Impact of changes in the target P450 CYP51 enzyme associated with altered triazole-sensitivity in fungal pathogens of cereal crops

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Abstract
Control of diseases caused by fungi in both medicine and agriculture is heavily dependent on the use of triazoles. As a consequence, resistance to triazoles is a threat to both human health and the sustainability of agricultural production systems. In human pathogens, particularly Candida albicans, mutations encoding alterations in the target cytochrome P450 sterol 14α-demethylase (CYP51; where CYP is cytochrome P450) enzyme are the primary determinants of triazole resistance. In fungal pathogens of cereals, CYP51A1 modifications, some at positions known to contribute to a resistant phenotype in human pathogens, have also been identified in isolates with altered triazole-sensitivity. However, unlike medicine where resistance to triazoles is a major clinical problem, failures of triazoles to control crop diseases in the field are rare with mean population sensitivities generally remaining low, perhaps due to differences in the selection pressures imposed on human and cereal pathogen populations. Nonetheless, the biological potential for resistance exists, and the question remains as to whether widespread triazole resistance can develop in an important cereal pathogen.

Introduction
Triazole fungicides interfere with the biosynthesis of the predominant sterol in fungal membranes, ergosterol, by stoichiometric interaction of the N-4 substituents of the triazole ring to the haem iron of the cytochrome P450 sterol 14α-demethylase (CYP51; where CYP is cytochrome P450) enzyme and of the hydrophobic substituent with the apoprotein in the vicinity of the CYP51-binding site [1]. Triazoles have been the leading agents for the control of fungal diseases of plants, humans and animals for over 20 years. In the pharmaceutical sector, triazole products account for approximately half of the North American and European US$2.4 billion systemic antifungal market. In agriculture, they continue to dominate the cereal fungicide market with approx. 40% of the total area to which foliar fungicides are applied in the U.K., treated with a straight triazole product since 1990 (Figure 1).

Resistance to triazoles is now a major problem in controlling fungal pathogens of humans. For example, highly resistant strains of the opportunistic pathogen Candida albicans, which can cause serious clinical problems in immunocompromised patients, are now common [2]. Similarly, the use of triazoles to control Aspergillus fumigatus, a pathogen of increasing importance due to a high mortality rate in an immunodeficient patient, is also now compromised due to resistance [3]. In contrast, despite their long-term widespread use, triazole resistance in cereal pathogens has developed slowly, with loss of efficacy in practice still rare. However, the emergence of resistance in agro-economically important cereal pathogens to other commonly used systemic fungicides with different modes of action, for example the strobilurins, has renewed pressure on triazole chemistry and prompted concerns over the potential for resistance development.

Modification of the target protein is the most common mechanism of resistance to site-specific fungicides. CYP51 alteration, although generally found in combination with other mechanisms, has been associated with altered triazole-sensitivity in pathogens of both humans and plants with the impact of a number of specific mutations on triazole affinity functionally characterized. In this short review, we discuss CYP51 mutations correlated with altered triazole-sensitivity in three pathogens of cereal crops and compare and contrast these with mutations identified in the orthologous CYP51 genes of triazole-resistant strains of human fungal pathogens.

CYP51 modification in fungal pathogens of cereal crops
Blumeria graminis (cereal powdery mildew)
Isolates of both barley (B. graminis f. sp. hordei) and wheat (B. graminis f. sp. tritici) powdery mildews highly resistant to triadimenol, the most widely used triazole in the late 1970s and early 1980s, have been identified. Interestingly, four distinct categories of triadimenol resistance exists in U.K. populations of B. graminis f. sp. hordei [4] with the highest three levels of resistance controlled by a single major gene. In France, characterization of a small number of B. graminis...
f. sp. _hordei_ isolates from triazole-treated fields associated a point mutation in the _CYP51_ gene, encoding a replacement of tyrosine for phenylalanine at position 136 (Y136F), with resistance [5]. Subsequently, alteration of this residue was correlated with a high triadimenol resistance phenotype in _B. graminis_ f. sp. _hordei_ and _tritici_ isolates, although in both cases Y136F was also found in isolates with low resistance, leading to the suggestion that this mutation alone confers only low resistance [6]. In _C. albicans_, alteration of the equivalent codon (Y132H) is commonly associated with resistance to fluconazole [7], and furthermore, has been shown biochemically to alter affinity of CYP51 for triazoles [8]. When heterologously expressed in yeast, this substitution alone confers low levels of resistance, with high levels attained when Y132H is expressed in combination with additional CYP51 alterations [9]. Similarly, in _B. graminis_ f. sp. _hordei_, a combination of Y136F and substitution K147Q was identified in highly resistant isolates, with K147Q absent from isolates with lower levels of resistance [6].

**Oculimacula spp. (cereal eyespot fungi)**

_Oculimacula_ _acaformis_ (syn. _Tapesia acaformis_) and _Oculimacula yallundae_ (syn. _Tapesia yallundae_), causal agents of cereal eyespot disease of wheat and barley, are closely related species with distinct sensitivities to triazoles. _O. acaformis_ is intrinsically resistant to triazoles and sensitive to imidazoles, whereas _O. yallundae_ is sensitive to bothazole chemistries [10]. Comparative analysis of _CYP51_ sequences between the two species identified a number of polymorphisms at the amino acid level, although only one (F180L) is highly conserved among fungi and, therefore, suggested to potentially impact on triazole binding and confer the resistant phenotype of _O. acaformis_ [11]. Analysis of acquired resistance to triazoles and/or imidazoles in both species identified amino acid alterations, but again, none could be directly associated with distinct phenotypes [11]. Studies by Wood et al. [12] could also not relate _CYP51_ changes with reduced sensitivity of _O. yallundae_ isolates to the imidazole prochloraz, despite previous work identifying a single major resistance gene [13].

**Mycosphaerella graminicola** (septoria leaf blotch fungus)

_M. graminicola_ causes septoria leaf blotch, the most economically important foliar disease of wheat in North Western Europe. Currently, the only fully effective method of controlling this disease is by the programmed application of fungicides. In 2002, resistance to the strobilurin fungicides was detected in the U.K. and Ireland [14] and is now widespread, with approx. 90% of the U.K. pathogen population carrying the resistance-conferring allele [15]. Control is therefore now reliant on the triazoles, prompting concerns, in the absence of effective chemistries with alternative modes of action, that resistance could develop. In fact, studies have shown a recent clear erosion of triazole efficacy against _M. graminicola_ [16,17], so that higher doses are now required to achieve the effective disease control (HGCA Wheat Disease Management Guide 2006, http://www.hgca.com/document.aspx?fn=load&media_id=2664&publicationId=1291).

Although reduced azole-sensitivity in _M. graminicola_ is undoubtedly a polygenic trait conferred by a combination of several mechanisms [16,18], _CYP51_ alterations, many of which are at positions orthologous to those found in fluconazole-resistant _C. albicans_ isolates, have been identified and some are associated with high resistance factors [19]. Interestingly, a substitution at the equivalent residue to Tyr<sup>132</sup> of _C. albicans_, Y137F is present in _M. graminicola_ isolates without reductions in triazole-sensitivity, although, perhaps significantly, this substitution was always found as a single mutation, never in combination with other _CYP51_ modifications [19].

We have also identified _CYP51_ substitutions in _M. graminicola_ not previously described in triazole-resistant plant or human pathogenic fungi; for example _I381V_, which, interestingly, appears to be differentially selected by triazoles commonly used for _M. graminicola_ control (B.A. Fraaije, S.H. Kim and H.J. Cools, unpublished work). Figures 2(A) and 2(B) show the relationship between _I381V_ and the percentage of isolates of distinct EC<sub>50</sub> categories to two triazoles, epoxiconazole and tebuconazole, and a new class of azole chemistry, a triazolinthione, prothioconazole. A continuous distribution of sensitivities of _I381_ isolates to epoxiconazole, tebuconazole and prothioconazole is evident. In contrast, _Val<sup>131</sup>_ isolates are all considered either less sensitive or resistant to tebuconazole, but yet range across EC<sub>50</sub> groupings to epoxiconazole and prothioconazole, indicating a selectable advantage for _Val<sup>131</sup>_ isolates in the presence of tebuconazole. The residue equivalent to Ile<sup>321</sup> in the crystal _Mycobacterium tuberculosis_ _CYP51_ protein, Leu<sup>121</sup>, is predicted to form a substrate recognition site, and also lies within 4 Å (1 Å = 0.1 nm) of the haem-bound triazole.
Implications of CYP51 modifications

The emergence of high levels of resistance to triazoles in human pathogens, particularly *C. albicans*, coincident with the rise over the past 20 years in the number of immunodeficient patients on long-term triazole therapy for recurrent infections, has raised concerns that levels of resistance sufficient to compromise control might also emerge in an important plant pathogen, especially given the widespread use of triazoles in cereal crops. As has been illustrated in numerous studies, mechanisms known to confer high levels of resistance in human pathogens also function in plant pathogens, with for example analogous CYP51 mutations manifesting in both.

Failure of fluconazole in the treatment of candidiasis was correlated with the presence of an isolate with an MIC (minimum inhibitory concentration) of $\geq 64 \, \mu g \cdot ml^{-1}$ [21]. In fact, isolates of plant pathogens with similar levels of resistance have been detected, for example *M. graminicola* isolates in Germany in 1998 with MICs at approx. $100 \, \mu g \cdot ml^{-1}$ to cyproconazole [18], yet disease control failures with the triazole class of chemistry remain rare.

Differences in the selection pressures to which pathogens are exposed undoubtedly contribute to this discrepancy. Antifungal agents available for the control of human pathogens have been limited. For example, for 15 years, fluconazole has been the leading drug for treatment of mucosal and invasive candidiasis [22], with alternative triazoles such as itraconazole and the more recently introduced voriconazole and posaconazole generally only used if resistance to fluconazole is compromising treatment [23]. Non-triazole-based antifungals have only recently become available with the approval of caspofungin, which disrupts fungal cell walls by the inhibition of $\beta$-(1,3)-glucan synthesis [24]. In contrast, cereal growers have had a choice of both non-triazole [e.g. chloronitriles, dithiocarbamates, methyl benzimidazole carbamates and strobilurins (QoIs)] and triazole-based fungicides that are often used in alterations or mixtures. In fact, triazoles are no longer relied upon for control of some cereal pathogens in which resistance was previously considered a problem, for example *B. graminis*, which is currently controlled by host resistance and highly active mildewicides (e.g. quinoxyfen, metrofenone and proquinazid). Therefore selection to resistance in a plant pathogen by single-product treatment over successive seasons has, perhaps until recently, not necessarily occurred.

Additionally, in the clinical setting, long-term exposure of pathogens to a single triazole is common. Fluconazole therapy of HIV patients, for example, often lasts for several months [22]. In cereal crops, fungicide application numbers average two to three sprays per season. Pathogens such as *M. graminicola* are only exposed to triazoles intermittently during the spring and early summer. Consequently, fitness costs or perturbation of reproductive output incurred by the acquisition of high levels of triazole resistance are more likely to impact in the field when selection pressure is not continuous over the season.

There may also be important differences in the population biology and infection cycle of fungal pathogens in these contrasting host environments. While there is evidence for genetic exchange and recombination in *Candida*, clonal reproduction predominates in this fungus [25]. Work on the emergence of fluconazole resistance in isolates of *Candida* from immunocompromised patients suggested that the rapid development of resistance occurred by selection of a resistant clone from a heterogeneous population of cells [26]. Interestingly, resistant isolates became more sensitive after

ligand [20]. Therefore it seems reasonable to suggest a possible interaction of this residue with the triazole side chain.
serial transfer in the absence of the drug. A recent study genotyping isolates of *C. albicans* from HIV-positive patients in Africa concluded that the pathogen reproduces clonally, with only rare exceptions, and that the rate of transmission between hosts is extremely low [27]. Hence, in the clinical setting, antifungal drug resistance appears to repeatedly evolve in isolated populations [28].

In contrast, epidemics of leaf blotch on wheat, caused by *M. graminicola*, are initiated each season by airborne sexual ascospores in which recombination has taken place. The founding population therefore has a high level of genetic diversity. Subsequent multiplication of the disease in the crop is predominantly asexual [29]. Calculations of the latent period of *M. graminicola* (the time from infection to production of the next generation of inoculum) in relation to temperature throughout the growth of the wheat crop [30] suggest that only six or at most seven cycles of infection can take place in a single season, and of these only three are likely to be exposed to fungicide selection. At the end of the season, the pathogen population survives on crop debris in which sexual reproduction again takes place. This disease cycle suggests that there is ample opportunity for recombination and possible pyramiding of genes encoding different resistance mechanisms, but relatively little scope for repeated selection of the most resistant clones. Conversely, the intercrop survival period and subsequent sexual cycle may lose or disassociate combinations of genes conferring resistance, especially if such genes incur fitness penalties in the absence of selection by the fungicide.

These factors might, so far, have restricted the impact of target site changes on triazole performance in the field. However, increased reliance on triazoles, for example in controlling *M. graminicola*, may provide a selective environment in which high resistance levels conferred by multiple mechanisms, including CYP51 modification, becomes a major determinant for survival of an individual within a treated population.

**Conclusions**

Mutations in the target-encoding *CYP51* gene have been identified in pathogens of cereal crops with altered sensitivity to triazole fungicides. A number of identified mutations encode amino acid changes at positions equivalent to those known to affect a triazole-resistant phenotype in human pathogens, most notably Y132F/H in *C. albicans*. In contrast with the clinical setting, widespread azole resistance has yet to develop in an economically important cereal pathogen, in part due to diversity of chemistry that has been available for their control, but also differences in pathogen population biology and the degree of exposure to the fungicide. However, for some cereal pathogens, particularly *M. graminicola*, triazoles are increasingly relied upon as the number of alternative modes of action decreases and clearly *CYP51* mutations, similar to those evolving in human pathogens, may eventually impact on the capability of growers to control disease in the future.

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**References**


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