School of Science

Department of Environment and Agriculture

Adaptation of Barley Powdery Mildew (*Blumeria graminis* f. sp. *hordei*) in Western Australia to Contemporary Agricultural Practices.

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“To the best of my knowledge and belief this thesis contains no material previously published by any other person or accepted in the award of any other degree or diploma in any university except where due acknowledgment has been made.”

1.0 Abstract

The powdery mildew pathogen *Blumeria graminis* f. sp. *hordei* (*Bgh*) is a significant threat to barley production, but is controlled in most regions of the world through combinations of host genetics and fungicides. Unfortunately in recent years powdery mildew has emerged as the most damaging disease of barley in Western Australia (WA) where many of the currently available cultivars are susceptible to disease and therefore growers have had to rely almost exclusively on fungicides as the main method of disease control

On a global scale *Bgh* is known to exist in numerous races or pathotypes each with a defined set of avirulence genes. Knowledge of the avirulence genes in the local population can be used to elucidate which major plant resistance genes (*R*-genes) may prove useful in current or future breeding strategies. Here sixty isolates of *Bgh* were collected from nine locations across Australia. Each was used to infect a set of cultivars with known *R*-genes for *Bgh.* A total of 18 unique pathotypes were identified and virulence against many of the *R*-genes were present in at least one pathotype. Undefeated genes included *Mla-6*, *Mla-9*, *Ml-ra* and the combinations of *Mla-1* plus *Mla-A12* and *Mla-6* plus *Mla-14* and *Mla-13* plus *Ml- Ru3* together with recessive *mlo-5*. The data indicated some pathotype spatial differentiation suggesting local selection pressures and proliferation within different regions of WA.

Highly polymorphic microsatellite or short sequence repeat (SSR) markers are additional tools that can be used to characterise diversity in pathogen populations. The number of short intergenic or intragenic repeats often varies between individuals. Thus SSR markers can be used to screen collections of isolates to elucidate intrinsic genetic relatedness. Despite the widespread colonisation of *Bgh* in many agriculturally important global cropping regions, as yet no SSR markers have been defined. Here, eight polymorphic microsatellite loci were identified and characterised. Primer pairs amplifying the loci were then applied to 111 Australian isolates of *Bgh*. The number of alleles per locus ranged from 4 to 13, and Nei's genetic diversity ranged from 0.25 to 0.76. The SSR primers detected several clones and defined 97 unique haplotypes. There was little evidence for regional genotypic subdivision, suggesting the movement of *Bgh* in the major Australian cropping areas remains largely unrestricted. All data was consistent with high levels of genetic diversity, potentially resulting from random mating and spread within each region.

Recent reductions in the efficacy of triazole fungicides in WA suggest that resistance may have developed. Yields in affected paddocks were reduced by 25 to 40% and resulted in harvested grain being downgraded from malt to feed. To determine if *Cyp51* (encoding 14α-sterol demethylase; syn. *ERG11*) mutations were causing the reduction in triazole efficacy, the *in vitro* sensitivities of *Bgh* isolates to currently registered triazoles were obtained. *Cyp51* sequencing revealed five non-synonymous amino acid substitutions: Y136F, K171E, M301I, R327G and S509T. A clear association was established between combinations of mutations and altered levels of resistance to triazoles. Characterization of the mutations in a yeast expression system dissected the contribution of each mutation to the final resistant phenotype. Yeast genotypes harbouring the S509T mutation were less sensitive to all triazoles except fluquinconazole, which was more effective than other genotypes, exhibiting negative cross resistance. Protein structural modelling revealed a clear association between fungicide sensitivity and the heme cavity volume. Fluquinconazole docking studies confirmed the negative cross resistance. The *in silico* appraisal of binding efficiencies between specific triazoles and *Cyp51* variants promises to facilitate the design of resistance management strategies that counter select the most abundant mutations and considerably extend the useful life of this critical group of fungicides.

Previous European studies have predicted that the repeated use of fungicides of single mode of action can lead to fungicide resistance. In Australia, contemporary agricultural practices have resulted in the utilisation of barley cultivars often harbouring a single dominant resistance gene for *Bgh.* Unfortunately resistance mediated in this manner has often proved to be short-lived, breaking down in as soon as a single season after wide scale exploitation. In WA, high disease incidence on susceptible cultivars has necessitated the application of fungicides for *Bgh* control – with each registered formulation containing a triazole. This study details the rapid adaptation of WA barley powdery mildew isolates, where with disappointing predictability many resistance genes and fungicides no longer provide effective control.

2.0 Acknowledgements

This research was conducted through scholarships from the Grains Research and Development Corporation (GRDC, Australia) and Curtin University, Australia.

3.0 Publications

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3.2 List of Publications

Tucker M. A, Jayasena K, Ellwood SR and Oliver RP (2013), Pathotype Variation of Barley Powdery Mildew in Western Australia. *Australasian Plant Pathology.* 42(5):617-623. DOI: 10.1007/s13313-013-0226-y

Tucker M. A., Moffat C. S., Ellwood S. R., Tan K. C., Jayasena K. and Oliver R. P.(2014), Development of Genetic SSR Markers in *Blumeria graminis* f. sp. *hordei* and Application to Isolates from Australia. *Plant Pathology*. 64 (2): 337-343. DOI: 10.1111/ppa.12258.

Tucker M. A, Lopez-Ruiz, F, Jayasena, and Oliver, R.P (2014) Origin of Fungicide Resistant Barley Powdery Mildew in West Australia – Lessons to be Learned. In Ishii, H and Hollomon, D (eds.), *Fungicide Resistance in Plant Pathogens: Principals and a Guide to Practical Management*. Springer, Japan. (In Press).

Tucker M. A, Lopez-Ruiz, F., Cools H. J., Mullins J. G. L. and Oliver R. P. (2014), Accumulation of mutations in *Blumeria graminis* f. sp. *hordei CYP51* confers positive and negative cross-resistance to triazole fungicides. (Under Review in New Phytologist).

4.0 Statement of Contribution of Others

Every reasonable effort has been made to acknowledge the owners of copyright material. I would be pleased to hear from any copyright owner who has been omitted or incorrectly acknowledged.

5.0 Additional Publications

5.1 Tucker M. A, Lopez-Ruiz F, Cools H. J, Mullins J. G. L. and Oliver R. P, *Accumulation of Mutations in Blumeria graminis f. sp. hordei Cyp51 Confers Positive and Negative Cross-resistance to Triazole Fungicides*. 28th Fungal Genetics Conference, March 2015, Monterey, USA.

5.2 Lopez-Ruiz F, Mair M, Rybak K, Chang Y, Davidson J, Tucker M. A and Oliver R. P, 2014, *Baseline Sensitivity of a Historical Collection of Botrytis spp. Isolates to Major Fungicides in Australia*. in Dehne H. W, Deising H. B, Fraaije B, Gisi U, Hermann D, Mehl A, Oerke E. C, Russell P. E, Stammler G, Kuck K. H and Lyr H (eds.) Modern Fungicides and Antifungal Compounds VII, DPG Spectrum Phytomedizin, Germany, pp. 135-140.

5.3 Tucker M. A, Lopez-Ruiz F, Cools H. J, Mullins J. G and Oliver R. P, *Barley Powdery Mildew Fungicide Resistance; Are we at a New Cliff Edge?* Pesticide Resistance Conference, November 2014, Perth, Australia.

5.4 Tucker M. A. and Oliver R. P, *Extra land clearing negates organic expectations.* Ground Cover. Issue 107. Nov to Dec 2013.

5.5 Tucker M. A, Lopez-Ruiz F, Cools H. J, Mullins J. G. L. and Oliver R. P, *The impact of recently emerged mutations in Cyp51 in Blumeria graminis f. sp. hordei isolates in Western Australia.* 17th International Reinhardsbrunn Symposium Modern Fungicides and Antifungal Compounds, April 2013, Friedrichroda, Germany.

5.6 Lopez-Ruiz F, Mair W, Davidson J, Rybak K, Tucker M. A and Oliver R. P, *Exploring the Molecular Basis Underlying Fungicide Resistance in Botrytis spp. Infecting Pulses in Australia.* 17th International Reinhardsbrunn Symposium Modern Fungicides and Antifungal Compounds, April 2013, Friedrichroda, Germany.

5.7 Tucker M. A, Lopez-Ruiz F, Ellwood S. R, Cools H. J, Jayasena K and Oliver R. P, *Barley Powdery Mildew: An Epidemic Caused by Fungicide Resistance*, Pesticide Resistance Conference, September 2012, University of Western Australia, Perth, Australia.

5.8 Tucker M. A, Lopez-Ruiz F, Cools H. J, Jayasena K and Oliver R. P, *A new mutation in the cytochrome P45014α-demethylase gene (Cyp51) mediates triazole resistance in barley powdery mildew in Australia.* Combined Biological Sciences Meeting, September 2012, Adelaide, Australia.

5.9 Tucker M. A and Oliver R. P (2011) *Barley Powdery Mildew Resistance Confirmed in Western Australia.* WANTFA New Frontiers in Agriculture, June 2011 Issue.

6.0 Table of Contents

7.0 Introduction and Overview

7.1 General Introduction

7.11 Powdery Mildew of Barley

Powdery mildew fungi cause disease on most cultivated plants ranging from cereals and vegetables to fruit trees and ornamental plants. At present, 650 powdery mildew species have been defined infecting thousands of plant species (Braun, Cook et al. 2002). In cereals the disease is caused by *Blumeria graminis* which has adapted into separate *forma specialis* (ff. spp.) depending on host specificity. *Blumeria graminis* f. sp. *hordei* (*Bgh*) is classified in the fungal Division *Ascomycota* (producing non-motile spores, called ascospores, in an ascus), Class *Leotiomycetes* (having cylindrical asci, without operculum, that are developed in the apothecia), Order *Erysiphales* (with cleistothecia having asci arranged in a hymenial layer), Family *Erysiphaceae*, Genus *Blumeria* and Species *graminis* Forma specialis *hordei* (Braun, Cook et al. 2002). *Bgh* occurs throughout many of the barley growing regions of the world including southern Western Australia. Barley is the fourth most important crop in the world behind wheat, maize and rice (FAOSTAT 2014). In WA barley is of great economic importance producing on an average of 2.5M tonnes per annum (ABARES 2014). WA growers are renowned for producing high quality malt grade grain, the majority of which is exported, mainly for malt and Japanese shochu production but also for human and animal consumption (Paynter, Hills et al. 2011). In many parts of the world *Bgh* is controlled through combinations of host resistance and fungicide applications. In WA, foreign demand for malt grade grain has led to the majority of the barley cropping area being sown with mildew susceptible cultivars. Therefore growers in regions prone to infection have relied heavily on the application of fungicides for disease control.

7.12 Disease symptoms

Barley powdery mildew infections are characterised by white to grey fluffy pustules which can occur on all aerial parts of the plant from the stem to the leaves and awns (Figure 1). Initial symptoms are usually on the abaxial surface of lower leaves and take the form of yellow chlorotic flecks. The development of white fluffy pustules follows rapidly which sprout masses of asexual conidia from the adaxial leaf surface. Cleistothecia (fruiting bodies forming sexual

spores) may develop late in the season and can be found embedded in the mildew pustules as minute black dots (Figure 2).

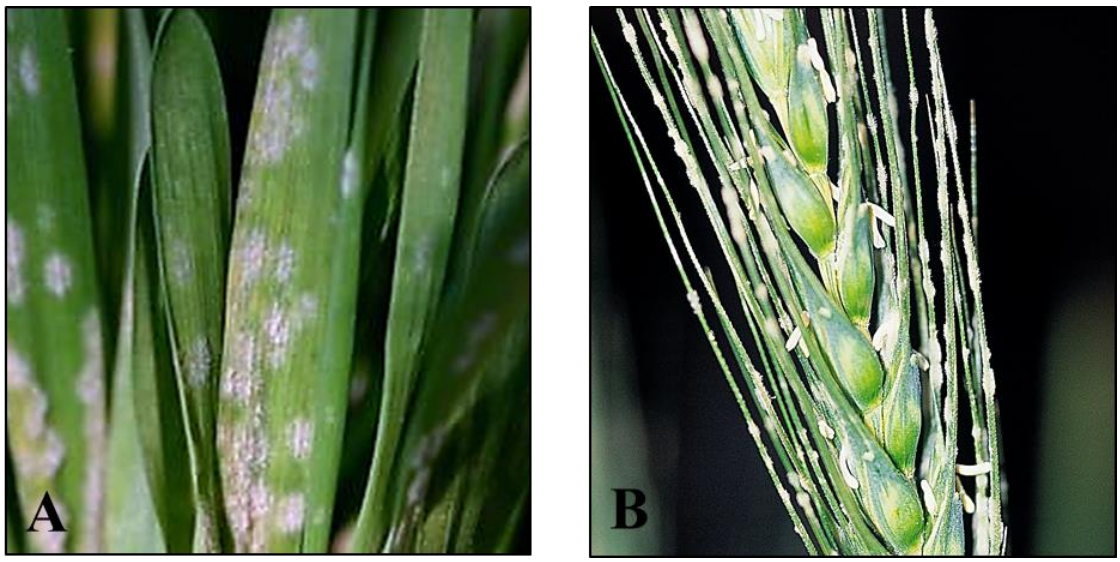


Figure 1. *Blumeria graminis* f. sp. *hordei* infecting barley (*Hordeum vulgare*).

Examples of pustule development on A) leaf tissue and B) awn.

In cultivars with some level of resistance to *Bgh* infection it is also common to observe localised brownish regions of cell necrosis triggered by the activation of the plant innate hypersensitive response (Figure 2). Here, cultivars undergo localised cell apoptosis in the region of infection thereby starving the biotrophic pathogen of essential plant derived nutrients.

In interactions that lead to disease (frequently called compatible interactions) or to immune responses (called incompatible interactions), it is not unusual to observe different stages of fungal development on a single leaf. The relative frequencies of the various developmental stages differ and this allows macroscopic discrimination of five infection types (ITs) ranging from IT0 to IT4 (Boyd, Smith et al. 1995; Thordal-Christensen, Gregersen et al. 2000). The coinciding developmental spectrum can be used to qualify the extent of interaction between host and pathogen and to gauge the level of host resistance or susceptibility.

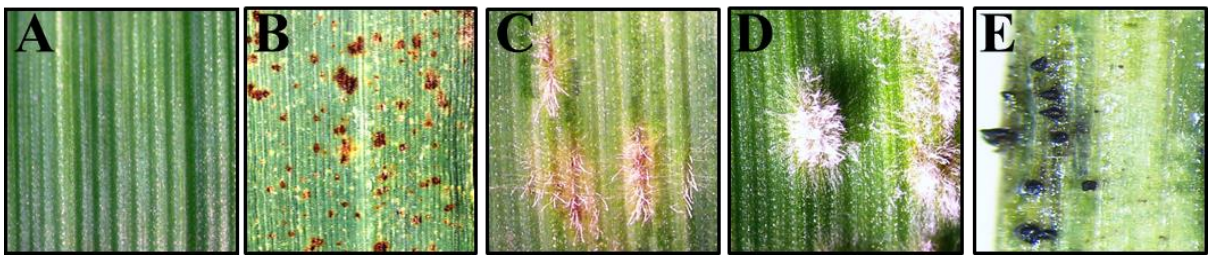


Figure 2. Various stages of development of *Blumeria graminis* f. sp. *hordei.*

A) Complete plant resistance (IT0), B) The plant innate hypersensitive response has triggered the death of cells at localised infection points resulting in necrotic flecks (IT1), C) Plant incomplete resistance characterised by patches of necrosis with some sporulation (IT2-IT3), D) Complete plant susceptibility characterised by profuse sporulation and total lack of necrosis (IT4) and E) Sexual fruiting bodies, cleistothecia, permits overwintering of pathogen on crop debris.

7.13 Life Cycle and Epidemiology

The dispersal of mildew occurs through the spread of asexual haploid conidia between susceptible host plants. The spores disseminate by wind and initiate a new infection cycle upon landing on neighbouring plants or by migrating up to hundreds of kilometres (Jørgensen, 1994; Thordal-Christensen et al., 2000). When a conidium lands on the leaf surface, infection structures begin to rapidly differentiate (Pascholati, Yoshioka et al. 1992). This begins with the development of two morphologically distinct germ tubes (Figure 3). The primary germ tube (PGT) appears approximately 2-4h after contact and is thought to be involved in adhesion and orientation, water acquisition and transduction of leaf derived signals (Heitefuss 2001; Eichmann and Hückelhoven 2008). Successful attachment drives the differentiation of the secondary tube, the appressorial germ tube (AGT), approximately four hours after initial contact. These early events on the epidermal surface of the leaf are of little relevance to the inherent susceptibility or resistance of the cultivar. The critical stage is the subsequent penetration phase, where the fungus attempts to enter the host cell wall via a penetration peg from the AGT (Eichmann and Hückelhoven 2008).

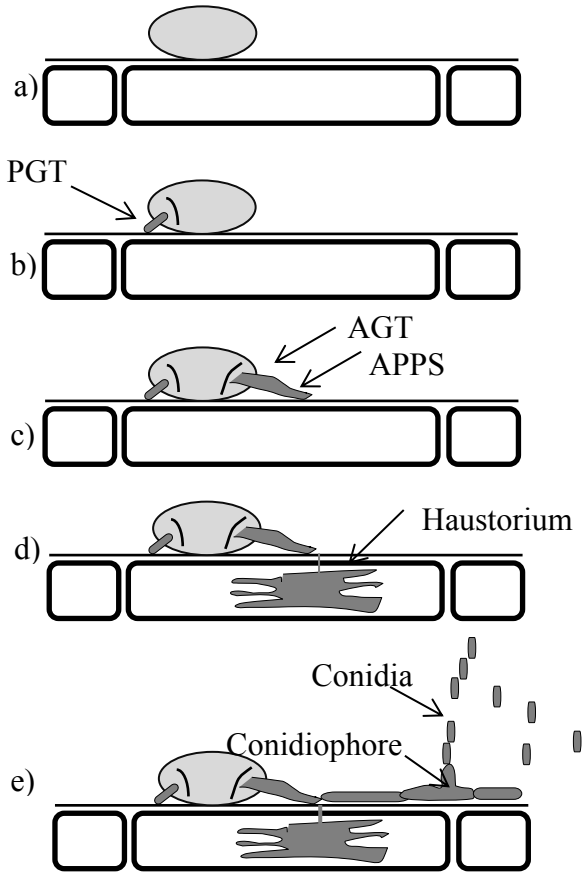


Figure 3. Diagram of the infection process of *Blumeria graminis* f. sp. *hordei.*

A) A conidia lands on the barley leaf epidermis. B) Approximately 2-4h after adhesion the primary germ tube (PGT) is formed. C) A secondary tube, the appressorial germ tube (AGT) swell at the tip to form the appressorium (APPS). D) In susceptible hosts, the APPS forms a small peg which penetrates the barley leaf epidermis. Haustoria are formed within cells that assimilate host derived nutrients. E) After approximately 72h after adhesion, conidiophore structures differentiate and form chains of asexual conidia. Adapted from Oliver (1998).

The appressorial germ tube swells at the tip producing a lobed appressorium (APPS). The APPS forms a small peg which penetrates the plant cell wall making contact with the plasma membrane of a leaf epidermal cell (Figure 3). Penetration through the epidermal cell wall is a critical stage in the infection process and plant defence mechanisms are induced in order to resist fungal entry at this point. Such mechanisms include a combination of site –directed cytoplasmic streaming, trafficking of Golgi bodies, accumulation of hydrogen peroxide (H_20_2) and papilla deposition (Bolwell and Wojtaszek 1997; von Ropenack, Parr et al. 1998). Papillae are collections of callose (β-1,3 glucan) that are assembled by the host acting as a physical barrier to penetration by the APPS peg. Interestingly it has been shown that papillae formation occurs irrespective of host resistance or susceptibility (Makepeace, Oxley et al. 2007). Heitfuss (2001) found that the chemical composition and speed of deposition of papillae plays an important role in the success or failure of subsequent intracellular haustorial formation.

Successful penetration leads to the formation of haustoria by invagination of the plant plasma membrane. These specialised feeding structures absorb and deprive the plant of chlorophyll, proteins and amino acids (Dik, Carver et al. 2002). The fungal demands for host derived nutrients, coupled with the associated decline in host photosynthetic ability ultimately results in reductions in yield and grain quality. After the successful establishment of intracellular haustoria, elongated secondary hyphae are formed on the host epidermal leaf surface (Figure 4). In susceptible cultivars, powdery colonies are formed on the adaxial surface of the leaf. Each colony is composed of both masses of mycelia and conidial chains which form from the conidiophore approximately 72h post infection (Boyd, Smith et al. 1995). Every successful infecting conidium has the potential to produce millions of asexual offspring. Each is then capable of spreading to other aerial parts of the plant and neighbouring hosts, where the infection cycle can initiate again. The asexual reproduction cycle has the potential to occur several times within a single season, depending on the efficacy of various control methods (Thordal-Christensen et al. 2000).

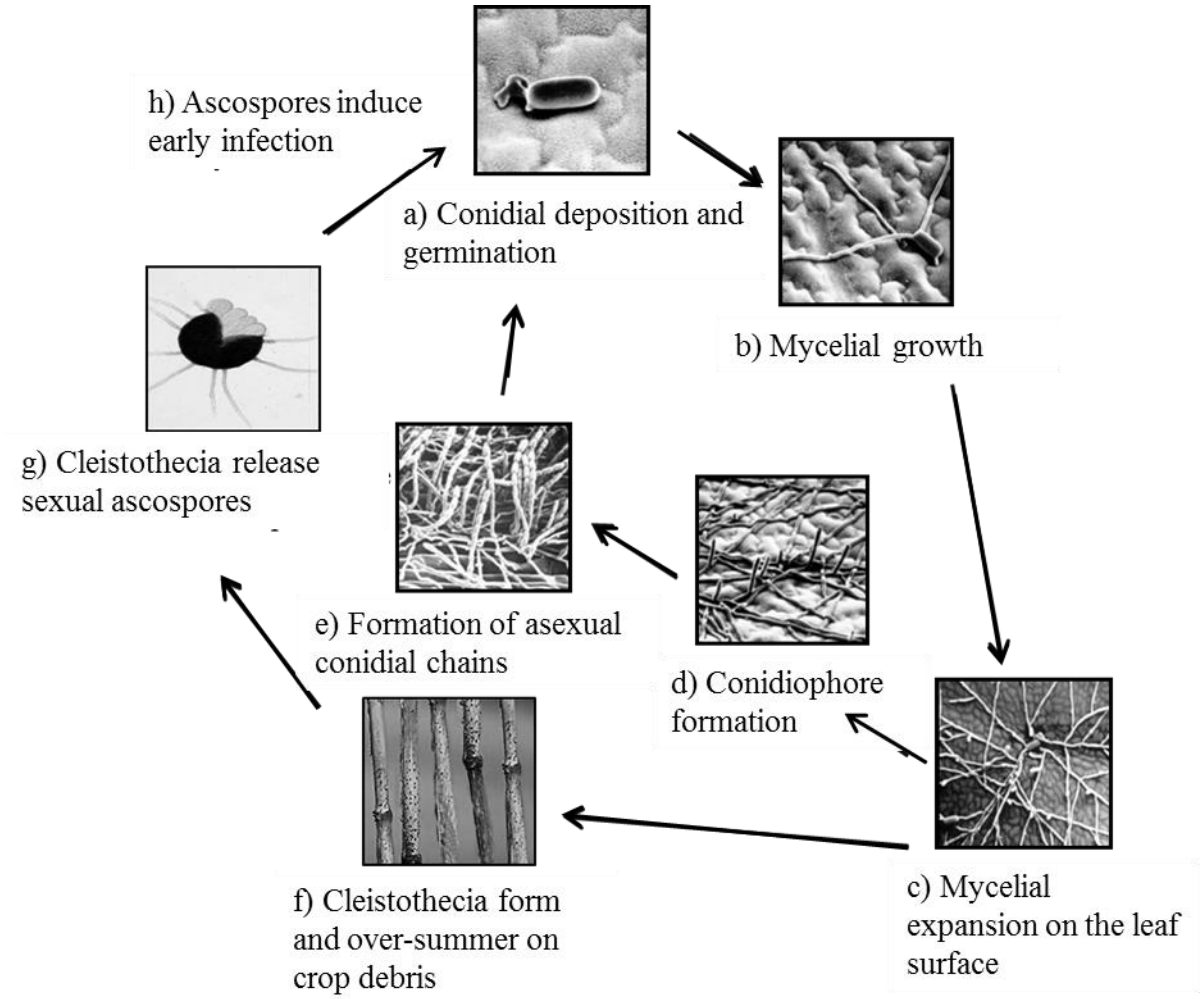


Figure 4. The polycyclic life-cycle of *Blumeria graminis* f. sp. *hordei.*

Bgh has two cycles of reproduction. The haploid asexual cycle is the predominant means by which powdery mildew proliferates, and the sexual cycle that allows for genetic recombination (a) In the asexual cycle conidia germinate and produce a series of germ tubes which may penetrate the epidermal cell wall of a susceptible host. Intracellular feeding structures known as haustoria are then produced. (b) Using plant derived nutrients conidia produce secondary hyphae which spread across the adaxial leaf surface. (c) Hyphae proliferate to form dense mats of mycelium. (d) Conidiophores from mycelia give rise to chains of asexual conidia. The mass of mycelium and conidial chains give the pathogen its fluffy powdery appearance. Conidia are dispersed by wind onto susceptible neighbouring barley plants where the cycle can initiate again. (f) The sexual cycle is a short diploid phase which may occur only once per season and is initiated by contact between hyphae of two different mating types. In the sexual cycle, fruiting bodies called cleistothecia are formed which permit the fungus to overwinter on susceptible stubble debris. (g) Under favourable conditions the cleistothecia burst releasing sexual progeny, known as ascospores, which can then initiate infection on newly planted crops in subsequent seasons.

During *Bgh's* life cycle the haploid asexual form prevails except for a short diploid phase which after mating results in the formation of cleistothecia. These structures are robust and provide a way for the fungus to remain dormant on barley stubble debris between cropping seasons. Under optimal temperature and moisture conditions the cleistothecia can burst releasing their sexual progeny known as ascospores, which can then initiate infection on susceptible cultivars in subsequent seasons. The divergent mating system has a considerable effect on how gene diversity is distributed within the total *Bgh* population. While asexual reproduction is the quickest means of exponential population growth, sexual reproduction provides an added advantage through the generation of novel genotypes or pathotypes through genetic recombination. With abundant asexual spore production, a relatively short latent period (approximately 7-10 days) and sexual recombination, *Bgh* is well-equipped to swiftly adapt to extensively utilised control methods.

7.14 Economic impact

Powdery mildew is one of the most common and destructive diseases of barley. Generally, losses are complex to estimate and depend on several factors such as climate, timing of disease onset, disease severity and the availability of effective control measures, all of which can vary greatly from one season to the next. In practice, when conditions for mildew are optimum, losses can reach as high as 40% (Dreiseitl and Platz 2012). Although it occurs in most if not all parts of the world where barley is grown, it is not considered to be a major problem in every region. In the UK and Europe, *Bgh* is controlled through coordinated uses of cultivar resistance, including *mlo* (refer to 7.2 for an explanation of *mlo*), and the application of fungicide when/if required. In Australia powdery mildew is ranked the second most destructive barley disease being a serious threat to production in southern cropping regions (Lord and Kehoe 2014). In WA, powdery mildew can be particularly devastating, where the predominance of highly virulent pathotypes coupled with widespread fungicide resistance has led to losses of $100M in severe seasons (Tucker, Lopez-Ruiz et al. 2015).

7.2 Literature Review and Discussion

7.21 Disease Management

Australian barley growers have a world-wide reputation for producing high quality malting barley. Fields of barley cover much of the south-west high rainfall WA cropping area and powdery mildew infection has proven to be a persistent threat to production in recent years (GRDC 2012). Generally, *Bgh* is controlled in other barley growing regions of the world through comprehensive integrated pest management strategies. Such strategies rely on host resistances, the timely application of effective fungicides and a range of cultural practices including stubble and green bridge removal. Historically in WA the majority of the barley cropping area has been sown with cultivars harbouring defeated resistance genes (Paynter, Hills et al. 2014). Australian fungicide registrations have meant that growers in regions prone to infection have had to rely on cultural practices and fungicides of a single mode of action for barley powdery mildew control.

7.211 Cultural Practices

Cultural disease management practices are essential components in any integrated pest management strategy. Cultural control practices are those undertaken by growers in an effort to minimise the spread, severity and onset of disease. This includes green bridge and stubble management (Pankhurst, McDonald et al. 2002), crop or pasture rotation (Peters, Sturz et al. 2003), optimisation of nitrogen input (Bainbridge 1974) and other general farm hygiene practices. Green bridge management involves the removal of mislaid host plants which can act as over-summer sources of inoculum. The widely adopted practice of no-till farming in WA results in the retention of crop residues and stubble from one season to the next (Bockus and Shroyer 1998). As with many other ascomycetes, *Bgh* form cleistothecia which can remain dormant on stubble for many seasons. In optimum conditions cleistothecia burst releasing ascospores which can initiate infection on newly planted barley seedlings. Foliar application of effective fungicides and the use of robust cultivar resistance genes have proven to be the most efficient means of controlling *Bgh* (Jayasena, Beard et al. 2008). The nitrogen level applied to barley crops also has a significant impact on disease intensity. Correlations have been drawn between the increased application of nitrogen and the increase of spore density per

mildew colony (Jensen and Munk 1997). As a result nitrogen should only be applied where necessary.

7.212 Breeding for Resistance

7.2121 Host Resistance

Both wild and cultivated plant species are constantly exposed to a plethora of pathogens. What determines whether a pathogen can successfully cause disease is dependent in many cases upon the presence and/or absence of genes in the pathogen and plant host. The different defence mechanisms employed by plants can be distinguished by the specificity of their action. In the interaction between barley and *Bgh*, four levels of resistance have been described; non-host resistance, race specific and major and minor non-race specific resistance (Jørgensen and Wolfe 1994). The first level of protection is non-host or basic resistance which protects against colonization by individual fungal species and is highly effective and durable (Thordal-Christensen 2003) In the case of powdery mildew, non-host resistance protects barley from infection by other *formae specialis* (*ff. spp.*) of *B. graminis* such as the wheat powdery mildew *Blumeria graminis* f. sp. *tritici*. This type of resistance is in contrast to race specific resistance which is expressed by only specific genotypes of a host plant and not the entire genus (Heath 2000).

A large volume of theoretical and experimental work has been conducted to elucidate the biological mechanisms behind such a robust form of plant innate resistance. Its appears as though defence responses in non-host plants are coordinated through programmed efforts between the formation of physical barriers to penetration (Trujillo, Troeger et al. 2004), the synthesis and accumulation of reactive oxygen species at attack sites (Bolwell and Wojtaszek 1997) and localized programmed cell death through initiation of a hypersensitive reaction (Ellis 2006).

7.21211 Race Specific Major *R* Gene Resistance

There are additional defence mechanisms that act to prevent fungal colonization even when basic compatibility has been established and the plant is a natural host for the pathogen. Barley presents a second level of defence that is governed by specific interactions between genes in both host plant and *Bgh*. In 1971, Henry Flor hypothesized the 'gene-for-gene' interaction following observations on the flax rust fungus, *Melampsora lini* (Flor 1971). Flor postulated the existence of complementary genetic elements, a dominant/semi-dominant major resistance gene (*R*) in the plant and a corresponding dominant avirulence gene (*Avr*) in the pathogen (Flor, 1971). If a plant resistance receptor recognises a pathogen avirulence effector (Figure 4a) a defence response will be activated conferring plant resistance and fungal avirulence (Caffier, Vallavielle-Pope et al. 1996). Conversely, the loss or alteration of either the *R* or *Avr* gene will result in disease (Figure 5b and c).

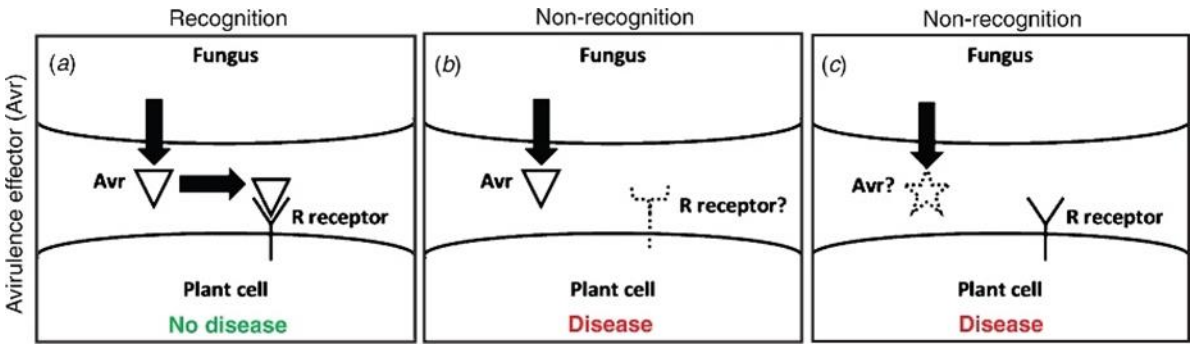


Figure 5. The gene-for-gene hypothesis in biotrophic pathogens.

(a) If the plant resistance gene receptor (R receptor) recognizes a corresponding fungal avirulence effector (Avr) host responses prevent disease. If either the corresponding plant R receptor (b) or the fungal Avr protein (c) are absent or non-functional, fungal colonization and hence disease can occur (Tan, Oliver et al. 2010).

At the species level, natural polymorphisms in *R* and *Avr* genes make it possible to discern plant lines and *Bgh* isolates, respectively. Barley has a large number of *R* genes for race specific resistance against *Bgh*. Jørgensen and Wolfe (1994) estimated that barley had 85 such *R* genes, located at 10 separate loci spanning several chromosomes. Landrace collections from centres of barley domestication mean there is a large pool of potentially resistant germplasm that could

be incorporated into barley breeding programs. Populations of *Bgh* are notorious for being composed of numerous races or pathotypes (Brown and Wolfe 1990; Bousset, Hovmøller et al. 2002; Aghnoum 2009). Each pathotype is defined on the basis of the presence of *Avr* genes and hence, their differential patterns of virulence on barley cultivars (Heitefuss 2001). With this knowledge, collections of *Bgh* can be screened on cultivars with known *R* genes to elucidate which avirulence genes are present in a population and which *R* genes barley cultivars harbour. Such screens have been used worldwide (Wolfe, Brändle et al. 1992; Müller, McDermott et al. 1996; Dreiseitl, Dinoor et al. 2006) although prior to the work of Tucker et al. (2013) no such differential tests employing the Pallas lines have been performed on Australian *Bgh* isolates. Studies conducted by Chan and Boyd (1992) utilized a number of cultivars from WA, the United States of American and the UK to screen for *Bgh* virulence's present in the WA population over the 1983-1984 cropping season. Cultivars containing the same single *R* genes as those used in the study herein were used including those containing *Ml-k, Ml-g, Mla-t, Ml-h, Ml-8, Mla-1, Mla-3, Mla-6, Mla -7, Mla-9, Mla-10, Mla-12* and *Mla-p1*. Unfortunately the cultivars used by Chan and Boyd (1992) for *Bgh* pathotype determination did not utilize an isogenic cultivar set and therefore any differences in virulence and susceptibility cannot entirely be attributed to the presence or absence of *R* genes and not from other cultivar characteristic or pleotropic effects.

There have been surveys conducted in many barley growing regions of the European Union employing, among others, several of the Pallas isogenic lines. Hovmøller *et al.* (2000) co-ordinated barley powdery mildew resistance surveys on a European scale from 1995-1999 in efforts to provide both temporal and spatial comparisons of virulence across national borders. The frequencies of virulence to cultivars possessing known *R* genes was assessed in barley powdery mildew populations, across thirteen geographically distinct countries. Resistance genes *Mla-1, Mla-3, Mla-6, Mla-7, Mla-9, Mla-12, Mla-13, Ml-k and Ml-La* were examined and changes in virulence frequency were noted from 1995 to 1999. Full data sets were generated for several sites including the Czech Republic, Denmark and the UK. Some genes such as *Mla-3*, *Mla-9* and *Ml-k* were the single dominant resistance gene present in a Pallas isoline and as such any *Bgh* virulence or avirulence towards these particular isolines could be directly attributed to the presence of the gene. Interestingly, virulence to resistance genes *Ml-k* and *Mla-9* decreased in frequency over the years surveyed in several locations, most strikingly virulence to the *Mla-9* gene which decreased in Latvian *Bgh* populations from 75% in 1995 to

50% in 1998. In this example, the decline of virulence towards the *Mla-9* gene may have been a direct result of concerted efforts in reducing the sowing of barley cultivars harbouring the *Mla-9* powdery mildew resistance gene. Any reduction in selection pressure that favours a particular *Bgh* pathotype over others, will correspondingly decrease the pathotypes local advantage. The dramatic decrease in pathotype frequency in as little as a few cropping seasons, emphasizes the importance of cultivar selection. Whereby the utilisation and rotation of barley cultivars with effective *R* genes *can* result in in a far more manageable *Bgh* population.

Unfortunately, the efficacy of dominant *R* genes is usually only short-lived. *Bgh* has many characteristics that support rapid adaption and a single nucleotide polymorphism in an *Avr* gene may result in non-recognition by the host resulting in successful fungal penetration leading to disease (Jørgensen and Wolfe 1994). In WA, foreign demand has led to the continual and widespread planting of a small number of cultivars which often share the same barley powdery mildew *R* gene (Dreiseitl and Platz 2012; Paynter, Hills et al. 2014; Tucker, Lopez-Ruiz et al. 2014). The combination of sowing such a large monoculture with *Bgh*'s propensity for adaptation will likely result in the breakdown of major *R* gene efficacy, which has been shown to occur even in one to two seasons (Lyngkjaer, Newton et al. 2000). Consequently many of the current WA cultivars have some degree of susceptibility to *Bgh* infection (Tucker, Jayasena et al. 2013). This has led to far reaching spread of *Bgh* resulting in devastating losses.

In addition to characterising isolates based on the presence or absence of *Avr* genes, microsatellite markers can also be valuable tools in studying genetic structure of a pathogen population. Microsatellites or SSRs (short sequence repeats) are short segments of genomic DNA composed of numerous nucleotide repeat motifs. The number of repeat motifs varies within the pathogen population because they are highly susceptible to slippage during DNA replication (Bruford and Wayne 1993). The length of SSR repeat motifs are heritable and can be used to establish genetic relatedness within a population. Specific SSR haplotypes can also be used to track or identify the origin of genetic mutations in other regions of the organism's genome. Screening of populations can provide insight into whether a genetic mutation, say conferring fungicide resistance, has occurred within a single isolate which is then passed to offspring, or has occurred and been selected for on multiple occasions both temporally and spatially.

7.21212 **Non-Race Specific Minor Gene Resistance**

In addition to major *R* gene resistance, researchers have been aware of non-race specific resistance for several decades in adult cultivars with defeated major *R* genes (Shaner and Finney 1977; Jones and Davies 1985; Shtaya, Marcel et al. 2006). This type of resistance is usually partial, allowing for a low rate of mildew infection and a prolonged latent period with an associated decrease in sporulation (Asad, Bai et al. 2014). Non-race specific resistance is highly variable, even between cultivars of the same plant species. Resistance mediated in this manner is often more durable then race-specific major gene resistance and as a consequence, there is increased interest in the development of breeding germplasm harbouring race non-specific resistances.

7.21213 Non-Race Specific Durable *mlo* Resistance

The presence of the dominant *Mlo* gene in barley is an absolute requirement for successful *Bgh* colonisation (Piffanelli, Zhou et al. 2002). Cultivars with loss-of-function alleles of *mlo* are therefore resistant to all known pathotypes of *Bgh* (Wolter, Hollricher et al. 1993). Owing to its broad-spectrum nature, the use of *mlo* in barley agriculture has remained a success story for over 45 years (Tacconi, Baldassarre et al. 2006). In the barley-*Bgh* pathosystem, papilla formation is the predominant mechanism to arrest fungal penetration in partial non-race specific resistance and in complete *Bgh* resistance mediated by the presence of *mlo*.

There have been many reports of *mlo*-associated negative pleiotropic effects causing reductions in yield compared to cultivars with functional *Mlo*. These include spontaneous deposition of papillae in unchallenged plants (Panstruga 2005), increases in susceptibility to necrotrophic pathogens including *Cochliobolis sativus* (Kumar, Hückelhoven et al. 2001) *Magnaporthe oryzae* (Jarosch, Kogel et al. 1999) and *Pyrenophora teres* (Brown and Rant 2013), decreases in symbiotic mycorrhizal colonisation (Marshall, Newton et al. 2009) as well as the onset of premature leaf senescence (Prell and Day 2001). Historically, Australian barley breeders have therefore tended to concentrate on the integration of major *R* genes (Dreiseitl and Platz 2012). European breeders have now produced elite *mlo* cultivars without any reported pleiotropic effects, with approximately 70% of spring barley cultivars now harbouring *mlo* (Lyngkjaer, Newton et al. 2000).

In WA, annual variety trials are conducted that test the suitability of new cultivars in each of the six main barley cropping regions. Recently three *mlo* containing varieties were examined; Westminster, Henley and Granger. These cultivars were bred using European spring barley germplasm and consistently performed better than the market leader cv. Baudin in regards to yield (NVT 2014). All three cultivars were resistant to *Bgh* and have now received malting accreditation (BarleyAustralia 2015). The key factor in lessening the impact of *Bgh* in WA is to reduce the area sown with susceptible cultivars. Currently many susceptible varieties are in high market demand and in some cases the premiums offered offset the majority of costs involved in controlling powdery mildew (Paynter, Hills et al. 2014). New malting cultivar options and those waiting accreditation will assist in decreasing the area sown to susceptible cultivars, although the enduring test will be gaining favour in the international market.

7.2122 Historical Cultivar Use in West Australia

Barley grain in Australia is an extremely important economic commodity, second only to wheat. In WA over 2.5M tonnes of grain is produced annually of which 85% is exported - contributing one sixth of the world's traded malting barley (ABARES 2014). The temperate climate of much of the WA barley cropping area lends itself to high yields, producing grain with excellent malting characteristics. Barley also represents a valuable rotational crop, which can assist in breaking host specific leaf and root disease cycles. There are a range of barley cultivars available to growers each with different characteristics of yield potential, agronomic features, disease resistance profiles and grain quality. Australian barley cultivars certified as malt, feed or food grades depending on their likely use and suitability post-harvest. Traditionally, growers decide which to grow depending on a number of factors. These include (*i*) the end price paid for seed and the likelihood that a particular cultivar will meet the required grade (*ii*) yield potential (*iii*) agronomic and disease resistance characteristics under specific environmental conditions and (*iv*) varietal market demand. Usually malt grade cultivars fetch a premium over feed or food varieties but factors such as weather and disease pressure, which vary season to season, can affect profit margins. Historically in WA particular barley cultivars have tended to fare better than others and have become market leaders in the industry. Each of the factors listed above are discussed below.

7.21221 End Price for Malt Grade Seed

Identifying the option that will lead to the greatest net return is complex and can vary dramatically from one season to the next. In some instances, the premium price paid for malt grade seed adequately compensates for the lower yield of some varieties when compared to a higher yielding feed variety. In other situations, the substantially higher yield of feed varieties, or the low possibility of a variety being sold as malt grade grain, may justify the choice of a feed variety. Historical data indicates that four cultivars consistently achieve higher sale prices than other varieties grown in WA. These are Baudin, Hamelin, Gairdner and Stirling which normally attract $12.60/t more than the average paid for barley grain. Baudin, the market leader fetching the highest price for barley grain per tonne, is rated highly susceptible with Hamelin, Gairdner and Stirling rated as susceptible to *Bgh* infection. Growers are most likely to choose cultivars that pay a premium post-harvest and thus these mildew susceptible cultivars have occupied on average 55% of the total of the WA barley growing area for nearly the last decade (CBH 2014).This has gifted the pathogen perfect conditions for proliferation and hence adaptation, key contributing factors to the prevalence of *Bgh* in WA.

7.21222 Cultivar Yield Potential

Another determining factor in selecting which cultivar to sow is the potential yield post-harvest. Each cultivar has certain agronomic characteristics which when grown under particular environment conditions will yield on average a certain amount of grain. Growers must compare the predicted sale price of grain with a cultivar's yield potential in selecting which cultivar to grow. Currently in WA the majority of malt grade cultivars (achieving higher end sale price over feed or food varieties) with the highest yield potential (Paynter, Hills et al. 2012) are susceptible to powdery mildew infection.

7.21223 Agronomic and Disease Resistance Characteristics

Ultimately agronomic and disease resistance characteristics will determine the total input costs associated with growing particular varieties. A grower must match any agronomic and disease resistance characteristics with the local environmental conditions in an effort to try and keep

costs afforded by fertiliser and fungicide applications to a minimum. Many additional post-seeding costs can be avoided by sowing cultivars that have genetic protection against local pests and disease (French 2003). Barley *mlo* cultivars can potentially be grown in WA mildew prone regions without the threat of yield losses from *Bgh* infection. A handful of *mlo* cultivars are currently undergoing field assessments for their suitability for growth in WA and early reports are positive (GRDC 2014). Despite this, exact quantification of the effect of *mlo* in varieties grown in WA cannot be gauged without more extensive and thorough trials. However, it should also be noted that the resistance profiles of the new *mlo* varieties are moderately resistant or moderately susceptible (Paynter and Hills 2011) to net type net blotch which is the predominant form of net blotch in WA (Lord and Kehoe 2014). Despite this *mlo* varieties may be a suitable option in reducing *Bgh* disease pressure and reduce the use of fungicides in controlling the disease.

7.21224 Market Demand

Market Demand is perhaps the major factor in determining which cultivar a grower will select. If there is not foreign demand for a particular malt grade cultivar, seed will only be sold at feed grade, substantially dropping the price/tonne paid. New high yielding cultivars are currently undergoing malt accreditation many of which have increased resistance to *Bgh*. (BarleyAustralia 2014). It is imperative that any new cultivars possess similar malting characteristics to Baudin in efforts to persuade international buyers to replace the seed in production. The replacement of *Bgh* susceptible varieties with high grade resistant varieties does take time as many of the susceptible varieties are in high market demand. Unfortunately, in some cases the premiums offered for premium malt grade seed offsets most of the cost of applying fungicides to combat the *Bgh* infection.

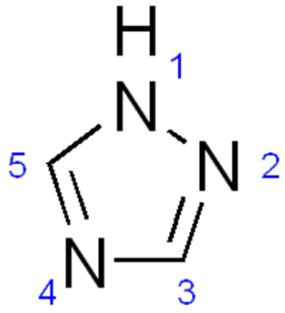
Each of the four factors that determine which cultivar growers will choose have contributed to the majority of WA barley cropping area being seeded with *Bgh* susceptible cultivars for the last decade (Paynter, Hills et al. 2011). This has provided conditions where *Bgh* can flourish and hence diversify and has now adapted to overcome many control methods.

7.213 Chemical Control

In the absence of fully effective host resistance, fungicides become essential for the maintenance of healthy crops and reliable yields of high-quality produce. Records date the use of sulphur in controlling powdery mildew back to 1824 when a number of basic chemicals were used on several important agricultural pathogens (Russell 2005). Since then many additional chemicals belonging to several different modes of action (MOA) have been found to be effective in both the eradication and protection of barley against *Bgh*. Despite the success of *mlo* in European agriculture, WA growers have relied upon the application of fungicides to control *Bgh* on susceptible cultivars (Tucker, Lopez-Ruiz et al. 2014). As of 2007, some 150 different fungicidal compounds are used in worldwide agriculture (Brent and Hollomon 2007). Regardless, triazoles have been the sole class of fungicides available for protecting WA barley crops since 1995 (APVMA 2014).

7.2131 Triazole Fungicides

Triazole fungicides are the most widely used class of antifungal agents for the control of medical (Snelders, Camps et al. 2012), veterinary (Beernaert, Pasmans et al. 2009) and agricultural fungal pathogens (Poole and Arnaudin 2014). The chemical structure of each triazole compound contains a characteristic five membered heterocyclic aromatic ring, composed of two carbon and three nitrogen atoms having (Figure 6). The 1,2,4 triazole isomer forms the backbone of many antifungal compounds with significant agricultural and medicinal applications (Schwinn 1984; Lass-Flörl 2012).



1,2,4 triazole

Figure 6. The 1,2,4 triazole isomer.

The 1,2,4 triazole isomer forms the chemical backbone for the most widely used group of antifungals in agriculture. Figure used with permission from ChemSpider.

Triazoles have a single-site MOA targeting the product of the *Cyp51* gene, the enzyme cytochrome P450 14α-demethylase. In fungi *Cyp51* has been shown to catalyse the C14-demethylation of a collection of structurally similar substrates including lanosterol and obtusifoliol (Lepesheva and Waterman 2004). The action of cytochrome P450 14α-demethylase has been well studied in most fungi where it catalyses the demethylation of lanosterol in the biosynthesis of ergosterol, an essential membrane sterol (Lepesheva and Waterman 2007; Weete, Abril et al. 2010). However, the role of cytochrome P450 14α-demethylase is less well characterised in a number of powdery mildew fungal species including *Blumeria graminis*. It has only been as recent as the last decade that studies by Weete *et al.* (2010) and Lepesheva and Waterman (2007) have examined the alternate action of *Cyp51* in a handful of *Blumeria* spp. In these fungi, sterol biosynthesis takes an altered pathway compared to that in yeast and other filamentous fungi. Here, lanosterol is first converted to 24-methylenelanosterol, which becomes the preferred substrate for C14-demethylation by *Cyp51* (Figure 7). A cascade of further enzymatic reactions then results in the production of the key membrane sterol in *Blumeria* spp., 24-methylcholesterol (Weete, Abril et al. 2010). In other filamentous fungi and yeast spp. *Cyp51* acts directly upon lanosterol, resulting in the production of zymosterol and eventually leading to the synthesis of ergosterol (Weete, Abril et al. 2010). For many years the main dogma of thought incorrectly assumed that the main sterol in the cellular membrane of all fungal species was ergosterol (Fuchs, Vries et al. ; Elewski 1993; Dupont, Lemetais et al. 2012). With the comparatively recent characterisation of 24-methycholesterol in *Blumeria* spp. further investigations into the membrane sterol content of additional filamentous fungal species may lead to the identification of additional, perhaps novel *Cyp51* substrates. What is common to all species studied thus far is that *Cyp51* plays a pivotal role in formation of key sterols in plant, mammal and fungal species. Membrane sterols are essential in maintaining optimum cellular function and integrity and as such their biosynthesis has remained a popular target for antifungal drugs for nearly 50 years (Russell 2005).

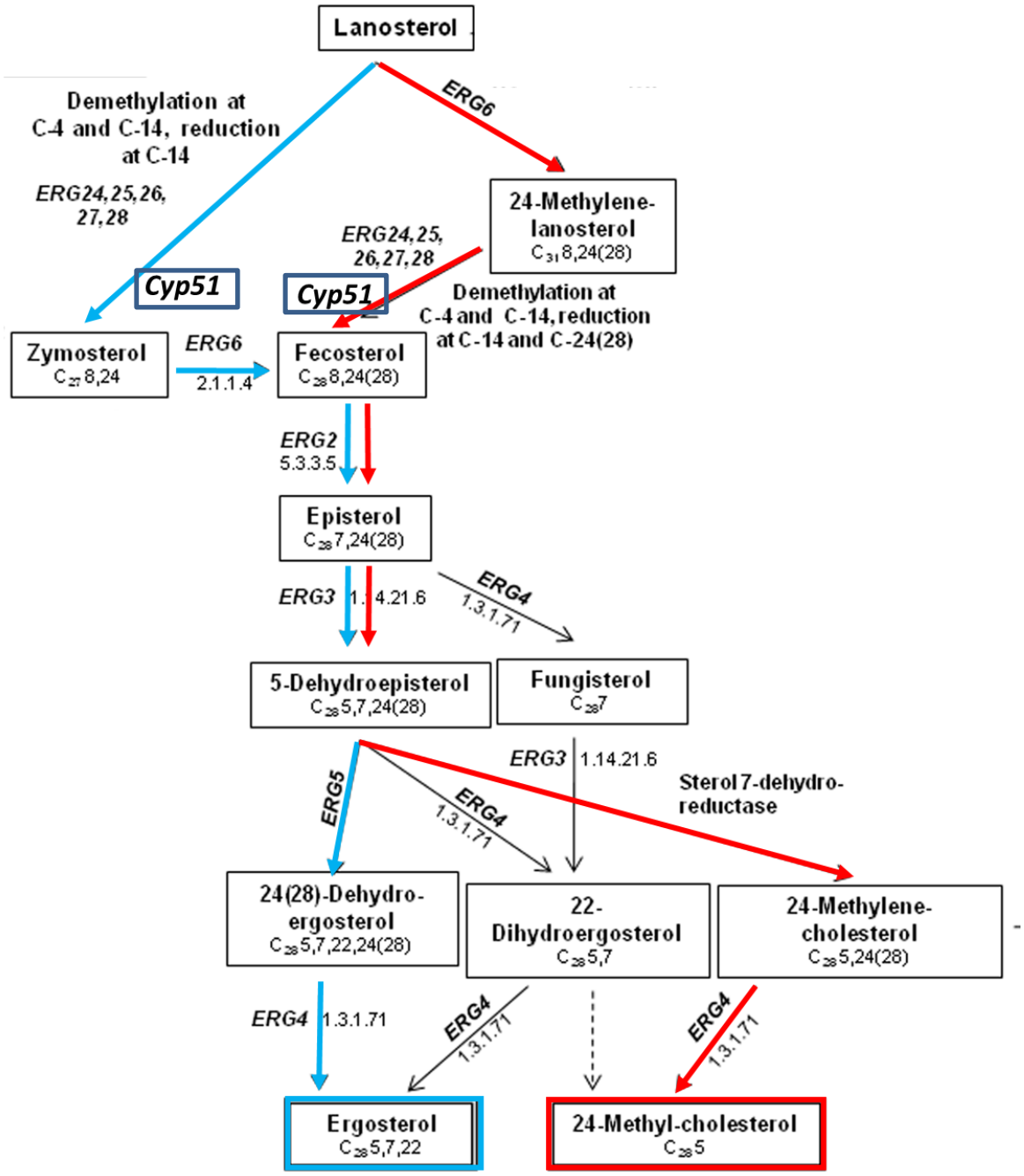


Figure 7. The sterol biosynthesis pathway in in filamentous fungi.

The *Cyp51* enzyme catalyses a three-step C14 demethylation (Waterman and Lepesheva 2005) in the cascade of reactions converting 24-methylenelanosterol to fecosterol in the synthesis of 24-methyl-cholesterol in *Blumeria* spp. (pathway indicated in red) and the conversion of lanosterol to zymosterol in the synthesis of ergosterol in other filamentous fungi (indicated in blue). Adapted from Weete *et al.* (2010).

In addition to *Cyp51*, other key enzymes involved in sterol biosynthesis have also become targets for other groups of antifungals. All fungicides targeting sterol biosynthesis are referred to collectively as sterol biosynthesis inhibitors (SBIs). This class of fungicide, encompassing the DMI group, is further categorised into four subclasses depending upon the enzymatic target (Figure 8). Amine fungicides, including the morpholines and spiro-ketalamines such as spiroxamine (recently granted a provisional permit for use in WA (APVMA 2014)), inhibit the biosynthesis of sterols by targeting two genes - *Erg24* encoding C14-reductase and *Erg2* encoding C8-isomerase. This group, comprised of seven compounds to date, has a limited spectrum of disease control compared to triazoles, although is particularly effective against the powdery mildews (Oliver and Hewitt 2014). The third class of DMIs are the hydroxyanilides. Hydroxyanilides also act on a key enzyme in sterol biosynthesis, coded for by the *Erg27* gene (Fillinger, Leroux et al. 2008). As yet only a single hydroxyanilide compound has been described, fenhexamid, which although has good activity on *Botrytis cinerea*, has only limited activity on other ascomycete pathogens (Oliver and Hewitt 2014). Therefore fenhexamid has limited application in broad acre cropping.

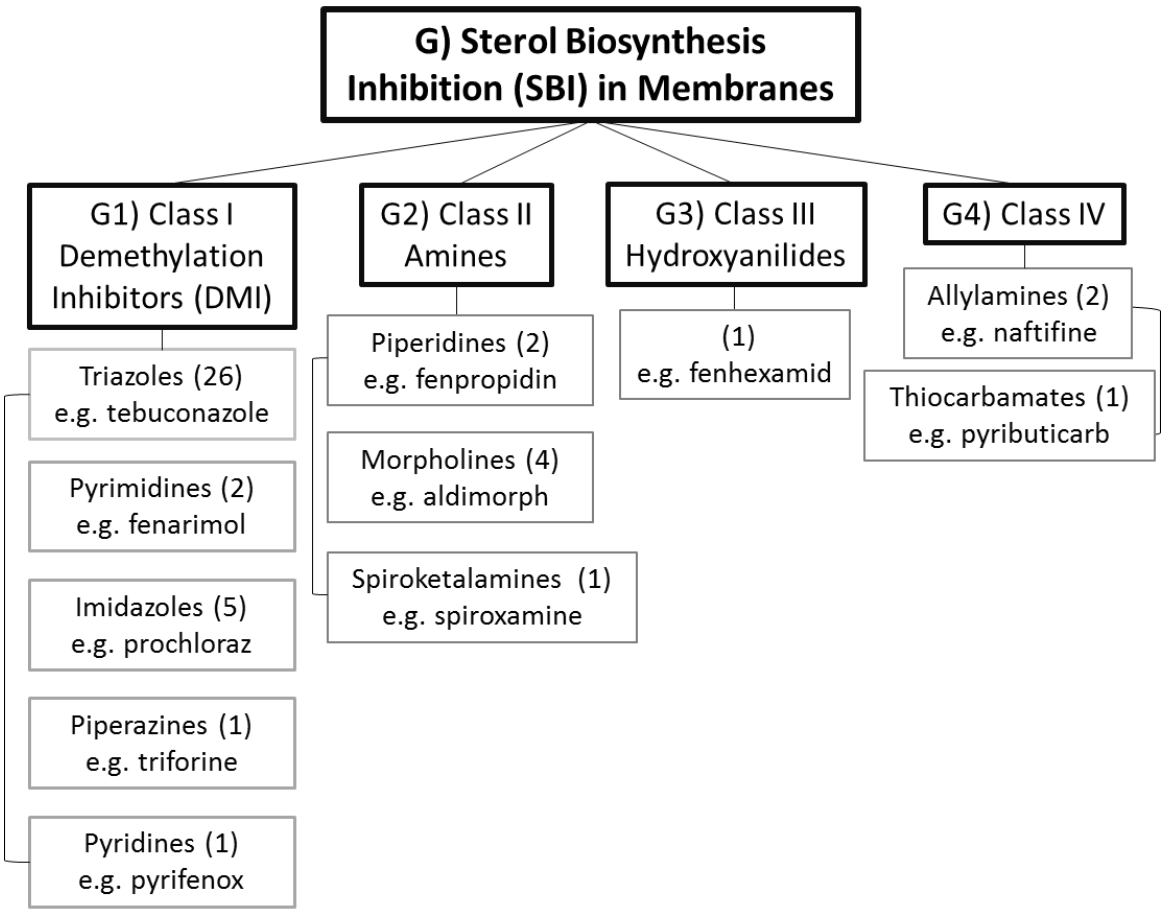


Figure 8. Classes of fungicides inhibiting membrane sterol biosynthesis (SBI).

G1-4 represent the sub-classifications as defined by the Fungicide Resistance Action Committee. Figures in parenthesis indicate the number of compounds in each subclass (FRAC 2014).

7.2132 Mode of Action

In silico structural modelling is now increasing being used to assess decreases in triazole susceptibility due to conformational changes in *Cyp51* (Bowyer and Denning 2014; Hoekstra, Garvey et al. 2014). Such investigations have primarily focused on differences in the binding of triazoles to both the wild-type and mutant *Cyp51* protein variants. (Parker, Warrilow et al. 2014). Ideally, triazole docking studies should be performed with the *Cyp51* protein from the species of interest following x-ray crystallisation. However, this is not always possible, given the membrane bound nature of many *Cyp51* enzymes present in filamentous fungi. As an alternate means, many in silico triazole docking studies have been performed by amalgamating known structures of a pool of crystallised *Cyp51* enzymes to produce a likely *Cyp51* model for the species of interest. One of the most extensively studied *Cyp* enzymes is *Cyp101* from *Pseudomonas putida,* being first described by Poulos *et al.* (1985). The crystallisation of additional *Cyp* enzymes since this time has led to the identification of characteristic protein folds that have thus far been shown in all *Cyp* enzymes (Hasemann, Kurumbail et al. 1995). In general 13 α-helices (A, B, B` and C-L) and 5 β-sheets (β1-β5) have been shown to contribute to the overall folding of *Cyp* enzymes, referred to as the P450 fold (Figure 9).

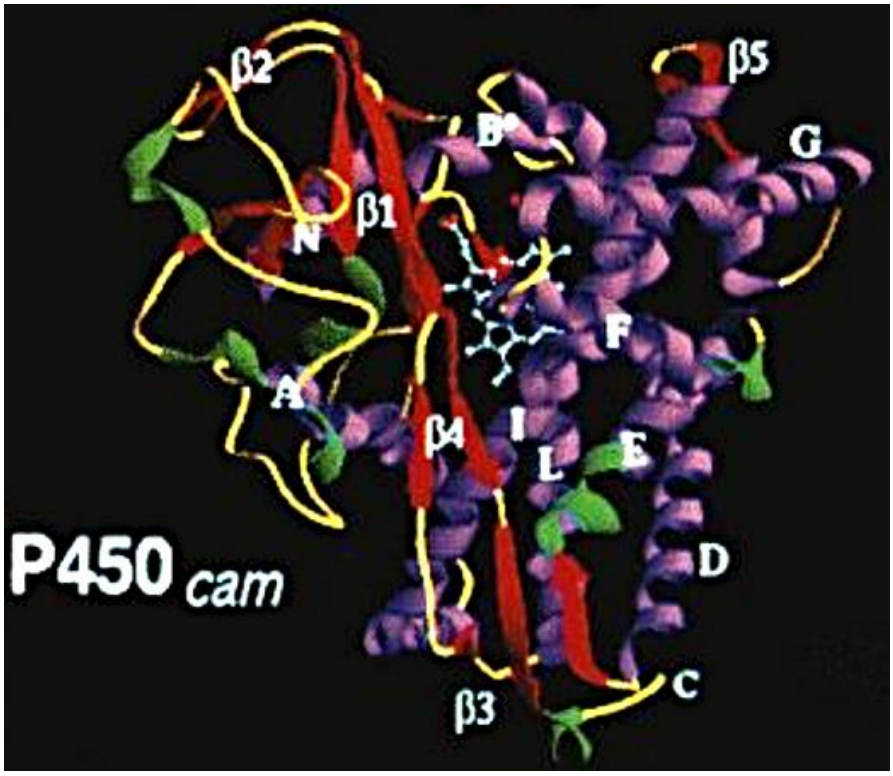


Figure 9. Ribbon diagram showing the overall fold and secondary structure of a *Cyp* enzyme from *Pseudomonas putida* ($P450_{cam}$).

The secondary structure shows 13 α-helices (A, B, B` and C-L) and 5 β-sheets (β1-β5) forming the characteristic P450 fold found to be common to all *Cyp* enzymes crystallised thus far. Adapted from (Hasemann, Kurumbail et al. 1995).

Cyp51 produced sterol 14α-demethylase is one of the most widely distributed P450s enzymes, with orthologues having been identified across all biological kingdoms (Lepesheva and Waterman 2004). Although the biological function of *Cyp51* is well conserved, the encoding target gene sequence can vary between kingdoms. On average, sequence identity is approximately 30%, but ranges from high (95%) in closely related species such as mammals to much lower (23%) when surveyed across all kingdoms (Lepesheva and Waterman 2007). In fungi, *Cyp51* sequence identity ranges from 45% between yeasts and filamentous fungi (*Candida glabrata* and *Aspergillus nidulans*) to 65% within filamentous fungal pathogens (*Aspergillus fumigatus* and *Penicillum digitatum*) (Lepesheva and Waterman 2011). Studies by Gotoh (1992) first identified several regions retaining a high degree of homology even over diverse kingdoms. Five putative substrate recognition sites (SRS) were hypothesized based on group-to-group alignment of eukaryotic *Cyp51* sequences to a previously crystallised bacterial sequence of *Pseudomonas putida.* Most of these SRS were confirmed with the crystallisation of *Cyp51* from *Mycobacterium tuberculosis* by Podust *et al.* (2001) and can be mapped over plant, fungal and bacterial species (Figure 10). Triazole antifungals have been shown to interact with the SRS of *Cyp51* in a number of fungal species (Yoshida and Aoyama 1991). Mutagenesis studies targeting select amino acids have correlated changes in SRS regions with decreases in triazole susceptibility (Cools, Parker et al. 2010), indicating the significance of the SRS in substrate recognition.

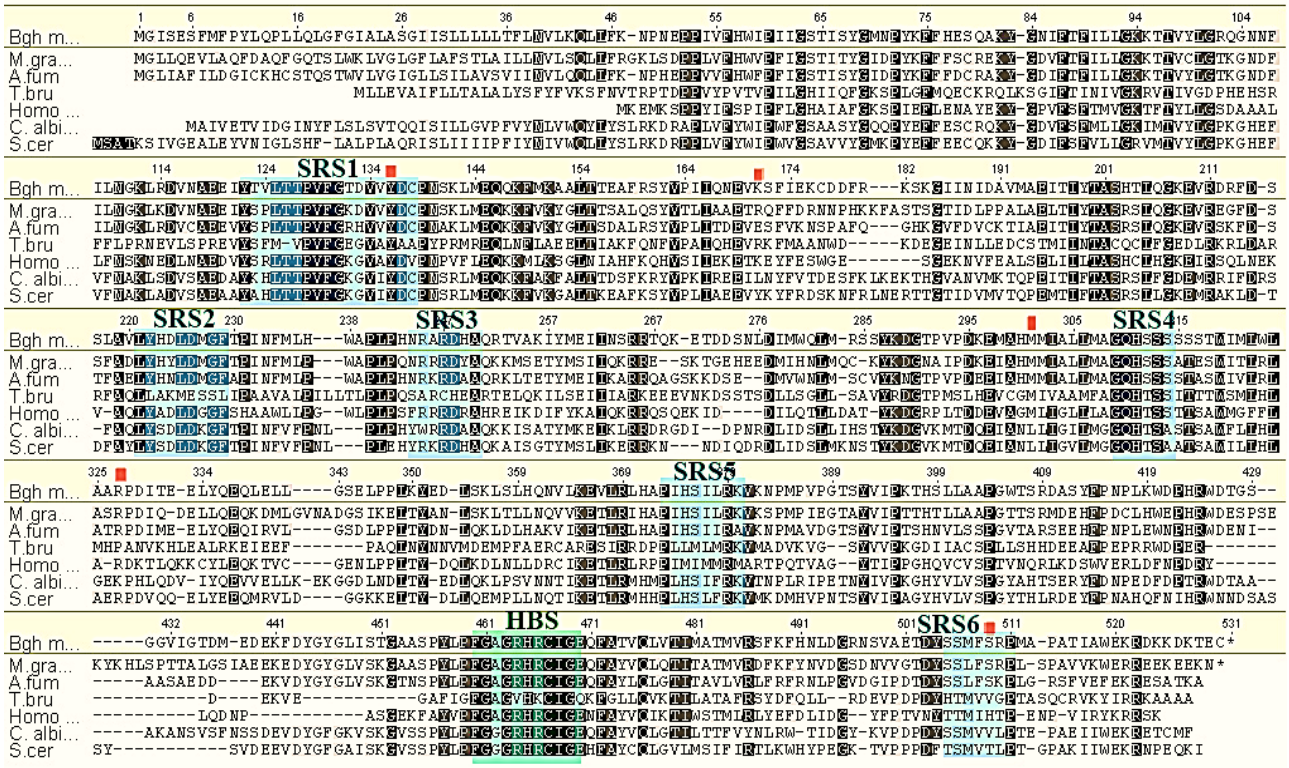


Figure 10. Sequence alignment of *Cyp51* proteins from animal, bacterial and fungal species.

Mutations discussed in this thesis are indicated with red boxes. Species include *Blumeria graminis* f. sp. *hordei* (*Bgh* m), *Zymoseptoria tritici* (M. gram), *Aspergillus fumigatus* (A. fum), *Trypanosoma brucei* (T. bru), *Homo sapiens* (Homo), *Candida albicans* (C. alb) and *Saccharomyces cerevisiae* (S.cer). Numbers refer to amino acid positions in *Bgh.* Amino acids conserved are shaded in black (100% homology), dark grey (83% homology), and light grey (66% homology). Substrate recognition sites (SRS) are shown in blue with the heme binding site shaded in green. Alignment generated with Clustal Omega.

Unfortunately, triazole resistance is now common in both the clinical (Denning, Park et al. 2011) and agricultural sectors (Cools, Fraaije et al. 2006) including powdery mildew populations from other barley growing regions of the world (Wyand and Brown 2005). The similarity in the structure of triazoles coupled with the specificity of the compounds mode of action, raises the issue of cross resistance, whereby a mutational change conferring insensitivity to one triazole may confer insensitivity to others. Cross resistance to a number of triazoles has been confirmed in several important fungal crop pathogens (Robbertse, Van Der Rijst et al. 2001; Cools and Fraaije 2008). Given that prior to 2012, all registered fungicides for barley powdery mildew control in WA contained a fungicide from the triazole class (APVMA 2014), the likelihood of detecting resistance in this pathogen population seems almost inevitable.

7.2133 Mechanisms of Fungicide Resistance.

Fungicide resistance is conferred through stable, heritable genetic mutations (Gisi, Waldner et al. 2007). Reductions in sensitivity appear in agricultural or clinical settings as the frequency of the mutation increases in the pathogen population (Ma and Michailides 2005). The breakdown of effective fungicidal activity via the increase of resistance in a pathogen population is thought to evolve through two distinct phases – genetic mutation followed by selection (Hobbelen, Paveley et al. 2014). An important point to note in the evolution of resistance is that genetic mutations occur randomly throughout an organism's genome and it is purely by chance that such a change will confer fungicide resistance. Thus it is theoretically possible that resistance mutations could be detected in fields that have previously been devoid of fungicides (Gisi, Chin et al. 2000). For fungicide breakdown to occur, an external agent must provide a mutant individual a selective advantage over the majority of the wild-type population. Under these conditions, resistant mutants will be selected and can then proliferate to become the dominant form in the pathogen population eventually leading to fungicide resistance (van den Bosch, Oliver et al. 2014).

Devoid of selection, mutations conferring fungicide resistance will only persist in pathogen populations in the absence of associated fitness costs. *B. cinerea* shares many of the same characteristics of *Blumeria* spp. meaning that adaptations encoding fungicide resistance can be very common in natural field and vineyard populations. Both Billard et al.(2012) and Veloukas (2013) examined the impact that mutations conferring fungicide have impacted on the survival capability of *B. cinerea* isolates. Resistance to the SBI fungicide fenhexamid, has been shown

to be conveyed by mutations in the *Erg27* gene, with the highest levels of resistance reported in field isolates harbouring one of F412S/I/V changes (Fillinger, Leroux et al. 2008). Surprisingly despite the repeated and prolonged period of application, fenhexamid has remained effective in the controlling *B. cinerea* despite the dominance of the F412 mutant genotype in French vineyards (Billard, Fillinger et al. 2012). Isogenic fenhexamid resistant strains of *B. cinerea* were generated, each carrying one of the naturally occurring F412 mutations to assess the effect that each change had on overall phenotype. Billard et al. (2012) found that there was a moderate but significant impact of *Erg27* F412 mutations on isolate survivability *in* vitro which may account for the continued effectiveness of fenhexamid despite the high frequency of resistance in the natural population.

Dual resistance to both succinate dehydrogenase inhibiting (SDHI) and quinone outside inhibiting (QoI) fungicides is now a common trait in many *B. cinerea* populations around the world (Bardas, Veloukas et al. 2010; Fernández-Ortuño, Chen et al. 2012; Amiri, Heath et al. 2013). Resistance to SDHI has been shown to be conveyed through mutations in *Sdh*B, *Sdh*C and/or *SdhD* genes (Bardas, Veloukas et al. 2010). The G143A substitution is the main mutation shown to be associated with QoI resistance (Ishii, Yano et al. 2007). Veloukas et al. (2014) assessed the impact that the G143A *CytB* mutation had on *B. cinerea* field isolates and in addition the fitness costs associated with the acquisition of additional *Sdh* mutations. The fitness costs associated with the presence of the *CytB* mutation have been reported as having variable effects on fitness. Ma and Uddin (2009) reported a select fitness advantage of wild type strains of *Magnaporthe oryzae* compared to *CytB* mutants, while in *Alternaria alternata* isolates did not exhibit any in vitro associated fitness costs that could be pertained to the presence of the G143A mutation (Karaoglanidis, Luo et al. 2010).For *B. cinerea*, the G143A *CytB* substitution does not exhibit any associated fitness costs (Veloukas, Kalogeropoulou et al. 2013). However, Veloukas et al. (2014) did observe that isolates carrying mutations in both *CytB* and *Sdh* genes decreased in frequency compared to wild-type *B. cinerea* isolates under fungicide selection. The fitness costs associated with the combination of mutations in *B. cinerea*, presents an opportunity to coordinate spray regimes that alternate SDHI applications with other MOA chemicals to delay or reduce the selection of SDHI resistance in the local population.

The identification and tracking of mutations throughout SSR haplotypes can be a useful means in determining the place of origin and spread of fungicide resistance within a pathogen population. If a resistance mutation is shown to be present only in very similar haplotypes, it

is quite possible that resistance is a result of a single mutational event that has been selected by fungicide use and then passed down through generations. However, if resistance is detected in a diverse range of SSR haplotypes, it is more likely that same genetic mutation occurred on multiple occasions and was subsequently selected for by temporally distinct fungicide applications. At this stage, the work performed by Tucker *et al.* (2014) indicates that the *Bgh* resistance mutations are found in numerous diverse SSR haplotypes possibly indicating multiple mutational and selection events. However, further investigations employing more SSR markers on many more *Bgh* isolates are required for this hypothesis to be conclusive.

Various molecular mechanisms have been shown to contribute to decreases in fungicide sensitivity (Figure 11), including alterations in target site (Cools, Mullins et al. 2011; Tucker, Lopez-Ruiz et al. 2015), changes in membrane composition (Löffler, Einsele et al. 2000) and increased active efflux (Da Silva Ferreira, Colombo et al. 2005). Unfortunately given *Bgh*'s propensity for adaptation, fungicide resistant populations are now widely reported in Europe (Bartlett, Clough et al. 2002; Fraaije, Butters et al. 2002; Baumler, Felsenstein et al. 2003).

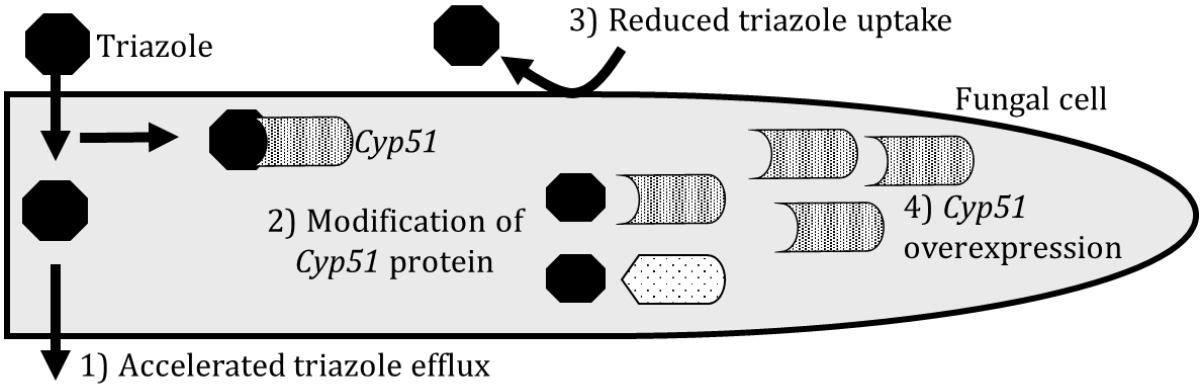


Figure 11. Resistance mechanisms to triazole fungicides.

In the absence if resistance, triazole antifungals (filled octagon) are taken up into the fungal cell and bind with *Cyp51.* Resistance to triazole fungicides has been shown to be conveyed by several defined mechanisms. The major mechanisms include 1) accelerated efflux out of the fungal cell the up-regulation of multidrug efflux transporter genes or duplication of ABC and MFS transporters; 2) through modification of the *Cyp51* substrate, via amino acid substitutions in the coding sequence or changes in promoter; 3) a reduction in the uptake of triazole into the fungal cell through changes in membrane permeability or through 4) overexpression of the target enzyme.

7.21321 Decreased Membrane Permeability and Changes in Cell-Wall Composition.

Changes in membrane composition have been found to limit the accumulation of intracellular antifungals. For example, Stergiopoulos *et al.*(2003) demonstrated that altered glycosylation of *Candida*. *albicans* surface proteins conferred some degree of resistance to triazole fungicides. In *C. albicans* the major phospholipids of the plasma membrane are phosphatidylcholine, phosphatidylethanolamine, phosphatidylinositol and phosphatidylserine. Löffler *et al.* (2000) found that the concentrations of phosphatidylcholine and phosphatidylethanolamine increased in triazole sensitive isolates whilst phosphatidylinositol and phosphatidylserine were increased in fungicide resistant *C. albicans.* Increases in the concentration of phospholipids in resistant isolates also brings about an increase in net negative charge (Löffler, Einsele et al. 2000). Löffler et al. (2000) postulated that changes in the composition of cell membrane phospholipids accompanied with a decrease in ergosterol concentration were key factors leading to a decrease in the accumulation of intracellular fungicides in *C. albicans* resistant isolates. In addition Sanglard et al. (1998) have shown that changes in cell wall protein characteristics also affects triazole diffusion. Mutant isolates of *C. albicans* have been shown to acquire some degree of resistance through altered glycosylation of cell surface proteins (Sanglard 2002). As yet no such alterations in membrane permeability have been reported in fungal crop pathogens including *Bgh.*

7.21322 Target Site Alteration

Perhaps the most widely reported mechanisms conferring reductions in triazole sensitivity are mutational changes in the triazole target protein, *Cyp51*. Most of the substitutions are present in highly conserved regions across the fungal kingdom which suggests the importance of these residues in the maintenance of *Cyp51* function throughout evolution (Figure 12). According to the molecular modelling of *C. albicans* in particular, these regions correspond to important functional domains of sterol C14α-demethylase and its interaction with the heme moiety, active site and an additional region postulated to play a role in the entry of the substrate into the binding pocket (Xiao, Madison et al. 2004).

A single nucleotide polymorphism or SNP is a variation in an organism's DNA sequence which results in a single base change. Nucleotide deletions, insertions or substitutions can each have

varying effects depending on where they occur and whether they have a corresponding impact on the translated protein. SNP's within both inter and intragenic regions are likely to have minimal or no effect on overall fitness. Point mutations that occur within the coding region or exon may have no effect if the corresponding SNP does not result in an amino acid change (synonymous mutation). If the SNP results in the replacement of an amino acid by another (non-synonymous mutation) it may result in a non-functional protein or alter its molecular and or biochemical characteristics. Studies by Délye *et al* (1998) examined homology between fungal, animal and plant kingdoms and identified two distinct *Cyp51* alleles in *Blumeria*: a DMI sensitive and a DMI insensitive. A SNP at amino acid position 136 was shown to distinguish between the two. The amino acid at position 136 is located within the highly conserved CR2 domain of all kingdoms and is thought to have a role in substrate binding (Délye, Bousset et al. 1998). Further studies by Wyand and Brown (2005) have characterised an additional SNP in the *Cyp51* gene which confers DMI insensitivity. In both cases the substitution of a single base pair results in the alteration of the translated protein: namely the exchange of a tyrosine for a phenylalanine at amino acid position 136, Y136F (Délye, Bousset et al. 1998) and the substitution of a lysine for a glutamine at 147, K147Q (Wyand and Brown 2005).

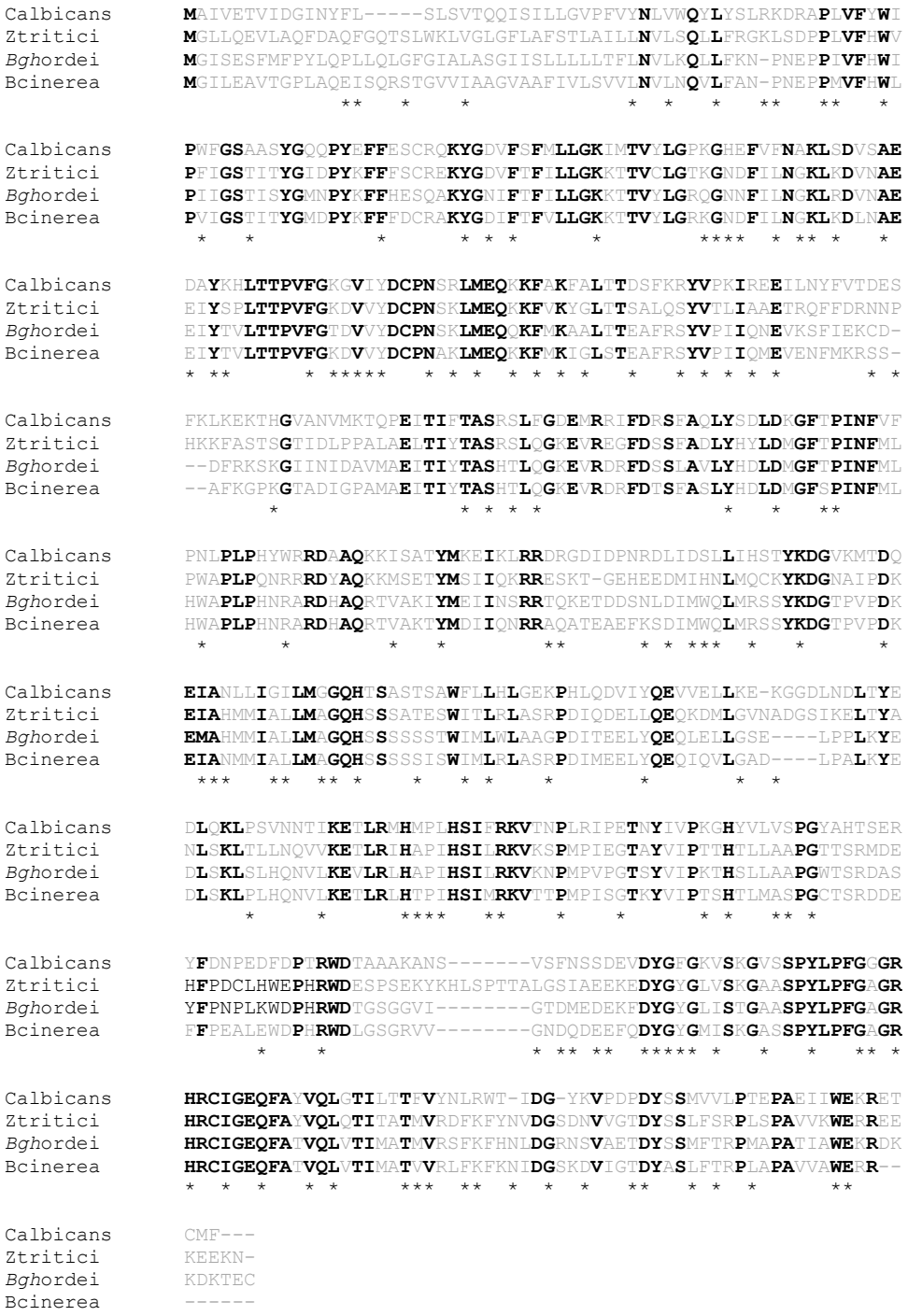


Figure 12. *Cyp51* protein alignment.

Cyp51 protein sequences of *Candida albicans* (Calbicans, Accession: AAF00597.1), *Zymoseptoria tritici* (Ztritici, Accession: AAU43734.1), *Blumeria graminis* f. sp. *hordei* (*Bgh*ordei, Accession: AIO08289) and *Botrytis cinerea* (Bcinerea, Accession: CCD54835.1). Asterisks indicate known mutations in one or more of the fungal species listed. Amino acids common to all included species are indicated in bold for *C. albicans* (Löffler, Kelly et al. 1997; Sanglard, Ischer et al. 1998; Sanglard and Odds 2002) *Z. tritici* (Cools and Fraaije 2013), *B. g. hordei* (Wyand and Brown 2005; Tucker 2015 unpublished) and *B. cinerea* (Albertini, Thebaud et al. 2002)

7.21323 Increased Target Gene Expression

A major mechanism for increasing fungal *Cyp51* expression is the presence of single insertions of tandem repeated sequences in the *Cyp51* promoter region. This mechanism causing resistance to a number of triazole fungicides has been observed in isolates of the phytopathogenic fungi *Penicillium digitatum* (Hamamoto, Hasegawa et al. 2000), *Blumeriella jaapii* (Ma, Proffer et al. 2006) and *Venturia inaequalis* (Schnabel and Jones 2001). Here, resistance was correlated with the presence of tandem repeats in the promoter region of *Cyp51* leading to enhanced target gene transcription. Transformation of a mutant *Cyp51* gene, which included the altered promoter region, rendered sensitive *P. digitatum* isolates resistant and increased *Cyp51* expression akin to levels found in resistant isolates (Hamamoto, Hasegawa et al. 2000). However, the extent to which this mechanism contributes to overall resistance is difficult to discern as increases in *Cyp51* expression was not always found in *V. inaequalis* resistant isolates harbouring identical promoter insertions (Schnabel and Jones 2001).

7.21324 Increased Active Efflux

ATP-binding cassettes (ABC) and major facilitator superfamily (MFS) transporters are membrane bound proteins present in all living organisms. These transporters secrete a large number of structurally and functionally distinct compounds into the extracellular matrix (Figure 8), thereby preventing intracellular accumulation (Stergiopoulos, Zwiers et al. 2003). Due to their indiscriminate rejection of cytotoxic moieties, the accumulation of many ABC or MFS membrane proteins in a single isolate results in resistance across many classes of fungicide; i.e. being multi-drug resistant (MDR). In phytopathogenic fungi the over-production of transporter proteins in MDR individuals may in part be due to up regulation of genes encoding ABC and MFS genes. In *S. cerevisiae*, Sanglard *et al.* (1996) identified several point mutations in loci concerned in transcriptional regulation which were correlated with a MDR phenotype. In clinical isolates of *C. albicans*, Sanglard and Odds (2002) reported a striking difference between the ABC and MFS transporters. It was found that ABC transporters can accept almost the entire class of triazoles used in medicine as substrates, whilst MFS transporters only accept fluconazole as a substrate. Such investigations into the use of increasing efflux as a mechanism of fungicide resistance have yet to be investigated in *Bgh* but have been reported in field isolates of *Z. tritici* (Leroux and Walker 2011).

8.0 Objectives

Usually, *Bgh* can be controlled through planting of resistant varieties (including those harbouring the *mlo* gene) and through the application of effective fungicides. However, incidences of fungicide and resistant cultivar breakdown have long been reported in other agriculturally important mildew prone areas of the world. History has shown that the wide scale planting of cultivars with dominant *R* genes and the continual use of fungicides with a single MOA, can lead the build-up *Bgh* isolates capable of overcoming such control measures. Despite this foreknowledge, barley growers in WA are now faced with an epidemic of highly virulent (Tucker, Jayasena et al. 2013) and fungicide resistant powdery mildew (Tucker, Lopez-Ruiz et al. 2015). The aim of the present study was to gain insight into the population of *Bgh* isolates by:

1. Determining the virulence genes present in a collection of WA *Bgh* isolates.
2. Analysing the population structure of *Bgh* isolates in West Australia.
3. Assessing the sensitivity levels of Australian isolates of *Bgh* to the currently registered triazole fungicides available for powdery mildew control in WA.
4. Isolating and characterising the sterol C14-α demethylase gene (*Cyp51*) from Australian isolates of *Bgh* differing in sensitivity to currently registered triazole fungicides.
5. Analysing the role that mutations in *Bgh Cyp51* play in resistance to triazole fungicides.
6. Understanding the structural implications that *Cyp51* mutations have on protein configurations and binding with triazole fungicides.

9.0 References

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10.0 Published Papers

10.1 Tucker M. A, Jayasena K, Ellwood SR and Oliver RP (2013), Pathotype Variation of Barley Powdery Mildew in Western Australia. *Australasian Plant Pathology*. 42(5):617-623. DOI: 10.1007/s13313-013-0226-y

Initial experiments, including the curation of pathotypes, were initiated prior to candidature. However confirmation of results, all data analysis, preparation of the manuscript and submission were completed during the PhD.

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Pathotype variation of barley powdery mildew in Western Australia

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Abstract Barley powdery mildew caused by the fungus *Blumeria graminis* f. sp. *hordei* (*Bgh*) has emerged as the most damaging disease of barley in Western Australia (WA). Many of the available cultivars display high levels of disease in the field when climatic conditions are conducive. As a result, fungicides have become the main method of disease control in the last 10 years. Different types and sources of genetic disease resistance are available but to optimise their deployment it is necessary to evaluate the spectrum of pathotypes present in the pathogen population. Sixty isolates of *Bgh* were collected in the 2009 season from 9 locations, single spored and characterised by infection on reference barley lines and cultivars. Eighteen unique pathotypes were resolved. Virulence against many of the *R*-genes in the reference lines was present in at least one pathotype. Isolates were virulent against 16 out of a total of 23 resistance gene combinations. Undefeated resistance genes included the major *R*-genes *Mla-6*, *Mla-9*, *Ml-ra* and the combinations of *Mla-1* plus *Mla-A12* and *Mla-6* plus *Mla-14* and *Mla-13* plus *Ml-Ru3* together with the recessive resistance gene *mlo-5*. There was significant pathotype spatial differentiation suggesting limited gene flow between different regions with WA or localised selection pressures and proliferation. On the basis of the results we recommend a number of strategies to manage powdery mildew disease levels within WA.

Keywords *Blumeria graminis* · Powdery mildew · Virulence · pathotype

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Introduction

Powdery mildew, caused by the fungus *Blumeria graminis f. sp. hordei*, results in major yield losses of barley (*Hordeum vulgare L.*) worldwide if uncontrolled. The disease is especially prevalent in moderate to temperate growing regions where yield losses can reach 40 % (Chaure *et al.* 2000). Along with cultural practices, the main control measures are the application of effective fungicides and the use of cultivars with genetic resistance. The challenge for both breeders and growers is the capacity of mildew populations to evolve virulent new forms on resistant cultivars together with fungicide resistant pathotypes (Wyand and Brown 2005). Powdery mildew has a number of characteristics that support rapid evolution, such as large numbers of asexual haploid spores, sexual recombination during the growing season, and airborne dispersal over large distances. Consequently finding effective and durable control measures to constrain powdery mildew fungi represents an important challenge in crop protection research.

There are a large number of mapped resistance genes that could provide protection against barley powdery mildew infection (Czembor and Johnston 1999). These include major dominant *R*-genes, operating at a gene-for-gene level (Flor 1971), the major recessive non-race specific resistance gene *mlo* (Buschges *et al.* 1997) and less well characterised minor genes (Yu *et al.* 2001). The recessive resistance gene *mlo* has remained undefeated after 50 years of use, but is associated with a yield penalty (Brown 2002). The use of major *R*-genes offers a rapid way to introgress resistance into current cultivars, but such resistance is seldom durable and is subject to a 'boom and bust' cycle (Hovmoller *et al.* 2000; McDonald and Linde 2002) when the pathogen population evolves via loss of the corresponding avirulence (*Avr*) gene. Such a strategy requires knowledge of the pathotypes of the pathogen present in the population. The introgression of a single *R*-gene is doomed to failure but

introgression of two or more *R*-genes, followed by pathotype surveys that detect virulence corresponding to the deployed *R*-genes, is potentially a viable strategy. As each *R*-gene is defeated, it must be replaced so that cultivars continue to carry one or more effective *R*-genes. As a first step in the process, the pathotypes of the population must be determined and monitored.

The objectives of this study were to i) determine the avirulence genes present in the WA *Bgh* population ii) determine which *R*-genes still provide protection against infection iii) assess the status of the *Bgh* resistant cultivars Dash and Barque iv) and provide a baseline of the frequencies of avirulence within the WA population for comparison in future surveys.

Materials and methods

Collection and maintenance of isolates

Isolates were sampled from nine locations throughout the barley growing region of Western Australia; Perth, Medina, Katanning, Broomehill, Mt Barker, Albany, Boxwood Hill, Gairdner and Esperance. In total 60 isolates were collected from August to October 2009. Tissue segments approximately 7 cm in length were excised from infected plants and inserted into slopes of water agar amended with 50 $mg.L^{-1}$ of benzimidazole (Chan and Boyd 1992). Conidia from each sample were shaken onto cv. Baudin grown under mildew free conditions using the tower inoculation method of Brown and Wolfe (1990) and maintained on benzimidazole agar plates in a controlled environment 20±2°C subject to a 12:12 h light: dark photoperiod. This process was then repeated with a single colony to obtain monoconidial cultures. Isolates were subcultured onto cv. Baudin at 7–10 day intervals and shaken 24 h prior to use to dislodge old conidia and ensure fresh inoculum for infection.

Inoculation of pallas differential lines and W.A. cultivars

Twenty three Pallas isolines (Kølster *et al*. 1986) and three current WA cultivars—Baudin, Barque and Dash were obtained from Department of Agriculture and Food Western Australia (DAFWA), South Perth, WA. Seedlings were potted in grade 2 vermiculite and grown in a mildew free controlled temperature environment subject to a 12 h fluorescent photoperiod at 400 $E.m^{-2}\ s^{-1}$. A single colony of each monoconidial isolate was used to inoculate the primary leaf of 10 days old seedlings. Leaf segments from each line were inoculated simultaneously using the settling tower method described previously and inserted into benzimidazole agar.

Virulence and pathotype designation

A five point (0 to 4) infection type (IT) scale was adapted from Czembor (2000) and used to assign a single infection type to each isolate/cultivar interaction 8 days post inoculation. Isolates that produced an infection type 3 or 4 were considered virulent. A selection of 16 differential Pallas lines was used to distinguish and group isolates into pathotypes. A pathotype encompasses isolates with identical pattern of virulence on the differentials. Analysis was conducted using the HaGis spread sheet for Automatic Habgood-Gilmor Calculation V.3.1 (Herrmann *et al*. 1999) to generate descriptive collection site parameters (virulence frequency, number of pathotypes, virulence complexity, and abundance and diversity parameters shown in Table 2).

Results

Pathotype complexity and distribution

In 2009 eighteen unique WA pathotypes from 60 isolates were identified, sampled from nine sites (Figure 1, Table 1), of which fourteen had more than one isolate.

Pathotypes 4 and 18 were the most abundant, encompassing in total eight isolates each and which showed virulence complexities of six and sixteen respectively (Table 2). Three pathotypes were found at more than one collection site whilst all remaining pathotypes were unique to their site of collection. The diversity parameters of the complete collection of isolates surveyed in 2009 are detailed in Table 2. The mean pathotype complexity, defined as the mean of virulence, per pathotype was 6.89. The most diverse sampling site was Mount Barker, from which a total of eight unique pathotypes were identified (Table 2). Virulence complexity of the pathogen collection (mean of the isolate complexity) was 7.98. However pathotype 18 collected from Esperance had a considerably higher virulence complexity of 15.

Isolate virulence frequency and complexity

The frequencies of virulence of all isolates on 26 barley lines varied from 0 % (no disease on 7 lines) to 100 % (complete susceptibility in lines P17 and P21 and to cv. Baudin). The *R*-genes that were present in the resistant lines were *Mla-1*, *Mla-A12*, *Mla-3*, *Mla-6*, *Mla-14*, *Mla-9*, *Mla-13*, *Ml-Ru3* and *Ml-ra* (Table 3).

There was no visible infection on the Pallas line harbouring *mlo-5*. The proportions of virulence were low to the resistance gene *Mla-23* (0.13) and to the combination of resistance genes *Mla-7* and *Ml-LG2* (0.13), *Mla-10* and *Ml-Du2* (0.13), *Mla-12* and *Ml-Em2* (0.13). The proportion

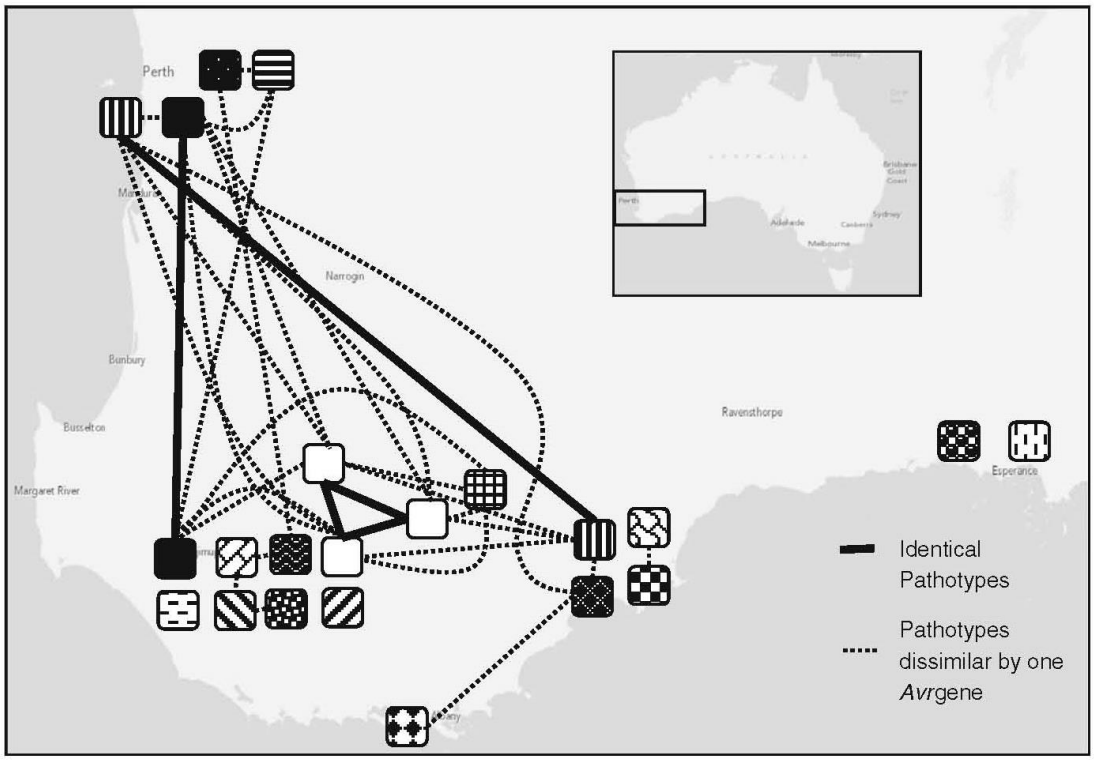

Fig. 1 Pathotype map of *Blumeria graminis* f. sp. *hordei* in Western Australia. Nine sample sites from west to east are Medina, Mount Barker, South Perth, Katanning, Albany, Broome Hill, Boxwood Hill, Gairdner and Esperance. Individual pathotypes are distinguished by patterned boxes. Identical pathotypes are linked with a solid line. Pathotypes dissimilar by a single *Avr* gene are linked by dotted lines

of virulence to *Mla-22* (0.87), *Ml-p1* (0.87), *Mla-t* (0.98) and *Mla-8* (0.98) were very high. The proportion of isolates virulent to the other *R*-genes ranged from 0.2 to 0.73. Resistance genes were classified as effective (0 % of isolates virulent) compromised (0 %>0.50 % of isolates virulent) and defeated (0.50 %>1.00 % of isolates virulent).

Table 1 Diversity parameters of the *Blumeria graminis* f. sp. *hordei* collection from Western Australia in 2009. Mean isolate complexity is defined as the mean of avirulence genes present in all isolates. Mean pathotype complexity is defined as the mean of avirulence in each pathotype

Parameter	
No. of isolates	60
No. of pathotypes	18
No. of pathotypes with frequency>1	14
Mean isolate complexity	7.98
Mean pathotype complexity	6.89
Diversity-Simple	0.30
Richness-Gleason	4.15
Diversity-Shannon	2.69
Diversity-Simpson	0.94

The virulence complexity of each pathotype was defined by the total number of virulence genes in that group. The lowest virulence complexity (3), with reference to the differentials lines used, was that of pathotype 11 represented by a single isolate. This isolate carried only *avr-a7*, *avr-aNo3* and *avr-a22* (Table 4). The highest virulence complexity (16) was found in a total of eight isolates in pathotype 18 *avra-7*, *avra-NO3*, *avr-LG2*, *avra-10*, *avra-Du2*, *avr-Em2*, *avra-22*, *avr-Ru2*, *avr-k*, *avr-nn*, *avrp1*, *avra-t*, *avr-g*, *avr-CP*, *avr-La*, *avr-h* and *avr-Ga* that corresponding to the postulated *R* gene in Barque (Dreiseitl and Platz 2012).

Discussion

In 2009 the estimated average annual losses to powdery mildew in Western Australia (WA) were $33M between 2000 and 2008 (Murray and Brennan 2010), but anecdotal evidence suggests losses have been far higher in recent

Table 2 Complexity of the nine sample sites of *Blumeria graminis* f. sp. *hordei* in Western Australia in 2009. Virulence complexity is defined as the mean of the avirulence genes of isolates collected from each location

Location	No. Pathotypes	Pathotypes	Average isolate virulence complexity
Albany	1	1	3.0
Gairdner	4	2, 3, 11, 12	4.0
Boxwood Hill	1	5	7.0
Broomehill	1	4	6.0
Mount Barker	9	4, 6, 9, 10, 13, 14, 15, 17	7.7
Katanning	1	4	5.0
Esperance	2	16, 18	15.0
South Perth	2	7, 8	6.4
Medina	2	3, 6	5.5

years. In 2011, 1.55 million hectares of barley were sown in WA (ABS 2012) with the majority seeded with cultivars that are highly susceptible or susceptible to powdery mildew infection. Baudin, a high yielding malt grade cultivar has been the dominant choice for growers for the past six seasons. This provided the perfect environment for the *Bgh* to proliferate, reaching epidemic proportions with losses estimated at $100M in the 2010 and 2011 cropping seasons. In addition to yield losses, much of the diseased crop was downgraded to feed quality, resulting in a typical loss of $200/ha.

At present there is a lack of high yielding malt grade cultivars with effective genetic resistance and hence fungicide application has been the main method of control. One economical and environmentally sustainable solution is to breed new cultivars with effective *Bgh* resistance genes.

Table 3 Differential Pallas lines, their genes for resistance to *Blumeria graminis* f. sp. *hordei* and proportion of corresponding virulent isolates among Western Australian isolates collected in 2009. An isolate was considered virulent with an IT of 3 or 4. Lines/resistance genes were classed as effective (0.00 % isolates virulent) compromised (0.00 %>0.50 % isolates virulent) and defeated (0.50 %>1.00 % isolates virulent)

Line/Cultivar	Resistance gene/s	Proportion of isolates virulent	
P01	*Mla-1 Mla-A12*	0.00	Effective
P02	*Mla-3*	0.00	
P03	*Mla-6, Mla-14*	0.00	
P8b	*Mla-9*	0.00	
P11	*Mla-13, Ml-Ru3*	0.00	
P14	*Ml-ra*	0.00	
P22	*ml-o5*	0.00	
P06	*Mla-7, Ml-LG2*	0.13	Compromised
P09	*Mla-10, Ml-Du2*	0.13	
P10	*Mla-12, Ml-Em2*	0.13	
P13	*Mla-23*	0.13	
P24	*Ml-h*	0.20	
P4a	*Mla-7, Ml-k, +?*	0.25	
P4b	*Mla-7, Mla-No3*	0.38	
P23	*Ml-La*	0.38	
P15	*Ml-Ru2*	0.65	Defeated
P18	*Ml-nn*	0.73	
P12	*Mla-22*	0.87	
P19	*Ml-p1*	0.87	
P20	*Mla-t*	0.98	
Pallas	*Ml-8*	0.98	
P17	*Ml-k*	1.00	
P21	*Ml-g, Ml-CP*	1.00	
Baudin	*Mla-8*[1]	1.00	
Barque	*Ml-Ga*[1]	0.54	
Dash	*Mla-7, Ml-k1, Ml-La*	0.00	

[1]Postulated by Dreiseitl and Platz 2012

Table 4 Virulence spectra of 18 pathotypes of Western Australian *Blumeria graminis* f. sp. *hordei* isolates. The number of isolates in each pathotype are indicated in parenthesis

Virulence (+) of the pathotypes to resistance genes and cultivar Barque.

Pathotype	*Mla-7, +?*	*Mla-7, Mla-No3*	*Mla-7, Ml-LG2*	*Mla-10, MlaDu2*	*Ml-Em2*	*Mla-22*	*Mla-23*	*Ml-Ru2*	*Ml-k*	*Ml-nn*	*Ml-p1*	*Mla-t*	*Ml-g, Ml-CP*	*Ml-La*	*Ml-h*	Barque
1 (1)										+		+	+			
2 (2)									+	+		+	+			
3 (6)									+	+	+	+	+			
4 (8)						+			+	+	+	+	+			
5 (3)						+			+	+	+	+	+			+
6 (2)						+			+	+	+	+	+	+		
7 (5)						+		+	+		+	+	+			
8 (4)						+			+		+	+	+	+		+
9 (2)						+		+	+	+	+	+	+			
10 (4)						+		+	+	+	+	+	+	+		+
11 (1)		+				+										
12 (1)		+				+										+
13 (3)		+				+		+	+			+	+			
14 (2)		+				+		+	+			+	+	+		
15 (1)		+				+		+	+			+	+	+		+
16 (4)	+	+				+		+	+	+	+	+	+	+		+
17 (3)	+	+				+		+	+	+	+	+	+		+	
18 (8)	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+

However, in order for this solution to be effective there is an absolute requirement for thorough knowledge of the virulence and hence pathotypes (isolates with the same patterns of virulence) within the mildew population. Virulence surveys of *Bgh* populations have been conducted in many countries around the world (Czembor 2000; Czembor and Johnston 1999; Dreiseitl 2008; Dreiseitl and Platz 2012; Hovmoller *et al*. 2000) but as yet the Western Australian *Bgh* population has not been extensively investigated. The Pallas near-isogenic lines used in this study were created by introgressing *R*-genes into the barley cultivar Pallas and are a set of 23 genetically near-identical lines differing only in their gene(s) for *Bgh* resistance (Kølster *et al*. 1986). By screening collections of isolates on the isolines the virulence present can be determined and hence one can ascertain which *R*-genes could be incorporated into future breeding programs for local *Bgh* control.

A number of cultivars are recommended to growers in the DAFWA 2013 barley variety guide (DAFWA 2012). Baudin is a sought-after malting variety and has remained one of the most widely grown for the past six years. It is very susceptible to powdery mildew infection which many believe has been the major contributing factor to recent epidemics. Trends predict that the popularity of Baudin will now begin to decline as the costs of effective disease control outweighs any end point profits.

Buloke is beginning to gain acceptance in international markets as an alternative to Baudin (DAFWA 2012). With moderate resistance to *Bgh* it was more widely grown in WA in 2011. Buloke is thought to contain two *R*-genes, *Mla-7* and *Ml-La* (Dreiseitl and Platz 2012). This survey indicates that both of these *R*-genes are compromised in WA. Recombination of virulent isolates or mutation would result in isolates capable of infection. Thus Buloke may be predicted to suffer from the well-established bust phase of the boom and bust cycle in the next few years.

Barque is a feed variety classed as resistant to powdery mildew. According to Dreiseitl and Platz (2012) this protection is provided by the presence of the *Ml-Ga* resistance gene. Although not included in this survey, this *R*-gene is also found in cultivars such as Capstan (MS), Commander (MR-MS) and Fleet (MR-MS). The disease resistance ratings of these cultivars are given in parenthesis, indicating that at least some isolates in the WA *Bgh* population have mutated to *avr-Ga* and as such this resistance gene is also predicted to be compromised.

Dash is suggested to have the genotype *Mla-7*, *Ml-k1* and *Ml-La* (Dreiseitl and Platz 2012) and is rated as resistant to powdery mildew in Western Australia (DAFWA 2012). However, this study has shown that these resistance genes are compromised, defeated and compromised respectively. This implies there are no isolates tested in this study in Western Australia that have lost the corresponding *Avr*-genes collectively. Alternatively losing all three of these *Avr*-genes may impose a fitness penalty (Brown 2002).

Hindmarsh is accredited as a food variety and carries the *Mla-8* and *Ml-La* resistance genes (Dreiseitl and Platz 2012). *Ml-a8* provides no protection against *Bgh* and *Ml-La* is now compromised. This correlates with the cultivar's moderate susceptibility towards powdery mildew infection (DAFWA 2012). If the *avr-a8+avr-La* genotype increases in the WA population Hindmarsh's susceptibility could increase to match that of Baudin.

Yagan has intermediate resistance to powdery mildew. This is governed by the presence of two major resistance genes, *Ml-Ch* and *Ml-ra* (Dreiseitl and Platz 2012). This study determined that *Ml-ra* still provides effective protection against *Bgh* in Western Australia; the *Ml-Ch* gene was not tested.

Our studies indicated that isolates carrying virulence to 16 out of the 22 single or combinations of *R*-genes studied herein are present in the WA population. Major R-gene breakdown has been observed in Europe but the extent in this study was surprising given WA's isolation (Brown 1994). Therefore we can predict that Buloke (and other varieties) will not provide long term resistance to powdery mildew. Therefore any strategy based on major *R*-genes must incorporate two or more of the following single *R*-genes *Mla-3*, *Mla-9*, *Ml-ra* and the combinations of *Mla-1* with *Mla-A12*, *Mla-6* with *Mla-14* or *Mla-13* with *Ml-Ru3*. Future surveys should be carried out to detect mutations to virulence. By testing the Pallas lines P01, P02, P03, P8b, P11 and P14 against a range of isolates, changes in virulence in the local population can be detected.

Experience from Europe suggests the best ways of achieving durable resistance is to use either *mlo* (Freialdenhoven *et al*. 1996) or combinations of minor genes. The recessive resistance gene *mlo* has remained effective for more than 50 years and is the mainstay of mildew control in European winter barley plantings. The yield penalties associated with *mlo* lines are significant (4.2 %, Kjaer *et al*. 1990) but need to be weighed against some productivity losses and the costs of fungicides. We therefore recommend that serious consideration be given to the utilisation of *mlo* in WA barley cultivars. One alternative to reduce the pleiotropic effects of *mlo* may be the incorporation of durable minor resistance genes effective against different isolates (Yu *et al*. 2001). These genes only allow low levels of mildew development and are common in plants, and while few have been isolated, many have been described in barley (Aghnoum and Niks 2011; Jones and Davies 1985).

We discovered significant spatial differentiation for the *Bgh* population – the highest diversity was at Mount Barker whilst the Perth population was a distinct subgrouping. *Bgh* is a highly mobile pathogen (Wyand and Brown 2003) and so it was surprising to see such differentiation, which may reflect local cultivar selection pressures. This finding indicates the necessity to carry out field trials in several

locations in order to accurately assess the cultivar resistance levels. Possibly the most promising result from this survey is the identification of resistance genes which still provide effective control of *Bgh*. The introduction of these and exotic genes into future barley breeding programs, along with an integrated fungicide regime, may allow the impact of *Bgh* to be ameliorated in WA.

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Development of genetic SSR markers in *Blumeria graminis* f. sp. *hordei* and application to isolates from Australia

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The barley powdery mildew pathogen, *Blumeria graminis* f. sp. *hordei* (Bgh), exists in numerous haplotypes and displays significant differences in fungicide sensitivity. It causes considerable yield losses throughout the world. Microsatellite SSRs are useful tools to study the population level and biogeographic aspects of intraspecific diversity, but so far none have been defined for Bgh. Here, eight polymorphic microsatellite loci were identified and characterized. Primer pairs amplifying the loci were then applied to 111 isolates of Bgh from Australia. The number of alleles per locus ranged from 4 to 13, and Nei's genetic diversity ranged from 0·25 to 0·76. The microsatellite primers detected several clones among the isolates and defined 97 unique haplotypes. There was little evidence for regional genotypic subdivision, suggesting that gene flow may not be restricted among geographic regions. All data was consistent with high levels of genetic diversity, potentially resulting from random mating and spread within each region.

Keywords: barley, microsatellite, population, powdery mildew

Introduction

Barley powdery mildew, caused by the filamentous ascomycete fungus *Blumeria graminis* f. sp. *hordei* (Bgh), is the most economically significant disease of barley in Western Australia (WA) (Murray & Brennan, 2010). Bgh has a number of characteristics that support rapid evolution, such as the production of large numbers of asexual progeny, annual sexual recombination and airborne conidial dispersal over large distances (McDonald & Linde, 2002). Consequently, finding effective and durable control measures represents an important challenge in crop protection research. To ensure the longevity of newly released resistant barley cultivars, knowledge of the genetic diversity of local Bgh populations and the mechanisms that lead to the development of new virulent Bgh strains is essential.

Population surveys of Bgh have been performed across many barley-growing regions of the world detailing both phenotypic and genotypic variation (Brown *et al.*, 1991; Hovmøller *et al.*, 2000; Tucker *et al.*, 2013). These studies used differential barley lines harbouring known resistance genes to ascertain virulences present in local Bgh populations. A comprehensive survey carried out in Europe over six years found that the majority of Bgh haplotypes had a strong association with collection region. With collection sites spanning from Finland to Hungary, Hovmøller *et al.* (2000) ascertained that the powdery mildew population was markedly different in the north compared to populations in southern Europe. Another survey crossing the British Isles detected several isolates sharing the same haplotype at multiple sites, collected 450 km apart (Brown *et al.*, 1991). However, the majority of the British Bgh population was highly diverse with the remaining isolates genotypically unique. The most recent survey conducted by Tucker *et al.* (2013) on West Australian Bgh isolates found significant spatial haplotype differentiation, but again this study focused on major virulence gene detection. To date, no Bgh population studies have been performed using neutral markers.

Microsatellite markers, otherwise referred to as simple sequence repeats (SSR), have been very informative in studying the genetic diversity of other important fungal species such as *Parastagonospora nodorum* (Stukenbrock *et al.*, 2005), *Zymoseptoria tritici* (Goodwin *et al.*, 2007), *Rhizoctonia solani* (Ferrucho *et al.*, 2013) and *Pyrenophora teres* (Ellwood *et al.*, 2010). Their abundance in the eukaryotic genome, genetic neutrality, co-dominance and multi-allelic nature means they can be employed to differentiate individuals and populations (Powell *et al.*, 1996). Here, the first instance of SSR markers for Bgh is described. The purpose of this study was (i) to define a set of polymorphic microsatellite markers using the first published Bgh genome sequence (Spanu *et al.*, 2010), (ii) to screen a collection of Australian Bgh isolates using the defined markers, and (iii) to compare Bgh isolates to determine whether there is any association between SSR haplotype and collection year, or region.

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Materials and methods

Isolate collection, maintenance and genomic DNA extraction

One hundred and eleven Bgh isolates were collected from seven naturally infected regions in Australia during the 2008–2011 growing seasons (Fig. 1). Multiple sampling trips were taken during each season, beginning 1–2 months after the first report of Bgh infection, continuing on a 3–4 week basis for the remainder of the season. Isolate numbers collected in WA were as follows: five isolates from the North region, 32 isolates from the Metro region, nine isolates from the East region, 11 isolates from the South-East region, 17 isolates from the Stirling Ranges, four isolates from the Fitzgerald River region and 26 isolates from the Esperance region. A further seven isolates from the east of Australia (Queensland, New South Wales and Tasmania) were grouped into an additional eighth region, and were supplied by the Department of Environment and Primary Industries, Victoria. Ten randomly collected leaf samples were obtained from infected barley within the same field at each sample site. Leaves were inserted into 15-mL tubes containing 3 mL 50 mg L^{-1} benzimidazole agar, which were capped for transportation. Isolates were single-spored, maintained and subcultured according to Tucker *et al.* (2013). All isolates are detailed in Table S1. For genomic DNA extraction, conidia of pure isolates were dislodged onto a glass plate and collected in Eppendorf tubes using sterile razor blades. Tubes were snap frozen in liquid nitrogen with the addition of lysis buffer (QIAGEN) prior to mechanical crushing in a high-speed mixer mill (MM301; Retsch Inc.). Genomic DNA extractions were performed using a BioSprint 15 DNA Plant Kit (QIAGEN) according to the manufacturer's specifications.

Microsatellite design and detection

The whole genome sequence of Bgh isolate DH14 (GenBank ID: ABSB00000000.2) (Brown & Simpson, 1994) was scanned for SSR repeats as per Ellwood *et al.* (2010). TANDEM REPEAT FINDER v. 4 (Bensen, 1999) parameters were set for the detection of imperfect di- or trinucleotide repeats with 60–100% identity. Primers were designed within flanking regions of SSRs of interest using PRIMER3 software (Rozen, 2000), with parameters set to a primer length of 18–22 bp, GC content 40–60%, and an optimal melting temperature of between 58 and 62°C for amplicons of 180–550 bp. A subset of 30 primer pairs was preselected on the basis of primer quality determined by NETPRIMER (Premier Biosoft) and an optimal motif repeat number of over eight units (Table 1).

Unlabelled primers were initially screened on genomic DNA extracted from 10 Bgh isolates collected from WA in 2010 from geographically distinct locations. A touchdown polymerase chain reaction was employed using the following thermal cycling conditions: 94°C/2 min; (94°C/30 s, 65°C/30 s –1°C/cycle, 72°C/60 s) × 5; (94°C/30 s, 50°C/30 s, 72°C/60 s) × 32, 72°C/5 min. PCR products were visualized on 2% agarose gels. All 30 primer pairs successfully amplified, and eight primer pairs that produced specific and reproducible bands were randomly selected for further screening on the remaining Bgh collection (Table 2). Forward primers of each polymorphic set were fluorescently labelled with either FAM (Geneworks), VIC or NED (Applied Biosystems) dyes, allowing PCR products to be multiplexed and capillary sequenced on a 3730xl DNA sequencer (Applied Biosystems) together with an internal size standard (Genescan LIZ 500; Applied Biosystems). Microsatellite chromatograms were analysed using the GENEMARKER (Soft Genetics) program.

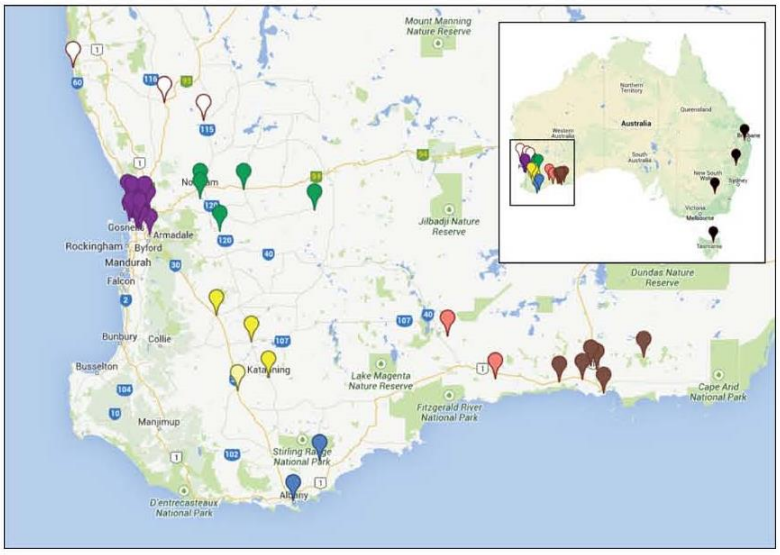

Figure 1 Collection sites of *Blumeria graminis* f. sp. *hordei* isolates (n – 111). For analysis, sites were grouped into eight geographic regions based on their proximity. Geographic regions indicated with colour, with number of isolates indicated in parenthesis, are as follows: red North (5); purple Metro (32); green East (9); yellow South East (11); blue Stirling Ranges (17); orange Fitzgerald River (4), brown Esperance (26). The eighth geographic range encompasses all isolates sampled from East Australia (7) as indicated in black (inset).

Table 1 Primer sequences and microsatellite loci as derived *in silico* from *Blumeria graminis* f. sp. *hordei* isolate DH14

Scaffold	Repeat motif	Primer sequences (5′–3′)	Tm (°C)	% GC	*In silico* product size (bp)
6[a]	$(TA)_{22}$	AGCTTGGGCGTTTGTATGTC	60·1	50·0	237
		CGCTTGTATTGAAAGTCTGGAA	59·4	40·9	
8[a]	$(CAA)_{9}$	CGTCGGTTCGTTACAGTTAGAT	58·3	45·5	239
		CCGATAAATCTCGTTCACTTCA	59·2	40·9	
9	$(GAC)_{10}$	GGAATTTGATGAAGTGGATCGT	60·2	40·9	247
		ACGAAGCAGCGACAGAGTAGAC	61·1	54·5	
9	$(AT)_{19}$	CCTGTTTCTAGGCTTTCTTCCA	59·9	45·5	250
		TTCAGTCTTGTCCTCCACTTCC	60·7	50·0	
10	$(AT)_{21}$	CAAATCTTCCGATTGAACCTGT	60·4	40·9	197
		GCACCTCGTCTTGAGTGAAAG	60·0	52·4	
10[a]	$(TCA)_{14}$	TGCATCTATACCAGCATCAAGG	60·1	45·5	251
		GGGCTGAACAGTCTTACTTGGA	60·7	50·0	
11	$(AAT)_{18}$	GCTTACCGTCTTCATGGTTCTG	61·0	50·0	206
		GTTAGGGTATGGCTGCAACTTT	59·6	45·5	
11	$(AGA)_{11}$	ATATTCTCAAGATGGCCGAAGA	60·1	40·9	270
		TTCCTTCATCACCTTCACTCC	59·1	47·6	
14[a]	$(CAT)_{8}$	TAGACACACACCTCCACAAAGG	60·1	50·0	256
		TGTACTGCGATTACGGGAAATA	59·5	40·9	
15[a]	$(AT)_{16}$	AATGCAGGTGGCTACAAACC	60·0	50·0	257
		GATAGGGTAATCCCAGACGACA	60·2	50·0	
17[a]	$(ACT)_{10}$	TCCAACTCTGTTACGCTCTTGA	60·1	45·5	282
		GCAAGCCCAAATGTTTCTCTA	59·4	42·9	
17[a]	$(CA)_{14}$	GGTATCCAGGGCCAATAAGTTT	60·4	45·5	254
		CGCTAACCTCTCACCTTTCCT	59·9	52·4	
18	$(AT)_{15}$	GGGCAATCTTGTAAGTGCAGTA	59·3	45·5	263
		GTCTCGGAAGCCACGAAAC	60·8	57·9	
18[a]	$(ATA)_{8}$	GGGAGATAGTGAAGGGCAAAG	60·1	52·4	233
		GGGACATGGTGACAACTACG	58·9	55·0	
19	$(AT)_{21}$	AAGTTAATGGGTGGGTCGAAG	60·2	47·6	243
		TAAGATCCTGGCTTTCATTTGC	60·6	40·9	
19	$(AAT)_{25}$	GGCTTACCGTCTTCATGGTT	59·1	50·0	242
		GGAATCAGGTTAGATGGGTCAAT	60·4	43·5	
20	$(ATT)_{22}$	AAGGAAAGCGAAATGATGAAAG	59·7	36·4	217
		GCCAAATCAGGACAATGAAATC	60·7	40·9	
20	$(AAT)_{16}$	CCAAATCGGGATAAATGAAATC	59·5	36·4	214
		CCATGTTGGAGCAAGGAAAG	60·6	50·0	
21	$(AC)_{16}$	GTCTTCTCAACCCACAAACTCC	60·0	50·0	251
		TAACTTTCATTGCTGACGACCA	60·7	40·9	
24	$(TTA)_{23}$	CCTAGAAACATAGCGGATGTCTG	60·2	47·8	383
		GCCAAGGCTAGATGAGATTTGA	60·7	45·5	
25	$(AAT)_{12}$	GCCAAATCAGGACAATGAAATC	60·7	40·9	199
		ATGTTGGAGCACAGAAGATGAA	59·7	40·9	
26	$(ATC)_{33}$	GAAATGATGATGTAGGTAGCGAAG	59·2	41·7	241
		GAGGGATTGGGACAAGTTTACA	60·2	45·5	
26	$(AAT)_{10}$	AATTACGGTTGAAAGGAACGTC	59·4	40·9	249
		AGACTGCAACTTCACGGGATTA	61·0	45·5	
27	$(CAT)_{10}$	TGCTTCCAATTTACCCTCTAGC	59·8	45·5	235
		CAGTAGTGGCAGACAGGATGAC	59·8	54·5	
27	$(ATC)_{14}$	TTGTCTCACACGCAAGTCAAAT	60·7	40·9	230
		TGATTCTGCACATGGATAGCTT	59·7	40·9	
28	$(TGA)_{9}$	AAACCATTCTCTTGATCCCAAA	59·8	36·4	233
		GCAGTTGGAAGGGCATAAATAA	60·3	40·9	
28	$(CT)_{20}$	AAAGAGCACTGTAGAAGGTGTGG	59·9	47·8	286
		CAATTTCATGTTCGATTCTTGG	59·5	36·4	
29	$(TA)_{22}$	CAGTAATTCAGGTCACAGGGAGA	60·5	47·8	252
		GAAGCCAATGTATGCTGTAAGG	58·8	45·5	
30	$(TC)_{12}$	ATTTGTGAGCTTTCGTCCAGTT	60·2	40·9	269
		TCACTTCTTTATTGGCGACTTG	59·4	40·9	
30	$(TAT)_{8}$	GTACGCACAAAGCTCAATTCG	60·8	47·6	245
		GCCAAATCAGGACAATGAAATC	60·7	40·9	

[a]Indicates markers used in this study.

Table 2 Features of microsatellites used to study the allelic diversity of a clone-corrected collection of Australian *Blumeria graminis* f. sp. *hordei* isolates ($n = 97$)

Locus	Scaffold	Primer sequences (5′–3′), forward[a] and reverse	Repeat motif	No. of alleles (N_a)	No. of repeat motifs	Nei's genetic diversity (H_E)	Effective number of alleles (N_e)
1	6	AGCTTGGGCGTTTGTATGTC CGCTTGTATTGAAAGTCTGGAA	$(TA)_{22}$	13	22–35	0·78	4·46
2	8	CGTCGGTTCGTTACAGTTAGAT CCGATAAATCTCGTTCACTTCA	$(CAA)_9$	7	6–16	0·77	4·28
3	10	TGCATCTATACCAGCATCAAGG GGGCTGAACAGTCTTACTTGGA	$(TCA)_{14}$	10	2–14	0·66	2·96
4	14	TAGACACACACCTCCACAAAGG TGTACTGCGATTACGGGAAATA	$(CAT)_8$	4	3–6	0·51	2·05
5	15	AATGCAGGTGGCTACAAACC GATAGGGTAATCCCAGACGACA	$(AT)_{16}$	4	11–14	0·43	1·74
6	17	TCCAACTCTGTTACGCTCTTGA GCAAGCCCAAATGTTTCTCTA	$(ACT)_{10}$	6	8–13	0·25	1·34
7	17	GGTATCCAGGGCCAATAAGTTT CGCTAACCTCTCACCTTTCCT	$(CA)_{14}$	7	4–15	0·60	2·48
8	18	GGGAGATAGTGAAGGGCAAAG GGGACATGGTGACAACTACG	$(ATA)_8$	5	6–10	0·67	3·02

[a]Forward primers were labelled fluorescently with either FAM, VIC or NED.

Statistical analysis

Automatic and manually curated allelic peak data was entered into PopGen v. 1.32 (University of Alberta) to characterize the genetic variation, observed number of alleles (N_a), effective number of alleles (N_e) and Nei's gene diversity (H_E) for each locus on a clone-corrected isolate set. Analysis was performed on the SSR profiles of isolates to determine genetic diversity of each region (Table 3). The fixation index (F_{ST}) between each paired region was determined by a *G*-test in FSTAT (Table 4, H_0 $F_{ST} = 0$, v. 2.9.3.2, University of Lausanne, Lausanne, Switzerland). Following 2800 permutations, in which the alleles of isolates from all regions were randomized, several pairwise F_{ST} values were identified as significant (Goudet, 1995). Principal component analysis (PCA) was performed on the clone-corrected data set (where duplicated genotypes are counted as a single clone) using UNSCRAMBLER v. 10.0.1 (Camo Software Inc.) as described previously (Gummer *et al.*, 2013). Full cross validation and a nonlinear iterative partial least squares algorithm were used for the analysis.

Table 3 Nei's genetic diversity (by region), obtained from the SSR profiles of 97 Australian isolates of *Blumeria graminis* f. sp. *hordei*

Region[a]	Nei's genetic diversity (H_E)
Fitzgerald River	0·344
Stirling Ranges	0·411
South East	0·419
Metro	0·426
Esperance	0·432
East	0·488
North	0·500
East Aust.	0·628
All WA regions ($n = 7$)	0·548
All Aust. regions	0·633

[a]WA, Western Australia; Aust., Australia.

Results

One hundred and four Bgh isolates were collected from southern WA and were grouped into seven geographic regions: North, Metro, East, South East, Stirling Ranges, Fitzgerald River and Esperance, based on proximity of collection (Fig. 1). An additional seven isolates from the east of Australia were grouped into the single region East Aust. All 30 primer pairs generated by PRIMER3 successfully amplified microsatellite regions in a subset of Bgh isolates (Table 1). From these, eight polymorphic microsatellites were randomly selected and were screened on all 111 Bgh isolates. Allelic peak data were analysed in GENEMAKER and 97 unique haplotypes were defined following clone-correction. No haplotype was detected in more than one region, and hence all isolates sharing the same haplotype originated from the same initial sample site and field. Six groups of isolates sharing the same multilocus haplotype were detected on multiple occasions and only one strain per haplotype was carried forward for further analysis.

The collection of Bgh isolates analysed in this study was highly polymorphic, with a total of 61 SSR alleles detected among the 97 isolates across all eight loci. The number of alleles per locus ranged from four to 13, with an average of seven. The number of SSR unit repeats at each locus was variable and ranged from two at locus 3, to 35 at locus 1. A summary of the genetic diversity data of the eight microsatellite loci is given in Table 2. Nei's genetic diversity within each region varied from 0·344 at Fitzgerald River to 0·628 in the East Aust. region. The overall genetic diversity of all regions surveyed was 0·633, with slightly less diversification within WA at 0·548 (Table 3). Regional differentiation due to genetic structure was also determined (F_{ST}, Table 4). The

Table 4 Pairwise fixation indices (F_{ST}, lower diagonal) with corresponding *P* values (upper diagonal) generated from SSR allele sizes of 111 Australian *Blumeria graminis* f. sp. *hordei* isolates from eight geographic regions

Region[a]	East Aust.	East	Esperance	Fitz River	Metro	North	South East	S Ranges
East Aust.	–	0	0	0·008	0	0·079	0	0
East	**0·150**	–	0	0·021	0	0·003	0·083	0·001
Esperance	**0·226**	**0·081**	–	0·002	0	0	0·002	0
Fitz River	0·178	0·137	0·190	–	0·001	0·046	0·028	0·001
Metro	**0·278**	**0·215**	**0·174**	**0·193**	–	0	0	0
North	0·107	0·201	**0·283**	0·166	**0·206**	–	0·009	0
South East	**0·213**	0·052	**0·076**	0·218	**0·250**	0·244	–	0·028
S Ranges	**0·173**	**0·143**	**0·133**	**0·273**	**0·341**	**0·301**	0·035	–

Both the significance (upper diagonal) and F_{ST} values (lower diagonal) were determined using a G-test in FSTAT (v. 2.9.3.2, University of Lausanne, Lausanne, Switzerland). Level of statistical significance is $\alpha - 0.0018$ after Bonferroni correction. *P* values were calculated following 2800 permutations. F_{ST} values of significance are indicated in bold.
[a]Geographic regions: East Aust., East Australia; Fitz River, Fitzgerald River; S Ranges, Stirling Ranges.

significance of F_{ST} among each region pair varied. FSTAT identified several pairwise comparisons of significance, which indicates that some regions could be genetically differentiated. For instance, the microsatellite profiles of isolates collected from the Metro region were consistently significantly different from those collected in all other regions. However, due to the small sample sizes these results should be interpreted with caution. The population structure was also examined by principal component analysis (PCA), and overall no distinct correlation between haplotype and region or year of collection was observed (Fig. 2), Two distinct clusters differentiated by PC1 were observed when grouping isolates both by region and year of collection. When loadings that influence PC1 were examined, the SSR at loci 1, 2 and 3 were discerned as key contributors to the observed variation (Fig. S1). Principal components 3 7 showed much less variation (data not shown).

Taken together, these results reaffirmed the low Bgh population structure in this study and reiterate that caution should be applied in assuming any SSR haplotype to region association.

Discussion

This study defines a set of SSR markers and their use in a preliminary study of Australian isolates of Bgh. Eight polymorphic microsatellite primer pairs were identified from the Bgh genome published by Spanu *et al.* (2010) and were used to screen a small collection of isolates from Australia. Although a relatively modest number of samples were analysed ($n = 111$), an average of seven alleles per microsatellite was detected, demonstrating their sensitivity and utility for use in future population and diversity studies.

The results show that isolates of Bgh in Australia exhibit a high degree of diversity at both the individual microsatellite level and haplotype. Ninety-seven unique Bgh haplotypes were detected, with 12·6% clones in a pool of 111 isolates. All isolates sharing the same multilocus haplotype were collected from the same initial sample field.

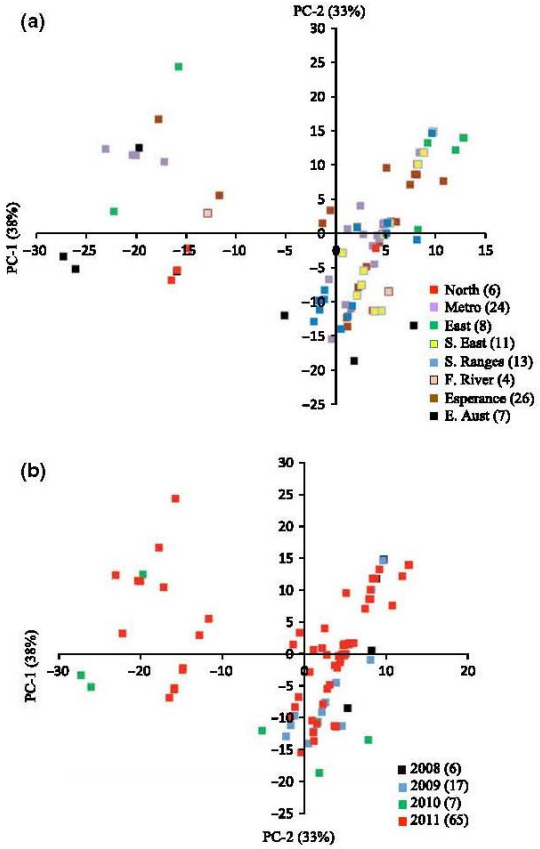

Figure 2 Principal component analysis of population structure among 97 clone-corrected individuals. The first two principal components (PCs) are shown with percentage variance indicated. Each isolate is represented by a single point. (a) Colours correspond to collection region, with numbers of isolates per region denoted in brackets. Australian region abbreviations are as follows: S. East, South East; S. Ranges, Stirling Ranges; F. River, Fitzgerald River; E. Aust, East Australia. Eigenvalues of each principal component are indicated. (b) Years of collection are distinguished by colour with number of isolates indicated in parenthesis.

As such, it is uncertain whether these isolates are true clones or progeny sharing the same SSR haplotype by chance. Isolates were analysed in eight regional groups based on the proximity of site of collection. The genetic diversity detected at each region ranged from 0·344 at the Fitzgerald River region to the most diverse in the East Aust. region. Given that isolates from the East Aust. region were collected over a very large area this high degree of diversity was to be expected and as such, isolates collected from this region should not be considered a concise population. Systematic and thorough sampling from sites in the eastern states in future studies may provide a clearer determination of the degree of genetic diversification of Bgh isolates in East Australia.

Pairwise comparisons between regions were made by determining a set of fixation indices (Table 4). It was established that isolates collected from the Metro region were significantly different to isolates collected from all remaining regions. This may be a factor of location; however, the small number of isolates analysed may be creating a false indication of genetic segregation. Studies by Horne *et al.* (2013) and Gerlach & Musolf (2000) also resulted in some F_{ST} values being statistically significant where other, very similar values, were not. Conversely, principal component analysis showed no real correlation between haplotype and collection region or year. There were however, two distinct clusters differentiated by PC1. The SSR at loci 1, 2 and 3 were the main contributors to the separation, although future work needs to be carried out to determine their significance.

The results obtained in this survey reflect those of previous barley powdery mildew surveys in Australia and around the world. Although only a modest number of isolates were used, SSR analysis showed great diversity within a collection of Australian Bgh isolates as was also shown in virulence surveys conducted in Europe by Brown *et al.* (1991) and Hovmøller *et al.* (2000). Isolates in these surveys were collected over a much larger area and hence geographical differentiation was apparent. Future Australian Bgh SSR studies would need to include more isolates collected from a wider area to determine such regional differentiation. The low level of genetic geographical differentiation shown in this study has several possible explanations. Geographic distances between regions in WA were in some cases modest, allowing movement of conidia by wind, through transportation on infected plant material and spread by agricultural vehicles. Dispersal by wind is probably the most likely cause. Generally, most conidia will be deposited close to where they are produced, although there is clear evidence that the asexual conidia can be transported transcontinentally (Brown & Hovmøller, 2002).

Another factor that appears to have significant impact on the genetic structure of Bgh in Australia is the mixed mating system. Pathogens undergoing regular sexual recombination pose a greater risk than those that undergo little or no recombination. Recombining pathogens can acquire new combinations of virulence alleles as rapidly as breeders can recombine resistance genes. Pathogens with mixed reproductive systems, as with Bgh, pose the highest risk of evolving new virulence and combinations of virulence. During the sexual cycle, many new combinations of alleles (haplotypes) are created through recombination. Combinations of alleles that are the most fit are held together through successive rounds of asexual reproduction and may increase to a high frequency in the field, which may lead to widespread virulence or fungicide resistance (Gisi *et al.*, 2002).

The high level of allele and haplotype diversity found in this collection of Australian Bgh isolates is perhaps a direct result of current barley growing practices and local legislation. Foreign demand for high quality WA grain has resulted in the majority of barley growing land being seeded with susceptible cultivars (DAFWA, 2012). These harbour major resistance genes against powdery mildew (Dreiseitl & Platz, 2012), many of which have now been defeated (Tucker *et al.*, 2013). Isolates of powdery mildew that are exposed to continual selection pressures via cultivars with effective major resistance genes, or through the application of fungicides over successive generations, will adapt more readily (Wolfe & McDermott, 1994). The high level of allele and haplotype diversity of Australian Bgh isolates may therefore result from the selection pressures of large-scale cropping of cultivars reliant on major resistance genes and the continual application of fungicides, which foster Bgh proliferation and hence diversification. Selection is the evolutionary force that can be most readily controlled through human intervention, and thus presents the most feasible point for hindering the evolutionary process. Agricultural systems that deploy major resistance genes, other forms of non-major gene resistance and fungicides in mixtures or rotations both temporally and spatially will reduce the efficiency of selection. This disruption of selection will act in slowing the rate of increase in the frequency of virulent or fungicide resistant mutants.

A population genetic structure analysis of such a damaging fungal pathogen is essential for optimizing a fungicide management strategy or barley breeding programme. Preliminary results indicate the remarkable diversity of haplotypes within WA along with substantial gene flow. This, coupled with Bgh evolutionary capabilities, presents a significant challenge in extending the efficacy of any newly introduced control methods. The current system that is aimed at maximizing barley production, subsequently reduces the range of options available for disease restraint because it also appears to maximize pathogen success. If current practices continue, the possibility of pathogen adaptation is almost certain. Knowledge of local Bgh genetic structure, and haplotype spread are essential for lessening the impact of this pathogen in WA. However, future studies with an increased number of markers and systematic isolate collections will provide more accurate conclusions as to the origin of evolution of a diverse population in an isolated area such as WA. Nonetheless, the SSR markers developed in this study proved to be a useful tool in discriminating isolates of Bgh.

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Supporting Information

Additional Supporting Information may be found in the online version of this article at the publisher's web-site.

Figure S1. The calculated negative and positive loading values contributing to the projections of PCA1 scores in Figure 2.

Table S1. Collection details and individual microsatellite allele lengths of *Blumeria graminis* f. sp. *hordei* isolates used in this study (n – 111).

10.3 Tucker M. A, Lopez-Ruiz, F, Jayasena, and Oliver, R.P (2014) Origin of Fungicide Resistant Barley Powdery Mildew in West Australia – Lessons to be Learned. In Ishii, H and Hollomon, D (eds.), *Fungicide Resistance in Plant Pathogens: Principles and a Guide to Practical Management*. Springer, Japan. (In Press).

Origin of Fungicide Resistant Barley Powdery Mildew in West Australia – Lessons to be learned.

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1.1 Abstract

The risk of fungicide resistance is greatest with pathogens with short dormant periods, with both sexual and asexual reproduction cycles, with large population sizes and when fungicides of a single mode of action (MOA) are repeatedly used. Most of the barley growing area in the west of Australia (WA) has been seeded with powdery mildew- (*Blumeria graminis* f. sp. *hordei* (*Bgh*)) susceptible cultivars for the last 10-15 years. Fungicides from the triazole group dominate the market and are used repeatedly as both seed and foliar treatments. Field failures have been observed leading to losses estimated at AU$100m annually since 2007. Reduced efficacy has often been found to result from alterations in the gene encoding triazole target 14α-sterol demethylase (*CYP51* syn. *ERG11*). Clear associations were found between accumulations of *CYP51* mutations and reductions in triazole sensitivity. The combination of susceptible cultivars, conducive environmental conditions and the repeated use of a single MOA has lead with disappointing predictability to perhaps the most costly fungicide resistance epidemic in history.

1.2 Introduction

Powdery mildew is a common fungal disease of many crops (Eichmann and Hückelhoven 2008). In grasses, the disease caused by *Blumeria* (syn. *Erysiphe*) *graminis* is divided into formae speciales (f. sp.) each colonizing individual genera of the grass family. *Blumeria graminis* f. sp. *hordei* (*Bgh*) is the causal agent of powdery mildew of barley, *Hordeum vulgare* (Pedersen, Rasmussen et al. 2002). Barley powdery mildew (BPM) is one of the most damaging diseases of barley throughout the world, with the potential to cause considerable yield losses (Jørgensen 1992; Tacconi, Baldassarre et al. 2006). *Bgh* can infect all green tissue of the host plant and is characterised by white fluffy pustules. In acute disease outbreaks, these pustules tend to coalesce, so that a severely infected barley crop may appear yellow at a distance.

Germplasm exploited in European spring cultivars provides complete protection against BPM infection. The MLO protein negatively regulates plant defences against powdery mildew, so cultivars carrying *mlo* alleles are resistant against all pathotypes of *Bgh*. The use of *mlo* in spring cultivars has remained a success story in agriculture for nearly 40 years (Kim, Panstruga et al. 2002). However, there are reported downsides to the use of *mlo* (Bjørnstad and Aastveit 1990). Cultivars with some *mlo* alleles are reported to have enhanced susceptibility to other fungal pathogens (Kumar, Hückelhoven et al. 2001) and it is claimed that *mlo* cultivars exhibit a yield penalty even in the absence of any infection (McGrann,

Stavrinides et al. 2014). It is for these reasons that breeders have been reluctant to include *mlo* germplasm in Australian barley breeding programs (Dreiseitl and Platz 2012).

Breeders in WA have historically focussed on malt quality and many cultivars released over the last few decades have been susceptible to BPM. Therefore, growers in regions prone to infection have relied heavily on the application of fungicides for disease control. Triazole fungicides are the most widely used class of antifungal agents for the control of fungal diseases of humans, animals (Chamilos and Kontoyiannis 2005) and plants (Poole and Arnaudin 2014). This fungicide class dominates those registered for barley diseases in WA, being contained in all registered foliar formulations until 2013 (Table 1). Triazole resistance is now widely reported in both clinical and agricultural sectors including powdery mildew populations from other barley growing regions of the world (Wyand and Brown 2005). The use of formulations containing only one class of fungicide coupled with the popularity of BPM susceptible cultivars was a perfect recipe for fungicide resistance. Growers in WA are now faced with an epidemic of highly virulent (Tucker, Jayasena et al. 2013) and fungicide-resistant BPM (Tucker, Lopez-Ruiz et al. 2015). Here we discuss the etiology which has led to such an epidemic and discuss future prospects for the billion dollar barley industry in Australia.

2.2 Historical cultivar use in west Australia

Australian barley growers have a world-wide reputation for producing a reliable supply of high quality malting barley. Annually, WA produces on average 2.5 million tonnes of barley grain (ABARES 2014) of which the majority is exported for malting, shochu production, human consumption and animal feed (Paynter, Hills et al. 2012). The Wheat Belt is the common name applied to the arable area of WA, and typically has 4Mha of wheat, 1.5Mha of barley and 1.5Mha of other crops (mainly canola and lupins). Barley production covers much of the Wheat Belt and powdery mildew infection has proven to be a massive threat to production in recent years (GRDC 2012). Field surveys and crop reports have mapped the pathogen's spread over the cereal cropping area with extremely high levels of disease pressure particularly in the cooler, high rainfall areas along the south coast (Figure 1).

There are a range of barley cultivars available to growers, each with different characteristics of yield potential, agronomic features, disease resistance profiles and grain quality and are marketed as malt, feed or food grades depending on their intended use post-harvest. Growers decide which cultivar to grow depending on a number of factors including (*i*) the end price paid for grain (*ii*) and the likelihood that a particular cultivar will meet the required grade (*iii*) likely yield potential and (*iv*) agronomic and disease resistance characteristics. Deliveries that meet malt grade specifications receive a premium price which normally outweighs the lower yield achievable compared to feed or food cultivars. Factors such as weather and disease pressure, which vary season to season, can result in downgrading of malt cultivars.

Malt grade deliveries of cultivars Baudin, Hamelin, Gairdner and Stirling attract about AU$37/t more than food or feed grades (DailyGrain 2014). The market leader for export malt, Baudin, is highly susceptible to powdery mildew (Paynter, Hills et al. 2012) as it possesses only the *Ml-a8* resistance gene which has now been completely defeated by *Bgh* in WA (Tucker, Jayasena et al. 2013). The three other cultivars of high popularity harbour the same resistance gene and are thus susceptible (Table 2).

On a typical farm of 4500ha where 67% is cropped (Rowe 2012) growers will earn an extra AU$300,000 year if their deliveries achieve malt grade (based on the average yield of

2.91t/ha (NVT 2014)). The mildew susceptible cultivars have occupied on average 95% of the total barley growing area for most of the last decade (Figure 2, (CBH 2014)). This has gifted the pathogen perfect conditions for proliferation and hence adaptation, key contributing factors to the current BPM epidemic.

Recently three *mlo* cultivars have been examined in WA. These cultivars, Westminster, Henley and Granger, which were bred using European spring barley germplasm, consistently performed better than the market leader Baudin in regards to yield (NVT 2014). All three were resistant to BPM infection but have so far not yet obtained malt certification. The effect of *mlo* on other pathogens and yields in WA conditions clearly needs further study. New high yielding cultivars which have increased resistance to BPM infection are currently undergoing malt accreditation (BarleyAustralia 2014). If accepted, we can expect that they will replace Baudin and the other susceptible cultivars as long as they possess a full suite of desirable characteristics.

2.3 Fungicide use in west Australia

2.3.1 Triazole use and the outbreak of resistance

Fungicide use in Australian broad acre cropping has risen sharply over the last decade. According to a ten year review published by Murray and Brennan (2010), nearly 95% of the land sown to barley in mildew-prone areas of WA received some form of fungicide treatment, with greater than 35% having multiple applications each season. The registration and use of formulations is tightly controlled by Australian government bodies and product labels specify the crop, specific diseases and the permitted minimum and maximum doses. Although there are numerous different MOA available (FRAC 2014), prior to 2013 all registered formulations for barley powdery mildew in WA contained a triazole (Table 1; Figure 3). Triazole fungicides have a specific single-site MOA, targeting the product of the *CYP51* gene (sterol 14α-demethylase) thereby disrupting the synthesis of Ergosta-5,24(24^1)-diene-3β-ol, the main sterol component of cell membranes in powdery mildews (Loeffler, Butters et al. 1992). Resistance to this class is now well documented in both clinical and agricultural fungal species where it can be acquired by (*i*) alterations in the *CYP51* gene which disturbs binding of the triazole at the target site (Mullins, Parker et al. 2011), (*ii*) *CYP51* overexpression thereby increasing levels of sterol 14α-demethylase (Ma, Proffer et al. 2006) and (*iii*) an increased efflux of triazoles mediated through the presence of membrane-bound transported proteins (Da Silva Ferreira, Luiz Capellaro et al. 2004). As yet mutations in *Bgh CYP51* have been reported only in UK isolates of *Bgh* that were less sensitive to the triazole fungicide triadimenol (Wyand and Brown 2005).

For almost a decade, growers in WA have been reporting a decline in the efficacy of the widely used triazole tebuconazole (GRDC 2012). First registered in 1995 (APVMA 2014) (Table 1), tebuconazole provided protective, eradicative and curative action against many barley diseases common in WA including rusts (*Puccinia* spp.) and powdery mildew (MacBean 2012). Few new products were registered for BPM control from this time until 2004 when the expiry of patents saw the market flood with formulations from international manufacturers, supplying chemicals at a fraction of the previous price (Figure 3) (Paton 2014). The first strobilurin (QoI) fungicide, azoxystrobin, was introduced in 2004 as a mixture with a triazole, cyproconazole (APVMA 2014). Additional formulations containing QoIs were incorporated in subsequent years but always mixed in patent formulations containing various triazole partners. BPM resistance to QoI fungicides has been detected in

European populations (Bäumler, Felsenstein et al. 2003) but as yet has not been found in Australia (Tucker, unpublished).

Local reports of triazole field failures have been circulating since 2005. Despite this, new formulations that were devoid of triazole fungicides were not registered until seven years later. The 2013 cropping seasons saw the first spiroketal-amine product registered in a formulation containing spiroxamine (Table 1, Figure 3) (APVMA, 2014). Spiroxamine also targets a key enzyme in the ergosterol biosynthesis pathway, *ERG24* encoding C14 sterol reductase (Jia et al. 2002). Although, both triazole and spiroketal-amine fungicides target the same essential biochemical pathway, no cross resistance between compounds of the two groups has been reported (FRAC, 2014).

Did this widespread and prolonged use triazoles contribute to the build-up of resistant BPM isolates in WA? To answer this we began a study in 2009 to examine whether resistance to triazoles had evolved in the *Bgh* population in WA.

2.3.2 Genetics of triazole resistance

Several studies have dissected the relationship between mutational changes in the triazole target gene, *CYP51*, with triazole efficacy failures in the field. To date, two mutational changes in *CYP51* have been reported in BPM isolates from the UK. A tyrosine to phenylalanine substitution at amino acid 136 (Y136F) and a lysine to glutamine change at amino acid 147 (K147Q), were found in isolates that had reduced sensitivity to the triazole triadimenol (Wyand and Brown 2005). To determine whether mutational events were causing loss of efficacy in WA fields, a pilot study was initiated in 2009 whereby isolates of *Bgh* were phenotyped in response to tebuconazole and their *CYP51* sequence was obtained. In 2009 the Y136F mutation was identified in WA isolates, although no correlation could be drawn between the presence of the mutation and the failure of tebuconazole in the field as wild-type *Bgh* isolates were never found (Tucker, Lopez-Ruiz et al. 2015).

CYP51 sequences were obtained from additional isolates in subsequent seasons and further mutations were identified. The Y136F mutation was found to be ubiquitous throughout Australia. WA isolates harboured additional modifications of which a change from serine to threonine at amino acid 509 (S509T) was the most significant (Tucker, Lopez-Ruiz et al. 2015).Isolates harbouring the S509T mutation were consistently less sensitive to all formulations tested (Figure 4). The most commonly used triazole, tebuconazole, was most badly affected by the mutation and this presumably explains its loss of field efficacy. The prevalence of the S509T mutation increased from non-existent in isolates collected in 2009 to almost universal in WA in 2011 (Figure 5). A homology search revealed that the S509T mutation aligned with the S524T change in *Zymoseptoria tritici*, previously characterised as conveying reductions in triazole sensitivity (Cools and Fraaije 2013). We conclude that tebuconazole has selected for the Y136F/S509T mutant which is capable of proliferation even in the presence of field rates of tebuconazole.

2.3.3 Strategies for combatting triazole resistance of *Bgh*

Genetic changes occur randomly in the field. They only become a problem when a resistant mutant form proliferates to become dominant in the population (van den Bosch, Oliver et al. 2014). It is therefore quite feasible that the *Bgh CYP51* mutations Y136F and S509T were present in the WA population long they were first detected in 2009 and 2010 respectively.

Microsatellite studies have determined that these mutations occurred numerous times and in numerous sites around the state (Tucker unpublished). Hence, we conclude that the increase in frequency has been caused by successive selection for Y136F/S509T mutants.

Until recently, triazoles dominated the market for foliar fungicides in WA. Products that also contain a QoI were introduced in 2004 and 2008 (Table 1). A formulation containing spiroxamine, registered in 2013, is the only product available that does not contain a triazole. In contrast in the UK 50 compounds having 15 different MOA are registered for use on spring barley (FRAG 2014). There are clearly many fungicides that are not currently accessible to WA growers. Furthermore, five of the fungicides registered in the UK are multi-site inhibitors, which have proven to have a low risk of resistance development. There is an immediate need to fast track the registration of chemicals with new MOA that have already been thoroughly assessed for use on many barley diseases.

The primary strategy recommended to manage resistance to any fungicide class is to mix or alternate with another fungicide with a different mode of action (Brent and Hollomon 2007). The at-risk fungicide, which usually provides great disease control, can be protected against resistance breakdown by the inclusion of an active with a different MOA, either in a tank mix or alternating spray applications. The partner compound could ideally be a multi-site inhibitor, presumed to have a low resistance risk, or an unrelated single-site fungicide. The concept behind this arises from the expectation that mutants have evolved resistance to one fungicide MOA would still be sensitive to a different MOA (van den Bosch, Oliver et al. 2014) . Thus there is an absolute requirement for registrations of numerous MOA that can be rotationally applied in attempt to suppress resistance build-up.

The widespread use of highly susceptible cultivars and the repeated use of a single mode of action fungicide was a perfect recipe for an epidemic of fungicide resistance. For a period of nine years there have been reports on field failure of tebuconazole in controlling BPM outbreaks (GRDC 2012). During successive seasons, BPM infections have been highly damaging and widespread resulting in reductions of yield from 2.91 t/ha to 1.76 t/ha and downgrading of grain to feed quality (NVT 2014). Annually an average of 1.3m ha (ABARES 2014) is planted in WA and receival ports indicate that 55% of barley grain taken was susceptible to BPM infection (CBH 2014). Given these figures we estimate that the epidemic of highly virulent and tebuconazole resistant *Bgh* has caused at least AU$100M anually.in losses in WA alone. This may well be the most damaging and costly fungicide resistance outbreak ever recorded.

3.1 Conclusions and Future Prospects

In the case of BPM, the factors that have been shown to contribute to fungicide resistance had been previously well recognised (Wyand and Brown 2005). BPM resistance to triazole fungicides has been documented since1981 (Fletcher and Wolfe 1981; FRAC 2013). Tebuconazole was registered for *Bgh* control in WA, 14 years later. Strategies for resistance management such combining or alternating triazoles with a different MOA, decreasing dose (van den Bosch, Paveley et al. 2011) or spraying before complete crop infection, could have been introduced.

Unfortunately for a number of diseases, including BPM, the rate of loss of effective fungicides threatens to exceed the rate of introduction (van den Bosch, Oliver et al. 2014) and this is very much the present situation in Australia. Maintaining an array of effective

fungicides with many different MOA is critical. Many fungicides with different MOA are registered overseas and these could be made available to Australian growers. This would provide more options for resistance management. As well as new fungicides, an ongoing program monitoring in shifts in resistance in the pathogen population would be needed to prolong the effective life of any newly introduced formulation.

Table 1. The year of first registration of the foliar active compounds permitted for barley powdery mildew control in Western Australia.

Active Ingredient/s[a]	Year first registered
Tebuconazole	1995
Flutriafol	1997
Propiconazole	1998
Triadimefon	1998
Azoxystrobin + Cyproconazole	2004
Propiconazole + Cyproconazole	2004
Epoxiconazole	2005
Epoxiconazole + **Pyraclostrobin**	2008
Tebuconazole + Flutriafol	2009
Tebuconazole + Prothioconazole	2009
Azoxystrobin + Tebuconazole	2012
Spiroxamine	2013

[a]Active ingredients other than triazoles are indicated in bold type (APVMA 2014).

Table 2. Popular cultivars grown in the west of Australia with resistance genes and powdery mildew susceptibility indicated.

Cultivar	**Resistance genes**[a]	**Relevant virulence present in WA *Bgh***[b]	**Powdery mildew susceptibility**[c]
Baudin	*Ml-a8*	Yes	VS
Vlamingh	*Ml-a8*	Yes	S
Gairdner	*Ml-g*	Yes	S
Hamelin	*Ml-g*	Yes	S
Stirling	None	-	S
Bass	*Ml-a8*,	Yes,	MS
Hindmarsh	*Ml-a8, Ml-La*	Yes, Yes	MRMS
Buloke	*Ml-a7,Ml-La*	Yes, Yes	MR

[a] As postulated by Dreiseitl and Platz (2012)
[b] Results published by Tucker et al. (2013)
[c] Mildew susceptibility ratings by the Department of Food and Agriculture Western Australia

Figure captions

Figure 1: Map of the Western Australian Wheat Belt. Grey shading indicates areas that barley is grown with mildew densities indicated in black.

Figure 2. Percent of Western Australian growing area seeded with barley cultivars from 2005 to 2013. Colour blocking indicates resistance profile to barley powdery mildew and are as follows; Very susceptible – black, susceptible – dark grey, moderately resistant – light grey and resistant – white. Letters indicate cultivars (a) Schooner, (b)Barque, (c) Dash, (d) Bass, (e) Yagan, (f) Scope and (g) Hindmarsh. Data obtained from CBH grower estimates 2005-2013.

Figure 3: Number of registered formulations for use on barley powdery mildew from 1995 to 2014 (APVMA, 2014). Single triazole formulations are shaded grey. Formulations mixing two triazoles are indicated with thick black lines and formulations containing fungicides with a different MOA to triazoles are unfilled. Letters indicate formulations containing (a) spiroxamine, (b) axoxystrobin and tebuconazole, (c) flutriafol and tebuconazole (d) epoxiconazole and pyraclostrobin, (e) epoxiconazole (f) cyproconazole and propiconazole (g) azoxystrobin and cyproconazole and (h) triadimefon.

Figure 4: Average EC_{50} (μg mL^{-1}) of a collection of Australian *Bgh* isolates that are either S509 (black area) or T509 (grey area) in *CYP51*. Each axis represents a triazole EC_{50}.Teb – tebuconazole, Epoxi – epoxiconazole, Propi – propiconazole, Triad – triadimefon, Prothio – prothioconazole, Flut – flutriafol or Cyp – cyproconazole. Outermost ring corresponds to 25 μg mL^{-1} with each preceding ring representing a 5 μg mL^{-1} reduction as indicated.

Figure 5: The proportion of *Bgh* isolates with mutant T509 *CYP51* alleles collected in Australia over a five year period.

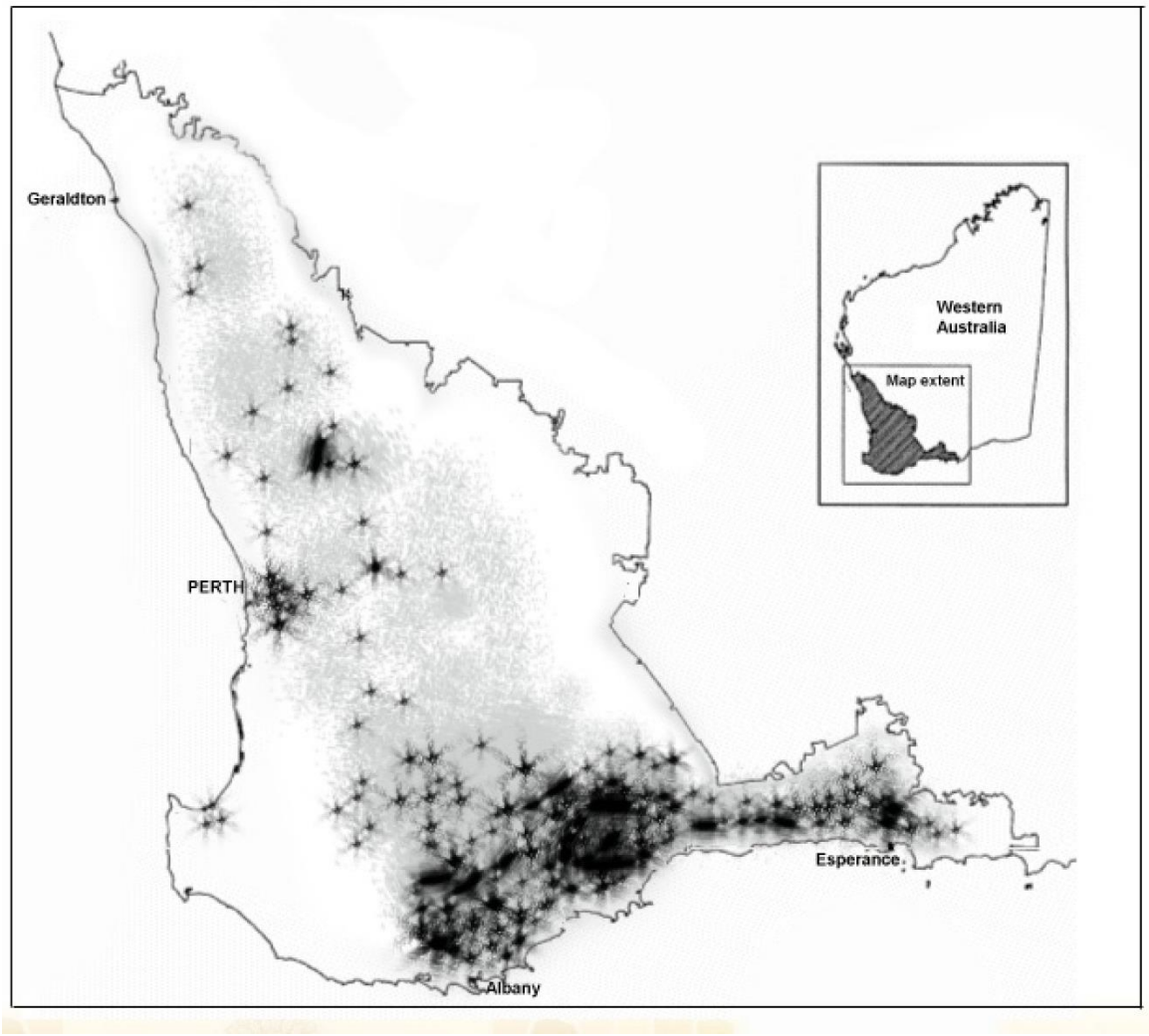
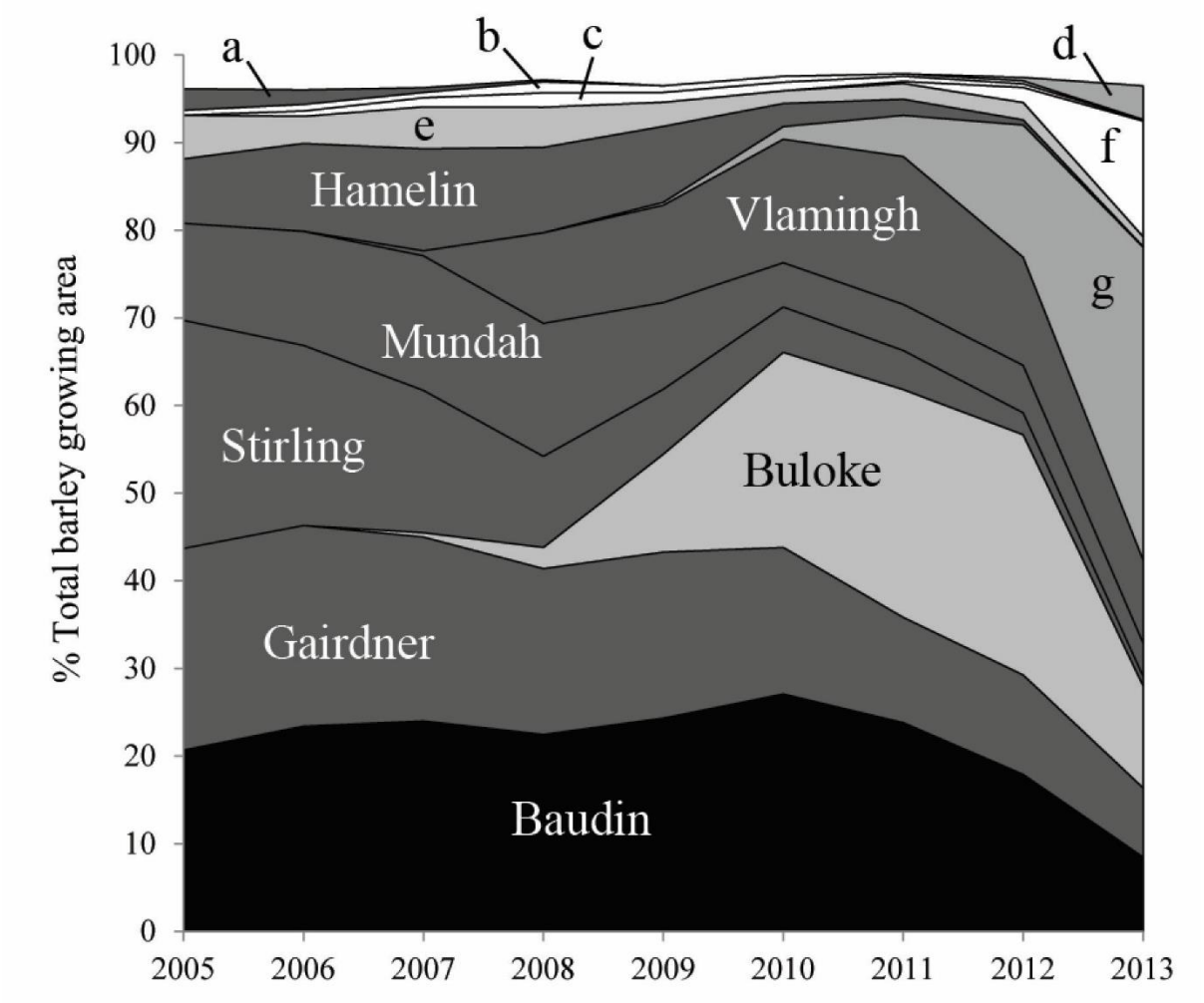

Geraldton
PERTH
Esperance
Albany
Western
Australia
Map extent

Figure 1.


a
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c
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g
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Vlamingh
Mundah
Stirling
Buloke
Gairdner
Baudin
% Total barley growing area
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2005
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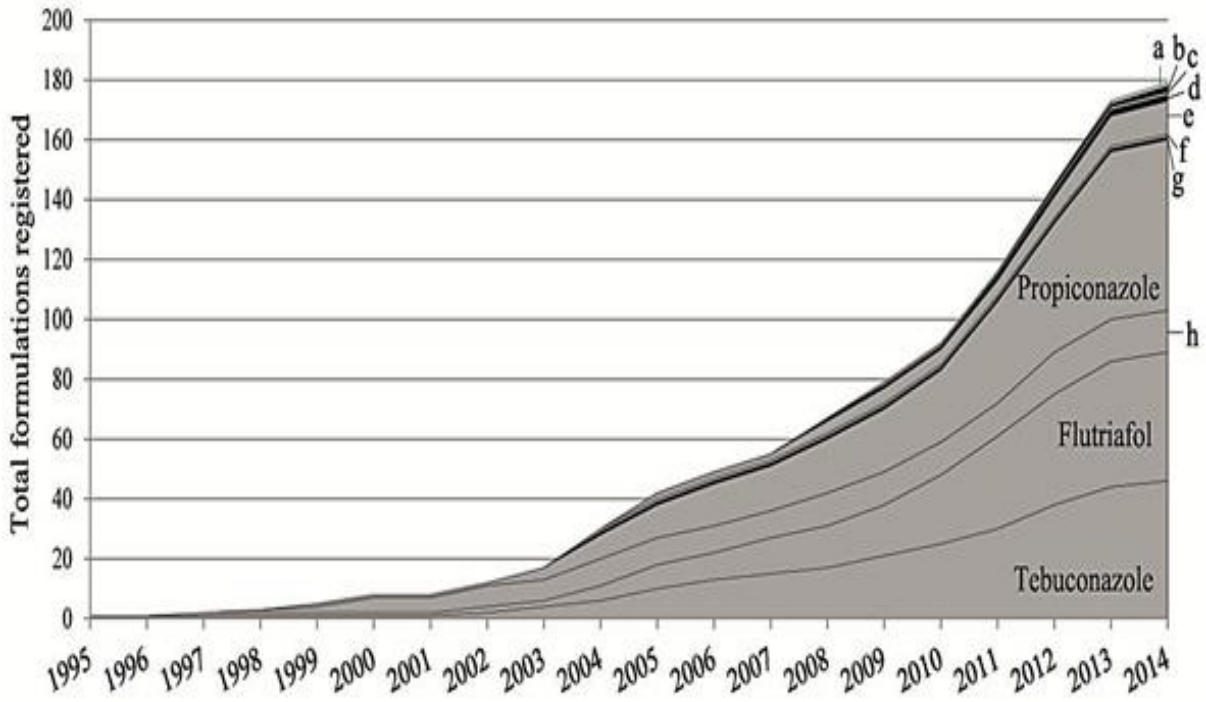
Figure 2.


Total formulations registered
200
180
160
140
120
100
80
60
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20
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Propiconazole
Flutriafol
Tebuconazole

Figure 3.

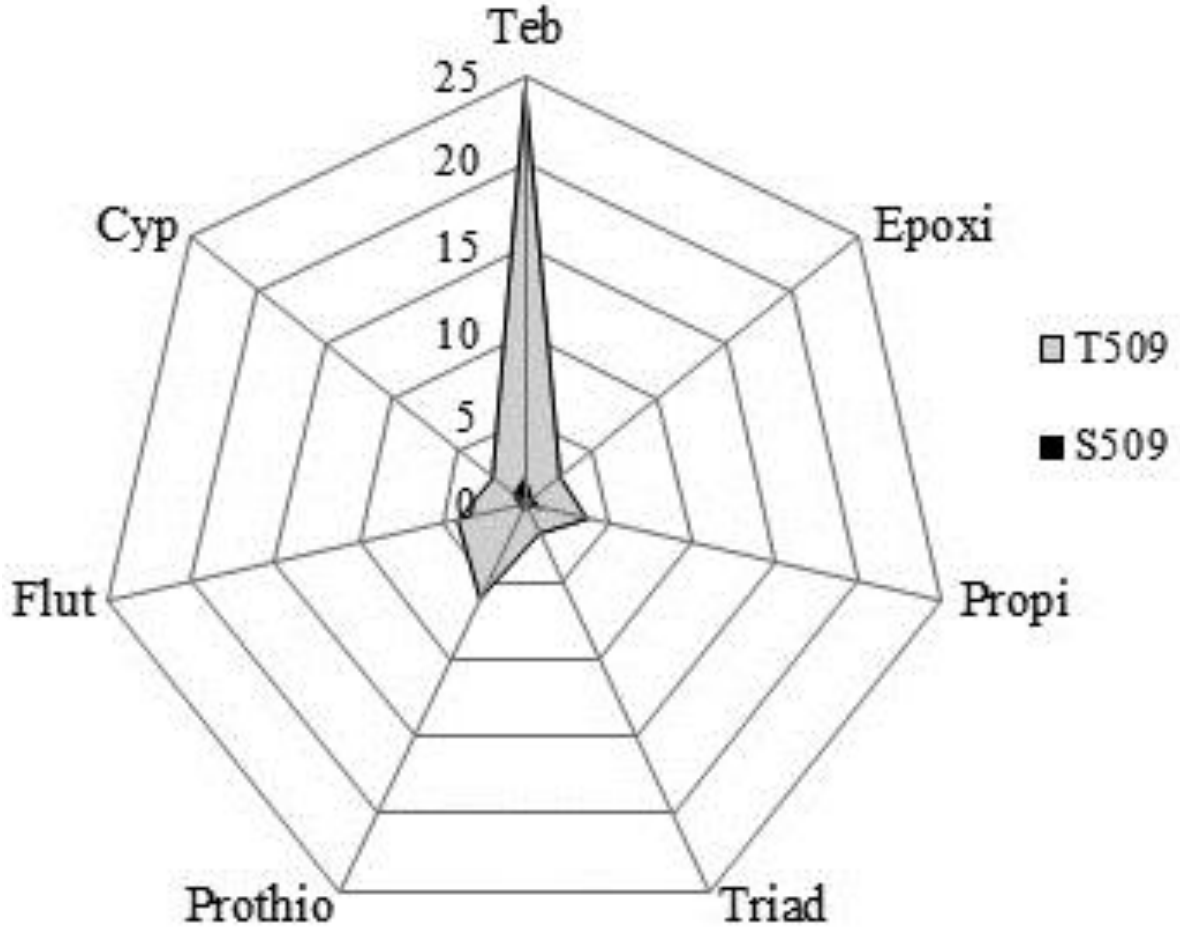
Teb
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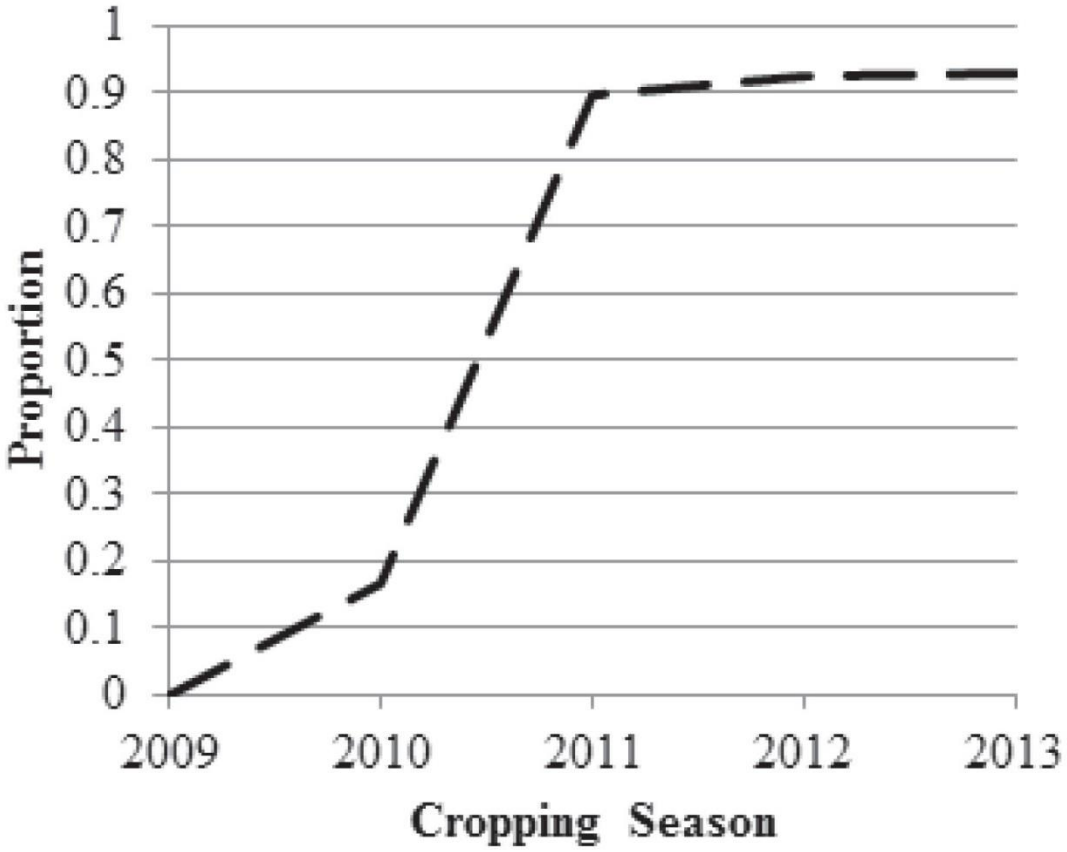
Figure 4.


1
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0.5
0.4
0.3
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0.1
0
Proportion
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2010
2011
2012
2013
Cropping Season

Figure 5.

ABARES. 2014. "Australian Crop Report." Australian Bureau of Agricultural and Resource Economics and Sciences Accessed 15th October. http://www.agriculture.gov.au/abares/publications/display?url=http://143.188.17.20/anrdl/DAFFService/display.php?fid=pb_aucrpd9aba_20141202_11a.xml.

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Accumulation of mutations in *Blumeria graminis* f. sp. *hordei CYP51* confers positive and negative cross-resistance to triazole fungicides.

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Summary

- 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. Reductions in the efficacy of triazole fungicides in controlling *Bgh* have recently been reported in the west of Australia (WA). Our study examined the sensitivity of *Bgh* isolates to triazoles and determined the molecular basis for loss of efficacy.
- The triazole target gene, *CYP51,* was sequenced from *Bgh* isolates and five amino acid substitutions were found or which four were novel.
- A clear association was established between combinations of mutations and altered levels of resistance to triazoles with both negative and positive cross-resistance observed. *In vitro* results were confirmed by *in silico* protein docking studies.
- The *in silico* appraisal of binding efficiencies between specific triazoles and *CYP51* variants promises to facilitate the design of resistance management strategies that counterselect the most abundant mutations and considerably extend the useful life of this critical group of fungicides.

Keywords:

Triazole, fungicide, negative cross-resistance, *Blumeria graminis* f. sp. *hordei* (barley powdery mildew), *CYP51*.

Introduction

Blumeria graminis f. sp. *hordei* (*Bgh*) is an ascomyceteous fungus causing barley (*Hordeum vulgare L.*) powdery mildew. In conducive seasons this biotrophic pathogen has the potential to reduce yields by as much as 20% (Murray and Brennan 2010). In most places *Bgh* is well controlled by host genetics (both the durable recessive *mlo* gene (Buschges, Hollricher et al. 1997) and combinations of minor genes) and fungicides (from the triazole, quinone and succinate dehydrogenase inhibiting (SDHI) classes as well as a number of specific mildewcides (FRAG 2014)). Since 1995 the majority of the west Australian (WA) barley area has been planted to susceptible cultivars and there has been a steep increase in triazole fungicide use since 1995. In 2009, 85 % of barley crops were treated with one or more sprays almost exclusively of triazole fungicides (Murray and Brennan 2010; ABARES 2014).

Triazole fungicides have been in the forefront of control of fungal pathogens of humans, animals and plants for nearly 40 years (Brent and Hollomon 2007). These fungicides interrupt the biosynthesis of ergosterol, an essential component of fungal cell membranes, by inhibiting the cytochrome P450 14α-sterol demethylase (*CYP51*) (Dupont, Lemetais et al. 2012). Resistance is now common in human pathogens, including *Candida* spp. (Hull, Parker et al. 2012; Xiang, Liu et al. 2013) and *Aspergillus fumigatus* (Lelièvre, Groh et al. 2013), and is a serious problem in agricultural systems (Cools and Fraaije 2013). Fungicide resistance has been associated with the alteration and overexpression of the *CYP51* protein and enhanced triazole efflux (Sanglard, Kuchler et al. 1995; Nakaune, Adachi et al. 1998; Gisi, Chin et al. 2000; Ma and Michailides 2005; Cools and Fraaije 2008; Hulvey, Popko Jr et al. 2012; Cools and Fraaije 2013).

Two earlier studies have noted changes in *Bgh CYP51* coding sequence (Y136F and K147Q) present in isolates less responsive to triazoles (Délye, Bousset et al. 1998; Wyand and Brown 2005). As K147Q was never found alone and Y136F was found in isolates with both low and high levels of triadimenol resistance, the exact sensitivity shift afforded by each was unclear. In these cases field resistance arose despite a complex pattern of simultaneous use of many fungicide modes of action (MOA) and the widespread use of other pathogen counter measures. In contrast, in WA the vast majority of the area was sown to susceptible cultivars and only a single class of fungicide was used (Tucker, Lopez-Ruiz et al. 2014). This has allowed us to chart the development of a major epidemic.

Many growers from southern WA reported a reduction in the effectiveness of triazoles in controlling barley powdery mildew outbreaks (GRDC 2012). Tebuconazole-containing formulations have been popular due to their low cost and spectrum of activity (Tucker, Lopez-Ruiz et al. 2014). Since 2005, accounts of mildew infection on barley sprayed with tebuconazole formulations have extended over much of the southern agricultural region with the frequency of reports increasing in recent years (Lord and Kehoe 2014).

In this study we have determined the sensitivity of Australian *Bgh* isolates to triazole fungicides registered in WA. Sequencing of the *CYP51* coding region in a subset of isolates revealed four unique genotypes. We have characterised the impact of these mutations on triazole sensitivity by *in vitro* assays and by heterologous expression in *Saccharomyces cerevisiae*. The results link variations in triazole sensitivities to changes in the *CYP51* coding region that are predicted to alter the heme binding pocket. Protein structural modelling and triazole docking was able to rationalise the observation of both positive and negative cross resistance. The results clearly demonstrate how misuse of pesticides leads directly to resistance in *Bgh* and validate how molecular methods accelerate the discovery of solutions.

Materials and Methods

Blumeria graminis f. sp. *hordei*

Isolates

One hundred and nineteen *Bgh* isolates were collected from Australia from 2009 to 2013 (Figure 1, Supporting Information 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 according to Tucker (2013).

Fungicide sensitivity assays

Fungicide sensitivities were determined *in vitro* by assessing growth of 18 *Bgh* isolates on susceptible (cv. Baudin) barley leaves inserted into fungicide-amended media with a no fungicide control. Commercial formulations of triazoles currently registered for *Bgh* control – Laguna (720g L^{-1} tebuconazole, Sipcam), Flutriafol (250g L^{-1} flutriafol, Imtrade Australia), Opus (125g L^{-1} epoxiconazole, Nufarm), Alto (100g L^{-1} cyproconazole, Nufarm), Tilt (418g

L^{-1} propiconazole, Syngenta), Proline (410g L^{-1} prothioconazole, Bayer Crop Science), Triad 125 (125g L^{-1} triadimefon, Farmoz) and Jockey Stayer (167g L^{-1} fluquinconazole, Bayer Crop Science) were incorporated into agar amended with 50mg L^{-1} of benzimidazole (Chan and Boyd 1992). Middle sections of 10 day old seedlings were excised with each tip inserted abaxial side up into fungicide amended agar. Each isolate was used to inoculate three replicates on subsequent weeks with conidia dislodged 24h before use to promote fresh growth. Conidal suspensions were collected on glossy black paper and blown into a 1.5m infection tower to ensure even inoculation. Following seven days growth at 20±2°C subject to a 12:12 h light :dark photoperiod the growth of each isolate and fungicide concentration was assessed using a 0-4 infection type (IT) scale adapted from Czembor (2000). Each pustule formation (or lack thereof) was assigned an IT and the average for each isolate and fungicide concentration pair was determined. Both the average IT and concentration was log transformed, % inhibition calculated and plotted against the transformed concentration to determine the regression equation and correlation coefficient. The mean 50% effective concentration (EC_{50}) and strength of the linear relationship between the two variables was determined. Resistance factors (RF) were calculated as the fold change in the average EC_{50} of T509 *CYP51* isolates compared to S509 isolates (Supporting Information Table S2). Data analysis was conducted in JMP, v10 (SAS Institute Inc. Cary, NC).

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 (Supporting Information Table S3) covering the length of the *Bgh CYP51* (*Bgh51*) gene (Supporting Information Fig. S1). Both the coding sequence and promoter region of 76 isolates were sequenced using Sanger sequencing and aligned in Geneious v 5.5 (Biomatters). A homology search was conducted between the Australian *Bgh CYP51* genotypes with other published *CYP51* variants. All sequences have been submitted to GenBank (Accession no. KM016902, KM016903, KM016904 and KM016905). By employing a high-throughput method of S509 and T509 allele detection ((digesting the amplicon of *Bgh51*_3F and *Bgh51*_3R with Hpy8I) (Supporting Information Table S3)), the *CYP51* 509 genotype of all 119 isolates was obtained.

Yeast Phenotyping

Strains and complementation of transformants

Synthesis of the wild type (wt) DH14 (Accession no. AJ313157) *CYP51* gene (*Bgh51wt*) was carried out by GENEWIZ Inc. (South Plainfield, NJ). Terminal 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 the synthesized *Bgh51wt* into the pYES3/CT vector (Invitrogen, Carlsbad, CA). The full length *Bgh51wt* gene in pYES-*Bgh51wt* was sequenced to ensure the validity. Transformations into *S. cerevisiae* strain YUG37:*erg11* (*MAT*a *ura3-52 trp1-63 LEU2::tTa tetO-CYC1::ERG11*) with native *Cyp51* gene under the control of a *tetO-CYC1*promoter, repressed in the presence of doxycycline (Parker, Merkamm et al. 2008). All complementation assays were preformed according to Cools *et al* (2010) with photographs taken following 72h of growth at 20°C (Supporting Information 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).

Comparative growth rate assay of transformants

The growth rate of transformants was assessed using Gen 5 data analysis software (BioTek Instruments, Inc. Winooski, VT) where duplicate cultures of replicate transformants were grown in SD GAL + RAF medium (SD medium) overnight at 30°C. One hundred microliters of each overnight culture, at 10^5 cells ml^{-1}, was used to inoculate 3 wells containing 200µl SD medium ±3µg ml^{-1} doxycycline. Cultures were incubated without light at 30°C, and the optical density at 600nm (OD_{600}) was measured every 15min for 12 days in a Synergy^{TM} HT Multi-Mode Microplate Reader (BioTek Instruments, Inc Winonski, VT). The mean maximum growth rate for each strain ± doxycycline was determined on the basis of the greatest increase in OD over a 2h period (Supporting Information Table S4).

Fungicide sensitivity assays

Sensitivity assays were carried out as described by Cools *et al.* (2010) using pure samples of tebuconazole, cyproconazole, propiconazole, epoxiconazole, fluquinconazole, triadimefon, flutriafol and desthio-conazole with a fungicide free control. It should be noted that as prothioconazole must be metabolised in plant tissue for it to be active (Parker, Warrilow et al. 2013), its desthio-conazole metabolite was used in all yeast assays.

Structural modelling

Structural modelling of *Bgh51wt* and mutant forms was undertaken using an automated homology modelling platform as previously described for *Z. tritici CYP51* (Mullins, Parker et al. 2011). Ligand docking of epoxiconazole and fluquinconazole were also carried out as previously described (Mullins, Parker et al. 2011). The volume of the heme cavity of the wild type and variant protein models was determined using Pocket-Finder (Leeds, UK) which is a pocket detection algorithm based on Ligsite (Hendlich, Rippmann et al. 1997).

Results

DNA sequencing

To determine whether mutational events caused the variance in the response of WA *Bgh* isolates to triazoles, primers were designed covering both the coding and promoter region of the single *CYP51* gene (Becher and Wirsel 2012) in *Bgh* (Supporting Information Table S3, Supporting Information Fig. S1). The *Bgh51wt* DH14 sequence was used as a reference (Spanu, Abbott et al. 2010). No indels were found in the promoter region of any isolate (data not shown). The *Cyp51* coding region was sequenced from 76 Australian isolates collected over from 2009 to 2013, including three from the east of Australia. Several synonymous and non-synonymous changes were identified (Supporting Information Fig. S3). All three isolates from the east of Australia carried two synonymous changes at nucleotides 81 and 1475, which were absent in WA isolates. All the Australian isolates carried the tyrosine to phenylalanine mutation at amino acid position 136 (Y136F) previously seen by Delye *et al.* (Délye, Bousset et al. 1998) and Wyand and Brown (Wyand and Brown 2005). Further non-synonymous mutations were found; K171E, M301I, R327G and S509T in various combinations (Figure 2). Considering only the non-synonymous changes, four novel *Bgh51* genotypes were distinguished. Isolates collected in WA were either F136/T509 (genotype 2) or F136/I301/G327/T509 (genotype 4) while isolates from other Australian states were either F136/E171 (genotype 3) or F136 (genotype 1). Mutations I301 and G327 were consistently found in the same isolates (Figure 2).

There was both spatial and temporal variation in the frequency of genotypes (Figure 1, Supporting Information Table S1). All isolates collected in 2009 were wild type at *CYP51* position 509. The proportion of S509 isolates collected declined dramatically over subsequent seasons to as low as 0.07 in 2011 (data not shown). Ninety nine of the 116 WA isolates

analysed contained the T509 mutation. These mutants were found in all major WA barley growing areas (Figure 1).

Triazole sensitivities of *Bgh* isolates

The sensitivities of *Bgh* isolates were determined using detached barley leaves inserted into triazole-amended agar. The results varied between genotype and fungicide (Figure 3, Supporting Information Fig. S4). There were no significant differences between isolates of genotype 1and 3 or between isolates that were genotype 2 or 4. For this reason we focussed on whether isolates contained the S509T mutation. Isolates of genotype 1 and 3 were consistently more sensitive to all foliar fungicides tested. The RFs were highest for genotypes 2 and 4 in the presence of tebuconazole and flutriafol (Figure 3). For fluquinconazole (used in WA solely as a seed formulation), the T509 isolates were marginally more sensitive suggesting negative cross resistance to this compound. Unfortunately, due to quarantine restrictions we were not able to phenotype the wild type DH14 isolate.

Heterologous expression in yeast

The *Bgh51wt* gene (i.e. as found in DH14) was synthesized and cloned into *S. cerevisiae* YUG37:*erg*11 with a doxycycline repressible promoter. The *S. cerevisiae Bgh51wt* transformant was able to grow in the presence of doxycycline (Supporting Information Fig. S2). There was no significant difference in the growth rates of all variants in the absence of doxycycline. However two *S. cerevisiae Bgh51* variants had significantly lower rates of growth in the presence of doxycycline (pYES-*Bgh51*_Y136F/S509T/R327G and pYES-*Bgh51*_Y136F/M301I/R327G/S509T) and were removed from all further *in vitro* analysis.

The triazole sensitivities of *S. cerevisiae* strains expressing *Bgh51* variants which restored growth on doxycycline-amended medium were determined (Supporting Information Table S5) and resistance factors were calculated (Table 1). Modest RFs were associated with the solo K171E and M301I mutations. RFs for the S509T mutation varied from 0.5 for fluquinconazole to 12.4 for propiconazole. Resistance for the solo Y136F mutation, which has been found in nature in *Bgh*, were also modest except for fluquinconazole having a RF of 9.7, confirming the marginal cross resistance detected in the detached leaf assays. The combination of F136 and T509 had much larger RFs of 340.6 for propiconazole and 33.2 for tebuconazole.

For many mutations fluquinconazole displayed outlier results. The RF for Y136F was strongly positive (9.7) whereas the RF for all other mutation combinations was 0.1 to 0.5 indicating greater sensitivity than the wild type construct

Structural modelling

The mutant protein variants of all *Bgh51* genotypes were structurally modelled (Supporting Information Fig S5). The effect of each mutational change on the volume of the heme cavity and distance from key amino acids around the cavity entrance was determined (Table 2). Modest volume increases were observed with the solo mutations; a 17.7% increase with K171E and 39.6% increase in volume with Y136F. Mutation S509T was an exception, with an increase in volume of 73.2% compared to that of the wild type model. The combination of F136/T509 gave a more substantial increase of 83.9%.

Table 2 also shows the estimated distances between Y222 and S506, which measures along the region of the access channel adjacent to the triazole binding site. Unlike the volume of the heme cavity, single mutational changes such as K171E and M301I decreased the diameter of the access channel by 7.8% and 6.6% respectively, compared to the wild type. The major conformation change is brought about through the acquisition of the Y136F mutation, which restricts the channel opening of the wild type model by 28.5%. The combination of F136 and T509 in a single model only slightly decreases the channel over the Y136 model to 28.7% of that of *Bgh51wt.*

The predicted binding pocket size and the diameter of the access channel were combined to elucidate the overall change of each genotype compared to the wild type. It appears that each of the two mechanisms can operate singly or in combination. This gives a possible explanation as to why the Y136F mutation, which brings about only a modest increase in cavity volume, results in notable resistance to tebuconazole. This is possibly due to the constriction of the access channel (from Y222 to S506) from 17.4Å to 6.5Å. The conformation of a loop of beta turn running from S505 to F508 is markedly different to that of the wild type, with the result that it projects into the cavity. A similar constriction is observed for the F136/T509 mutant (Supporting Information Fig. S5b). 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

S509. This supports the idea that the structural changes brought about by the Y136F mutation on its own may exert selective pressure on the 509 position, leading to the F136/T509 mutant.

Fluquinconazole docking studies were carried out to elucidate the mechanistic reasons for the cross resistance patterns (Figure 4b). In the wild type structure, the binding site of fluquinconazole is bordered by amino acids Y122 and Y222. It appears that the position of Y122 is particularly important in establishing the correct orientation of fluquinconazole so as to be coordinated by the heme. This arrangement is disrupted in the Y136F mutant, where Y122 and S506 prevent fluquinconazole accommodation (Figure 4b). With the Y136F/S509T mutant, Y122 is positioned similarly to the wild type, allowing accommodation of fluquinconazole as in the wild type. Here S506 borders the binding site and is predicted to interact with the fluquinconazole ligand (Figure 4c). Thus it appears the relative inconsistency of the Y136F mutant and enhanced selection of the Y136F/S509T double mutant can be explained by the 3D docking results.

Discussion

Studies best exemplified by the wheat pathogen *Z. tritici*, have discussed the relationship between mutational changes in *CYP51* with failures of triazole fungicides in the field. Triazoles have been used since the first registration of triadimefon in 1973 in the UK (Russell 2005). Twenty years after its introduction, *Z. tritici* isolates were found with *CYP51* changes conferring reductions in sensitivity (Cools and Fraaije 2013). Subsequently, numerous azoles have been introduced and 34 additional *CYP51* mutations have been identified (Cools and Fraaije 2013). This long history and the recent identification of mutations has made it difficult to discern cause and effect. The situation in WA is simple: triazole use has been widespread since 2004 and the first reports of resistance date from 2005.

Analysis of *Cyp51*of Australian *Bgh* isolates collected from 2009 to 2013 revealed four genotypes. The *in vitro* sensitivities of isolates from different genotypes varied between the triazoles tested. *Bgh* isolates with genotypes harbouring the S509T mutation were less sensitive to all the foliar fungicides used on barley in WA. In the case of fluquinconazole (which is only currently registered in WA as a seed treatment) isolates harbouring the T509 *CYP51* allele were more sensitive than their S509 counterparts. The Y136F mutation was found in all isolates examined including those from the east of Australia. As yet there have been no reports of field failure in this region.

This mutation was previously correlated with strong resistance to triadimenol (Wyand and Brown 2005). We cannot test the wild type *CYP51 Bgh* isolate in Australia due to quarantine restrictions. Y136F expression in yeast showed modest but significant decreases in sensitivity to most triazoles (Table 1). We therefore assume that there would be small differences in field efficacy that are unlikely to be noticeable. This suggests that even the limited fungicide use in the east of Australia has been sufficient to select for this mutation (Murray and Brennan 2010). If the new S509T mutation integrates into eastern populations of *Bgh* or is selected *in situ*, tebuconazole breakdown is inevitable and may no longer be an option for disease control in Australia.

Figure 3 compares the *in vitro* EC_{50} with the field rates for triazole foliar fungicides. Registered maximal application rates range from 125g ha $^{-1}$ for tebuconazole, flutriafol, propiconazole and triadimefon to 65g ha $^{-1}$ for epoxionzaole, cyproconazole and prothioconazole. It is clear that the high EC_{50} for tebuconazole means that strains harbouring the Y136F/S509T genotype are essentially resistant to field rates of this fungicide as the dose rate safety margin is less than for other registered compounds In yeast, assays are more precise and there was a consistent pattern whereby large RFs were associated with strains carrying both the Y136F and S509T mutations for all triazoles except fluquinconazole.

A search was conducted on the *CYP51* mutations in other fungal species reported as conferring a reduction in triazole sensitivity. The *Bgh51* amino acid sequence of Australian genotypes was aligned with *Z. tritici CYP51* (Figure 2). Mutational changes at the amino acids 136, 301, 327 and 509 fall in regions conserved between *Bgh* and *Z. tritici* (Becher and Wirsel 2012). Amino acids 136 and 509 in *Bgh51* correspond to 137 and 524 in *Z. tritici* which have previously been correlated with reductions in triazole sensitivity (Cools, Mullins et al. 2011). In our studies the combination of Y136F/S509T encoded a *CYP51* with a marked decrease in sensitivity to tebuconazole that can account for the field failure (Figure 3). The relationship between the combination of these two mutations and reduced efficacy was confirmed by the yeast expression data. Increases in heme cavity volume and restriction of the access channel in Y136F/S509T protein models correlate well with the significant RF obtained (Figure 5).

Both positive and negative selection was observed in the *in vitro Bgh* and yeast expression studies. The widespread use of tebuconazole on very susceptible cultivars appears to account for the selection of the more damaging Y136F/S509T combination since 2005 (APVMA

2014). The prior selection of Y136F, although insignificant in field terms, paved the way for the selection of further mutations in isolates, capable of surviving field application rates of the most widely used fungicides.

Structural modelling suggests that there are two main mechanisms that underpin the emergence of triazole resistance associated with mutational changes in *Bgh51*. The first mechanism is similar to that observed in *Z. tritici CYP51* (Mullins, Parker et al. 2011), 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 RFs reported in table 1. It is likely that any increase in heme cavity volume would perturb the orientation of the triazole ligand and hence its binding to the heme. This therefore isolates the smaller to medium size triazole ligands such as tebuconazole and epoxiconazole.

The second mechanism at play 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 Y222 and S312 appears to correlate well with resistance to tebuconazole. The narrowing of the access channel between Y222 and S506 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 percent change in the heme cavity volume is multiplied by the percent change in the distance between Y222 and S506 (Figure 5). All the variants that contain F136 demonstrate a substantially reduced distance between Y222 and S506 (Table 2). When one of the mechanisms is employed, moderate resistance factors are observed (F136 (access channel narrowing); T509 (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 F136/T509 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 triazole 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* genotype is present in the field.

One of the major resistance strategies used for fungicides is to mix active compounds with different MOA (van den Bosch, Oliver et al. 2014). The concept here is that isolates with mutations conferring resistance to one fungicide will most likely still be sensitive to the second mixing partner (van den Bosch, Oliver et al. 2014).Negative cross-resistance within a single MOA group has been shown *in vitro* with mutant *Z. tritici* isolates which are highly resistant to tebuconazole but fully susceptible to prochloraz (Fraaije, Cools et al. 2007; Leroux, Albertini et al. 2007). The negative cross-resistance shown in both the *Bgh in vitro* (Figure 3) and yeast expression studies (Table 1) was confirmed using *in silico* protein docking studies. Here the single Y136F mutation substantially impaired the binding of fluquinconazole (Figure 4). In contrast, the binding of fluquinconazole at the docking site of the Y136F/S509T protein model was much akin to that of the wild-type. Fluquinconazole would be expected to efficiently select for Y136F but *counter select* the more T509 genotypes. We therefore suggest that fluquinconazole be trialled as a means to control this epidemic. Nonetheless there is an immediate need for the registration of formulations with novel MOA and breeding of cultivars with durable resistance. Even if fluquinconazole effectively counter selects the S509T mutation, its efficacy would more than likely be short lived given *Bgh's* propensity for adaptation (data not shown). However these studies do provide a means of testing the likely efficacy of any currently available triazole and will permit the *in silico* exploration of novel triazoles. The affinity for the wild type and any mutant *CYP51* variants can now be compared thereby fast tracking the design, development and registration of new compounds not affected by current mutations.

The widespread use of highly susceptible varieties and the repeated use of a single MOA fungicide was a perfect recipe for an epidemic of fungicide resistance. A review covering the decade from 1999 to 2009 estimated that *Bgh* in WA caused losses of AU$30M p.a. (Murray and Brennan 2010). The area sown to barley was approximately 1.3 m ha (ABARES 2014) and 55% of the delivered grain was from cultivars rated as susceptible to powdery mildew infection (CBH 2014). The epidemic of highly virulent (Tucker, Jayasena et al. 2013) and tebuconazole resistant *Bgh* in WA is estimated to have reduced average yield from 2.91 t/ha to 1.76 t/ha (NVT 2014) and reduce the quality of grain from malt to feed grade. Based on these figures, and an average grain price of $268/t for malt barley and $231/t for feed (DailyGrain 2014), the epidemic of WA tebuconazole resistant *Bgh* caused at least an average of AU$100M p.a. from 2007 to 2010.

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Abbreviations

Cyp	Cyproconazole
Desthio	Desthioconazole
Epoxi	Epoxiconazole
Fluquin	Fluquinconazole
Flut	Flutriafol
Propi	Propiconazole
Prothio	Prothioconazole
Teb	Tebuconazole
Triad	Triadimefon

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Figure Legends

Figure 1: Sample sites of *Bgh* isolates collected from Australia. Black triangles indicate mutant T509 *CYP51* isolates (n = 119). Grey triangles indicate isolates with *CYP51* genotype S509 (n = 24). Numbers within triangles indicate isolates collected at each site.

Figure 2: Sequence alignment of fragments of the *CYP51* protein family. Changes found in Australian *Bgh* isolates from wild-type DH14 (GenBank: CAC85622, Wyand and Brown, 2005) are indicated in black. Numbers represent amino acid positions with assigned genotypes (Gen) given. *Bgh* isolates Pshk1 and Pshk2 from west Australia , Frnk1 from New South Wales and Strl1 from Tasmania are aligned with *Zymoseptoria tritici* (*Z. tr*) isolate ST1 (GenBank: AAU43734, Cools and Parker *et al.* 2010). Boxes indicate conserved regions.

Figure 3: Box plots of the EC_{50} ($\mu g\ mL^{-1}$) of a collection of Australian *Bgh* isolates that are wild-type (filled) and mutant (empty) at amino acid position 509 of *CYP51*. Numbers indicate the resistance factor (RF) of T509 over S509 isolates. Field application rates ($g\ ha^{-1}$) are given as black bars.

Figure 4: Docking of fluquinconazole in *Blumeria graminis* f. sp. *CYP51*.
A) Wild type *CYP51*, showing bound fluquinconazole (in light green, centre) and steric interaction with Y122 (surface shown as mesh). B) The Y136F mutant, showing encroachment of Y122 and S506 (surface shown as mesh) upon the docking site of fluquinconazole, indicating that the compound cannot be bound at that location. C) The Y136F-S509T mutant, showing orientation of Y122 similar to wild type and predicted interaction with S506 (shown in yellow).

Figure 5: Correlation between tebuconazole resistance factor (RF) of pYES-*Bgh51*_Y136F/S509T and the product of the change in the volume of the heme cavity (ΔHCV) with the change in distance between amino acids Y222 and S506 (ΔY222-S506).

Supporting Information

Supporting Information Table S1: Details of collection and *CYP51* genotype of the seventy-three *Bgh* isolates sequenced using Sanger technology in this study.

Supporting Information Table S2: Average *in vitro* EC_{50} and resistance factors of T509 and S509 *Bgh51* isolates when exposed to currently registered triazoles in WA.

Supporting Information Table S3: Primers used for sequencing and site-directed mutagenesis.

Supporting Information Fig. S1: Binding position of primers used to sequence the length of *Bgh51* including the *5*' promoter region.

Supporting Information Fig. S2: Complementation of *S. cerevisiae* strain YUG37:*erg11* with wild type (*Bgh51wt*) and mutated variants.

Supporting Information Table S4: Growth rate analysis of *S. cerevisiae* YUG37:*erg11* transformants.

Supporting Information Fig. S3: Nucleotide alignment of the *CYP51* gene of five isolates of *Bgh.*

Supporting Information Fig. S4: Average *in vitro* EC_{50} (μg mL^{-1}) of Australian *Bgh* isolates from four distinct genotypes 1 – F136, 2 – F136/T509, 3 – F136/E171 and 4 – F136/I301/G327/T509.

Supporting Information Table S5: EC_{50} and resistance factors of the pYES-*Bgh51* yeast mutants when exposed to currently registered triazoles in Western Australia.

Supporting Information Fig. S5: Structural modelling of *Bgh CYP51*.

Table 1: Resistance factors of *S. cerevisiae* YUG37:*erg11* transformants.

Construct[a]	Resistance Factors							
	Teb	Epoxi	Propi	Desthio	Cyp	Flut	Triad	Fluquin
pYES-*Bgh51*_Y136F	1.1	3.7	1.6	3.3	1.0	1.4	1.5	9.7
pYES-*Bgh51*_K171E	0.9	1.4	0.6	2.1	0.9	1.0	0.9	0.2
pYES-*Bgh51*_M301I	0.9	1.8	2.1	0.2	0.7	0.5	0.5	0.1
pYES-*Bgh51*_S509T	3.7	7.3	12.4	1.2	2.1	2.7	3.6	0.5
pYES-*Bgh51*_Y136F/K171E	1.3	2.4	1.9	0.9	0.9	1.3	3.3	0.2
pYES-*Bgh51*_Y136F/S509T	33.2	18.5	340.6	0.8	3.2	10.5	23.8	0.2
pYES-*Bgh51*_Y136F/S509T/M301I	2.3	4.9	2.8	0.1	0.8	1.9	5.7	<0.1

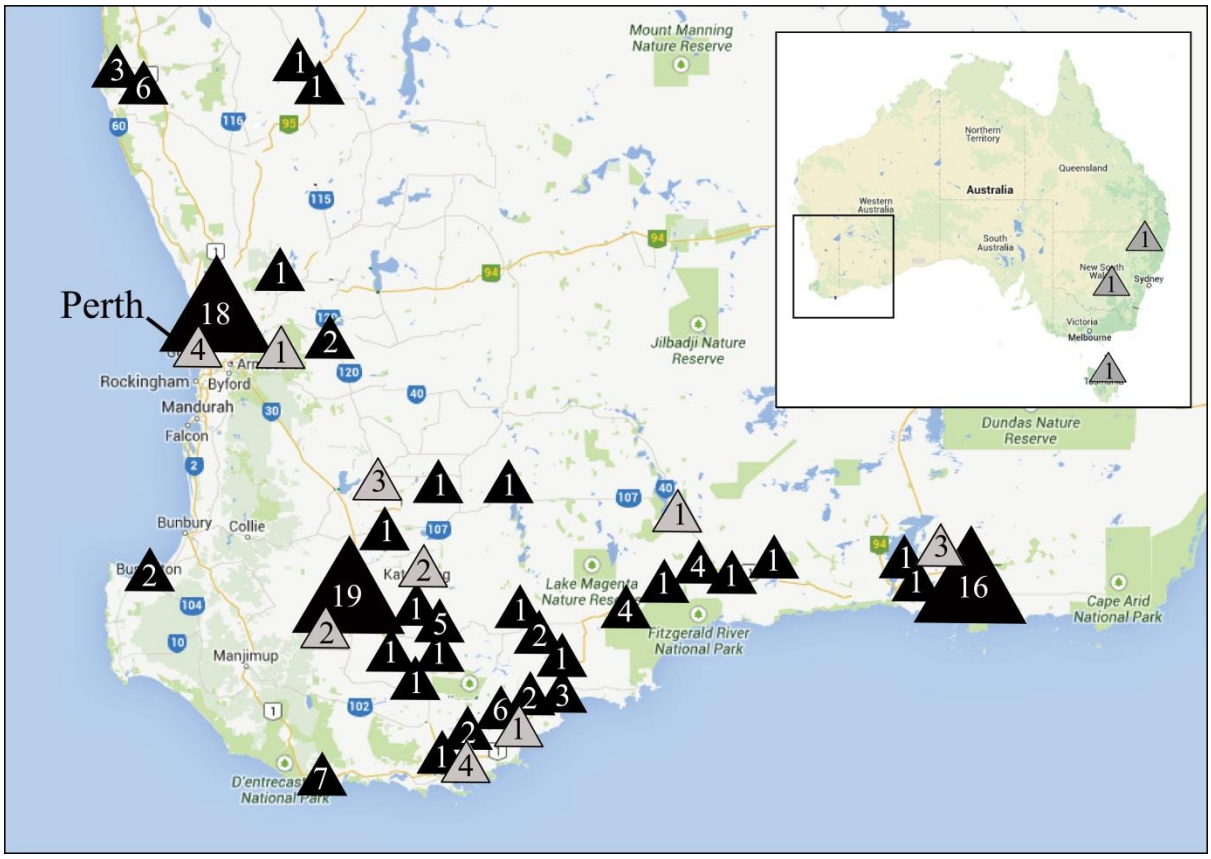
[a]Numbers refer to amino acid positions in *Bgh*. Resistance factors (RF) were calculated from the mean EC_{50} values of eight independent replicates. RF <1 indicates greater sensitivity than the wild-type construct. No growth was observed for the pYES3/CT (vector) construct.

Table 2: Measurements of heme cavity volume and key inter-residue distances in wild-type genotype Y136/K171/M301/R327/S509 (WT) and mutant *Bgh CYP51*.

CYP51 model	Heme cavity volume (Å^3)	ΔHCV[a] from WT	Distance Y222-S312[b]	ΔY222-S312 from WT	Product ΔHCV x ΔY222-S312
Wild-type	1809	-	12.862		
F136	2526	+39.6%	9.202	-28.5%	0.113
E171	2130	+17.7%	11.861	-7.8%	0.014
I301	2573	+42.2%	12.015	-6.6%	0.028
G327	2607	+44.1%	12.074	-6.1%	0.027
T509	3134	+73.2%	10.233	-20.4%	0.149
F136/E171	2334	+29.0%	10.870	-15.5%	0.045
F136/T509	3327	+83.9%	9.294	-28.7%	0.241
F136/I301/ G327/T509	2181	+20.6%	9.960	-22.6%	0.047

[a]ΔHCV – change in heme cavity volume.

[b]A measure of the diameter of the access channel adjacent to the triazole binding site.



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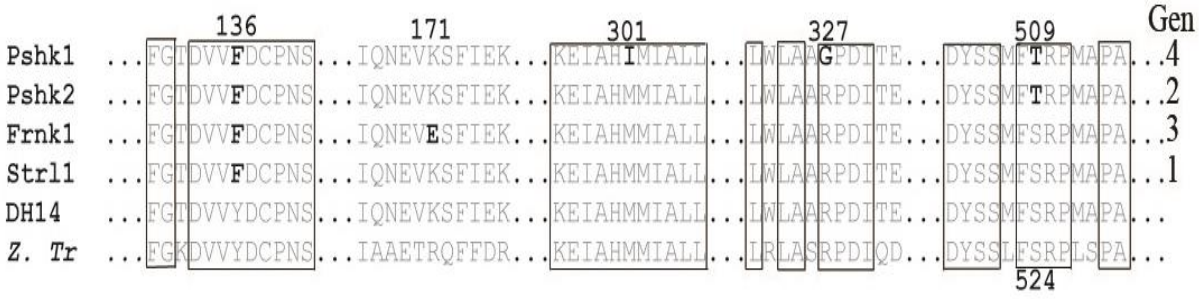

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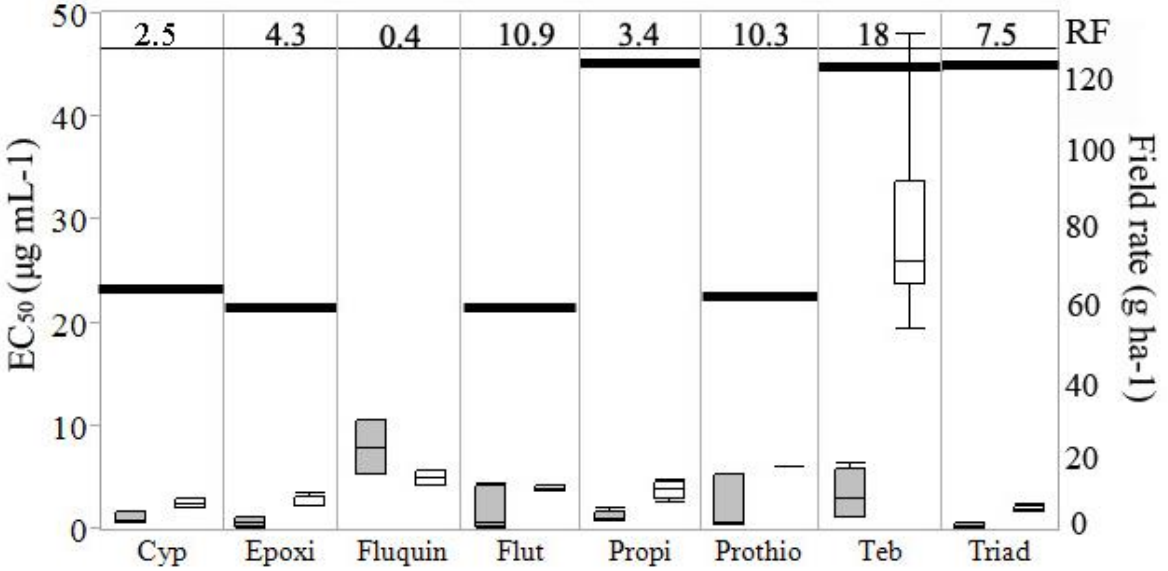

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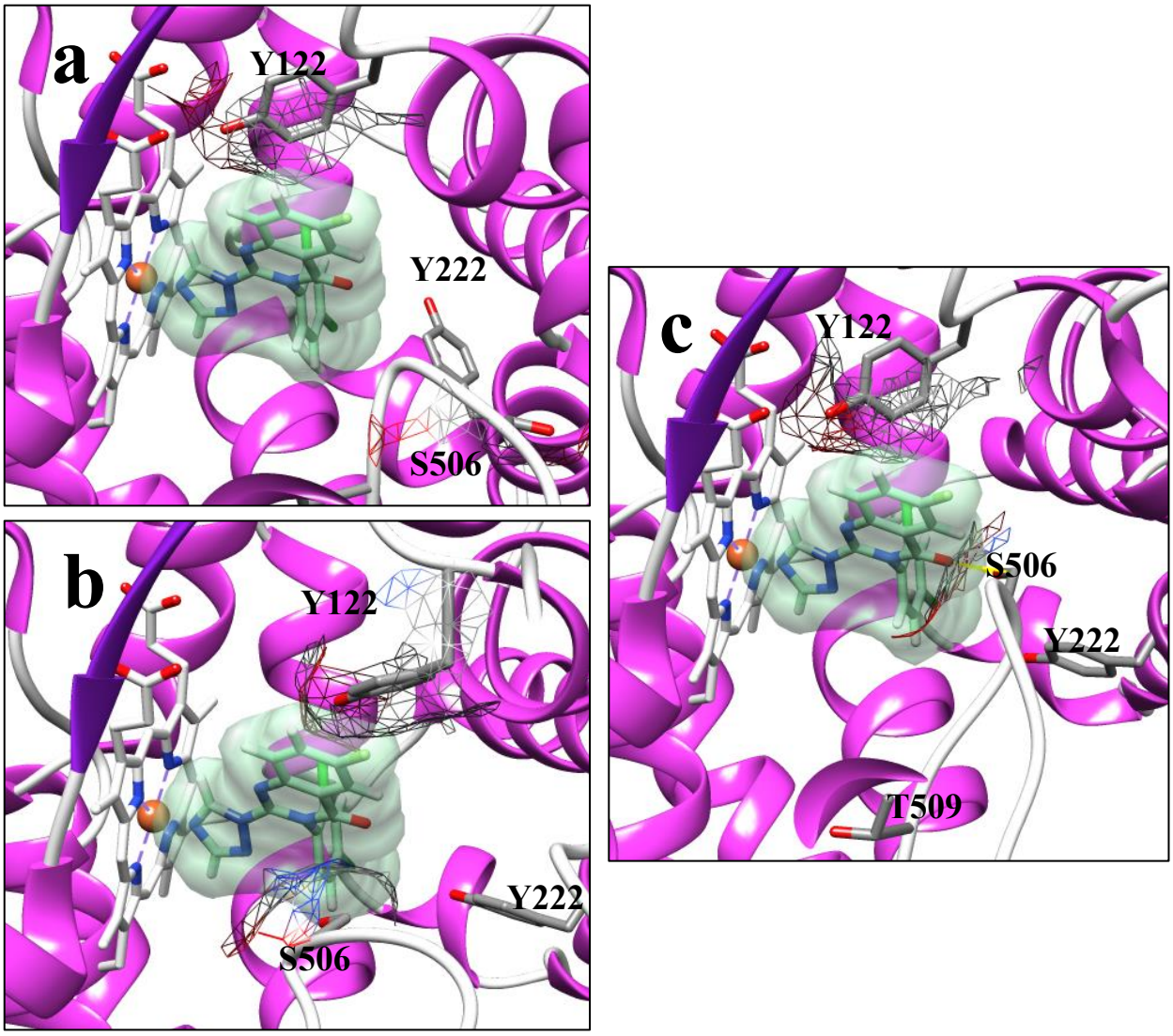

A) Wild type *CYP51*, showing bound fluquinconazole (in light green, centre) and steric interaction with Y122 (surface shown as mesh). B) The Y136F mutant, showing encroachment of Y122 and S506 (surface shown as mesh) upon the docking site of fluquinconazole, indicating that the compound cannot be bound at that location. C) The Y136F-S509T mutant, showing orientation of Y122 similar to wild type and predicted interaction with S506 (shown in yellow).

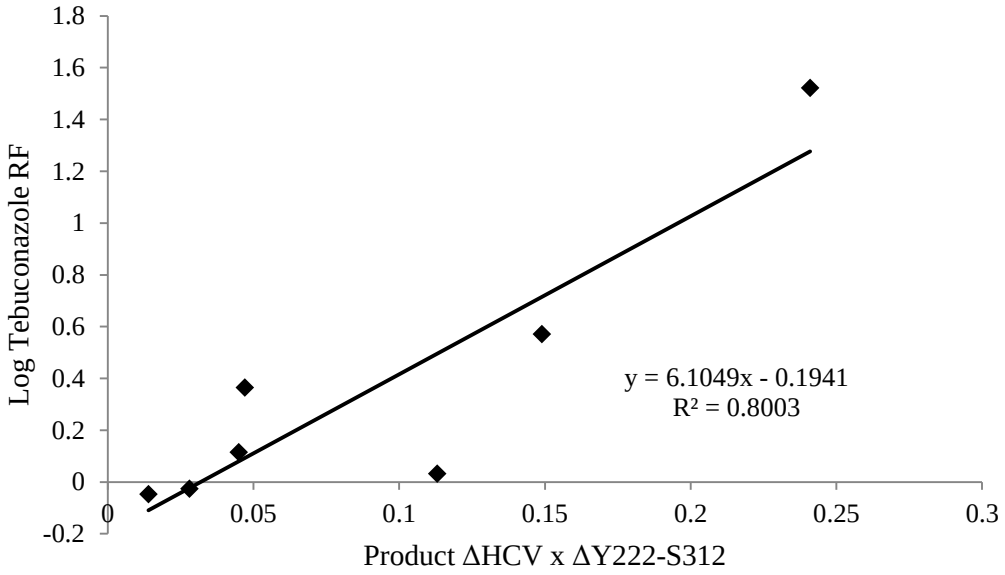

Fig. 5: Correlation between tebuconazole resistance factor (RF) of pYES-*Bgh51*_Y136F/S509T and the product of the change in the volume of the heme cavity (ΔHCV) with the change in distance between amino acids Y222 and S312 (ΔY222-S312).

Accumulation of mutations in *Blumeria graminis* f. sp. *hordei CYP51* confers positive and negative cross-resistance to triazole fungicides.

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Summary

- 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. Reductions in the efficacy of triazole fungicides in controlling *Bgh* have recently been reported in the west of Australia (WA). Our study examined the sensitivity of *Bgh* isolates to triazoles and determined the molecular basis for loss of efficacy.
- The triazole target gene, *CYP51,* was sequenced from *Bgh* isolates and five amino acid substitutions were found of which four were novel.
- A clear association was established between combinations of mutations and altered levels of resistance to triazoles with both negative and positive cross-resistance observed. *In vitro* results were confirmed by *in silico* protein docking studies.
- The *in silico* appraisal of binding efficiencies between specific triazoles and *CYP51* variants promises to facilitate the design of resistance management strategies that counterselect the most abundant mutations and considerably extend the useful life of this critical group of fungicides.

Keywords:

Triazole, fungicide, negative cross-resistance, *Blumeria graminis* f. sp. *hordei* (barley powdery mildew), *CYP51*.

Introduction

Blumeria graminis f. sp. *hordei* (*Bgh*) is an ascomyceteous fungus causing barley (*Hordeum vulgare L.*) powdery mildew. In conducive seasons this biotrophic pathogen has the potential to reduce yields by as much as 20% (Murray and Brennan 2010). In most places *Bgh* is well controlled by host genetics (both the durable recessive *mlo* gene (Buschges, Hollricher et al. 1997) and combinations of minor genes) and fungicides (from the triazole and succinate dehydrogenase inhibiting (SDHI) classes as well as a number of specific mildewcides (FRAG 2014)). Since 1995 the majority of the West Australian (WA) barley area has been planted to susceptible cultivars and there has been a steep increase in triazole fungicide use since 1995. In 2009, 85% of barley crops were treated with one or more sprays almost exclusively of triazole fungicides (Murray and Brennan 2010; ABARES 2014).

Triazole fungicides have been in the forefront of control of fungal pathogens of humans, animals and plants for nearly 40 years (Brent and Hollomon 2007). These fungicides interrupt the biosynthesis of ergosterol, an essential component of fungal cell membranes, by inhibiting the cytochrome P450 14α-sterol demethylase (*CYP51*) (Dupont, Lemetais et al. 2012). Resistance is now common in human pathogens, including *Candida* spp. (Hull, Parker et al. 2012; Xiang, Liu et al. 2013) and *Aspergillus fumigatus* (Lelièvre, Groh et al. 2013), and is a serious problem in agricultural systems (Cools and Fraaije 2013). Fungicide resistance has been associated with the alteration and overexpression of the *CYP51* protein and enhanced triazole efflux (Sanglard, Kuchler et al. 1995; Nakaune, Adachi et al. 1998; Gisi, Chin et al. 2000; Ma and Michailides 2005; Cools and Fraaije 2008; Hulvey, Popko Jr et al. 2012; Cools and Fraaije 2013).

Two earlier studies have noted changes in *Bgh CYP51* coding sequence (Y136F and K147Q) present in isolates less responsive to triazoles (Délye, Bousset et al. 1998; Wyand and Brown 2005). As K147Q was never found alone and Y136F was found in isolates with both low and high levels of triadimenol resistance, the exact sensitivity shift afforded by each was unclear. In these cases field resistance arose despite a complex pattern of simultaneous use of many fungicide modes of action (MOA) and the widespread use of other pathogen counter measures. In contrast, in WA the vast majority of the area was sown to susceptible cultivars and only a single class of fungicide was used (Tucker, Lopez-Ruiz et al. 2014). This has allowed us to chart the development of a major epidemic.

Many growers from southern WA reported a reduction in the effectiveness of triazoles in controlling barley powdery mildew outbreaks (GRDC 2012). Tebuconazole-containing formulations have been popular due to their low cost and spectrum of activity (Tucker, Lopez-Ruiz et al. 2014). Since 2005, accounts of mildew infection on barley sprayed with tebuconazole formulations have extended over much of the southern agricultural region with the frequency of reports increasing in recent years (Lord and Kehoe 2014).

In this study we have determined the sensitivity of Australian *Bgh* isolates to triazole fungicides registered in WA. Sequencing of the *CYP51* coding region in a subset of isolates revealed four unique genotypes. We have characterised the impact of these mutations on triazole sensitivity by *in vitro* assays and by heterologous expression in *Saccharomyces cerevisiae*. The results link variations in triazole sensitivities to changes in the *CYP51* coding region that are predicted to alter the heme binding pocket. Protein structural modelling and triazole docking was able to rationalise the observation of both positive and negative cross resistance. The results clearly demonstrate how misuse of pesticides leads directly to resistance in *Bgh* and validate how molecular methods accelerate the discovery of solutions.

Materials and Methods

Blumeria graminis f. sp. *hordei*

Isolates

One hundred and nineteen *Bgh* isolates were collected from Australia from 2009 to 2013 (Figure 1, Supporting Information 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 according to Tucker (2013).

Fungicide sensitivity assays

Fungicide sensitivities were determined *in vitro* by assessing growth of 18 *Bgh* isolates on susceptible (cv. Baudin) barley leaves inserted into fungicide-amended media with a no fungicide control. Commercial formulations of triazoles currently registered for *Bgh* control – Laguna (720g L^{-1} tebuconazole, Sipcam), Flutriafol (250g L^{-1} flutriafol, Imtrade Australia), Opus (125g L^{-1} epoxiconazole, Nufarm), Alto (100g L^{-1} cyproconazole, Nufarm), Tilt (418g

L^{-1} propiconazole, Syngenta), Proline (410g L^{-1} prothioconazole, Bayer Crop Science), Triad 125 (125g L^{-1} triadimefon, Farmoz) and Jockey Stayer (167g L^{-1} fluquinconazole, Bayer Crop Science) were incorporated into agar amended with 50mg L^{-1} of benzimidazole (Chan and Boyd 1992). Middle sections of 10 day old seedlings were excised with each tip inserted abaxial side up into fungicide amended agar. Each isolate was used to inoculate three replicates on subsequent weeks with conidia dislodged 24h before use to promote fresh growth. Conidal suspensions were collected on glossy black paper and blown into a 1.5m infection tower to ensure even inoculation. Following seven days growth at 20±2°C subject to a 12:12 h light :dark photoperiod the growth of each isolate and fungicide concentration was assessed using a 0-4 infection type (IT) scale adapted from Czembor (2000). Each pustule formation (or lack thereof) was assigned an IT and the average for each isolate and fungicide concentration pair was determined. Both the average IT and concentration was log transformed, % inhibition calculated and plotted against the transformed concentration to determine the regression equation and correlation coefficient. The mean 50% effective concentration (EC_{50}) and strength of the linear relationship between the two variables was determined. Resistance factors (RF) were calculated as the fold change in the average EC_{50} of T509 *CYP51* isolates compared to S509 isolates (Supporting Information Table S2). Data analysis was conducted in JMP, v10 (SAS Institute Inc. Cary, NC).

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 (Supporting Information Table S3) covering the length of the *Bgh CYP51* (*Bgh51*) gene (Supporting Information Fig. S1). Both the coding sequence and promoter region of 76 isolates were sequenced using Sanger sequencing and aligned in Geneious v 5.5 (Biomatters). A homology search was conducted between the Australian *Bgh CYP51* genotypes with other published *CYP51* variants. All sequences have been submitted to GenBank (Accession no. KM016902, KM016903, KM016904 and KM016905). By employing a high-throughput method of S509 and T509 allele detection ((digesting the amplicon of *Bgh51*_3F and *Bgh51*_3R with Hpy8I) (Supporting Information Table S3)), the *CYP51* 509 genotype of all 119 isolates was obtained.

Yeast Phenotyping

Strains and complementation of transformants

Synthesis of the wild type (wt) DH14 (Accession no. AJ313157) *CYP51* gene (*Bgh51wt*) was carried out by GENEWIZ Inc. (South Plainfield, NJ). Terminal 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 the synthesized *Bgh51wt* into the pYES3/CT vector (Invitrogen, Carlsbad, CA). The full length *Bgh51wt* gene in pYES-*Bgh51wt* was sequenced to ensure the validity. Transformations into *S. cerevisiae* strain YUG37:*erg11* (*MAT*a *ura3-52 trp1-63 LEU2::tTa tetO-CYC1::ERG11*) with native *Cyp51* gene under the control of a *tetO-CYC1*promoter, repressed in the presence of doxycycline (Parker, Merkamm et al. 2008). All complementation assays were preformed according to Cools *et al* (2010) with photographs taken following 72h of growth at 20°C (Supporting Information 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).

Comparative growth rate assay of transformants

The growth rate of transformants was assessed using Gen 5 data analysis software (BioTek Instruments, Inc. Winooski, VT) where duplicate cultures of replicate transformants were grown in SD GAL + RAF medium (SD medium) overnight at 30°C. One hundred microliters of each overnight culture, at 10^5 cells ml^{-1}, was used to inoculate 3 wells containing 200µl SD medium ±3µg ml^{-1} doxycycline. Cultures were incubated without light at 30°C, and the optical density at 600nm (OD_{600}) was measured every 15min for 12 days in a Synergy^{TM} HT Multi-Mode Microplate Reader (BioTek Instruments, Inc Winonski, VT). The mean maximum growth rate for each strain ± doxycycline was determined on the basis of the greatest increase in OD over a 2h period (Supporting Information Table S4).

Fungicide sensitivity assays

Sensitivity assays were carried out as described by Cools *et al.* (2010) using pure samples of tebuconazole, cyproconazole, propiconazole, epoxiconazole, fluquinconazole, triadimefon, flutriafol and desthio-conazole with a fungicide free control. It should be noted that as prothioconazole must be metabolised in plant tissue for it to be active (Parker, Warrilow et al. 2013), its desthio-conazole metabolite was used in all yeast assays.

Structural modelling

Structural modelling of *Bgh51wt* and mutant forms was undertaken using an automated homology modelling platform as previously described for *Z. tritici CYP51* (Mullins, Parker et al. 2011). Ligand docking of epoxiconazole and fluquinconazole were also carried out as previously described (Mullins, Parker et al. 2011). The volume of the heme cavity of the wild type and variant protein models was determined using Pocket-Finder (Leeds, UK) which is a pocket detection algorithm based on Ligsite (Hendlich, Rippmann et al. 1997).

Results

DNA sequencing

Eighteen *Bgh* isolates were assessed for susceptibility to currently registered fungicides for barley powdery mildew control in WA. Distinct patterns of resistance were observed and the triazole target gene – *Cyp51* was sequenced to ascertain whether mutational events caused the variance in the response to triazoles. Primers were designed covering both the coding and promoter region of the single *CYP51* gene (Becher and Wirsel 2012) in *Bgh* (Supporting Information Table S3, Supporting Information Fig. S1). The *Bgh51wt* DH14 sequence was used as a reference (Spanu, Abbott et al. 2010). No indels were found in the promoter region of any isolate (data not shown). The *Cyp51* coding region was sequenced from 76 Australian isolates collected over from 2009 to 2013, including three from the east of Australia. Several synonymous and non-synonymous changes were identified (Supporting Information Fig. S3). All three isolates from the east of Australia carried two synonymous changes at nucleotides 81 and 1475, which were absent in WA isolates. All the Australian isolates carried the tyrosine to phenylalanine mutation at amino acid position 136 (Y136F) previously seen by Delye *et al.* (Délye, Bousset et al. 1998) and Wyand and Brown (Wyand and Brown 2005). Further non-synonymous mutations were found; K171E, M301I, R327G and S509T in various combinations (Figure 2). Considering only the non-synonymous changes, four novel *Bgh51* genotypes were distinguished. Isolates collected in WA were either F136/T509 (genotype 2) or F136/I301/G327/T509 (genotype 4) while isolates from other Australian states were either F136/E171 (genotype 3) or F136 (genotype 1). Mutations I301 and G327 were consistently found in the same isolates (Figure 2).

There was both spatial and temporal variation in the frequency of genotypes (Figure 1, Supporting Information Table S1). All isolates collected in 2009 were wild type at *CYP51*

position 509. The proportion of S509 isolates collected declined dramatically over subsequent seasons to as low as 0.07 in 2011 (data not shown). Ninety nine of the 116 WA isolates analysed contained the T509 mutation. These mutants were found in all major WA barley growing areas (Figure 1).

Triazole sensitivities of *Bgh* isolates

The sensitivities of *Bgh* isolates were determined using detached barley leaves inserted into triazole-amended agar. The results varied between genotype and fungicide (Figure 3, Supporting Information Fig. S4). There were no significant differences between isolates of genotype 1and 3 or between isolates that were genotype 2 or 4. For this reason we focussed on whether isolates contained the S509T mutation. Isolates of genotype 1 and 3 were consistently more sensitive to all foliar fungicides tested. The RFs were highest for genotypes 2 and 4 in the presence of tebuconazole and flutriafol (Figure 3). For fluquinconazole (used in WA solely as a seed formulation), the T509 isolates were marginally more sensitive suggesting negative cross resistance to this compound. Unfortunately, due to quarantine restrictions we were not able to phenotype the wild type DH14 isolate.

Heterologous expression in yeast

The *Bgh51wt* gene (i.e. as found in DH14) was synthesized and cloned into *S. cerevisiae* YUG37:*erg*11 with a doxycycline repressible promoter. The *S. cerevisiae Bgh51wt* transformant was able to grow in the presence of doxycycline (Supporting Information Fig. S2). There was no significant difference in the growth rates of all variants in the absence of doxycycline. However two *S. cerevisiae Bgh51* variants had significantly lower rates of growth in the presence of doxycycline (pYES-*Bgh51*_Y136F/S509T/R327G and pYES-*Bgh51*_Y136F/M301I/R327G/S509T) and were removed from all further *in vitro* analysis.

The triazole sensitivities of *S. cerevisiae* strains expressing *Bgh51* variants which restored growth on doxycycline-amended medium were determined (Supporting Information Table S5) and resistance factors were calculated (Table 1). Modest RFs were associated with the solo K171E and M301I mutations. RFs for the S509T mutation varied from 0.5 for fluquinconazole to 12.4 for propiconazole. Resistance for the solo Y136F mutation, which has been found in nature in *Bgh*, were also modest except for fluquinconazole having a RF of 9.7, confirming the marginal cross resistance detected in the detached leaf assays. The

combination of F136 and T509 had much larger RFs of 340.6 for propiconazole and 33.2 for tebuconazole.

For many mutations fluquinconazole displayed outlier results. The RF for Y136F was strongly positive (9.7) whereas the RF for all other mutation combinations was 0.1 to 0.5 indicating greater sensitivity than the wild type construct

Structural modelling

The mutant protein variants of all *Bgh51* genotypes were structurally modelled (Supporting Information Fig S5). The effect of each mutational change on the volume of the heme cavity and distance from key amino acids around the cavity entrance was determined (Table 2). Modest volume increases were observed with the solo mutations; a 17.7% increase with K171E and 39.6% increase in volume with Y136F. Mutation S509T was an exception, with an increase in volume of 73.2% compared to that of the wild type model. The combination of F136/T509 gave a more substantial increase of 83.9%.

Table 2 also shows the estimated distances between Y222 and S506, which measures along the region of the access channel adjacent to the triazole binding site. Unlike the volume of the heme cavity, single mutational changes such as K171E and M301I decreased the diameter of the access channel by 7.8% and 6.6% respectively, compared to the wild type. The major conformation change is brought about through the acquisition of the Y136F mutation, which restricts the channel opening of the wild type model by 28.5%. The combination of F136 and T509 in a single model only slightly decreases the channel over the Y136 model to 28.7% of that of *Bgh51wt.*

The predicted binding pocket size and the diameter of the access channel were combined to elucidate the overall change of each genotype compared to the wild type. It appears that each of the two mechanisms can operate singly or in combination. Notably the change in heme cavity volume of the less sensitive S509T mutant had an increase of 73.2% over that of the wild-type, compared to an increase of 39.6% for the Y136F mutant. The inverse relationship was observed between decreases in the diameter of the substrate access channel Here S509T mutation, which rendered constructs less sensitive to Tebuconazole, resulted in a 20.4% restriction in the diameter of the access channel over the wild-type compared to a more marked restriction of -28.5% in the more sensitive Y136F mutant. However these two protein

characteristics combined in the single Y136F + S509T genotype may well be responsible for the exceptional RF observed especially in the case of Tebuconazole. In addition conformation of a loop of beta turn running from S505 to F508 is markedly different in the Y136F genotype to that of the wild type, with the result that it projects into the cavity. A similar constriction is observed for the F136/T509 mutant (Supporting Information Fig. S5b). 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 S509. This supports the idea that the structural changes brought about by the Y136F mutation on its own may exert selective pressure on the 509 position, leading to the F136/T509 mutant.

Fluquinconazole docking studies were carried out to elucidate the mechanistic reasons for the cross resistance patterns (Figure 4b). In the wild type structure, the binding site of fluquinconazole is bordered by amino acids Y122 and Y222. It appears that the position of Y122 is particularly important in establishing the correct orientation of fluquinconazole so as to be coordinated by the heme. This arrangement is disrupted in the Y136F mutant, where Y122 and S506 prevent fluquinconazole accommodation (Figure 4b). With the Y136F/S509T mutant, Y122 is positioned similarly to the wild type, allowing accommodation of fluquinconazole as in the wild type. Here S506 borders the binding site and is predicted to interact with the fluquinconazole ligand (Figure 4c). Thus it appears the relative inconsistency of the Y136F mutant and enhanced selection of the Y136F/S509T double mutant can be explained by the 3D docking results.

Discussion

Studies best exemplified by the wheat pathogen *Z. tritici*, have discussed the relationship between mutational changes in *CYP51* with failures of triazole fungicides in the field. Triazoles have been used since the first registration of triadimefon in 1973 in the UK (Russell 2005). Twenty years after its introduction, *Z. tritici* isolates were found with *CYP51* changes conferring reductions in sensitivity (Cools and Fraaije 2013). Subsequently, numerous azoles have been introduced and 34 additional *CYP51* mutations have been identified (Cools and Fraaije 2013). This long history and the recent identification of mutations has made it difficult to discern cause and effect. The situation in WA is simple: triazole use has been widespread since 2004 and the first reports of resistance date from 2005.

Analysis of *CYP51*of Australian *Bgh* isolates collected from 2009 to 2013 revealed four genotypes. The *in vitro* sensitivities of isolates from different genotypes varied between the triazoles tested. *Bgh* isolates with genotypes harbouring the S509T mutation were less sensitive to all the foliar fungicides used on barley in WA. In the case of fluquinconazole (which is only currently registered in WA as a seed treatment) isolates harbouring the T509 *CYP51* allele were more sensitive than their S509 counterparts. The Y136F mutation was found in all isolates examined including those from the east of Australia. As yet there have been no reports of field failure in this region.

This mutation was previously correlated with strong resistance to triadimenol (Wyand and Brown 2005). We cannot test the wild type *CYP51 Bgh* isolate in Australia due to quarantine restrictions. Y136F expression in yeast showed modest but significant decreases in sensitivity to most triazoles (Table 1). We therefore assume that there would be small differences in field efficacy that are unlikely to be noticeable. This suggests that even the limited fungicide use in the east of Australia has been sufficient to select for this mutation (Murray and Brennan 2010). If the new S509T mutation integrates into eastern populations of *Bgh* or is selected *in situ,* tebuconazole breakdown is inevitable and may no longer be an option for disease control in Australia.

Figure 3 compares the *in vitro* EC_{50} with the field rates for triazole foliar fungicides. Registered maximal application rates range from 125g ha $^{-1}$ for tebuconazole, flutriafol, propiconazole and triadimefon to 65g ha $^{-1}$ for epoxionzaole, cyproconazole and prothioconazole. It is clear that the high EC_{50} for tebuconazole means that strains harbouring the Y136F/S509T genotype are essentially resistant to field rates of this fungicide as the dose rate safety margin is less than for other registered compounds. In yeast, assays are more precise and there was a consistent pattern whereby large RFs were associated with strains carrying both the Y136F and S509T mutations for all triazoles except fluquinconazole.

A search was conducted on the *CYP51* mutations in other fungal species reported as conferring a reduction in triazole sensitivity. The *Bgh51* amino acid sequence of Australian genotypes was aligned with *Z. tritici CYP51* (Figure 2). Mutational changes at the amino acids 136, 301, 327 and 509 fall in regions conserved between *Bgh* and *Z. tritici* (Becher and Wirsel 2012). Amino acids 136 and 509 in *Bgh51* correspond to 137 and 524 in *Z. tritici* which have previously been correlated with reductions in triazole sensitivity (Cools, Mullins et al. 2011). In our studies the combination of Y136F/S509T encoded a *CYP51* with a marked

decrease in sensitivity to tebuconazole that can account for the field failure (Figure 3). The relationship between the combination of these two mutations and reduced efficacy was confirmed by the yeast expression data. Increases in heme cavity volume and restriction of the access channel in Y136F/S509T protein models correlate well with the significant RF obtained (Figure 5). A high RF was also observed for the Y136F/S509T *Bgh Cyp51* construct when expressed in the yeast system, the results of which did not reflect data obtained in the *in planta* assays nor colloquial observations made in the field. Perhaps the reason propiconazole is still providing sufficient mildew control in the field is due to the large safety margin between the dose required for effective control and the dose registered for use.

Both positive and negative selection was observed in the *in vitro Bgh* and yeast expression studies. The widespread use of tebuconazole on very susceptible cultivars appears to account for the selection of the more damaging Y136F/S509T combination since 2005 (APVMA 2014). The prior selection of Y136F, although insignificant in field terms, paved the way for the selection of further mutations in isolates, capable of surviving field application rates of the most widely used fungicides.

Although all triazole compounds contain the 1, 2, 4 triazole ring, the adjoining structures are highly variable and directly reflect specificity of action. After examination of the structure of the triazoles employed in this study, it is clear that several compounds have chemical elements that are common to more than one compound (Supporting Information Fig S6). Tebuconazole, Cyproconazole, Epoxiconazole and Triadimefon all comprise the usual triazole backbone with an adjoining six carbon unsaturated heterocyclic ring with an attached chlorine molecule. Flutriafol, Epoxiconazole, and Fluquinconazole have the same six membered ring except with an attached Fluorine atom. Propiconazole is the only fungicide tested that possesses a 1, 3 dioxolane ring joined to the characteristic 1, 2, 4 triazole backbone. Theoretically, the presence of this unique triazole moity may affect propiconazole binding and/or specificity in the yeast system compared with the *in planta* assays, possibly resulting in the increased RF for Propiconazole in the heterologous expression system.

Structural modelling suggests that there are two main mechanisms that underpin the emergence of triazole resistance associated with mutational changes in *Bgh51*. The first mechanism is similar to that observed in *Z. tritici CYP51* (Mullins, Parker et al. 2011), 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 RFs reported in table 1. It is likely that any increase in heme cavity volume would perturb the orientation of the triazole ligand and hence its binding to the heme. This therefore isolates the smaller to medium size triazole ligands such as tebuconazole and epoxiconazole.

The second mechanism at play 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 Y222 and S312 appears to correlate well with resistance to tebuconazole. The narrowing of the access channel between Y222 and S506 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 percent change in the heme cavity volume is multiplied by the percent change in the distance between Y222 and S506 (Figure 5). All the variants that contain F136 demonstrate a substantially reduced distance between Y222 and S506 (Table 2). When one of the mechanisms is employed, moderate resistance factors are observed (F136 (access channel narrowing); T509 (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 F136/T509 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 triazole 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* genotype is present in the field.

One of the major resistance strategies used for fungicides is to mix active compounds with different MOA (van den Bosch, Oliver et al. 2014). The concept here is that isolates with mutations conferring resistance to one fungicide will most likely still be sensitive to the second mixing partner (van den Bosch, Oliver et al. 2014).Negative cross-resistance within a single MOA group has been shown *in vitro* with mutant *Z. tritici* isolates which are highly resistant to tebuconazole but fully susceptible to prochloraz (Fraaije, Cools et al. 2007; Leroux, Albertini et al. 2007). The negative cross-resistance shown in both the *Bgh in vitro* (Figure 3) and yeast expression studies (Table 1) was confirmed using *in silico* protein

docking studies. Here the single Y136F mutation substantially impaired the binding of fluquinconazole (Figure 4). In contrast, the binding of fluquinconazole at the docking site of the Y136F/S509T protein model was much akin to that of the wild-type. Fluquinconazole would be expected to efficiently select for Y136F but *counter select* the more T509 genotypes. We therefore suggest that fluquinconazole be trialled as a means to control this epidemic. Nonetheless there is an immediate need for the registration of formulations with novel MOA and breeding of cultivars with durable resistance. Even if fluquinconazole effectively counter selects the S509T mutation, its efficacy would more than likely be short lived given *Bgh's* propensity for adaptation (data not shown). However these studies do provide a means of testing the likely efficacy of any currently available triazole and will permit the *in silico* exploration of novel triazoles. The affinity for the wild type and any mutant *CYP51* variants can now be compared thereby fast tracking the design, development and registration of new compounds not affected by current mutations.

The widespread use of highly susceptible varieties and the repeated use of a single MOA fungicide was a perfect recipe for an epidemic of fungicide resistance. A review covering the decade from 1999 to 2009 estimated that *Bgh* in WA caused losses of AU$30M p.a. (Murray and Brennan 2010). The area sown to barley was approximately 1.3 m ha (ABARES 2014) and 55% of the delivered grain was from cultivars rated as susceptible to powdery mildew infection (CBH 2014). The epidemic of highly virulent (Tucker, Jayasena et al. 2013) and tebuconazole resistant *Bgh* in WA is estimated to have reduced average yield from 2.91 t/ha to 1.76 t/ha (NVT 2014) and reduce the quality of grain from malt to feed grade. Based on these figures, and an average grain price of $268/t for malt barley and $231/t for feed (DailyGrain 2014), the epidemic of WA tebuconazole resistant *Bgh* caused at least an average of AU$100M p.a. from 2007 to 2010.

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Abbreviations

Cyp	Cyproconazole
Desthio	Desthioconazole
Epoxi	Epoxiconazole
Fluquin	Fluquinconazole
Flut	Flutriafol
Propi	Propiconazole
Prothio	Prothioconazole
Teb	Tebuconazole
Triad	Triadimefon

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Figure Legends

Figure 1: Sample sites of *Bgh* isolates collected from Australia. Black triangles indicate mutant T509 *CYP51* isolates (n = 119). Grey triangles indicate isolates with *CYP51* genotype S509 (n = 24). Numbers within triangles indicate isolates collected at each site.

Figure 2: Sequence alignment of fragments of the *CYP51* protein family. Changes found in Australian *Bgh* isolates from wild-type DH14 (GenBank: CAC85622, Wyand and Brown, 2005) are indicated in black. Numbers represent amino acid positions with assigned genotypes (Gen) given. *Bgh* isolates Pshk1 and Pshk2 from west Australia , Frnk1 from New South Wales and Strl1 from Tasmania are aligned with *Zymoseptoria tritici* (*Z. tr*) isolate ST1 (GenBank: AAU43734, Cools and Parker *et al.* 2010). Boxes indicate conserved regions.

Figure 3: Box plots of the EC_{50} ($\mu g\ mL^{-1}$) of a collection of Australian *Bgh* isolates that are wild-type (filled) and mutant (empty) at amino acid position 509 of *CYP51*. Numbers indicate the resistance factor (RF) of T509 over S509 isolates. Field application rates ($g\ ha^{-1}$) are given as black bars.

Figure 4: Docking of fluquinconazole in *Blumeria graminis* f. sp. *CYP51*.
A) Wild type *CYP51*, showing bound fluquinconazole (in light green, centre) and steric interaction with Y122 (surface shown as mesh). B) The Y136F mutant, showing encroachment of Y122 and S506 (surface shown as mesh) upon the docking site of fluquinconazole, indicating that the compound cannot be bound at that location. C) The Y136F-S509T mutant, showing orientation of Y122 similar to wild type and predicted interaction with S506 (shown in yellow).

Figure 5: Correlation between tebuconazole resistance factor (RF) of pYES-*Bgh51*_Y136F/S509T and the product of the change in the volume of the heme cavity (ΔHCV) with the change in distance between amino acids Y222 and S506 (ΔY222-S506).

Supporting Information

Supporting Information Table S1: Details of collection and *CYP51* genotype of the seventy-three *Bgh* isolates sequenced using Sanger technology in this study.

Supporting Information Table S2: Average *in vitro* EC_{50} and resistance factors of T509 and S509 *Bgh51* isolates when exposed to currently registered triazoles in WA.

Supporting Information Table S3: Primers used for sequencing and site-directed mutagenesis.

Supporting Information Fig. S1: Binding position of primers used to sequence the length of *Bgh51* including the *5*' promoter region.

Supporting Information Fig. S2: Complementation of *S. cerevisiae* strain YUG37:*erg11* with wild type (*Bgh51wt*) and mutated variants.

Supporting Information Table S4: Growth rate analysis of *S. cerevisiae* YUG37:*erg11* transformants.

Supporting Information Fig. S3: Nucleotide alignment of the *CYP51* gene of five isolates of *Bgh.*

Supporting Information Fig. S4: Average *in vitro* EC_{50} (μg mL^{-1}) of Australian *Bgh* isolates from four distinct genotypes 1 – F136, 2 – F136/T509, 3 – F136/E171 and 4 – F136/I301/G327/T509.

Supporting Information Table S5: EC_{50} and resistance factors of the pYES-*Bgh51* yeast mutants when exposed to currently registered triazoles in Western Australia.

Supporting Information Fig. S5: Structural modelling of *Bgh CYP51*.

Table 1: Resistance factors of *S. cerevisiae* YUG37:*erg11* transformants.

Construct containing mutation/s[a]	Resistance Factors							
	Teb	Epoxi	Propi	Desthio	Cyp	Flut	Triad	Fluquin
pYES-*Bgh51*_Y136F	1.1	3.7	1.6	3.3	1.0	1.4	1.5	9.7
pYES-*Bgh51*_K171E	0.9	1.4	0.6	2.1	0.9	1.0	0.9	0.2
pYES-*Bgh51*_M301I	0.9	1.8	2.1	0.2	0.7	0.5	0.5	0.1
pYES-*Bgh51*_S509T	3.7	7.3	12.4	1.2	2.1	2.7	3.6	0.5
pYES-*Bgh51*_Y136F/K171E	1.3	2.4	1.9	0.9	0.9	1.3	3.3	0.2
pYES-*Bgh51*_Y136F/S509T	33.2	18.5	340.6	0.8	3.2	10.5	23.8	0.2
pYES-*Bgh51*_Y136F/S509T/M301I	2.3	4.9	2.8	0.1	0.8	1.9	5.7	<0.1

[a]Numbers refer to amino acid positions in *Bgh*. 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 (vector) construct.

Table 2: Measurements of heme cavity volume and key inter-residue distances in wild-type genotype Y136/K171/M301/R327/S509 (WT) and mutant *Bgh CYP51*.

CYP51 genotype	Heme cavity volume (Å^3)	ΔHCV[a] from WT	Distance Y222-S312[b]	ΔY222-S312 from WT	ΔHCV x ΔY222-S312
Wild-type	1809	-	12.862		
Y136F	2526	+39.6%	9.202	-28.5%	0.113
K171E	2130	+17.7%	11.861	-7.8%	0.014
M301I	2573	+42.2%	12.015	-6.6%	0.028
R327G	2607	+44.1%	12.074	-6.1%	0.027
S509T	3134	+73.2%	10.233	-20.4%	0.149
Y136F/K171E	2334	+29.0%	10.870	-15.5%	0.045
Y136F/S509T	3327	+83.9%	9.294	-28.7%	0.241
Y136F/M301I/R327G/S509T	2181	+20.6%	9.960	-22.6%	0.047

[a]ΔHCV – change in heme cavity volume.

[b]A measure of the diameter of the access channel adjacent to the triazole binding site.

11.0 Appendices

11.1 Statements from Co-Authors

To Whom It May Concern,

I, Madeline Tucker, contributed to marker design, conceived and carried out all the experiments, interpreted and analysed the data and wrote the manuscript for the paper entitled Development of Genetic SSR Markers in *Blumeria graminis* f. sp. *hordei* and Application to Isolates from Australia Published in Plant Pathology.

Madeline Tucker

I, as a Co-Author, endorse that this level of contribution by the candidate indicated above is appropriate.

Caroline Moffat

Kar Chun Tan

Simon Ellwood

Kithsiri Jayasena

Richard Oliver

To Whom It May Concern,

I, Madeline Tucker, conceived, designed and performed the experiments, conducted all statistical analysis and interpretation of data and wrote the manuscript for the paper entitled Pathotype Variation of Barley Powdery Mildew in Western Australia published in Australasian Plant Pathology.

Madeline Tucker

I, as a Co-Author, endorse that this level of contribution by the candidate indicated above is appropriate.

Kithsiri Jayasena

Simon Ellwood

Richard Oliver

To Whom It May Concern,

I, Madeline Tucker, conducted all historical investigations, sourced all data, interpreted and analysed the data and wrote the manuscript for to the book chapter entitled "Origin of fungicide resistant barley powdery mildew in West Australia – Lessons to be learned." *Fungicide Resistance in Plant Pathogens: Principals and a Guide to Practical Management*. Springer, Japan

Madeline Tucker

I, as a Co-Author, endorse that this level of contribution by the candidate indicated above is appropriate.

Fran Lopez-Ruiz

Kithsiri Jayasena

Richard P. Oliver

To Whom It May Concern,

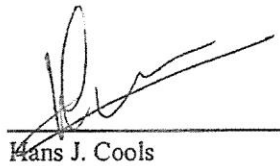
I, Madeline Tucker, contributed to the conception and design of experiments, performed laboratory experiments, conducted statistical analysis, interpreted data and wrote the manuscript for the paper entitled "Accumulation of mutations in *Blumeria graminis* f. sp. *hordei CYP51* confers positive and negative cross-resistance to triazole fungicides."

Which is currently under review.

Madeline Tucker

I, as a Co-Author, endorse that this level of contribution by the candidate indicated above is appropriate.

Fran Lopez-Ruiz



Hans J. Cools

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