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Secretion of natural and synthetic toxic compounds from filamentous fungi by membrane transporters of the ATP-binding cassette and major facilitator superfamily

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Abstract

This review provides an overview of members of the ATP-binding cassette (ABC) and major facilitator superfamily (MFS) of transporters identified in filamentous fungi. The most common function of these membrane proteins is to provide protection against natural toxic compounds present in the environment of fungi, such as antibiotics produced by other microorganisms. In plant pathogenic fungi, these transporters can also be an important determinant of virulence on host plants by providing protection against plant defence compounds or mediating the secretion of host-specific toxins. Furthermore, they play a critical role in determining base-line sensitivity to fungicides and other antimycotic agents. Overexpression of some of these transporters can lead to the development of resistance to chemically-unrelated compounds, a phenomenon described as multidrug resistance (MDR). This has been observed in a variety of organisms and can impose a serious threat to the effective control of pathogenic fungi.

Abbreviations: ABC – ATP-binding cassette; Acc. – accession number; DAS – diacetoxyscirpenol; DMI – demethylation inhibitor; EST – expressed sequence tag; MDR – multidrug resistance; MFS – major facilitator superfamily; MRP – multidrug resistance-related protein; NBF – nucleotide-binding fold; PDR – pleiotropic drug resistance; PDREs – pleiotropic drug resistance elements; SBI – sterol biosynthesis inhibitor; TMD – trans-membrane domain.

Introduction

In nature, filamentous fungi are constantly exposed to a wide variety of toxic compounds originating from various sources. In their living environment they encounter numerous antibiotic compounds produced by other microorganisms. Plant pathogenic fungi must also be able to resist the toxic effects of plant defence products such as phytoalexins and phytoanticipins. In addition, the advent of chemical disease control over the past decades led to an increased exposure of fungi to fungicides. On the other hand, fungi may also need to handle toxicants of endogenous origin such as antibiotics and mycotoxins that provide the producing organism with a competitive advantage in its ecological habitat. In this context, the following questions can be raised: 'Which strategies have organisms developed to protect themselves against exposure to exogenous toxic products?' and 'How do toxin producing organisms manage to protect themselves against auto-toxicity of endogenous toxins?' The answers to both questions relate to mechanisms of selective toxicity and can be of a qualitative or quantitative nature. Qualitative factors relate to the presence of the target site of a toxic compound in sensitive organisms and its absence in resistant ones. Quantitative factors determine the effective concentration of the cytotoxic agent that can be built up at the target site as a result of uptake, transport, storage and

natural metabolism. Differences in affinity of the target site in target and non-target organisms are also important quantitative factors.

Functions of ABC and MFS transporters in filamentous fungi

Quantitative factors that influence the accumulation of toxicants in cells were only discovered in the last couple of decades and are described as members of the ATP-binding cassette (ABC) and the major facilitator superfamily (MFS) of membrane transporters. These transporters have a remarkably broad substrate specificity and are able to transport a wide variety of natural and synthetic toxic products of either endogenous or exogenous origin. In this way, they prevent or reduce accumulation of these products inside cells and hence, avoid or minimise their toxic action (Nelissen et al., 1997; Bauer et al., 1999; Pao et al., 1998). Furthermore, overexpression of these transporters is known to play an essential role in resistance of cells to chemically-unrelated compounds. This phenomenon is described as multidrug resistance (MDR) and is a serious threat to the effectiveness of drugs (Fling et al., 1991; Juliano and Ling, 1976).

The physiological functions of ABC and MFS transporters in fungi are also highly significant. These transporters provide protection against toxic compounds present in their natural habitat or prevent the cytoplasmatic accumulation of toxic secondary metabolites produced by the fungus itself. In addition, in plant pathogenic fungi these transporters may act as virulence or pathogenicity factors if they mediate secretion of virulence factors, such as host-specific toxins, or provide protection against plant defence compounds during pathogenesis (De Waard, 1997) (Figure 1). If the sexual stage of a fungus is present, ABC transporters can also be involved in secretion of mating type factors and thus, contribute to the genetic diversity of a fungal population. Such transporters have already been characterised in *Saccharomyces cerevisiae* and *Schizosaccharomyces pombe* (Christensen et al., 1997; Kuchler et al., 1989).

ABC and MFS transporters also function in protection against synthetic toxic compounds, such as fungicides and other antimycotic agents (De Waard, 1997). Over the past few years, the widespread use of antifungal compounds has resulted in the development of fungicide resistance in several fungal species. Studies on azole-resistant strains of *Candida albicans* and other fungal pathogens of medical importance indicated a decreased accumulation of these compounds in cells, mediated by ABC and MFS transporters. In many of these cases, exposure to a single antimycotic agent resulted in simultaneous resistance to a number

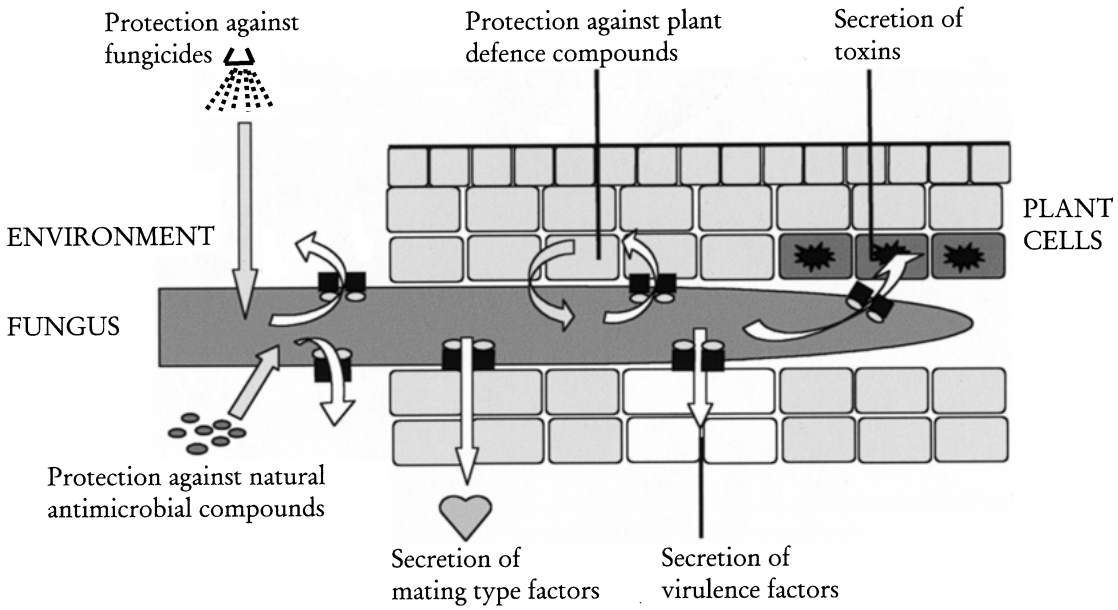

Figure 1. Functions of ABC and MFS transporters in filamentous fungi.

of chemically unrelated compounds. Overexpression of ABC and MFS genes was closely linked with such MDR phenotypes (Albertson et al., 1996; Fling et al., 1991; Franz et al., 1998; Sanglard et al., 1995; 1997; White, 1997). Reduced accumulation of azole fungicides in *Aspergillus nidulans* and *Penicillium italicum* suggested that a similar mechanism attributed to resistance development in these fungi (De Waard and Van Nistelrooy, 1980; 1984a,b; 1988). Recent studies support the hypothesis that ABC and MFS transporters are indeed involved in MDR in filamentous fungi (Del Sorbo et al., 2000).

The aim of this review is to describe ABC and MFS transporters from filamentous fungi, with a function in protection against natural toxic compounds and fungicides. This review will also focus on recent data that demonstrate the role of ABC and MFS transporters in secretion of host-specific toxins and mycotoxins.

ABC transporters

ABC transporters comprise one of the largest protein families known to date, operating in a wide variety of organisms from bacteria to man (Higgins, 1992). They are located in the outer plasma membrane or in membranes of intracellular compartments, such as the vacuoles, endoplasmatic reticulum, peroxisomes, and mitochondria. ABC transporters are capable of transporting a wide variety of cytotoxic, hydrophobic agents, ranging from ions to macromolecules, against a concentration gradient (Ambudkar et al., 1999; Bauer et al., 1999; Del Sorbo et al., 2000; Theodoulou, 2000). The energy needed for transport is generated by the hydrolysis of ATP and for this reason ABC transporters are characterised as primary active transport systems (Azzaria et al., 1989). ABC transporters are also described as P-glycoprotein, PDR (pleiotropic drug resistance) proteins or MDR proteins.

The structural unit of an ABC transporter is composed of two homologous halves each containing six trans-membrane 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] motif, and the ABC signature [L-S-G-G-(X)3-R-hydrophobic-X-hydrophobic-A] (Ames et al., 1989; Walker et al., 1982). The catalytic activity of these sites with respect to coupling and hydrolysis of ATP provides the energy necessary for transport of substrates (Azzaria et al., 1989). Both NBFs can have ATPase activity, stimulated by the presence of the substrate and in the case of full size transporters interaction between the two homologous halves seems to be necessary for transport (al Shawi and Senior, 1993; Ambudkar et al., 1992; Loo and Clarke, 1994). The TMDs of ABC proteins are less conserved as compared to the NBFs. They might form a pore across the lipid bilayer of membranes (Rosenberg et al., 1997) and are known to play a role in determining the substrate specificity of the transporters. More specific, TMDs 4, 5, 6, 10, 11, and 12 and the extra-cellular loops connecting them are thought to be closely linked with substrate binding and transport (Greenberger, 1993; Loo and Clarke, 1995; Safa et al., 1990; Zhang et al., 1995).

ABC transporters can be classified into different clusters based on their topology. The majority of these proteins have a $[TMD_6\text{-}NBF]_2$ or $[NBF\text{-}TMD_6]_2$ topology. 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 (Decottignies and Goffeau, 1997; Theodoulou, 2000). Multidrug resistance-related proteins (MRP) are ABC transporters with a $TMD_n[TMD_6\text{-}NBF]_2$ topology. They are characterised by the presence of an additional trans-membrane spanning domain of approximately 200 amino acids at the N-terminus of the protein and the presence of a putative 'Regulatory' (R) or 'Connector' domain between the two homologue halves, thought to act in regulation of the protein (Tusnady et al., 1997) (Figure 2). Some representatives of this group of transporters have been identified as glutathione S-conjugate pumps involved in cellular detoxification and other processes (Ishikawa, 1992; Ishikawa et al., 1997).

ABC transporters include both uptake and efflux systems. In general, they exhibit a 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, flavanoids, 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 (Ambudkar et al., 1999; Bauer et al., 1999; Del Sorbo et al., 2000; Theodoulou, 2000). Most of these compounds have a positive charge at physiological pH, are hydrophobic and enter the cells through passive diffusion (Gottesman and Pastan, 1993). The way that these compounds are transported is not

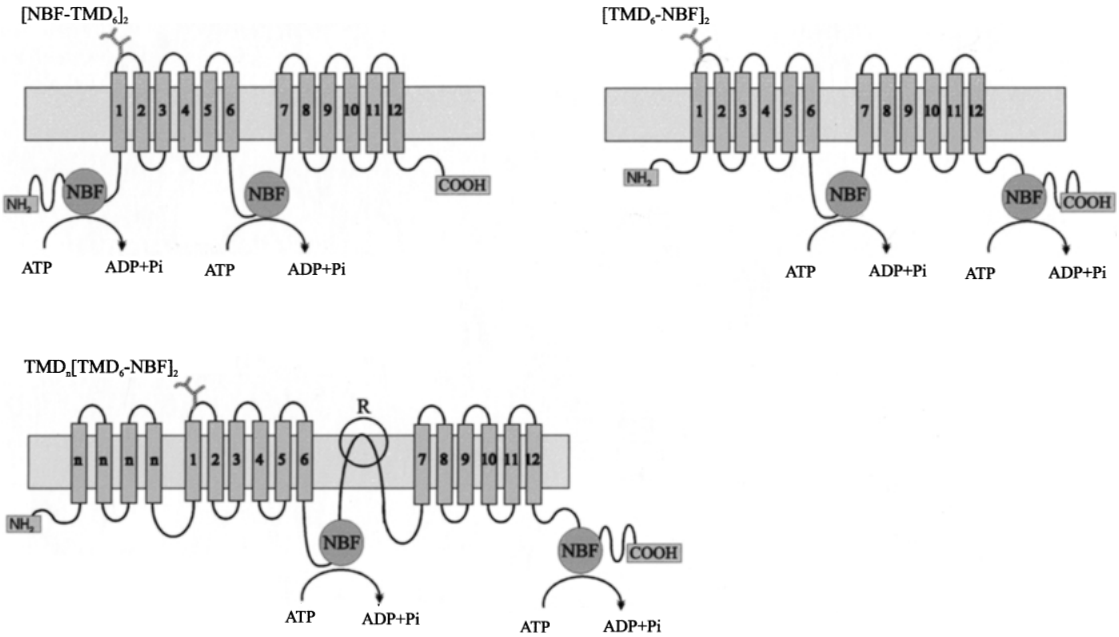

Figure 2. Schematic representation of ABC transporters with the $[NBF\text{-}TMD_6]_2$ and $[TMD_6\text{-}NBF]_2$ topology. MRP proteins are ABC transporters with a $TMD_n[TMD_6\text{-}NBF]_2$ topology. TMDs are indicated as boxes and are numbered. The NBFs are indicated as circles. 'R' is the 'regulatory' or 'connector' domain of the MRP-like proteins.

fully understood and several models have been proposed. Early models suggested that ABC proteins act as 'biological pumps' interacting directly with their substrates for their removal from the cytoplasm (Gottesman and Pastan, 1988). However, recent studies indicate that detection and excretion of toxic agents takes place at the membrane level before toxic concentrations can be built up in the cytoplasm. In this case, drugs are removed directly from the membranes into the extra-cellular space, and therefore, ABC transporters have been described as 'hydrophobic membrane vacuum cleaners' (Gottesman and Pastan, 1993; Raviv et al., 1990). However, it is also assumed that, initially, a small amount of the drug might be necessary to reach the cytoplasm in order to activate the cell defence mechanism and induce production of these pumps. A second 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 released into the outer environment (Higgins and Gottesman, 1992). Yet, other models propose that ABC proteins indirectly promote decreased intracellular accumulation of drugs by altering biophysical properties of membranes (Roepe, 1994; Wadkins, 1997).

In silico analysis of the *S. cerevisiae* genome identified at least 30 different ABC transporters (Taglicht and Michaelis, 1998). Other genome sequencing programmes reported the presence of 56 ABC proteins in *Drosophila melanogaster*, 56 in *Caenorhabditis elegans*, 129 in *Arabidopsis thaliana,* and 48 in *Homo sapiens* (http://www.humanabc.org/). Evidence suggests that an equally high number of ABC proteins are present in filamentous fungi. Complete sequencing of the *Neurospora crassa* genome revealed the existence of 39 putative ABC proteins, while in *Cochliobolus heterostrophus, Fusarium sporotrichioides*, and *Botrytis cinerea* 51, 54, and 46 ABC proteins were identified, respectively (Yoder and Turgeon, 2001). The function of a limited number of these proteins has been elucidated, but the vast majority remains to be investigated.

ABC transporters from filamentous fungi involved in protection against natural toxic compounds and fungicides

Aspergillus spp.

Several ABC transporter genes from *A. nidulans* have been described (Table 1). *AtrA* and *atrB* encode

Table 1. ABC transporter genes from *A. nidulans*

ABC gene	Acc.	Reference
atrA	Z68904	Del Sorbo et al., 1997
atrB	Z68905	Del Sorbo et al., 1997
atrC	AF071410	Andrade et al., 2000b
*atrC2**	AF082072	Angermayr et al., 1999
atrD	AF071410	Andrade et al., 2000b
atrE	AJ309280	Andrade et al., 2000
atrF	AJ309281	Andrade et al., 2000
atrG	AJ309282	Andrade et al., 2000
abcA	—	Do Nascimento et al., 1999
abcB	—	Do Nascimento et al., 1999
abcC	—	Do Nascimento et al., 1999
abcD	—	Do Nascimento et al., 1999

*Renamed. Original name was also *atrC*.

proteins with a [NBF-TMD_6]$_2$ topology, while *atrC* and *atrD* encode proteins with a [TMD_6-NBF]$_2$ topology. 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 sterol-demethylation-inhibiting (DMI) fungicides (Andrade et al., 2000; Del Sorbo et al., 1997). 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 (Del Sorbo et al., 1997). Functional analysis by gene replacement in *A. nidulans* demonstrated that atrB is involved in protection against compounds from all major classes of fungicides and natural toxic compounds. These include anilinopyrimide, benzimidazole, phenylpyrrole, phenylpyridylamine, DMI, and strobilurin fungicides, as well as the plant alkaloid camptothecin and the phytoalexin resveratrol (Andrade et al., 2000a). Disruption of *atrD* in *A. nidulans* resulted in a hypersensitive phenotype to cycloheximide, the cyclosporin derivative PSC 833, nigericin, and valinomycin (Andrade et al., 2000b). The results show that some of the *A. nidulans* ABC transporters play a role in defence against a wide range of natural toxic products and fungicides.

Since ABC transporters can play a role in protection of fungi against the activity of DMI fungicides, efforts have been undertaken to identify ABC transporter genes in *A. flavus* and *A. fumigatus*, two important opportunistic pathogens involved in human aspergillosis. *AflMDR1* (Acc. U62931) is the only ABC transporter gene cloned from *A. flavus*. It encodes a transporter with a [TMD_6-NBF]$_2$ predicted topology

Table 2. ABC transporter genes from *A. fumigatus*

ABC gene	Acc.	Reference
AfuMDR1	U62934	Tobin et al., 1997
AfuMDR2	U62936	Tobin et al., 1997
ADR1	—	Slaven et al., 1999
abcA	AJ417501	—
atrF	AJ311940	—

but no function for this protein has been reported yet. ABC transporter genes cloned from *A. fumigatus* are listed in Table 2. *AfuMDR1* encodes a protein with a predicted [TMD_6-NBF]$_2$ topology while *abcA* and *atrF* encode proteins with a [NBF-TMD_6]$_2$ topology. Interestingly, *AfuMDR2* encodes a protein with only four putative TMDs and a single NBF. Heterologous expression of AfuMDR1 in *S. cerevisiae* resulted in enhanced resistance to cilofungin, a (1,3)-β-D glucan synthase inhibitor, but not to other antimycotics tested. *A. fumigatus* is insensitive to cilofungin, suggesting that this property is due to the ability of AfuMDR1 to provide protection against this compound (Tobin et al., 1997). Increased levels of *ADR1* expression were found in an itraconazole-resistant isolate of *A. fumigatus* that does not accumulate this compound, implying the involvement of ADR1 in itraconazole efflux (Slaven et al., 1999). Reduced cellular accumulation of this compound has also been described for other itraconazole-resistant isolates of this fungus (Denning et al., 1997). Hence, increased drug efflux activity seems to be a common mechanism of resistance in *A. fumigatus*.

Botrytis cinerea

B. 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 (Table 3). The basal level of transcripts of these genes vary from undetectable (*BcatrC*, *BcatrJ*, *BcatrN*), to low (*BcatrA*, *BcAtrB*, *BcatrE*, *BcatrG*, *BcatrK*), and high (*BcatrF*, *BcatrH*, *BcatrI*) (Vermeulen et al., 2001). Treatment with fungicides can increase transcript levels of several of these genes. *BcatrB* encodes a protein with a [NBF-TMD_6]$_2$ topology. Increased transcript levels of this gene are observed after treatment with phytoalexins, phenylpyrrole, anilinopyrimide, and dicarboximide fungicides (Schoonbeek et al., 2001; Vermeulen et al., 2001). Functional analysis by means of gene

Table 3. ABC transporter genes from *B. cinerea*

ABC gene	Acc.	Reference
BcatrA	Z68906	Del Sorbo and De Waard, 1996
BcatrB	AJ006217	Schoonbeek et al., 1999
BcatrC	AF241315	Vermeulen et al., 2001
BcatrD	AJ272521	Vermeulen et al., 2001
BcatrE	AF238224	Vermeulen et al., 2001
BcatrF	AF238230	Vermeulen et al., 2001
BcatrG	AJ278038	Vermeulen et al., 2001
BcatrH	AF241313	Vermeulen et al., 2001
BcatrI	AF238229	Vermeulen et al., 2001
BcatrJ	AF238228	Vermeulen et al., 2001
BcatrK[*]	AF238227	Vermeulen et al., 2001
BMR1[*]	AB028872	Nakajima et al., 2001
BcatrL	—	Vermeulen et al., 2001
BcatrM	—	Vermeulen et al., 2001
BcatrN	AF238226	Vermeulen et al., 2001

[*] *BcatrK* and *BMR1* are identical.

disruption showed that Δ*BcatrB* mutants display increased sensitivity to resveratrol, a plant defence product in grapevine, while virulence tests showed a slight reduction in virulence on grapevine leaves as compared to the wild-type control. These results suggest a role for BcatrB in virulence of *B. cinerea* by providing protection against resveratrol. In addition, Δ*BcatrB* mutants exhibit increased sensitivity to the phenylpyrrole fungicides fenpiconil and fludioxonil, while mutants overexpressing this gene show decreased sensitivity to these compounds, suggesting an additional role for BcatrB in fungicide sensitivity of *B. cinerea* (Schoonbeek et al., 2001). *BcatrD* encodes a protein with a $[\text{NBF-TMD}_6]_2$ 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 DMI, dicarboximide, and benzimidazole fungicides, as well as with the antibiotic cycloheximide (Hayashi et al., 2001). 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 DMIs and accumulate relatively high amounts of 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 DMI fungicides (Hayashi et al., 2001). *BMR1* (*BcatrK*) encodes a protein with a $[\text{NBF-TMD}_6]_2$ topology. Δ*BMR1* mutants display increased sensitivity to the antibiotic polyoxin and the organophosphorous fungicide iprobenfos, which implies that BMR1 is an additional MDR transporter of this fungus (Nakajima et al., 2001).

Gibberella pulicaris

The necrotrophic fungus *G. pulicaris* (anamorph *Fusarium sambucinum*), the cause of dry rot disease of potatoes, infects tubers through open wounds. Recently, the ABC transporter gene *Gpabc1* (Acc. AJ306607), encoding a protein with a $[\text{NBF-TMD}_6]_2$ topology, was cloned and characterised from this fungus. Wounding of potato tissues induces the production of phytoalexins, such as rishitin and lubimin. Treatment of the fungus with either of these two compounds induced rapid expression of *Gpabc1*. Pathogenicity tests revealed that Δ*Gpabc1* mutants are unable to colonise potato slices. Additionally, these mutants are hypersensitive to rishitin and lubimin, suggesting that they are incapable of coping with the toxic effect of phytoalexins produced at the infection site. Thus, Gpabc1 is essential for pathogenicity of *G. pulicaris* on potato tubers by providing protection against plant defence compounds (Fleissner et al., 2002).

Magnaporthe grisea

M. grisea is a major pathogen of rice. The ABC transporter gene *ABC1* (Acc. AF032443) was identified through an insertional mutagenesis screen for pathogenicity mutants. The encoded protein has a $[\text{NBF-TMD}_6]_2$ topology. Δ*ABC1* mutants have normal growth on agar media but showed a complete loss of virulence on barley and rice plants. Histopathological analysis of the infection process on rice showed that the Δ*ABC1* mutants, although capable of forming appressoria, failed to produce extensive infection hyphae and died shortly after penetration of the epidermal cells. Expression analysis of the Δ*ABC1* mutants after treatment with several compounds demonstrated that *ABC1* transcript levels are strongly induced by the DMI fungicides miconazole and metconazole, the rice phytoalexin sakuranetin, and the protein synthesis inhibitor hygromycin. Yet, Δ*ABC1* mutants do not show increased sensitivity to any of these compounds. Thus, the exact role of ABC1 during pathogenesis needs to be established. The most probable explanation for the loss of virulence of the deletion mutants is that ABC1 provides protection against antimicrobial compounds present in barley and rice cells, although the compound(s) involved remain to be identified (Urban et al., 1999).

Mycosphaerella graminicola

Five ABC transporter genes have been cloned and sequenced from *M. graminicola* (anamorph *Septoria tritici*) the causal agent of *Septoria tritici* leaf blotch, one of the most important diseases of wheat (Table 4). The encoded ABC proteins all exhibit the $[NBF\text{-}TMD_6]_2$ configuration. Expression of *MgAtr3* was not detected under any conditions tested. However, *MgAtr1*, *MgAtr2*, *MgAtr4*, and *MgAtr5* display distinct expression profiles when treated with a range of compounds known to be either substrates or inducers of ABC transporters. These include DMIs, natural toxic compounds such as the plant defence compounds eugenol and psoralen, and the antibiotics cycloheximide and neomycin. The expression pattern of the genes also depends on the morphological state, yeast-like cells or mycelium, of the fungus (Stergiopoulos et al., 2002a; Zwiers and De Waard, 2000). Heterologous expression of *MgAtr1*, *MgAtr2*, *MgAtr4*, and *MgAtr5* genes in a multiple knockout strain of *S. cerevisiae* showed that the products of these genes transport a wide range of chemically-unrelated compounds and possess an extensive overlap in substrate specificity. Their substrate range includes synthetic compounds such as DMIs, natural compounds such as the plant metabolites berberine and camptothecin, and the mycotoxin diacetoxyscirpenol (DAS). The function of *MgAtr1-5* in virulence of *M. graminicola* on wheat was investigated with knockout mutants. Analysis of the transformants 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. No further phenotypes were observed for any of the mutants and compounds tested. This could be due to redundancy of ABC transporters with similar substrate specificity (Zwiers, 2002). Thus, the possibility that some of these transporters are involved in protection against natural and synthetic toxic compounds cannot be excluded. All transformants were tested for virulence on wheat seedlings. Δ*MgAtr4* mutants displayed reduced virulence as compared to the wild-type control strain. Northern analysis on interaction RNA isolated from wheat infected with the wild-type isolate and the Δ*MgAtr4* mutant showed a low build-up of biomass of the Δ*MgAtr4* mutant on wheat (Stergiopoulos et al., 2002b). The findings indicate a role for this protein in virulence.

Table 4. ABC transporter genes from *M. graminicola*

ABC gene	Acc.	Reference
MgAtr1	AJ243112	Zwiers and De Waard, 2000
MgAtr2	AJ243113	Zwiers and De Waard, 2000
MgAtr3	AF364105	Stergiopoulos et al., 2002a
MgAtr4	AF329852	Stergiopoulos et al., 2002a
MgAtr5	AF364104	Stergiopoulos et al., 2002a

Penicillium digitatum

P. digitatum is the causal agent of the citrus green mould. Five ABC transporter genes have been cloned from this fungus (Table 5). *PMR1* was cloned from a DMI-resistant isolate that is also cross-resistant to the unrelated chemicals cycloheximide, 4-nitroquinoline-*N*-oxide (4NQO), and acriflavine. The gene has a $[NBF\text{-}TMD_6]_2$ topology and shares a high degree of identity with *atrA* and *atrB* from *A. nidulans*. Basal level of *PMR1* transcription was higher in DMI-resistant isolates of *P. digitatum* than in sensitive ones. Treatment of the fungus with the DMI fungicide triflumizole induced *PMR1* transcription in sensitive and DMI-resistant strains. Disruption of *PMR1* in sensitive and DMI-resistant isolates resulted in increased sensitivity to DMIs as well as to camptothecin, phloretin, and oligomycin (Nakaune et al., 1998; 2002). Reintroduction of *PMR1* in Δ*PMR1* mutants derived from DMI-resistant strains resulted in decreased sensitivity to DMIs. However, reintroduction of *PMR1* in Δ*PMR1* mutants derived from DMI-sensitive strains did not result in decreased sensitivity. These results suggest that although PMR1-mediated efflux of DMIs plays an important role in determining sensitivity of *P. digitatum* to DMIs, it does not, alone, explain the difference in sensitivity between DMI-sensitive and resistant isolates (Hamamoto et al., 2000). *PMR5* encodes a protein with a $[NBF\text{-}TMD_6]_2$ topology and shares a high degree of identity with *PMR1* from *P. digitatum*, *atrB* from *A. nidulans* and *BcatrB* from *B. cinerea*. In contrast to *PMR1*, transcription of *PMR5* is strongly induced by benzimidazoles, dithianon, and resveratrol but not by DMIs. Furthermore, Δ*PMR5* mutants display increased sensitivity to benzimidazole fungicides and dithianon as well as to plant products such as

Table 5. ABC transporter genes from *P. digitatum*

ABC gene	Acc.	Reference
PMR1	AB010442	Nakaune et al., 1998
PMR3	—	Nakaune et al., 2001
PMR4	—	Nakaune et al., 2001
PMR5	AB060639	Nakaune et al., 2001; 2002

camptothecin and resveratrol. The results demonstrate that PMR5 and PMR1 possess distinct substrate specificity and have an important role in providing protection of the fungus against a range of natural and synthetic toxic compounds (Nakaune et al., 2002).

Other filamentous fungi

ABC transporter genes from other fungi include two genes, coded *LMABC1* and *LMABC2,* from *Leptosphaeria maculans* (anamorph *Phoma lingam*), the causal agent of blackleg disease of crucifers. Both genes encode proteins with the $[NBF\text{-}TMD_6]_2$ topology. *LMABC1* is strongly induced after treatment of the fungus with an analogue of the brassinin phytoalexin methyl-4-chlorobenzyldithiocarbamate and the azole fungicide miconazole. Increased transcripts of *LMABC2* are observed after treatment of the fungus with cycloheximide and the phytotoxin sirodesmin PL. Functional complementation of a *S. cerevisiae* ABC transporter null mutant, demonstrated that LMABC2 is able of transporting cycloheximide and 4NQO. Thus, it is possible that this gene plays a role in MDR, thereby protecting the fungus against natural and synthetic toxic compounds (Taylor and Condie, 1999).

Four ABC transporter genes have been cloned from *Venturia inaequalis*, the causal agent of the apple scab disease (Table 6). *ViABC1* and *ViABC2* encode proteins with a $[NBF\text{-}TMD_6]_2$ topology, while *ViABC3* and *ViABC4* encode proteins with a $[TMD_6\text{-}NBF]_2$ topology. Northern blot analysis revealed that *ViABC4* has a high basal level of expression indicating that this gene might be involved in basic metabolism (Schnabel and Jones, 2001).

ABC transporters involved in transport of toxins

Although data on the role of fungal ABC proteins in the secretion of endogenous produced toxins are limited, it is clear that ABC transporters can at least provide protection against toxins produced by other fungi. Bissinger and Kuchler (1994) reported the first evidence that ABC transporters can function in protection against mycotoxins. They showed that *PDR5* (Acc. L19922), a well-known ABC transporter gene from *S. cerevisiae*, can provide protection against sporidesmin, an epidithidioxopiperazine mycotoxin produced by the fungus *Phytomyces chartarum*. Δ*PDR5* mutants are supersensitive to sporidesmin as well as to a number of unrelated drugs, suggesting that PDR5 is involved in cellular detoxification processes. Trichothecene mycotoxins can act as virulence factors (Desjardins et al., 1996). The expression level of *PDR5* in *S. cerevisiae* correlates with sensitivity to exogenous trichothecenes. Transgenic tobacco plants transformed with *PDR5* were less sensitive to the trichothecene 4,15-diacetoxyscirpenol (Muhitch et al., 2000).

Table 6. ABC transporter genes from *V. inaequalis*

ABC gene	Acc.	Reference
ViABC1	AF227914	Schnabel et al., 2001
ViABC2	AF227915	Schnabel et al., 2001
ViABC3	AF375878	Schnabel et al., 2001
ViABC4	AF375879	Schnabel et al., 2001

Preliminary data have been reported for sirodesmins, which are non-selective toxins produced by the plant pathogenic fungus *L. maculans*. Increased expression levels of *LMABC2* are found after treatment with sirodesmin. The fungus is also known to produce the host-specific phytotoxin phomalide, suggesting that LMABC2 is involved in secretion of such compounds (Taylor and Condie, 1999). Further functional analysis of these ABC genes with respect to secretion of toxins is under investigation.

Heterologous expression of *MgAtr1* and *MgAtr*4 from *M. graminicola* in a *S. cerevisiae* strain with multiple non-functional ABC genes, demonstrated that both genes confer decreased sensitivity to the trichothecene DAS, a mycotoxin produced by *Fusarium graminearum*. Kema et al. (1996) proposed that formation of necrotic lesions on wheat leaves infected with *M. graminicola* is associated with secretion of phytotoxic compounds produced by the pathogen. Although such compounds have not yet been characterised, it might be possible that MgAtr1 and MgAtr4 are involved in secretion of host-specific toxins produced by the fungus. The reduced virulence phenotype of the Δ*MgAtr4* mutants supports this hypothesis (Stergiopoulos et al., 2002b).

Furthermore, it has been shown that MRP transporters of *H. sapiens* are capable of energy-dependent transport of aflatoxin B1 and its glutathione conjugates (Loe et al., 1997). These reports suggest that protection of organisms against aflatoxins can be mediated via efflux activity of ABC transporters.

Structure–function relationships among ABC transporters

We performed a phylogenetic analysis of ABC transporters from filamentous fungi sharing the

$[NBF\text{-}TMD_6]_2$ topology. Multiple sequence alignments were made using the ClustalW programme and a dendrogram was generated by parsimony as calculated using PROTPARS (Figure 3). A cluster of ABC proteins involved in protection against natural toxic compounds can be distinguished. This cluster includes atrB from *A. nidulans,* BcatrB from *B. cinerea*, MgAtr5 from *M. graminicola*, and PMR5 from *P. digitatum*. These transporters are induced and/or transport plant-defence related compounds, with the stilbene resveratrol and the plant alkaloid camptothecin as the most striking examples. It is possible that this branch of ABC transporters represents a group of orthologs with a similar function. A second cluster seems to be constituted by ABC proteins involved in protection against DMIs. This cluster includes BcatrD from *B. cinerea*, atrE and atrG from *A. nidulans*, and PMR1 from *P. digitatum*. BcatrD and PMR1 are specifically involved in protection against synthetic toxic compounds such as DMIs, while *atrG* and *atrE* are induced by DMIs (Andrade, 2000). Similarities in smaller clusters can also be found. For example, Gpabc1 from *G. pulicaris* and ABC1 from *M. grisea* are both pathogenicity factors. MgAtr1 from *M. graminicola* and BcatrA from *B. cinerea* cluster together and are both induced by cycloheximide. The same holds true for atrA from *A. nidulans* and MgAtr4 from *M. graminicola* that are both strongly induced by cycloheximide and imazalil.

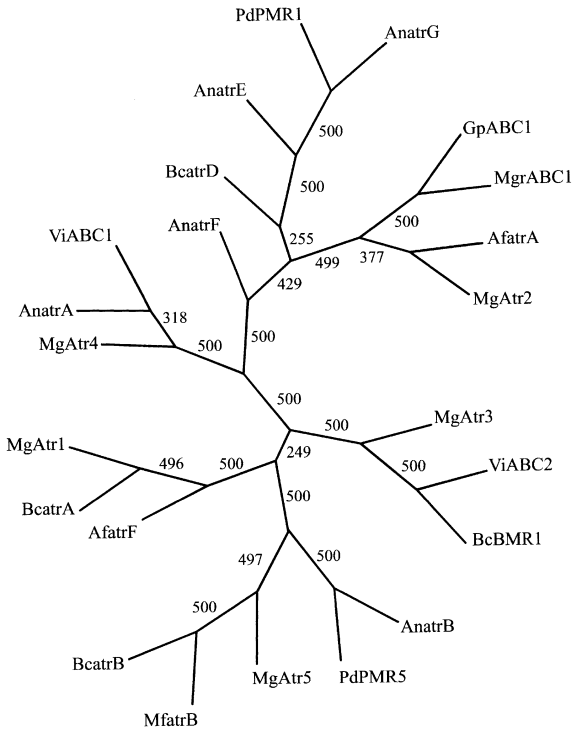

Figure 3. Phylogenetic unrooted tree of 23 ABC transporters with the $[NBF\text{-}TMD_6]_2$ topology from filamentous fungi. The tree was based on parsimony as calculated using PROTPARS. Alignments were performed using the ClustalW alignment programme. The code of the proteins mentioned in the tree are proceeded by two or three letters, indicating the source organism: An: *Aspergillus nidulans*, Af: *Aspergillus fumigatus*, Bc: *Botrytis cinerea*, Gp: *Gibberella pulicaris*, Mf: *Monilinia fructicola*, Mg: *Mycosphaerella graminicola*, Mgr: *Magnaporthe grisea*, Pd: *Penicillium digitatum*, Vi*: Venturia inaequalis*.

MFS transporters

MFS transporters comprise the largest protein family, present from bacteria to higher eukaryotes. They facilitate the uniport, symport, or antiport of various compounds using the energy from electrochemical gradients across membranes. For this reason MFS transporters are classified as secondary transport systems (Marger and Saier, 1993; Pao et al., 1998; Paulsen et al., 1996).

In general, MFS transporters consist of 12 or 14 TMDs arranged into two homologous halves, joined together by a large cytoplasmatic loop between TMDs 6 and 7 (Henderson, 1993; Kilty and Amara, 1992; Paulsen and Skurray, 1993) (Figure 4). Similarities between the two halves of the protein suggest that MFS transporters with 12 TMDs evolved

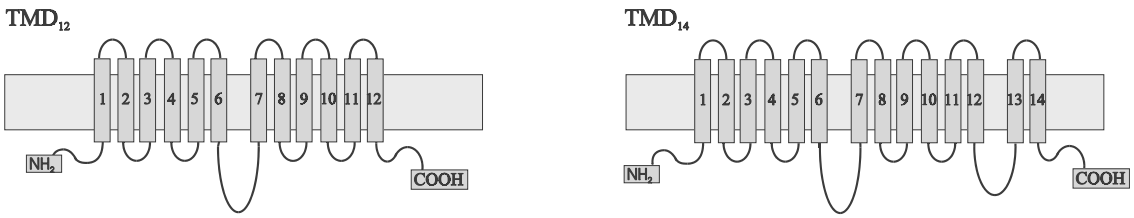

Figure 4. Schematic representation of MFS transporters with 12 and 14 TMDs. The TMDs are indicated in boxes and are numbered.

from a duplication event of a gene encoding a protein with 6 TMDs, while transporters with 14 TMDs emerged by additional acquisition of 2 TMDs at the C-terminal domain of the protein (Pao et al., 1998; Paulsen and Skurray, 1993; Rubin et al., 1990). Mutational analysis with respect to functionality of the two halves of these proteins suggests that the N-terminal domain is primarily involved in proton translocation while the C-terminal domain is engaged in substrate binding and recognition (Griffith et al., 1992). Unlike ABC proteins, MFS transporters do not possess well-defined conserved motifs. An overview of this superfamily classified at least 18 distinct families. Sequence alignment identified the presence of a conserved motif of 13 amino acids between TMDs 2 and 3 [G-[RKPATY]-L-[GAS]-[DN]-[RK]-[FY]-G-R-[RK]-[RKP]-[LIVGST]-[LIM]]. This motif is thought to be involved in promoting conformational changes in the protein upon substrate binding, allowing trafficking of substrates through the membrane. The motif may also act as a gate, regulating substrate transport from and to the cytoplasm. Additional sequence motives present in members of specific families of the MFS transporters can also be found, although the significance and conservation of such motives among the different MFS families is not yet fully defined (Pao et al., 1998).

Although functional analysis of most MFS transporters is still in its initial stages, reports suggest that the function and substrate specificity of these proteins can be impressively broad. MFS transporters are involved in the trafficking of sugars, drugs, polyols, vitamins, neurotransmitters, Krebs-cycle metabolites, phosphorylated glycolytic intermediates, amino acids, peptides, osmolites, iron-siderophores, nucleosides and organic and inorganic anions and cations. In this way, they facilitate cell functions such as the uptake of nutrients, secretion of cell cycle metabolites, protection against endogenous and exogenous toxic compounds, and maintenance of an electrochemical gradient across membranes. They are also involved in sporulation of fungi and yeasts, cell to cell communication and pathogenesis. MFS transporters differ from ABC proteins by the fact that they only mediate transport of relatively small-sized molecules.

The phylogenetic classification of MFS proteins into families revealed a close relation between structure and function. Thus, members of different families have specific substrate specificity and predictions about the function of novel members of a specific family can be made (Nelissen et al., 1997; Pao et al., 1998; Paulsen et al., 1996). In this respect, transporters of sugars and drugs comprise by far the largest families. For example, genome analysis of *S. cerevisiae* revealed 186 MFS transporters. A total of 28 of these proteins play a role in MDR while 34 were classified as sugar transporters (Nelissen et al., 1997).

MFS transporters involved in secretion of host-specific toxins

The first MFS transporter involved in secretion of host-specific toxins was reported by Pitkin et al. (1996). They found that the *TOXA* (Acc. AAB36607) gene product from *Cochliobolus carbonum*, encoding a MFS transporter with 10–13 predicted TMDs, secretes the host-specific cyclic tetrapeptide HC-toxin. Interestingly, *TOXA* occurs in two linked copies flanking the *HTS1* gene that encodes the central enzyme in HC-toxin biosynthesis. This suggests that there is a cluster of genes responsible for HC-toxin production. The *TOXA* genes have only been found in fungal strains that produce the HC-toxin. Mutants with a single-disrupted copy of *TOXA* still produce the HC-toxin and are virulent on maize. However, attempts to disrupt both copies of the *TOXA* gene were unsuccessful, suggesting that the encoded protein acts in self-protection against the HC-toxin. Thus, TOXA is essential for survival and virulence on host plants of HC-toxin producing strains.

Many species of the fungal genus *Cercospora* spp. including the soybean pathogen *C. kikuchii*, produce the phytotoxic polyketide cercosporin. Sequencing of cDNA clones generated from mRNA transcripts that were specifically induced under light-conditions led to the identification of *CFP* (Acc. AAC78076). This gene encodes a MFS transporter with 14 predicted TMDs that is involved in cercosporin transport. Δ*CFP* mutants do not produce cercosporin, have reduced virulence on soybean, and show increased sensitivity to this toxin. Complementation of these mutants with a functional *CFP* copy restores the parental phenotype. The results indicate that CFP is a cercosporin transporter involved in virulence and self-protection against this toxin (Callahan et al., 1999; Upchurch et al., 2001).

MFS transporters involved in secretion of mycotoxins

Most of the genes involved in trichothecene biosynthesis by *Fusarium* spp. are located within a gene cluster. In *F. sporotrichioides*, this cluster includes

Tri12 (Acc. AF11355), an MFS gene encoding a transporter with 14 predicted TMDs. Mutants of *S. cerevisiae* transformed with *Tri12* have decreased sensitivity to trichothecene. Disruption of *Tri12* in *F. sporotrichioides* reduced secretion of trichothecene and the Δ*Tri12* mutants showed impaired *in vitro* growth. Thus, Tri12 acts as a trichothecene transporter and plays a role in self-protection of *F. sporotrichioides* against trichothecenes (Alexander et al., 1999).

Recently, the *A. flavus* MFS gene *aflT* was identified in a gene cluster involved in aflatoxin biosynthesis (Acc. AC087725). The protein encoded by *aflT* is highly homologous to the HC-toxin transporter TOXA from *C. carbonum*. These observations suggest that *aflT* encodes an aflatoxin transporter (Chang et al., 1999).

The information available, indicates that secretion of endogenously-produced mycotoxins proceeds via MFS transporters, while protection against exogenous trichothecenes and aflatoxins seems to be mediated via ABC transporters. This is a striking difference, which might relate to the evolution of these proteins.

MFS transporters with other functions

Fragments of three MFS genes from *B. cinerea* were cloned from an expressed sequence tag (EST) library of this fungus grown under nitrogen starvation conditions. These genes were coded *Bcmfs1* (Acc. AF238225), *Bcmfs2* (Acc. AF241312), and *Bcmfs4* (Acc. AF238231). *Bcmfs1* encodes a protein with 14 predicted TMDs, which has highest homology to the MFS transporters Apafl1 from *A. parasiticus*, CFP1 from *C. kikuchii*, and TOXA from *C. carbonum*. DMI-resistant strains of *B. cinerea* show an increased expression of *Bcmfs1*. Δ*Bcmfs1* mutants display an increased sensitivity to the plant defence compound camptothecin and the toxin cercosporin. Mutants overexpressing *Bcmfs1* show an increased tolerance to these natural toxic compounds as well as to several DMIs, the dicarboximide fungicide iprodione, and the fungicides fenhexamide and captan. Deletion of *Bcmfs1* in a Δ*BcatrD* mutant increased the sensitivity of this mutant to DMIs. These results demonstrate that *Bcmfs1* is a multidrug transporter. Bcmfs1 is the first fungal MFS transporter identified with a function in MDR, capable of transporting both natural toxic compounds and fungicides (Hayashi et al., 2002).

In many cases, fungal MFS transporters involved in secretion of host-specific toxins are located in gene clusters responsible for the biosynthesis of these products. However, this is not always true as the *Gibberella fujikuroi* MFS transporter gene *smt* (Acc. AJ272424) is located adjacent to the gibberellin (GA) biosynthesis pathway genes but not involved in secretion of GAs from this fungus. *Smt* is probably a member of the sugar-transporter family as expression of this gene is induced by sugar alcohols, such as sorbitol, mannitol, and myoinositol (Voss et al., 2001).

Concluding remarks

State of the art

In eukaryotes, all well-characterised drug efflux mechanisms can be ascribed to the activity of ABC and MFS transporters. These transporters belong to superfamilies that are large and ancient, being thought to date back more that 3 billion years. It is suggested that the subfamilies of drug transporters evolved from families with other transport capacities and that members of these parent families may have functioned as transporters of nutrients into the cell or as exporters of biosynthetic macromolecules (Saier et al., 1998).

The ABC transporters characterised in filamentous fungi seem to be specifically involved in transport of exogenous substrates. These can be toxic compounds of either natural or synthetic origin. The rapid production of these transporters upon exposure to cytotoxic agents suggests that these proteins function as a first line of defence for the survival of the fungus. This protection mechanism is highly significant, as ABC transporters possess a broad range of substrate specificity and thus, can provide protection against toxic compounds of different chemical classes. This is a fundamental difference compared to other defence mechanisms, which are effective only against a particular class of compounds or even a single molecule. In plant pathogenic fungi, ABC transporters also provide protection against plant defence compounds, and thus, act as virulence factors. No ABC transporters that secrete endogenously produced virulence factors such as host-specific toxins have yet been identified. ABC transporters from filamentous fungi also have an important role in influencing base-line sensitivity to fungicides. Furthermore, overproduction of ABC proteins can result in MDR to different classes of fungicides. This has been reported especially for pathogens of medical importance (St Georgiev, 2000; Vanden Bossche et al., 1998). Yet, the risk of MDR in fungal pathogens of agricultural importance seems to

be limited (De Waard et al., 1995). This discrepancy is ascribed to a relatively low fitness of MDR strains by which they are unable to compete with wild-type isolates under field conditions (De Waard et al., 1982; Dekker, 1981).

Transporters of endogenous toxins most frequently belong to the MFS transporters. This may be explained by the fact that transport of such substrates should be constitutive as endogenously synthesised toxicants can be produced during the major part of the lifetime of the fungus. In this case, maintenance of MFS transport systems may be more cost-effective than the ABC transporter systems. MFS proteins are also involved in transport of exogenous substrates, such as cytotoxic compounds and thus, can function as a protection mechanism of cells. These MFS transporters are usually members of a specific 12 TMDs subfamily and are also implicated in MDR. Hence, activation of different efflux mechanisms seems necessary to ensure successful protection of cells from toxicants. For example, in a recent study on fluconazole-resistant strains of *C. albicans*, overexpression of ABC and MFS transporter genes operated concurrently in 85% of all the resistant strains tested (Perea et al., 2001).

Practical implications

The observation that ABC and MFS transporters influence base-line sensitivity to fungicides, are responsible for MDR and act as virulence factors implies that these transporters constitute an attractive target for chemical control. In this context, inhibitors of ABC and MFS transporters may improve the efficacy of chemical control and reduce virulence of plant pathogenic fungi.

Inhibition of ABC transporter activity can occur through compounds that are termed chemosensitizers or MDR modulators. These modulators may have little or no intrinsic cytotoxic action but inhibit ABC transporter-mediated drug export through competitive inhibition of transport or through interaction with other binding sites of the transporter. Compounds that block the generation of ATP in cells can also inhibit the activity of the ABC proteins. This approach has already been validated in clinical trials aimed at the reversal of MDR in tumor cells (Avendano and Menendez, 2002). Mixtures of agricultural fungicides and ABC transporter modulators may also be synergistic. This has been demonstrated for mixtures of DMIs and respiratory inhibitors, such as oligomycin and dicyclohexylcarbodiimide (De Waard and Van Nistelrooy, 1982; 1984a,b).

This review has described how ABC proteins with high sequence homologies can have similar functions. This observation implies that it should be feasible to develop inhibitors of specific ABC transporters. Such targeted inhibition is of particular interest for the cluster of DMI transporters and for the ones involved in virulence of plant pathogens. Inhibition of specific ABC transporters could also result in selectivity between target and non-target organisms, which is, for instance, required during plant pathogenesis.

Efflux mechanisms can interfere with screening programmes aimed at the discovery of new fungitoxic lead compounds. The reason is that test organisms have an intrinsic insensitivity to test compounds as a consequence of efflux systems that reduce accumulation of the compounds in cells. In this case, promising lead compounds would be missed. Thus, the use of hypersensitive fungal strains in high-throughput screening processes for the identification of new antifungal compounds is important. Single and multiple knockout mutants of ABC genes with a hypersensitive phenotype could be very useful for this purpose. Furthermore, to prevent fungicide resistance development based upon increased efflux activity, candidate compounds should not act as substrates of ABC transporters. This implies that these compounds must have the same toxic activity to wild-type, disruption, and overexpression mutants of ABC transporters. Hence, such mutants should also be included in the optimisation of new fungicides.

Many fungal species are known to produce antibiotics and other important metabolic compounds. Secretion of such compounds by the producing organisms might be mediated via efflux pumps such as ABC and MFS transporters. For example, penicillin secretion by *A. nidulans* may be influenced by the ABC transporter atrD (Andrade et al., 2000b). This implies that the production of secondary metabolites could be increased by overexpression of specific transporters.

Future perspectives

The number of ABC and MFS transporters cloned from filamentous fungi rapidly increases and genome-sequencing programmes will provide an even larger amount of data regarding sequences of ABC and MFS transporters. Although the role of some of these proteins has been elucidated, the function of the majority of transporters is still based on speculations

derived from sequence homologies and expression data. Additional data on phenotypic characterisation of gene disruption and/or overexpression mutants are certainly needed.

In many cases, ABC and MFS proteins have a remarkable broad substrate specificity that may overlap. This abundance of transporters presents serious difficulties in the functional analysis of gene disruption mutants. For this reason, overexpression mutants might be more helpful in the characterisation of the substrate specificity of the transporters.

Expression data show that ABC or MFS transporter genes can respond to the same stimuli, such as cytotoxic agents. This might suggest a general stress response and/or a common regulatory mechanism for these transporters. Yet, the way in which fungi regulate expression of ABC or MFS transporters is still unknown. Several studies have indicated that the promoter activity of the human P-glycoprotein may be modulated via the so-called Ras signal transduction pathway (Bosch and Croop, 1996). No evidence is available for the presence in filamentous fungi of a signal transduction pathway or a specific transcription factor that could regulate transporter activity. The way cells perceive potential substrates may be closely related with such a transduction pathway. Specific substrate receptors in membranes or membrane disturbance caused by the presence of the substrate might possibly lead to activation of such a transduction pathway. Alternatively, it might be possible that the transporter itself is involved in recognition of substrates and thus regulate its own expression.

Regulation of ABC transporter gene expression in *S. cerevisiae* is described as the PDR network. In this system, expression of several ABC transporters is under the regulatory control of PDR1 and PDR3. These proteins are members of the bi-nuclear Ga14p-like $Zn(II)_2Cys_6$ class of transcription factors that regulate transcription through *cis*-acting elements. These elements (5′-TCCG/aC/tGG/cA/g-3′) were coded as PDREs (pleiotropic drug resistance elements) and are present in all known PDR1/PDR3 target sites including MFS and ABC transporter genes (Bauer et al., 1999; Wolfger et al., 2001). Thus, it would be interesting to search for PDR1/PDR3 homologues in filamentous fungi and for putative PDREs elements. In *M. graminicola* disruption of single ABC genes did not result in increased sensitivity to any of the compounds tested. However, heterologous expression studies in *S. cerevisiae* shows that the encoded proteins posses an overlapping specificity in transport of substrates and northern blot analysis reveals that they are also induced by several common compounds (Stergiopoulos et al., 2002a; Zwiers, 2002; Zwiers and De Waard, 2000). Such observations support the idea that regulation of these transporters might proceed via a common transduction pathway.

An interesting question remains how ABC and MFS transporters or putative cell membrane receptors recognise and transport compounds from diverse chemical classes. This is especially relevant for xenobiotics since these were only developed in the last three decades. Despite this, several ABC and MFS transporters effectively transport these compounds. This may be due to a low substrate specificity of multidrug transporters. However, it is also possible that synthetic compounds mimic natural analogues. For example, DMI fungicides structurally resemble sterols and for this reason might be perceived as such by cells.

In summary, fungal ABC and MFS transporters present an exciting field of research since it combines fundamental aspects with various practical applications. Advances in molecular biology techniques especially with respect to fungal transformation and high-throughput functional analysis will strongly stimulate this type of research for the near future and may lead to the discovery of new disease control agents.

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References

al Shawi MK and Senior AE (1993) Characterization of the adenosine triphosphatase activity of Chinese hamster P-glycoprotein. Journal of Biological Chemistry 268: 4197–4206

Albertson G, Niimi M, Cannon R and Jenkinson H (1996) Multiple efflux mechanisms are involved in *Candida albicans* fluconazole resistance. Antimicrobial Agents and Chemotherapy 40: 2835–2841

Alexander NJ, McCormick SP and Hohn TM (1999) TRI12, a trichothecene efflux pump from *Fusarium sporotrichioides*: gene isolation and expression in yeast. Molecular and General Genetics 261: 977–984

Ambudkar S, Lelong I, Zhang J, Cardarelli C, Gottesman M and Pastan I (1992) Partial Purification and Reconstitution of the Human Multidrug-Resistance Pump: Characterization of the Drug-Stimulatable ATP Hydrolysis. Proceedings of the National Academy of Sciences of United States of America 89: 8472–8476

Ambudkar SV, Dey S, Hrycyna CA, Ramachandra M, Pastan I and Gottesman MM (1999) Biochemical, cellular, and pharmacological aspects of the multidrug transporter. Annual Review of Pharmacology and Toxicology: 39361–39398

Ames GFL, Nikaido K, Groarke J and Petithory J (1989) Reconstitution of periplasmic transport in inside-out membrane vesicles: energization by ATP. Journal of Biological Chemistry 264: 3998–4002

Andrade AC (2000) ABC transporters and multidrug resistance in *Aspergillus nidulans.* PhD Thesis Wageningen University, Wageningen, the Netherlands, ISBN 90-5808-279-2

Andrade AC, Del Sorbo G, Van Nistelrooy JGM and De Waard MA (2000a) The ABC transporter AtrB from *Aspergillus nidulans* mediates resistance to all major classes of fungicides and some natural toxic compounds. Microbiology 146: 1987–1997

Andrade AC, Van Nistelrooy JGM, Peery RB, Skatrud PL and De Waard MA (2000b) The role of ABC transporters from *Aspergillus nidulans* in protection against cytotoxic agents and in antibiotic production. Molecular and General Genetics 263: 966–977

Avendano C and Menendez JC (2002) Inhibitors of multidrug resistance to antitumor agents (MDR). Current Medical Chemistry 9: 159–193

Azzaria M, Schurr E and Gros P (1989) Discrete mutations introduced in the predicted nucleotide-binding sites of the *mdr1* gene abolish its ability to confer multidrug resistance. Molecular and Cellular Biology 9: 5289–5297

Bauer BE, Wolfger H and Kuchler K (1999) Inventory and function of yeast ABC proteins: about sex, stress, pleiotropic drug and heavy metal resistance. Biochimica Biophysica Acta, Biomembranes: 217–236

Bissinger PH and Kuchler K (1994) Molecular cloning and expression of the *Saccharomyces cerevisiae* Sts1 gene product – a yeast ABC transporter conferring mycotoxin resistance. Journal of Biological Chemistry 269: 4180–4186

Bosch I and Croop J (1996) P-glycoprotein multidrug resistance and cancer. Biochimimica Biophysica Acta 1288: F37–F54

Callahan TM, Rose MS, Meade MJ, Ehrenshaft M and Upchurch RG (1999) CFP, the putative cercosporin transporter of *Cercospora kikuchii*, is required for wild type cercosporin production, resistance, and virulence on soybean. Molecular Plant–Microbe Interactions 12: 901–910

Chang P-K, Yu J, Bhatnagar D and Cleveland TE (1999) The carboxy-terminal portion of the aflatoxin pathway regulatory protein AFLR of *Aspergillus parasiticus* activates GAL1::lacZ gene expression in *Saccharomyces cerevisiae*. Applied and Environmental Microbiology 65: 2508–2512

Christensen PU, Davey J and Nielsen O (1997) The *Schizosaccharomyces pombe* mam1 gene encodes an ABC transporter mediating secretion of M-factor. Molecular and General Genetics 255: 226–236

De Waard MA, Van Nistelrooy JGM, Langeveld CR, Van Kan JAL and Del Sorbo G (1995) Multidrug resistance in filamentous fungi. 11th International Rheinhardsbrunn Symposium: Modern Fungicides and Antifungal Compounds (pp 293–300) Friedrichroda, Germany

De Waard MA (1997) Significance of ABC transporters in fungicide sensitivity and resistance. Pesticide Science 51: 271–275

De Waard MA, Groeneweg H and Van Nistelrooy JGM (1982) Laboratory resistance to fungicides which inhibit ergosterol biosynthesis in *Penicillium italicum*. Netherlands Journal of Plant Pathology: 99–112

De Waard MA and Van Nistelrooy JGM (1982) Antagonistic and synergistic activities of various chemicals on the toxicity of fenarimol to *Aspergillus nidulans*. Pesticide Science: 279–286

De Waard MA and Van Nistelrooy JGM (1980) An energy-dependent efflux mechanism for fenarimol in a wild-type strain and fenarimol-resistant mutants of *Aspergillus nidulans*. Pesticide Biochemistry Psysiology 13: 255–266

De Waard MA and Van Nistelrooy JGM (1984a) Differential accumulation of fenarimol by a wild-type isolate and fenarimol-resistant isolates of *Penicillium italicum*. Netherlands Journal of Plant Pathology: 143–153

De Waard MA and Van Nistelrooy JGM (1984b) Effects of phthalimide fungicides on the accumulation of fenarimol by *Aspergillus nidulans*. Pesticide Science: 56–62

De Waard MA and Van Nistelrooy JGM (1988) Accumulation of SBI fungicides in wild-type and fenarimol-resistant isolates of *Penicillium italicum.* Pesticide Science 22: 371–382

Decottignies A and Goffeau A (1997) Complete inventory of the yeast ABC proteins. Nature Genetics 15: 137–145

Dekker J (1981) Impact of fungicide resistance of disease control toxicity. British Crop Protection Conference: pests and diseases (pp 857–863) Brighton, England

Del Sorbo G, Andrade AC, Van Nistelrooy JG, Van Kan JA, Balzi E and De Waard MA (1997) Multidrug resistance in *Aspergillus nidulans* involves novel ATP-binding cassette transporters. Molecular and General Genetics 254: 417–426

Del Sorbo G, Schoonbeek H and De Waard MA (2000) Fungal transporters involved in efflux of natural toxic compounds and fungicides. Fungal Genetics and Biology 30: 1–15

Denning DW, Venkateswarlu K, Oakley KL, Anderson MJ, Manning NJ, Stevens DA, Warnock DW and Kelly SL (1997) Itraconazole resistance in *Aspergillus fumigatus*. Antimicrobial Agents and Chemotherapy 41: 1364–1368

Desjardins AE, Proctor RH, Bai G, McCormick SP, Shaner G, Buechley G and Hohn TM (1996) Reduced virulence of trichothecene-nonproducing mutants of Gibberella zeae in wheat field tests. Molecular Plant–Microbe Interactions 9: 775–781

Do Nascimiento AM, Terenzi MF, Goldman MMH and Goldman GH (1999) A novel ATP-binding cassette transporter involved in multidrug resistance in the filamentous fungus *Aspergillus nidulans*. Fungal Genetics Newsletter 46: 44

Fleissner A, Sopalla C and Weltring K (2002) An ATP-binding cassette multidrug-resistance transporter is necessary for tolerance of *Gibberella pulicaris* to phytoalexins and virulence on potato tubers. Molecular Plant–Microbe Interactions 15: 102–108

Fling ME, Kopf J, Tamarkin A, Gorman JA, Smith HA and Koltin Y (1991) Analysis of a *Candida albicans* gene that encodes a novel mechanism for resistance to benomyl and methotrexate. Molecular and General Genetics 227: 318–329

Franz R, Kelly SL, Lamb DC, Kelly DE, Ruhnke M and Morschhauser J (1998) Multiple molecular mechanisms contribute to a stepwise development of fluconazole resistance in clinical *Candida albicans* strains. Antimicrobial Agents and Chemotherapy 42: 3065–3072

Gottesman MM and Pastan I (1988) The multidrug transporter, a double-edged sword. Journal of Biological Chemistry 263: 12163–12166

Gottesman MM and Pastan I (1993) Biochemistry of multidrug resistance mediated by the multidrug transporter. Annual Review of Biochemistry 62: 385–427

Greenberger LM (1993) Major photoaffinity drug labeling sites for iodoaryl azidoprazosin in P-glycoprotein are within, or immediately C-terminal to, transmembrane domains 6 and 12. Journal of Biological Chemistry 268: 11417–11425

Griffith JK, Baker ME, Rouch DA, Page MG, Skurray RA, Paulsen IT, Chater KF, Baldwin SA and Henderson PJ (1992) Membrane transport proteins: implications of sequence comparisons. Current Opinion in Cell Biology 4: 684–695

Hamamoto H, Hasegawa K, Nakaune R, Lee YJ, Makizumi Y, Akutsu K and Hibi T (2000) Tandem repeat of a transcriptional enhancer upstream of the sterol 14alpha-demethylase gene (CYP51) in *Penicillium digitatum*. Applied and Environmental Microbiology 66: 3421–3426

Hayashi K, Schoonbeek H and De Waard MA (2002) *Bcmfs1*, a novel ABC transporter from *Botrytis cinerea*, provides tolerance to the natural toxic compounds camptothecin and to fungicides (submitted)

Hayashi K, Schoonbeek H, Sugiura H and De Waard MA (2001) Multidrug resistance in *Botrytis cinerea* associated with decreased accumulation of the azole fungicide oxpoconazole and increased transcription of the ABC transporter gene *BcatrD*. Pesticide Biochemistry and Physiology 70: 168–179

Henderson P (1993) The 12-transmembrane helix transporters. Current Opinion in Cell Biology 5: 708–721

Higgins CF and Gottesman M (1992) Is the multidrug transporter a flippase? Trends in Biochemical Sciences 17: 18–21

Higgins CF (1992) ABC transporters: from microorganisms to man. Annual Review of Cell Biology 8: 67–113

Ishikawa T (1992) The ATP-dependent glutathione S-conjugate export-pump. Trends in Biochemical Sciences 17: 463–468

Ishikawa T, Li ZS, Lu YP and Rea PA (1997) The GS-X pump in plant, yeast, and animal cells: structure, function, and gene expression. Bioscience Reports 17: 189–207

Juliano RL and Ling V (1976) A surface glycoprotein modulating drug permeability in Chinese hamster ovary cell mutants. Biochimica Biophysica Acta 455: 152–162

Kema GHJ, Yu D, Rijkenberg FHJ, Shaw MW and Baayen RP (1996) Histology of the pathogenesis of *Mycosphaerella graminicola* in wheat. Phytopathology 86: 777–786

Kilty JE and Amara SG (1992) Families of twelve transmembrane domain transporters. Current Opinion in Biotechnology 3: 675–682

Kuchler K, Sterne RE and Thorner J (1989) *Saccharomyces cerevisiae* STE6 gene product: A novel pathway for protein export in eukaryotic cells. The EMBO Journal 8: 3973–3984

Loe DW, Stewart RK, Massey TE, Deeley RG and Cole SP (1997) ATP-dependent transport of aflatoxin B1 and its glutathione conjugates by the product of the multidrug resistance protein (MRP) gene. Molecular Pharmacology 51: 1034–1041

Loo TW and Clarke DM (1994) Reconstitution of drug-stimulated ATPase activity following co-expression of each half of human P-glycoprotein as separate polypeptides. Journal of Biological Chemistry 269: 7750–7755

Loo TW and Clarke DM (1995) Covalent modification of human P-glycoprotein mutants containing a single cysteine in either nucleotide-binding fold abolishes drug-stimulated ATPase activity. Journal of Biological Chemistry 270: 22957–22961

Marger MD and Saier MHJ (1993) A major superfamily of transmembrane facilitators that catalyse uniport, symport and antiport. Trends in Biochemical Sciences 18: 13–20

Muhitch MJ, McCormick SP, Alexander NJ and Hohn TM (2000) Transgenic expression of the TRI101 or PDR5 gene increases resistance of tobacco to the phytotoxic effects of the trichothecene 4,15-diacetoxyscirpenol. Plant Science 157: 201–207

Nakajima M, Suzuki J, Hosaka T, Hibi T and Akutsu K (2001) Functional analysis of an ATP-binding cassette transporter gene in *Botrytis cinerea* by gene disruption. Journal of General Plant Pathology 67: 212–214

Nakaune R, Adachi K, Nawata O, Tomiyama M, Akutsu K and Hibi T (1998) A novel ATP-binding cassette transporter involved in multidrug resistance in the phytopathogenic fungus *Penicillium digitatum*. Applied and Environmental Microbiology 64: 3983–3988

Nakaune R, Hamamoto H, Imada J, Akutsu K and Hibi T (2002) A novel ABC transporter gene, *PMR5*, is involved in multidrug resistance in the phytopathogenic fungus *Penicillium digitatum*. Molecular Genetics and Genomics: Published online: 22 February

Nelissen B, De Wachter R and Goffeau A (1997) Classification of all putative permeases and other membrane plurispanners of the major facilitator superfamily encoded by the complete genome of *Saccharomyces cerevisiae*. FEMS Microbiology Reviews 21: 113–134

Pao SS, Paulsen IT and Saier MH, Jr. (1998) Major facilitator superfamily. Microbiology and Molecular Biology Reviews 62: 1–34

Paulsen IT, Brown MH and Skurray RA (1996) Proton-dependent multidrug efflux systems. Microbiology Reviews 60: 575–608

Paulsen IT and Skurray RA (1993) Topology, structure and evolution of two families of proteins involved in antibiotic and antiseptic resistance in eukaryotes and prokaryotes – an analysis. Gene 132: 155

Perea S, Lopez-Ribot JL, Kirkpatrick WR, McAtee RK, Santillan RA, Martinez M, Calabrese D, Sanglard D and Patterson TF (2001) Prevalence of molecular mechanisms of resistance to azole antifungal agents in *Candida albicans* strains displaying high-level fluconazole resistance isolated from human immunodeficiency virus-infected patients. Antimicrobial Agents and Chemotherapy 45: 2676–2684

Pitkin JW, Panaccione DG and Walton JD (1996) A putative cyclic peptide efflux pump encoded by the TOXA gene of the plant-pathogenic fungus *Cochliobolus carbonum*. Microbiology 142: 1557–1565

Raviv Y, Pollard H, Bruggemann E, Pastan I and Gottesman M (1990) Photosensitized labeling of a functional multidrug transporter in living drug-resistant tumor cells. Journal of Biological Chemistry 265: 3975–3980

Roepe P (1994) Indirect mechanism of drug transport by P-glycoprotein. Trends in Pharmacological Sciences 15: 445–446

Rosenberg MF, Callaghan R, Ford RC and Higgins CF (1997) Structure of the multidrug resistance P-glycoprotein to 2.5 nm resolution determined by electron microscopy and image analysis. Journal of Biological Chemistry 272: 10685–10694

Rubin RA, Levy SB, Heinrikson RL and Kezdy FJ (1990) Gene duplication in the evolution of the two complementing domains of gram-negative bacterial tetracycline efflux proteins. Gene 87: 7–13

Safa AR, Stern RK, Choi K, Agresti M, Tamai I, Mehta ND and Roninson IB (1990) Molecular basis of preferential resistance to colchicine in multidrug-resistant human cells conferred by Gly-185 fwdarw Val-185 substitution in P-glycoprotein. Proceedings of the National Academy of Sciences of the United States of America 87: 7225–7229

Saier MH, Jr., Paulsen IT, Sliwinski MK, Pao SS, Skurray RA and Nikaido H (1998) Evolutionary origins of multidrug and drug-specific efflux pumps in bacteria. The FASEB Journal 12: 265–274

Sanglard D, Ischer F, Monod M and Bille J (1997) Cloning of *Candida albicans* genes conferring resistance to azole antifungal agents: Characterization of CDR2, a new multidrug ABC transporter gene. Microbiology 143: 405–416

Sanglard D, Kuchler K, Ischer F, Pagani JL, Monod M and Bille J (1995) Mechanisms of resistance to azole antifungal agents in *Candida albicans* isolates from AIDS patients involve specific multidrug transporters. Antimicrobial Agents and Chemotherapy 39: 2378–2386

Schnabel G and Jones LA (2001) Fungicide resistance genes in *Venturia inaequalis*. Modern fungicides and antifungal compounds. 13th International Reinhardsbrunn Symposium: Modern Fungicides and Antifungal Compounds, Friedrichroda, Germany

Schoonbeek H, Del Sorbo G and De Waard MA (2001) The ABC transporter BcatrB affects the sensitivity of *Botrytis cinerea* to the phytoalexin resveratrol and the fungicide fenpiclonil. Molecular Plant–Microbe Interactions 14: 562–571

Slaven JW, Anderson MJ, Sanglard D, Dixon GK, Bille J, Roberts IA and Denning DW (1999) Fungal Genetics Newsletter 46: 64

St Georgiev V (2000) Membrane transporters and antifungal drug resistance. Current Drug Targets 1: 261–284

Stergiopoulos I, Gielkens MMC, Goodall SD, Venema K and De Waard MA (2002a) ABC transporter genes from the wheat pathogen *Mycosphaerella graminicola*. Gene 289: 141–149

Stergiopoulos I, Zwiers L-H and De Waard MA (2002b) ABC transporters involved in pathogenesis of *Mycosphaerella graminicola*. 6th European Conference of Fungal Genetics (p 250) Pisa, Italy

Taglicht D and Michaelis S (1998) *Saccharomyces cerevisiae* ABC proteins and their relevance to human health and disease. Methods in Enzymology 292: 130–162

Taylor JL and Condie J (1999) Characterisation of ABC transporters from the fungal phytopathogen *Leptosphaeria maculans*. 9th International Congress on Molecular Plant–Microbe Interactions (p L71) Amsterdam, the Netherlands

Theodoulou FL (2000) Plant ABC transporters. Biochimica Biophysica Acta, Biomembranes 1465: 79–103

Tobin MB, Peery RB and Skatrud PL (1997) Genes encoding multiple drug resistance-like proteins in *Aspergillus fumigatus* and *Aspergillus flavus*. Gene 200: 11–23

Tusnady GE, Bakos E, Varadi A and Sarkadi B (1997) Membrane topology distinguishes a subfamily of the ATP-binding cassette (ABC) transporters. FEBS Letters 402: 1–3

Upchurch RG, Rose MS and Eweida M (2001) Over-expression of the cercosporin facilitator protein, CFP, in *Cercospora kikuchii* up-regulates production and secretion of cercosporin. FEMS Microbiology Letters 204: 89–93

Urban M, Bhargava T and Hamer JE (1999) An ATP-driven efflux pump is a novel pathogenicity factor in rice blast disease. The EMBO Journal 18: 512–521

Vanden Bossche H, Dromer F, Improvisi I, Lozano-Chiu M, Rex JH and Sanglard D (1998) Antifungal drug resistance in pathogenic fungi. Medical Mycology 36: 119–128

Vermeulen T, Schoonbeek H and De Waard MA (2001) The ABC transporter BcatrB from *Botrytis cinerea* is a determinant of the phenylpyrrole fungicide fludioxonil. Pest Management Science 57: 393–402

Voss T, Schulte J and Tudzynski B (2001) A new MFS-transporter gene next to the gibberellin biosynthesis gene cluster of *Gibberella fujikuroi* is not involved in gibberellin secretion. Current Genetics 39: 377–383

Wadkins RM RP (1997) Biophysical aspects of P-glycoprotein-mediated multidrug resistance. International Review of Cytology 171: 121–165

Walker JE, Saraste M, Runswick MJ and Gay NJ (1982) Distantly related sequences in the alpha- and beta-subunits of ATP synthase, myosin, kinases and other ATP-requiring enzymes and a common nucleotide binding fold. The EMBO Journal 1: 945–951

White T (1997) Increased mRNA levels of ERG16, CDR, and MDR1 correlate with increases in azole resistance in *Candida albicans* isolates from a patient infected with human immunodeficiency virus. Antimicrobial Agents and Chemotherapy 41: 1482–1487

Wolfger H, Mamnun YM and Kuchler K (2001) Fungal ABC proteins: pleiotropic drug resistance, stress response and cellular detoxification. Research in Microbiology 152: 375–389

Yoder OC and Turgeon BG (2001) Fungal genomics and pathogenicity. Current Opinion in Plant Biology 4: 315–321

Zhang XP, Collins KI and Greenberger LM (1995) Functional evidence that transmembrane 12 and the loop between transmembrane 11 and 12 form part of the drug-binding domain in P-glycoprotein encoded by MDR1. Journal of Biological Chemistry: 5441–5448

Zwiers L-H (2002) ABC transporters of the wheat pathogen *Mycosphaerella graminicola.* PhD Thesis Wageningen University, Wageningen, the Netherlands, ISBN 90-5808-613-5

Zwiers LH and De Waard MA (2000) Characterization of the ABC transporter genes *MgAtr1* and *MgAtr2* from the wheat pathogen *Mycosphaerella graminicola*. Fungal Genetics and Biology 30: 115–125