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Constraints on the evolution of azole resistance in plant pathogenic fungi

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The durability of azole fungicides in controlling agriculturally important pathogenic fungi is unique amongst modern single site fungicides. Today, azoles are still relied on to control pathogens of many crops including cereals, fruits and vegetables, canola and soybeans. Significantly, this widespread use continues despite many reports of azole-resistant fungal strains. In this review, recent reports of azole resistance and the mechanisms associated with resistant phenotypes are discussed. The example of the complex evolution of the azole target sterol 14α-demethylase (CYP51) enzyme in modern European populations of the wheat pathogen *Mycosphaerella graminicola* is used to describe the quantitative and epistatic effects on fungicide sensitivity and enzyme function of target site mutations, and to explore the hypothesis that constraints on CYP51 evolution have ensured the longevity of azoles. In addition, the threats posed by alternative resistance mechanisms causing cross-resistance to all azoles or even unrelated fungicides are discussed, and postulations are made on how using new genomic technologies to gain a greater understanding of azole resistance evolution should enhance the ability to control azole-resistant strains of plant pathogenic fungi in the future.

Keywords: CYP51, epitasis, fungicide resistance, *Zymoseptoria tritici*

Introduction

Azoles (imidazoles and triazoles) are the largest group of sterol 14α-demethylation inhibiting (DMI) fungicides and the most widely used class of antifungal agents for the control of pathogenic fungi of humans and plants, dominating the agricultural fungicide market since their introduction in the 1970s. In contrast to other single site fungicides, and despite their widespread long-term use, control failures with azoles are rare. When resistance occurs, resistance levels are often low and cross-resistance between members of the azole class incomplete. Therefore, disease control can be maintained by the use of more active compounds of the same class. Recently, more studies of azole resistance in plant pathogenic fungi have started to define the molecular mechanisms underlying less sensitive or resistant (here defined as reductions in sensitivity that may cause control failures in the field) phenotypes. Together with studies of human pathogens, these investigations have defined three primary mechanisms of azole resistance. These are (i) mutations in the target-encoding *CYP51* gene resulting in decreased affinity of the protein for inhibitors, (ii) over-expression of the target *CYP51* gene most frequently caused by insertions in the predicted promoter regions, and (iii) increased efflux caused by the over-expression of genes encoding membrane transporters. These mechanisms can combine, and resistance levels are often determined by combinations of CYP51 amino acid alterations, *CYP51* gene over-expression and/or increased efflux (Cools & Fraaije, 2013). Furthermore, increasing genome sequence information has revealed that many filamentous fungi, particularly filamentous Ascomycetes (subphylum Pezizomycotina), possess two or more paralogous *CYP51* genes (Becher *et al.*, 2011). Species with multiple *CYP51*s are intrinsically less sensitive to some azoles, and mutations conferring acquired resistance to effective azoles are usually restricted to one paralogue, most often *CYP51A* (Becher & Wirsel, 2012).

The most recent studies describing the impact of genetic changes conferring azole resistance on fungicide sensitivity and the efficacy of azole sprays in the field will be discussed. Based on these data, the proposition that the longevity of azole fungicides in agriculture is a product of the costs and trade-offs associated with the genetic alterations conferring a less sensitive phenotype will be explored, with the hypothesis that these preclude the development of widespread resistance to all azoles, and should ensure this group of fungicides, if they are available to growers, remain an important class of antifungal agent in the future.

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Cost and trade-offs associated with azole resistance mechanisms

CYP51 alteration

Azoles inhibit sterol 14α-demethylase (CYP51). This P450 enzyme is essential for the biosynthesis of sterols, critical components of cell membranes that are considered prerequisite for the evolution of eukaryotes. Therefore, CYP51 activity is widely viewed as the ancestral

Plant Pathology

P450 activity (Kelly & Kelly, 2013). Many *CYP51* mutations associated with altered azole fungicide sensitivity phenotypes have been reported in fungi, some at the equivalent positions in both human and plant pathogens, and others unique to one species or genus (Becher & Wirsel, 2012). Unlike target site mutations causing resistance to other single site fungicides, *CYP51* mutations often specifically affect individual, or a subset of azole compounds, with cross-resistance across the whole class generally incomplete. For example, substitutions at the equivalent residue to Y136 are the most frequently reported CYP51 alterations in azole-resistant isolates of human and plant pathogenic fungi (Becher & Wirsel, 2012). In agriculture, Y136F was first reported in grape and cereal powdery mildews resistant to triadimenol and propiconazole (Table 1; Délye *et al.*, 1997, 1998; Wyand & Brown, 2005). However, isolates of other fungi carrying substitutions at the equivalent residue, for example the septoria leaf blotch pathogen *Mycosphaerella graminicola* (Y137F; Leroux *et al.*, 2007; Cools *et al.*, 2011) and *Puccinia triticina* (Y134F; Stammler *et al.*, 2009), the wheat brown rust pathogen, although resistant to triadimenol, can be controlled by newer azoles such as epoxiconazole. Modelling of the *M. graminicola* CYP51 confirms this differential effect, as the exchange to F137 pushes this residue into an obstructive position specifically prohibiting the binding of triadimenol (Mullins *et al.*, 2011). In Europe, cereal powdery mildews are generally controlled by mildewicides and host resistance. However, in Australia, where azoles are the only systemic fungicide class registered for use on cereals, incomplete cross-resistance between azoles is now relied on for powdery mildew control (M. Tucker and R. Oliver, Curtin University, Australia, personal communication).

Other *CYP51* mutations confer contrasting effects on azole sensitivity. For example, *M. graminicola* isolates carrying the substitution V136A are less sensitive to the imidazole prochloraz, but sensitive to tebuconazole, whereas isolates with I381V have lower sensitivities to most triazoles, particularly tebuconazole, but remain sensitive to prochloraz (Fraaije *et al.*, 2007; Leroux *et al.*, 2007). Furthermore, until recently V136A and I381V had not been found in combination. Consequently, it was proposed this trade-off could be exploited to prevent the further evolution of azole resistance by using mixtures or alternations of azoles that are differentially affected by V136A and I381V (Cools & Fraaije, 2008). However, in modern *M. graminicola* populations the sequential accumulation of *CYP51* mutations has generated CYP51 variants with both V136A and I381V, often combined with the more recently emerged substitutions D134G and/or S524T (Leroux & Walker, 2011; Cools & Fraaije, 2013). Isolates carrying these CYP51 variants are becoming more common as they are less sensitive to the most widely used azoles epoxiconazole and prothioconazole, and also prochloraz, although the presence of V136A seems to maintain isolate sensitivity to tebuconazole.

In fact, the accumulation of *CYP51* mutations seems to be critical for the stepwise evolution of new resistant phenotypes of *M. graminicola* in response to the introduction of progressively more active azoles. However, the sequence and frequency by which *CYP51* mutations emerge, driven by azole selection, is constrained by effects on enzyme activity. For example, western European populations of *M. graminicola* are dominated by isolates with CYP51 variants carrying V136A and/or I381V, combined with changes at residues Y459–Y461 (Stammler *et al.*, 2008), and different combinations of these mutations can confer decreased sensitivity to all azoles currently registered for septoria leaf blotch control (Cools *et al.*, 2011; Cools & Fraaije, 2013). Heterologous expression in *Saccharomyces cerevisiae* showed that when introduced as single amino acid substitutions, V136A and I381V destroy the capacity of the *M. graminicola* CYP51 protein to complement yeast CYP51, indicating a loss of sterol 14α-demethylase activity. However, this impairment of function can be restored by combining V136A and I381V with changes at Y459–Y461 (Cools *et al.*, 2010). Therefore, the rise of mutations encoding alterations at residues Y459–Y461 in the mid- to late 1990s (Cools & Fraaije, 2013) was a precondition to the diversity of CYP51 variants and azole resistance phenotypes observed in modern *M. graminicola* populations. The most recent *M. graminicola* CYP51 variants, for example, have up to eight amino acid alterations compared to the wild type. The identification of the equivalent mutations in isolates of the banana pathogen *Mycosphaerella fijiensis* (Y461–Y463, Table 1; Cañas-Gutiérrez *et al.*, 2009) may suggest a propensity for alterations in this region in fungi related to *M. graminicola*. However, although rare in plant pathogenic fungi, amino acid changes in this region have been found in azole-resistant isolates of human pathogenic fungi not closely related to *M. graminicola*, including *Candida albicans* and *Aspergillus fumigatus*.

CYP51 over-expression

As a mechanism of acquired resistance to fungicides, increased expression of the target-encoding gene is unique to the azoles. In plant pathogens it is quite common, with *CYP51* over-expression contributing to azole-resistant phenotypes in *Venturia inaequalis* (Table 1; Schnabel & Jones, 2001), *Penicillium digitatum* (Sun *et al.*, 2013), *Cercospora beticola* (Bolton *et al.*, 2012), *Monilinia fructicola* (Luo & Schnabel, 2008), *Blumeriella jaapii* (Ma *et al.*, 2006), *P. triticina* (Stammler *et al.*, 2009) and *M. graminicola* (Cools *et al.*, 2012). Increases in expression are most often constitutive and are frequently caused by alterations in the predicted regulatory regions. For example, higher *CYP51* expression was associated with insertions in the predicted promoter regions of *V. inaequalis* (Schnabel & Jones, 2001), *M. fructicola* (Luo & Schnabel, 2008), *M. graminicola* (Cools *et al.*, 2012) and *P. digitatum* (Sun *et al.*, 2013).

Table 1 Examples of azole-resistant field isolates of plant pathogenic fungi

Organism	Crop	Azole sensitivity phenotype	Mechanism(s)	Comments[a]	Selected reference(s)
Blumeria graminis f.sp. *hordei*	Barley	Varying levels of resistance to triadimenol and propiconazole. Positive cross-resistance to other azoles	CYP51 alterations (Y136F, K147Q)	CYP51 alterations accumulate to confer highest levels of resistance. In Europe *B. graminis* f.sp. *hordei* is currently controlled by mildewicides and host resistance	Délye *et al.* (1998); Wyand & Brown (2005)
Blumeria graminis f.sp. *tritici*	Wheat	Varying levels of resistance to triadimenol and propiconazole. Positive cross-resistance to other azoles	CYP51 alteration (Y136F)	In Europe *B. graminis* f.sp. *tritici* is currently controlled by mildewicides and host resistance	Kuck & Mehl (2004); Wyand & Brown (2005)
Botrytis cinerea	Various fruit and vegetables	Reduced azole sensitivity and cross-resistance to unrelated fungicides (multiple drug resistance (MDR))	Increased efflux (*MfsM2* and *Mrr1* alteration)	Resistance levels low. Azoles not currently relied on	Kretschmer *et al.* (2009)
Cercospora beticola	Sugar beet	Cross-resistant to epoxiconazole and flutriafol. Reduced tetraconazole, prothioconazole and difenoconazole sensitivity	Constitutive *CYP51* over-expression (*CbCYP51*)	Azoles used to control *C. beticola*	Nikou *et al.* (2009); Bolton *et al.* (2012)
Erysiphe necator	Grapevine	Varying levels of resistance to triadimenol. Restricted cross-resistance to mycobutanil and fenarimol	CYP51 alteration (Y136F)	Isolates carrying Y136F are least sensitive to triadimenol. Most azoles still effective although highly resistant isolates in the USA do not have a *CYP51* mutation	Délye *et al.* (1997); Gadoury *et al.* (2012)
Monilinia fructicola	Stone fruit	Resistant to propiconazole. Reduced sensitivity to tebuconazole and fenbuconazole	Constitutive *CYP51* over-expression (*MfCYP51* promoter insert 'Mona')	Isolates resistant to propiconazole can be controlled by higher doses or azoles with greater intrinsic activity	Holb & Schnabel (2007); Chen *et al.* (2012)
Mycosphaerella fijiensis	Banana	Varying levels of resistance to propiconazole. Cross-resistance between propiconazole and cyproconazole, but not imazalil	CYP51 alterations (Y136F, A313G, A381G, Y461D, G462A, Y463D, Y463H and Y463N). Inserts in the predicted *MfCYP51* promoter also found	Most resistant isolates found in areas with highest numbers of sprays. Newer azoles (e.g. epoxiconazole) remain very effective	Cañas-Gutiérrez *et al.* (2009); Chong *et al.* (2011)
Mycosphaerella graminicola	Wheat	Varying levels of resistance to all azoles registered for *M. graminciola* control	CYP51 alterations (>30 reported, e.g. D134G, V136A, Y137F, A379G, I381V, Y459D, Y461H, ΔY459/G460, S524T). Constitutive *MgCYP51* over-expression. Increased efflux suggested	Although some recent isolates are less sensitive to epoxiconazole and prothioconazole, these compounds remain an important component of disease control	Leroux & Walker (2011); Cools & Fraaije (2013)
Oculimacula acuformis	Wheat	Intrinsically resistant to some triazoles. Acquired resistance to the imidazole prochloraz. Sensitive to prothioconazole	Mechanism on intrinsic or acquired resistance unknown, although *CYP51* sequence variation suggested	Prothioconazole remains effective	Leroux *et al.* (2013)
Oculimacula yallundae	Wheat	Acquired resistance to some azoles. Sensitive to prothioconazole	Mechanism of acquired resistance unknown, although efflux pump activity suggested	Prothioconazole remains effective	Leroux *et al.* (2013)
Penicillium digitatum	Citrus	Resistant to imazalil. Cross-resistant to prochloraz,	Constitutive *CYP51* over-expression (*PdCYP51A* or *PdCYP51B* promoter	Imazalil widely used. High doses control resistant isolates	Nakaune *et al.* (1998); Ghosoph

(continued)

Table 1 (continued)

Organism	Crop	Azole sensitivity phenotype	Mechanism(s)	Comments[a]	Selected reference(s)
		myclobutanil and propiconazole	insert, transposable element). ABC transporter *PMR1* over-expression		*et al.* (2007); Sun *et al.* (2013)
Podosphaera fusca	Cucumber	Lower sensitivities to triadimenol and fenarimol. Cross-resistance between triadimenol and myclobutanil. No cross-resistance between fenarimol and triadimenol or myclobutanil	No *CYP51* over-expression. CYP51 alteration suggested	Azole fungicides, in mixture with alternative modes of action, still recommended for *P. fusca* control	López-Ruiz *et al.* (2010, 2011)
Puccinia triticina	Wheat	Varying sensitivity of European isolates to epoxiconazole	CYP51 alteration (Y134F) and *CYP51* over-expression identified in sensitive and less sensitive isolates	Deceased sensitivity phenotypes are still rare. Azoles remain very effective	Stammler *et al.* (2009)
Rhynchosporium commune	Barley	Positive cross-resistance between propiconazole, tebuconazole, epoxiconazole. Incomplete cross-resistance to prothioconazole	Presence of a second *CYP51* paralogue (*RcCYP51A*). Mechanism responsible for more recent shifts unknown	Prothioconazole remains very effective	Hawkins *et al.* (2011)
Sclerotinia homoeocarpa	Turf grass	Reduced sensitivities to propiconazole. Sensitivities between azoles as well as to plant growth regulators highly correlated	Induced *CYP51* over-expression. Constitutive and induced efflux pump (*ShatrD*) over-expression	Azoles still relied on for *S. homoeocarpa* control, resistance to unrelated fungicides a possibility	Ok *et al.* (2011); Hulvey *et al.* (2012)
Venturia inaequalis	Apple	Resistance to myclobutanil and reduced sensitivity to fenbuconazole and difenoconazole reported. Cross-resistance between azoles. Some local variation	Constitutive *CYP51* (*CYP51A1)* over-expression caused by a promoter insert	Azoles still used although other modes of action are available	Schnabel & Jones (2001); Pfeufer & Ngugi (2012)

[a]Current control strategies.

These inserts vary in size, often have the signatures of transposable elements, and contain powerful promoter sequences (Sun *et al.*, 2013). For some fungi, including *C. beticola* (Bolton *et al.*, 2012) and *P. triticina* (Stammler *et al.*, 2009), the genetic changes responsible for over-expression are currently unknown.

CYP51 over-expression may offer some selective advantages in comparison to other resistance mechanisms. Unlike alterations of CYP51 primary sequence, changes in sensitivity of individuals over-expressing *CYP51* are not compound-specific, with complete cross-resistance between the azoles common (Table 1). However, resistance levels are generally lower than those caused by target site alteration, and therefore higher doses or more active compounds can be effective against isolates over-expressing *CYP51* (Table 1). Recently, *M. graminicola* isolates over-expressing azole-resistant forms of the *CYP51* gene have been identified. This combination of target site mutation and over-expression causes a pan-azole-resistant phenotype, with high levels of resistance to compounds specifically affected by mutations (Cools *et al.*, 2012), leading to the concern that over-expression of the most resistant CYP51 variants could confer a phenotype that may affect the field performance of all azoles in the future.

Enhanced fungicide efflux

Enhanced fungicide efflux, leading to resistance to multiple unrelated drugs, a so-called multidrug-resistant (MDR) phenotype, is viewed as a major threat to the control of fungal pathogens of humans (Gulshan & Moye-Rowley, 2007). Indeed in pathogenic yeasts, for example *Candida glabrata*, *Candida krusei* and *C. albicans*, the impact of over-expression of ATP binding cassette (ABC) or major facilitator superfamily (MFS) transporter genes on intrinsic and acquired resistance to multiple antifungals is well established (Thakur *et al.*, 2008; Morschhäuser, 2010). However, in filamentous fungi, particularly pathogens of plants, the importance of efflux pump activity is less clear. For example, genes encoding ABC or MFS transporters have been identified in plant pathogenic fungi, and their capacity to export azoles by heterologous expression and targeted knockout studies has been demonstrated (de Waard *et al.*, 2006). Genome-wide transcriptional studies have

identified ABC transporter genes responsive to azole treatment (Becher *et al.*, 2011), and the use of putative chemical efflux antagonists has shifted resistant phenotypes, as well as sensitizing wildtype isolates (Roohparvar *et al.*, 2002; Leroux & Walker, 2011). Yet, although MDR phenotypes of plant pathogens, including *M. graminicola* and *Oculimacula yallundae*, have been proposed to impact on fungicide performance (Table 1; Leroux & Walker, 2011; Leroux *et al.*, 2013), to date, only in *Botrytis cinerea* has a genetic mechanism conferring enhanced efflux to multiple fungicides been characterized and shown to impact on the performance of fungicides in the field (Table 1; Kretschmer *et al.*, 2009). The factors suggested as responsible for the limited occurrence of MDR in the field, including large population size, the restricted number of sprays per season and the occurrence of refugia, rely on fitness costs in the absence of fungicide selection (de Waard *et al.*, 2006). However, as shown in experimentally evolved populations of *C. albicans* (Cowen *et al.*, 2001), the biological potential to compensate for fitness costs associated with an MDR phenotype exists.

Multiple CYP51s

As more genomes of plant pathogenic fungi become available, the influence of multiple target site-encoding genes on intrinsic and acquired fungicide resistance is becoming more apparent (Cools & Hammond-Kosack, 2013). Multiple *CYP51* paralogues have been identified in a number of plant pathogens including species of *Aspergillus*, *Fusarium*, *Penicillium* and *Rhynchosporium* (Becher *et al.*, 2011; Hawkins *et al.*, 2011; Liu *et al.*, 2011). In *Fusarium graminearum*, heterologous expression and gene knockout studies have shown two of the three *CYP51* paralogues, *CYP51A* and *CYP51B*, to be functionally redundant, both encoding sterol 14α-demethylases, but *CYP51A* is rapidly induced upon ergosterol depletion caused by azole treatment, and is thus responsible for the intrinsically low sensitivity of *F. graminearum* to some azole fungicides (Fan *et al.*, 2013). Although tebuconazole resistance in four isolates of *F. graminearum* and *Fusarium asiaticum* was not associated with *CYP51* changes (Yin *et al.*, 2009), numerous studies in *A. fumigatus* (Becher & Wirsel, 2012) and *P. digitatum* (Sun *et al.*, 2013) have shown acquired resistance to effective azoles is caused largely by mutations and/or over-expression of the *CYP51A* paralogue. This implies that carrying additional *CYP51*s may offer an advantage under selection by azoles, as any costs or trade-offs associated with changes in protein structure or gene over-expression of one paralogue are circumvented by the presence of an unchanged enzyme with paralogous wildtype activity.

Conclusions and perspectives

Early studies seeking to predict the risk of resistance to azoles using experimentally evolved fungal populations of, for example, *Cladosporium cucumerinum* (Fuchs & Drandarevski, 1976), concluded that resistance under practical conditions was unlikely as spore formation, mycelial growth rate and germination were all retarded in resistant isolates. Although the conclusions ultimately proved incorrect, these studies identified a possible cost to azole resistance. With the molecular tools now available, it is known that target site changes contributing to an azole-resistant phenotype have interacting effects, and mutations that alone may be deleterious can be maintained in populations in the presence of compensatory mutations. The evolution of *CYP51* in European *M. graminicola* populations under selection by azoles is an elegant demonstration of these epistatic effects, where the phenotypic consequences of individual mutations are dependent on the genetic background in which they occur (Poelwijk *et al.*, 2007). In addition, as target site resistance is generally compound-specific, the occurrence of particular mutations reflects the history of azole use. Therefore, a requirement for multiple mutations to confer resistance, and the diversity of azoles available to growers, have extended the effective life of this chemistry and have ensured that newly developed compounds, for example the recently introduced prothioconazole, can still have a profitable share of the market, despite the existence of azole-resistant strains of target pathogens. A current example is the potential use of prothioconazole to control azole-resistant strains of Asian soybean rust, *Phakopsora pachyrhizi* (Koga *et al.*, 2011; Schmitz *et al.*, 2013).

In the future, it may be that mechanisms that can provide cross-resistance across the azole class, for example *CYP51* over-expression and enhanced fungicide efflux activity, will become more common, although the infrequency of these mechanisms in azole-resistant field isolates in current populations of plant pathogens suggests fitness costs (Leroux *et al.*, 2013). Whether additional mutations will alleviate these costs in the future is unknown. However, as genome-wide analysis of different plant pathogens becomes increasing feasible (Cools & Hammond-Kosack, 2013), and we are able to identify genetic changes in azole-resistant strains and gain a greater understanding of the interactions between them, it may be possible to potentiate the activity of azoles or alternative fungicides in the future by inhibiting compensatory mechanisms.

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Conflicts of interest

The authors have no conflicts of interest to declare.

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