Analysis of mutations in West Australian populations of Blumeria graminis f. sp. hordei CYP51 conferring resistance to DMI fungicides

Madeline A Tucker, Francisco Lopez-Ruiz, Hans J Cools, Jonathon GL Mullins, Kithsiri Jayasena and Richard P Oliver

Abstract

BACKGROUND: Powdery mildew caused by Blumeria graminis f. sp. hordei (Bgh) is a constant threat to barley production but is generally well controlled through combinations of host genetics and fungicides. An epidemic of barley powdery mildew was observed from 2007 to 2013 in the West Australian grain belt.

RESULTS: We collected isolates across Australia, examined their sensitivity to demethylation inhibitor (DMI) fungicides and sequenced the Cyp51B target gene. Five amino acid substitutions were found, of which four were novel. The most resistant haplotypes increased in prevalence from 0% in 2009 to 16% in 2010 and 90% in 2011. Yeast strains expressing the Bgh Cyp51 haplotypes replicated the altered sensitivity to various DMIs and these results were complemented by in silico protein docking studies.

CONCLUSIONS: The planting of very susceptible cultivars and the use of a single fungicide mode of action was followed by the emergence of a major epidemic of barley powdery mildew. Widespread use of DMI fungicides led to the selection of Bgh isolates carrying both the Y137F and S524T mutations, which, as in Zymoseptoria tritici, account for resistance factors varying from 3.4 for propiconazole to 18 for tebuconazole, the major azoles used at that time in WA.

Keywords: triazoles; DMI; fungicide; Blumeria graminis f. sp. hordei (barley powdery mildew); CYP51; cross resistance

1 INTRODUCTION

Blumeria graminis f. sp. hordei (Bgh) is an ascomycete fungus causing barley (Hordeum vulgare L.) powdery mildew. In conducive seasons this biotrophic pathogen can reduce yields by as much as 20% but is generally well controlled by host genetics including the durable recessive mlo gene, dominant major R-genes and combinations of minor genes. In cases in which genetic disease resistance cannot be combined with optimum malting characteristics, fungicides can be used. Many classes of fungicides have been used to control powdery mildews but the pathogen has a marked propensity to develop resistance rapidly.

Barley was grown on ~ 1.3 million hectares in West Australia (WA) and yielded 1.5–3 million tonnes from 2000 to 2011. Since 1995 the majority of the barley area has been planted to cultivars with excellent malting quality but low disease resistance. To combat the increasing incidence of diseases, including powdery mildew, there has been a steep increase in fungicide use. In 2009, 85% of barley crops were treated with one or more fungicide application (both seed and foliar) taken from a list of registered chemicals consisting of almost exclusively of sterol demethylation inhibitors (DMIs). These fungicides interrupt the biosynthesis of ergosterol (and other mycosterols in powdery mildews) by inhibiting cytochrome P450 14α-sterol demethylase (CYP51). Resistance is now common in human pathogens, including Candida spp. and Aspergillus fumigatus, and is a serious problem in agricultural systems. Fungicide resistance has been associated...
with a number of mechanisms including the alteration and overexpression of the Cyp51 gene(s) as well as enhanced DMI efflux.\textsuperscript{13,15,16}

The most commonly observed mechanism of resistance is non-synonymous changes in the gene sequence of Cyp51.\textsuperscript{13} A large number of non-synonymous changes have been observed in Cyp51A and B genes of various fungal pathogens. A unified nomenclature for these changes has been proposed and is adopted in this report.\textsuperscript{17} Two earlier studies examining DMI resistance in \textit{Bgh} in Europe identified two changes in Cyp51, Y137F and K148Q (equivalent to Y136F and K147Q).\textsuperscript{18,19} Isolates with only Y137F exhibited both low and high levels of triadimenol resistance, and K148Q was only ever found in combination with Y137F. Hence, the exact sensitivity shift afforded by each mutation remained unclear.

Tebuconazole-containing formulations were registered for barley mildew from 1995 in West Australia\textsuperscript{20} and were followed soon after by other DMI actives, mainly flutriafol, triadimenol and propiconazole. Initially, they provided good control of leaf rust, powdery mildew and other diseases.\textsuperscript{5} Since 2005, WA farmers have reported reduced efficacy of DMIs in controlling barley powdery mildew outbreaks.\textsuperscript{21} Accounts of mildew infection on barley treated with tebuconazole formulations in particular extended over much of the southern WA agricultural cropping region with the frequency of reports greatly increasing after 2009.

In this study, we determined the sensitivity of Australian \textit{Bgh} isolates to DMI fungicides registered in WA for use on barley. Sequencing of the \textit{CYP51} coding region in a subset of isolates revealed five mutational changes defining four unique haplotypes. The fungicide sensitivities of isolates representing each haplotype were determined both by screening on fungicide-treated detached leaves and heterologous expression of the respective mutation in \textit{Saccharomyces cerevisiae}. Our results link variations in DMI sensitivity to changes in \textit{CYP51}. \textit{In silico} protein structural modelling demonstrated the conformational changes afforded by mutations, suggesting significant effects on DMI sensitivity, and was able to rationalize our observations of partial cross-resistance (see Oliver and Hewitt\textsuperscript{14} pp. 129–130). A brief report on some this data has been published previously.\textsuperscript{22}

2 MATERIALS AND METHODS

2.1 Isolates

One hundred and nineteen \textit{Bgh} isolates were collected from 2009 to 2013 (Fig. 1, Table S1). Isolates from Wagga Wagga, Tamworth (New South Wales) and Launceston (Tasmania) were supplied by the Department of Environment and Primary Industries, Victoria. Isolate purification, sub-culturing and assessments of growth were performed as described.\textsuperscript{23}

2.2 Fungicide sensitivity assays

Fungicide sensitivities were determined by assessing growth of isolates on susceptible (cv. Baudin) barley leaves inserted into fungicide-amended media. Commercial formulations of DMIs currently registered for \textit{Bgh} control – Laguna (720 g L\textsuperscript{-1} tebuconazole, Sipcam, Geelong, Victoria, Australia), Flutriafol (250 g L\textsuperscript{-1} flutriafol, Imtrade, Perth WA, Australia), Opus (125 g L\textsuperscript{-1} epoxiconazole, Nufarm), Alto (100 g L\textsuperscript{-1} cyproconazole, NuFarm, Laverton, Victoria, Australia), Tilt (418 g L\textsuperscript{-1} propiconazole, Syngenta, North Ryde, NSW, Australia), Proline (410 g L\textsuperscript{-1} prothioconazole, Bayer Cropscience, Kallaroo, WA, Australia), Triad 125 (125 g L\textsuperscript{-1} triadimefon, Farmoz (Adama) St Leonards, NSW, Australia) and Jockey Stayer (167 g L\textsuperscript{-1} fluquinconazole, Bayer Crop Science) – were incorporated into agar amended with 50 mg L\textsuperscript{-1}
of benimidazole.24 Middle sections of 10-day-old seedlings were excised with each tip inserted abaxial side up into fungicide amended agar. A wide range of concentrations was tested to identify a specific set of six needed to calculate an accurate half-maximal effective concentration (EC₅₀) for each product. Each isolate was inoculated onto three replicates on successive weeks with conidia dislodged 24 h before use to promote fresh growth. Conidia were collected on glossy black paper and blown into a 1.5 m infection tower to ensure even inoculation. Following 7 days incubation at 20 ± 2 °C in a 12:12 h light/dark photoperiod, the growth of each isolate was assessed using a 0–4 infection type (IT) scale adapted from Czembor.23 Each pustule formation was assigned an IT score and the average for each isolate and fungicide concentration was determined. Both the average IT and concentration was log transformed, per cent inhibition calculated and plotted to determine the regression equation and correlation coefficient. Mean EC₅₀ values with associated errors were calculated for each Bgh Cyp51 haplotype (Fig. 3). All EC₅₀ values were log₁₀-transformed prior to statistical analysis. Data analysis was conducted in JMP, v. 10 (SAS Institute Inc., Cary, NC, USA).

2.3 CYP51 sequencing

DNA isolations were performed using a BioSprint 15 DNA Plant Kit (Qiagen) following the manufacturer’s instructions. The wild-type Bgh DH14 isolate (GenBank accession no. AJ313157) was used to design primers (Table S2) covering the entire length of the Bgh CYP51 (Bgh51) gene including the promoter region (Fig. S1). The amplimers of 76 isolates were sequenced using Sanger sequencing and aligned in Geneious v. 5.5 (Biomatters). All sequences have been submitted to GenBank (Accession no. KM016902, KM016903, KM016904 and KM016905). A high-throughput method of S524 and T524 allele detection was devised (digesting the amplicon of Bgh51 WT and Bgh51 3R with Hpy8I; Table S2), and used to determine the CYP51 S524 haplotype of all 119 isolates.

2.4 Yeast phenotyping

2.4.1 Strains and complementation of transformants

Synthesis of the wild-type DH14 (Accession no. AJ313157) CYP51 gene (Bgh51WT) was carried out byGENEWIZ Inc. (South Plainfield, NJ, USA). Restriction enzyme recognition sites for Kpn1 and EcoR1 were added at the 5’- and 3’-ends respectively. The pYES-Bgh51WT expression plasmid was constructed by cloning Bgh51WT into pYES3/CT (Invitrogen, Carlsbad, CA, USA). pYES-Bgh51WT was sequenced to confirm the fidelity and transformed into S. cerevisiae YUG37-erg11 (MATa ura3-52 trp1-63 LEU2::To tetO-CYC1::ERG11) with its native Cyp51 gene under the control of a tetO-CYC1 promoter, repressed in the presence of doxycycline.26 All complementation assays were performed according to Cools et al.27 with photographs taken following 72 h of growth at 20 °C (Fig. S2). Mutations found in Bgh51 of Australian isolates were introduced into pYES-Bgh51WT through a QuickChange II site-directed mutagenesis kit (Stratagene, La Jolla, CA, USA).

2.4.2 Comparative growth rate assay of transformants

The growth rate of transformants was assessed using the GenS data analysis software (BioTek Instruments, Inc., Winooski, VT, USA) where duplicate cultures of replicates transformants were grown in SD GAL + RAF medium (SD medium) overnight at 30 °C. One hundred microliters of each overnight culture, at 10⁶ cells mL⁻¹, was used to inoculate three wells containing 200 μL SD medium ±3 μg mL⁻¹ doxycycline. Cultures were incubated without light at 30 °C, and OD₆₀₀ was measured every 15 min for 12 days in a Synergy™ HT Multi-Mode Microplate Reader (BioTek). The mean maximum growth rate for each strain ± doxycycline was determined on the basis of the greatest increase in OD over a 2 h period (Table S4).

2.4.3 Fungicide sensitivity assays

Sensitivity assays were carried out as described by Cools et al.27 using pure samples of tebuconazole, cyproconazole, propiconazole, epoxiconazole, fluquinconazole, triadimefon, flutriafol and prothioconazole-desthio with a fungicide-free control. Because prothioconazole must be activated in plant tissue,28 prothioconazole-desthio was used in all yeast assays.

2.4.4 Structural modelling

Structural modelling of Bgh51WT and mutant forms and ligand docking of epoxiconazole and fluquinconazole were undertaken using an automated homology modelling platform as described previously for Zymoseptoria tritici CYP51.29 The volume of the heme cavity of the wild-type and variant protein models was determined using Pocket-Finder (Leeds, UK) based on Ligsite.30

3 RESULTS

3.1 DNA sequencing

A trial set of Bgh isolates was assessed for sensitivity to DMI fungicides in use in WA to control powdery mildew using a detached leaf assay. Substantial variation in resistance was observed. Because of the laboriousness of this phenotyping assay, we decided to sequence the CYP51 gene first and then determine the fungicide sensitivity of isolates from each haplotype class. Primers were designed spanning both the coding and promoter region of the single Cyp51B gene31 in Bgh (Table S3, Fig. S1). The Bgh51WT DH14 sequence was used as a reference.32 Cyp51S was sequenced from 76 Australian isolates collected from 2009 to 2013, including three from eastern Australia. No indels were found in the promoter but two synonymous and five non-synonymous changes were identified in the coding region (Fig. 2). All Australian isolates carried the previously seen tyrosine to phenylalanine mutation at amino acid position 136 (Y137F).18,19 All three isolates from the east of Australia carried two synonymous changes at nucleotides 81 and 1475, which were absent in WA isolates. Further non-synonymous mutations leading to amino acid changes were found; K172E (K171E), M304I (M303I), R330G (R327G) and S524T (S509T) in various combinations (Fig. 2). Considering only the non-synonymous changes, four novel Bgh51 haplotypes were distinguished. Isolates collected in WA were either F137/T524 (haplotype 2) or F137/I304/G330/T524 (haplotype 4), whereas isolates from other Australian states were either F137/E172 (haplotype 3) or F137 (haplotype 1). Mutations I304 and G330 were consistently found together in the same isolates (Fig. 2). There was both spatial and temporal variation in the frequency of haplotypes (Fig. 1, Table S1). All isolates collected in 2009 were wild-type at Cyp51 position S524. The proportion of mutant T524 isolates dramatically increased over subsequent seasons; 99 of the 116 WA isolates collected in 2011 contained the T524 mutation. These mutants were found in all major WA barley-growing areas (Fig. 1).

3.2 DMI sensitivities of Bgh isolates

The sensitivities of 18 Bgh isolates (two isolates from haplotypes 2 and 3; seven from haplotypes 1 and 4) were determined using
The 3.3 Heterologous expression in yeast

Y137

The wild-type haplotypes 1 and 3 form most of the DMI tested. The mean EC50 values for S524 isolates (haplotypes 1 and 3) or between isolates with the same haplotype ranged from 1.88 for propiconazole to 8.06 for tebuconazole. However, the resistance factor (RF) for tebuconazole was much larger (RF = 340.6) than for fluquinconazole, which had a RF of 30.6 for propiconazole.

There was no significant differences in the mean EC50 values of isolates WA_1, WA_2, NSW and TAS are from Western Australia (1 and 2), Wagga Wagga and Launceston respectively.

Figure 2. Sequence alignment of fragments of the Cyp51 protein family. Changes found in Australian Bgh isolates to that of the wild-type DH14 (GenBank Accession AY730587).17 Isolates WA_1, WA_2, NSW and TAS are from Western Australia (1 and 2), Wagga Wagga and Launceston respectively.

Figure 3. Box plots of the EC50 (µg mL−1) of a collection of Bgh isolates having one of four Cyp51 haplotypes identified in Australia. Haplotype 1 (black), F137; haplotype 2 (strips), F137/T524; haplotype 3 (blank), F137/E172; and haplotype 4 (crosshatch), F137/I304/R330/T524. Bars represent mean EC50 (µg mL−1) of haplotypes, with error bars indicated. Cyp, cyproconazole; Epo, epoxiconazole; Flua, fludioxonil; Flut, fluquinconazole; Flut, flutriafol; Propi, propiconazole; Prothio, prothioconazole; Teb, tebuconazole; Triad, triadimenol.

detached barley leaves inserted into DMI-amended agar. The results varied between haplotype and fungicide (Fig. 3, Fig. S4). There was no significant differences in the mean EC50 values of SS24 isolates (haplotypes 1 and 3) or between isolates with the T524 mutation (haplotypes 2 and 4). Isolates of haplotypes 2 and 4 were found to have significantly higher mean EC50 values than haplotypes 1 and 3 for most of the DMIs tested. The mean EC50 values for T524 haplotypes ranged from 1.88 µg mL−1 for triadimenol, 3.73 µg mL−1 for propiconazole to 29.88 µg mL−1 for tebuconazole, whereas the SS24 isolates had mean EC50 values of 0.59, 1.09 and 1.7 µg mL−1 respectively. The estimated resistance factors ranged from 3.41 for propiconazole to 17.6 for tebuconazole. However, for fluquinconazole (used solely in WA in seed dressing formulations) mutant T524 haplotypes were marginally more sensitive (EC50 4.73 µg mL−1 compared with 8.06 µg mL−1; resistance factor (RF) = 0.58). Unfortunately, because of quarantine restrictions, we were not able to phenotype the Bgh CYP51 DH14 isolate carrying the wild-type Y137 allele.

3.3 Heterologous expression in yeast

The Bgh51wt gene was synthesized and cloned into S. cerevisiae YUG37 erg11 with a doxycycline repressible promoter. The S. cerevisiae Bgh51wt transformant was able to grow in the presence of doxycycline (Fig. S2) and for most variants there was no significant difference in the growth rates in the absence of doxycycline. Two S. cerevisiae Bgh51 variants (pYES-Bgh51_Y137F/S524T/R330G and pYES-Bgh51_Y137F/M304I/R330G/S524T) had significantly lower rates and were therefore removed from all further in vitro analysis.

The DMI sensitivities of S. cerevisiae strains expressing Bgh51 variants which restored growth on doxycycline-amended medium were determined (Table S4) and resistance factors were calculated (Table 1). Modest RF values were associated with the solo K172E and M304I mutations. RF values for the S524T mutation varied from 0.5 for fluquinconazole to 12.4 for propiconazole. The combination of F137 and T524 had much larger RF values of 340.6 for propiconazole and 33.2 for tebuconazole. RF values for fluquinconazole were < 1.0 except for the solo Y137F construct with a calculated RF of 9.7.

3.4 Structural modelling

Protein variants of all Bgh51 haplotypes were modelled in silico (Fig. S5). The effect that each mutational change had on the volume of the heme cavity containing the DMI binding site and the morphological changes to the cavity access channel were determined (Table 2). Modest volume increases in binding cavity were observed with the introduction of the solo mutations; a 17.7% increase with K172E and 39.6% increase in volume with Y137F. Mutation S524T was an exception, with an increase in the volume of the heme cavity by 73.2% compared with that of the wild-type model. The combination of F137/T524 gave an even more substantial increase in volume of 83.9%. Table 2 also shows the estimated distances between amino acids Y222 (Y222) and S521 (S506), which span the entrance to the channel leading to the DMI binding site. Modelling simulations predicted that all Bgh CYP51 mutations observed in WA would cause a restriction in the diameter of the access channel when compared to wild-type Bgh CYP51. The most dramatic decrease was observed with the introduction of the Y137F mutation, which caused a 28.5% decrease in channel diameter compared with the wild-type model. The combination of Y137F and S524T in a single model did not result in a further significant restriction (Table 2).

Further morphological changes were observed that may impact DMI binding. In particular conformation of a loop of beta-turn running from F508 to F523 (F508) is markedly different in the Y137F haplotype from that of the wild-type, with the result that it projects into the cavity. A similar constriction is observed for the F137/T524 mutant (Fig. S5). However, in this case, it is also accompanied by a substantial increase in cavity volume (Table 2), consistent with the exceptional resistance factors observed. It is interesting to note that this loop is adjacent to S524. This supports the idea that the structural changes brought about by the Y137F
### Table 1. Resistance factors of Saccharomyces cerevisiae YUG37: erg11 transformants

| Construct containing mutation(s) | Prothioxonazole | Epoxiconazole | Cyproconazole | Flutriafol | Triadimefon | Fluquinconazole |
|----------------------------------|-----------------|---------------|---------------|------------|-------------|----------------|
| pYES-1 Bgh51 Y137F               | 1.1             | 1.4           | 1.0           | 1.0        | 1.0         | 1.0            |
| pYES-1 Bgh51 K172E               | 1.6             | 2.1           | 0.6           | 0.9        | 0.2         | 0.1            |
| pYES-1 Bgh51 S524T               | 3.7             | 21            | 2.1           | 0.7        | 2.7         | 0.2            |
| pYES-1 Bgh51 Y137F/K172E         | 3.3             | 2.1           | 1.2           | 1.2        | 1.3         | 0.9            |
| pYES-1 Bgh51 Y137F/S524T         | 3.7             | 21            | 1.9           | 2.4        | 1.9         | 0.8            |
| pYES-1 Bgh51 Y137F/S524T/R330G   | 3.7             | 21            | 1.9           | 2.4        | 1.9         | 0.8            |
| pYES-1 Bgh51 Y137F/S524T/M304I   | 3.7             | 21            | 1.9           | 2.4        | 1.9         | 0.8            |
| pYES-1 Bgh51 Y137F/M304I/R330G/S524T | 3.7             | 21            | 1.9           | 2.4        | 1.9         | 0.8            |

Resistance factors (RF) were calculated from the mean EC$_{50}$ values of eight independent replicates. RF < 1 indicates greater sensitivity than the wild-type construct. No growth was observed for the pYES3/CT construct.

These constructs grew significantly more slowly after doxycycline removal and were therefore not analysed further.

Fluquinonazole docking studies were carried out to elucidate the mechanistic reasons for the contrasting cross-resistance patterns (Fig. 4). In the wild-type structure, the binding site of fluquinconazole is bordered by amino acids Y123 and Y226. It appears that the position of Y123 is particularly important in establishing the correct orientation of fluquinconazole so as to be coordinated by the heme. This arrangement is disrupted in the Y137F mutant, where Y123 and S521 prevent fluquinconazole accommodation (Fig. 4B). With the Y137F/S524T mutant, Y123 is positioned similarly to the wild-type, allowing accommodation of fluquinconazole as in the wild-type. Here, S521 borders the binding site and is predicted to interact with the fluquinconazole ligand (Fig. 4). Thus, it appears the relative inconsistency of the Y137F mutant and enhanced selection of the Y137F/S524T double mutant can be explained by the 3D docking results.

### DISCUSSION

Studies best exemplified by those in the wheat pathogen *Zymoseptoria tritici* have dissected the relationship between mutational changes in CYP51 with failures of DMI fungicides in the field. DMIs have been used since the first registration in the UK of triadimefon in 1973. Twenty years later, *Z. tritici* isolates were found with CYP51 changes conferring reductions in sensitivity. Subsequently, numerous DMIs have been introduced and additional CYP51 mutations have been identified. This long history of chemical use and the comparatively recent identification of mutations has made it difficult to discern cause and effect. The situation in WA is far simpler: DMI use has been widespread only since 2004 with the first reports of resistance dating from 2005. Furthermore, usage in WA has been dominated by first-generation DMIs and mainly tebuconazole and propiconazole formulations.

Analysis of the single CYP51 gene of Australian *Bgh* isolates collected from 2009 to 2013 revealed four haplotypes. The sensitivities of isolates from different haplotypes on detached leaves varied between the DMIs tested. *Bgh* isolates with haplotypes harbouring the S524T mutation were less sensitive to all the foliar fungicides used on barley in WA and more sensitive to fluquinconazole. The Y137F mutation was found in all isolates examined including those from the east of Australia, where as yet, there have been no reports of DMI field failure. Previous phenotypic tests correlated the presence of the Y137F mutation with strong resistance to triadimenol. We were unable import the wild-type CYP51 *Bgh* isolate DH14 into Australia due to quarantine restrictions. However, Y137F expression in the heterologous yeast system showed only modest decreases in sensitivity to most DMIs including triadimenol. The ubiquity of Y137F in Australia suggests two possibilities: (i) the limited fungicide use in eastern Australia has been sufficient to select for this mutation, or (ii) the wild-type CYP51 haplotype has never been present in Australia.

A search was conducted on the CYP51 mutations in other fungal species reported as conferring a reduction in DMI sensitivity. The *Bgh*1 amino acid sequence of Australian haplotypes was aligned with Z. tritici CYP51 (Fig. 2). Mutational changes at amino acids 137, 304, 330 and 524 fall in regions conserved between *Bgh* and *Z. tritici*. Amino acids 136 and 509 in *Bgh* correspond to 137 and 524 in *Z. tritici*, which have previously been correlated with alterations in DMI sensitivity. The combination of Y137F mutation on its own may exert selective pressure on the S524 position, leading to the F137T/S524 mutant.
Table 2. Measurements of heme cavity volume and key inter-residue distances in wild-type haplotype Y137/K172/M304/R330/S524 (WT) and mutant Bgh CYP51

| CYP51 haplotype | Heme cavity volume (Å³) | ΔHCVa from WT (%) | Diameter channel to binding site b | ΔChannel diameter from WT | ΔHCV x ΔChannel diameter |
|-----------------|-------------------------|------------------|-----------------------------------|--------------------------|--------------------------|
| Wild-type       | 1809                    | –                | 12.862                            | –                        | 0.113                    |
| Y137F           | 2526                    | +39.6            | 9.202                             | –28.5                    | 0.113                    |
| K172E           | 2130                    | +17.7            | 11.861                            | –7.8                     | 0.014                    |
| M304I           | 2573                    | +42.2            | 12.015                            | –6.6                     | 0.028                    |
| R330G           | 2607                    | +44.1            | 12.074                            | –6.1                     | 0.027                    |
| S524T           | 3134                    | +73.2            | 10.233                            | –20.4                    | 0.149                    |
| Y137F/K172E     | 2334                    | +29.0            | 10.870                            | –15.5                    | 0.045                    |
| Y137F/S524T     | 3327                    | +83.9            | 9.294                             | –28.7                    | 0.241                    |
| Y137F/M304I/R330G/S524T | 2181         | +20.6            | 9.960                             | –22.6                    | 0.047                    |

a ΔHCV – change in heme cavity volume.

b Distance between key amino acids Y123–S315 which border the entrance to the DMI binding site.

Figure 4. Docking of fluquinconazole in Blumeria graminis f. sp. hordei CYP51. (A) Wild-type CYP51, showing bound fluquinconazole (in light green, centre) and steric interaction with Y123 (surface shown as mesh). (B) The Y137F mutant, showing encroachment of Y123 and S521 (surface shown as mesh) upon the docking site of fluquinconazole, indicating that the compound cannot be bound at that location. (C) The Y137F-S524T mutant, showing orientation of Y123 similar to wild-type and predicted interaction with S521 (shown in yellow).

and S524T was associated with substantial RFs in both the Z. tritici strains and the yeast transformants. This study did not test fluquinconazole or the sole Y137F haplotype in the yeast system.

In the current study, the combination of Y137F and S524T encoded a CYP51 with a marked decrease in sensitivity to tebuconazole and propiconazole in both mildew and the yeast system. This may be sufficient to account for the field failure (Fig. 3). Increases in heme cavity volume and restriction of the access channel in Y137F/S524T protein models correlate well with the significant RF obtained (Fig. 5). A high RF for propiconazole was also observed for the Y137F/S524T Bgh CYP51 construct when expressed in the yeast system.

Structural modelling suggests that there are two main mechanisms that underpin the emergence of DMI resistance associated with mutational changes in Bgh51. The first mechanism is similar to that observed in Z. tritici CYP51,29 where the gross volume of the heme cavity increases with successive mutations (Table 2). There appears to be a correlation between the increase in cavity volume and the RF values reported in Table 1. It is likely that any increase in heme cavity volume would perturb the orientation of the DMI ligand and hence the heme binding properties. This differentiates the smaller DMI ligands such as tebuconazole and epoxiconazole.

The second mechanism provides a means of linking structural changes with phenotypic changes in a measurable way. Changes
in distances between specific pairs of residues that border the cavity result in changes to the diameter of the access channel. The limiting of the binding surface between Y226 and S314 (S312) appears to correlate well with resistance to tebuconazole. The narrowing of the access channel between Y226 and S521 correlates particularly well, especially when tempered by consideration of the effects of each variant on the cavity volume. This is demonstrated by the result obtained when the product of the per cent change in the heme cavity volume is multiplied by the per cent change in the distance between Y226 and S521 (Fig. 5). All the variants that contain F137 demonstrate a substantially reduced distance between Y226 and S521 (Table 2). When one of the mechanisms is employed, moderate resistance factors are observed: F137 (access channel narrowing); T524 (substantial increase in cavity volume). Although, when both mechanisms act together there is a strong correlation between the structural changes and the very high resistance factors of the F137/T524 mutants in the presence of tebuconazole. The in silico creation of Bgh51wt and mutant CYP51 protein variants opens the possibility of future docking studies employing novel or unregistered DMI fungicides. This will allow the prediction the effectiveness of any new product prior to in planta testing. Furthermore, we can now recommend bespoke spray regimes depending on which Bgh51 haplotype is present in the field even if the benefit might only be transient.

One of the major anti-resistance strategies used for fungicides is to mix active compounds with different MOA because isolates with mutations conferring resistance to one fungicide will most likely still be sensitive to the second.35 This strategy isolates with mutations conferring resistance to one fungicide cides is to mix active compounds with different MOA because transient.

The widespread use of high malt quality but very susceptible barley varieties and the repeated use of fungicide with a single mode of action was a perfect recipe for an epidemic of powdery mildew in WA. A report covering the decade from 1999 to 2009 estimated that Bgh caused losses of AU$30 million each year in WA.1 We have estimated that in the period from 2007 to 2010 a population of highly virulent32 and DMI-resistant Bgh caused losses of AU$100 million each year5 Since 2010, the barley area in WA has grown from 1.3 to 1.95 million ha and the total yield has grown from a decadale average of ~ 2 million tonnes to 5.1 million tonnes in 2017 as new varieties and fungicides have been introduced.38 It is reasonable to suggest that some of these increases are due to greater farmer confidence in the control of powdery mildew.

ACKNOWLEDGEMENTS
This work was funded by a PhD studentship to MAT. We would like to thank Harry Zhang for his technical assistance and Simon Ellwood and Ryan Fowler from the Department of Environment and Primary Industries, Victoria, for supplying isolates. We also thank Rohan Rainbow of the Australian Grains and Research Development Corporation for support.

SUPPORTING INFORMATION
Supporting information may be found in the online version of this article.

REFERENCES
1 Murray G and Brennan J, Estimating disease losses to the Australian barley industry. Australas Plant Pathol 39:85–96 (2010).
2 Buschges R, Holricher K, Panstruga R, Simons M, Wolter M, Frijters A et al., The barley Mlo gene: a novel control element of plant pathogen resistance. Cell 88:695–705 (1997).
3 Brent KJ, Hollomon DW, G.C.P. Federation, Fungicide resistance: the assessment of risk, Global Crop Protection Federation Brussels, Belgium (1998).
4 Giron MK, van den Bosch F, Powers SJ and Paveley ND, Fungicide resistance risk assessment based on traits associated with the rate of pathogen evolution. Pest Manag Sci 71:207–215 (2015).
5 Tucker MA, Lopez-Ruiz F, Jaya S and Oliver RP, Origin of the fungicide-resistant barley powdery mildew in Western Australia: lessons to be learned, in Fungicide Resistance in Plant Pathogens, ed. by HHDW I. Springer, Tokyo, Japan, pp. 329–340 (2015).
6 IPBARES. Australian Crop Report, Australia Co. Australian Bureau of Agricultural and Resource Economics and Sciences: Canberra (2014).
7 Brent K and Hollomon DW, Fungicide Resistance: The Assessment of Risk. Fungicide Resistance Action Committee, Brussels, Belgium (2007).
8 Senior U, Hollomon DW, Lowe SS and Baldwin BC, Sterol composition and resistance to DMI fungicides in Erysiphe graminis. Pestic Sci 45:57 –67 (1995).
9 Dupont S, Lemetsais G, Ferreira T, Cayot P, Gervais P and Bene L, Ergosterol biosynthesis: a fungal pathway for life on land? Evolution 16:2961–2968 (2012).
10 Xiang MJ, Liu JY, Ni PH, Wang S, Shi C, Wei B et al., Erg11 mutations associated with azole resistance in clinical isolates of Candida albicans. FEMS Yeast Res 13:386–393 (2013).
11 Hull CM, Parker JE, Bader O, Weig M, Gross U, Warnoll AGS et al., Facultative sterol uptake in an ergosterol-deficient clinical isolate of Candida glabrata harboring a missense mutation in Erg11 and exhibiting cross-resistance to azoles and amphotericin B. Antimicrob Agents Chemother 56:4223–4232 (2012).
12 Lelièvre L, Groh M, Angebault C, Maherauc AC, Didier E and Bougnoux ME, Azole resistant Aspergillus fumigatus: an emerging problem. Med Mal Infect 43:139–145 (2013).
13 Cools HJ, Harkins NJ and Fraaije BA, Constraints on the evolution of azole resistance in plant pathogenic fungi. Plant Pathol 62:36–42 (2013).
14 Oliver RP and Hewitt HG, *Fungicides in Crop Protection*, 2nd edn. CAB, Wallingford, UK (2014).

15 Omrane S, Sghyer A, Audén C, Lanen C, Duplaix C, Walker AS et al., Fungicide efflux and the MgMFS1 transporter contribute to the multidrug resistance phenotype in *Zymoseptoria tritici* field isolates. *Environ Microbiol* **17**:2805–2823 (2015).

16 Cools HJ and Fraaije BA, Are azole fungicides losing ground against Septoria wheat disease? Resistance mechanisms in *Mycosphaerella graminicola*. *Pest Manag Sci* **72**:1449–1459 (2016).

17 Dèlye C, Bousset L and Corio-Costet MF, PCR cloning and detection of point mutations in the ebucarol 14α-demethylase (CYP51) gene from *Erysiphe graminis* f. sp. *hordei*, a ‘recalcitrant’ fungus. *Curr Genet* **34**:399–403 (1998).

18 Wyand RA and Brown JK, Sequence variation in the CYP51 gene of *Blumeria graminis* associated with resistance to sterol demethylase inhibiting fungicides. *Fungal Genet Biol* **42**:726–735 (2005).

19 APVMA, *Public Chemical Registration Information Systems Search*. Available: https://portal.apvma.gov.au/pubcris?p_auth=ZdZn7ZW&pp_id=pubcrisportlet_WARN_pubcrisportlet&pp_lifecycle=1&pp_state=normal&pp_mode=view&pp_col_id=column-1&pp_col_pos=2&pp_col_count=48&pubcrisportlet_WARN_pubcrisportlet_id=44205&pubcrisportlet_WARN_pubcrisportlet_javax.portlet.action=viewProduct [20 June 2014].

20 GRDC, *Barley Powdery Mildew Fact Sheet: Control Strategies for Powdery Mildew*. CORETEXT, Western Region (2012). Available: https://grdc.com.au/resources-and-publications/all-publications/factsheets/2012/12/barley-powder-mildew-2012. 2012/12/barley-powder-mildew-2012.

21 Tucker MA, Lopez-Ruiz F, Jayasena K and Oliver RP, Origin of fungicide-resistant barley powdery mildew in Western Australia: lessons to be learned, in *Fungicide Resistance in Plant Pathogens: Principles and Guide to Practical Management*, ed. by Ishi H and Holloman D, Springer, Japan, pp. 329–340 (2015).

22 Tucker MA, Jayasena K, Ellwood SR and Oliver RP, Pathotype variation of barley powdery mildew in Western Australia: *Australas Plant Pathol* **42**:617–623 (2013).

23 Chan KC and Boyd WJR, Pathogenic variation of powdery mildew of barley in Western Australia. *Aust J Agr Res* **43**:79–85 (1992).

24 Czembor JH, Resistance to powdery mildew populations of barley landraces from Morocco. *Australas Plant Pathol* **29**:137–148 (2000).

25 Parker JE, Merkamm M, Manning NJ, Pompon D, Kelly SL and Kelly DE, Differential azole antifungal efficacies contrasted using a *Saccharomyces cerevisiae* strain humanized for sterol 14α-demethylase at the homologous locus. *Antimicrob Agents Chemother* **52**:3597–3603 (2008).

26 Cools HJ, Parker JE, Kelly DE, Lucas JA, Fraaije BA and Kelly SL, Heterologous expression of mutated ebucarol 14α-demethylase (CYP51) proteins of *Mycosphaerella graminicola* to assess effects on azole fungicide sensitivity and intrinsic protein function. *Appl Environ Microbiol* **76**:2866–2872 (2010).

27 Parker JE, Warrillow AGS, Cools HJ, Fraaije BA, Lucas JA, Rigdova K et al., Prothioconazole and prothioconazole-desthio activities against *Candida albicans* sterol 14α-demethylase. *Appl Environ Microbiol* **79**:1639–1645 (2013).

28 Mullins JGL, Parker JE, Cools HJ, Togawa RC, Lucas JA, Fraaije BA et al., Molecular modelling of the emergence of azole resistance in *Mycosphaerella graminicola*. *PLoS One* **6**:e20973 (2011). Available: https://doi.org/10.1371/journal.pone.0020973.

29 Hendrich M, Rippmann F and Barnickel G, LIGSITE: automatic and efficient detection of potential small molecule-binding sites in proteins. *J Mol Graph Model* **15**:359–363 (1997).

30 Becher R and Wirsel SGR, Fungal cytochrome P450 14α-demethylase (CYP51) and azole resistance in plant and human pathogens. *Appl Microbiol Biotechnol* **95**:825–840 (2012).

31 Spanu PD, Abbott JC, Amselem J, Burgess TA, Soanes DM, Stüber K et al., Genome expansion and gene loss in powdery mildew fungi reveal tradeoffs in extreme parasitism. *Science* **330**:1543–1546 (2010).

32 Russell PE, A century of fungicide evolution. *J Agric Sci* **143**:11–25 (2005).

33 Cools HJ, Mullins JGL, Fraaije BA, Parker JE, Kelly DE, Lucas JA et al., Impact of recently emerged sterol 14α-demethylase (CYP51) variants of *Mycosphaerella graminicola* on azole fungicide sensitivity. *Appl Environ Microbiol* **77**:3830–3837 (2011).

34 Van Den Bosch F, Paveley N, Van Den Berg F, Hobbel P and Oliver R, Mixtures as a fungicide resistance management tactic. *Phytopathology* **104**:1264–1273 (2014).

35 Leroux P, Albertini C, Gautier A, Gredt M and Walker AS, Mutations in the CYP51 gene correlated with changes in sensitivity to sterol 14α-demethylase inhibitors in field isolates of *Mycosphaerella graminicola*. *Pest Manag Sci* **63**:688–698 (2007).

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

37 Australia GloW, *GIWA Crop Report: Australia* (2019). Available: http://www.giwa.org.au/2019 and http://www.giwa.org.au/2010-crop-reports.