

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

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## 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.

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**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%<sup>1</sup> but is generally well controlled by host genetics including the durable recessive *mlo* gene,<sup>2</sup> 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.<sup>3,4</sup>

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.<sup>5</sup> 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).<sup>1,6</sup>

DMI fungicides have been in the forefront of control of fungal pathogens of humans, animals and plants for nearly 50 years.<sup>7</sup>

These fungicides interrupt the biosynthesis of ergosterol (and other mycosterols in powdery mildews) by inhibiting cytochrome P450 14 $\alpha$ -sterol demethylase (CYP51).<sup>8,9</sup> Resistance is now common in human pathogens, including *Candida* spp.<sup>10,11</sup> and *Aspergillus fumigatus*,<sup>12</sup> and is a serious problem in agricultural systems.<sup>13,14</sup> Fungicide resistance has been associated

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with a number of mechanisms including the alteration and overexpression of the *Cyp51* gene(s) as well as enhanced DMI efflux.<sup>13,15,16</sup>

The most commonly observed mechanism of resistance is non-synonymous changes in the gene sequence of *Cyp51*.<sup>13</sup> 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.<sup>17</sup> Two earlier studies examining DMI resistance in *Bgh* in Europe identified two changes in *Cyp51*, *Y137F* and *K148Q* (equivalent to *Y136F* and *K147Q*).<sup>18,19</sup> 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<sup>20</sup> 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.<sup>5</sup> Since 2005, WA farmers have reported reduced efficacy of DMIs in controlling barley powdery mildew outbreaks.<sup>21</sup> 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 *Bgh* isolates to DMI fungicides registered in WA for use on barley. Sequencing of the *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 *Saccharomyces cerevisiae*. Our results link variations in DMI sensitivity to changes in *CYP51*. *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<sup>14</sup> pp. 129–130). A brief report on some this data has been published previously.<sup>22</sup>

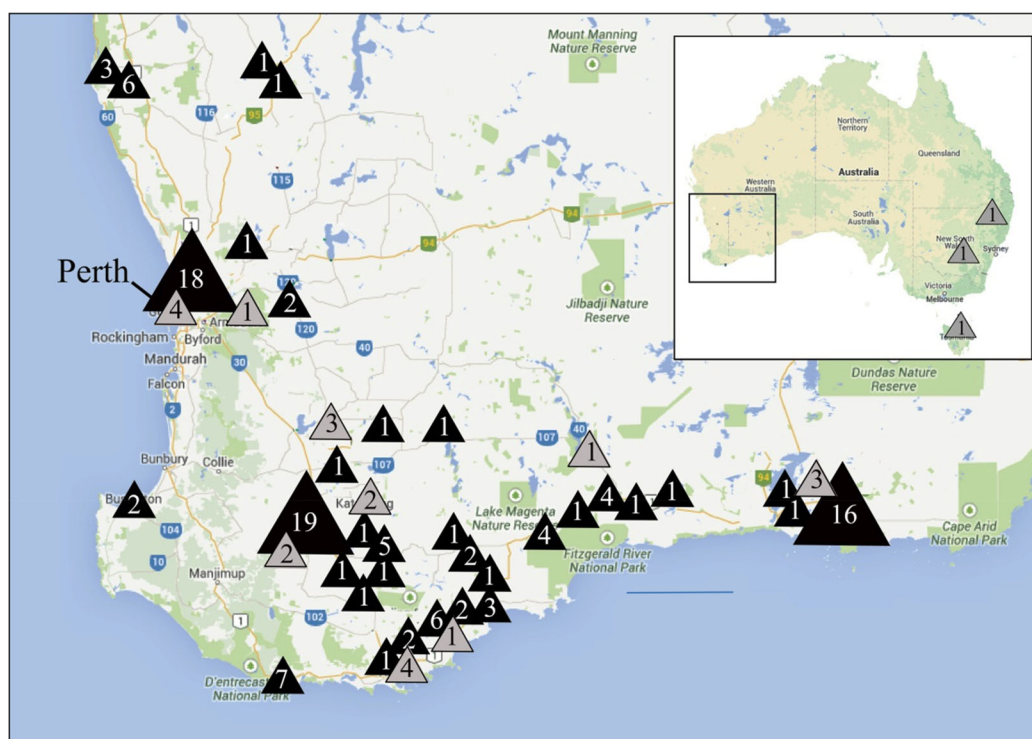
## 2 MATERIALS AND METHODS

### 2.1 Isolates

One hundred and nineteen *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.<sup>23</sup>

### 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 *Bgh* control – Laguna (720 g L<sup>-1</sup> tebuconazole, Sipcam, Geelong, Victoria, Australia), Flutriafol (250 g L<sup>-1</sup> flutriafol, Imtrade, Perth WA, Australia), Opus (125 g L<sup>-1</sup> epoxiconazole, Nufarm), Alto (100 g L<sup>-1</sup> cyproconazole, NuFarm, Laverton, Victoria, Australia), Tilt (418 g L<sup>-1</sup> propiconazole, Syngenta, North Ryde, NSW, Australia), Proline (410 g L<sup>-1</sup> prothioconazole, Bayer Cropscience, Kallaroo, WA, Australia), Triad 125 (125 g L<sup>-1</sup> triadimefon, Farmoz (Adama) St Leonards, NSW, Australia) and Jockey Stayer (167 g L<sup>-1</sup> fluquinconazole, Bayer Crop Science) – were incorporated into agar amended with 50 mg L<sup>-1</sup>



**Figure 1.** Sample sites of *Bgh* isolates collected from Australia. Black triangles indicate mutant *T524 CYP51* isolates ( $n = 119$ ). Grey triangles indicate isolates with *CYP51* haplotype *S524* ( $n = 24$ ). Numbers within triangles indicate isolates collected at each site. Scale bar, 100 km. The map is from [https://www.westernaustralia.com/en/plan\\_your\\_trip/Pages/WA\\_maps.aspx#](https://www.westernaustralia.com/en/plan_your_trip/Pages/WA_maps.aspx#/).

of benzimidazole.<sup>24</sup> 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 ( $EC_{50}$ ) 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  $20 \pm 2^\circ\text{C}$  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.<sup>25</sup> 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  $EC_{50}$  values with associated errors were calculated for each *Bgh* *Cyp51* haplotype (Fig. 3). All  $EC_{50}$  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 amplicons 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\_3F* and *Bgh51\_3R* with Hpy8I; Table S2), and used to determine the *CYP51* 524 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 by GENEWIZ 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::tTa tetO-CYC1::ERG11*) with its native *Cyp51* gene under the control of a *tetO-CYC1* promoter, repressed in the presence of doxycycline.<sup>26</sup> All complementation assays were performed according to Cools *et al.*<sup>27</sup> with photographs taken following 72 h of growth at  $20^\circ\text{C}$  (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 Gen5 data analysis software (BioTek Instruments, Inc., Winooski, VT, USA) where duplicate cultures of replicate transformants were grown in SD GAL + RAF medium (SD medium) overnight at  $30^\circ\text{C}$ . One hundred microliters of each overnight culture, at  $10^5$  cells  $\text{mL}^{-1}$ , was used to inoculate three wells containing 200  $\mu\text{L}$  SD medium  $\pm 3 \mu\text{g mL}^{-1}$  doxycycline. Cultures were incubated

without light at  $30^\circ\text{C}$ , and  $OD_{600}$  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  $\pm$  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.*<sup>27</sup> 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,<sup>28</sup> 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.<sup>29</sup> The volume of the heme cavity of the wild-type and variant protein models was determined using Pocket-Finder (Leeds, UK) based on Ligsite.<sup>30</sup>

## 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* gene<sup>31</sup> in *Bgh* (Table S3, Fig. S1). The *Bgh51wt* DH14 sequence was used as a reference.<sup>32</sup> *Cyp51* 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).<sup>18,19</sup> 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 (M301I), 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 524. 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

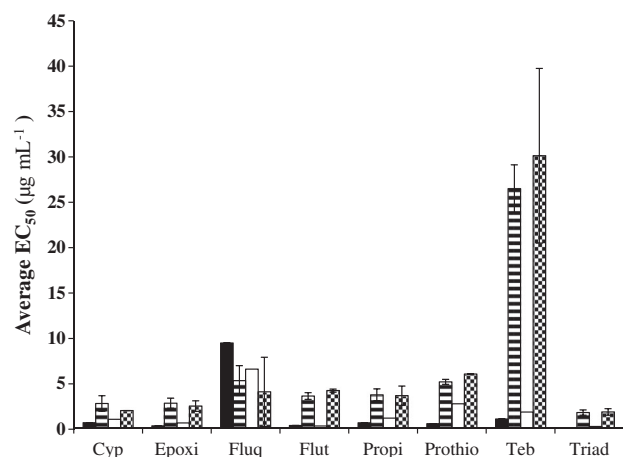


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WA_1  ...FGTDVVFDCPNS...IQNEVKSFIEK...KEIAHIMIALL...LWLAAGPDITE...DYSSMFTRPMAPA...
WA_2  ...FGTDVVFDCPNS...IQNEVKSFIEK...KEIAHMMIALL...LWLAARPDITE...DYSSMFTRPMAPA...
NSW   ...FGTDVVFDCPNS...IQNEVESFIEK...KEIAHMMIALL...LWLAARPDITE...DYSSMFSRPMAPA...
TAS   ...FGTDVVFDCPNS...IQNEVKSFIEK...KEIAHMMIALL...LWLAARPDITE...DYSSMFSRPMAPA...
DH14  ...FGTDVVYDCPNS...IQNEVKSFIEK...KEIAHMMIALL...LWLAARPDITE...DYSSMFSRPMAPA...
Z. tri ...FGKDVVYDCPNS...IAAETRQFFDR...KEIAHMMIALL...LRLASRPDIQD...DYSSLFSRPLSPA...
.      137          172          304          330          524

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**Figure 2.** Sequence alignment of fragments of the Cyp51 protein family. Changes found in Australian *Bgh* isolates to that of the wild-type DH14 (ABS01000011.1) are indicated in bold. Numbers above represent amino acid positions in the *Bgh* and below in the archetype *Zymoseptoria tritici* isolate ST1 (GenBank Accession AY730587).<sup>17</sup> 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  $EC_{50}$  ( $\mu\text{g mL}^{-1}$ ) of a collection of *Bgh* isolates having one of four Cyp51 haplotypes identified in Australia. Haplotype 1 (black), *F137*; haplotype 2 (stripes), *F137/T524*; haplotype 3 (blank), *F137/E172*; and haplotype 4 (crosshatch), *F137/I304/R330/T524*. Bars represent mean  $EC_{50}$  ( $\mu\text{g mL}^{-1}$ ) of haplotypes, with error bars indicated. Cyp, cyproconazole; Epoxi, epoxiconazole; Fluq, fluquinconazole; Flut, flutriafol; Propi, propiconazole; Prothio, prothioconazole; Teb, tebuconazole; Triad, triadimefon.

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  $EC_{50}$  values of *S524* 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  $EC_{50}$  values than haplotypes 1 and 3 for most of the DMIs tested. The mean  $EC_{50}$  values for *T524* haplotypes ranged from  $1.88 \mu\text{g mL}^{-1}$  for triadimefon,  $3.73 \mu\text{g mL}^{-1}$  for propiconazole to  $29.88 \mu\text{g mL}^{-1}$  for tebuconazole, whereas the *S524* isolates had mean  $EC_{50}$  values of  $0.59$ ,  $1.09$  and  $1.7 \mu\text{g mL}^{-1}$  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 [ $EC_{50}$   $4.73 \mu\text{g mL}^{-1}$  compared with  $8.06 \mu\text{g mL}^{-1}$ ; 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 *Y226* (*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 *S520* (*S505*) 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)	Resistance factors						
	Tebuconazole	Epoxiconazole	Propiconazole	Prothioconazole-desmethio	Cyproconazole	Flutriafol	Triadimefon
pYES- <i>Bgh51_Y137F</i>	1.1	3.7	1.6	3.3	1.0	1.4	1.5
pYES- <i>Bgh51_K172E</i>	0.9	1.4	0.6	2.1	0.9	1.0	0.9
pYES- <i>Bgh51_M304I</i>	0.9	1.8	2.1	0.2	0.7	0.5	0.5
pYES- <i>Bgh51_S524T</i>	3.7	7.3	12.4	1.2	2.1	2.7	3.6
pYES- <i>Bgh51_Y137F/K172E</i>	1.3	2.4	1.9	0.9	0.9	1.3	3.3
pYES- <i>Bgh51_Y137F/S524T</i>	33.2	18.5	340.6	0.8	3.2	10.5	23.8
pYES- <i>Bgh51_Y137F/S524T/M304I</i>	2.3	4.9	2.8	0.1	0.8	1.9	5.7
pYES- <i>Bgh51_Y137F/S524T/R330G<sup>a</sup></i>							
pYES- <i>Bgh51_Y137F/M304I/R330G/S524T<sup>a</sup></i>							

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

<sup>a</sup> These constructs grew significantly more slowly after doxycycline removal and were therefore not analysed further.

mutation on its own may exert selective pressure on the 524 position, leading to the *F137/T524* mutant.

Fluquinconazole 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.

## 4 DISCUSSION

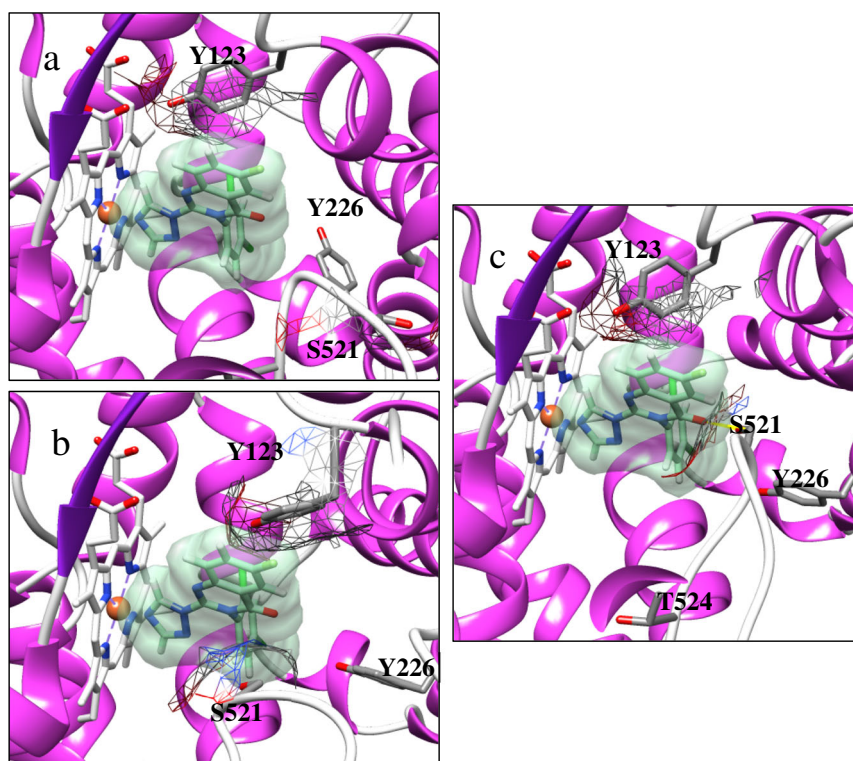
Studies best exemplified by those in the wheat pathogen *Zymoseptoria tritici* have dissected the relationship between mutational changes in CYP51 with failures of DMI fungicides in the field. DMIs have been used since the first registration in the UK of triadimefon in 1973.<sup>33</sup> Twenty years later, *Z. tritici* isolates were found with CYP51 changes conferring reductions in sensitivity.<sup>13</sup> Subsequently, numerous DMIs have been introduced and 34 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 solo tebuconazole and propiconazole formulations.<sup>22</sup>

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.<sup>18,19</sup> We were unable to 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 (Table 1). This suggests that Y137F would lead to only small reductions in the DMI field efficacy. 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 *Bgh51* 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*.<sup>31</sup> Amino acids 136 and 509 in *Bgh51* correspond to 137 and 524 in *Z. tritici*, which have previously been correlated with alterations in DMI sensitivity.<sup>34</sup> The combination of Y137F

**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 (Å <sup>3</sup> )	ΔHCV <sup>a</sup> from WT (%)	Diameter channel to binding site <sup>b</sup>	ΔChannel diameter from WT	ΔHCV × ΔChannel diameter
Wild-type	1809	–	12.862		
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

<sup>a</sup> ΔHCV – change in heme cavity volume.<sup>b</sup> Distance between key amino acids Y226–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).

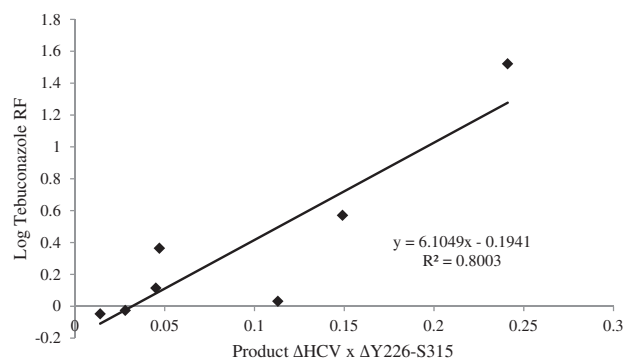
and S524T was associated with substantial RFs in both the *Z. tritici* strains and the yeast transformants. This study did not test fluquinconazole or the solo Y137F haplotype in the yeast system.

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,<sup>29</sup> 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





**Figure 5.** Correlation between tebuconazole resistance factor (RF) of pYES-*Bgh51\_Y137F/S524T* and the product of the change in the volume of the heme cavity ( $\Delta\text{HCV}$ ) with the change in distance between amino acids Y226 and S315 ( $\Delta\text{Y226-S312}$ ).

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 of 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.<sup>35</sup> This strategy requires that there is no positive cross resistance between the two fungicides and so generally rules out mixtures of the same MOA. However some cases of negative cross-resistance within a single MOA group has been shown with *Z. tritici* isolates which are highly resistant to tebuconazole but fully susceptible to prochloraz.<sup>36,37</sup> The negative cross-resistance shown in both the *Bgh* (Fig. 3) and yeast expression studies (Table 1) was confirmed using *in silico* protein docking studies. Here the single Y137F mutation substantially impaired the binding of fluquinconazole (Fig. 4). By contrast, the binding of fluquinconazole at the docking site of the Y137F/S524T protein model was more akin to that of the wild-type. Exploitation of negative cross-resistance as a resistance management is attractive in principle but would be rendered inoperable if cross-resistant isolates were to evolve.

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.<sup>1</sup> We have estimated that in the period from 2007 to 2010 a population of highly virulent<sup>23</sup> and DMI-resistant *Bgh* caused losses of AU\$100 million each year.<sup>5</sup> 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 decadal average of ~2 million tonnes to 5.1 million tonnes in 2017 as new varieties and fungicides have been introduced.<sup>38</sup> It is reasonable to suggest that some of these increases are due to greater farmer confidence in the control of powdery mildew.

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## SUPPORTING INFORMATION

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

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