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

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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* CYP51s (FgCYP51s) were heterologously expressed in *Saccharomyces cerevisiae*. Single and double *FgCYP51* deletion mutants were generated and the functions of the FgCYP51s 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 ∆FgCYP51B and ∆FgCYP51BC 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, a critical component 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 theazole (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*.
Fungi with multiple CYP51s are intrinsically resistant to some azoles, although some remain effective. For example, *A. fumigatus* is well controlled by itraconazole and voriconazole, whereas azoles, although some remain effective. For example, the intrinsic sensitivity to some azoles in most common mechanism of resistance in *Fusarium* species, and is ubiquitous across the genus, as demonstrated by its use as a reliable phylogenetic marker (Fernández-Ortuno et al., 2010).

Fungi with multiple CYP51s 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 CYP51s is most frequently mediated by changes in the CYP51A parologue. 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 (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 parologue in some species, for example *A. flavus* and *A. terreus*, respectively. Uniquely, the third *CYP51* parologue 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-Ortuno et al., 2010).

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 (Ehrenshtaff et al., 1995). Approximately 50 ascii per strain/mutant were scraped from the plates and physically broken to determine ascospore formation.

Heterologous expression of *FgCYP51* genes in *S. cerevisiae*

Heterologous expression of *FgCYP51* genes in *S. cerevisiae* followed the method described by Cools et al. (2010). *FgCYP51* genes were amplified from cDNA of...
F. graminearum isolate Fg1955 (Nicolaïsen 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-fold dilutions of a starting concentration of 1 × 10⁶ 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 × 10⁶ 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 polyethylene glycol (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 genetin. 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 × 10⁴ 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 × 10⁶ cells ml⁻¹ (Table S2) (Cools et al., 2010). The absorbance was measured at 630 nm using a FLUOS-star 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 (EC₅₀) 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 EC₅₀ at 24 h of incubation. In vitro gene expression of ΔFgCYP51 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⁻ΔΔC method with F. graminearum actin and β-tubulin as reference genes.

| Strain         | Description                        | Reference              |
|---------------|-----------------------------------|------------------------|
| Fg1955        | Wild-type, Finland origin         | Nicolaïsen 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 deletion mutant      | 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             |
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. Non-saponifiable 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 × 10⁴ 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 (Diagnostic, 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 × 10⁴ spores ml⁻¹ of wild-type PH-1 or Δ*FgCYP51* 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 × 10⁵ ml⁻¹ conidia of wild-type isolate PH-1, sterile water control or a Δ*FgCYP51* 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 Δ*FgCYP51* 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 × 10⁵ 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 byazole fungicides

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.
Heterologous expression of \( \text{FgCYP51} \) genes in \( S. \text{cerevisiae} \) strain YUG37::\( \text{erg11} \)

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

**In vitro growth characteristics of \( \text{FgCYP51} \) deletion strains**

There were no differences in growth rate between mutants \( \Delta \text{FgCYP51A}, \Delta \text{FgCYP51B}, \Delta \text{FgCYP51C}, \Delta \text{FgCYP51AC} \) and \( \Delta \text{FgCYP51BC} \) (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 \( \text{FgCYP51} \) 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 \( (\Delta \text{FgCYP51AC} \) and \( \Delta \text{FgCYP51BC} \) 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 \( \Delta \text{FgCYP51AC} \) and \( \Delta \text{FgCYP51BC} \) 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).

\( \text{FgCYP51B} \) 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 \( \Delta \text{FgCYP51B} \) and \( \Delta \text{FgCYP51BC} \) and, when perithecia were physically broken, no ascospores were found (Fig. 3b). Ascospores produced by \( \Delta \text{FgCYP51A}, \Delta \text{FgCYP51C} \) and \( \Delta \text{FgCYP51AC} \) showed wild-type morphology and germinated normally (data not shown).
Table 3 Azole sensitivity of *Fusarium graminearum* isolates and *Saccharomyces cerevisiae* YUG37:erg11 transformants

| Isolate/yeast transformant | EC<sub>50</sub> (µg ml<sup>−1</sup>)<sup>a</sup> | Prochloraz | Tebuconazole | Epoxiconazole | Cycloheximide |
|---------------------------|---------------------------------------------|------------|--------------|---------------|--------------|
| Fg1955<sup>b</sup>        | 0.0264 ± 0.0025                              | 0.3410 ± 0.0010 | 1.4550 ± 0.1050 | –             |
| pYES-Fg51A<sup>c</sup>    | 0.0043 ± 0.0003                              | 0.0063 ± 0.0003 | 0.0261 ± 0.0089 | 0.0537 ± 0.0005 |
| pYES-Fg51B<sup>c</sup>    | 0.0160 ± 0.0031                              | 0.0351 ± 0.0054 | 0.0103 ± 0.0044 | 0.0590 ± 0.0005 |

<sup>a</sup>EC<sub>50</sub> values (µg ml<sup>−1</sup>) are means of EC<sub>50</sub> from two independent replicates ± SD.

<sup>b</sup>*Fusarium graminearum* isolate.

<sup>c</sup>*Saccharomyces cerevisiae* YUG37:erg11 transformants expressing FgCYP51 genes.

–, means not measured.

(b) PH-1 ΔFgCYP51ΔA ΔFgCYP51ΔB ΔFgCYP51ΔC ΔFgCYP51AC ΔFgCYP51BC

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) Ascospor 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<sup>a</sup> | Conidiation<sup>b</sup> (10<sup>5</sup> spores per plate) | Conidia germination<sup>c</sup> (%) |
|---------------|--------------------------|-------------------------------------------------|-----------------------------------|
| PH-1          | 0.0145 ± 0.0005<sup>**</sup> | 38.2 ± 5.6<sup>**</sup> | 94.7 ± 0.8<sup>**</sup> |
| ΔFgCYP51B     | 0.0135 ± 0.0019<sup>**</sup> | 27.5 ± 4.8<sup>**</sup> | 94.4 ± 1.1<sup>**</sup> |
| ΔFgCYP51A     | 0.0124 ± 0.0011<sup>**</sup> | 33.8 ± 7.4<sup>**</sup> | 94.6 ± 0.4<sup>**</sup> |
| ΔFgCYP51C     | 0.0112 ± 0.0002<sup>**</sup> | 28.7 ± 3.8<sup>**</sup> | 94.7 ± 0.5<sup>**</sup> |
| ΔFgCYP51AC    | 0.0114 ± 0.0001<sup>**</sup> | 18.1 ± 3.2<sup>**</sup> | 94.4 ± 0.6<sup>**</sup> |
| ΔFgCYP51BC    | 0.0124 ± 0.0002<sup>**</sup> | 19.4 ± 2.5<sup>**</sup> | 94.8 ± 0.7<sup>**</sup> |

<sup>a</sup>Values represent the greatest increase in the optical density at 600 nm (OD<sub>600</sub>) 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).

<sup>b</sup>Conidia produced from 7 d of incubation on synthetic nutrient-poor agar (SNA) plate with 10<sup>6</sup> conidia inoculation.

<sup>c</sup>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 inazole sensitivity between ΔFgCYP51A and ΔFgCYP51AC, confirming that FgCYP51C has no effect on azole sensitivity.
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 Paracoccidioides 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)-tri-enol, was less abundant in all ΔFgCYP51 mutants relative to the wild-type, with the greatest reduction detected in ΔFgCYP51BC. Two additional 14-demethylated sterols (4,4,14-trimethyl ergosta-tienol 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 rearrangement of double bonds by ERG2 (Δ^-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)-tri-enol and ergosta-8,24(28)-dienol.
Table 6 Ergosterol biosynthesis inhibitor (EBI) sensitivity of Fusarium graminearum wild-type isolate PH-1 and FgCYP51 gene deletion mutants

| Strain/mutant | Amphotericin B | Terbinafine | Fenhexamid | Fenpropimorph | Carbenazim |
|---------------|----------------|-------------|------------|---------------|------------|
|               | EC50 (µg ml⁻¹) | RF          | EC50 (µg ml⁻¹) | RF          | EC50 (µg ml⁻¹) | RF          |
| PH-1          | 0.1034 ± 0.0125*; | 1           | 0.3650 ± 0.0323*; | 1           | 2.7029 ± 0.5793*; | 1   |
| ΔFgCYP51A     | 0.0915 ± 0.0131*; | 0.9         | 0.3800 ± 0.0749*; | 1.0         | 2.4775 ± 0.4657*; | 0.9   |
| ΔFgCYP51B     | 0.0921 ± 0.0206*; | 0.9         | 0.3263 ± 0.0223*; | 0.9         | 3.9425 ± 0.3495*; | 1.5   |
| ΔFgCYP51C     | 0.0930 ± 0.0143*; | 0.9         | 0.4339 ± 0.1127*; | 1.2         | 3.5325 ± 0.6481*; | 1.3   |
| ΔFgCYP51AC    | 0.0826 ± 0.0124*; | 0.8         | 0.5830 ± 0.1422*; | 1.6         | 3.3625 ± 0.8714*; | 1.2   |
| ΔFgCYP51BC    | 0.0919 ± 0.0114*; | 0.9         | 0.5574 ± 0.0740*; | 1.5         | 5.4333 ± 0.7164*; | 7.0   |

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

| Compound | Systematic name | Common name | Retention time (min) | Relative composition (%)\(^a\) in the different ΔFgCYP51 mutants |
|----------|-----------------|-------------|----------------------|---------------------------------------------------------------|
| 14-methylated sterols | 4,4,14-trimethyl cholesterol-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 |
| 8,24-dienol | 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 ± 0.03; 14.26 ± 2.78 |
| 4,4,14-trimethyl ergosta-trienol | 4,14-trimethyl ergosta-dienol | 37.8 | ND; ND; 0.10 ± 0.02; ND; ND; 0.05 ± 0.09 |
| 4,14-trimethyl ergosta-dienol | 4,14-dimethyl ergosta-8,14,24(28)-triienol | 38.1 | ND; ND; 0.08 ± 0.07; ND; ND; 0.04 ± 0.06 |

*Relative compositions of sterols are means of EC50 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 EC50 compared with wild-type PH-1.

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 increased in PH-1 compared with PH-1 ΔFgCYP51A.
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. 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 × 10⁶ 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⁻ΔΔCt method with actin as the reference gene and PH-1 as a calibrator. The error bars show ± SE between three biological replicates.

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.
infection was measured for ΔFgCYP51BC mutants, demonstrating a strongly 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 bleached, 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

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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.

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**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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**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 × 10⁴ *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.
ERG27 expression between wild-type PH-1 and ΔFgCYP51 mutations 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 parologue 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 ΔfCYP51B mutant than the ΔfCYP51A 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 (Warrillow 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-24-enol) 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., 2011).
Amino acid substitutions and CYP51 over-expression are the most common mechanisms of resistance to azoles in filamentous ascomycetes. In fungi with multiple CYP51s, it is the CYP51A parologue that is most commonly altered, for example in A. fumigatus and P. digitatum (Ghosph et al., 2007; Mellado et al., 2007). In addition, single gene deletion has confirmed that CYP51A is involved in intrinsic azole resistance, for example to flucytosine in A. fumigatus (Mellado et al., 2005), and azole affinity studies have shown that the A. fumigatus protein has lower affinity than A. fumigatus CYP51B 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 (AfCYP51AC), 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 glisoxole in F. graminearum and 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).

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 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, ΔAfCYP51C mutants had less 4,4-dimethyl ergosta-8,14,24(28)-triienol, the product of CYP51, and accumulated the sterol intermediates episterol, ergosta-5,7,24(28)-triienol 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 ΔAfCYP51AC 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, ΔAfCYP51AC and ΔAfCYP51BC mutants, the number of infected spikelets per wheat ear was reduced significantly relative to inoculations with wild-type PH-1, ΔAfCYP51C and ΔAfCYP51BC. In addition, not all the grain in bleached spikelets had a rough, shrivelled appearance, although infected grains were pink in wheat ears inoculated with ΔAfCYP51C, ΔAfCYP51AC and ΔAfCYP51BC, rather than the grey in plants inoculated with PH-1, ΔAfCYP51C and ΔAfCYP51BC (Fig. 7c). The racahis of wheat heads infected with ΔAfCYP51C, ΔAfCYP51AC and ΔAfCYP51BC were dark brown, in contrast with the bleached racahis of plants inoculated with PH-1, ΔAfCYP51C and ΔAfCYP51BC. The blocking of fungal growth from inoculated spikelets to adjacent spikelets is correlated with an unidentified brown substance deposited in the racachis 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 ΔAfCYP51C, ΔAfCYP51AC and ΔAfCYP51BC during wheat ear infection is impaired.

In contrast with wheat ear infection, there were no differences in virulence between wild-type PH-1 and ΔAfCYP51C on Arabidopsis, in which the trichothecene mycotoxin DON is not required for fungal infection (Cuzick et al., 2008), although colonization by the double ΔAfCYP51AC and ΔAfCYP51BC mutants was impaired. This suggests that altered DON production is
responsible for the decreased virulence of the ΔFgCYP51C mutant on wheat ears. However, unlike mutants unable to produce DON, for example TR15 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, TR14 and TR15 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.

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Supporting Information
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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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