RESEARCH LETTER

Sterol content analysis suggests altered eburicol 14α-demethylase (CYP51) activity in isolates of *Mycosphaerella graminicola* adapted to azole fungicides

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Received 5 January 2009; accepted 30 April 2009.
Final version published online 20 May 2009.

DOI:10.1111/j.1574-6968.2009.01645.x

Editor: Holger Deising

Keywords
Septoria tritici; azole fungicide; eburicol 14α-demethylase; CYP51; sterol content.

Abstract

The recent decline in the effectiveness of some azole fungicides in controlling the wheat pathogen *Mycosphaerella graminicola* has been associated with mutations in the *CYP51* gene encoding the azole target, the eburicol 14α-demethylase (CYP51), an essential enzyme of the ergosterol biosynthesis pathway. In this study, analysis of the sterol content of *M. graminicola* isolates carrying different variants of the *CYP51* gene has revealed quantitative differences in sterol intermediates, particularly the CYP51 substrate eburicol. Together with *CYP51* gene expression studies, these data suggest that mutations in the *CYP51* gene impact on the activity of the CYP51 protein.

Introduction

Azole (imidazole and triazole) fungicides interrupt biosynthesis of ergosterol, an essential sterol of fungal cell membranes, by inhibiting the cytochrome P450 eburicol 14α-demethylase (CYP51) (Yoshida & Aoyama, 1987). This inhibition prevents demethylation of eburicol, the principal CYP51 substrate in most filamentous fungi, resulting in a depletion of ergosterol and a concomitant accumulation of nonfunctional 14α-methyl sterols, including eburicol. The accumulation of 14α-methyl sterols and the depletion of ergosterol itself, perturbs membrane function and prevents further fungal growth.

Azoles have been the leading agents for the control of fungal pathogens of humans, animals and plants for over 30 years. Resistance to this class of chemistry is now common in human pathogens, including *Candida* spp. (Kontoyiannis & Lewis, 2002) and *Aspergillus fumigatus* (Warnock *et al.*, 1999), and is a serious problem in clinical antifungal therapy. As a consequence, the molecular basis of azole resistance is best understood in these pathogens. Common mechanisms of azole resistance in *Candida albicans* are alteration of the CYP51 target protein (Sanglard *et al.*, 1998; Marichal *et al.*, 1999) and enhanced efflux of azole outside the cell mediated by the over expression of energy-dependent efflux proteins (Sanglard *et al.*, 1995; Perea *et al.*, 2001). Altered ergosterol biosynthesis, although a less common mechanism of azole resistance, has been reported for *C. albicans* (Nolte *et al.*, 1997). These isolates, cross resistant to fluconazole and amphotericin B, have reduced levels of ergosterol and accumulate sterols lacking a $C^{5(6)}$ double bond, a result of null mutations in the gene (*erg3*) encoding the sterol $C^{5(6)}$ desaturase (Kelly *et al.*, 1995, 1996). However, these isolates grew poorly *in vivo* (Nolte *et al.*, 1997), probably as a result of using 14α-methylated sterols rather than ergosterol in cell membrane synthesis.

Azole resistance in plant pathogens has developed more slowly, and rarely leads to a loss of field efficacy. Recently,

however, there has been a notable decline in the effectiveness of a number of azoles in controlling *Mycosphaerella graminicola* so that some compounds no longer control the disease (Clark, 2006). This fungus causes *Septoria* leaf blotch, the most economically important foliar disease of winter wheat in North West Europe. Studies suggest alterations in the target protein, caused by mutations in the *CYP51* gene, are responsible for this recent decline in sensitivity in *M. graminicola* populations (Cools & Fraaije, 2008). Some CYP51 substitutions identified in *M. graminicola* isolates, less sensitive to azoles, are at equivalent residues to those found in resistant strains of *C. albicans*, for example Y137F (Cools *et al.*, 2005; Leroux *et al.*, 2007), whereas others, including V136A (Leroux *et al.*, 2007), A379G (Leroux *et al.*, 2007) and I381V (Cools *et al.*, 2005; Fraaije *et al.*, 2007; Leroux *et al.*, 2007) are unique to *M. graminicola*.

While studies have demonstrated clear correlations between CYP51 alterations and altered azole sensitivity in *M. graminicola* (Fraaije *et al.*, 2007; Leroux *et al.*, 2007), the effects of these changes on azole affinity or CYP51 activity have yet to be elucidated. In this study, we established the impact of alterations in the CYP51 protein of *M. graminicola* on sterol biosynthesis by analysing the sterol content of *M. graminicola* field isolates carrying different variants of the *CYP51* gene.

Materials and methods

Culturing of *M. graminicola* isolates

Isolates (Table 1) were grown in triplicate on yeast peptone dextrose (YPD) agar (ForMedium, Norwich, UK) at 15 °C for 7 days. Spores from each culture were harvested and diluted to a concentration of 4.5×10^5 spores mL^{-1} in 100 mL YPD broth (ForMedium) and incubated for 72 h at 21 °C, with shaking at 220 r.p.m. Replicate fungal cultures were collected by vacuum filtration through 8-μM pore nitrocellulose filters (Millipore, Billerica), snap frozen in liquid nitrogen and freeze dried before free sterol and RNA extractions.

Azole sensitivity testing

Sensitivity assays were modified from Fraaije *et al.* (2007). A 100-μL aliquot of 2 × potato dextrose broth (PDB; Sigma-Aldrich Inc., St. Louis) amended with decreasing concentrations of epoxiconazole (50, 16.7, 5.6, 1.9, 0.62, 0.21, 0.069, 0.023, 0.008, 0.0025 and 0.0008 mg L^{-1}), tebuconazole (50, 20, 8, 3.2, 1.3, 0.51, 0.2, 0.082, 0.033, 0.013 and 0.0052 mg L^{-1}) or prochloraz (10, 3.3, 1.1, 0.37, 0.12, 0.041, 0.014, 0.0046, 0.0015, 0.0005 and 0.00017 mg L^{-1}) was added to wells of flat-bottomed microtitre plates (TPP 92696 test plates, Trasadingen, Switzerland). After 7 days of growth at 15 °C on YPD to ensure yeast-like growth, isolates were suspended in 5 mL of sterile distilled water. Aliquots of 100 μL of isolate spore suspensions (1×10^5 spores mL^{-1}) were added to each well. Plates were incubated for 4 days at 23 °C, and growth measured at $A_{630\,nm}$ using a Fluorstar Optima microplate reader (BMG Labtech GmbH, Offenberg, Germany). Fungicide sensitivities were determined as 50% effective concentration (EC_{50}) using a dose–response relationship.

CYP51 sequencing

CYP51 genes of *M. graminicola* isolates IPO323, Berdun 17, R6-31, Berdun 13, Berdun 10, Berdun 4, V103.9, V115.3, V209.10, V209.9, V212.2, V214.7 and V202.1 (Table 1; NCBI GenBank accessions: FJ196446–FJ196457) were amplified and sequenced using primers, PCR conditions and protocols

Table 1. Isolates of *Mycosphaerella graminicola* used for sterol content analysis

		Mean EC_{50} (mg L^{-1})			
Isolate designation	Location and year of isolation	Epoxiconazole	Tebuconazole	Prochloraz	CYP51 alteration(s)*
IPO323	North Brabant, the Netherlands; 1981	0.071	0.491	0.382	None (wild type)
Berdun 17	Aragon, Spain; 2006	0.066	0.319	0.097	None (wild type)
R6-31	Hertfordshire, UK; 2006	0.958	1.36	1.86	Y137F
Berdun 13	Aragon, Spain; 2006	ND	ND	ND	Y137F
Berdun 10	Aragon, Spain; 2006	0.081	0.273	0.121	L50S
Berdun 4	Aragon, Spain; 2006	0.121	0.562	0.293	G460D
V103.9	Kent, UK; 2006	1.26	0.492	2.19	L50S, V136A, Y461H
V115.3	Kent, UK; 2006	0.817	4.12	0.704	L50S, I381V, Y461H
V209.10	Kent, UK; 2006	1.825	5.99	0.657	L50S, I381V, Y461H
V209.9	Kent, UK; 2006	0.735	0.712	2.48	V136A, S188N, ΔY459/G460
V212.2	Kent, UK; 2006	1.28	4.41	2.58	V136A, S188N, ΔY459/G460
V214.7	Kent, UK; 2006	1.66	9.17	0.107	L50S, S188N, A379G, I381V, ΔY459/G460, N513K
V202.1	Kent, UK; 2006	1.05	10.1	0.104	L50S, S188N, A379G, I381V, ΔY459/G460, N513K

*Based on predicted protein sequence for isolate ST1 (accession number AY730587).
ND, not determined.

outlined in Cools *et al.* (2005). Amplifications were carried out using Phusion high-fidelity DNA polymerase to ensure the validity of sequences (Finnzymes, Espoo, Finland).

Total sterol extraction and derivatization

Sterol extraction and analysis was adapted from Hey *et al.* (2006). Fifty milligrams of lyophilized tissue from each biological replicate was extracted with 5 mL of 2 : 1 (v/v) chloroform : methanol containing 50 μg dihydrocholesterol (internal standard) at 55 °C, for 30 min. The cooled mixture was filtered and the solvent removed under a stream of gaseous nitrogen. To the lipid residue obtained, 1 mL toluene and 2 mL sodium methoxide (0.5 M) in methanol were added and the sample heated at 60 °C for 30 min. Once cooled, 2 mL of 14% boron trifluoride in methanol was added and the sample heated at 60 °C for a further 10 min. Once cooled, 2–3 mL diethyl ether and 5 mL deionized water were added. The ether layer was removed to a clean vial and the aqueous phase re-extracted with a further 2–3 mL diethyl ether and both extracts combined. After backwashing with 5 mL water, the organic phase was then dried, overnight, over anhydrous sodium sulphate and filtered through glass wool. The filtrate was collected in a fresh vial and the solvent was removed under a stream of gaseous nitrogen. The dry residue was resuspended in 300 μL toluene and silylated with 200 μL *N*-methyl-*N*-(trimethylsilyl)trifluoroacetamide (MSTFA) at 50 °C for 10 min. All samples (isolates/treatment combinations) were prepared and analysed in triplicate. Reagents were obtained from Sigma-Aldrich (Gillingham, Dorset, UK) unless otherwise stated.

GC-MS analysis

GC-MS analysis was carried out on a Hewlett Packard (Palo Alto, CA) 5890 Series Gas Chromatograph coupled to a 5970 Series Mass Selective Detector and fitted with 7373 automatic liquid sampler and Zebron ZB5 column with 5 m Guardian (Phenomenex, Macclesfield, UK; 30 m × 0.25 mm internal diameter × 0.25 μm film). One-microlitre splitless injections were given at an inlet temperature of 280 °C and 105 kPa head pressure. The oven was set to an initial temperature of 100 °C for 2 min), then ramped at 10 °C min^{-1} to 320 °C and held for 6 min. The GC interface was 290 °C and 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^{-1} scan rate. Data analysis was conducted 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, NY). Peak areas were normalized to the internal standard.

To eliminate the contribution from coeluting hydrocarbons, reconstituted total ion chromatogram (TIC) peak areas were calculated by dividing the integrated extracted ion chromatogram peak areas of individual sterols by the quantification of the ion's contribution to the TIC, allowing direct comparison of the relative concentrations, within and between samples, to be made (equal reconstituted peak areas representing approximately equal concentrations).

For ergosterol, lanosterol, cholesterol and dihydrocholesterol, identifications were confirmed using authentic standards. For all other sterols, putative identifications were made on the basis of literature relative retention times combined with matching the experimentally obtained spectra to those in the National Institute of Standards and Technology (NIST) Spectral Library.

CYP51 expression

Levels of *CYP51* transcript were measured with real-time reverse transcription (RT)-PCR. Roughly 30-mg powdered freeze dried fungal material from each biological replicate 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 millilitre of TRIZOL reagent (Invitrogen, Paisley, UK) was then added and run through the FastPrep once again. TRIZOL was used according to the manufacturer's protocol, and further RNA purification was achieved with an overnight 4-M lithium chloride precipitation.

Reverse transcription of the first strand of cDNA was carried out with the Superscript III RT-PCR kit (Invitrogen) following the manufacturer's protocol using oligo d(T) primers and 5 μg total RNA per reaction. A one in 10 dilution of this reaction was used as the template for the real-time PCR analysis.

Real-time PCR reactions were carried out with Platinum SYBR green PCR mix with ROX (Invitrogen) and a modified version of the supplier's protocol, using 5 μL cDNA solution in a 25-μL reaction and CYP51-specific primers from Cools *et al.* (2007). The cycling conditions used were 50 °C for 2 min, 95 °C for 2 min, and then 40 replicates of 95 °C for 15 s, 58 °C for 30 s and 72 °C for 36 s. Each reaction was carried out in triplicate, in the ABI PRISM 7500 sequence detection system (Applied Biosystems, Foster City, CA). Relative quantification values were calculated with the $2^{-\Delta\Delta C_t}$ method using the constitutively expressed housekeeping β-tubulin gene as an endogenous control, and gene expression levels in the 'wild-type' isolate IPO323 untreated sample were used as calibrators. Transcript abundance was measured in triplicate for each isolate.

Statistical analysis

A linear model and ANOVA were used to estimate the effect of each amino acid alteration on the relative efficiency of the demethylation step calculated as the logarithm of the

product of eburicol content (*E*) and *CYP51* transcript abundance (*C*). Not all combinations of alterations were present. Therefore, it was possible to ascribe the variation in calculated enzyme efficiency to alterations in more than one way. The regression fit provided by all subsets of the mutations was used to determine economical descriptions of the data by effects of individual mutations. Model selection took into account Mallow's C_p, the proportion of the variance accounted for by the model and *F*-tests of the effect of deleting a factor from the model. Calculations were performed in GENSTAT v10 (VSN International, Oxford).

Results

Isolate azole sensitivities

Correlations between azole sensitivities and CYP51 alterations were consistent with previous studies (Cools *et al.*, 2005; Fraaije *et al.*, 2007; Leroux *et al.*, 2007). Isolates carrying substitution I381V were resistant to tebuconazole and considered sensitive to prochloraz, whereas isolates carrying substitution V136A were resistant to prochloraz and, with the exception of V212.2, more sensitive than isolates carrying I381V to tebuconazole. EC_{50} values for isolate R6-31, carrying substitution Y137F, were higher to all azoles than for wild-type isolates or those carrying L50S or G460D alone (Table 1).

Identification of sterols

Ergosterol, lanosterol, cholesterol and dihydrocholesterol were identified with authentic standards. Ergosta-7,22-dienol, fungisterol, episterol, 4,4-dimethylfecosterol and eburicol were identified according to the retention time and mass spectra data published or present in the NIST MS database (Table 2). However, due to lack of comparisons, other sterols could not be identified with certainty. These are designated as unknown sterols. It was possible to identify unknown sterols 1 and 2 as desmethyl sterols through MS (data not shown). Mass spectra of ergosterol and its stereoisomer have closely related fragmentation therefore the stereochemistry of the isomer is unknown. Studies by Joseph-Horne *et al.* (1996) of *M. graminicola* predicted the isomer to have an α-hydroxy moiety at C-3 rather than the usual β-hydroxy, and as no further evidence can be presented here, the same tentative designation is maintained.

Sterol content related to *CYP51* sequence

Quantitative differences in the level of different sterols were detected, particularly eburicol levels, although these could not be directly related to predicted CYP51 amino acid sequence. There were no significant differences in ergosterol content between isolates ($P = 0.05$), suggesting that all isolates are able to produce functional levels of ergosterol *in vitro* (Table 3).

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

CYP51 expression and relationship with eburicol content

Expression of *CYP51* was analysed from the same samples as sterol content. *CYP51* transcript was detected in all isolates, with the lowest level in IPO323 to which relative expression in all other isolates was calibrated. The highest levels of *CYP51* transcript were detected in isolates V103.9 and

Table 2. Designation of sterols by relative retention times and authentic samples

GC peak	Retention time	RRt* Moretti *et al.* (2003)	RRt* Loeffler & Hayes (1990)	RRt* Barrero *et al.* (1998)	Designation
1	24.89	–	–	–	Unknown 1
2	25.21	–	–	1	Cholesterol†
3	25.32	1	1	–	Dihydrocholesterol†,‡
4	25.68	–	–	–	Ergosterol isomer
5	25.91	–	–	–	Nonsterol
6	25.98	1.19	1.096	1.12	Ergosterol†
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†
12	27.57	1.62	1.313	1.422	Eburicol
	R^2	0.9991	0.9993	0.9962	

*RRt, relative retention time of the TMS derivative with respect to the TMS-ether of dihydrocholesterol or cholesterol.

†Identified with authentic sample.

‡Dihydrocholesterol (containing low levels of cholesterol) used as surrogate standard.

Table 3. Sterol content of *Mycosphaerella graminicola* isolates

	Isolate sterol content (mg g^{-1})											
Sterols ± SE ($n = 3$)	Berdun 17	IPO323	Berdun 10	Berdun 4	Berdun 13	R6-31	V103.9	V115.3	V209.10	V209.9	V212.2	V202.1	V214.7
Unknown sterol 1	0.018	0.042*	0.029	0.016	0.046*	0.019	0.019	0.023	0.017	0.018	0.019	0.018	0.015
	5.1E − 03	1.7E − 03	2.5E − 03	2.8E − 03	5.2E − 03	5.9E − 03	4.1E − 03	6.3E − 03	3.5E − 03	6.5E − 03	5.1E − 03	3.6E − 03	1.7E − 03
Cholesterol†	0.015	0.016	0.013	0.016	0.005*	0.017	0.014	0.015	0.016	0.015	0.015	0.015	0.016
	2.1E − 03	2.8E − 03	1.8E − 04	7.7E − 04	4.8E − 03	1.6E − 03	6.1E − 04	5.5E − 04	6.2E − 04	4.8E − 04	1.2E − 03	6.5E − 04	9.9E − 04
Ergosterol isomer	1.152*	0.883	0.919	1.205*	0.997	0.819	0.667*	0.893	0.785	0.844	0.778	0.856	0.93
	0.032	0.034	0.048	0.042	0.097	0.037	0.083	0.082	0.116	0.054	0.068	0.086	0.093
Ergosterol	1.105	1.007	0.929	1.056	1.012	0.955	0.784	1.047	0.819	0.934	1.01	1.107	0.881
	0.050	0.061	0.048	0.012	0.113	0.067	0.126	0.142	0.111	0.091	0.079	0.083	0.111
Ergosta-7,22-dienol	0.096	0.09	0.078	0.084	0.066	0.116*	0.049	0.074	0.109	0.048	0.053	0.049	0.089
	2.2E − 03	5.8E − 03	6.1E − 03	6.8E − 03	7.9E − 03	9.1E − 03	6.3E − 03	9.1E − 03	0.029	6.3E − 03	6.9E − 03	9.0E − 03	0.016
Unknown sterol 2	0.047*	0.041	0.029	0.050*	0.032	0.020*	0.02	0.029	0.024	0.018*	0.023	0.051*	0.033
	1.9E − 03	1.8E − 03	9.4E − 04	4.4E − 03	5.4E − 03	2.7E − 03	5.5E − 03	4.5E − 03	3.7E − 03	2.6E − 03	2.2E − 03	8.3E − 03	6.8E − 03
Episterol	0.288*	0.172	0.223*	0.264*	0.168	0.094	0.011*	0.264*	0.21	0.038*	0.050*	0.074*	0.155
	0.022	3.0E − 03	0.011	0.016	0.021	6.7E − 03	2.4E − 03	0.021	0.066	9.3E − 03	7.3E − 03	0.011	0.018
Fungisterol	0.117	0.111	0.12	0.1	0.094	0.06	0.014*	0.121*	0.104	0.027*	0.026*	0.049	0.093
	0.010	4.3E − 03	0.010	7.8E − 03	8.2E − 03	3.2E − 03	1.4E − 03	0.011	0.029	2.8E − 03	2.3E − 03	7.9E − 03	0.012
Lanosterol	4.22E − 03	0.116	1.50E − 03	0.003	0.157*	0.006	0.028	0.148*	0.13*	0.028	0.033	0.02	0.003
	4.4E − 04	0.011	7.8E − 04	4.8E − 04	0.034	1.8E − 03	7.9E − 03	0.088	0.039	9.2E − 03	9.6E − 03	4.1E − 03	2.7E − 04
Eburicol	3.50E − 03	0.034	0.004	0.042	0.256	0.067	0.079	0.195	0.036	0.149	0.278	0.06	0.017
	4.3E − 04	2.2E − 03	6.5E − 04	0.012	0.021	0.022	0.029	0.120	0.013	0.044	0.194	4.2E − 03	1.8E − 03

*Significantly different from the mean of all isolates ($P < 0.05$).
†Dihydrocholesterol used as surrogate standard, in which cholesterol occurs in low quantities.

V209.9 (Table 4). *CYP51* expression levels could not be directly linked to eburicol levels (Table 4).

Mycosphaerella graminicola isolates grouped according to their CYP51 variant efficiency $1/C \times E$ (where C = *CYP51* expression, E = eburicol content). Highest values were obtained for the wild-type isolates Berdun 17 and IPO323, and isolates carrying single alterations L50S (Berdun 10) and G460D (Berdun 4). Lowest values were obtained for isolates carrying the CYP51 alteration, V136A (isolates V103.9, V209.9 and V212.2), which had high levels of both *CYP51* gene expression and eburicol content (Table 4). SE of *CYP51* expression was large in two of the isolates (V103.9 and Berdun 17), but this did not affect the overall $1/C \times E$ classifications.

Statistical analysis of the impact of CYP51 alterations

Three alterations I381V, V136A and Y137F together explained 44% of the variation in the log of eburicol content significant at $P = 0.08$, 0.01 and 0.03, respectively. I381V and V136A together explained 61% of the variation in log transcript abundance, with individual significance at $P = 0.07$ and 0.002. Y137F, I381V and V136A also accounted for 89.3% of the variance for $1/C \times E$ data. All three terms were significant at $P < 0.001$ if dropped individually from the model, indicating that changes in eburicol demethylation efficiency are mainly due to these substitutions. There was no statistical evidence that any of the alterations present compensate for one another. No nonadditive effects of protein alterations on this efficiency measure were observed.

Discussion

Total lipid extraction and derivatization followed by GC-MS was used to analyse the sterol content of *M. graminicola* isolates carrying different variants of the *CYP51* gene predicted to encode amino acid alterations associated with altered azole sensitivity. Previous experiments (data not shown) had shown a significant increase in eburicol, the substrate of CYP51, in isolates treated with azole, confirming that azole fungicides inhibit eburicol demethylation in *M. graminicola*.

It was hypothesized that CYP51 changes may affect intrinsic enzyme activity, resulting in eburicol accumulation. The sterol content of 13 *M. graminicola* isolates with a total of eight different combinations of CYP51 alterations was analysed. Quantitative variations in sterol content between isolates were detected, but their differences did not appear to be related to CYP51 amino acid sequence. Specifically, increased eburicol content could not be directly correlated with CYP51 alterations and there was as much variation in eburicol content between isolates carrying the same CYP51 variant as between isolates with different CYP51s. Azole resistance can be caused by *CYP51* overexpression, the most notable case being in azole-resistant *Penicillium digitatum* isolates, which constitutively overexpressed *CYP51* 100-fold (Hamamoto *et al.*, 2000). However, in this study, the observed variation in *CYP51* expression was considerably lower and could not be directly correlated with either azole sensitivity or CYP51 sequence, evidence that CYP51 sequence, not expression level, is the most important factor in determining azole resistance in *M. graminicola*.

Using a measurement of eburicol demethylation efficiency, based on a combined measure of *CYP51* expression levels and eburicol content ($1/C \times E$), isolates with the same CYP51 grouped together. Furthermore, statistical analyses revealed CYP51 substitution V136A had the largest effect on the measure of efficiency, with substantial effects also

Table 4. Grouping of *Mycosphaerella graminicola* isolates according to the CYP51 efficiency measure, $1/C \times E$

Isolates	CYP51 alterations	Eburicol content (mg g^{-1})*	*CYP51* expression†	$1/C \times E$
Berdun 17	None	3.50E − 03	2.59 (±2.75)	110.23
IPO323	None	0.034	1 (±0.10)	29.02
Berdun 10	L50S	4.30E − 03	3.54 (±0.71)	65.61
Berdun 4	G460D	0.042	1.48 (±0.23)	16.21
Berdun 13	Y137F	0.256	1.34 (±0.58)	2.91
R6-31	Y137F	0.067	5.12 (±0.84)	2.93
V115.3	L50S, I381V, Y461H	0.195	4.37 (±0.96)	1.17
V209.10	L50S, I381V, Y461H	0.036	15.71 (±3.33)	1.77
V202.1	L50S, S188N, A379G, I381V, ΔY459/G460, N513K	0.06	6.78 (±2.80)	2.46
V214.7	L50S, S188N, A379G, I381V, ΔY459/G460, N513K	0.017	15.08 (±2.01)	3.91
V103.9	L50S, V136A, Y461H	0.079	23.07 (±12.53)	0.55
V209.9	V136A, S188N, ΔY459/G460	0.149	26.2 (±8.15)	0.26
V212.2	V136A, S188N, ΔY459/G460	0.278	5.84 (±0.57)	0.62

*Milligrams of sterol per gram fungal biomass.

†Relative to IPO323 untreated.

C, CYP51 expression; E, eburicol content; ±, SE ($n = 3$).

produced by I381V and Y137F. Those variants with substitutions L50S and G460D appear to encode proteins with unaltered efficiency. This is consistent with the impact of these amino acid changes on azole sensitivity and the location of these alterations in the predicted protein (Cools & Fraaije, 2008). Although these data do not clarify the effects of the CYP51 alterations on the biochemical properties of the enzyme, they clearly indicate that changes in activity have occurred. $1/C \times E$ also suggests that the accumulation of eburicol, caused by reduced enzyme activity, can be accommodated by increased gene expression.

It is possible that CYP51 amino acid alterations selected by azole treatment are reducing the intrinsic activity of CYP51 particularly as accumulated eburicol does not appear to be converted to the toxic sterol intermediate 14α-methyl-3,6-diol in *M. graminicola*. Studies of *Ustilago maydis*, also detected higher levels of 14α-methylated sterols, including eburicol, in isolates with reduced azole sensitivity with normal levels of ergosterol, suggesting leaky CYP51 activity (Joseph-Horne *et al.*, 1995). Further studies are underway to determine the precise impact of individual CYP51 amino acid alterations on intrinsic enzyme activity and azole binding.

Acknowledgements

Work carried out by T.P.B. was part of a CASE postgraduate studentship funded by the Biotechnology and Biological Sciences Research Council (BBSRC) of the United Kingdom, BASF and the Home Grown Cereals Authority (HGCA). Part of this work was supported by BBSRC project BB/E02257X/1. Rothamsted Research receives grant-aided support from the BBSRC. The authors would like to thank Sandy Hey for technical assistance and Kim Hammond-Kosack for critical reading of the manuscript.

References

Barrero AF, Oltra JE, Poyatos JA, Jiménez D & Oliver E (1998) Phycomysterols and other sterols from the fungus *Phycomyces blakesleeanus*. *J Nat Prod* **61**: 1491–1496.

Clark WS (2006) *Septoria tritici* and azole performance. In fungicide resistance: are we winning the battle but losing the war? *Aspects Appl Biol* **8**: 127–132.

Cools HJ & Fraaije BA (2008) Are azole fungicides losing ground against *Septoria* wheat disease? Resistance mechanisms in *Mycosphaerella graminicola*. *Pest Manag Sci* **64**: 681–684.

Cools HJ, Fraaije BA & Lucas JA (2005) Molecular mechanisms correlated with changes in triazole sensitivity in isolates of *Mycosphaerella graminicola*. *Proceedings of the BCPC Congress, Crop Science and Technology 2005*, Vol. 1, pp. 267–274. BCPC, Alton, UK.

Cools HJ, Fraaije BA, Bean TP, Antoniw J & Lucas JA (2007) Transcriptome profiling of the response of *Mycosphaerella graminicola* isolates to an azole fungicide using cDNA microarrays. *Mol Plant Pathol* **8**: 639–651.

Fraaije BA, Cools HJ, Kim S-H, Motteram J, Clark WS & Lucas JA (2007) A novel substitution I381V in the sterol 14α-demethylase (CYP51) of *Mycosphaerella graminicola* is differentially selected by azole fungicides. *Mol Plant Pathol* **8**: 245–254.

Hamamoto H, Hasegawa K, Nakaune R, Jin Lee Y, Makizumi Y, Akutsu K & Hibi T (2000) Tandem repeat of a transcriptional enhancer upstream of the sterol 14α-demethylase gene (*CYP51*) in *Penicillium digitatum*. *Appl Enivron Microb* **66**: 3421–3426.

Hey SJ, Powers SJ, Beale MH, Hawkins ND, Ward JL & Halford NG (2006) Enhanced seed phytosterol accumulation through expression of a modified HMG-CoA reductase. *Plant Biotechnol J* **4**: 219–229.

Joseph-Horne T, Hollomon DW, Loeffler RST & Kelly SL (1995) Altered P450 activity associated with direct selection for fungal azole resistance. *FEBS Lett* **374**: 174–178.

Joseph-Horne T, Hollomon D, Manning N & Kelly SL (1996) Investigation of the sterol composition and azole resistance in field isolates of *Septoria tritici*. *Appl Enivron Microb* **62**: 184–190.

Kelly SL, Lamb DC, Corran AJ, Baldwin BC & Kelly DE (1995) Mode of action and resistance to azole antifungals associated with the formation of 14α methylergosta-8,24(28)-dien-3β,6α-diol. *Biochem Bioph Res Co* **207**: 910–915.

Kelly SL, Lamb DC, Kelly DE, Loeffler J & Einsele H (1996) Resistance to fluconazole and amphotericin in *Candida albicans* from AIDS patients. *Lancet* **348**: 1523–1524.

Kontoyiannis DP & Lewis RE (2002) Antifungal drug resistance of pathogenic fungi. *Lancet* **359**: 1135–1144.

Leroux P, Albertini C, Gautier A, Gredt M & Walker AS (2007) Mutations in the cyp51 gene correlated with changes in sensitivity to sterol 14α-demethylation inhibitors in field isolates of *Mycosphaerella graminicola*. *Pest Manag Sci* **63**: 688–699.

Loeffler RST & Hayes AL (1990) Sterols of the plant pathogenic fungi *Botrytis cinerea* and *Pyrenophora teres*. *Phytochem* **26**: 3423–3425.

Marichal P, Koymans L, Willemsens S, Bellens D, Verhasselt P, Luyten W, Borgers M, Ramaekers FCS, Odds F & Vanden Bossche H (1999) Contribution of mutations in the cytochrome P450 14α-demethylase (Erg11p, Cyp51p) to azole resistance in *Candida albicans*. *Microbiology* **145**: 2701–2713.

Moretti M, Arnoldi A, D'Agostina A, Farina G & Gozzo F (2003) Characterization of field-isolates and derived DMI-resistant strains of *Cercospora beticola*. *Mycol Res* **107**: 1178–1188.

Nolte FS, Parkinson T, Falconer DJ, Dix S, Wiiliams J, Gilmore C, Geller R & Wingard JR (1997) Isolation and characterization of fluconazole- and amphotericin B-resistant *Candida albicans* from blood of two patients with leukaemia. *Antimicrob Agents Ch* **41**: 196–199.

Perea S, Lopez-Ribot JL, Kirkpatrick WR, McAtee RK, Santillan RA, Martinez M, Calabrese D, Sanglard D & Patterson TF (2001) Prevalence of molecular mechanisms of resistance to azole antifungal agents in *Candida albicans* strains displaying high-level fluconazole resistance isolated from human immunodeficiency virus-infected patients. *Antimicrob Agents Ch* **45**: 2676–2684.

Sanglard D, Kuchler K, Ischer F, Pagani JL, Monod M & Bille J (1995) Mechanisms of resistance to azole antifungals in *Candida albicans* from AIDS patients involves specific multidrug resistance transporters. *Antimicrob Agents Ch* **39**: 2378–2386.

Sanglard D, Ischer F, Koymans L & Billie J (1998) Amino acid substitutions in the cytochrome P450 lanosterol 14α-demethylase (CYP51A1) from azole-resistant *Candida albicans* clinical isolates contribute to resistance to antifungal agents. *Antimicrob Agents Ch* **42**: 241–253.

Warnock DW, Arthington-Skaggs BA & Li RK (1999) Antifungal drug susceptibility testing and resistance in *Aspergillus*. *Drug Resist Update* **2**: 326–334.

Yoshida Y & Aoyama Y (1987) Interaction of azole antifungal agents with cytochrome P-45014DM purified from *Saccharomyces cerevisiae* microsomes. *Biochem Pharmacol* **36**: 229–223.