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Review

Impact of fungal drug transporters on fungicide sensitivity, multidrug resistance and virulence†

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Abstract: Drug transporters are membrane proteins that provide protection for organisms against natural toxic products and fungicides. In plant pathogens, drug transporters function in baseline sensitivity to fungicides, multidrug resistance (MDR) and virulence on host plants. This paper describes drug transporters of the filamentous fungi *Aspergillus nidulans* (Eidam) Winter, *Botrytis cinerea* Pers and *Mycosphaerella graminicola* (Fückel) Schroter that function in fungicide sensitivity and resistance. The fungi possess ATP-binding cassette (ABC) drug transporters that mediate MDR to fungicides in laboratory mutants. Similar mutants are not pronounced in field resistance to most classes of fungicide but may play a role in resistance to azoles. MDR may also explain historical cases of resistance to aromatic hydrocarbon fungicides and dodine. In clinical situations, MDR development in *Candida albicans* (Robin) Berkhout mediated by ABC transporters in patients suffering from candidiasis is common after prolonged treatment with azoles. Factors that can explain this striking difference between agricultural and clinical situations are discussed. Attention is also paid to the risk of MDR development in plant pathogens in the future. Finally, the paper describes the impact of fungal drug transporters on drug discovery.

Keywords: ABC transporters; ATP-binding cassette transporters; fungicides; multidrug resistance; *Aspergillus nidulans*; *Botrytis cinerea*; *Mycosphaerella graminicola*

1 INTRODUCTION

During evolution, fungi evolved mechanisms of insensitivity or resistance to protect themselves against a wide variety of toxic compounds. The latter can be natural toxins such as antibiotics produced by micro-organisms in their living environment, or plant defence products, particularly relevant for plant pathogens. In addition, the advent of chemical control of plant diseases and mammalian mycoses challenges fungi to a constantly increasing number of synthetic antifungal compounds (drugs, fungicides and antimycotics). Fungi may also need to protect themselves from toxicants of endogenous origin such as antibiotics and mycotoxins that give the producing organism a competitive advantage in its ecological habitat.

Mechanisms of insensitivity or resistance to fungitoxic compounds can relate to qualitative factors such as the absence or presence of a sensitive target site or to quantitative factors such as uptake, transport, storage and metabolism. A quantitative factor involved in sensitivity to natural toxic products and fungicides is drug transporters located in the membranes of fungi.[1,2] Drug transporters in plasma membranes have the potency to secrete antifungal compounds back into the outer environment. In this way, drug transporters prevent the accumulation of compounds up to fungitoxic concentrations at their target site inside mycelial cells and hence prevent or reduce their toxic action. ATP-binding cassette (ABC) transporters are the most important drug transporters involved

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in the protection of fungi against antifungals.[2] The first fungal ABC transporter characterised was PDR5 from *Saccharomyces cerevisiae* Meyer ex Hansen.[3] The protein is a classical multidrug transporter and can confer resistance to many unrelated toxicants.[4] Readers interested in an inventory of ABC proteins in this organism and the diverse cellular functions revealed are referred to an overview by Bauer *et al*.[5]

The goal of this paper is to describe the physiological functions of ABC transporters identified in the model fungus *Aspergillus nidulans* (Eidam) Winter and in the plant pathogens *Botrytis cinerea* Pers and *Mycosphaerella graminicola* (Fückel) Schröter. In this context the paper focuses on the ability of drug transporters to provide protection of fungi against antibiotics, plant defence compounds and fungicides, their role in multidrug resistance (MDR) and their function in virulence on host plants. The paper also describes ABC transporters from *Candida* species, since the significance of MDR development in the treatment of mycoses under clinical situations is more severe than in agriculture. Potential factors contributing to this difference are discussed.

2 ABC TRANSPORTERS

ABC transporter proteins comprise one of the largest families of transport proteins known to date, operating in a wide variety of organisms from bacteria to man.[6] The proteins are located in the outer plasma membrane or in membranes of intracellular compartments, such as the vacuoles, endoplasmatic reticulum, peroxisomes and mitochondria, and are capable of transporting a wide variety of agents, ranging from ions to macromolecules, against a concentration gradient.[2,7,8] The energy needed for transport is provided by the hydrolysis of ATP and, for this reason, ABC transporters are characterised as primary active transport systems (Fig. 1A). ABC transporters were originally described in the literature as P-glycoproteins and are also known as PDR (pleiotropic drug resistance) proteins or MDR proteins. ABC transporters became well known in the 1990s because of their vital role in multidrug resistance of human tumours, resulting in the simultaneous loss in activity of multiple unrelated anticancer drugs. At present, MDR is also a serious threat to loss in activity of drugs used in medical treatment against human pathogens.[9–11]

The structural unit of a eukaryotic ABC transporter is most commonly composed of two homologous halves each containing six transmembrane domains (TMDs) and a conserved nucleotide-binding fold (NBF). The NBFs of ABC transporters are located in the cytoplasm. They are distinguished by the presence of highly conserved amino acid sequences, called the Walker A (G-(X)4-G-K-(T)-(X)6-I/V) and Walker B (R/K-(X)3-G-(X)3-L-(hydrophobic)4-D) motifs, and the ABC signature (L-S-G-G-(X)3-R-hydrophobic-X-hydrophobic-A).[12] The catalytic activity of these sites with respect to coupling and hydrolysis of ATP

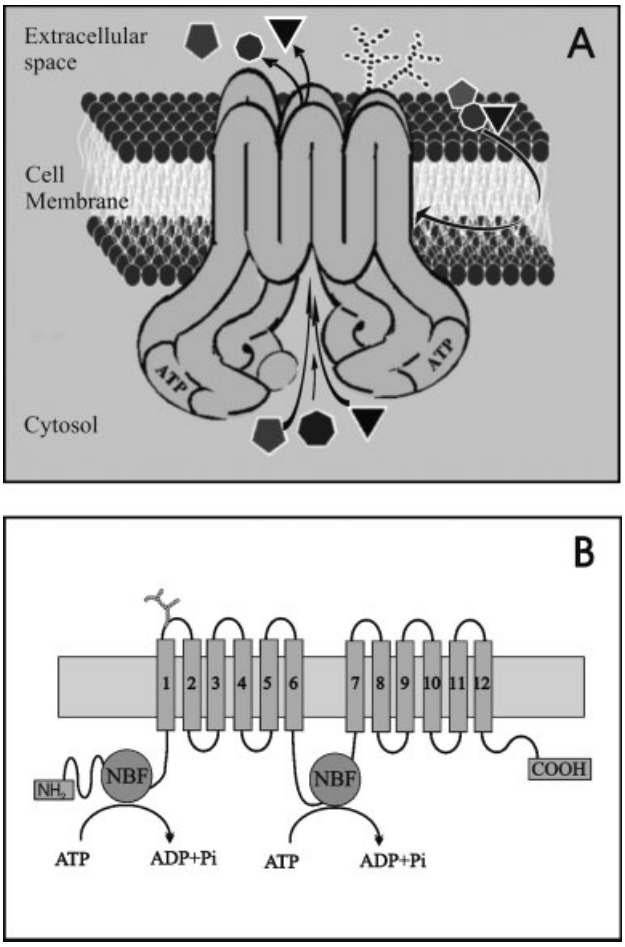

Figure 1. Schematic representation of (A) a three-dimensional model of an ATP-binding cassette (ABC) protein in a lipid bilayer membrane and (B) a two-dimensional model of a transporter with pleiotropic drug resistance (PDR) topology showing the two homologous halves each comprising a nucleotide-binding fold (NBF) and six transmembrane domains.

provides the energy necessary for the transport of substrates. The TMDs of ABC proteins are less conserved than the NBFs. They might form a pore across the lipid bilayer of membranes and are known to play a role in determining the substrate specificity of the transporters. For instance, in the human MDR1 protein, TMDs 4, 5, 6, 10, 11 and 12 and the extracellular loops connecting them are thought to be linked with substrate binding and transport.[13] Binding of substrates to substrate-binding sites of transporters supposedly triggers ATP hydrolysis and drug transport.

ABC transporters can be classified into different clusters based on their topology.[2] The majority of the eukaryotic proteins have a $[TMD_6\text{-}NBF]_2$ (MDR-type transporter) or an $[NBF\text{-}TMD_6]_2$ topology (PDR-type topology; Fig. 1B). However, half-sized transporters with a single TMD_6-NBF or NBF-TMD_6 configuration have also been described and are assumed to function after dimerisation.[14] Multidrug resistance-related proteins (MRPs) are ABC transporters with a $TMD_n[TMD_6\text{-}NBF]_2$ topology. They are characterised by the presence of an additional

transmembrane-spanning domain of approximately 200 amino acids at the *N*-terminus of the protein and by the presence of a putative 'regulatory' (R) or 'connector' domain between the two homologue halves, thought to act in the regulation of the protein. Representatives of this group of transporters have been identified as glutathione *S*-conjugate pumps involved in cellular detoxification and other processes.

ABC transporters include both uptake and efflux systems. In general, they exhibit broad substrate specificity, although transporters with specific substrates also occur. The broad range of substrates for these proteins includes alkaloids, lipids, peptides, steroids, sterols, terpenoids, flavonoids, sugars, inorganic anions and heavy metal chelates. Synthetic compounds such as fungicides, anticancer drugs and other therapeutic or disease control agents have also been described.[2,7] Most of these compounds have a positive charge at physiological pH, are of hydrophobic nature and enter the cells through passive diffusion.[15] The way these compounds are perceived and transported is not yet fully understood and several models have been proposed. Perception may be the consequence of non-specific stress, since the compounds alter the biophysical properties of membranes. Alternatively, drug sensor proteins can be involved that bind structurally unrelated compounds. Such proteins have been demonstrated in bacteria.[16] It may not be excluded that drug transporter proteins themselves can also act as receptor proteins. One of these effects probably triggers a signalling pathway resulting in fast expression of ABC transporter genes and subsequently drug efflux activity (Fig. 2).[17–19] Early transport models suggest that ABC proteins act as 'biological pumps' interacting directly with their substrates for their removal from the cytoplasm. However, recent studies indicate that detection and excretion of toxic agents take place at the membrane level before toxic concentrations can build up in the cytoplasm. In this case, drugs are removed directly from the membranes into the extracellular space and therefore ABC transporters are characterised as 'hydrophobic membrane vacuum cleaners'.[15] A third model suggests that ABC transporters have a flippase activity, translocating drugs from the inner leaflet of the lipid membrane bilayer to the outer one and subsequently releasing them into the outer environment.[20]

In silico analysis of the *S. cerevisiae* genome identified at least 28 different ABC transporters. Other genome-sequencing programmes reported the presence of 56 ABC proteins in *Drosophila melanogaster* Meig, 56 in *Caenorhabditis elegans* (Maupas) Dougherty, 129 in *Arabidopsis thaliana* Heynhoe and 49 in *Homo sapiens* L. (http://www.nutrigene.4t.com/humanabc.htm, accessed 28 June 2005). Sequence data suggest that an equally high number of ABC proteins are present in filamentous fungi. Complete sequencing of the *Neurospora crassa* Shear & Dodge, *Fusarium graminearum* Schwabe, *Magnaporthe grisea* (Hebert) Barr and *A. nidulans* genomes revealed the presence of 70, 58, 35 and 49 putative ABC proteins respectively (http://www.broad.mit.edu/annotation/fgi/, accessed 13 September 2005). The function of a limited number of these proteins has been elucidated, but the vast majority of them remain to be investigated.

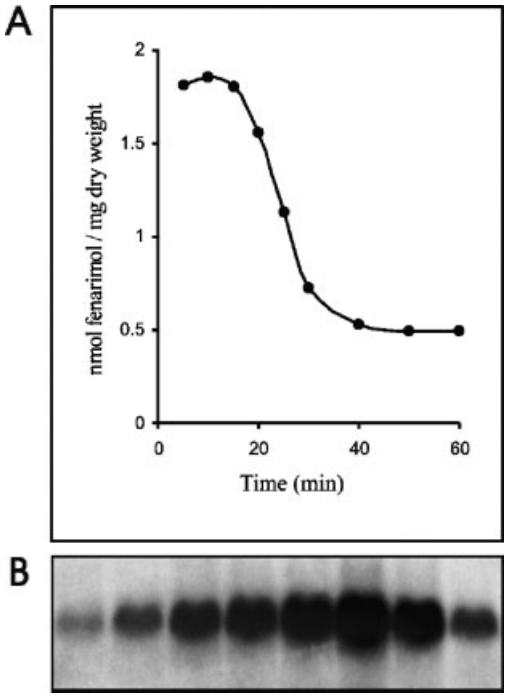

Figure 2. (A) Time-course efflux activity of the azole fungicide fenarimol from germlings of *Aspergillus nidulans* and (B) time-course induction of expression of *atrB* upon treatment of germlings with the azole fungicide imazalil.[17,19]

3 DRUG TRANSPORTERS IN *Aspergillus nidulans*

Several ABC transporter genes from *A. nidulans* have been described. *AtrA* and *atrB* encode proteins with an $[NBF\text{-}TMD_6]_2$ topology, while *atrC* and *atrD* encode proteins with a $[TMD_6\text{-}NBF]_2$ topology.[19,21] Expression of these genes is up-regulated by a range of natural and synthetic toxic compounds, such as the secondary plant metabolites pisatin and reserpine, the antibiotic cycloheximide and azole fungicides (also described in the literature as sterol demethylation inhibitors). Heterologous expression of *atrB* in an ABC transporter-deficient mutant strain of *S. cerevisiae* showed that yeast transformants carrying this gene are resistant to the antibiotic cycloheximide and a number of other drugs, suggesting a role for atrB in MDR. Functional analysis by gene replacement in *A. nidulans* demonstrated that *atrB* is involved in protection against compounds from all major classes of fungicide and natural toxic compounds (Fig. 3A). These include anilinopyrimidine, benzimidazole, phenylpyrrole, phenylpyridylamine, azole and strobilurin fungicides as well as the plant alkaloid camptothecin and the phytoalexin resveratrol.[22] Disruption of *atrD* in *A. nidulans* resulted in a phenotype hypersensitive to the antibiotics cycloheximide, the

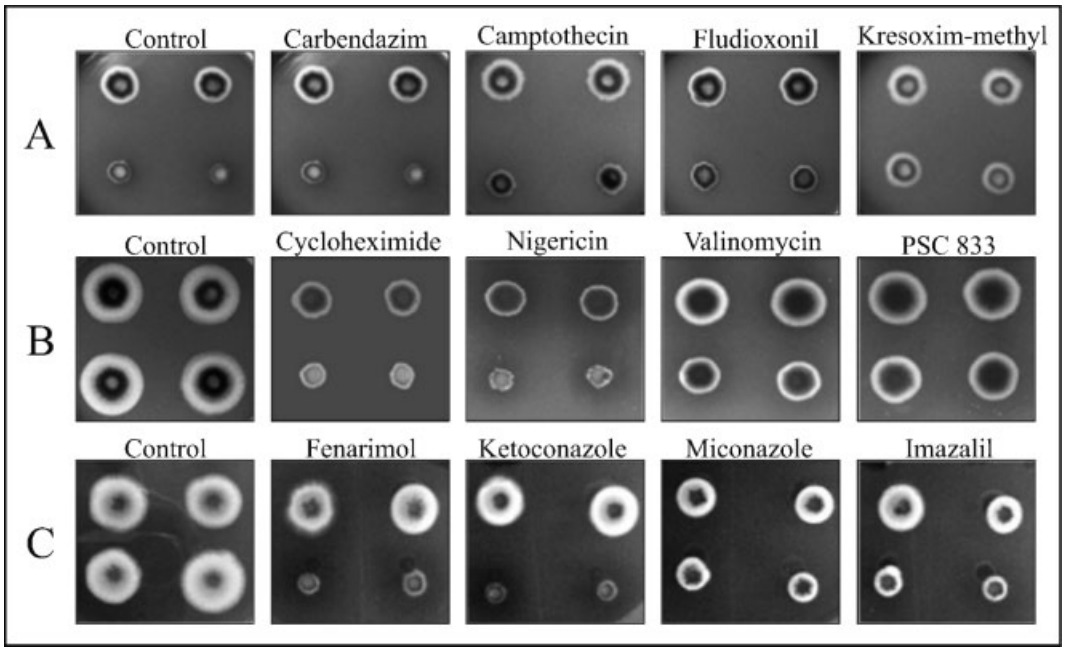

Figure 3. Radial growth of deletion mutants of (A) *atrB*, (B) *atrD* and (C) *atrG* from *Aspergillus nidulans* on medium amended with antifungals at sublethal concentrations. In each box the two colonies at the top are control transformants with intact *atr* genes and the two colonies at the bottom are independent deletion mutants[21,22] (Andrade AC, unpublished).

cyclosporin derivative PSC 833, nigericin and valinomycin (Fig. 3B). Recently, we demonstrated that *atrG* specifically mediates sensitivity to azole fungicides (Fig. 3C). These results show that at least three *A. nidulans* ABC transporters play a role in defence against natural toxic products and fungicides.

4 DRUG TRANSPORTERS IN *Botrytis cinerea*

Botrytis cinerea (teleomorph *Botryotinia fuckeliana*) is the causal agent of the grey mould disease that attacks a wide variety of crop plants and causes serious economic losses. Several ABC transporter genes have been cloned from this fungus. The basal transcript level of these genes varies from undetectable (*BcatrC, BcatrJ, BcatrN*) to low *(BcatrA, BcatrB, BcatrE, BcatrG, BcatrK*) and high (*BcatrF, BcatrH, BcatrI*).[23] Treatment with fungicides can increase transcript levels of several of these genes. *BcatrB* encodes a protein with an $[NBF\text{-}TMD_6]_2$ topology. Increased transcript levels of this gene are observed after short exposure (15–60 min) to antibiotics (e.g. phenazines), phytoalexins (e.g. resveratrol) and phenylpyrrole, anilinopyrimidine and dicarboximide fungicides.[23–25] Functional analysis by means of gene disruption showed that Δ*BcatrB* mutants display increased sensitivity to resveratrol, a plant defence compound in grapevine, while virulence tests showed a slight reduction in virulence on detached grapevine leaves as compared with the wild-type control. These results indicate that BcatrB is a virulence factor of *B. cinerea* on grapes by providing protection against resveratrol.[24] In addition, Δ*BcatrB* mutants exhibit increased sensitivity to the phenylpyrrole fungicides fenpiclonil and fludioxonil, indicating that drug transporters can play a role in baseline sensitivity to fungicides. Mutants overexpressing *BcatrB* show decreased sensitivity to phenylpyrroles, suggesting a possible role for BcatrB in fungicide resistance.[23] *BcatrD* also encodes a protein with an $[NBF\text{-}TMD_6]_2$ topology. This gene exhibits a high level of basal expression in germlings of *B. cinerea* and its transcript level is up-regulated by treatment with azole, dicarboximide and benzimidazole fungicides as well as the antibiotic cycloheximide.[26,27] A positive correlation between increased transcript levels of *BcatrD* and resistance to azole fungicides was observed. Replacement mutants of *BcatrD* exhibit increased sensitivity to several azoles and accumulate relatively high amounts of the azole fungicide oxpoconazole. Likewise, mutants overexpressing *BcatrD* show a positive correlation between *BcatrD* expression level and decreased sensitivity to this compound. These results indicate that *BcatrD* is a determinant of sensitivity of *B. cinerea* to azoles (Fig. 4). *BMR1* (*BcatrK*) encodes a protein with an $[NBF\text{-}TMD_6]_2$ topology. Δ*BMR1* mutants display increased sensitivity to the antibiotic polyoxin and the organophosphorus fungicide iprobenfos, which implies that BMR1 is an additional MDR transporter of this fungus.[28]

5 DRUG TRANSPORTERS IN *Mycosphaerella graminicola*

Five ABC transporter genes (*MgAtr1–MgAtr5*) have been cloned and sequenced from the plant pathogenic fungus *M. graminicola* (anamorph *Septoria tritici*), the causal agent of leaf blotch on wheat.[29,30] The encoded ABC proteins all exhibit the $[NBF\text{-}TMD_6]_2$ topology. *MgAtr1, MgAtr2, MgAtr4* and *MgAtr5* display distinct expression profiles when treated with a

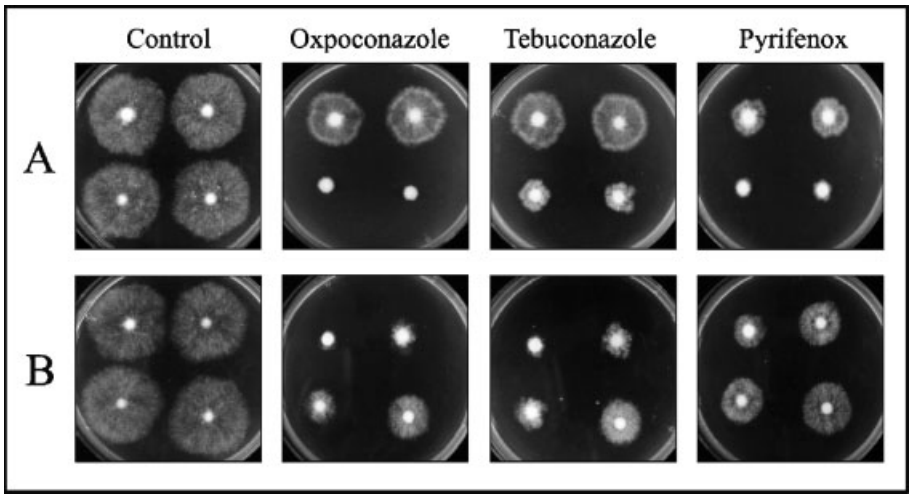

Figure 4. Radial growth of (A) deletion and (B) overexpression mutants of *atrD* from *Botrytis cinerea* on medium amended with azole fungicides. (A) In each box the two colonies at the top are control transformants with an intact *atrD* gene and the two colonies at the bottom are independent deletion mutants. (B) In each box the top-left colony represents the control transformant strain and the top-right, bottom-left and bottom-right colonies represent mutants with low, medium and high degrees of overexpression of *atrD* respectively.[27]

range of compounds known to be either substrates or inducers of ABC transporters. These include azoles, natural toxic compounds such as the plant defence compounds eugenol and psoralen, and the antibiotics cycloheximide and neomycin. The expression pattern of the genes is also influenced by the morphological state, yeast-like or mycelial, of the fungus. Heterologous expression of *MgAtr1, MgAtr2, MgAtr4* and *MgAtr5* in a strain of *S. cerevisiae* with multiple non-functional ABC transporter genes showed that the products of these genes transport a wide range of chemically unrelated compounds and possess an extensive overlap in substrate specificity (Table 1).[31] Their substrate range includes synthetic compounds such as azoles and natural toxic compounds such as the plant metabolites berberine and camptothecin and the mycotoxin diacetoxyscirpenol.

Table 1. Sensitivity to antifungals of *Saccharomyces cerevisiae* strain AD12345678 lacking multiple drug transporter genes and expressing *Mycosphaerella graminicola* ABC transporter genes *MgAtr1, MgAtr2, MgAtr4* or *MgAtr5*[31]

Compound	*MgAtr1*	*MgAtr2*	*MgAtr4*	*MgAtr5*
Fungicides				
Cyproconazole	+[a]	+	+	−
Propiconazole	+	+	+	−
Tebuconazole	+	+	+	−
Lipids				
Ergosterol	+	+	+	−
Progesterone	+	+	+	−
Plant metabolites				
Berberine	+	−	−	+
Camptothecin	+	−	−	+
Antibiotics				
Cycloheximide	+	−	−	−
Various				
Diacetoxyscirpenol	+	−	+	−
Rhodamine 6G	−	+	+	−

[a] The symbols '+' and '−' indicate respectively decreased and unaltered sensitivity to antifungals as compared with the pYes2-transformed control strain.

The role of *MgAtr1–MgAtr5* in sensitivity to the azole fungicide cyproconazole was studied by expression analysis in laboratory isolates with decreased sensitivity to that fungicide. One of the laboratory mutants possessed a constitutive overexpression of *MgAtr1*, and *MgAtr1* disruptants of this mutant were hypersensitive to cyproconazole (Fig. 5), suggesting that overexpression of *MgAtr1* could also be relevant in reduced sensitivity of field isolates.[32] The function of *MgAtr1–MgAtr5* in virulence of *M. graminicola* on wheat was investigated with knockout mutants.[33] Δ*MgAtr4* mutants displayed reduced virulence as compared with the wild-type control strain. Northern analysis on RNA isolated from wheat infected with the wild-type isolate and the Δ*MgAtr4* mutant, as well as microscopical analysis, showed a low build-up of biomass of the Δ*MgAtr4* mutant on wheat (Fig. 6). These findings indicate a role for this protein in virulence.[33] Analysis of the transformants also showed that Δ*MgAtr5* mutants have a small increase in sensitivity to the putative wheat defence compound resorcinol, suggesting a role for this transporter during pathogenesis.[31] No further phenotypes were observed for any of the mutants and compounds tested. This could be due to redundancy of ABC transporters with a similar substrate range. Thus the possibility that some of these transporters are involved in protection against natural and synthetic toxic compounds cannot be excluded.

6 DRUG TRANSPORTERS IN *Candida albicans*

Candida albicans is an opportunistic pathogen important in humans and the major cause of oropharyngeal candidiasis in immunodepressed patients. The azole fungicide fluconazole has been the most commonly used fungicide in the treatment of the disease.[34]

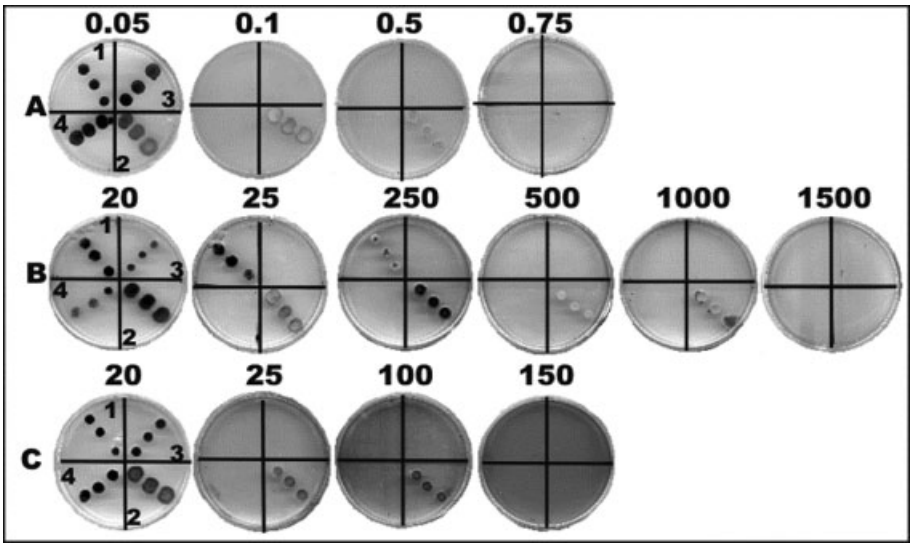

Figure 5. Growth of *Mycosphaerella graminicola* wild-type strain (quadrant 1), a laboratory-generated azole-resistant strain (quadrant 2) and two independent *MgAtr1* knockout mutants of a laboratory-generated azole-resistant strain (quadrants 3 and 4) on medium amended with (A) the azole fungicide cyproconazole, (B) cycloheximide and (C) rhodamine 6G.[32]

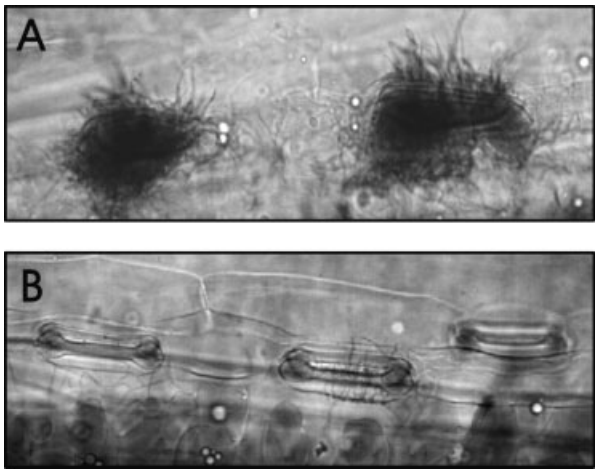

Figure 6. Comparison of virulence of a wild-type and *MgAtr4* disruption mutants of *Mycosphaerella graminicola* on wheat seedling 17 days post-inoculation. (A) Wild-type strain showing dense systemic infection of the leaf apoplast and abundant fungal biomass in substomatal cavities. (B) Mutant strain showing limited intercellular growth in the apoplast and hardly any fungal biomass in stomatal cavities.[33]

Other azoles used in the chemotherapy of candidiasis are itraconazole and ketoconazole. ABC transporters implicated in azole sensitivity and resistance in *C. albicans* are described as *CDR* genes. *CDR1* encodes the best-characterised multidrug transporter.[35] It has the $[NBF\text{-}TMD_6]_2$ topology, and complementation of a hypersensitive *PDR5*-deficient mutant of *S. cerevisiae* with *CDR1* restores wild-type insensitivity to azoles, cycloheximide and chloramphenicol. Deletion of both *CDR1* alleles in *C. albicans* results in a strain hypersensitive to the same toxicants and to allylamine and morpholine fungicides.[9] Heterologous expression of CDR2 in the hypersensitive PDR5-deficient mutant of *S. cerevisiae* showed that the same classes of fungicide also act as substrate for CDR2. Deletion of both *CDR2* alleles in *C. albicans* did not cause hypersensitivity to the toxicants. This could only be observed if *CDR2* was deleted in a mutant with a *CDR1*-deficient background, suggesting that overexpression of CDR1 can compensate for loss of CDR2.[10] Both *CDR1* and *CDR2* are under the control of the same transcriptional regulator *CAP1*.[36]

Clinical isolates of *C. albicans* and other *Candida* species with azole resistance have been described frequently. Most of these isolates were obtained after prolonged use of azoles for treatment of candidiasis, particularly in immunodepressed patients. These isolates show a clear correlation between reduced *in vitro* sensitivity and clinical failure of disease control despite proper delivery of adequate amounts of fungicide to the site of treatment. The clinical, cellular and molecular factors that contribute to resistance have been reviewed frequently.[11,37–39] Resistance to azoles in clinical isolates of *C. albicans* can be mediated by mechanisms such as target site modifications of sterol 14 alpha-demethylase, increased expression of the target site and increased expression of the major facilitator transporter MDR1.[40–42] However, a majority of azole-resistant isolates overexpress *CDR1* and *CDR2* and display increased efflux of azoles. They also exhibit an MDR phenotype, since the isolates are cross-resistant to the unrelated fungicides terbinafine and caspofungin.[43] Hence distinct mechanisms of resistance may operate simultaneously and these can contribute to a stepwise development of high resistance levels to azoles in *C. albicans*.[39,41] Similar phenomena have been described for other pathogenic yeast species such as *C. glabrata*.[37]

7 IMPACT OF DRUG TRANSPORTERS ON MDR IN AGRICULTURAL PRACTICE

7.1 Cases of MDR in practice

In agricultural practice, obvious cases of MDR to fungicides in plant pathogens are restricted. A classical

example of an MDR phenotype is the cross-resistance in *B. cinerea* and other pathogens to aromatic hydrocarbons (e.g. chloroneb, dicloran, tolclofos-methyl), dicarboximides (e.g. iprodione, procymidone, vinclozolin) and other fungicides.[44] The levels of resistance to these compounds were so high that failure in disease control occurred. Many putative mechanisms of resistance were suggested for aromatic hydrocarbon- and dicarboximide-resistant mutants of *B. cinerea*, but the actual mechanism has never been fully elucidated. At the time that these mechanisms were investigated, MDR based on increased efflux by drug transporters was not yet known, but, based on present knowledge, we suggest that drug transporters may have played a role in these cases of fungicide resistance.

More recently, *B. cinerea* from French vineyards showed MDR phenotypes for various classes of fungicide. AniR2 and AniR3 isolates possessed cross-resistance to anilinopyrimidines (e.g. cyprodinyl, mepanipyrim, pyrimethanil), dicarboximides, phenylpyrroles (e.g. fenpiclonil, fludioxonil) and other fungicides.[45] AniR3 isolates were also cross-resistant to azole fungicides.[45] Both types of isolate exhibited low resistance levels and seemed to be effectively controlled in practice. Circumstantial evidence indicates that active efflux is involved.[46] Since BcatrB and BcatrD are described as a true multidrug transporter and an azole transporter respectively, it is possible that these ABC transporters function in the MDR phenotype of AniR2 and AniR3 isolates.[23,26,27] Since *B. cinerea* has a family of ABC transporters that are not functionally analysed completely, it is possible that other ABC transporters can also be responsible for MDR of the AniR2 and AniR3 isolates.

In Europe, pathogens of wheat constitute another major target for disease control by fungicides from different classes of compound. Azoles are one of the most important groups, having been applied for at least 20 years.[47] Two major foliar pathogens are *Blumeria graminis* Speer f. sp *tritici* Marchal and *M. graminicola*. High levels of field resistance in *B. graminis* f. sp *tritici* can probably be ascribed to point mutations in the *cyp51* gene encoding the target site of azoles.[48] Field resistance in *M. graminicola* to azole fungicides has not been reported. However, field populations of the pathogen in France and Germany exhibit a more than 100-fold difference in sensitivity to cyproconazole.[49] Azole resistance in laboratory mutants can be associated with expression of specific ABC transporter genes, particularly *MgAtr1*.[32] For that reason, expression of *MgAtr1–MgAtr5* was also studied in the French and German field isolates. No correlation between azole sensitivity and transcript level of any of the *MgAtr* genes tested was observed.[49] Studies with UK isolates showed similar results, although some isolates from a field with a 40-fold reduction in sensitivity to the azoles epoxiconazole and flusilazole revealed strongly increased transcript levels of *MgAtr3*.[50] These results suggest that multiple ABC transporters can contribute to reduced sensitivity to azoles in field isolates of *M. graminicola*. It might be that other types of drug efflux transporter also play a role in reduced sensitivity of field isolates. The full genome of *M. graminicola* was sequenced in 2005 and, as a result, all ABC and MFS drug transporter genes as well as other putative genes in azole resistance can be identified. Screening of their expression by means of microarray analysis may reveal genes that play a more obvious role in resistance than the ones identified so far.

MDR in practice is also described for azole-resistant isolates of *Penicillium digitatum* (Pers) Sacc. These isolates are cross-resistant to unrelated chemicals and show an up-regulation of gene expression of the ABC transporter gene *PMR1*.[51] Other ABC transporter genes involved in drug resistance in *P. digitatum* have also been described.[52] The distinct role of these various transporters in MDR still remains to be elucidated.

7.2 Stepwise evolution of resistance and drug transporters

Circumstantial evidence for a role of multidrug transporters in fungicide resistance is also provided by the observation of stepwise evolution of resistance in practice and predisposition of fungicide sensitivity by previous selection with unrelated fungicides. Stepwise evolution of fungicide resistance became known after the introduction of azole fungicides. One of the first cases was described for azole sensitivity in *Sphaerotheca fuliginea* (Schlecht ex Fr) Poll. A stepwise decrease in sensitivity to imazalil and fenarimol over a 3 year period was observed in The Netherlands.[53] At present the field performance of these compounds in the control of cucumber powdery mildew is low. Similarly, stepwise evolution of decreased sensitivity to fungicides has been described for other mildew pathogens such as *B. graminis* f. sp *hordei*, *B. graminis* f. sp *tritici* and the leaf blotch pathogen *M. graminicola*.[47,49,54,55] A possible explanation for stepwise evolution of resistance is polygenic resistance related to ABC transporter genes as described for azole fungicides *in vitro* in laboratory-generated mutants of *A. nidulans* and *Nectria haematococca* Berk & Broome.[56,57] Ample evidence is provided that azole-resistant mutants of these organisms indeed overexpress multiple ABC transporter genes and/or display reduced accumulation of azoles in the mycelium owing to increased efflux activity. Stepwise development of resistance to azoles related to reduced accumulation of these fungicides was also observed in laboratory-generated mutants of *P. italicum* Wehmer.[58,59] However, it should be kept in mind that stepwise development of resistance does not necessarily imply multiple ABC transporter genes, since this phenomenon can also be based on a combination of different mechanisms as observed in *C. albicans* and *M. graminicola*.[32,41]

7.3 Predisposition and drug transporters

Predisposition of fungicide sensitivity by previous selection with unrelated fungicides has been described

in *Venturia inaequalis* (Cooke) Winter.[60] This pathogen developed stepwise resistance to dodine in the USA in the 1960s.[61] In *V. inaequalis*, four levels of reduced sensitivity were detected, suggesting the additive action of at least two genes for dodine resistance.[62] Dodine resistance in *N. haematococca* is determined by four unlinked loci and, in recombinants, these genes have an additive effect on resistance. The presence of resistance genes was correlated with increased efflux activity of dodine or its metabolites from mycelium.[63] These results suggest that resistance in fungi to dodine is a polygenic trait related to increased drug transporter activity. In the course of these studies on dodine, resistance attention was probably never paid to an MDR phenotype of these resistant isolates. However, in the 1990s, resistance to azoles developed to significantly higher frequencies within dodine-resistant populations than in dodine-sensitive ones.[60] Recently, isolates of *V. inaequalis* from New York state demonstrated a correlation in sensitivity to azoles and anilinopyrimidines. The correlation was most significant in subpopulations of the pathogen previously treated over 10 years with azoles but not with anilinopyrimidines. Subpopulations differed in azole sensitivity, and it might be that at least one of the genes conferring azole resistance also lowered sensitivity to anilinopyrimidines and that a common resistance mechanism is involved (Köller, personal communication). Predisposition was also described in *Uncinula necator* (Schw) Burr to the QoI fungicide azoxystrobin in fungal populations with decreased sensitivity to the azole fungicide myclobutanil.[64] In our view these cases of predisposition to fungicides may represent cases of multidrug resistance in practice. Circumstantial evidence to support this hypothesis is that the degrees of resistance involved are relatively low, which is quite common for resistance mediated by drug transporters. In addition, it is known that laboratory mutants of *A. nidulans* overexpressing the ABC transporter gene *atrB* display MDR to azoles, anilinopyrimidines and QoI fungicides.[22] These observations suggest that a similar overexpression of transporter gene(s) may have happened in the above-mentioned cases of predisposition to fungicides. It is important to investigate predisposition in more detail, since validation of the hypothesis that ABC transporters are involved in this phenomenon would implicate that drug transporters are more important determinants in fungicide sensitivity and resistance than ever anticipated.

8 DISCUSSION

8.1 MDR in practice

The main reason for the limited relevance of MDR in agricultural practice is probably that evolution of resistance is dominated by mutants with changed target site modifications. In general, mutants with target site modifications have a decreased affinity for the fungicide and are common for most classes of fungicide (benzimidazoles, carboximides, dicarboximides, QoI fungicides). Populations with a high frequency of such mutants can often be selected within a few years of selection pressure in polycyclic pathogens with a high propagation rate. Other major determinants in rapid evolution of populations composed of such mutants are a high level of resistance associated with these single-gene mutations and a normal fitness as compared with the wild-type isolate. Obviously, such mutant populations will mask subpopulations composed of mutants with low levels of resistance and a fitness penalty.

This reasoning implies that fungicide resistance in plant pathogens based on alternative mechanisms will only become detectable in practice in exceptional cases when resistance based on target site modifications cannot evolve readily. This situation is probably applicable for azole fungicides, since target site modifications in field-resistant isolates have only been described for *B. graminis* f. sp *hordei* and *U. necator* but never for other pathogens such as *M. graminicola*.[48,50] The underlying reason for this situation is not known, but it might be that target site mutations of *CYP51* resulting in azole resistance are lethal or strongly reduce comparative fitness. This hypothesis is corroborated by early findings that the degree of resistance to azoles in laboratory mutants of *Cladosporium cucumerinum* Ell & Arthur correlates negatively with virulence and in *A. nidulans* with saprophytic growth.[65] In view of this situation it is no surprise that multiple mechanisms of resistance to azoles in various fungi have been reported. A mechanism reported for azole-resistant field isolates of *P. digitatum* and *V. inaequalis* is overexpression of *CYP51* caused by tandem repeats in its promoter region.[66,67] Another mechanism that can operate in plant pathogens is increased expression of drug transporters, associated with increased azole efflux and decreased accumulation of these compounds at their target site. Under practical conditions this mechanism has only been reported for azole-resistant isolates of *P. digitatum* (Section 7.1).[52] Also described in Section 7.1 is circumstantial evidence for a role of this mechanism in particular azole-resistant field isolates of *B. cinerea* and *M. graminicola*. This evidence is mainly based on expression analysis. These results should be interpreted with care and one should consider that conclusive evidence in this respect may be difficult to achieve, because multiple resistance mechanisms may operate simultaneously. An additional difficulty in studying azole resistance in *B. cinerea* and *M. graminicola* is their significant phenotypic variability, also with respect to baseline sensitivity to fungicides, and, related to this, the inability to identify the corresponding wild-type mother isolates of azole-resistant field isolates which are required to determine the degree of reduced azole sensitivity.

Azole resistance in the human pathogenic yeast *C. albicans* evolves readily in patients undergoing

Table 2. Putative epidemiological and operational factors involved in ABC transporter-mediated resistance development to azole fungicides in plant pathogens and *Candida albicans*

Factor	Plant pathogens	*C. albicans*
Population size	Large; not limited to a treated field	Can be restricted to a single patient undergoing drug therapy
Duration of life cycle	Days–weeks	Hours
Refugia for survival of wild-type population during treatment	Present	Limited–absent
Long-distance mobility of propagules	Possible between fields	More difficult between patients
Frequency of application	Limited number of sprays per season	Long-term treatment regimens for weeks or months
Mode of application	Sprays that allow escape of sensitive subpopulation	Oral treatment that reduces escape of sensitive subpopulation
Selection of mutants that compensate for loss of fitness	Not readily possible because periods without selection allow competition with wild-type isolates	Possible since elimination of wild-type population can annul competition
Multiple attack	Fungicide mixtures and rotational use of fungicides with different resistance mechanisms	Exclusive use of fluconazole for whole treatment period
Host	No host plant conditions known that would favour MDR	Favoured by absence of immune system in patients

antifungal therapy (Section 6). In this context, resistance based on increased expression of ABC transporters is common and an obvious question is why this situation is so different from azole resistance in plant pathogens. In general, the rapid emergence of resistance to azoles in *C. albicans* (including resistance mediated by ABC transporters) is ascribed to the net effect of the (small) pathogen population size and high mutation rate.[39] The mutation rate in plant pathogens for azole resistance is also high and hence cannot explain the slow resistance development mediated by ABC transporters in plant pathogens.[57] A major difference between the two groups of pathogens is the population size, being extremely large for many plant pathogens. Therefore we suppose that the large size of plant pathogen populations constitutes a major factor in slow resistance development to azoles in plant pathogens. Additional epidemiological and operational factors possibly involved as well are summarised in Table 2. We suggest that a combination of these factors and the observation that ABC transporter mutants often possess a fitness penalty in the absence of selection pressure significantly delays the selection of such azole-resistant populations in plant pathogens.

We believe that, in the long run, MDR in plant pathogens will develop more frequently in agriculture. This might be the result of stepwise selection of MDR mutants with higher levels of resistance that can better cope with full application rates of fungicides. This may be relevant for azole sensitivity in pathogens such as *M. graminicola*. Recent surveys indicate that sensitivity to azoles in populations of this pathogen gradually reduced up to 20-fold in particular locations in France and Germany.[55] In 2003 in the UK a field population with a 40-fold reduction in sensitivity to the azoles epoxiconazole and flusilazole was detected.[50] Such shifts will gradually reduce the performance of azole fungicides, especially that of the weaker members.

In the long run, MDR mutants of plant pathogens may also have a higher chance of restoring normal fitness by compensatory mutations. Compensatory mutations not only occur in yeasts but have also been described for phenylpyrrole-resistant mutants of *A. nidulans* (Fig. 7).[68] So far, underlying mechanisms related to these mutations are not known.

Possibly, the simultaneous application of fungicides from different chemical classes constitutes an additional risk for MDR development in plant pathogens, since this situation prevents or delays the evolution of mutants with target site modifications but may not be effective against MDR mutants. Multiple attacks by diverse fungicides are common practice in anti-resistance strategies for disease control in major agricultural crops such as banana, cereals, rice and grapevine. These strategies imply the use of fungicide mixtures and rotational use of these compounds. If the hypothesis is valid, it might be that the selection of MDR mutants of *B. cinerea* in French vineyards relates to such a multiple attack by fungicides. It might also be relevant for pathogens such as *Mycosphaerella musicola* Mulder (banana), *M. graminicola* (wheat) and *Pyricularia oryzae* Cavara (rice). This situation might have serious consequences for future disease control with new classes of chemical because of predisposition to new fungicides as described in Section 7.3.

8.2 Functional analysis of drug transporter genes

To date, functional analysis of drug transporters has mainly been performed for individual transporter genes, cloned with the help of heterologous probes or PCR using degenerated primers. In future, sequences of fungal genomes will become available in increasing number, which will facilitate the cloning of drug transporter genes more easily. Sequences are usually not helpful to predict the physiological function of

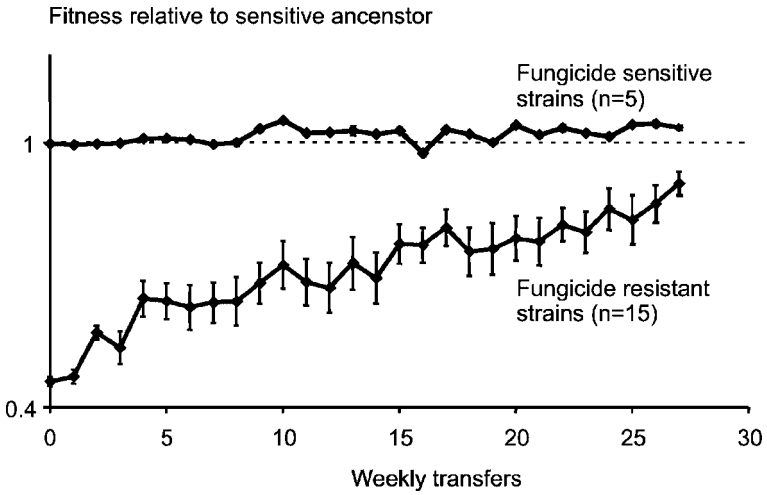

Figure 7. Radial growth rate of a wild-type strain of *Aspergillus nidulans* and a fludioxonil-resistant mutant relative to that of the wild-type strain upon weekly transfers on minimal medium (without fungicide). Note that fitness loss in the fludioxonil-resistant strain is compensated in time. During transfers the initial degree of fludioxonil resistance was retained. Genetic analysis of a fludioxonil-resistant mutant revealed interactions between three fitness compensatory mutations.[68]

the encoded proteins. However, it might be that the phylogenetic relationship of the proteins may provide some predictive value. This has already been shown for ABC transporters of *A. nidulans* involved in transport of azole fungicides.[69] It is anticipated that these types of study will identify many more putative ABC transporter genes involved in MDR than have been described so far.

8.3 Impact of drug transporters on fungicide discovery

Since drug transporters reduce the activity of fungicides, fungal mutants that lack drug transporters may become fungicide-hypersensitive and can be used as tester strains in fungicide discovery screening programmes. We have constructed a triple knockout mutant of *A. nidulans* with non-functional *atrB, atrD* and *atrG* genes. The mutant has an increased sensitivity to many unrelated fungicides, antibiotics and to natural antifungal compounds from plants. We propose this mutant as a useful tester strain in high-throughput screening for lead compounds of agricultural fungicides and antimycotics (Fig. 8).[70] Similar suggestions were made to discover new antimycotics.[11] Multiple disruption mutants are also useful in structure–activity studies and may be used as a tool to explain why fungi with a sensitive target site have a whole-cell insensitivity to fungicides and cannot be controlled under field conditions.[70]

A rational approach in the search for new antifungals would also be the development of compounds that inhibit ABC transporters involved in fungicide activity or virulence. These compounds are described in the medical literature as blockers or modulators of ABC transporters and are tested in human chemotherapy as synergists of drugs or as compounds that annihilate MDR in cancer cells.[71] Similarly, such compounds could be developed as synergists of agricultural fungicides or as compounds that

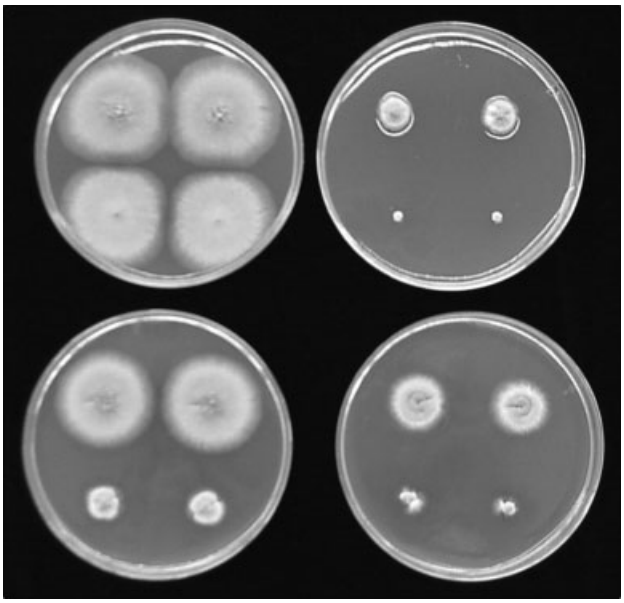

Figure 8. Radial growth of *Aspergillus nidulans* on (top left) control medium and medium amended with (top right) fludioxonil, (bottom left) nigericine and (bottom right) fenarimol. In each petri dish the two colonies at the top represent control transformant strains and the two colonies at the bottom represent independent mutants with non-functional *atrB, atrD* and *atrG ABC* transporter genes.

annul MDR in plant pathogens (Fig. 9). Such compounds have been described.[72–74] Recently, it was demonstrated that combinations of azole or strobilurin fungicides with a hydroxyflavone derivative shifted fungicide-resistant *Pyrenophora tritici-repentis* (Died) Dreschler isolates back to normal sensitivity levels and prevented infection of wheat leaves.[75] The feasibility of developing ABC modulators for use in practice needs to be investigated further.

Another approach would be to develop modulators that inhibit the activity of ABC transporters involved in fungal virulence on host plants. The role of drug transporters in virulence on a range of host

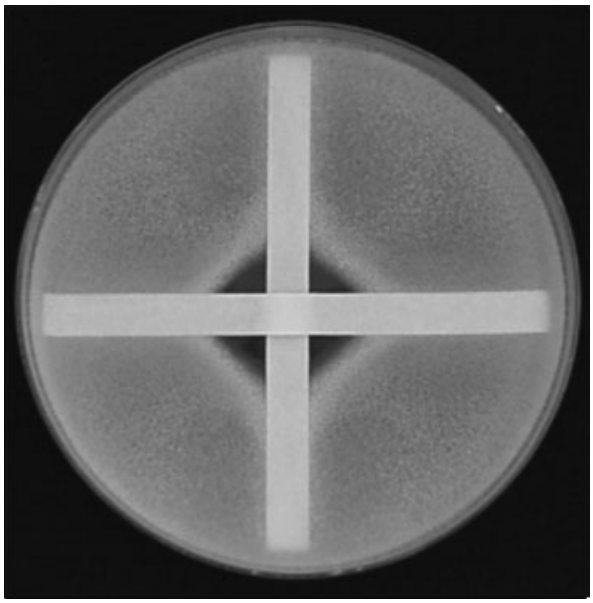

Figure 9. Cross-paper strip experiment showing that chlorpromazine (impregnated in horizontal strip), known as a modulator of ABC transporter activity, can annihilate resistance to the azole fungicide oxpoconazole (impregnated in vertical strip) in a *BcatrD* overexpression mutant of *Botrytis cinerea*.[74]

plants has now been demonstrated for ABC and MFS transporters in a range of important plant pathogens.[2] It is anticipated that this number will increase, especially in pathogens of host plants known to produce phytoalexins. Inhibition of such transporters would enhance accumulation of plant defence compounds in fungal cells and hence exploit the natural defence reaction of host plants. Such compounds do not necessarily need to be fungitoxic *in vitro*, and hence development of such compounds might result in the discovery of disease control agents that do not have a direct fungitoxic activity. A prerequisite for the discovery of these compounds is, of course, selective activity between the target and the non-target organism. This may be feasible, since modulators used as medical drugs can have a selective activity to cancer cells in humans.

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