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Molecular evolution and mechanisms of fungicide resistance in plant pathogenic fungi

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1 Introduction

Chemicals have been used to reduce crop damage since Greek and Roman times. In the seventeenth century, lime and salt were used as seed treatment to prevent bunt on wheat. In the nineteenth century, sulphur and copper were used in foliar sprays and seed treatments to control a range of plant pathogens. In 1882, the famous Bordeaux mixture, a mixture of copper sulphate and hydrated lime, was developed to control grapevine mildew. Until the 1940s, chemical disease control continued to rely on inorganic chemical preparations which were prepared by the user and had to be applied in enormous amounts. After the war the agrochemical industry started to develop, and new chemicals such as dithiocarbamates and phthalimides appeared on the market. These compounds are still used as foliar sprays and seed treatments today. Fungicides used from the 1940s to the 1960s were multi-site inhibitors which affected

many fungal enzymes, had generally broad-spectrum effectiveness against many target species, were non-systemic because they did not move through the plant, and only had protectant activity, being ineffective against established infection. They had to be applied in large quantities and very frequently, often weekly, and they tended to be toxic not only to the crop or plant to which they were applied but also to the environment and the user.

In the 1960s and 1970s, the fungicide market expanded dramatically with the release of an entirely new type of fungicides. These were systemic fungicides which could be taken up by and travelled through plant tissues. These new formulations all had single-site modes of action which were highly specific to target pathogens but not host crop plants. They were more effective, less toxic to people and the natural environment and could be used as eradicants as well as protectants. This revolutionised crop protection and the main group of fungicides which are used today are all single-site fungicides. The history of fungicides has been reviewed in detail by Morton and Staub (2008) and Hollomon (2015).

The main disadvantage of single-site mode of action fungicides is that resistance in pathogen populations tends to evolve shortly after the introduction of a product. Resistance may be either qualitative or quantitative. Qualitative resistance is characterised by an abrupt loss of effectiveness of the fungicide, resulting in control failure. Isolates in the population tend to fall into two groups, resistant and sensitive. Quantitative resistance is characterised by a gradual decline in disease control with variable degrees of resistance in the pathogen population, resulting in a correspondingly gradual decline in fungicide performance.

This chapter reviews the groups of fungicides which have been most widely used in agriculture and horticulture over the last 50 years and summarises the mechanisms of resistance to each class of compounds. We address resistance to compounds active against fungi *sensu stricto* but not those used specifically to oomycete pathogens. Single-site fungicides target essential fungal cell processes. As fungal cells have many biochemical features and processes in common with animal cells the number of useful target proteins are limited. The main targets for fungicide development are the integrity of the fungal cell and fungal respiration. We focus on powdery mildew fungi because these pathogens are notorious for the speed with which they have evolved resistance to many groups of systemic fungicides. Why they are so prone to evolving resistance is not known but reasons may include the fact that they are haploid, so mutations to resistance are immediately exposed to natural selection by the fungicide, their short life cycle in their asexual phase, and the plasticity of their genomes (Spanu et al., 2010, Wicker et al., 2013, Bhosle et al., 2019, Bindschedler et al., 2016). Moreover, very unusually for a eukaryote, all reproductive cells of powdery mildew are exposed to the atmosphere and thus

to mutagens including ultra-violet wavelengths of sunlight, chemical pollutants and cosmic rays (Arnold, 2018).

2 Methyl benzimidazole carbamate: fungicides which target the cytoskeleton

Three important classes of fungicides target structural features of the fungal cell. MBC fungicides target the cytoskeleton whereas the azole and amine groups affect damage the integrity of fungal membranes.

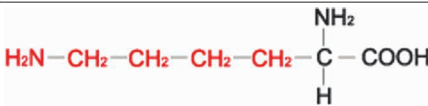
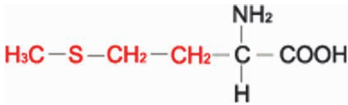
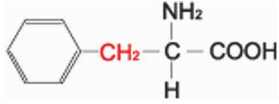
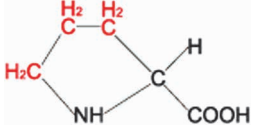
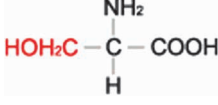
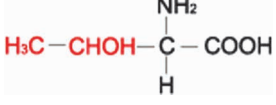
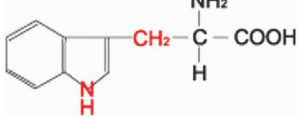
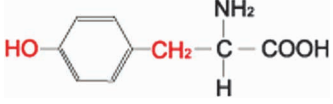
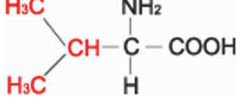
The cytoskeleton provides structure and shape to eukaryotic cells. An important part of the cytoskeleton consists of microtubules, which are polymers of tubulin proteins. There are two types of tubulin forming the microtubule, α -tubulin, encoded by the *Tub1* gene, and β -tubulin, encoded by *Tub2*. Methyl benzimidazole carbamate (MBC) or benzimidazole fungicides, which target β -tubulin, were introduced in the early 1970s and quickly became widely used in agriculture. They are effective against a broad range of ascomycete and basidiomycete pathogens of cereals, grapes, fruits and vegetables but are ineffective against oomycetes. They were the first single-site fungicides developed. Resistance to MBCs developed very soon after their introduction and has now been reported in over 100 species of plant pathogenic fungi (Hawkins and Fraaije, 2016, Vela-Corcía et al., 2018).

Resistance was first reported in cucurbit powdery mildew (Schroeder and Providenti, 1969) followed by *Botrytis* in grapevine (Ehrenhardt et al., 1973) then cereal powdery mildew (Vargas, 1972, 1973). As resistance started to emerge in the field, mutagenesis studies in model organisms such as brewer's yeast, *Neurospora crassa* and *Aspergillus nidulans* (teleomorph *Emericella nidulans*) were quickly set up to assess mechanisms and risks of resistance in advance of resistance becoming widespread in the field. These experiments led to the identification of the target of MBCs being β -tubulin and the identification of mutations responsible for resistance (Thomas et al., 1985, Fujimura et al., 1992, Fujimura et al., 1994, Orbach et al., 1986).

Several mutations in *Tub2* are associated with resistance, most of which are at amino acid 198 and 200. The most common substitutions are F200Y (phenylalanine mutated to tyrosine at residue 200) and E198A/K/G (glutamic acid mutated to alanine, lysine or glycine at residue 198); see Table 1 for amino acid codes (Hawkins and Fraaije, 2016, Vela-Corcía et al., 2018). In the cucurbit powdery mildew fungus *Podosphaera xanthii* (which may be synonymous with *P. fusca*; (Pérez-García et al., 2009)), resistance in the field was identified in the early 2000s (McGrath, 2001). No crystal structure for fungal β -tubulin is available yet but Vela-Corcía et al. (2018) were able to use the mammalian crystal structure from *Bos taurus* (cattle) and heterologous expression of β -tubulin in *E. coli* to explore the mechanism of resistance in *P. xanthii* to two

Table 1 Amino acid codes and properties

Amino acid	Code	Properties	Structure
Alanine	A	Hydrophobic (non-polar)	
Arginine	R	Polar charged basic	
Asparagine	N	Polar uncharged	
Aspartic acid	D	Polar charged acid	
Cysteine	C	Polar uncharged	
Glutamic acid	E	Polar charged acid	
Glutamine	Q	Polar uncharged	
Glycine	G	Polar uncharged	
Histidine	H	Polar charged	
Isoleucine	I	Hydrophobic	
Leucine	L	Hydrophobic	

Amino acid	Code	Properties	Structure
Lysine	K	Polar charged basic	
Methionine	M	Hydrophobic	
Phenylalanine	F	Hydrophobic	
Proline	P	Hydrophobic	
Serine	S	Polar uncharged	
Threonine	T	Polar uncharged	
Tryptophan	W	Hydrophobic	
Tyrosine	Y	Hydrophobic	
Valine	V	Hydrophobic	

molecules, carbendazim, an MBC fungicide, and diethofencarb, an N-phenyl carbamate, which is a herbicide with additional activity against several fungi. In *P. xanthii*, resistance is conferred by the substitution of glutamic acid to alanine at position 198 (E198A). This study postulated that the E198A mutation induces subtle structural rearrangements of the entire protein, specifically that the resistant β -tubulin protein had four β -sheet strands which were not present in the sensitive protein. Furthermore, residues 198 and 200 did not appear to be part of the binding site.

Protein homology modelling in *P. xanthii* suggested that residue 198 is located deep inside the protein making it inaccessible to the fungicide; rather, the carbendazim binding site involves residues S138 and T178, while diethiofencarb binds to S178 and Y222. It was predicted that carbendazim binds to β -tubulin via three hydrogen bonds, one at amino acid residue S138 and two at T178 and that the E198A mutation results in T178 being twisted out, precluding the establishment of two of the three hydrogen bonds between carbendazim and the β -tubulin target. A similar mechanism was predicted for diethiofencarb, with the fungicide binding the protein via two hydrogen bonds at residues S138 and Y222. The modeling predicted that the E198A mutation results in the movement of Y222, preventing the formation of one of these hydrogen bonds. Site-directed mutagenesis of residues 138 and 178 was performed with both residues mutated to alanine. The resultant mutant proteins underwent dramatic misfolding in the absence of the fungicide and T178A was unable to bind to the fungicide (Fig. 1).

Resistance to MBC fungicides has been detected in several powdery mildew species. Indeed, resistance appeared in grapevine powdery mildew *Erysiphe necator* (anamorph *Uncinula necator*) in California vineyards in the late 1970s but the underlying mutations have not been investigated. This is probably due

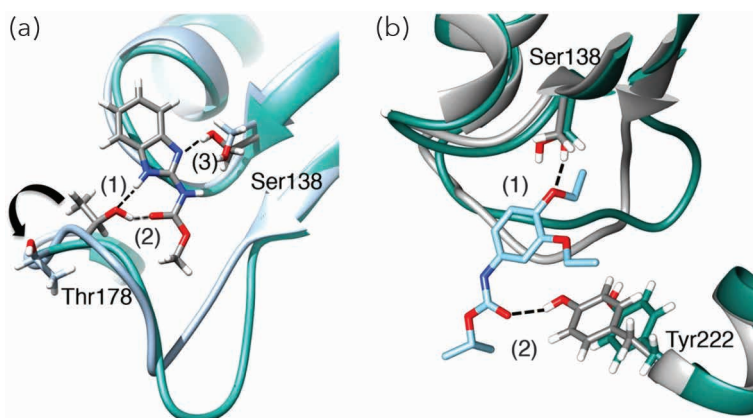


Figure 1 Molecular docking of MBC fungicides to superpositions of MBC-sensitive and resistant *Podosphaera xanthii* β -tubulin models. (a) Docking of carbendazim to MBC-sensitive *P. xanthii* β -tubulin (in cyan) via three hydrogen bonds (dashed lines) involving residues Threonine 178 (T178) and Serine 138 (S138). Thr178 is twisted out (arrow) in the MBC-resistant β -tubulin model (gray) preventing carbendazim binding to T178. (b) Docking of diethiofencarb to MBC-resistant β -tubulin model (cyan) via two hydrogen bonds that involve residues Ser138 and Tyr222 (Y222). Diethiofencarb cannot bind to MBC-sensitive β -tubulin (gray) due to movement of Tyr222. This figure is reproduced from Vela-Corcía et al. (2018) and is included in the article's Creative Commons CC-BY Licence.

to the low importance of MBC use in grapevine mildew management (Pearson and Taschenberg, 1980, Kunova et al., 2021). In cucumber mildew *P. fusca* (syn. *P. xanthii*), resistance in the field was identified in the early 2000s (McGrath, 2001) and study of the β -tubulin gene sequence revealed that the E198A mutation conferred resistance to MBC fungicides in *P. fusca* (Vela-Corcía et al., 2014).

3 Azoles: inhibitors of sterol demethylation

The sterol synthesis pathway is the target of several classes of fungicide because ergosterol is specific to fungi whereas animals cells have cholesterol and fungi cannot survive without ergosterol. Like other sterols, ergosterol is an essential component of fungal cell membranes, determining the fluidity, permeability and activity of the membrane. The two main classes of agricultural fungicide which attack the sterol synthesis pathway are azoles and amines. In addition, terbinafine, used widely in medicine, inhibits the ERG1 squalene monooxygenase, an important step in the pathway to sterol, steroid and triterpenoid synthesis.

3.1 Activity of azoles

Since their introduction in the 1970s, azole antifungals have been the mainstay of antifungal treatments in human and veterinary medicine as well as arable agriculture and horticulture. They are the largest class of all fungicides. They have broad-spectrum activity and have remained largely effective against many medical and agricultural pathogens. Most agricultural azole fungicides fall into the triazole sub-class, in contrast to medical antifungals, most of which are imidazoles. A notable agricultural imidazole is the broad-spectrum fungicide prochloraz. Other, smaller groups of sterol C14 demethylation inhibitor fungicides have also been important, including the piperazine compound triforine, the pyrimidine fenarimol and the pyridine pyrifenoxy (Kuck et al., 1995), but they are not considered further here.

Although they are generally considered to be safe products, azole fungicides used in medicine and agriculture have been reported to have off-target effects on mammalian CYP enzymes other than CYP51, especially CYP19 and also CYP11 and CYP17 (Trösken et al., 2006); also see summaries in (Warrillow et al., 2013) and (Munkboel et al., 2019). Several of these CYP enzymes are involved in sex hormone synthesis; for example, CYP19A1, known as aromatase or oestrogen synthase, is involved in the conversion of androgens to oestrogens. At high doses, azole fungicides have generally small effects on mammalian reproduction (Zarn et al., 2003). As the European Union has banned agricultural (but not medical) fungicides with any endocrine disrupting effect at all, regardless of their value in food production, understanding the interaction

of azoles with the active site of CYP51 in fungi is of critical importance to food security, particularly in Europe. Knowledge of the molecular mechanism and evolution of azole resistance is valuable in achieving this. An example of the fruits of applying molecular information to fungicide development is mefentrifluconazole (trade name Revysol), invented by BASF AG, which has high inhibition of fungal CYP51 and low inhibition of human CYP19 with no evidence of endocrine disruption (Tesh et al., 2019). This compound is particularly interesting because it has a flexible structure which allows it to bind to the binding pocket even when the shape of the pocket has been distorted by mutation, so the fungicide retains its efficacy (Strobel et al., 2020).

Azoles interfere with the fungal enzyme lanosterol 14 α -demethylase, known as CYP51 or ERG11 (Van Den Bossche et al. 1984). Fungal CYP51 catalyzes a key step in the biosynthesis of ergosterol, a component of the fungal membrane that mediates membrane permeability and fluidity (Daum et al., 1998). CYP51 belongs to the cytochrome P450 monooxygenase (CYP) super-family. It is one of the most ancient families of proteins, exists in all biological kingdoms and has a narrow function: to remove the 14 α methyl group from sterol precursors. CYP51 proteins have narrow specificity for their sterol substrates, so specificity of an azole fungicide for the enzyme in a fungus rather than its plant or animal host depends on interactions between side-chains of the fungicide and amino acids in the CYP51 protein (Parker et al., 2014).

3.2 Cyp51 sequence variation

Widespread use of azoles in both medicine and agriculture has resulted in reduced sensitivity in many fungi. The most common cause of reduced sensitivity is point mutations in the protein-coding sequence of the *Cyp51* gene. In pathogens such as *Candida albicans* and *Zyloseptoria tritici* (formerly *Mycosphaerella graminicola*), numerous mutations have been identified (Cools et al., 2011, Warrilow et al., 2019). Over 140 mutations have been reported in *C. albicans* (reviewed by Morio et al., (2010), most of which clustered in three hot-spot regions encoding amino acids 105 to 165, 266 to 287 and 405 to 488 (Marichal et al., 1999). At least 23 of these substitutions have been shown to be involved in reduced sensitivity to azoles (Morio et al., 2010). Similarly in *Z. tritici*, 22 substitutions associated with reduced sensitivity to azoles had been identified in field isolates by 2011 (Cools and Fraaije, 2008, Leroux et al., 2007, Stammler et al., 2008, Zhan et al., 2006, Jørgensen and Heick, 2021).

Many substitutions in *Z. tritici* are at amino acid residues homologous to those altered in *C. albicans*. In fact, the most common mutations are located at equivalent residues in many fungi. For example, Y137F in *Z. tritici* is homologous to Y132 in *C. albicans*, Y134 in *Puccinia triticina* (brown or leaf rust of wheat) and Y136 in *Blumeria graminis* (powdery mildew of cereals and

grasses). To facilitate comparison between different fungi which are targets of the same fungicide, a system of unified nomenclature has been introduced (Mair et al., 2016). For each main class of fungicide, a well-studied archetype species was chosen, then orthologous amino acids in all species were assigned numerical labels based on the positions of the equivalent amino acids in the archetype protein. *Z. tritici* is the reference archetype for CYP51. Note that for β -tubulin, none of the fungal species discussed in Section 2 have insertions or deletions in amino acid sequences with respect to the archetype species, *Aspergillus nidulans*.

In contrast to *C. albicans*, *Z. tritici* and many other well-studied fungi, very few mutations have evolved in powdery mildews. The first mutation to be identified in powdery mildews was Y136F (Délye et al., 1998, Délye et al., 1997a, Délye et al., 1997b), corresponding to Y137F in the unified nomenclature (Mair et al., 2016), hereafter written [Y137F]. This mutation was first identified in grapevine mildew, *E. necator*, and was subsequently found in *B. graminis* (Wyand & Brown, 2005). This is the most common mutation for resistance to azoles and it is found in many fungi including the ascomycetes *C. albicans*, *Z. tritici* and powdery mildews and the basidiomycete rust fungi (Mair et al., 2016). [Y137] is conserved in all known wild-type CYP51 proteins in fungi and animals. It is located in the CR2 domain which is highly conserved and thought to be involved in substrate recognition (Fig. 2). This domain corresponds to Substrate Recognition Site 1 (SRS1) in human CYP51 (Podust et al., 2001, Leroux and Walker, 2011). In *E. necator*, Y136F is the only mutation found to be associated with azole resistance to date but in cereal powdery mildews other mutations have been discovered.

A larger study of barley powdery mildew expanded on the work of Délye et al. (1998), using more isolates with a wider range of resistance levels and identified a second mutation K147Q [K148] (Wyand and Brown, 2005). In plant pathogens, this mutation has only been detected in barley powdery mildew *Blumeria graminis* f.sp. *hordei* (*Bgh*) but has also been identified in *C. albicans* where it was associated with resistance to fluconazole in particular but had a limited effect on responses to itraconazole and voriconazole (Flowers et al., 2015).

Another four mutations were subsequently identified in Australia in *Bgh* isolates sampled between 2009 and 2013 (Tucker et al., 2020). These were K171E [K172], M301I [M304], R327G [R330] and S509T [S524T]. The latter three mutations are in regions conserved between *Bgh* and *Z. tritici*. The S509T mutation is of particular significance because it has spread very quickly in the Australian *Bgh* population and seems to confer the most significant increase in resistance to DMIs in Australia (Tucker et al., 2020), in particular to the triazole tebuconazole. This mutation is common in other fungi such as *Z. tritici* and is associated with decreased sensitivity to all commonly used azoles (Cools et al., 2011).

In *B. graminis*, as in *C. albicans* and *Z. tritici*, mutations in CYP51 have occurred sequentially, a process which was correlated with increasing resistance to DMI (Wyand and Brown, 2005, Tucker et al., 2020, Warrilow et al., 2019, Cools et al., 2011, Leroux et al., 2007). Resistance to DMI developed quickly but gradually in powdery mildews. Low levels of resistance first appeared in the late 1970s (Walmsley-Woodward et al., 1979). Subsequently, moderate resistance was recorded in 1982, followed by high resistance in 1984 and very high resistance in 1986 (Blatter et al., 1998, Brown et al., 1991a, Brown and Wolfe, 1991). In Australia in 2009, isolates showing moderate to high levels of resistance to tebuconazole were collected, all of which had the Y136F mutation. Higher resistance was associated with the combination of Y136F and a second mutation, S509T. The latter was first identified in *Bgh* in 2010 in Australia and by 2011 it had become universal there. Isolates carrying the doubly mutant genotype F136+T509 displayed increased resistance to all the formulations of DMIs tested including tebuconazole, suggesting that not only had resistance increased but the number of formulations affected had increased too (Tucker et al., 2015, Tucker et al., 2020). The same phenomenon has been observed in *Z. tritici* where isolates with the homologous genotype F137+T524 were identified in the early 2000 and quickly became universal. These strains showed reduced sensitivity to all azoles tested (Cools et al., 2011). The F137 genotype subsequently disappeared from the European population of *Z. tritici* (Stammler et al., 2008). The S524T mutation, on the other hand, has remained prominent in *Z. tritici*, in which this mutation appears to affect fungicide resistance most strongly when it is combined with another mutation such as L50S, D134G, V136A or Y461S (Cools and Fraaije, 2013). In a yeast heterologous expression system, the Y137F mutation alone (Y136F in *B. graminis*) caused a small decrease in DMI sensitivity but the presence of both Y137F and S524T (S509T in *B. graminis*) produced a much larger reduction in sensitivity to both tebuconazole and propoconazole (Tucker et al., 2020). Similarly, in *C. albicans* double substitutions have also been shown to greatly affect tolerance to tebuconazole compared to single mutations (Warrilow et al., 2019).

The CYP51 protein has been widely studied and crystal structures of the CYP51 in bacteria and fungi are now available (Podust et al., 2001, Hargrove et al., 2017, Hargrove et al., 2015, Keniya et al., 2018). All CYP51 proteins possess a typical P450 fold with a conserved core formed by α -helices and β -sheets. Helices E, I, J, K and L are located around the haem cavity (Figs 2 and 3). The active site which comprises the haem group is deeply embedded in the interior of the protein. In bacteria, Podust et al. (2001) have shown that azoles bind directly to the active site of the CYP51 with theazole ring of the fungicide perpendicular to the porphyrin group, such that theazole's nitrogen atom is coordinated to the haem iron. In bacteria, it is thought that the substrate

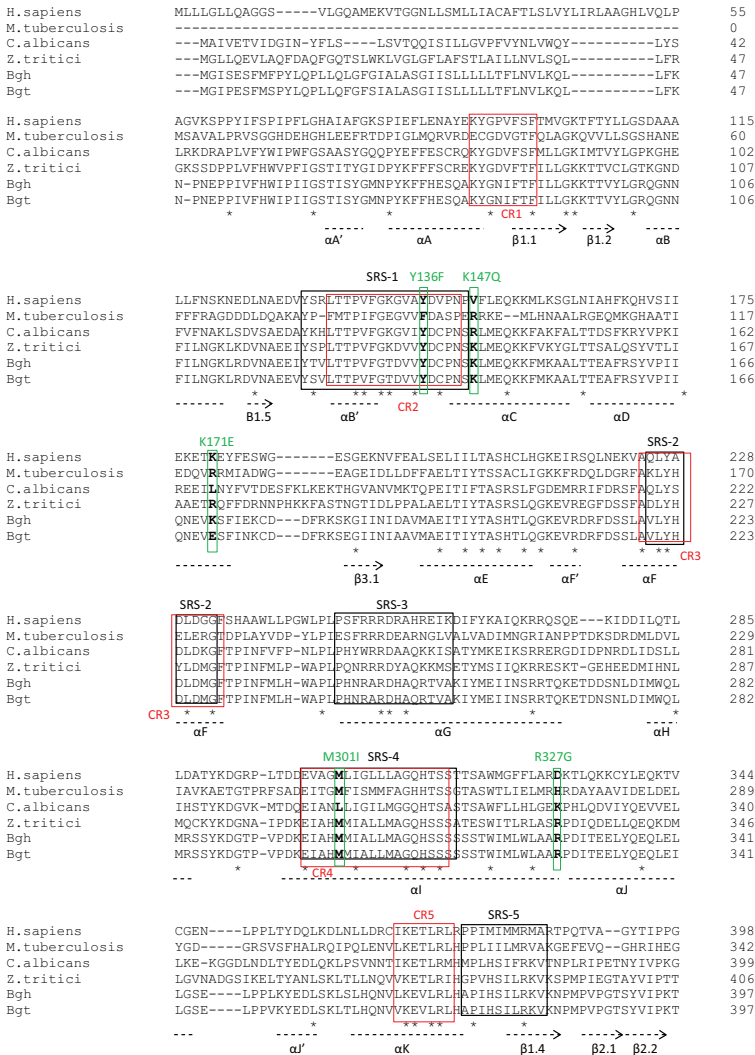


Figure 2 Amino acid sequence alignment of the *Cyp51* gene family. Sequences were downloaded as Fasta files from UniprotKB at <https://www.uniprot.org>. The alignment was carried out with ClustalOmega algorithm using the following Uniprot entries: *Homo sapiens* entry Q1658, *Mycobacterium tuberculosis* strain ATCC25618/H37Rv entry P9WPP9, *Candida albicans* strain SC5314/ATCC MYA-2876 entry P10613, *Zymoseptoria tritici* strain ST99CH_3D1 entry AOA1B1H4M, *Blumeria graminis* f.sp. *hordei* (*Bgh*) isolate DH14 entry N1J881 and *B. graminis* f.sp. *tritici* (*Bgt*) isolate JIW2 entry QZ77V5. Residues conserved across the *Cyp51* family are marked with an asterisk *. Gotoh’s predicted substrate recognition sites (SRSs) as are boxed in black (Gotoh, 1983). Conserved regions (CR) as identified by Délye et al. (1998) are boxed in red. Loci of *Bgh* mutations associated with resistance to azoles are boxed in green and the mutated amino acid highlighted in bold. α indicates α -helices and β indicates β -sheets as illustrated in Figure 2 of Leroux and Walker (2011).

H. sapiens	HQVCVSPTVNQRKDSWVERLDFNPDRLQDN-----PAS	433
M. tuberculosis	DLVAASPAISNRIPEDFDPDFVFPARYEQRQEDL-----	378
C. albicans	HYVLVSPGYAHTSERYFDNPEDFDPTRWDTAAAKAN----SVSFNSDEVDYFGKVK	454
Z. tritici	HTLLAAPGTTSRMDEHFPDCLHWEPHRWDESPSEKYKHLSPPTALGSAIEEKEDYGLVSK	466
Bgh	HSLLAAPGWTSRDASYFPNPLKWDPHRWDTGSGGVIQTDME----DEKFDYGYGLIST	451
Bgt	HSLLAAPGWTSRDASYFPNPLKWDPHRWDTGSGGVIQTDME----DEKFDYGYGLIST	451
	---> * * * * *	
	β1.3 αK'	
H. sapiens	GEKFAYVFPFGAGRHRICIGENFAYVQIKTIWSTMLRLRYEFDLIDGYF--PTVNYITMIIITFP	491
M. tuberculosis	LNRWITNIPFGAGRHRICVGAFAIMQIKAIKIFSVLLREYEFEMAQPP--ESYRNDHSMKVVQL	437
C. albicans	GVSSPYLPFGGRRHRICIGEQFAYVQLGTILITTFVYNLRWT-ID-GYKVPDPDYSMMVQLP	512
Z. tritici	GAASPYLPFGAGRHRICIGEQFAYVQLQTIATMVRDFKFNVDGSKVVGTDYSSLSFSSRP	526
Bgh	GAASPYLPFGAGRHRICIGEQFATVQLVTIMATMVRSFKFNHLDGRNSVAETDYSMSFSRP	511
Bgt	GAASPYLPFGAGRHRICIGEQFATVQLVTIMATMVRSFKFNHLDGRNSVAETDYSMSFSRP	511
	CR6 ***** * ** * *	
	-----> ----->	
	αL β3.3 β4.1	
H. sapiens	ENPV-IRYKRRSK-----	503
M. tuberculosis	AQPACVYRRRTGV----	451
C. albicans	TEPAEIIWEKRETCMF---	528
Z. tritici	LSPAVVKWERREEKEKN-	542
Bgh	MAPATIAWEKRDKKDKTEC	530
Bgt	MAPATIAWEKRDKKDKTEC	530
	* * *	
	---> ----->	
	β4.2 β3.2	

Figure 2 (Continued)

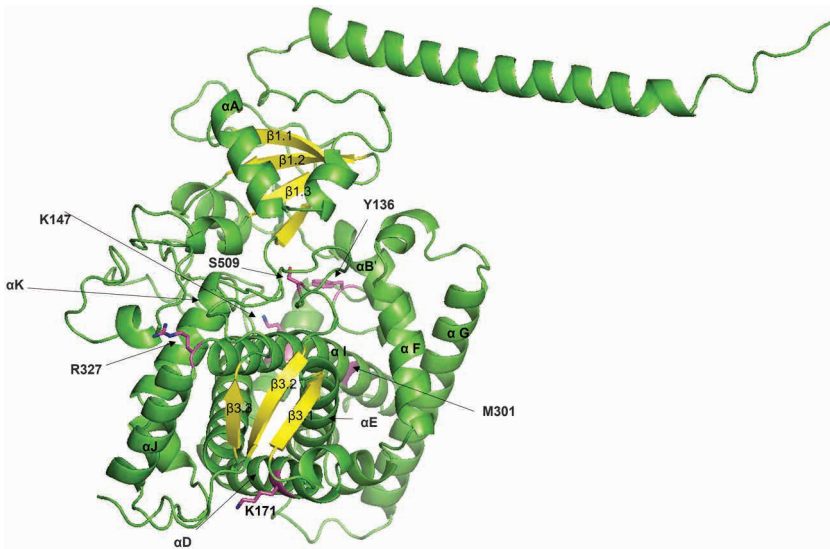


Figure 3 AlphaFold prediction of the structure of the azole-sensitive form of the *Blumeria graminis* f.sp. *hordei* CYP51 protein. β-sheets are in yellow. α-helices are in green. Amino acid residues which are sites of known mutations associated with azole resistance (Section 3) are in purple. The AlphaFold model was created by Oliver Powell.

enters the binding cavity through Entrance Channel 1 which runs parallel to the haem plane. Further characterisation of crystal structures in human have shown that eukaryotic and bacterial CYP51 proteins have different access channels (reviewed by Becher and Wirsal, 2012). Furthermore, some fungi such as *C.*

albicans have an additional external loop between residues 428 and 459. In *C. albicans*, substitutions associated with azole resistance have been mapped to the catalytic site (Y132F/H [Y137]), and the region below the haem (K143R [K148]) (Flowers et al., 2012).

The substitution of tyrosine by phenylalanine as Y136F [Y137F] in *B. graminis* results in a small but significant change. Tyrosine and phenylalanine have similar structures except that tyrosine has a hydroxyl group which is missing in phenylalanine (Table 1). The loss of the hydroxyl group is thought to increase the hydrophobicity at the border of the catalytic pocket without changing its configuration (Délye et al., 1997b). Tucker et al. (2020) showed that in *Bgh* the Y136F mutation resulted in an increased volume of the binding cavity and a reduction of the diameter of the access channel. It is thought that greater binding cavity volume may perturb the orientation of the fungicide molecule. The reduction of the diameter of the access channel is thought to affect the ability of larger fungicide molecules to access the haem cavity. In *Z. tritici*, the Y137F substitution was also shown to result in an overall increase in the size of the binding pocket. The F137 residue appeared to be in an obstructive position which may prevent the binding of triadimenol but not other azoles. This might explain why the frequency of F137 in *Z. tritici* has fallen as tebuconazole has largely been replaced by more modern triazoles for use on arable crops. F137 may also have an effect on Y459, causing it to move away from the position where it can bind to azole molecules. As the frequency of F137 in *Z. tritici* has declined in Europe, mutations which included a deletion around position Y459-Y461 have since become dominant in this fungus (Mullins et al., 2011).

K147Q [K148] in *Bgh* results in the substitution of glutamine for lysine. Both residues are similar (Table 1) but lysine is more polar. Similarly in *C. albicans*, the homologous mutation K143R results in the substitution of glutamine for arginine. Both arginine and lysine are thought to increase the net positive charge of the β loop where they are located. In *C. albicans*, K143R has been mapped to the other side of the haem group from the docking site of fluconazole (Flowers et al., 2015). It is thought a net positive charge may affect the orientation of the substrate in the binding cavity (Wyand and Brown, 2005).

Similarly, the change from serine to threonine at amino acid 509 [S524T] in *Bgh* is small because threonine only differs from serine by the addition of a methyl group (Table 1). S509T resulted in the greatest increase in cavity volume recorded by Tucker et al. (2020). More strikingly, the combination of F136 [F137] and T509 [T524] increased the volume of the cavity even further. In *Z. tritici*, the [S524T] mutation is thought to cause the loss of two β -sheets in the vicinity of the binding cavity, leading to changes in the conformation of the cavity, making it more open and thereby reducing its affinity for the azole (Cools et al., 2011).

3.3 Expression of *Cyp51*

A second mechanism of resistance to azole involves increased expression of the *Cyp51* gene. In vitro, over-expression of *Cyp51* in *Saccharomyces cerevisiae* conferred resistance to fluconazole (Kontoyiannis et al., 1999). In vivo, increased expression of *Cyp51* has been shown to be linked to azole resistance in several fungi. In *Penicillium digitatum*, transcription was enhanced by the insertion of a 126-bp sequence tandemly repeated five times in the promoter of the *Cyp51* gene. Strains with the five repeats were resistant to a range of azoles whereas strains with only one copy of the enhancer sequence were sensitive. In the resistant strains, constitutive expression of *Cyp51* was about 100-fold higher than in azole-sensitive strains (Hamamoto et al., 2000). In *Venturia inaequalis*, a 553-bp insertion upstream of the *Cyp51A* gene was identified in myclobutanil-resistant isolates with high levels of *Cyp51* gene expression (Schnabel and Jones, 2001). Similarly, in the cherry leaf spot pathogen *Blumeriella jaappi*, the insertion of a retrotransposon sequence into the promoter was identified in azole-resistant isolates. This insertion was associated with a 5 to 12-fold increase in expression of the *Cyp51* gene (Ma et al., 2006). In *Z. tritici* a 120-bp insertion in the promoter of the *Cyp51* gene was also associated with over-expression of CYP51 and increased resistance to azoles (Cools et al., 2012).

A microarray experiment conducted by Dunkel et al. (2008) in *C. albicans* identified a transcription factor called *Upc2* as a regulator of *Cyp51*. This study showed that a gain of function mutation in *Upc2* resulted in over-expression of *Cyp51* and other genes in the ergosterol biosynthesis pathway and in higher resistance to azoles and other fungicides involved in the ergosterol pathway such as terbinafine.

In powdery mildews, enhanced expression of *Cyp51* has been associated with increased azole resistance. In *E. necator*, Frenkel et al. (2015) compared isolates from the US and Chile and found that those with the Y136F mutation had higher expression of *Cyp51*. A second mutation was identified in the *Cyp51* DNA sequence at base 1119 (*a1119c*). This synonymous mutation, which did not change the protein sequence, was associated with higher expression of the gene and increased resistance to azoles. This may have been the result of genetic hitch-hiking, with the (*a1119c*) mutation occurring by chance in a genotype carrying another mutation, as yet uncharacterised, which increased *Cyp51* expression, possibly in the promoter. Azole resistance in the *c1119* isolates was comparable to that of isolates with the F136 genotype.

3.4 Copy number variation

A second study by Jones et al. (2014) investigated isolates from seven vineyards in California and found that *Cyp51* copy number varied greatly. Isolates with

high *Cyp51* copy number were significantly more likely to be present in azole-treated vineyards and all isolates with high copy number had the Y136F mutation suggesting that higher copy number only became advantageous once the resistance allele has been acquired. A further study by Rallos and Baudoin (2016) confirmed the existence of the silent mutation (*a1119c*) in American vineyards and its association with over-expression of *Cyp51* and azole resistance. Interestingly, this study found isolates with two alleles with either the Y or F amino-acids at residue 136, demonstrating gene duplication or increased copy number.

Duplication of the *Cyp51B* gene was also identified in a study of *B. graminis* f.sp. *tritici* (*Bgt*; powdery mildew of wheat) by Arnold (2018). Among US and UK isolates, those with both F and Y at residue 136 [137] were identified. In the UK population, these heteroallelic isolates were also heteroallelic at position 509 [524] with F136 always linked to T509 and Y136 to S509. In US isolates, the T509 mutation did not occur so heteroallelic isolates were either F136/S509 or Y136/S509. Heteroallelic isolates from the US had greater resistance to tebuconazole and prothioconazole on average than homoallelic isolates with Y136 only and similar resistance as homoallelic F136 isolates. In the UK population, heteroallelic Y136+S509 / F136+T509 isolates were significantly more resistant to both azoles than homoallelic F136 isolates, which in turn were more resistant than the Y136 isolates. *Cyp51* expression was similar in all heteroallelic isolates, whether from the US or the UK, to that of homoallelic F136 isolates. Copy number and gene expression was higher in UK isolates than in the US isolates, which was correlated with higher resistance to both triazoles. These findings indicate that in *Bgt*, increased resistance to azoles is associated with the presence of the Y136F mutation, increased *Cyp51* gene expression and higher copy number. Stronger resistance is associated with the combination of the Y136F and S509T mutations, especially in heteroallelic isolates with both Y136+S509 and F136+T509 copies of CYP51. Remarkably, no other coding sequence mutation was detected in these isolates. The small number of ORF mutations in *Cyp51* in *B. graminis* is in striking contrast to *Z. tritici* and *C. albicans*.

Copy number variation mediating resistance to azoles is not unique to powdery mildews. In *Candida glabrata*, increased expression of *Cyp51* was associated with increased resistance to azoles. In this instance, over-expression resulted from higher copy number in azole-resistant isolates in which the entire chromosome containing the *Cyp51* gene was duplicated (Marichal et al., 1997). Similarly, aneuploidy involving the formation of an isochromosome composed of the two left arms of chromosome 5, including the *Cyp51* locus, was associated with azole resistance in *C. albicans* (Selmecki et al., 2006).

3.5 Paralogue divergence

In Pezizomycotina, a subphylum of Ascomycota which comprises many plant and most human pathogens, Becher et al. (2011) found great variation not only in the copy number but also in the subtypes of *Cyp51* genes. Phylogenetic analysis revealed three clades of CYP51 proteins, A, B and C encoded by paralogues genes *Cyp51A*, *B* and *C*. Clades A and B are the most common (Becher et al., 2011). Indeed, both CYP51A and CYP51B have been found in fungi such as *Aspergillus fumigatus* (Mellado et al., 2001), *A. nidulans* (Ferreira et al., 2005) and *Magnaporthe oryzae* (Yan et al., 2011). Other fungi such as *Aspergillus oryzae* (Ferreira et al., 2005) and *Fusarium* spp. have three paralogues of the CYP51 enzyme (Deng et al., 2007), CYP51A, B and C. There is striking variation within *Aspergillus*, in which some species such as *A. fumigatus* and *A. nidulans* have only one A- or B-type of CYP51 protein while other species have undergone recent duplication of one protein type. In *A. oryzae* and *A. terreus*, the *Cyp51A* gene has been duplicated and in *A. terreus* CYP51B has been duplicated. It is not known why some fungi have multiple paralogues and others do not, but knocking out one of the paralogues in *A. fumigatus* and *F. graminearum* resulted in increased sensitivity to azoles suggesting that additional paralogues may play a role in reducing sensitivity (Mellado et al., 2005, Fan et al., 2013). Furthermore, in *Rhynchosporium commune* (formerly *R. secalis*), *Cyp51A* had been lost from multiple lineages but re-emerged in the *R. commune* population when azoles were introduced in UK fields; this re-emergence was associated with increased resistance to azoles (Hawkins et al., 2014). In *A. flavus*, *A. oryzae* and *A. terreus*, phylogenetic studies have shown that the *Cyp51* gene (*Cyp51A* in the first two pathogens and *Cyp51B* in the latter) have recently been duplicated. Cereal powdery mildews have the *Cyp51B* paralogue gene (Mair et al., 2016).

3.6 Over-expression of efflux pumps

Finally, the third mechanism of resistance to azoles involves the over-expression of plasma membrane efflux pumps. Fungi can become resistant to multiple unrelated antifungal compounds with different modes of action by increasing the rate at which they transport them out of their cells. This phenomenon is called multi-drug resistance or MDR. It became an important clinical problem in the 1990s when cancer tumors suddenly became resistant to chemotherapeutic drugs (Gottesman and Pastan, 1993). There are two families of efflux pumps, the major facilitator super-family (MFS) and the ATB-binding cassette (ABC) pumps. MFS pumps have a limited range of substrates whereas ABC pumps have broader specificity. ABC transporters are found in all organisms. In prokaryotes they are involved in both the uptake and the efflux of compounds whereas in

eukaryotes their function seems limited to efflux (Driessen et al., 2000). ABC transporters have been identified in many organisms and linked to resistance to drugs such as azoles. In clinical *Candida* isolates, over-expression of both ABC and MFS efflux pumps is the major mechanism for high-level resistance to azoles. Efflux pumps genes *Cdr1*, *Cdr2* (which encode ABC transporters) and *Md1* (encoding MFS transporters) are involved with azole resistance in *Candida* species (White, 1997). In vitro, some azole-resistant *C. albicans* strains expressed multiple pumps (Rogers and Barker, 2003). In *A. nidulans*, two genes, *atrA* and *atrB*, encoding ABC transporters were identified. Transcription of both was enhanced within a few minutes of treatment by several drugs including azole fungicides. Heterologous expression of *atrB* in an ABC-transporter-deficient yeast strain restored multi-drug resistance (Del Sorbo et al., 1997). A further study by deletion and over-expression mutants showed that *atrB* functions as a multi-drug transporter with affinity to substrates belonging to all major classes of fungicides including azoles (Andrade et al., (2000).

The role of ABC transporters in fungicide resistance has been documented in plant pathogens. In *Botrytis cinerea*, the *BcatrD* gene was cloned and its function characterised using gene-replacement and over-expression mutants. Replacement mutants where *BcatrD* had been replaced by the selectable marker hygromycin exhibited increased sensitivity to oxpoconazole and accumulated high amounts of the fungicide. Furthermore, mutants with the highest expression levels of *BcatrD* were the most resistant to oxpoconazole (Hayashi et al., 2002). Similarly in *Z. tritici*, ABC transporter encoding genes *MgAtr1* and *MgAtr2* have been cloned and characterised. Both were upregulated after treatment with cyproconazole (Zwiers and De Waard, 2000). Stergiopoulos et al. (2002) expanded on these experiments by cloning and characterising three more transporter genes, *Mgatr3*, *Mgatr4* and *Mgatr5* and showing that both *Mgatr4* and *Mgatr5* are upregulated by exposure to cyproconazole. Leroux and Walker (2011) tested the effect of known inhibitors of efflux pumps on resistance to azoles and other drugs, finding that changes in the sequence of *Cyp51* combined with over-expression of efflux pumps resulted in MDR in *Z. tritici*.

MFS pumps have not been as well-studied as ABC pumps but they have been shown to play a role in MDR in plant pathogens. In *Z. tritici* for example, *MgMfs1*, has been identified from an expressed sequenced tag (EST) screen. Heterologous over-expression of *Mgmfs1* in a hypersensitive strain of *S. cerevisiae* yeast resulted in increased resistance to several fungicidal compounds including azoles (Roohparvar et al., 2007).

In powdery mildews, the role of efflux transporters in mediating fungicide resistance has not been studied in depth. Frenkel et al. (2015) identified three ABC transporters and one MFS homologues in the genome of *E. necator* but found no evidence that they contributed to resistance to an azole fungicide, myclobutanil.

4 Amines: inhibitors of sterol reductase and sterol isomerase

Heterocyclic amine fungicides include morpholines, piperidines and spiroketalamines. They are especially effective against powdery mildews and rusts, and are used as both eradicators and protectants (Baloch and Mercer, 1987, Mercer, 1991). Amines are highly effective in inhibiting mycelial growth and often cause abnormal mycelium formation (Mercer, 1991). Like the azoles, morpholines target the ergosterol biosynthesis pathway but at two target enzymes: Δ^{14} -reductase (ERG24) (Lorenz and Parks, 1992) and $\Delta^8 \Delta^7$ -isomerase (ERG2) (Ashman et al., 1991) (Fig. 4). Inhibition of either Δ^{14} -reductase or $\Delta^8 \Delta^7$ -isomerase results in the depletion of ergosterol and the accumulation of toxic sterols such as ignosterol and fecosterol (Lorenz and Parks, 1991, Marcireau et al., 1990). Studies in *S. cerevisiae* and *Ustilago maydis* have shown that different morpholines act differently on each enzyme. For example, in yeast fenpropimorph mainly inhibits Δ^{14} -reductase whereas tridemorph mainly inhibits $\Delta^8 \Delta^7$ -isomerase (Baloch et al., 1984, Baloch and Mercer, 1987). In *C. albicans*, *Erg24* is involved in pathogenicity and resistance to fenpropimorph but not to tridemorph (Jia et al., 2002). Like *Cyp51*, some species have evolved to have two paralogues of *Erg24*. In *Fusarium*, *Egr24a* and *Erg24b* have been identified and disruption of the ERG24B protein results in hypersensitivity to fenpropidin and fenpropimorph (Liu et al., 2011).

In the UK, the main amine fungicides used have been the morpholines fenpropimorph, dodemorph and tridemorph and the piperidine fenpropidin. Dodemorph and tridemorph were introduced in the late 1960s for control of powdery mildew on diverse crops but the introduction of fenpropimorph and fenpropidin in the mid-1980s produced a step-change in the ability of farmers to control mildew even on the most susceptible varieties, such as Golden Promise spring barley. Within a few years, however, decreased sensitivity was identified in barley mildew in Scotland where these molecules had been used regularly. Indeed by 1988, Brown et al. (1991b) sampled isolates with reduced sensitivity to both fenpropimorph and fenpropidin in Scottish barley fields. Application of either of these fungicides selected isolates with reduced sensitivity to both of them, whereas application of tridemorph selected against resistance to fenpropidin (Brown and Evans, 1992). Resistance was incomplete and there was still moderately good field control of mildew by doses of fungicides lower than the recommended field application rate. To date this is still the case and fenpropimorph is still in use today to control cereal powdery mildew in the field.

In 2014, a population of *Bgt* with moderate resistance to fenpropimorph was identified in a glasshouse in the UK containing experimental populations of wheat (Arnold, 2018). Sequencing of the genes encoding the target enzymes of amine fungicides identified a mutation in the open reading frame of the *Erg24* gene in *Bgt*, V295L. A fenpropimorph-resistant *Bgh* isolate retained

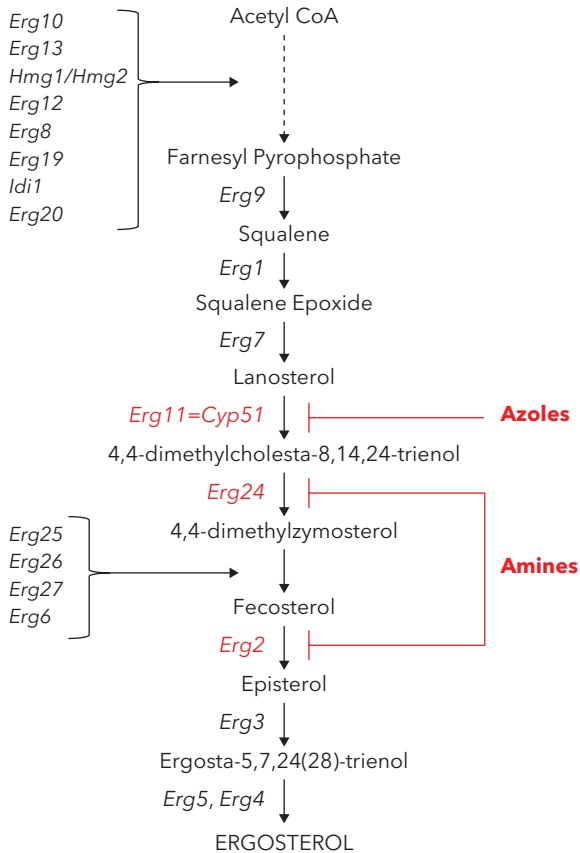


Figure 4 Summary of the ergosterol biosynthesis pathway in fungi. Fungicides which target the pathway are on the right-hand side and genes encoding key enzymes are on the left. Azoles and amines and genes for their respective target enzymes are highlighted in red.

from the study by Brown et al. (1992) had a D291N mutation in *Erg24*. No mutation was identified in *Erg2* in either *Bgh* or *Bgt*. To our knowledge, this is the only evidence of mutation in the ERG24 protein linked to fungal resistance. In *C. albicans*, over-expression of either *Erg24* or *Erg2* in the lab resulted in resistance to morpholines (Luna-Tapia et al., 2015) but this potential mechanism of resistance has not yet been studied in natural or clinical strains of the fungus.

5 Succinate dehydrogenase inhibitors: inhibitors of respiration at Complex II

Aerobic respiration happens all the time in animal, plants and fungi as a process by which organisms use oxygen to turn food into energy. This

process involves the transfer of electrons through the mitochondrial membrane via a series of protein complexes. The respiration process starts with the oxidization of NADH into NAD by the first protein complex, which is at Complex I. This releases an electron which enters the respiration chain. Electrons then travel through protein Complexes II, III and IV until they reach the final acceptor, molecular oxygen (Fig. 5). There are three main classes of fungicides which target fungal respiration: the succinate dehydrogenase inhibitors (SDHIs) which target Complex II, and the Quinone-outside inhibitors (QoIs) and Quinone-inside inhibitors (QiI), which target different sites in Complex III.

SDHIs block the fungal respiration process by binding to the ubiquinone reduction site of Complex II of the respiratory chain also known as succinate dehydrogenase (SDH) or succinate:ubiquinone reductase (SQR). This group of fungicides have a carboxamide structure and are thus sometimes known as carboxamides. The first formulation, Carboxin, introduced in 1966 (Schmeling and Kulka, 1966) had a limited spectrum of action and it was mainly used to control smuts. Subsequently, new molecules were developed between 1971 and 1997 but the range remained limited until the release of boscalid in 2003 (Matheron and Porchas, 2004, Stammler et al., 2008, Stammler and Speakman, 2006). Boscalid was the first member of this class of fungicide to control a broad range of pathogens of fruit, vegetables and cereals, which was achieved by

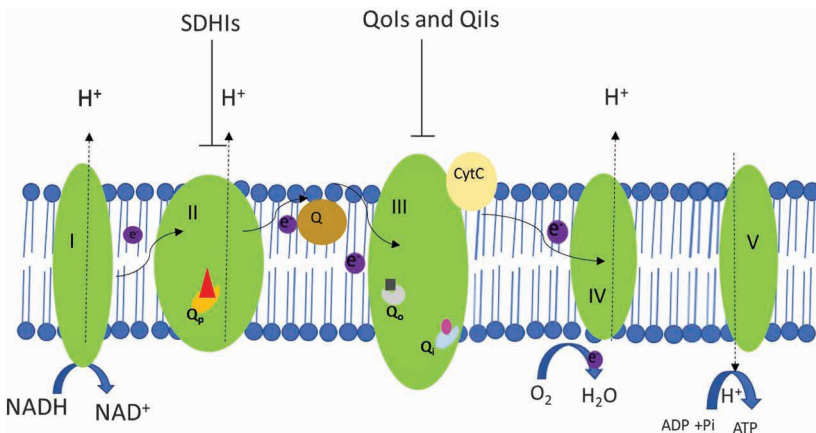


Figure 5 Schematic representation of the mitochondrial respiration chain. I, II, III and IV are the different protein complexes of the electron transfer chain. V is the ATP synthase complex. Q is the ubiquinone pool. CytC is the peripheral protein cytochrome c. Electron transfer route is shown with black arrows. The Qp binding site in Complex II is delineated by a red triangle representing Qp-inhibitor molecules (SDHIs). The Qo and Qi binding sites in Complex III are delineated by a dark gray square and a pink circle respectively representing Qo and Qi-inhibitor molecules respectively (QoI and QiIs).

significant modifications of the carboxamide molecule (Glättli et al., 2010). The discovery of boscalid triggered a renaissance of SDHIs and a new generation of SDHIs with broader specificity were released from 2010 onwards. SDHIs have been on the market for over 40 years and over the last 20 years, have become the class of fungicide with the most compounds released onto the market for crop disease control.

SDHIs are a structurally diverse class of fungicide with a broad spectrum of action. They all have in common an amide bond, which is strictly conserved among SDHIs and defines the orientation of the molecule. It is essential for the hydrogen-bond interactions in the ubiquinone-binding site of SDH. The SDH enzyme is a mitochondrial heterodimer formed by four subunits: SDHA (flavoprotein), SDHB (iron sulfur subunit), SDHC (cytochrome b560 subunit) and SDHD (cytochrome b small subunit). SDHC and DARE membrane proteins which anchor the SDHAB dimer to the membrane of the mitochondria. Amino acid sequences of the *SdhA* and *SdhB* subunits are highly conserved among fungal species in contrast to the *SdhC* and *SdhD* units which are diverse. The SDH enzyme transfers succinate-derived electrons to the ubiquinone pool of the respiratory chain and as such SDH is an essential component of the respiratory chain (Cecchini, 2003, Huang and Millar, 2013, Hägerhäll, 1997). All crop protection SDHIs target the hydrophobic ubiquinone-binding pocket (Qp) formed by residues of the subunits B, C and D. The fungicide binds to the Qp site, thus blocking access for the substrate and preventing further cycling of succinate oxidation. Crystal structures of SDH have been determined (Yankovskaya et al., 2003, Sun et al., 2005, Huang et al., 2006) and several homology models and docking predictions have been drawn for common plant pathogens such as *Z. tritici* and *B. cinerea* (Fraaije et al., 2012, Scalliet et al., 2012, Ruprecht et al., 2009, Amiri et al., 2020). Key amino acids which form the Qp pocket have been identified in *SdhB*, *SdhC* and *SdhD* (Horsefield et al., 2006, Hägerhäll, 1997, Sierotzki and Scalliet, 2013). The reference species for numbering of *Sdh* gene sequences in relation to fungicide resistance mutations is *Pyrenophora teres* (Mair et al., 2016). In *Z. tritici* for example, W224 [W234] in *SdhB*, Y130 [Y146] in *SdhD* and S83[S72] in *SdhC* all establish hydrogen bonds with the central amid bond and are directly involved with ubiquinone binding and catalysis while A87 [A87] in *SdhC* and H267 [H277] in *SdhB* are both buried deep in the binding pocket and are thought to interact with the core section of SDHI fungicides (Sierotzki and Scalliet, 2013).

More than 40 amino acid mutations encoded by *SdhB*, *SdhC* and *SdhD* have been linked to decreased sensitivity to SDHIs in a wide range of pathogenic fungi, notably *B. cinerea*, *Z. tritici*, *Sclerotinia sclerotiorum* (*S. sclerotiorum*) and *Alternaria alternata*. The most frequent mutations have been reported in *SdhB* at 277 [H277R/Y/N/L] (Sang and Lee, 2020), where H227R and H227Y are the

most common changes. Less frequent are mutations at codon 230 [P230F/L/T] and 235 [N235 I/T] in *SdhB*. *SdhC* is the subunit where the most mutations have been identified across fungi as a whole with 14 mutations, while 6 mutations have been identified in *SdhD* (reviewed by Sang and Lee (2020) and Vielba-Fernández (2021)).

In powdery mildews, decreased sensitivity to boscalid has been observed sporadically in grapevine mildew *E. necator* since 2014. Cherrad et al. (2018) studied isolates from French vineyards resistant to boscalid in 2015 and identified two mutations in *SdhB* associated with resistance homologous to those reported in other fungi, H242R/Y [H277R/Y]. Resistance was specific to boscalid and did not affect the other SDHs tested, fluxapyroxad and fluopyram. No other mutations were identified in *SdhC* although, in Germany (Graf, 2017) identified two mutations in *SdhC*, both at residue 169 in *E. necator* [G169 D/S]. In cucurbit mildew *P. xanthii*, decreased sensitivity to a range of SDHs including boscalid has been reported in the US, Japan and Spain (Vielba-Fernández et al., 2021, Miyamoto et al., 2020, McGrath, 2008, Keinath et al., 2018). In Japan, boscalid was introduced to control pathogens such as gray mold (*B. cinerea*) and sclerotinia rot (*S. sclerotiorum*) on cucumber and was not specifically targeted at the control of powdery mildews. Nonetheless, resistance to boscalid occurred in *P. xanthii* in 2008 and 2009, probably because of the selection pressure from fungicide applications to control other diseases. Four new generation SDHs have been registered specifically to control cucurbit powdery mildew in Japan from 2010 onwards and various levels of resistance have developed to all of them, from moderate to very high (Miyamoto et al., 2020). Gene sequencing showed that mutations in *SdhD* S121P [S118], H137R [H134R] and *SdhC* A84V [S73 P/V], G172D [G159] and G151R [G138] were associated with decreased sensitivity to SDHs in Japan and Spain (Miyamoto et al., 2020, Vielba-Fernández et al., 2021). It is worth noting that, in these studies, less than 50% of the isolates tested showed decreased sensitivity to any of the SDHs used in the experiments. Even so, polymorphism in the level of sensitivity would still be detrimental to crop management if the more resistant genotypes were selected by continued use of the fungicide.

To our knowledge, there has been no report of resistance to SDHs in cereal powdery mildews to date. Cereal powdery mildews are not the primary target for this class of fungicide (AHDB, 2022). Nevertheless, given that SDHs are widely used on cereals against *Z. tritici* of wheat, *Rhynchosporium commune* of barley and other diseases, *B. graminis* is being exposed to them. Future studies of responses of cereal powdery mildew to SDHs will need to compare current isolates with those in historic collections sampled before the introduction of boscalid.

6 Quinone-outside inhibitors: inhibitors of respiration at Complex III

A second group of fungicides that target fungal respiration is Qols, also known as the strobilurin group. Qols were introduced to the market in 1996 and quickly became one of the most important classes of agricultural fungicide, accounting for 20% of the global fungicide market within the first 10 years of introduction (Bartlett et al., 2002). Qols are active against all major classes of fungal pathogen although control levels vary. They have been of particular value against powdery and downy mildews and brown rusts (Balba, 2007). They revolutionised disease control of grapevine mildew because, for the first time, both powdery and downy mildews could be controlled using one fungicide even though the causal organisms are in different kingdoms of eukaryote. In cereals, Qols are associated with increased yield and grain quality. This is called the 'greening effect', which seems to be caused by delayed senescence (Bartlett et al., 2002). It has also been shown that azoxystrobin (one of the first Qols, released in 1996) inhibits the production of ascospores and oospores of grapevine powdery and downy mildews, potentially reducing the evolutionary potential of these pathogens in vineyards (Godwin and Cortesi, 1999, Vercesi et al., 2001).

The Qol group binds to the quinol oxidation (Qo) site of the cytochrome *bc1* protein complex, also known as Complex III of the respiration chain. Complex III is formed by 10 to 11 polypeptides in eukaryotes with the catalytic site of the protein being formed by 3 subunits: cytochrome *b*, cytochrome *c1* and the Rieske iron sulfur protein (ISP) (Becker et al., 1981). The catalytic mechanism is called the Q-cycle and it requires two distinct binding sites, Qo, the ubiquinol oxidation site, and Qi, the ubiquinone reduction site. Both sites are located within the cytochrome *b* subunit but on opposite sides of the mitochondrial membrane, Qo on the outside of the mitochondrion and Qi on the inside (Fig. 5). The Qol fungicides target the Qo site whereas some natural products such as Antimycin A and a new fungicide, fempicoxamid, target the Qi site. All the Complex III subunits are encoded by the nucleus except cytochrome *b*, which is encoded by the mitochondrial genome. Qols bind tightly to the Qo pocket preventing binding of ubiquinol. This inhibition blocks the transfer of electron between cytochrome *b* and cytochrome *c1*, leading to energy deficiency in the fungal cell (Fisher et al., 2020, Vielba-Fernández et al., 2021).

Resistance to Qols emerged very quickly in many pathogens including powdery mildews. In Europe, Qols were introduced in 1996 to control cereal powdery mildews and by 1998 resistant wheat isolates of *Bgt* were identified in Germany with high levels of resistance (resistance factor >50) (Chin et al., 2001). In Japan, a rapid decline of Qol efficacy against cucumber powdery mildew

was reported within two years of QoI introduction to the market (Grasso et al., 2006). In the US, QoIs were introduced in 1998 to control grapevine downy and powdery mildews (*Plasmopora viticola* and *E. necator* respectively). In 1999, Wong and Wilcox (2002) found that two per cent of the *E. necator* population was resistant to low doses of azoxystrobin. Within a few years, a much higher proportion of the pathogen population was resistant (90% in Virginia) to much higher doses of azoxystrobin (resistance factor >100) resulting in control failure (Baudoin et al., 2008). Resistance persisted for at least 4 years after QoIs were no longer applied to control *E. necator* (Rallos et al., 2014). Evidence for similar strong selection for resistance has been established in other species of powdery mildews such as sugar beet and apple powdery mildews (reviewed by Vielba-Fernández et al. (2020).

In *E. necator*, the resistance described earlier was caused by the substitution of glycine by alanine at position 143 in the Cytochrome b protein (G143A) which corresponds to G143A in the reference archetype species for *Z. tritici*. Mutations in the *Cytb* gene have been found in two regions corresponding to amino acids 120-155 and 255-280 of the Qo site (Degli Esposti et al., 1993, Sierotzki et al., 2000, Fisher and Meunier, 2001). In the folded cytochrome b protein, these domains are close to each other and important for substrate binding (Brasseur et al., 1996, Geier et al., 1994). The G143A substitution is by far the most common to QoI-resistance, having been found in over 25 species of phytopathogenic ascomycetes and oomycetes including *Mycosphaerella* (including *Zymoseptoria*), *Alternaria*, *Blumeria* and other fungal species (reviewed by Fernández-Ortuño et al. (2010). Interestingly, A143 is the wild-type residue in basidiomycetes that naturally produce QoIs, such as *Mycena galopoda* (Kraiczy et al., 1996). G143A is associated with complete or qualitative resistance. It is thought to result in steric hindrance between the fungicide and the cytochrome b but does not hinder interaction of the protein with the ubiquinol substrate (Esser et al., 2004). (Steric hindrance at a given atom in a molecule is the congestion caused by the physical presence of surrounding ligands which slow down or prevent a reaction at the atom.) It is thought that G143A has spread very rapidly among pathogen populations because it confers a high level of resistance without a negative effect on enzyme activity (Rallos et al., 2014).

Not all pathogen species have evolved the G143A mutation, however. In all rust species, resistance has never developed. Grasso et al. (Grasso et al., 2006) identified an intron located immediately after the G143 codon which was predicted to alter splicing and thus to prevent this mutation from occurring. In some powdery mildew species such as *P. xanthii*, both the G143 and A143 alleles of *Cytb* co-exist in the same cell, a phenomenon called heteroplasmy (Vielba-Fernández et al., 2018). In sexual crosses, *B. graminis*, which is a hermaphrodite, inherits the maternal genotype at *Cytb* and in a cross between a QoI-sensitive isolate with G143 and a resistant one with A143, all progeny of

any one chasmothecium, the sexual structure of powdery mildews, had either G143 or A143 (Robinson et al., 2002).

Other common substitutions in pathogenic fungi and oomycetes including downy mildews and rusts are F129L (phenylalanine to leucine) and G137R/S (glycine to arginine or serine) (Sierotzki et al., 2007, Gisi et al., 2002), which both confer less resistance than G143A. The side chain of F129 is oriented toward the hydrophobic cavity that facilitates substrate access to Qo. It is thought that F129 is involved with van der Waals and aromatic-aromatic interactions between the QoI fungicide and the bc1 complex proteins. The F129L substitution is thought to destabilise both types of interactions between the bound fungicide and the bc1 proteins (Esser et al., 2004). Like G143A, it is thought that F129L does not affect vitality or fitness of isolates carrying the mutation (Sierotzki et al., 2007, Esser et al., 2004). This is in contrast with mutations at G137, located at the N-terminal region of helix cd1. It is thought that the G137R/S mutation results in the distortion of the loop connecting helices C and cd1 by arginine or serine, which are bulkier than glycine. In yeast, the G137R mutation caused a significant reduction in respiratory growth (Fisher and Meunier, 2001). In phytopathogenic fungi, mutations at G137 have been identified in the lab but have remained rare in the field (Sierotzki et al., 2007, Standish et al., 2016, Standish et al., 2018).

7 Conclusion

Despite improvements in agronomy and breeding (Brown 2021), fungicides continue to play an integral role in sustaining food production. The challenges of climate change and an ever-growing human population means that fungicides will still be needed for the foreseeable future. Yet resistance to modern single-target fungicides is now common, particularly in agriculture where many important pathogens have developed reduced sensitivity to many of the major classes of systemic fungicides. These compounds now need to be used carefully to maintain efficacy. The most common mechanism for resistance to all the major classes of fungicide used in agriculture today is mutation in the target gene. A single amino acid substitution can alter the conformation of the binding site and thus reduce affinity for the fungicide. These mutations are often conserved between pathogens. Some fungi have evolved more complex mechanisms of resistance to the most commonly used fungicides such as the azoles, where pathogens have evolved resistance through a combination of mechanisms such as mutation of the target gene, copy number variation and over-expression of the target gene, as well as changes in transport processes. There is a constant arms race between pathogen evolution and fungicide discovery, and new fungicides are needed urgently, both for agriculture and medicine (Fisher et al., 2018). The advent

of new technologies, such as protein modelling, prediction of substrate and inhibitor docking, and real-time pathogen detection may help the chemical industry develop compounds with safer chemistries and new, more durable modes of action.

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