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Fan, J., Urban, M., Parker, J. E., Brewer, H. C., Kelly, S. L., Hammond-Kosack, K. E., Fraaije, B. A., Liu, X. and Cools, H. J. 2013. Characterization of the sterol 14alpha-demethylases of Fusarium graminearum identifies a novel genus-specific CYP51 function. *New Phytologist.* 198 (3), pp. 821-835.

The publisher's version can be accessed at:

- https://dx.doi.org/10.1111/nph.12193

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Research

Characterization of the sterol 14α-demethylases of *Fusarium graminearum* identifies a novel genus-specific CYP51 function

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Received: *19 November 2012*
Accepted: *15 January 2013*

New Phytologist (2013) **198:** 821–835
doi: 10.1111/nph.12193

Key words: azole target, CYP51 function, cytochrome P450 evolution, deoxynivalenol production, fungicide resistance, *Fusarium* head scab/blight, sterol biosynthesis, wheat infection.

Summary

- *CYP51* encodes the cytochrome P450 sterol 14α-demethylase, an enzyme essential for sterol biosynthesis and the target of azole fungicides. In *Fusarium* species, including pathogens of humans and plants, three *CYP51* paralogues have been identified with one unique to the genus. Currently, the functions of these three genes and the rationale for their conservation within the genus *Fusarium* are unknown.
- Three *Fusarium graminearum CYP51*s (*FgCYP51*s) were heterologously expressed in *Saccharomyces cerevisiae*. Single and double *FgCYP51* deletion mutants were generated and the functions of the *FgCYP51*s were characterized *in vitro* and *in planta*.
- *FgCYP51A* and *FgCYP51B* can complement yeast *CYP51* function, whereas *FgCYP51C* cannot. *FgCYP51A* deletion increases the sensitivity of *F. graminearum* to the tested azoles. In *ΔFgCYP51B* and *ΔFgCYP51BC* mutants, ascospore formation is blocked, and eburicol and two additional 14-methylated sterols accumulate. *FgCYP51C* deletion reduces virulence on host wheat ears.
- *FgCYP51B* encodes the enzyme primarily responsible for sterol 14α-demethylation, and plays an essential role in ascospore formation. *FgCYP51A* encodes an additional sterol 14α-demethylase, induced on ergosterol depletion and responsible for the intrinsic variation in azole sensitivity. *FgCYP51C* does not encode a sterol 14α-demethylase, but is required for full virulence on host wheat ears. This is the first example of the functional diversification of a fungal *CYP51*.

Introduction

The cytochrome P450 sterol 14α-demethylase (CYP51, syn. ERG11) is an essential enzyme in the biosynthesis of sterols, critical components of cell membranes of all eukaryotic organisms required for the regulation of membrane fluidity and permeability (Parks *et al.*, 1995; Lepesheva & Waterman, 2007). Of the many sterols identified in fungi, ergosterol is the most common and is required for fungal growth (Rodriguez *et al.*, 1985; Weete *et al.*, 2010). Consequently, CYP51 is a widely exploited target for the control of fungal pathogens of humans and plants, with the azole (imidazole and triazole) fungicides being the leading class of antifungals for over three decades (Sheehan *et al.*, 1999). Surprisingly, despite their widespread long-term use and single-site mode of action, incidences of resistance to azole fungicides are rare. More commonly, reductions in sensitivity are reported with cross-resistance within the azole class often incomplete. To date, three mechanisms of resistance to azoles predominate in filamentous fungi: mutations in the *CYP51* gene encoding amino acid alterations, resulting in decreased affinity of the protein for inhibitors (Loffler *et al.*, 1997; Sanglard *et al.*, 1998; Wyand & Brown, 2005; Cools *et al.*, 2010); over-expression of the target *CYP51* gene, most frequently caused by insertions in the predicted promoter regions (Mellado *et al.*, 2007; Cools *et al.*, 2012); and over-expression of genes encoding efflux pumps (Sanglard *et al.*, 1995; Kretschmer *et al.*, 2009). These mechanisms can combine, and therefore resistance levels in fungal strains are often determined by combinations of CYP51 amino acid alterations, *CYP51* gene over-expression and/or enhanced efflux (Perea *et al.*, 2001; Mellado *et al.*, 2007; Cools *et al.*, 2012).

Until recently, *CYP51* was thought to exist as a single gene in all phyla. Mammalian genomes, for example, contain one *CYP51*, with some nonfunctional pseudogenes identified (Rozman *et al.*, 1996). However, increasing genome sequence information has shown that this is not the case in all kingdoms. To date, multiple *CYP51* genes have been found in plants, including rice (12), oats (two), tobacco (two) and *Arabidopsis*

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thaliana (two) (Lepesheva & Waterman, 2007). Filamentous fungi, particularly Ascomycetes, often possess two or more *CYP51* paralogues, for example in *Penicillium digitatum* (two), *Aspergillus fumigatus* (two), *A. nidulans* (two), *A. flavus* (three), *Magnaporthe oryzae* (two) and species of *Fusarium*, including *F. verticillioides*, *F. oxysporum* f. sp. *lycopersici* and *F. graminearum* (three). Molecular phylogenetic analysis has shown that the *CYP51* genes of fungi within the subphylum Pezizomycotina fall into three clades, designated A, B and C (Becher *et al.*, 2011). All species possess a *CYP51* in clade B (*CYP51B*). Species with multiple paralogues carry an additional *CYP51* in clade A (*CYP51A*), with duplications of *CYP51A* or *CYP51B* generating the third paralogue in some species, for example *A. flavus* and *A. terreus*, respectively. Uniquely, the third *CYP51* paralogue in *Fusarium* species forms a distinct clade, *CYP51C*. The *CYP51C* gene is found exclusively in *Fusarium* species, and is ubiquitous across the genus, as demonstrated by its use as a reliable phylogenetic marker (Fernández-Ortuño *et al.*, 2010).

Fungi with multiple *CYP51*s are intrinsically resistant to some azoles, although some remain effective. For example, *A. fumigatus* is well controlled by itraconazole and voriconazole, whereas fluconazole is ineffective. Species of *Fusarium*, for example *F. solani*, are resistant to commonly used medical azoles, including fluconazole, voriconazole and the recently introduced posaconazole (Nucci & Anaissie, 2007). Deletion of *CYP51A* increases the intrinsic sensitivity to some azoles in *M. oryzae* (e.g. tebuconazole and prochloraz), *A. fumigatus* (e.g. fluconazole and ketoconazole) and *F. graminearum* (e.g. tebuconazole and prochloraz) (Mellado *et al.*, 2005; Liu *et al.*, 2011; Yan *et al.*, 2011). Furthermore, resistance to effective azoles in fungi with multiple *CYP51*s is most frequently mediated by changes in the *CYP51A* paralogue. For example, over-expression of *CYP51A* has been reported for resistant isolates of *P. digitatum* (Hamamoto *et al.*, 2000; Ghosoph *et al.*, 2007), and mutation of *AfCYP51A* is the most common mechanism of resistance in *A. fumigatus* isolates (Diaz-Guerra *et al.*, 2003; Mellado *et al.*, 2005), which, when combined with *AfCYP51A* over-expression, confers a multi-azole-resistant phenotype (Mellado *et al.*, 2007; Snelders *et al.*, 2008).

The control of *F. graminearum*, the most important pathogen causing *Fusarium* head blight (FHB) or head scab disease on wheat and barley, is primarily dependent on effective azole fungicides (e.g. tebuconazole, metconazole and prothioconazole). Effective application of azoles reduces the content of the harmful trichothecene mycotoxin deoxynivalenol (DON) in wheat grains (Beyer *et al.*, 2006; Paul *et al.*, 2008). However, an increased level of DON has been detected after treatment with sublethal doses of prothioconazole both *in vitro* and *in planta* (Audenaert *et al.*, 2010), and trichothecenes accumulate in grain samples after treatment with tebuconazole (Kulik *et al.*, 2012). In addition, a study using the enhanced green fluorescent protein gene (*egfp*) as a reporter demonstrated activation of the *F. graminearum* trichothecene biosynthetic *TRI5* gene by sublethal concentrations of tebuconazole (Ochiai *et al.*, 2007); both *TRI4* and *TRI5* transcript levels were higher after tebuconazole treatment in culture (Kulik *et al.*, 2012). Currently, although stress responses and enhanced secondary metabolism have been proposed, the mechanism(s) responsible for altered mycotoxin production after azole treatment is unknown.

Functional analysis of multiple *CYP51*s has identified roles additional to primary sterol biosynthesis. In oats, a *CYP51* homologue, *AsCyp51H10*, is dispensable for sterol biosynthesis, but is required for the synthesis of avenacins, antimicrobial compounds unique to the genus *Avena* (Qi *et al.*, 2006). Heterologous expression in yeast identified *AsCyp51H10* as the first CYP51 not classified as a sterol demethylase (Kunii *et al.*, 2012). Recent analysis of the 12 *CYP51*s of rice has differentiated some genes as *CYP51H*, a group likely to have functions beyond sterol biosynthesis (Inagaki *et al.*, 2011). Previous work on the *CYP51* genes of *F. graminearum* (*FgCYP51*) has demonstrated that the deletion of individual *FgCYP51* genes can reduce conidiation, but otherwise causes no changes in *in vitro* morphology, mycelial growth rate or ergosterol content (Liu *et al.*, 2011).

In this study, we have determined the competence of the three paralogous *FgCYP51* genes to act as sterol 14α-demethylases by heterologous expression in *S. cerevisiae* strain YUG37:*erg11*, which carries a regulatable promoter controlling native *CYP51* expression, and found that *FgCYP51C* cannot complement the CYP51 function of the yeast gene. We generated single (*ΔFgCYP51A*, *ΔFgCYP51B* and *ΔFgCYP51C*) and combined (*ΔFgCYP51AC* and *ΔFgCYP51BC*) *CYP51* deletion mutants and characterized their function *in vitro* and *in planta*. We report distinct roles of the three *FgCYP51* genes, with the *FgCYP51C* gene specifically required for full virulence on host wheat ears, but not on *Arabidopsis* floral tissue or the fruits of apple and tomato.

Materials and Methods

Fungal strains, growth assays and sporulation tests

Fusarium graminearum isolates (Table 1) used in this study were routinely cultured on synthetic nutrient-poor agar (SNA) plates, as described previously (Urban *et al.*, 2002). Conidiation assays were performed on SNA plates, and the spores were germinated in YPS medium (0.3% (w/v) yeast extract, 0.3% (w/v) bactopeptone and 20% (w/v) sucrose) (Cuzick *et al.*, 2008). Colony morphology was assayed on potato dextrose agar (PDA) plates in the dark at 22°C. Growth rate tests were conducted in potato dextrose broth (PDB) and Czapek Dox liquid medium (CZD) using an amended 96-well microtitre plate method. Absorbance measurements were made every 8 h for 96 h at 22°C (Fraaije *et al.*, 2007). Carrot agar plates were used for perithecia formation and ascospore production (Ehrenshaft *et al.*, 1995). Approximately 50 asci per strain/mutant were scraped from the plates and physically broken to determine ascospore formation.

Heterologous expression of *FgCYP51* genes in *S. cerevisiae YUG37::erg11*

Heterologous expression of *FgCYP51* genes in *S. cerevisiae* YUG37::*erg11* followed the method described by Cools *et al.* (2010). *FgCYP51* genes were amplified from cDNA of

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Table 1 Fungal strains/mutants used in this study

Strain	Description	Reference
Fg1955	Wild-type, Finland origin	Nicolaisen *et al.* (2009)
PH-1	Wild-type, USA origin	Cuomo *et al.* (2007)
ΔFgCYP51A4	CYP51A gene deletion mutant	This study
ΔFgCYP51A5	CYP51A gene deletion mutant	This study
ΔFgCYP51B3	CYP51B gene deletion mutant	This study
ΔFgCYP51B4	CYP51B gene deletion mutant	This study
ΔFgCYP51C2	CYP51C gene deletion mutant	This study
ΔFgCYP51C3	CYP51C gene deletion mutant	This study
ΔFgCYP51AC1	CYP51AC gene deletionmutant	This study
ΔFgCYP51AC3	CYP51AC gene deletion mutant	This study
ΔFgCYP51BC2	CYP51BC gene deletion mutant	This study
ΔFgCYP51BC3	CYP51BC gene deletion mutant	This study
ΔFgCYP51BC4	CYP51BC gene deletion mutant	This study

F. graminearum isolate Fg1955 (Nicolaisen *et al.*, 2009) with the primers shown in Supporting Information Table S1 designed and cloned into the yeast expression vector pYES3/CT (Invitrogen). Plasmids were transformed into *S. cerevisiae* strain YUG37::*erg11* (Revankar *et al.*, 2004) using an S.c. EasyComp transformation kit (Invitrogen) with the pYES3/CT vector as a negative control. The complementation efficiency of two independent transformants per construct was screened on Synthetic Drop-out (SD) galactose (GAL) + raffinose (RAF) medium containing 3 μg ml^{-1} doxycycline (Sigma-Aldrich) by droplet inoculation with 5 μl of cell suspensions (six five-fold dilutions of a starting concentration of 1×10^6 cells ml^{-1}) for each transformant and incubation for 6 d at 30°C. The growth rate of YUG37::*erg11* transformants was determined by inoculation of liquid SD GAL + RAF medium containing 6 μg ml^{-1} doxycycline with 100 μl of a cell suspension of 1×10^6 cells ml^{-1} (grown for 24 h at 30°C). Absorbance was measured every 4 h for 96 h at 30°C and the growth rate was calculated at the linear growth stage.

Generation of gene deletion mutants

FgCYP51 gene deletion mutants were generated in *F. graminearum* strain PH-1 (NRRL 31084) (Cuomo *et al.*, 2007), the genome of which has been sequenced, using polyethyleneglycol (PEG)-mediated protoplast transformation of split-marker PCR fragments (Catlett *et al.*, 2003). The primers used to amplify the flanking sequences for each gene are listed in Table S1. PCR products were transformed into protoplasts of wild-type PH-1 to delete single *FgCYP51* genes. Transformants were selected with 75 μg ml^{-1} hygromycin B or geneticin. The split hygromycin markers of the *FgCYP51A* and *FgCYP51B* genes were transformed into the *FgCYP51C* gene deletion mutants carrying a geneticin selectable marker to generate the double deletion mutants, *ΔFgCYP51AC* and *ΔFgCYP51BC*. Candidate transformants were screened using 5′ flank and 3′ flank PCRs to confirm the targeted deletion of the genes (Fig. S1). An additional PCR to amplify the 5′ flank and 3′ flank of the nontargeted *FgCYP51* gene confirmed that the remaining *FgCYP51* genes were not disrupted. Double deletion of *FgCYP51A* and *FgCYP51B* was considered to be lethal.

Fungicide sensitivity testing

Fungicide sensitivities were tested using 96-well microtitre plate sensitivity assays (Fraaije *et al.*, 2007). Briefly, spore suspensions of *Fusarium* isolates with 2×10^4 spores ml^{-1} were added to PDB amended with serial diluted concentrations of fungicides (Table S2). For *S. cerevisiae* transformants, SD GAL + RAF medium containing 6 μg ml^{-1} doxycycline (Sigma-Aldrich) and amended with the serial diluted concentrations of fungicides was inoculated with 1×10^6 cells ml^{-1} (Table S2) (Cools *et al.*, 2010). The absorbance was measured at 630 nm using a FLUOstar OPTIMA microplate reader (BMG Labtech GmbH, Offenburg, Germany) after 96 h of incubation at room temperature for *F. graminearum* and at 30°C for *S. cerevisiae* transformants. Fungicide sensitivities were determined as the 50% effective concentration (EC_{50}) using a dose–response relationship according to the BMG Labtech Optima Software. Three independent replicates were performed for statistical analysis.

Gene expression analysis

Total RNA was extracted from freeze–dried samples with TRIzol reagent (Invitrogen), followed by a 4 M lithium chloride purification and DNase I treatment. For *in vitro FgCYP51* expression analysis, RNA was extracted from mycelia of isolate Fg1955 grown in PDB for 24, 48, 72 and 96 h in the absence of azole, or after treatment with tebuconazole, epoxiconazole or prochloraz at the calculated EC_{50} at 24 h of incubation. *In vitro* gene expression of Δ*FgCYP51* mutants used RNA extracted after 48 h of growth in PDB, compared with expression in wild-type parent strain PH-1. For *in planta* expression analysis, RNA was extracted from inoculated wheat ears harvested at 7 d post-inoculation (dpi). First-strand cDNA was prepared using the SuperScript III first-strand synthesis system with oligo(dT) (Invitrogen). Real-time PCR amplifications were performed on an ABI 7500 sequence detection system (Applied Biosystems, Foster City, CA, USA) using SYBR Green I fluorescent dye detection. The relative quantities (RQs) were calculated using the $2^{-\Delta\Delta C_t}$ method with *F. graminearum actin* and *β-tubulin* as reference genes

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and the statistical analysis of three biological replicates. The primers used for quantitative PCR are listed in Table S1.

Sterol extraction and analysis

Fusarium graminearum strains PH-1 and Δ*FgCYP51* mutants were collected after growth on PDB medium at 23°C for 48 h. Nonsaponifiable lipids were extracted as described previously (Kelly *et al.*, 1995). Briefly, pellets were resuspended in 2.5 ml of methanol, followed by the addition of 1.5 ml of 60% (w/v) KOH and 1 ml of 5 mg ml^{-1} pyrogallol (in methanol). Samples were refluxed at 90°C for 2 h, and then extracted twice with hexane and dried in a rotary evaporator (Heto, Allerod, Denmark). Samples were derivatized by the addition of 100 μl of 90% bis(trimethylsilyl)-trifluoroacetamide (BSTFA) – 10% trimethylsilyl (TMS) (Sigma-Aldrich) and 50 μl of anhydrous pyridine (Sigma-Aldrich) and heating for 2 h at 80°C. Gas chromatography-mass spectrometry was performed using a VG12-250 mass spectrometer (VG Biotech, Mumbai, India) with splitless injection and chemical ionization. Data were analysed using an MSD Enhanced ChemStation (Agilent Technologies), and TMS esters of individual sterols were identified by reference to relative retention times, mass ions and fragmentation patterns. Data present the relative composition in total sterol in each sample. Three biological replicates were performed.

Plant disease assays

Wheat ear infection, gene expression and DON concentration measurement Wheat (*Triticum aestivum* L., cv Bobwhite) plants were grown and infected by point inoculation as described previously (Urban *et al.*, 2003). The 11th and 12th flowering spikelets counted from the stem were inoculated with 5 μl of 5×10^4 spores per spikelet or mock inoculated with sterile water. The number of infected spikelets was recorded at 3, 7, 11, 16 and 21 dpi. For measurements of DON concentration and *in planta* gene expression assays, 14 flowering spikelets were inoculated per ear. The infected spikelets were harvested at 7 dpi for gene expression analyses and at 10 dpi for DON measurements. Samples were lyophilized and infected spikelets were ground to a fine powder in liquid nitrogen using a mortar and pestle. The powder was resuspended in water as described previously (Urban *et al.*, 2003). After incubation at 30°C for 30 min with shaking at 80 rpm, the solutions were centrifuged and the supernatants were analysed quantitatively for DON content using an EZ-Quant Vomitoxin (DON) plate kit (Diagnostix, Ontario, Canada), according to the manufacturer's instructions. Three wheat ears of different wheat plants were inoculated for each strain/mutant in each replicate experiment. Three independent replicate experiments (total of nine ears per strain/mutant inoculation) were carried out and analysed statistically.

***Arabidopsis* floral inoculations** *Arabidopsis* plants (ecotype Landsberg) were grown and inoculated as described previously (Cuzick *et al.*, 2008). Five-week-old flowering plants were spray inoculated with *c.* 0.5 ml of conidial suspensions of 1×10^5 spores ml^{-1} of wild-type PH-1 or *ΔFgCYP51* strains, and incubated in a 100% relative humidity box. Each isolate was sprayed onto three plants per experiment, with two experimental replicates performed (total of six plants per strain/mutant inoculation), with sterile water as a negative control. Disease symptoms on apical flowers and siliques were assessed using the *Fusarium*–Arabidopsis disease (FAD) scoring system (Urban *et al.*, 2002), with the modification of combining old and new silique scores. Floral and silique scores were added to give the total FAD score shown in Table S3.

Infection assays on apple fruit sections The ability of Δ*FgCYP51* mutants to colonize apple fruit sections relative to strain PH-1 was tested on cv Braeburn. After surface sterilization with 70% ethanol, apples were sliced into 8-mm-thick sections and inoculated in three discrete areas with a 5-μl droplet of a spore suspension containing 5×10^5 ml^{-1} conidia of wild-type isolate PH-1, sterile water control or a *ΔFgCYP51* gene mutant. In each experiment, three apple slices were inoculated for each mutant. Three independent experiments were carried out. Apple slices were sealed in a 9-cm Petri dish and incubated at 28°C for 6 d.

Infection assays on tomato fruits To assay the colonization of ripe tomato fruits (cultivar Encore) by *ΔFgCYP51* mutants, three wounds were made with a hyperdermic needle (BD microlance 23G 10.6×25) on 70% ethanol surface-sterilized tomatoes. Fruits were inoculated with 3×5 μl of 5×10^5 ml^{-1} conidia suspensions and incubated at 28°C in a sealed plastic container at 100% humidity for 6 d (Urban *et al.*, 2003). For each strain/mutant, three tomatoes were inoculated in each of three independent experiments. Mock inoculations were with sterile distilled water.

Statistical analysis

Data were analysed from two or three independent replicates using ANOVA. Following a significant *F*-test result ($P < 0.0001$ for plant infection, $P < 0.05$ for fungicide sensitivity tests and *in vitro*/*in planta* gene expression), means of independent replicates were compared using least-significant differences (LSD). No transformation of the data was required. $P < 0.05$ was considered to be significant. Greek letters were used to mark statistically significant differences ($P < 0.05$).

Results

FgCYP51A expression is induced by azole fungicides *in vitro*

In the absence of fungicide, transcript levels of *FgCYP51A* and *FgCYP51B* were highest at 72 h of incubation (Fig. 1). *FgCYP51C* expression did not change significantly across the time course. Twenty-four hours after treatment (48 h after inoculation) with subinhibitory concentrations of azoles, the expression of *FgCYP51A* was induced up to 30-fold (prochloraz and tebuconazole) or 100-fold (epoxiconazole) relative to the untreated control. *FgCYP51B* and *FgCYP51C* transcript levels did not change significantly after azole treatment.

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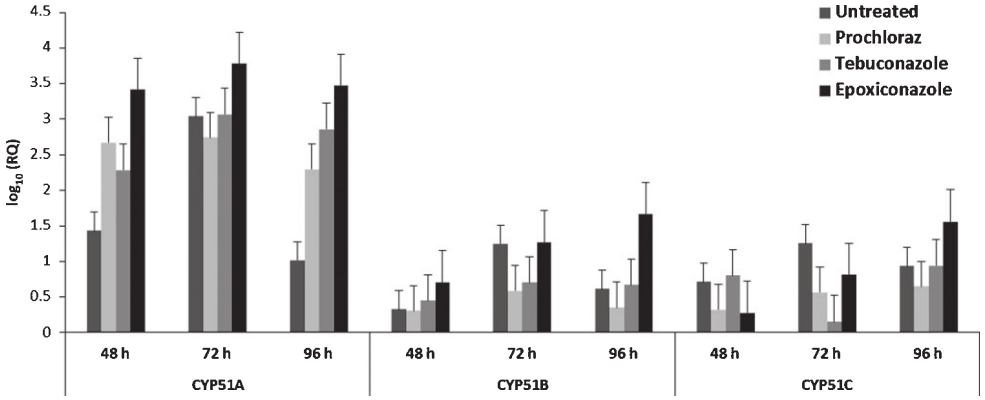

Fig. 1 Time course of *in vitro* constitutive and induced *FgCYP51* expression. Growth of *Fusarium graminearum* was in potato dextrose broth (PDB) liquid medium. Treatment with prochloraz, tebuconazole or epoxiconazole at EC_{50} concentration in the linear growth stage at 24 h of incubation, followed by sample collection at 24, 48, 72 and 96 h of incubation. The relative quantification (RQ) of the *FgCYP51A*, *FgCYP51B* and *FgCYP51C* genes was analysed using the $2^{-\Delta\Delta G}$ method with *actin* as the reference gene and untreated 24 h of incubation as a calibrator. The error bars show ± SE of three biological replicates.

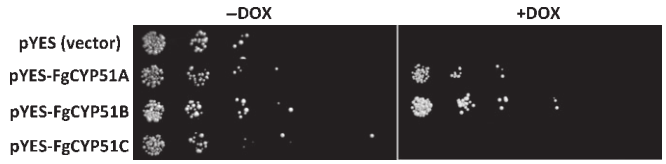

Fig. 2 Complementation of *Saccharomyces cerevisiae* strain YUG37::*erg11* with *FgCYP51A*, *FgCYP51B* or *FgCYP51C*. The growth of cells (six five-fold dilutions of a starting concentration of 1×10^6 cells ml^{-1}) in the absence (–DOX) and presence (+DOX) of doxycycline is shown. Yeast expression vector pYES3/CT without the *FgCYP51* gene was transformed into the yeast strain as a negative control.

Heterologous expression of *FgCYP51* genes in *S. cerevisiae* strain YUG37::*erg11*

FgCYP51A, *FgCYP51B* and *FgCYP51C* were expressed in *S. cerevisiae* strain YUG37::*erg11*, in which native *CYP51* gene (*ScCYP51*) expression is under the control of a doxycycline-repressible promoter (Revankar *et al.*, 2004). *FgCYP51A* and *FgCYP51B* genes were able to complement *ScCYP51* function; however, no growth was observed with transformants expressing *FgCYP51C* on doxycycline amended medium, a phenotype similar to the empty vector control (Fig. 2). Yeast transformants expressing *FgCYP51A* grew more slowly than those expressing *FgCYP51B* in the presence of doxycycline (Table 2). Yeast transformants expressing *FgCYP51A* were less sensitive to epoxiconazole than to tebuconazole and prochloraz. However, the sensitivity of *FgCYP51B* transformants was similar to all three azoles (Table 3). There was no difference in cycloheximide sensitivity, a non-azole fungicide, of yeast transformants expressing *FgCYP51A* relative to *FgCYP51B*.

In vitro growth characteristics of *FgCYP51* deletion strains

There were no differences in growth rate between mutants *ΔFgCYP51A*, *ΔFgCYP51B*, *ΔFgCYP51C*, *ΔFgCYP51AC* and *ΔFgCYP51BC* (Table 4) and the wild-type parental strain PH-1 in PDB or CZD (Table 4). Nor were there morphological differences on SNA plates under UV light (data not shown), or for single *FgCYP51* deletion strains on PDA incubated in the dark (Fig. 3a), confirming the findings of Liu *et al.* (2011). However, the morphology of double gene deletion mutants uniquely generated in this study (*ΔFgCYP51AC* and *ΔFgCYP51BC*) on PDA incubated in the dark (Fig. 3a) was different. Growth was slower and the colony pigment was yellow rather than the pink of wild-type and single gene deletion strains. Although *ΔFgCYP51AC*> and *ΔFgCYP51BC* strains produced around half the number of conidia of the PH-1 strain, conidial morphologies (data not shown) and germination rates were normal (Table 4).

Table 2 Growth rate of yeast transformants expressing *FgCYP51* genes of *Fusarium graminearum*

	Growth rate maximum[a]	
Transformants	–DOX[b]	+DOX
pYES-Fg51B	8.6 ± 0.2	3.5 ± 0.1[c]
pYES-Fg51A	8.7 ± 0.2	1.6 ± 0.1[d]
pYES-Fg51C	11.4 ± 0.0	0.1 ± 0.0[d]
pYES (Vector)	10.7 ± 1.4	0.0 ± 0.0[d]

[a]Values represent the greatest increase in the optical density at 600 nm (OD_{600}) in the absence or presence of doxycycline over a 4-h period measurement, and are means of two transformants from two independent replicates ± SD.
[b]Calculation at linear growth period (16–28 h).
[c]Calculation at linear growth period (26–52 h).
[d]Calculation at linear growth period (40–76 h).

FgCYP51B is essential for ascospore production

Perithecia were produced by all strains with no morphological differences relative to PH-1. However, 30 d after inoculation, no ascospores oozed from perithecia of *ΔFgCYP51B* and *ΔFgCYP51BC* and, when perithecia were physically broken, no ascospores were found (Fig. 3b). Ascospores produced by *ΔFgCYP51A*, *ΔFgCYP51C* and *ΔFgCYP51AC* showed wild-type morphology and germinated normally (data not shown).

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Table 3 Azole sensitivity of *Fusarium graminearum* isolates and *Saccharomyces cerevisiae* YUG37:*erg11* transformants

Isolate/yeast transformant	EC_{50} (μg ml^{-1})[a]			
	Prochloraz	Tebuconazole	Epoxiconazole	Cycloheximide
Fg1955[b]	0.0264 ± 0.0025	0.3410 ± 0.0010	1.4550 ± 0.1050	–
pYES-Fg51A[c]	0.0043 ± 0.0003	0.0063 ± 0.0003	0.0261 ± 0.0089	0.0537 ± 0.0005
pYES-Fg51B[c]	0.0160 ± 0.0031	0.0351 ± 0.0054	0.0103 ± 0.0044	0.0590 ± 0.0005

[a]EC_{50} values (μg ml^{-1}) are means of EC_{50} from two independent replicates ± SD.
[b]*Fusarium graminearum* isolate.
[c]*Saccharomyces cerevisiae* YUG37:*erg11* transformants expressing *FgCYP51* genes.
–, means not measured.

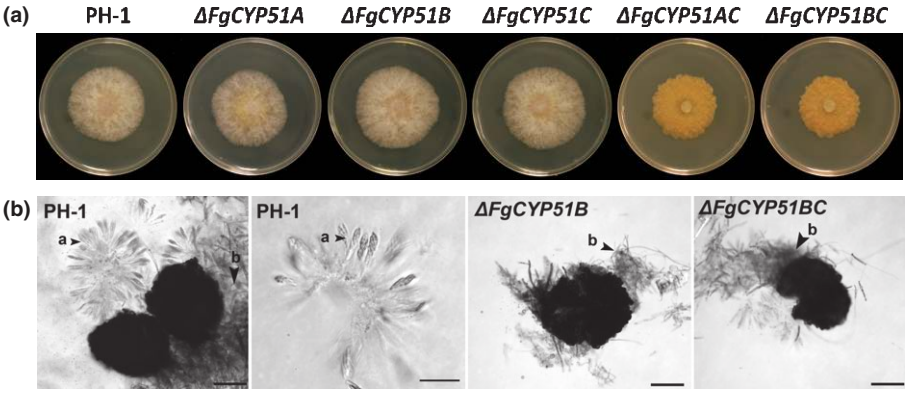

Fig. 3 *In vitro* characteristics of *Fusarium graminearum* wild-type PH-1 and *FgCYP51* gene deletion mutants. (a) Hyphal morphology after incubation on potato dextrose agar (PDA) in the dark. (b) Ascospore formation in perithecia of *F. graminearum* wild-type PH-1 and *ΔFgCYP51B* and *ΔFgCYP51BC* gene deletion mutants. Arrows show: a, asci with eight ascospores; b, hyphae forming the black pigmented perithecia.

Table 4 Phenotype characteristics of *FgCYP51* gene deletion mutants

Strain/mutant	Growth rate[a]		Conidiation[b] (10^5 spores per plate)	Conidia germination[c] (%)
	CZD medium	PDB medium		
PH-1	$0.0145 \pm 0.0005^{\alpha*}$	$0.3664 \pm 0.0046^{\alpha}$	$38.2 \pm 5.6^{\alpha}$	$94.7 \pm 0.8^{\alpha}$
ΔFgCYP51B	$0.0135 \pm 0.0019^{\alpha}$	$0.3499 \pm 0.0705^{\alpha}$	$27.5 \pm 4.8^{\beta}$	$94.4 \pm 1.1^{\alpha}$
ΔFgCYP51A	$0.0124 \pm 0.0011^{\alpha}$	$0.3560 \pm 0.0141^{\alpha}$	$33.8 \pm 7.4^{\alpha\beta}$	$94.6 \pm 0.4^{\alpha}$
ΔFgCYP51C	$0.0112 \pm 0.0002^{\alpha}$	$0.3304 \pm 0.0032^{\alpha}$	$28.7 \pm 3.8^{\alpha\beta}$	$94.7 \pm 0.5^{\alpha}$
ΔFgCYP51AC	$0.0114 \pm 0.0001^{\alpha}$	$0.3627 \pm 0.0060^{\alpha}$	$18.1 \pm 3.2^{\gamma}$	$94.4 \pm 0.6^{\alpha}$
ΔFgCYP51BC	$0.0124 \pm 0.0002^{\alpha}$	$0.3301 \pm 0.0024^{\alpha}$	$19.4 \pm 2.5^{\gamma}$	$94.8 \pm 0.7^{\alpha}$

[a]Values represent the greatest increase in the optical density at 600 nm (OD_{600}) in Czapek Dox medium (CZD) or potato dextrose broth (PDB) medium over an 8-h period and are means for two transformants from two independent replicates ± SD. The growth rate in CZD medium was calculated at the linear growth period (36–72 h), and the growth rate in PDB medium was calculated at the linear growth period (24–60 h).
[b]Conidia produced from 7 d of incubation on synthetic nutrient-poor agar (SNA) plate with 10^4 conidia inoculation.
[c]Percentage of germinated conidia was measured after 16 h of incubation in YPS medium at room temperature.
*Data were analysed using ANOVA ($P < 0.05$, F test). Different Greek letters are used to mark statistically significant differences between strains/mutants ($P < 0.05$, least-significant difference (LSD)).

Contribution of *FgCYP51A* and *FgCYP51B* to intrinsic differences in *F. graminearum* azole sensitivity

According to the sensitivity of *ΔFgCYP51A* and *ΔFgCYP51B* mutants, the seven azole fungicides tested can be divided into two groups (Table 5). Sensitivity to group 1, which includes tebuconazole, epoxiconazole, propiconazole and imazalil, is only affected significantly by *FgCYP51A* deletion. For example, *ΔFgCYP51A* strains are > 30-fold more sensitive to epoxiconazole than are wild-type and *ΔFgCYP51B* strains. Group 2 includes metconazole, prochloraz and difenoconazole. Deletions of both *FgCYP51A* and *FgCYP51B* increased the sensitivity to these compounds. There was no significant difference in the sensitivity of single *FgCYP51C* gene deletion mutants to all the azoles tested relative to the wild-type PH-1, in contrast with previous reports (Liu *et al.*, 2011). In addition, there were no significant differences in azole sensitivity between *ΔFgCYP51A* and *ΔFgCYP51AC*, confirming that *FgCYP51C* has no effect on azole sensitivity.

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Table 5 Azole sensitivity of *Fusarium graminearum* wild-type isolates PH-1 and *FgCYP51* gene deletion mutants[a]

	Metconazole		Prochloraz		Difenoconazole		Tebuconazole		Epoxiconazole		Propiconazole		Imazalil	
Strain/mutant	EC_{50} (μg ml^{-1})	RF	EC_{50} (μg ml^{-1})	RF	EC_{50} (μg ml^{-1})	RF	EC_{50} (μg ml^{-1})	RF	EC_{50} (μg ml^{-1})	RF	EC_{50} (μg ml^{-1})	RF	EC_{50} (μg ml^{-1})	RF
PH-1	$0.0284 \pm 0.0022^{\alpha*}$	1	$0.0250 \pm 0.0026^{\alpha}$	1	$0.6350 \pm 0.0600^{\alpha}$	1	$0.2044 \pm 0.0220^{\alpha}$	1	$0.4327 \pm 0.0422^{\beta}$	1	$0.4629 \pm 0.0536^{\beta}$	1	$0.4963 \pm 0.0415^{\alpha}$	1
ΔFgCYP51A	$0.0132 \pm 0.0029^{\beta}$	0.5	$0.0099 \pm 0.0020^{\gamma}$	0.4	$0.2855 \pm 0.0400^{\gamma}$	0.5	$0.0668 \pm 0.0137^{\beta}$	0.3	$0.0139 \pm 0.0036^{\gamma}$	0.03	$0.0908 \pm 0.0031^{\gamma}$	0.2	$0.0668 \pm 0.0249^{\beta}$	0.1
ΔFgCYP51B	$0.0083 \pm 0.0009^{\gamma}$	0.3	$0.0116 \pm 0.0017^{\gamma}$	0.5	$0.4528 \pm 0.0513^{\beta}$	0.7	$0.1783 \pm 0.0623^{\alpha}$	0.8	$0.3893 \pm 0.0188^{\beta}$	0.9	$0.4273 \pm 0.0391^{\beta}$	0.9	$0.5710 \pm 0.1160^{\alpha}$	1.1
ΔFgCYP51C	0.0298 ± 0.0024^{a}	1.0	$0.0240 \pm 0.0039^{\alpha}$	1.0	$0.5348 \pm 0.0784^{\alpha}$	0.8	$0.2284 \pm 0.0420^{\alpha}$	1.1	$0.3923 \pm 0.0320^{\beta}$	0.9	$0.4492 \pm 0.0792^{\beta}$	1.0	$0.5770 \pm 0.0790^{\alpha}$	1.1
ΔFgCYP51AC	$0.0158 \pm 0.0006^{\beta}$	0.6	$0.0136 \pm 0.0012^{\gamma}$	0.5	$0.2960 \pm 0.0423^{\gamma}$	0.5	$0.1153 \pm 0.0084^{\beta}$	0.6	$0.0268 \pm 0.0013^{\gamma}$	0.06	$0.0613 \pm 0.0070^{\gamma}$	0.1	$0.0406 \pm 0.0131^{\beta}$	0.1
ΔFgCYP51BC	$0.0140 \pm 0.0037^{\beta}$	0.5	$0.0190 \pm 0.0034^{\beta}$	0.8	$0.3683 \pm 0.0359^{\beta\gamma}$	0.6	$0.2053 \pm 0.0394^{\alpha}$	1.0	$0.5470 \pm 0.0351^{\alpha}$	1.3	$0.5703 \pm 0.0526^{\alpha}$	1.2	$0.5066 \pm 0.0688^{\alpha}$	1.0

[a]EC_{50} (μg ml^{-1}) values are the means of two transformants from three independent replicates ± SD. Mean resistance factor (RF), representing the sensitivity change of each isolate, was calculated as the fold change in EC_{50} compared with wild-type PH-1.
*Data were analysed using ANOVA ($P < 0.05$, *F* test). Different Greek letters are used to mark statistically significant differences between strains/mutants ($P < 0.05$, least-significant difference (LSD)).

Deletion of *FgCYP51B* and *FgCYP51C* decreases sensitivity to fenhexamid, an erg27 inhibitor

Testing the sensitivity to other ergosterol biosynthesis inhibitors (EBIs, Table 6) revealed no differences in amphotericin B sensitivity, an inhibitor that binds to ergosterol (Gray *et al.*, 2012), between PH-1 and the *FgCYP51* deletion mutants, suggesting no differences in ergosterol content between strains. *ΔFgCYP51B* and *ΔFgCYP51C* mutants were significantly less sensitive to fenhexamid, an erg27 inhibitor, relative to the wild-type strain. The *ΔFgCYP51BC* double mutants were least sensitive. There was no effect of *FgCYP51A* deletion on fenhexamid sensitivity. For all single *FgCYP51* deletion mutants, there was no difference in sensitivity to the erg1 inhibitor terbinafine relative to the wild-type strain PH-1. However, the double gene deletion mutants, *ΔFgCYP51AC* and *ΔFgCYP51BC*, were significantly less sensitive to terbinafine. There were no differences in sensitivity between mutant strains and the wild-type to the other EBIs tested, or to the β-tubulin assembly inhibitor carbendazim.

Sterol profiles of PH-1 and the *ΔFgCYP51* strains

Fourteen sterols were detected in the mycelia of the wild-type *F. graminearum* isolate PH-1 (Table 7). The most abundant sterol was ergosterol (72.98%), followed by 4,4-dimethyl ergosta-8,24(28)-dienol (5.24%), eburicol (3.46%), lanosterol (2.15%), 4-methyl ergosta-8,24-dienol (4.01%), brassicasterol (2.46%) and other minor sterols (< 2%). A proposed ergosterol biosynthesis pathway, based on the compounds detected in *FgCYP51* deletion mutants and the wild-type PH-1 (Table 7), and the reported sterol biosynthesis pathways of *Candida albicans*, *A. fumigatus* and *Paracoccidiodes brasiliensis*, is shown in Fig. 4 (Visbal *et al.*, 2003; Alcazar-Fuoli *et al.*, 2008; Martel *et al.*, 2010a; Weete *et al.*, 2010).

The *ΔFgCYP51B* and *ΔFgCYP51BC* mutants accumulated significant amounts of eburicol, the CYP51 substrate commonly seen in filamentous fungi (Bean *et al.*, 2009). There was no difference in eburicol content in the *ΔFgCYP51A* mutant relative to PH-1 and, in the *ΔFgCYP51C* and *ΔFgCYP51AC* mutants, the eburicol content was reduced relative to PH-1. The product of CYP51, 4,4-dimethyl ergosta-8,14,24(28)-trienol, was less abundant in all *ΔFgCYP51* mutants relative to the wild-type, with the greatest reduction detected in *ΔFgCYP51BC*. Two additional 14-methylated sterols (4,4,14-trimethyl ergosta-trienol and 4,4,14-trimethyl ergosta-dienol) were only detected in *ΔFgCYP51B* and *ΔFgCYP51BC*. These are likely to have arisen from accumulated eburicol, through the desaturation of bonds in the sterol backbone by ERG4 (C-24 reductase) and/or ERG5 (C-22 desaturase) or, possibly, these actions combined with a rearrangement of double bonds by ERG2 (Δ^{8-7} isomerase). In addition, the content of episterol was less in both *ΔFgCYP51B* and *ΔFgCYP51BC*, although higher in *ΔFgCYP51A*, *ΔFgCYP51C* and *ΔFgCYP51AC* mutants. Other 14-demethylated sterols more abundant in *ΔFgCYP51C* and *ΔFgCYP51AC* mutants relative to PH-1 included episterol, ergosta-5,7,24(28)-trienol and ergosta-

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Table 6 Ergosterol biosynthesis inhibitor (EBI) sensitivity of *Fusarium graminearum* wild-type isolate PH-1 and *FgCYP51* gene deletion mutants[a]

	Amphotericin B		Terbinafine		Fenhexamid		Fenpropimorph		Carbendazim	
Strain/mutant	EC_{50} (μg ml^{-1})	RF	EC_{50} (μg ml^{-1})	RF	EC_{50} (μg ml^{-1})	RF	EC_{50} (μg ml^{-1})	RF	EC_{50} (μg ml^{-1})	RF
PH-1	$0.1034 \pm 0.0125^{\alpha}$*	1	$0.3650 \pm 0.0323^{\alpha}$	1	$2.7029 \pm 0.5793^{\alpha}$	1	$63.9457 \pm 3.7561^{\alpha}$	1	$0.8249 \pm 0.0062^{\alpha}$	1
ΔFgCYP51A	$0.0915 \pm 0.0131^{\alpha}$	0.9	$0.3808 \pm 0.0749^{\alpha}$	1.0	$2.4775 \pm 0.4657^{\alpha}$	0.9	$61.7000 \pm 5.6303^{\alpha}$	1.0	$0.8213 \pm 0.0173^{\alpha}$	1.0
ΔFgCYP51B	$0.0921 \pm 0.0206^{\alpha}$	0.9	$0.3263 \pm 0.0223^{\alpha}$	0.9	$3.9425 \pm 0.3495^{\beta}$	1.5	$55.4000 \pm 3.1633^{\alpha}$	0.9	$0.8000 \pm 0.0085^{\alpha}$	1.0
ΔFgCYP51C	$0.0930 \pm 0.0143^{\alpha}$	0.9	0.4339 ± 0.1127^{a}	1.2	$3.5325 \pm 0.6481^{\beta}$	1.3	$56.6333 \pm 9.0779^{\alpha}$	0.9	$0.8318 \pm 0.0113^{\alpha}$	1.0
ΔFgCYP51AC	$0.0826 \pm 0.0124^{\alpha}$	0.8	$0.5830 \pm 0.1422^{\beta}$	1.6	$3.3625 \pm 0.8714^{\beta}$	1.2	$59.6000 \pm 9.1597^{\alpha}$	0.9	$0.8213 \pm 0.0217^{\alpha}$	1.0
ΔFgCYP51BC	$0.0919 \pm 0.0114^{\alpha}$	0.9	$0.5574 \pm 0.0740^{\beta}$	1.5	$5.4333 \pm 0.7164^{\gamma}$	2.0	$69.2333 \pm 16.5599^{\alpha}$	1.1	$0.7977 \pm 0.0146^{\alpha}$	1.0

[a]EC_{50} values (μg ml^{-1}) are means of EC_{50} of two transformants from three independent replicates ± SD. Mean resistance factor (RF), representing the sensitivity change of each isolate, was calculated as the fold change in EC_{50} compared with wild-type PH-1.
*Data were analysed using ANOVA ($P < 0.05$, *F* test). Different Greek letters are used to mark statistically significant differences between strains/mutants ($P < 0.05$, least-significant difference (LSD)).

Table 7 Relative composition (%) of sterols of *Fusarium graminearum* wild-type PH-1 and *FgCYP51* gene deletion mutants

Compound			Relative composition (%)[a] in the different *ΔFgCYP51* mutants					
Systematic name	Common name	Retention time (min)	PH-1	*ΔFgCYP51A*	*ΔFgCYP51B*	*ΔFgCYP51C*	*ΔFgCYP51AC*	*ΔFgCYP51BC*
14-methylated sterols								
4,4,14-trimethyl cholesta-8,24-dienol	Lanosterol	36.0	2.15 ± 0.26	1.99 ± 0.10	1.76 ± 0.17	1.51 ± 0.02	1.58 ± 0.07	1.43 ± 0.04
4,4,14-trimethyl ergosta 8,24-dienol	Eburicol	38.8	3.46 ± 0.65	3.33 ± 0.39	**12.22 ± 1.59**	1.05 ± 0.15	1.24 ± 035	**14.26 ± 2.78**
4,4,14-trimethyl ergosta-trienol		37.8	ND	ND	**0.10 ± 0.02**	ND	ND	**0.05 ± 0.09**
4,4,14-trimethyl ergosta-dienol		38.1	ND	ND	**0.08 ± 0.07**	ND	ND	**0.04 ± 0.06**
14-demethylated sterols								
4,4-dimethyl ergosta-8,14,24(28)-trienol		39.7	0.51 ± 0.11	0.32 ± 0.02	0.31 ± 0.01	0.23 ± 0.05	0.25 ± 0.04	0.09 ± 0.16
4,4-dimethyl ergosta-8,24(28)-dienol		40.2	5.24 ± 1.32	3.55 ± 0.38	3.76 ± 0.39	2.86 ± 0.74	2.45 ± 0.10	1.79 ± 0.62
4-methyl ergosta-8,24-dienol		36.7	4.01 ± 0.77	2.04 ± 0.27	3.08 ± 0.32	3.18 ± 0.73	2.34 ± 0.11	1.18 ± 0.99
Ergosta-8,24-dienol	Fecosterol	32.8	0.54 ± 0.06	0.56 ± 0.01	0.35 ± 0.01	0.67 ± 0.14	0.68 ± 0.05	0.13 ± 0.23
Ergosta-7,24(28)-dienol	Episterol	34.5	1.62 ± 0/05	2.87 ± 0.39	0.93 ± 0.10	2.75 ± 0.72	2.52 ± 0.30	0.98 ± 0.36
Ergosta-5,7,24(28)-trienol		33.6	1.53 ± 0.39	0.05 ± 0.09	1.44 ± 0.05	2.41 ± 0.13	2.09 ± 0.64	0.56 ± 0.48
Ergosta-5,7-dienol		33.9	1.73 ± 0.13	1.78 ± 0.25	0.99 ± 0.12	2.41 ± 0.15	2.63 ± 0.24	1.44 ± 0.38
Ergosta-5,7,22,24(28)-tetraenol		30.3	0.88 ± 0.74	1.44 ± 0.33	0.83 ± 0.23	0.94 ± 0.13	0.78 ± 0.09	0.56 ± 0.34
Ergosta-5,8,22,24(28)-tetraenol		32.3	0.97 ± 0.06	1.05 ± 0.05	0.85 ± 0.02	1.15 ± 0.07	1.12 ± 0.04	0.30 ± 0.29
Ergosta-5,22,24(28)-trienol		30.0	1.53 ± 0.02	1.61 ± 0.02	1.77 ± 0.01	1.56 ± 0.09	1.41 ± 0.15	1.47 ± 0.18
Ergosta-5,22-dienol	Brassicasterol	29.7	2.46 ± 0.01	2.43 ± 0.08	2.72 ± 0.18	3.31 ± 0.30	2.74 ± 0.48	2.57 ± 0.25
Ergosta-5,7,22-trienol	Ergosterol	31.7	72.98 ± 2.53	74.74 ± 0.80	68.49 ± 0.91	75.66 ± 2.35	77.45 ± 2.12	72.85 ± 6.00

[a]Relative compositions of sterols are means of the percentage of the total sterol mass for the isolates from three independent replicates ± SD. ND, not detected.
Accumulated 14-methylated sterols are shown in bold.

5,7-dienol. Although the composition of sterol intermediates differed between the *ΔFgCYP51* mutants and PH-1, there were no substantial differences in ergosterol content, a finding consistent with the similar *in vitro* growth rates of the various *ΔFgCYP51* mutants and PH-1.

Expression of genes in the ergosterol biosynthesis pathway

The relative expression of genes involved in the ergosterol biosynthesis pathway was measured after 48 h of incubation in PDB (Fig. 4a). *FgCYP51A* gene expression was almost 10-fold

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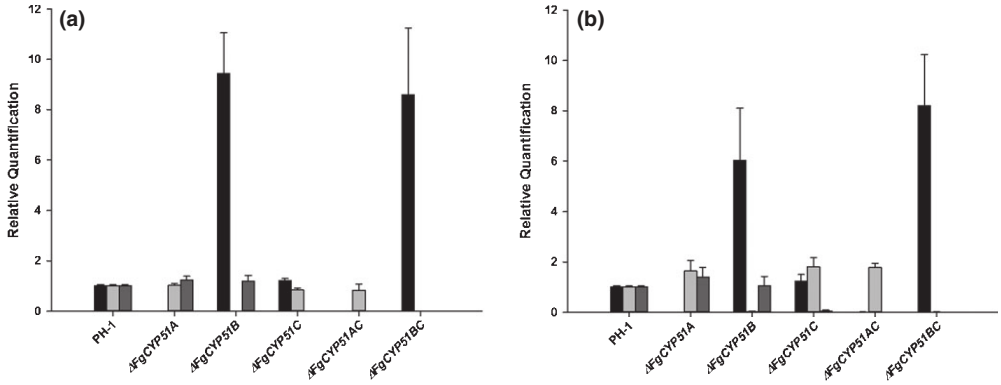

Fig. 4 *FgCYP51* expression in wild-type PH-1 and *FgCYP51* gene deletion mutants *in vitro* and *in planta*. (a) *FgCYP51* gene expression *in vitro*. Mycelia were collected after 48 h of incubation in potato dextrose broth (PDB) medium inoculated with 1×10^4 spores ml^{-1}. (b) *FgCYP51* gene expression *in planta*. Fourteen spikelets were inoculated on each ear and collected at 7 d post-inoculation (dpi). The relative expression levels of *FgCYP51A* (black bars), *FgCYP51B* (light grey bars) and *FgCYP51C* (dark grey bars) genes were analysed with the $2^{-\Delta\Delta C_t}$ method with *actin* as the reference gene and PH-1 as a calibrator. The error bars show + SE between three biological replicates.

higher in *ΔFgCYP51B* and *ΔFgCYP51BC* mutants relative to PH-1. When present, the expression of *FgCYP51B* and *FgCYP51C* was unchanged in the *ΔFgCYP51* mutants relative to PH-1. Of the other genes involved in ergosterol biosynthesis, there were no significant differences in the expression of *ERG2*, *ERG3A* or *ERG4* in any mutants relative to PH-1 (Fig. S2). *ERG7* and *ERG27* expression was significantly higher in the *ΔFgCYP51A* and *ΔFgCYP51BC* mutants. The expression of *ERG1*, encoding squalene epoxidase, the first enzyme in ergosterol biosynthesis, was significantly lower in the single *ΔFgCYP51A* and *ΔFgCYP51C*, and double *ΔFgCYP51AC* and *ΔFgCYP51BC* mutants, which are less sensitive to terbinafine, an *erg1* inhibitor (Table 6). *ERG3B* expression, encoding a C-5 (6) desaturase, was lower in *ΔCYP51AC* and *ΔCYP51BC* mutants. There were no significant differences in *actin* or *β-tubulin* gene expression (Fig. S3).

FgCYP51C is required for full virulence on wheat ears

In wheat ear infection assays, four of the *FgCYP51* gene deletion mutants showed reduced virulence relative to wild-type PH-1 (Fig. 5). There were no significant differences ($P < 0.05$, LSD) in *ΔFgCYP51A* infection over the time course relative to the wild-type. This suggests that *FgCYP51A* is not essential for wheat ear infection. The percentage ear infection caused by *ΔFgCYP51B* was significantly less than that of the wild-type at 16 dpi ($P < 0.05$, LSD), but infection levels were similar at 21 dpi, indicating that *FgCYP51B* gene deletion delays infection. Deletion of *FgCYP51C* decreased significantly ear infection at both 16 dpi and 21 dpi ($P < 0.05$, LSD), suggesting that *FgCYP51C* is required for full virulence on wheat ears. The virulence of *ΔFgCYP51AC* was similar to *ΔFgCYP51C*, supporting the assertion that *FgCYP51A* has no effect on infection. The strongest reduction in wheat ear

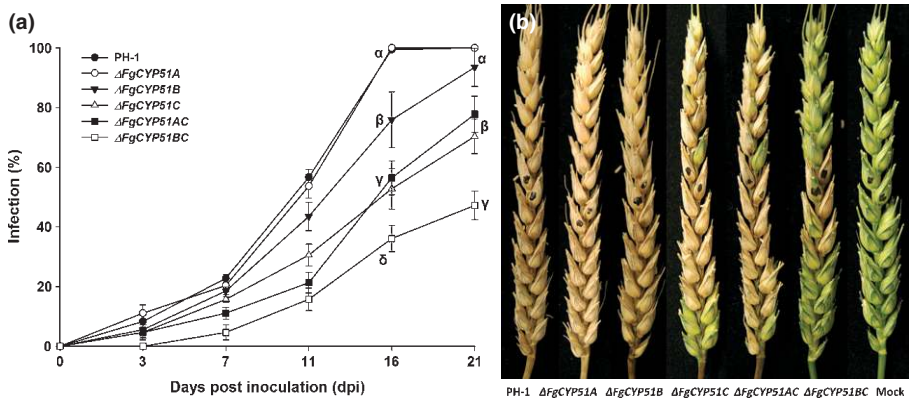

Fig. 5 Infection of wheat ears with wild-type PH-1 and *FgCYP51* gene deletion mutants. The 11th and 12th spikelets (shown by the black dots) from the bottom of each ear were droplet inoculated with conidia. The number of infected spikelets was recorded at 3, 7, 11, 16 and 21 d post-inoculation (dpi). Mock inoculation with distilled water was the negative control. Three wheat ears of different wheat plants were inoculated per strain in each replicate experiment. Data were analysed from three independent experiments using ANOVA ($P < 0.001$, F test). Greek letters show the statistically significant differences between different strains on the same day ($P < 0.05$, least-significant difference (LSD)). Error bars show ± SE. (a) Wheat ear infection over the time course. (b) Infected wheat ears at 21 dpi.

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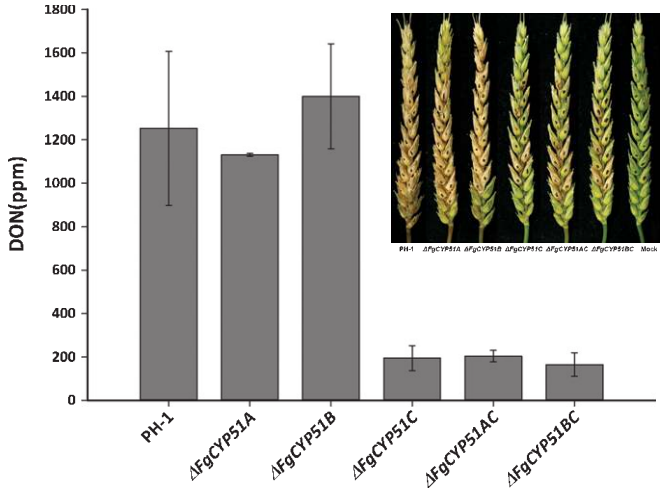

Fig. 6 Quantification of deoxynivalenol (DON) content in wheat spikelets infected with *Fusarium graminearum* PH-1 and *FgCYP51* gene deletion mutants at 10 d post-inoculation (dpi). Fourteen spikelets of each ear were inoculated and harvested after 10 d. The concentration of DON (ppm) per infected dry weight was measured by an EZ-Quant Vomitoxin (DON) plate kit (Diagnostix). Mock inoculated was used as a negative control. Error bars show ± SE between three biological replicates.

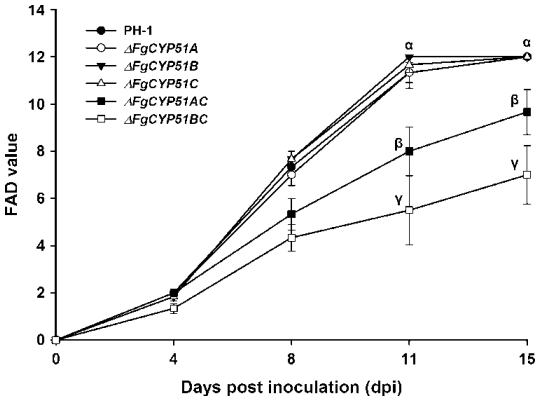

Fig. 8 Pathogenicity of PH-1 and *FgCYP51* gene deletion mutants on *Arabidopsis* floral tissue. The apical inflorescences of 5-wk-old *Arabidopsis* plants (ecotype Landsberg) were spray inoculated with *c.* 5×10^4 *Fusarium graminearum* spores per plant. *Arabidopsis–Fusarium* disease (FAD) scores for each plant were recorded at 4, 8, 11 and 15 d post-inoculation (dpi). Three plants were inoculated per *F. graminearum* strain/mutant. Data were analysed from two experimental replicates using ANOVA ($P < 0.001$, F test). Greek letters show the statistically significant differences among different strains on the same day ($P < 0.05$, least-significant difference (LSD)). Error bars show ± SE between biological replicates.

infection was measured for *ΔFgCYP51BC* mutants, demonstrating a strong combined effect of the absence of both *FgCYP51C* and *FgCYP51B* on virulence. In addition, wheat spikelets inoculated with *ΔFgCYP51C*, *ΔFgCYP51AC* and *ΔFgCYP51BC* mutants contained five-fold less DON at 10 dpi than those inoculated with PH-1 (Fig. 7). There was no significant difference in the amount of DON in wheat ears inoculated with *ΔFgCYP51A* and *ΔFgCYP51B* (Fig. 6).

The grains in wild-type PH-1, *ΔFgCYP51A* and *ΔFgCYP51B* infected wheat ears were shrivelled and bleached (Fig. 7c). Not all the grains of ears inoculated with *ΔFgCYP51C*, *ΔFgCYP51AC* and *ΔFgCYP51BC* were infected, although some spikelets were bleached (Fig. 7a). Rachises infected with PH-1, *ΔFgCYP51A* and *ΔFgCYP51B* were all bleached, whereas those infected with *ΔFgCYP51C*, *ΔFgCYP51AC* and *ΔFgCYP51BC* were a dark-brown colour (Fig. 7b). In addition, grains in *ΔFgCYP51C*, *ΔFgCYP51AC* and *ΔFgCYP51BC* infected ears had a pink pigment. This was absent from ears infected with PH-1 and the *ΔFgCYP51A* and *ΔFgCYP51B* mutants (Fig. 7c).

Gene expression in infected wheat ears

Similar to the *in vitro* assay, *FgCYP51A* expression was highest in wheat ears inoculated with *ΔFgCYP51B* and *ΔFgCYP51BC* (Fig. 4b). Single *FgCYP51A* and *FgCYP51C* gene deletions did not affect the expression of *FgCYP51A* and *FgCYP51B in planta* (Fig. 4b). The expression of *TRI* genes, involved in DON production, was measured in inoculated wheat ears (Fig. S4). Although the RQs of *TRI4*, *TRI5* and *TRI15* transcripts varied between *ΔFgCYP51* mutants, these differences were not significant. There were no significant differences in *ERG1*, *ERG7* and

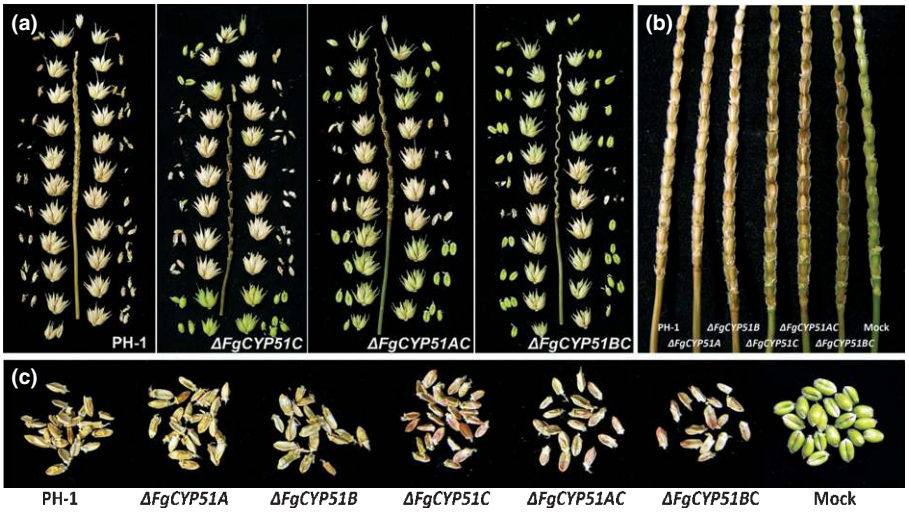

Fig. 7 Dissection of wheat ears infected with *Fusarium graminearum* PH-1 and *FgCYP51* gene deletion mutants. The 11th and 12th spikelets from the bottom of each ear were inoculated with conidia or sterile water as a control. Inoculated ears were dissected at 21 d post-inoculation (dpi). (a) Dissection of infected wheat ears infected with PH-1 and *ΔFgCYP51C*, *ΔFgCYP51AC* and *ΔFgCYP51BC* mutants. (b) Infected rachis. (c) Infected grains.

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ERG27 expression between wild-type PH-1 and *ΔFgCYP51* mutants in infected wheat ears (Fig. S4).

Virulence on dicotyledonous hosts: *Arabidopsis*, tomato fruit and apple fruit sections

In contrast with wheat ear infection, there were no significant differences in disease values between the wild-type and *ΔFgCYP51A*, *ΔFgCYP51B* and *ΔFgCYP51C* on *Arabidopsis* plants, a host which does not require DON mycotoxin for colonization (Cuzick *et al.*, 2008). Both *ΔFgCYP51AC* and *ΔFgCYP51BC* were significantly less virulent than wild-type PH-1 on *Arabidopsis* (Fig. 8), consistent with the virulence of these mutants on wheat ears. In addition, 8 d after inoculation, less aerial mycelia were visible and more green siliques were present on *Arabidopsis* plants infected with *ΔFgCYP51AC* and *ΔFgCYP51BC* in comparison with PH-1 and *ΔFgCYP51A*, *ΔFgCYP51B* and *ΔFgCYP51C* (Fig. S5). All mutants infected tomato fruits and apple fruit sections, with phenotypes similar to that of wild-type PH-1 (Figs S6, S7).

Discussion

The recent increase in genome sequence information has revealed that many fungi, particularly ascomycetes, carry more than one copy of the azole fungicide target encoding gene, *CYP51*. In the Pezizomycotina, a subphylum of the ascomycetes that includes important pathogens of animals and plants, such as *Aspergillus* spp., *M. oryzae*, *Mycosphaerella graminicola* and *Fusarium* spp., *CYP51* paralogues have been classified into three phylogenetic clades, designated A, B and C (Becher *et al.*, 2011), with the *CYP51C* paralogue only found in *Fusarium* species. The *CYP51C* clade is ubiquitous across the genus *Fusarium* and, consequently, can be used as a reliable phylogenetic marker for the identification of different species (Fernández-Ortuño *et al.*, 2010). A previous study has reported that the deletion of individual *F. graminearum CYP51* genes (*FgCYP51A*, *FgCYP51B* or *FgCYP51C*) has no effect on colony morphology, vegetative growth rate or ergosterol content, although conidiation is reduced in all mutants and deletion of the *FgCYP51A* and *FgCYP51C* genes increases azole sensitivity (Liu *et al.*, 2011). In this study, by heterologous expression in yeast and systematic characterization of the impact of individual (*FgCYP51A*, *FgCYP51B*, *FgCYP51C*) and double (*FgCYP51AC* and *FgCYP51BC*) gene deletions on *in vitro* growth, fungicide sensitivity, total sterol composition and virulence on wheat ears and other plants, we describe distinct roles for the *FgCYP51* paralogues of *F. graminearum*.

FgCYP51B encodes the primary sterol 14α-demethylase and is essential for ascospore production

The three *FgCYP51* genes of *F. graminearum* isolate Fg1955 were heterologously expressed in *S. cerevisiae* strain YUG37::*erg11*, which has been used previously to assess the impact of *CYP51* mutations on *Mycosphaerella graminicola* azole sensitivity and enzyme function (Cools *et al.*, 2010, 2011) and to analyse the role of *AfCYP51A* and *AfCYP51B* genes in *A. fumigatus* (Martel *et al.*, 2010b). *FgCYP51A* and *FgCYP51B* were able to substitute *ScCYP51* function, whereas *FgCYP51C* could not (Fig. 2). In addition, transformants expressing FgCYP51B grew faster than those expressing FgCYP51A (Table 2). This suggests that the FgCYP51B protein is a more effective sterol 14α-demethylase than FgCYP51A in yeast.

FgCYP51 gene deletion did not impact on ergosterol content, in accordance with Liu *et al.* (2011). However, the abundance of intermediate sterols was different in all mutants relative to the wild-type. The specific accumulation of eburicol in *ΔFgCYP51B* and *ΔFgCYP51BC* suggests that the overall eburicol demethylation activity is perturbed in mutants lacking FgCYP51B. This is in contrast with those deficient in FgCYP51A or FgCYP51C activity, although the product of CYP51, 4,4-dimethyl ergosta-8,14,24(28)-trienol, was significantly less abundant in all *ΔFgCYP51* mutants relative to the wild-type. These data are consistent with studies of *A. fumigatus*. Deletions of either *AfCYP51A* or *AfCYP51B* blocked C14-demethylation, but far more eburicol accumulated in the *AfCYP51B* mutant than the *AfCYP51A* mutant (Alcazar-Fuoli *et al.*, 2008). Furthermore, substrate binding studies of AfCYP51 proteins expressed in *Escherichia coli* detected strong binding with purified AfCYP51B using eburicol and lanosterol, in contrast with AfCYP51A (Warrilow *et al.*, 2010). As a consequence of accumulated eburicol, two additional novel 14-methylated sterol intermediates (4,4,14-trimethyl ergosta-trienol and 4,4,14-trimethyl ergosta-dienol) were detected in *ΔFgCYP51B* and *ΔFgCYP51BC* (Fig. S8). These data suggest that Pezizomycotina *CYP51B*, including FgCYP51B, is central to effective sterol C14-demethylation.

Ascospores forcibly ejected from mature perithecia, formed by the overwintering fungus on field debris, are the primary source of inoculum for *F. graminearum* epidemics (Parry *et al.*, 1995; Trail *et al.*, 2005). In this study, no ascospores were formed in *ΔFgCYP51B* and *ΔFgCYP51BC* mutants (Fig. 3b), although all the *FgCYP51* gene deletion mutants produced superficially normal perithecia. This finding demonstrates that *FgCYP51B* is specifically required in the development of the sexual stage, a role that cannot be fulfilled by the up-regulation of *FgCYP51A*. Similarly, the delayed colonization of wheat ears by *ΔFgCYP51B* suggests that FgCYP51A cannot fully complement *FgCYP51B* function during wheat infection.

FgCYP51A encodes an inducible sterol 14α-demethylase that determines azole sensitivity

In the absence of fungicide treatment, the relative transcript quantities of *FgCYP51A* and *FgCYP51B* were highest at 72 h of incubation, decreasing at 96 h during growth in rich medium (PDB). This pattern of expression is coincident with rapid fungal growth, which is linear between 24 and 60 h of incubation, and into stationary phase after 72 h of incubation (data not shown). The relative transcript quantities of *FgCYP51A* changed most over this time course. Previous studies have shown an increase in *CYP51A* gene expression after azole treatment and *CYP51B* deletion *in vitro* in *F. graminearum* (Liu *et al.*, 2010; Becher *et al.*,

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2011) and *M. oryzae* (Yan *et al.*, 2011). We report similar results, with *FgCYP51A* expression induced over 100-fold by azoles *in vitro*, and *c.* 10-fold by *FgCYP51B* deletion both *in vitro* and *in planta*. The enhanced transcription of *FgCYP51A* on exposure to azoles suggests that this gene is not only responsive to chemical or genetic perturbation of *FgCYP51B* activity, but also other stresses induced by fungicide treatment. For example, in *S. cerevisiae*, *ScCYP51* expression is higher during growth on glucose, in the presence of haem, under oxygen-limiting growth conditions and during exposure to anaerobic conditions (Turi & Loper, 1992).

Amino acid substitutions and *CYP51* over-expression are the most common mechanisms of resistance to azoles in filamentous ascomycetes. In fungi with multiple *CYP51*s, it is the *CYP51A* paralogue that is most commonly altered, for example in *A. fumigatus* and *P. digitatum* (Ghosoph *et al.*, 2007; Mellado *et al.*, 2007). In addition, single gene deletion has confirmed that *AfCYP51A* is involved in intrinsic azole resistance, for example to fluconazole in *A. fumigatus* (Mellado *et al.*, 2005), and azole affinity studies have shown that the AfCYP51A protein has lower affinity than AfCYP51B for a wide range of azoles (Warrilow *et al.*, 2010). In *F. graminearum*, it has been suggested previously that different azole fungicides target different FgCYP51s (Liu *et al.*, 2011). In this study, deletion of *FgCYP51C*, either alone or with *FgCYP51A* (*ΔFgCYP51AC*), had no effect on azole sensitivity. Deletion of *FgCYP51B* caused an increase in sensitivity to some azoles, with sensitivities to metconazole and prochloraz particularly affected. These data conflict with those of Liu *et al.* (2011), who reported increased sensitivity to tebuconazole and prochloraz of *ΔFgCYP51C* mutants, with *FgCYP51B* deletion having no impact on azole sensitivity. The reason for this discrepancy is unclear, although the different methodologies used for fungicide sensitivity testing and the different origins of the strains used in gene deletion studies may have contributed. However, clearly, in this study, an interaction of azole fungicides with *FgCYP51B* is consistent with the assertion that this paralogue is the primary sterol 14α-demethylase in *F. graminearum*. In both studies, however, the deletion of *FgCYP51A* increased the sensitivity to all azoles tested and, particularly, epoxiconazole. Considering that *F. graminearum* isolate Fg1955 is least sensitive to epoxiconazole, *FgCYP51A* expression is inducible on azole exposure and yeast transformants expressing *FgCYP51A* are least sensitive to epoxiconazole (Table 3), it can be concluded that the intrinsically lower sensitivity to some azoles in *F. graminearum* is primarily determined by *FgCYP51A*.

FgCYP51C is a novel genus-specific *CYP51* gene required for full virulence on wheat ears

In the *S. cerevisiae* heterologous expression system, *FgCYP51A* and *FgCYP51B* were able to substitute for *ScCYP51* function. By contrast, *FgCYP51C* could not complement *ScCYP51*. This suggests that FgCYP51C cannot function as a sterol 14α-demethylase. This loss or diversification of function is probably caused by substitutions in conserved putative substrate recognition sites (SRSs) of FgCYP51C. The predicted FgCYP51 amino acid sequences are sufficiently identical (over 40%) to be considered as members of the same P450 family (Liu *et al.*, 2011). Analysis of residues conserved in eukaryotic CYP51s (Fig. S9, Lepesheva & Waterman, 2011) identified two residues (N304 and T305) unique to FgCYP51C. Although the importance of these residues in the function of FgCYP51C is unknown, substitutions T315N or S316T of rat CYP51, equivalent to N304 and T305, caused significant reductions in lanosterol demethylase activity (Nitahara *et al.*, 2001). Deletion of *FgCYP51C* had no impact on *in vitro* fungal morphology, growth rate, conidiation and spore germination at almost all vegetative stages, perithecia production, ascospore formation or azole sensitivity. In addition, there was no difference in eburicol or ergosterol content in *ΔFgCYP51C* mutants. However, *ΔFgCYP51C* mutants had less 4,4-dimethyl ergosta-8,14,24(28)-trienol, the product of CYP51, and accumulated the sterol intermediates episterol, ergosta-5,7,24(28)-trienol and ergosta-5,7-dienol, products of ERG2, ERG3 and ERG4 activity, respectively (Fig. S8). This suggests that FgCYP51C can impact indirectly on sterol 14α-demethylation, ERG2, ERG3 and ERG4 activity. There was no difference in *ERG2*, *ERG3A*, *ERG3B* and *ERG4* gene expression in single *ΔFgCYP51C* mutants relative to wild-type PH-1 *in vitro*. However, the *ERG3B* gene was expressed less in both *ΔFgCYP51AC* and *ΔFgCYP51BC* mutants, which grew more slowly and produced less aerial mycelia on rich medium in the dark and when inoculated on wheat ears and *Arabidopsis* floral tissues. The *CYP51* gene is required for aerobic viability in *S. cerevisiae*, *C. albicans* and *C. glabrata*. In a *CYP51*-deficient mutant, aerobic growth can be restored by null mutation or deletion of *ERG3* (Bard *et al.*, 1993; Kelly *et al.*, 1993, 1995; Geber *et al.*, 1995). However, to date, there is no evidence for *ERG3*-mediated azole resistance in filamentous fungi.

After inoculation with the *ΔFgCYP51C*, *ΔFgCYP51AC* and *ΔFgCYP51BC* mutants, the number of infected spikelets per wheat ear was reduced significantly relative to inoculations with wild-type PH-1, *ΔFgCYP51A* and *ΔFgCYP51B*. In addition, not all the grain in bleached spikelets had a rough, shrivelled appearance, although infected grains were pink in wheat ears inoculated with *ΔFgCYP51C*, *ΔFgCYP51AC* and *ΔFgCYP51BC*, rather than the grey in plants inoculated with PH-1, *ΔFgCYP51A* and *ΔFgCYP51B* (Fig. 7c). The rachises of wheat heads infected with *ΔFgCYP51C*, *ΔFgCYP51AC* and *ΔFgCYP51BC* were dark brown, in contrast with the bleached rachises of plants inoculated with PH-1, *ΔFgCYP51A* and *ΔFgCYP51B*. The blocking of fungal growth from inoculated spikelets to adjacent spikelets is correlated with an unidentified brown substance deposited in the rachis node in the additional wheat line CS-7EL, which carries resistance to FHB on the long arm of chromosome 7E(7EL) (Miller *et al.*, 2011). These data suggest that hyphal development of *ΔFgCYP51C*, *ΔFgCYP51AC* and *ΔFgCYP51BC* during wheat ear infection is impaired.

In contrast with wheat ear infection, there were no differences in virulence between wild-type PH-1 and *ΔFgCYP51C* on *Arabidopsis*, in which the trichothecene mycotoxin DON is not required for fungal infection (Cuzick *et al.*, 2008), although colonization by the double *ΔFgCYP51AC* and *ΔFgCYP51BC* mutants was impaired. This suggests that altered DON production is

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responsible for the decreased virulence of the *ΔFgCYP51C* mutant on wheat ears. However, unlike mutants unable to produce DON, for example *TRI5* gene mutants, which cause only discrete eye-shaped lesions on spikelets and fail to infect the rachis (Cuzick *et al.*, 2008), *ΔFgCYP51C*, *ΔFgCYP51AC* and *ΔFgCYP51BC* mutants are able to infect beyond the inoculated spikelet. In addition, *TRI4* and *TRI5* gene expression is not altered significantly in wheat ears inoculated with *ΔFgCYP51C*, *ΔFgCYP51AC* and *ΔFgCYP51BC* relative to the wild-type. Therefore, rather than a biosynthetic requirement for DON biosynthesis, FgCYP51C is likely to have an indirect effect. A relationship between the sterol and trichothecene biosynthesis pathways has been reported previously. They share a common precursor, farnesol pyrophosphate, and the global regulator *TRI6*, located in the core *TRI* gene cluster. For example *ERG9* (FGSG_09381), encoding squalene synthase, the first step of sterol biosynthesis, was down-regulated in the *ΔTRI6* strain under nitrogen-deprived conditions, and *ERG25* (FGSG_10666) was up-regulated, although there was no impact on *FgCYP51C* expression (Nasmith *et al.*, 2011).

Conclusion

We have identified distinct functions of the three *CYP51* paralogues of *F. graminearum*. *FgCYP51B*, as the most conserved *CYP51* gene in all fungi, encodes the enzyme primarily responsible for sterol 14α-demethylation, a role essential for ascospore formation. *FgCYP51A*, found in many human and agricultural pathogens, is induced by azoles and environmental stress, encodes a sterol 14α-demethylase with the capacity to compensate for disruption of FgCYP51B function, and is responsible for intrinsic variation in sensitivity to different azoles. *FgCYP51C*, a *Fusarium*-specific *CYP51* gene, no longer functions as a sterol 14α-demethylase, but rather is specifically required for full virulence on host wheat ears. This is the first example of functional diversification of a fungal *CYP51* gene.

Acknowledgements

The authors thank Nichola Hawkins and John Lucas (Rothamsted Research) for comments on the manuscript, and Mogens Nicolaisen (Aarhus University, Denmark) for supplying the isolate Fg1955. At Rothamsted Research, all experiments involving transgenic strains of *F. graminearum* were conducted in biological containment facilities under FERA licence number PHSI 181/6695 and with appropriate approval from the UK Health and Safety Executive. Jieru Fan was supported by an award of a Postgraduate Study Abroad Program Sponsored by the China Scholarship Council. Rothamsted Research receives grant-aided support from the Biotechnology and Biological Sciences Research Council (BBSRC) of the UK.

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Supporting Information

Additional supporting information may be found in the online version of this article.

Fig. S1 The spilt-marker deletion strategy of *FgCYP51* genes in *Fusarium graminearum* isolate PH-1.

Fig. S2 *In vitro* transcript levels of *ERG* genes.

Fig. S3 Relative quantification of *actin* and *β-tubulin* gene expression *in vitro*.

Fig. S4 Relative quantification of gene expression in infected wheat ears.

Fig. S5 Infection of Arabidopsis floral tissue with PH-1 and *FgCYP51* gene deletion mutants.

Fig. S6 Pathogenicity of PH-1 and *FgCYP51* gene deletion mutants on apple sections.

Fig. S7 Infection of tomato fruits with PH-1 and *FgCYP51* gene deletion mutants.

Fig. S8 The proposed sterol biosynthesis pathway in *Fusarium graminearum*. The sterol pathway based on compounds detected in the wild-type PH-1 and *FgCYP51* gene deletion mutants incubated in PDB media for 48 h. The proposed pathway in wild-type PH-1 is shown in solid arrows. The pathway after *FgCYP51B* deletion is shown with dashed arrows in the dashed box. The two additional sterols, 4,4,14-trimethyl ergosta-trienol and 4,4,14-trimethyl ergosta-dienol, in the dashed box were identified as TMS esters with *m/z* 510 and *m/z* 512.

Fig. S9 Alignment of predicted FgCYP51 amino acid sequences.

Table S1 Primers used in this study

Table S2 Concentrations of fungicides used in azole sensitivity testing

Table S3 The *Fusarium*–Arabidopsis disease scoring system

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