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Transcriptome profiling of the response of *Mycosphaerella graminicola* isolates to an azole fungicide using cDNA microarrays

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SUMMARY

Resistance to azole antifungals is a major problem in the control of diseases caused by fungal pathogens of both humans and plants. Potential for the development of azole resistance in the wheat leaf blotch pathogen *Mycosphaerella graminicola*, the causal agent of the most economically significant foliar disease of wheat in north-western Europe, is now of particular concern after the recent emergence of widespread resistance to quinone outside inhibitor fungicides. Using a cDNA microarray representing around 25% of the genome, we have profiled the transcriptional response of *M. graminicola* to epoxiconazole, currently the most widely used azole fungicide on cereal crops. By comparing the transcription profiles of two *M. graminicola* isolates with contrasting sensitivities to epoxiconazole we show qualitative and quantitative differences in differentially expressed genes, including those involved in ergosterol biosynthesis, mitochondrial respiration and transport mechanisms. This represents the first study investigating the response of a plant pathogenic fungus to a fungicide using cDNA microarray technology.

INTRODUCTION

Septoria leaf blotch, caused by the ascomycete fungus *Mycophaerella graminicola* (anamorph: *Septoria tritici*) has been the most economically important fungal pathogen of winter wheat in the UK for over 20 years, causing estimated yield losses of £30 million per annum (Hardwick *et al*., 2001). None of the current commercially available wheat cultivars is fully resistant to the disease. Therefore, the programmed application of fungicides is the only effective control strategy. Groups of site-specific systemic fungicides including the methyl benzimidazole carbamates (MBCs), quinone outside inhibitors (QoIs) and demethylation inhibitors (DMIs) have been used extensively against the disease since the early 1970s. However, the pathogen has quickly adapted and resistance to a number of chemical classes, including the MBCs and QoIs, is now widespread in UK populations (Fraaije *et al*., 2003, 2005). Resistance to the azole group of DMI fungicides has not yet emerged. However, owing to the current lack of alternative chemistry to control the disease, and the consequent increased reliance on azoles, there is concern that resistance to this group could develop, particularly in light of the recent decline in efficacy of a range of azoles in controlling Septoria leaf blotch (Clark, 2006).

Azole resistance in plant pathogenic fungi has previously developed slowly and loss of efficacy in practice remains rare. By contrast, resistance to azoles in human pathogens is a major problem, particularly in immunosuppressed patients in whom control of recurrent infections caused by opportunistic pathogens, for example *Candida albicans*, is commonly compromised by resistance. The adaptation of human fungal pathogens to azoles demonstrates the biological potential for resistance development, thereby providing a paradigm for the evolution to resistance in other pathogens, including those of plants. Consequently, studies of the mechanisms underlying changes in azole sensitivity in plant pathogens have to date focused on those well-characterized in human pathogens, principally *C. albicans*.

The most frequently identified azole resistance mechanism in *C. albicans* is up-regulation of active efflux proteins, ATP-binding cassette (ABC) transporters and major facilitators (MFs), which reduce the intracellular concentration of the fungicide (Perea *et al*., 2001). Active efflux has also been shown to contribute to azole resistance in plant pathogens, although identified most frequently in laboratory-generated mutants. Such laboratory mutants of *M. graminicola* resistant to azoles with altered expression of ABC transporter genes have been shown to revert to an azole-sensitive phenotype when transporter genes are disrupted (Zwiers *et al*., 2002). Efflux pump over-expression has only been shown to confer directly azole resistance in field isolates of *Penicillium italicum* (Nakuane *et al*., 1998). Studies of *M. graminicola* field isolates collected in the 1990s showed differences in both basal and induced levels of ABC transporter gene transcript, although no correlation between increased expression and azole sensitivity was evident (Stergiopoulos *et al*., 2003). Similarly, studies of more recent *M. graminicola* field isolates from the UK

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failed to establish any direct relationship between over-expression and decreased azole sensitivity (Cools *et al.*, 2005a). Therefore, the contribution of active efflux to azole resistance in field populations of plant pathogens, although implicated, remains unclear.

The most common mechanism of resistance to site-specific fungicides is modification of the target protein. The target for azole fungicides is the cytochrome P450 sterol 14α-demethylase (CYP51), an essential enzyme of the ergosterol biosynthesis pathway, the predominant sterol in the membranes of fungi. Alterations in CYP51 have been associated with changes in azole sensitivity in fungal pathogens of humans and plants, although the impacts of these changes are best characterized in the human pathogen *C. albicans* with a number of *CYP51* mutations functionally characterized (Kelly *et al.*, 1999; Lamb *et al.*, 2000; Sanglard *et al.*, 1998). Mutations in *CYP51* encoding changes at residues equivalent to those known to affect an azole resistant phenotype in *C. albicans* have also been identified in plant pathogens, most notably the equivalent to substitution Y132F/H in powdery mildews (Delye *et al.*, 1997, 1998; Wyand and Brown, 2005). This substitution has also been identified in *M. graminicola* (Y137F) (Cools *et al.*, 2005b). Our studies have also identified CYP51 alterations not previously associated with azole resistance in human or plant pathogenic fungi. Substitution I381V, for example, appears to be differentially selected by members of the azole class of fungicides (Fraaije *et al.*, 2007). In *C. albicans*, combinations of *CYP51* mutations confer greater reductions in azole sensitivity than single mutations. In plant pathogens mutations also appear to be accumulating, for example in *Blumeria graminis* f. sp. *hordei* (Wyand and Brown, 2005) and *M. graminicola* (Cools *et al.*, 2005b; Fraaije *et al.*, 2007), although as isolates with identical *CYP51* mutations can have different azole sensitivities, the exact impact of target site changes on azole efficacy in the field remains unknown (Cools *et al.*, 2006).

In this study, we have used cDNA microarrays to profile the transcriptional response of *M. graminicola* to epoxiconazole, currently the azole most widely used to control this disease. We have compared the expression profiles of an azole-sensitive and less sensitive *M. gramincola* isolate to a single discriminatory dose to determine whether decreased azole sensitivity is conferred by mechanisms other than mutations in the *CYP51* gene. To our knowledge, this is the first investigation into the response of a plant pathogenic fungus to a fungicide using microarrays. We describe the effect of epoxiconazole treatment on genes involved in ergosterol biosynthesis, mitochondrial respiration and toxicant transport. Furthermore, we report both qualitative and quantitative differences in basal and induced gene expression between *M. graminicola* isolates with different *CYP51* mutations and contrasting sensitivities to azoles.

RESULTS

Characterization of isolates

The calculated EC_{50} to epoxiconazole for isolate G303 is around 25-fold higher than sensitive isolate IPO323. *CYP51* genes of IPO323 and G303 have been previously sequenced (Cools *et al.*, 2005b), with a number of encoded amino acid changes identified in G303. Some of these alterations have been correlated with reduced azole sensitivity (Cools *et al.*, 2005a,b; Fraaije *et al.*, 2007). EC_{50} values for Rothamsted, Hertfordshire, isolates obtained in 2006 ranged from 0.164 mg/L (isolate R6-40) to 2.19 mg/L (R6-31). Multiple *CYP51* mutations were identified in all isolates (Table 1). All isolates grew at similar rates *in vitro* (data not shown).

Constitutive differences in gene expression between IPO323 and G303

Ten genes were more highly expressed, beyond a two-fold cut off, in less sensitive isolate G303 compared with sensitive isolate IPO323 (Table 2). These include those encoding a drug transporter (unisequence Id: mga1012f) protein, a cell surface glycoprotein (mg[0175]), thought to be a flocculin, stress response protein rds1 (mg[0194]), a *myo*-inositol transport protein (mgb17a02f), a mitochondrial transport protein (mgb12c11f), and an unknown gene encoding a homologue of the antibiotic response protein CipA of *Aspergillus nidulans* (mgc03e06f).

Table 1 *M. graminicola* isolates used in this study.

Isolate	Year	Location	Epoxiconazole EC_{50} (mg/L)	CYP51 alteration(s)
IPO323	1981	Netherlands	0.06	none
G303	2003	Kent	1.47	L50S, D138G, S188N, A379G, I381V, ΔY459-G460, N513K
R6-31	2006	Hertfordshire	2.19	Y137F
R6-32	2006	Hertfordshire	0.698	L50S, S188N, I381V, ΔY459-G460, N513K
R6-40	2006	Hertfordshire	0.164	L50S, I381V, Y459D
R6-55	2006	Hertfordshire	0.976	L50S, I381V, Y461H

Table 2 Genes differentially expressed in isolate G303 compared with isolate IPO323 in the absence of epoxiconazole.

Functional category (MIPS)	EST ID	Unisequence ID*	Best match; accession no.	Mean fold change in expression
cell wall biogenesis	mga0371	mga0371	*N*-acetyl-β-D-glucosaminidase (exochitinase); AAL78815	+2.1
drug transporters	mga1012f	mga1012f	multidrug resistance protein; EAA28910	+3.4
electron transport proteins	mga0964	mga0964	subunit IV of cytochrome c oxidase; EAA33815	+2.1
mitochondrial transport	mgb12c11f	mgb12c11f	protein of the inner mitochondrial membrane, required for import of mitochondrial matrix proteins; EAA36389	+2.4
organization of cell wall	mga0959f	mg[0175]	cell surface flocculin (glycoprotein); T45462	+3.4
	mgc02g01f	mg[0558]	homologue of clock controlled protein 6 (*N. crassa*) (cell surface glycoprotein); Q01302	+2.1
protein modification	mg12h01f	mg12h01f	nuclear protein arginine methyltransferase; EAA34674	+2.1
sugar, carbohydrate and metabolite transporters	mgb17a02f	mgb17a02f	myo-inositol transport protein; EAA28903	+2.6
stress response	mga0334	mg[0194]	stress response protein (rds1); CAD21425	+3.2
unclassified protein	mgc03e06f	mg[1000]	homologue of CipA (*E. nidulans*), associated with antibiotic response; CAC87270	+2.8

*Sequences available at http://cogeme.ex.ac.uk/

Table 3 Genes differentially expressed in isolate IPO323 after 24 h exposure to 2 mg/L epoxiconazole.

Functional category (MIPS)	EST ID	Unisequence ID*	Best match; accession no.	Mean fold change in expression
tetracyclic and pentacyclic triterpene (steroids and hopanoids) biosynthesis	mgc01h02f	mg[0887]	C-4 sterol methyl oxidase; EAA33004	+4.2
	mga0888f	mga0888f	C14-sterol reductase; O13597	+7.7
	mgc01f02f	mg[0869]	sterol C-22 desaturase; BAC01140	+5.9

*Sequences available at http://cogeme.ex.ac.uk/

Gene expression in response to epoxiconazole treatment

Genes up-regulated in response to 24 h exposure to a lethal (Table 1) dose of epoxiconazole in isolate IPO323, beyond a two-fold cut-off, all encoded components of the sterol biosynthesis pathway: C-4 sterol methyl oxidase (*erg25*, mg[0887]), C14-sterol reductase (*erg24*, mga0888f) and C-22 sterol desaturase (*erg5*, mg[0869]) (Table 3). Expression of *erg5*, *erg24* and *erg25* in isolate G303 also increased after treatment with a non-lethal (Table 1) dose of epoxiconazole, although transcript levels were lower than those detected for IPO323 (Table 4). Furthermore, mga1391f encoding hydroxymethylglutaryl-CoA synthase (HMG-CoA synthase, *erg13*), an enzyme involved in mevalonate synthesis, a precursor of ergosterol biosynthesis, is up-regulated in G303. The orthologous gene is similarly responsive in *C. albicans* (De Backer *et al.*, 2001). Genes other than those encoding proteins involved in sterol biosynthesis up-regulated in G303 include a putative hexose transporter, mg[0271], a ubiquinol cytochrome c reductase subunit, mg[1446], and an unknown protein homologous to FAO1 of *Cryptococcus neoformans*, mgc03g08f.

Hierarchical clustering of genes involved in sterol biosynthesis

Hierarchical clustering of genes in the same functional category (MIPS) associated genes by relative expression profile. This enabled genes that were not differentially expressed beyond the stringent two-fold cut-off to be analysed. Cluster analysis confirmed sterol biosynthesis genes *erg25* (mg[0887]), *erg24* (mga0888f) and *erg5* (mg[0869]) as up-regulated in response to 24 h epoxiconazole treatment (Fig. 1A). In addition, expression of genes encoding a sterol isomerase (mga1231f) and the sterol C-24 reductase (mgc19a12f) were identified by clustering as azole induced. Expression of mgc19a12f, encoding erg4, the sterol C-24 reductase, is only induced in G303, perhaps reflecting continued ergosterol production in this isolate. Genes originally annotated as involved in sterol biosynthesis that are unaffected or down-regulated by azole treatment include mg[1270] encoding a farnesyl diphosphate synthetase (erg20), mg[0650] suggested to encode the sterol C-24 methyltransferase (erg6) and other sterol-associated proteins, for example mg1265f encoding a hydroxysteroid dehydrogenase. However, Blastx searches fail to identify

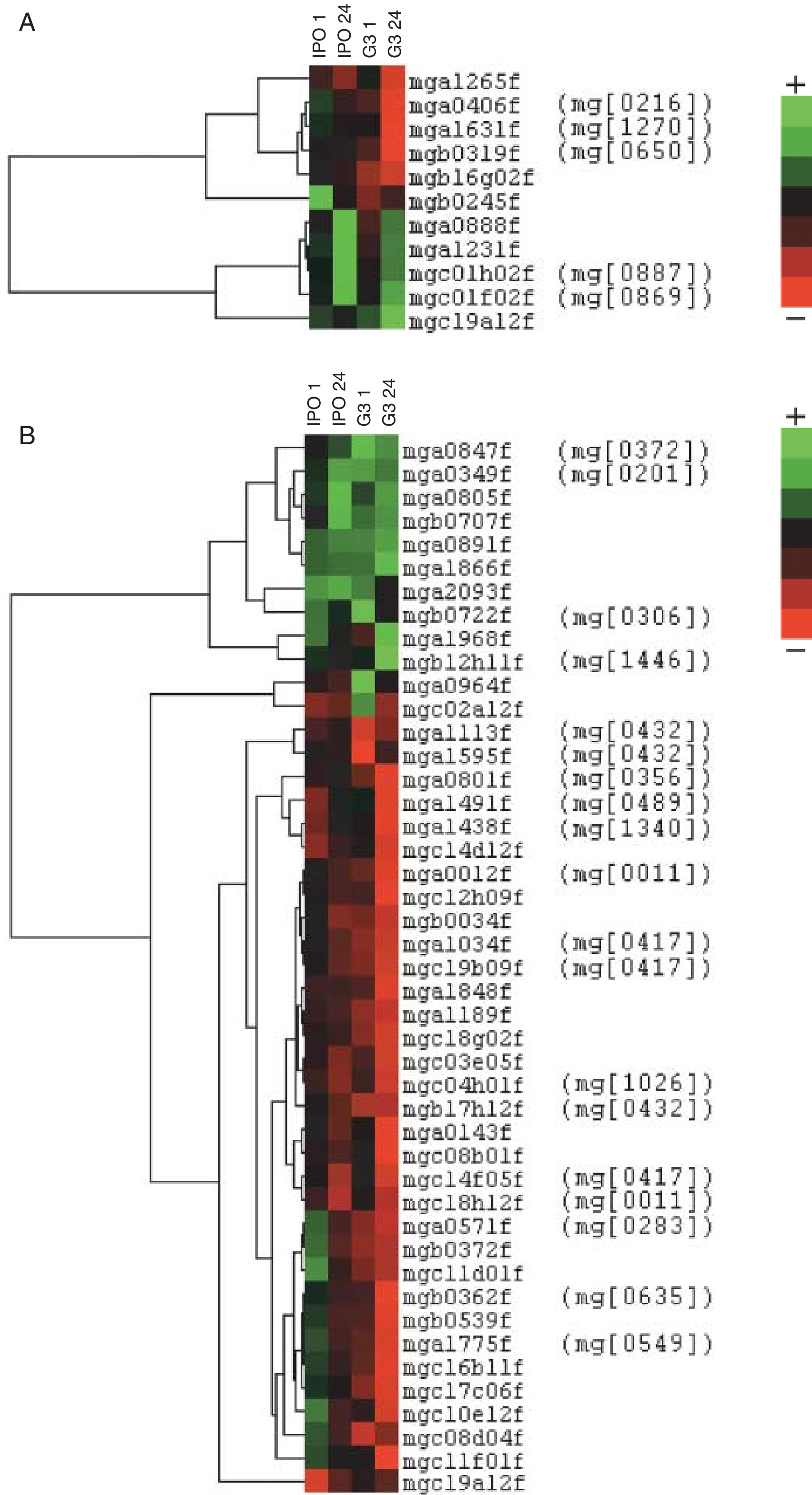

Fig. 1 Hierarchical clustering showing the patterns of expression of genes involved in sterol biosynthesis (A) and electron transport (B) in isolate IPO323 after 1 h (IPO 1) and 24 h (IPO 24) and G303 after 1 h (G3 1) and 24 h (G3 24) of epoxiconazole treatment. Colour bars display normalized $\log_2$ (green/red) ratio, with untreated IPO323 as the reference point. EST IDs are shown. Unisequence IDs are given in parentheses.

Table 4 Genes differentially regulated in isolate G303 after 24 h exposure to 2 mg/L epoxiconazole.

Functional category (MIPS)	EST ID	Unisequence ID*	Best match; accession no.	Mean fold change in expression
electron transport proteins	mgb12h11f	mg[1446]	ubiquinol cytochrome-c reductase subunit; P48503	+2.1
isoprenoid biosynthesis	mga1391f	mga1391f	hydroxymethylglutaryl-CoA synthase, functions in mevalonate synthesis; T49718	+2.2
sugar, carbohydrate and metabolite transporters	mgb0930f	mg[0271]	hexose transporter; EAA28833	+2.1
tetracyclic and pentacyclic triterpene (steroids and hopanoids) biosynthesis	mgc01h02f	mg[0887]	C-4 sterol methyl oxidase; EAA33004	+2.9
	mga0888f	mga0888f	C14-sterol reductase; O13597	+3.6
	mgc01f02f	mg[0869]	sterol C-22 desaturase; BAC01140	+4.5
unclassified protein	mgc03g08f	mgc03g08f	homologue of FAO1 (*Cryptococcus neoformans*); AAN75712	+2.8

*Sequences available at http://cogeme.ex.ac.uk/

most of these genes (mga1265, mg[0216], mgb16g02f and mgb0245f) as sterol-associated. The exceptions are mg[1270], which has homology to *erg20* of *Gibberella zeae* (2e-50), and mg[0650], homologous to *erg6* of *Neurospora crassa* (1e-63).

Hierarchical clustering of genes encoding components of the mitochondrial electron transport chain

Clustering of genes encoding components of the mitochondrial electron transport chain revealed a number up-regulated in response to epoxiconazole (on average around 1.5-fold), particularly in isolate IPO323 (Fig. 1B). Interestingly, most azole-responsive genes are mitochondrially encoded. For example, subunits 1–7 of the NADH-ubiquinone oxidoreductase complex are mitochondrially encoded in fungi and higher eukaryotes. All of these represented on the microarray (subunits 1 (mg[0372]), 2 (mg[0201]; mga0891f), 4 (mga1866f; mga2093f) and 5 (mga0805f)) are up-regulated in response to epoxiconazole treatment. The remaining NADH-ubiquinone oxidoreductase subunits are nuclear encoded. These include the 51-kDa (mg[1340]), 78-kDa (mga[0489]) and 20.8-kDa (mgc02a12f) subunits, all either unaffected or down-regulated in this study. Similarly, cytochrome *b* (mgb0707f) and subunit III of the cytochrome c oxidase (mg[0306]) are mitochondrially encoded, with smaller subunits (e.g. subunit V (mgc14d12f), VI (mga1848f)) encoded in the nucleus.

Validation of microarray data by quantitative RT-PCR and analysis of Rothamsted 2006 isolates

Genes selected for analysis by quantitative RT-PCR were those either constitutively up-regulated in isolate G303 (mga1012f (Fig. 2A) and mg[0194] (Fig. 2B)), involved in sterol biosynthesis (mga0888f (Fig. 2C), and the *CYP51/erg11* azole target (Fig. 2D), absent from the microarray) and responsive to epoxiconazole, but not directly involved in sterol biosynthesis (mg[0271] (Fig. 2E) and mgc03g08f (Fig. 2F)). In general, quantitative RT-PCR results correlated well with microarray data, although for some genes, particularly mga0888f, fold changes in expression were greater, perhaps reflecting the greater dynamic range of real-time PCR.

Analysis of the expression of mg1012f (Fig. 3A) and epoxiconazole-responsive genes mg[0271] (Fig. 3B) and mgc03g08f (Fig. 3C) in *M. graminicola* isolates with varying azole sensitivities obtained from an untreated field at Rothamsted in 2006 revealed differences in constitutive and azole-induced transcript levels between isolates. For example, a high basal level of mga1012f expression was only detected in R6-55, with expression slightly reduced after epoxiconazole treatment, similar to results for G303. In the remaining isolates mga1012f expression was generally unaffected by azole. Expression of mg[0271] was induced by epoxiconazole treatment in all isolates; however, transcript levels varied between isolates with highest basal (6.2-fold) and induced (9.8-fold) expression levels detected for the least sensitive isolate R6-31. Mean expression levels of mgc03g08f, the predicted iron/ascorbate oxidoreductase gene, varied greatly between isolates. A 53-fold increase (relative to the calibrator sample) in transcript level was detected after epoxiconazole treatment in isolate R6-55. In no other isolates was mgc03g08f expression induced to the same extent.

DISCUSSION

Microarray transcriptional profiling of the response of human fungal pathogens to antifungals has already proved successful in identifying novel genes and/or gene families expressed upon exposure to these compounds and involved in the acquisition of resistance (De Backer *et al.*, 2001; Ferreira *et al.*, 2006; Rogers and Barker, 2003). This is the first study using microarray technology to profile the response of a plant pathogen when exposed to a fungicide. By analysing the expression of expressed sequence

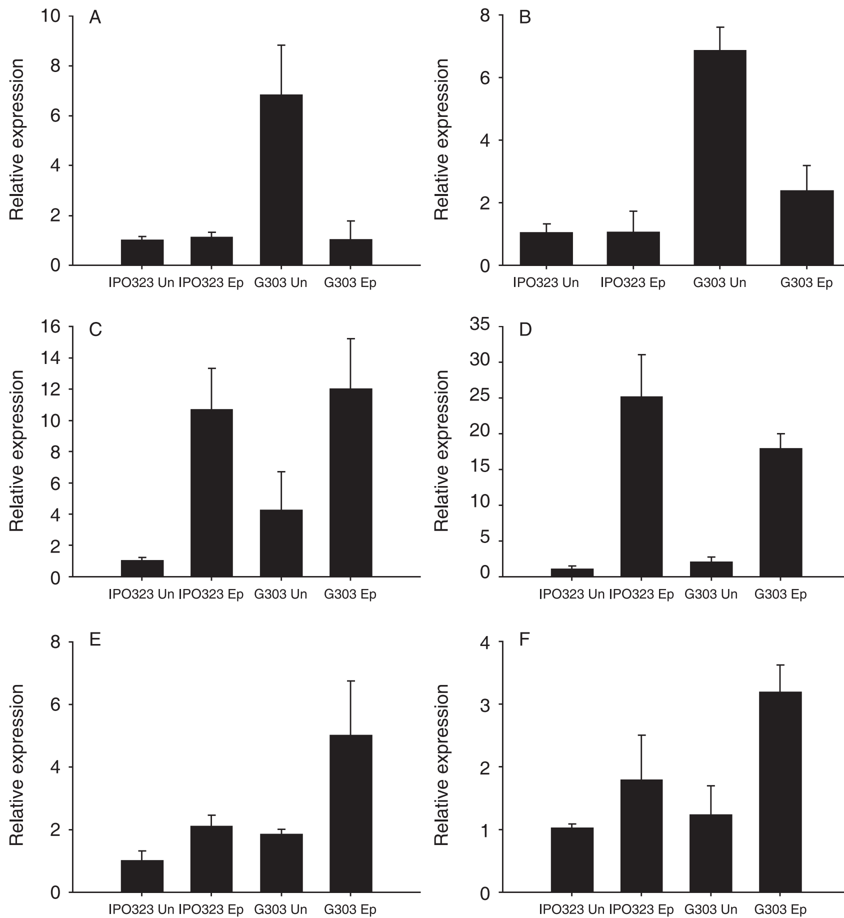

Fig. 2 Validation of microarray results using quantitative RT-PCR for genes identified as differentially expressed in microarray experiments. Shown are relative expression values in untreated IPO323 (IPO323 Un) and after 24 h of epoxiconazole treatment (IPO323 Ep) and untreated G303 (G303 Un) and after 24 h of epoxiconazole treatment (G303 Ep). Unisequences IDs of genes analysed are mga1012f (A), mg[0194] (B), *CYP51/erg11* (C, not represented on the microarray), mga0888f (D), mg[0271] (E) and mg03g08f (F). Mean of two replicate experiments with standard deviations are shown.

tags (ESTs), predicted to cover a quarter of the genome of the wheat leaf blotch pathogen *M. graminicola*, upon exposure to the azole fungicide epoxiconazole, we have confirmed the effect of this compound on ergosterol biosynthesis, and, in addition, demonstrated an impact on the expression of components of the respiratory chain, predominantly those encoded in the mitochondrial genome. Comparison of the expression profile of an isolate less sensitive to azole (G303), carrying mutations in the target-encoding *CYP51* gene, with the sensitive isolate (IPO323), both in the absence and in the presence of epoxiconazole, has identified differentially expressed genes that potentially have a role in reducing azole sensitivity in G303. Subsequent quantitative RT-PCR analyses of the expression of these candidate genes in four more recently collected isolates with varying levels of azole sensitivity suggest these genes may also contribute to a less sensitive phenotype in other *M. graminicola* isolates.

Response of genes involved in ergosterol biosynthesis

Genes encoding components of the ergosterol biosynthesis pathway that are represented on the microarray were consistently the most responsive to treatment, confirming that this pathway is the primary target for epoxiconazole and responds to changes in ergosterol levels. Most ergosterol biosynthesis genes were up-regulated in both isolates. The exceptions were *erg4* (mgc19a12f), encoding the sterol C-24 reductase, which was only responsive in isolate G303, *erg20* (mg[1270]) encoding a farnesyl diphosphate synthetase, and *erg6* (mg[0650]), encoding the sterol C-24 methyltransferase. Expression of both *erg20* and *erg6* was unaffected by azole treatment in isolate IPO323 and decreased in G303.

The sterol C-24 reductase catalyses the final step in ergosterol biosynthesis. Therefore, differential expression of *erg4* between isolates in the presence of epoxiconazole may reflect differences in the capacity of isolates to complete ergosterol biosynthesis. Consistent with this study, expression of *erg20* is not affected by azole treatment in *Saccharomyces cerevisiae* (Bammert & Fostel 2000) and *C. albicans* (De Backer *et al.*, 2001). The observed effect of epoxiconazole treatment on *erg6* expression, however, conflicts with previous studies of both *S. cerevisiae* (Agarwal *et al.*, 2003; Bammert & Fostel, 2000) and *C. albicans* (De Backer *et al.*, 2001). This discrepancy with earlier studies and other data presented here, particularly the up-regulation of *erg5*, *erg24* and

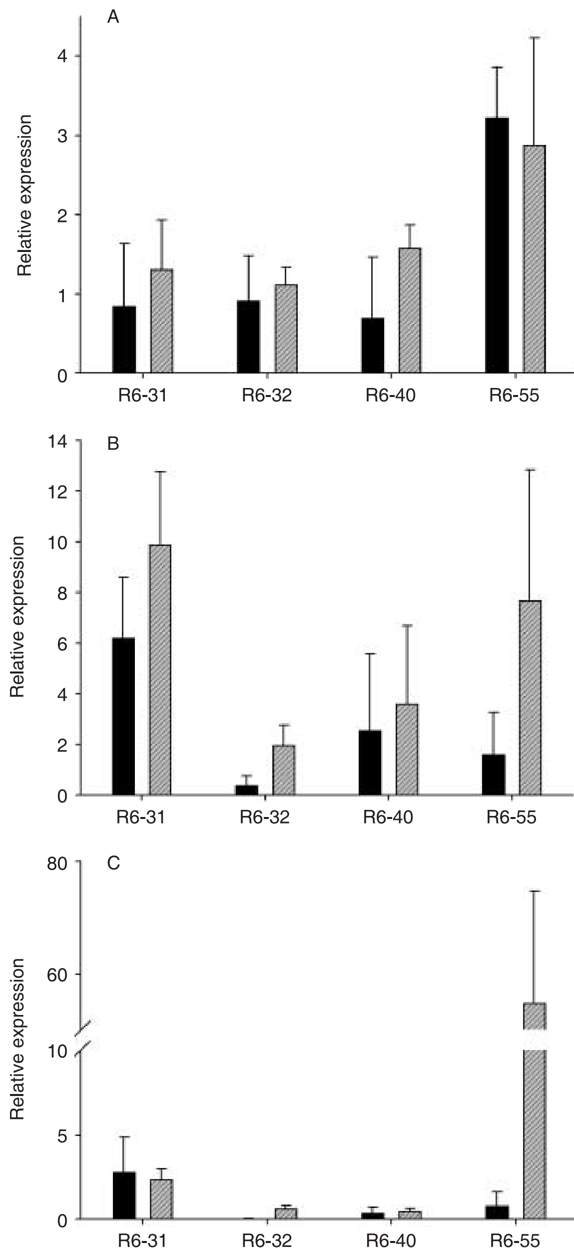

Fig. 3 Quantitative RT-PCR analysis of the expression of mga1012f (A), mg[0271] (B) and mgc03g08f (C) in isolates obtained from Rothamsted, Hertfordshire, in 2006 (R6-31, R6-32, R6-40 and R6-55). Expression analyses were carried out on untreated (filled) and 24 h epoxiconazole-treated (shaded) samples using untreated IPO323 as the reference. Mean of two replicate experiments and standard deviations are shown. For clarity, a break (10–50) has been inserted into the *y*-axis of graph C.

erg25 in response to azole, suggests this gene may not be correctly annotated and requires further analysis to establish its precise function. In *Aspergillus fumigatus*, the only filamentous fungus to be analysed after antifungal treatment by microarray thus far, expression of *erg24* and *erg25* also increased upon exposure to voriconazole (Ferreira *et al.*, 2006), although unlike this (as shown by quantitative RT-PCR) and other studies (Agarwal *et al.*, 2003; De Backer *et al.*, 2001; Liu *et al.*, 2005) expression of both *CYP51/erg11* paralogues in *A. fumigatus* decreased upon treatment. Amongst the remaining genes predicted to encode components of the ergosterol biosyntheisis pathway, EST mga1231f, suggested to encode a sterol isomerase, is up-regulated upon azole exposure. Although homology searches suggest this gene does not encode erg2, evidence here does suggest mga1231f responds to changes in ergosterol levels.

The mechanisms responsible for *erg* gene up-regulation upon azole exposure remain unclear. However, consistent with other studies of *S. cerevisiae* (Agarwal *et al.*, 2003; Bammert and Fostel, 2000) and *C. albicans* (De Backer *et al.*, 2001; Liu *et al.*, 2005), greatest changes in gene expression levels in *M. graminicola* after azole treatment were detected for genes encoding components functioning downstream of *CYP51*/*erg11* (Fig. 4). This suggests that *erg* gene expression is induced specifically by ergosterol depletion, as has previously been proposed (Agarwal *et al.*, 2003; De Backer *et al.*, 2001; Liu *et al.*, 2005). Alteration of *erg* gene expression, particularly up-regulation of *erg11/CYP51*, has been shown to confer azole resistance both in human (Rogers and Barker, 2003; White, 1997) and in plant pathogenic fungi (Hamamoto *et al.*, 2000; Schnabel and Jones, 2001). In this study, no constitutive differences in *erg* gene expression were evident in microarray studies when the less sensitive isolate G303 was compared with the sensitive isolate IPO323. After 24 h exposure to azole, levels of *erg* gene up-regulation were similar between isolates. Quantitative RT-PCR analysis did suggest some differences in basal *erg11*/*CYP51* and *erg24* transcript levels. For example, *erg11*/*CYP51* expression appeared around two-fold higher in G303. However, in the absence of corroborative microarray data, it is difficult to conclude an impact of altered *erg* gene expression on reducing azole sensitivity in G303.

Response of genes involved in mitochondrial electron transport

The relationship between ergosterol biosynthesis and mitochondrial respiration has long been established. For example, certain steps of ergosterol biosynthesis are dependent on mitochondrial development in yeast (Daum *et al.*, 1998). Yet the effect of azoles on mitochondrial function is not clearly defined. In this study, hierarchical clustering revealed that a number of genes encoding components of the mitochondrial electron transport chain were up-regulated (between 1.5- and 1.8-fold) by epoxiconazole treatment. Microarray and *erg* mutant studies of *S. cerevisiae* (Bammert and Fostel, 2000) also demonstrated increased transcript levels of respiratory chain components after azole treatment, including members of the cytochrome c oxidase and cytochrome c reductase complexes and subunits of ATP synthase. Unlike the

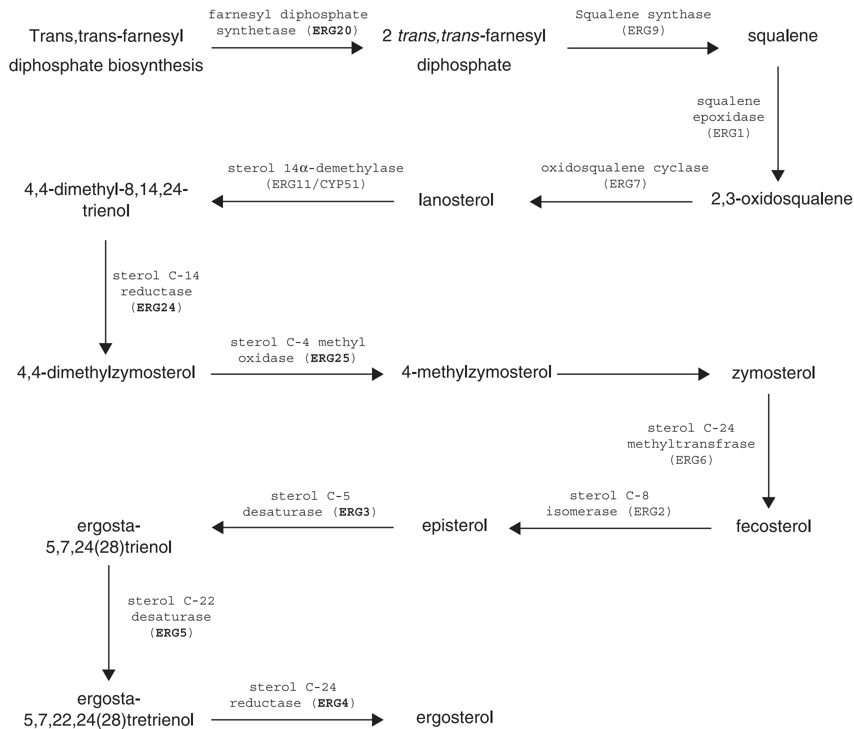

Fig. 4 Schematic of the ergosterol biosynthesis pathway from farnesyl pyrophosphate. Genes represented on the microarray are shown in bold type.

findings presented here, however, Bammert and Fostel (2000) also observed increased transcript levels of known oxidative stress response genes, leading to the suggestion that perturbation of ergosterol biosynthesis by mutation or azole, and the consequent accumulation of sterol precursors, indirectly affects mitochondrial electron transport, leading to the generation of reactive oxygen species (ROS). Other microarray studies of fungi including *C. albicans* and *A. fumigatus* have not observed changes in electron transport gene expression upon azole exposure; however, studies of the transcriptional response of the bacterial pathogen *Mycobacterium tuberculosis* to azole treatment did identify responsive components of the respiratory chain (Boshoff *et al.*, 2004).

Studies of petite mutants of *S. cerevisiae* and *Candida glabrata* provide additional evidence for an interaction between azole and mitochondria. In both *S. cerevisiae* (Kenna *et al.*, 1989) and *C. glabrata* (Sanglard *et al.*, 2001) azoles have been shown to be potent inducers of petite mutagenesis and, furthermore, petite mutants are highly resistant to azoles (Brun *et al.*, 2004; Sanglard *et al.*, 2001). Brun *et al.* (2004) suggested that azoles induce petite mutagenesis by directly inhibiting mitochondrial function, causing an accumulation of ROS and leading to mitochondrial damage. A direct interaction of azoles with components of the respiratory chain has previously been demonstrated using isolated mitochondria from *C. albicans* (Shigematsu *et al.*, 1982).

Interestingly, most of the components of the respiratory chain which were up-regulated in response to epoxiconazole treatment in this study are mitochondrially encoded. The exceptions are the nuclear encoded NADH-ubiquinone oxidoreductase 49-kDa (mga1968) subunit and cytochrome c reductase subunit VII (mg[1446]), both of which are only responsive in isolate G303. Therefore, consistent with previous studies, epoxiconazole treatment of *M. graminicola* induces the expression of genes involved in mitochondrial respiration (Bammert and Fostel, 2000; Boshoff *et al.*, 2004); however, it remains to be investigated whether this induction is specific to mitochondrially encoded components of the respiratory chain.

Response of genes encoding transporter proteins

In this study, only two genes encoding transport proteins were differentially expressed between isolates, or induced upon epoxiconazole treatment. mga1012f, encoding a vacuolar glutathione S-conjugate ABC transporter, potentially involved in drug resistance, is constitutively over-expressed in isolate G303. Upon epoxiconazole exposure, however, expression of this gene decreases in this isolate, suggesting this gene is not contributing to reducing azole sensitivity in G303. Homology searches against the fungal genome database identify mga1012f as a vacuolar glutathione S-conjugate ABC transporter. These proteins are members of the multidrug resistance-related family of ABC transporters, primarily involved in the detoxification of metals (De Waard *et al.*, 2006). No transporters of this type have previously been associated with altered azole sensitivity, although

coexpression of an ABC transporter and a glutathione-S-transferase gene has been suggested to be involved in voriconazole detoxification in *A. fumigatus* (Ferreira *et al.*, 2006).

Microarray analysis demonstrated increased expression of mg[0271], encoding a putative hexose transporter, upon epoxiconazole treatment in G303. Expression of this gene was also shown subsequently by quantitative RT-PCR to be induced in isolate IPO323, although not to the same extent (around two-fold in IPO323 compared with six-fold in G303). Expression of this gene in four more recently obtained *M. graminicola* isolates appears to be similarly affected by azole treatment. Interestingly, the highest constitutive and induced transcript levels of mg[0271] were detected in the least sensitive isolate R6-31. Hexose transporters of the major facilitator superfamily have been previously implicated in resistance to drugs. D-Hexose transporters have been shown to modulate drug accumulation in *Xenopus* oocytes expressing murine *Mdr1b*, an ABC transporter that confers a multidrug-resistant phenotype (Vera *et al.*, 1991). *HXT9* and *HXT11*, two *S. cerevisiae* hexose transporter genes that have homology to mg[0271] (5e-12), have also been implicated in a multidrug-resistant phenotype (Nourani *et al.*, 1997). Both *HXT9* and *HXT11* are Pdr1p and Pdr3p targets, transcriptional regulators of ABC transporters required for drug resistance in yeast. Interestingly, although *HXT9* and *HXT11* are co-regulated with multidrug transporter genes, deletion of either gene decreases sensitivity to drugs, and, conversely, over-expression of *HXT11* increases sensitivity, leading to the suggestion that these proteins are not involved in drug efflux *per se*, but rather in modulating membrane permeability in cells which are over-expressing drug efflux ABC transporters (Nourani *et al.*, 1997).

Thirteen genes encoding toxicant efflux proteins, predominantly ABC transporters and MF proteins, are present on this *M. graminicola* microarray. The lack of transporters identified as azole-responsive in this study is therefore somewhat surprising. However, previous microarray studies of the transcriptional response of *S. cerevisiae* (Bammert and Fostel, 2000) and *C. albicans* (De Backer *et al.*, 2001) to azoles have also failed to detect significant up-regulation of efflux proteins. This is in contrast to earlier candidate gene approaches [e.g. the identification of *CDR1* induction in response to fluconazole in *C. albicans* (Hernaez *et al.*, 1998)]. Kontoyiannis and May (2001) suggested conditions of microarray studies, such as azole concentration and exposure time, were not optimal for detection of transporter gene expression. This might also apply to the current study, as only a single time point at a single fungicide concentration has been studied in detail. However, if a transporter is directly involved in exporting epoxiconazole out of the *M. graminiola* cell, it may simply be that it is not represented on this microarray. Up-regulation of hexose transporter (mg[0271]) in response to azole treatment in isolates with greatest reductions in azole sensitivity provides evidence for this, as studies have suggested that homologues of mg[0271] are involved in regulating membrane permeability in *S. cerevisiae* cells over-expressing efflux pumps (Nourani *et al.*, 1997).

Response of other genes

Azole resistance, for example in *C. albicans*, has been shown in both candidate gene (White, 1997) and microarray (Rogers and Barker, 2003) studies to be conferred, at least in part, by the constitutive over-expression of genes in resistant isolates. In this study, the expression of all genes identified as constitutively up-regulated in less sensitive isolate G303, for example mg[0194] and mgc03e06f, decreased upon epoxiconazole treatment. It seems unlikely therefore that these genes play a role in reducing azole sensitivity in G303, although they may confer an improved capacity of this isolate to compete, infect and reproduce compared with the sensitive isolate IPO323. For example, mg[0194] is a homologue of the yeast stress response protein rds1 (Ludin *et al.*, 1995). This gene has been shown to be highly expressed in pycnidiospores and late-stage *in planta* infection in *M. graminicola* isolate IPO323, but not *in vitro*, suggesting a specific role for this gene in reproduction and infection (Keon *et al.*, 2005a,b). EST mgc03e06f encodes a homologue of the antibiotic response protein CipA of *A. nidulans*. *CipA* expression in *A. nidulans* is responsive to concanamycin A produced by *Streptomyces* (Melin *et al.*, 2002). Therefore, the product of mgc03e06f may also afford *M. graminicola* protection against antibiotics produced by antagonistic microorganisms in the wheat phylloplane.

Microarray analyses identified one unclassified gene, mgc03g08f, as azole induced in G303. None were detected in studies of IPO323. This gene is annotated as a homologue of a *C. neoformans* mating-type locus marker, *FAO1* (Lengeler *et al.*, 2002), and has high homology to an iron/ascorbate oxidoreductase from *A. fumigatus*. Very high transcript levels of this gene (greater than 50-fold increase) were also detected in the less sensitive isolate R6-55 after epoxiconazole treatment. No members of this protein family have previously been associated with response to azole in studies of fungal pathogens. In plants, these proteins have been suggested to play a role in detoxifying ROS (Qiu *et al.*, 2004). Consistent with an effect of epoxiconazole on components of the mitochondrial respiratory chain, induction of mgc03g08f may be in response to changing the oxidative state of the cell. Further functional studies are required to determine the exact impact of the product of this gene on azole sensitivity.

CONCLUSIONS

Profiling the response of *M. graminicola* to epoxiconazole using a cDNA microarray representing around one-quarter of the genome has confirmed ergosterol biosynthesis as the primary target of this compound and, furthermore, demonstrated an additional effect on components of the mitochondrial respiratory

chain. The findings presented here are, in general, consistent with previous studies of the response of fungi to azoles. The reported anomalies, for example down-regulation of a putative *erg6* gene after azole treatment, are most probably a consequence of misannotation of the EST set. The forthcoming release of the *M. graminicola* annotated genome sequence should resolve these inconsistencies. Comparisons of constitutive and azole-induced expression profiles between an azole-sensitive and less sensitive isolate failed to identify a gene, for example a drug efflux protein, directly responsible for the reduced azole sensitivity phenotype. However, a gene encoding a hexose transporter has been shown to be more highly expressed in least sensitive isolates. Although probably not directly involved in azole efflux, this protein may modulate the membrane activity of isolates over-expressing an, as yet unidentified, efflux protein. In the future, studies using whole genome microarrays will facilitate the identification of genes, and therefore mechanisms (other than target site alteration) responsible for decreased azole sensitivity in *M. graminicola*.

EXPERIMENTAL PROCEDURES

Fungal isolates

M. graminicola isolates G303, R6-31, R6-32, R6-40 and R6-55 (Table 1) were obtained following the protocol described in Fraaije *et al.* (2007). Briefly, wheat leaves with lesions were stapled on to sterile moist filter paper, placed in up-turned Petri dishes and incubated overnight at 23 °C. Single cirri, emerging from pycnidia, were collected using sterile forceps and suspended in 30 μL of sterile distilled water. Suspensions were streaked on to yeast potato dextrose agar (YPD; ForMedium, Norwich, UK) amended with penicillin G sodium and streptomycin sulphate at 100 μg/mL and incubated for 5 days at 23 °C. Single colonies grew in the form of yeast-like budding cells. Single spore isolates were propagated by transferring single colonies to fresh YPD plates. Isolates were stored in 80% glycerol at –80 °C until epoxiconazole sensitivity testing.

Epoxiconazole sensitivity testing

Sensitivity assays were also according to Fraaije *et al.* (2007), with a few modifications. One hundred microlitres of 2× potato dextrose broth (PDB; Sigma-Aldrich, Inc., St. Louis, MO) amended with decreasing concentrations of epoxiconazole (30, 10, 3.3, 1.1, 0.37, 0.123, 0.041, 0.014, 0.005, 0.0015 and 0.0005 mg/L) was added to wells of flat-bottomed microtitre plates (TPP 92696 test plates, Trasadingen, Switzerland). After 7 days' growth at 15 °C on YPD to ensure yeast-like growth, isolates were suspended in 5 mL sterile distilled water. Aliquots of 100 μL of isolate spore suspensions (1×10^5 spores/mL) were added to each well. Plates were incubated for 4 days at 23 °C, and growth measured by absorbance at 630 nm using an MRX plate reader (Dynex Technologies, Chantilly, VA). Fungicide sensitivities were determined as 50% effective concentration (EC_{50}) using a dose–response relationship (Table 1).

CYP51 sequencing

CYP51 genes of *M. gramincola* isolates IPO323, G303, R6-31, R6-32, R6-40 and R6-55 were amplified and sequenced using the primers, PCR conditions and protocols outlined in Cools *et al.* (2005a). Amplifications were carried out using Phusion high-fidelity DNA polymerase to ensure the validity of sequences (Finnzymes, Espoo, Finland).

Growth of fungal cultures for microarray and quantitative RT-PCR analyses

M. graminicola isolate spore suspensions were added to 100 mL of PDB (Sigma) at a final concentration of 4×10^5 spores/mL. Cultures were grown at 23 °C with shaking (220 r.p.m.). After 48 h, with isolates in the linear phase of growth (data not shown), epoxiconazole was added to each culture at 2 mg/L, a concentration lethal to isolate IPO323 and around 50% effective (EC_{50}) for isolate G303. Isolate IPO323 has previously been treated at the approximate EC_{50} (0.1 mg/L) with no effect on expression profile (data not shown). This finding is in agreement with those of Jia *et al.* (2000). This treatment was therefore not continued. Tissue was harvested by filtration after either 1 or 24 h further growth at 23 °C with shaking (220 r.p.m.). Filtered tissue was snap frozen in liquid nitrogen and stored at −80 °C.

RNA extraction and microarray hybridization and scanning

Protocols for RNA extraction and microarray hybridization were essentially those described by Keon *et al.* (2005b) with some modification. Total RNA was extracted from freeze-dried *M. graminicola* tissue with TRIZOL reagent (Invitrogen, Carlsbad, CA) following the manufacturer's protocol. A subsequent overnight incubation of extracts in 4 M lithium chloride was used to purify RNA further. Labelling of isolated total RNA with reactive Alexafluor dyes was carried out using the Superscript Indirect cDNA Labelling System (Invitrogen) following the recommended protocol. Twenty micrograms of total RNA was used in each labelling reaction. Samples were labelled with either Alexafluor 555 (equivalent to Cy3) detected in the green channel, or Alexaflour 647 (equivalent to Cy5) detected in the red channel. Hybridization and washing steps were as outlined at the COGEME website (http://www.cogeme.man.ac.uk). Details of the *M. graminicola* microarray, including quality control experiments previously carried out, are described by Keon *et al.* (2005a,b). The annotated unigene set represented on the microarray is also available at

Table 5 Primers used for quantitative RT-PCR studies.

Target	Sequence (5′–3′)	
	Forward	Reverse
β-tubulin	AGAGAGCCTCGTTGTCAATGC	CGGTATGGGAACACTTCTCATCAG
CYP51/erg11	ACAACGAGCCCCATGGACGA	CGCCGATACATCTGCGTCGT
mga1012f	AGTCTAGCAGTGACGAGACCAGCAA	CTTGGTGTTCGACTTGCTCTCCTC
mg[0194]	CGCTGTCGATCAACGCTGTCTAT	CCTTGATGGGACCAGTGACAGAGT
mga0888f	ACCTCGCCTGGATCGCAAAG	TGGAGGCGCGGAAGATGTAGTA
mgc19a12f	CGCCCGGCAGTACGAACTCTA	TTGTTCCAGGCGTAAGTGGCTG
mg[0271]	TGCTCTGTGCACGTTTGCCTT	CATTACGCTCAGCCTTCTCCGA
mgc03g08f	ACGAACTTTGGCCTCAGTCAGGA	GTTCGGGAAGGAATTTGAAGACGT

the Phytopathogenic Fungi and Oomycete EST Database (http://cogeme.ex.ac.uk/). Microarray slides were scanned using an Axon 4000B (Axon Instruments, Foster City, CA) scanner with a spot size of 5 μm. Preliminary analysis of the data output using GenePix software (Axon Instruments) was used to optimize scanning parameters.

Microarray data analysis

Output data were analysed using the methods described by Keon *et al.* (2005b) with the exception that eight replicates (four replicates of two repeat experiments) of GenePix results files were analysed to determine constitutive gene expression differences between IPO323 and G303, and to compare expression data after 24 h of epoxiconazole exposure to untreated samples for both IPO323 and G303. Microarray data were normalized by per spot and per chip intensity-dependent (Lowess) normalization. Up- and down-regulated transcripts were determined by filtering on confidence at a *t*-test *P* value of 0.01 using the Benjamini and Hochberg multiple testing correction. Only genes with transcript levels differing two-fold or more were considered as differentially expressed. Blastx searches against the NCBI GenBank fungal genome database were used to verify annotations of differentially expressed genes.

Hierarchical clustering

Ratios of fluorescence (green/red) signals from eight replicate experiments after 24 h of epoxiconazole exposure, and four replicate experiments after 1 h of exposure (IPO323 treated/IPO323 untreated, and G303 treated/G303 untreated) were subtracted from fluorescent data generated for untreated IPO323. Data were logged to base two and stored in tab-delimited form. Cluster 3.0 (Eisen *et al.*, 1998) was used to cluster data hierarchically 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. Blastx searches against the NCBI GenBank fungal genome database were used to verify annotations of genes emerging from cluster analyses.

Quantitative RT-PCR analysis

Five micrograms of total RNA was reversed transcribed with oligo$(dT)_{20}$ using the SuperScript III First Strand Synthesis System (Invitrogen) according to the supplier's instructions. Quantitative RT-PCR reactions were carried using the SYBR Green Jumpstart Taq Ready Mix for Quantitative PCR (Sigma). cDNAs were diluted (1 : 10) and 5 μL used in a 25-μL reaction together with 0.25 μM of each primer (Table 5). Thermal cycling conditions were as per the manufacturer's recommendations, with an annealing temperature of 56 °C. Reactions were carried out on the ABI 7500 Real Time PCR System (Applied Biosystems) and data analysed using the 7500 SDS software (version 1.2.1, Applied Biosystems). All reactions were carried out in triplicate. Relative transcript abundances were calculated using the $2^{-[\Delta][\Delta]Ct}$ method (Muller *et al.*, 2002) with beta-tubulin as the endogenous control and untreated IPO323 as the calibrator sample. Data shown are the mean relative transcript abundances of two replicate experiments with standard deviations.

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