesis but, contrary to the situation in yeast, this results in the accumulation of eburicol and its precursors, not toxic sterol-derivatives. *M. graminicola* CYP51 has clearly been evolving as a response to azole fungicide use, with novel amino acid alterations appearing and accumulating in populations (Fraaije *et al.*, 2007; Leroux *et al.*, 2007). Analysis of sterol content showed higher levels of the sterol intermediate eburicol in resistant isolate R6-17 than in wild type isolates. As R6-17 has an altered CYP51 amino acid sequence, it was hypothesised that the increased level of eburicol in this isolate was caused by a reduction in CYP51 activity, as previously observed in isolates of *Ustilago maydis* (Joseph-Horne *et al.*, 1995). To test this theory, sterol content of wild type-isolates was determined alongside 11 isolates with different combinations of CYP51 alterations. Isolates with the same CYP51 variant did not directly group according to levels of eburicol. However, when *CYP51* expression was taken into account isolates could be grouped together according to their CYP51 variant. The exact formula used

(the inverse of *CYP51* transcript level multiplied by eburicol content) gives an estimate of CYP51 efficiency. It was demonstrated that the wild-type enzyme and those harbouring only substitution L50S have the highest efficiency. Conversely, CYP51 variants harbouring V136A had the lowest efficiency. The impact of further alterations on enzyme activity proved difficult to dissect as they exist as combinations, but it was possible to identify that A379G and N513K are likely to be compensatory alterations that reduce the loss of function caused by other alterations.

In *M. graminicola* the alteration I381V dramatically reduces sensitivity to tebuconazole (Fraaije *et al.*, 2007) and, as shown in this study, partially to epoxiconazole Amino acid 381 has equivalent residues in the structurally characterised *Mycobacterium tuberculosis* CYP51. The residue exists within 4 Å of bound substrate and is conserved in all known CYP51's as either leucine or isoleucine (Podust *et al.*, 2001; Podust *et al.*, 2004). I381V in *M. graminicola* is therefore a unique substitution at this residue. It may be the case that the mutations found only in *M. graminicola*, such as I381V, reduce CYP51 activity to a much larger extent than any reported in *C. albicans*. *M. graminicola* can cope with a reduction in activity because eburicol is not converted to the toxic 14α-methyl-3,6-diol. The equivalent reduction in CYP51 activity in *C. albican*s would have a larger fitness penalty because the sterol intermediates resulting from low CYP51 activity would be converted to toxic derivatives. This may explain why perhaps equivalent alterations have not been observed in these two species.

Accumulation of alterations in CYP51 certainly accounts for some of the recent decline in azole efficacy but unexplained variation in the *in vitro* sensitivity of individual isolates to azoles remains. Some isolates which harbour the same CYP51 variants differ in sensitivity. This must be caused by other resistance mechanisms acting in combination with CYP51 amino acid alterations. In *C. albicans,* overexpression of *CDR1* and/or *CaMDR1* are the most commonly observed mechanisms of resistance to fluconazole. Proteins from either the major facilitator, or ATP-binding cassette transporter superfamilies can actively export azoles from the cell, reducing intracellular accumulation and increasing resistance levels. *M. graminicola* has both ABC transporter and MFS transporter superfamilies. Some of these genes, including *MgAtr1* and *MgMfs1*, have been studied previously and shown capable of reducing cellular accumulation of cyproconazole (Zwiers *et al.*, 2000; Roohparvar *et al.*, 2007). *M. graminicola* therefore, has the cellular apparatus required to reduce accumulation of azoles. Consequently, this resistance mechanism investigated in one wild type

isolate and three isolates with reduced epoxiconazole sensitivity. Expression of these genes was studied both *in vitro* and *in planta* in the presence and absence of epoxiconazole. None of the 16 transporter genes studied in three resistant and one sensitive isolate was overexpressed to the extent observed in *C. albicans*. The lack of distinct up-regulation of a single gene may be explained by the fact that this selection of genes studied only represents a small proportion of all the transporters in the genome. However, it is also possible that the variation in resistance is caused by the accumulated effect of the products of these genes, in which case the impact of individual transporters would be difficult to dissect. In isolate G303, seven transporter genes were up-regulated in the presence of epoxiconazole, a change that could conceivably alter fungicide sensitivity. The findings in this project concur with a study of *Phytophthora infestans,* where expression of 54 transporter genes could not be correlated with fungicide sensitivity (Judelson *et al.*, 2006). It should also be noted that other plant pathogens, such as *Magnaporthe grisea* and *Botrytis cinerea,* have transporters able to reduce accumulation of fungicides but these have not reported as a cause of fungicide resistance (Urban *et al.*, 1999; Vermeulen *et al.*, 2001; Lee *et al.*, 2005; Bao Sun *et al.*, 2006).

Expression of the 16 transporter genes, as measured *in planta*, also proved to be inconclusive in defining differences in fungicide sensitivity but did raise an interesting point of discussion. Expression profiles of the transporter genes appeared to be more affected by the point in the infection process than by the presence or absence of epoxiconazole. Comparing *in vitro* and *in planta* expression data shows that transporter gene expression *in planta* is more regulated than *in vitro*. This suggests that transporter genes play a more important role in infection than in reducing fungicide sensitivity, and may partly explain why this is an uncommon resistance mechanism in plant pathogenic fungi. For example MFS transporter *Mgc1408f* showed a pattern of expression that suggested a role in the uptake of hexose sugars when they become available later in the infection cycle. It is also likely that any variation in resistance *in vitro* caused by variation in transporter gene expression may not operate *in planta*. If this is the case, it suggests either a fitness penalty that is not evident *in vitro* or a gain of function *in vitro* that cannot exist *in planta*. The most likely is the gain of function *in vitro,* where much more energy is available for consistent action of these enzymes. The fact that resistance through transporter gene overexpression has rarely been encountered in field isolates of plant pathogenic fungus suggests a fundamental difference between human and plant pathogens.

With continual monitoring of fungicide resistance it is possible to identify and study reductions in fungicide sensitivity that cause practical loss of disease control. Alongside this monitoring, molecular diagnostics and biochemical analysis can be used to study the biological mechanisms through which resistance develops. As a continuation of the work described in this thesis it will be possible to use biochemical analysis of enzyme function, to further dissect the impact of alterations in CYP51, and understand their effect on both fungicide binding and on enzyme function. Alterations can be studied in their natural combinations, by directly cloning cDNA from isolates harbouring the desired variant, or individual alterations can be assessed through site-directed mutagenesis of wild type *CYP51.* When the variant gene is isolated, it can be cloned into an expression vector and transformed into a strain of yeast containing a repressible endogenous *CYP51*. Once transformed, variation in fungicide sensitivity between yeast harbouring different CYP51 variants can be assessed and, protein can be purified for substrate and fungicide binding assays. In my view, work in this area holds the key to understanding why azole fungicides are the key method of controlling *Mycosphaerella graminicola* in spite of over two decades of evolution towards resistance.

Conclusions and knowledge transfer

There has been considerable industry interest in the project due to the concerns over declining performance of some azole fungicides against Septoria. Work in the project, complementary to other ongoing research at Rothamsted, has helped to clarify the mechanisms of insensitivity in the fungus, but also shown that the mechanisms identified to date do not confer any extreme phenotypes that might compromise the performance of the currently most effective azoles. This has on the one hand raised awareness of the nature of the problem, but at the same time dispelled fears that azoles might suffer the same catastrophic loss of efficacy that was seen with the QoIs.

Project outputs

Information from the project has been widely disseminated via scientific papers and posters (see below), presentations at conferences and agricultural shows (eg Cereals) as well as HGCA events. Relevant information arising from the project was also channelled through an existing LINK consortium involving agricultural consultancies (Velcourt and ADAS), PSD (Defra), Scottish Agricultural Colleges, and four of the main agrochemical industries (BASF, Bayer Crop Science, Du Pont, and Syngenta). BASF

were formally involved in the project as a CASE partner. Aspects of the work were also included in presentations made by members of the Fungicide Resistance Group at Rothamsted to AICC and the Rothamsted Research Association.

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