THE ROLE OF ATP-BINDING CASSETTE (ABC) TRANSPORTERS IN PATHOGENESIS AND MULTIDRUG RESISTANCE OF THE WHEAT PATHOGEN *MYCOSPHAERELLA GRAMINICOLA*

IOANNIS STERGIOPOULOS

Promotor:	Prof. Dr Ir P.J.G.M. de Wit, Hoogleraar Fytopathologie, in het bijzonder plant-pathogeen interacties, Wageningen Universiteit, Nederland
Co-promotor:	Dr Ir M.A. de Waard, Universitair Hoofddocent, Laboratorium voor Fytopathologie, Wageningen Universiteit, Nederland
Promotiecommissie:	Dr K-H. Kuck, Bayer Crop Science Research, Global Biology Fungicides, Agricultural Centre Monheim, Germany Dr G.H.J. Kema, Plant Research International, Wageningen, Nederland Prof. Dr M.R. Müller, Wageningen Universiteit, Nederland Prof. Dr L.H.W. van der Plas, Wageningen Universiteit, Nederland

IOANNIS STERGIOPOULOS

THE ROLE OF ATP-BINDING CASSETTE (ABC) TRANSPORTERS IN PATHOGENESIS AND MULTIDRUG RESISTANCE OF THE WHEAT PATHOGEN *MYCOSPHAERELLA GRAMINICOLA*

Proefschrift

ter verkrijging van de graad van doctor
op gezag van de rector magnificus
van Wageningen Universiteit,
Prof. Dr Ir L. Speelman
in het openbaar te verdedigen
op maandag 20 januari 2003
des namiddags te vier uur in de Aula

The role of ATP-binding cassette (ABC) transporters in pathogenesis and multidrug resistance of the wheat pathogen *Mycosphaerella graminicola*
Ioannis Stergiopoulos, 2003

Ph.D. Thesis Wageningen University, The Netherlands
With references – with summaries in English, Dutch, and Greek

ISBN 90-5808-787-5

To my parents, Spyros and Eleni
And my beloved brother, Fotis

To the land of Macedonia,
4000 years of Greek history

LIST OF ABBREVIATIONS

4NQO	4-nitroquinoline-*N*-oxide
ABC	ATP-binding cassette
AOA	α-aminooxy acetic acid
AOPP	α-aminooxy-*β*-phenylpropionic acid
BLAST	basic local alignment search tool
CCCP	carbonyl-cyanide m-chlorophenylhydrazone
cv	cultivar
DAS	diacetoxyscirpenol
DIMBOA	2,4-dihydroxy-7-methoxy-2*H*-1,4-benzoaxin-3(4*H*)-one
diol	C14α-methylergosta-8,24(28)-dien-3β,6α-diol
DMI	demethylation inhibitor
DMSO	dimethylsulfoxide
dpi	days post inoculation
EC_{50}	effective concentration inhibiting growth by 50%
EST	expressed sequence tag
HFAR	high-frequency azole resistance
IWF	intercellular washing fluid
MBC	methyl-benzimidazole carbamate
MDR	multidrug resistance
MeOH	methanol
MFS	major facilitator superfamily
MIC	minimum inhibitory concentration
MRP	multidrug resistance-related protein
NBD	nucleotide-binding domain
NBF	nucleotide-binding fold
OD_{600}	optical density at 600 nm
ORF	open reading frame
$P450_{14DM}$	cytochrome P450 sterol 14α-demethylase
PAL	phenylalanine ammonia-lyase
PCR	polymerase chain reaction
PDR	pleiotropic drug resistance

PDRE	pleiotropic drug resistance element
PEG	polyethylene glycol
P-gp	P-glycoprotein
RACE	rapid amplification of cDNA ends
RH	relative humidity
RT-PCR	reverse transcription PCR
TMD	trans-membrane domain
TMS	trans-membrane segment
UTR	untranslated region

CONTENTS

OUTLINE OF THE THESIS

ATP-binding cassette (ABC) transporters are membrane proteins that utilise the energy derived from the hydrolysis of ATP to drive the transport of compounds over biological membranes. They are members of one of the largest protein families to date, present in both pro- and eukaryotic organisms. ABC transporters play an essential role in multidrug resistance (MDR) of cancer cells to chemically unrelated compounds. ABC transporters involved in drug resistance have also been described in filamentous fungi. In plant pathogenic fungi ABC transporters may act as virulence factors if they mediate secretion of virulence factors or provide protection against plant defence compounds during pathogenesis. Such a role in pathogenesis has been demonstrated for the ABC transporters ABC1 from *Magnaporthe grisea*, BcatrB from *Botrytis cinerea*, and GpABC1 from *Gibberella pulicaris.*

In our laboratory ABC transporters from *Mycosphaerella graminicola* (Fückel) Schröter (anamorph state: *Septoria tritici* Rob.ex.Desm.), the causal agent of septoria tritici blotch of wheat, are studied. This disease can cause a significant reduction in yield. Typical disease symptoms are necrotic spots filled with the asexual pycnidia and sexual pseudothecia of the fungus. Formation of the necrotic lesions may be associated with secretion of phytotoxic compounds by the pathogen. On the other hand, wheat is known to produce plant defence compounds, such as 2,4-dihydroxy-7-methoxy-2*H*-1,4-benzoxazin-3(4*H*)-one (DIMBOA) and fluorescent compounds produced around infected stomata. Therefore, the fungus may have evolved specific ABC transporters that secrete toxins, or reduce the intracellular accumulation of plant defence compounds. Disease management of *M. graminicola* has widely involved the use of azole fungicides such as cyproconazole, propiconazole, and tebuconazole. The mode of action of these fungicides is based on inhibition of cytochrome P450 sterol 14α-demethylase ($P450_{14DM}$) activity, a key enzyme in the sterol biosynthetic pathway. In plant pathogenic fungi four major mechanisms of resistance to azoles have been reported. One of these is reduced accumulation of the fungicides in mycelium, attributed to an energy-dependent efflux mechanism mediated by ABC transporters. Other possible resistance mechanisms include mutations in the *CYP51* gene encoding $P450_{14DM}$ as well as overexpression of this gene.

Recently, Zwiers and De Waard (2000) cloned and characterised the ABC transporter genes *MgAtr1* and *MgAtr2* from *M. graminicola*. The present study is focused on cloning additional

ABC transporter genes from this pathogen and the analysis of their function in pathogenesis, protection against natural and synthetic toxic compounds, and resistance to azole fungicides in laboratory-generated mutants and field isolates of the fungus.

Chapter 1 describes *M. graminicola* and its importance in agriculture. In addition, mechanisms of resistance to azole fungicides are presented.

Chapter 2 comprises a review, describing the function of ABC transporters from filamentous fungi in pathogenesis and protection against natural and synthetic toxic compounds. Members of the major facilitator superfamily (MFS) of membrane transporters from filamentous fungi are also described, since these proteins can mediate similar functions in cells as ABC transporters.

Chapter 3 describes the cloning and characterisation of the ABC transporter genes *MgAtr3*, *MgAtr4*, and *MgAtr5* using a PCR-based approach. Sequence analysis showed that the encoded proteins exhibit a topology similar to that of MgAtr1 and MgAtr2 from *M. graminicola*. Northern analysis demonstrated that the genes display distinct but overlapping expression profiles when treated with a number of natural or synthetic toxic compounds known to be either inducers or substrates of ABC transporters.

In ***Chapter 4*** the role in MDR of *MgAtr1-MgAtr5* is studied. This was done by complementation of *Saccharomyces cerevisiae* mutants with the *M. graminicola* ABC transporter genes and by analysis of ABC transporter disruption or replacement mutants of *M. graminicola* with respect to sensitivity to natural and synthetic toxic compounds as well as antagonistic bacteria. Results indicate that ABC transporters from *M. graminicola* can play a role in protection of the fungus against natural and synthetic toxic compounds.

In ***Chapter 5*** the role of *MgAtr1-MgAtr5* as virulence factors during pathogenesis on wheat seedlings is studied. Disruption or replacement strains of *MgAtr1, MgAtr2, MgAtr3,* and *MgAtr5* displayed an unaltered phenotype in comparison to the wild-type control but virulence of *MgAtr4* disruption mutants was significantly reduced on seedlings of all wheat cultivars tested. Therefore, MgAtr4 is a virulence factor of *M. graminicola* during pathogenesis on wheat. This is the first virulence factor identified so far from this important plant pathogen.

Chapter 6 describes studies on mechanisms of resistance to azole fungicides in azole-resistant laboratory-generated mutants of *M. graminicola*. These include efflux mechanisms mediated by ABC transporters, overexpression of *CYP51,* and mutations in the coding

sequence of this gene. The results indicate that multiple mechanisms may be responsible for reduced sensitivity of the mutants to azoles.

Chapter 7 describes molecular mechanisms that account for variation in base-line sensitivity to azole fungicides in field isolates of *M. graminicola* and hence, complement results described in Chapter 6 for the field situation. Genetic analysis showed that azole sensitivity in *M. graminicola* is a polygenic trait. Overexpression of ABC transporter genes and *CYP51* may explain the reduced azole sensitivity of some field isolates, indicating that multiple mechanisms could account for differences in base-line sensitivity to azoles.

In ***Chapter 8*** the antimicrobial activity of the azole fungicides cyproconazole and propiconazole alone and in combination with ABC transporter modulators against *M. graminicola* is studied. Interactions in the mixtures are tested using the Colby and Wadley method with a wild-type *M. graminicola* isolate that showed moderate sensitivity to azole fungicides. Analysis with both methods showed that interactions between the compounds in most combinations tested are additive.

Chapter 9 presents a summarising discussion of the thesis.

CHAPTER 1

GENERAL INTRODUCTION:

Mycosphaerella graminicola

Molecular mechanisms of azole resistance in fungi

Mycosphaerella graminicola

Classification and Nomenclature

Mycosphaerella graminicola (Fückel) J. Schröt. in Cohn is the teleomorphic stage of *Septoria tritici* Roberge in Desmaz., causing the disease known as septoria tritici blotch of wheat. The asexual stage*, S. tritici,* was described by Roberge and published by Desmazières in 1842 (Desmazières, 1842). The sexual stage of this fungus was initially described by Fückel as *Sphaerella graminicola* (1865) and later classified by Schröter in the genus *Mycosphaerella* (1894). The debate on the classification of the sexual stage lasted until 1972 when Sanderson characterised *M. graminicola* as the perfect stage of the fungus (Sanderson, 1972; Sanderson, 1976).

Taxonomy and description of the fungus

M. graminicola is a member of the Order Dothideales (Ascomycota). It produces pseudothecia (70-100 μm) that enfold asci (30-40 x 11-44 μm), which contain eight ascospores (9-16 x 2.5-4.5 μm), consisting of two cells that are unequal in size (Sivanesan, 1990). The imperfect stage, *S. tritici,* is classified in the Order of Sphaeropsidales (Deuteromyces). It produces round, dark, brown to black pycnidia (60-200 μm), which contain threadlike pycnidiospores (1.7-3.1 x 39-86 μm), bearing 3 to 7 septations (Sivanesan, 1990). These are released from an opening at the tip of the pycnidium (ostiole) after a wet period of 30 minutes or longer. Release happens inside an extra-cellular matrix known as the ooze or cirrhus. The cirrhus consists of a thick, sticky substrate, enriched in sugars and proteins that enables spores to attach to the surface of their host and to remain viable under dry conditions (Fournet, 1969). The number of pycnidiospores released from a single pycnidium may vary from 5 to 10 x 10^3. Discharge of pycnidiospores from pycnidia progresses gradually in time, with the majority (40-60%) released during the first wetting period (Eyal, 1971).

Host range

Although bread (*Triticum aestivum*) and durum (*Triticum turgidum* subsp. *durum*) wheat are the primary hosts of *M. graminicola*, several other Graminaceous species may also function as alternative hosts of the pathogen. These include *Agropyron* spp., *Agrostis* spp., *Brachypodium* spp., *Bromus* spp., *Dactylis* spp., *Festuca* spp., *Glyceria* spp., *Hordeum* spp., *Poa* spp., *Secale*

cereale, and *Triticum* spp. Although such hosts may function as an alternative source of primary inoculum, their exact role in the epidemiology of *M. graminicola* needs to be established (Weber, 1922; Sprague, 1950; Williams and Jones, 1973; Brokenshire, 1975).

Disease symptoms

Wheat plants are susceptible to *M. graminicola* at any stage of their development. Although infections of leaves are most common, all aerial parts can be infected. Initial symptoms of the disease are irregular yellowish or chlorotic spots on the infected tissue that spread longitudinally between the veins. These symptoms appear five to six days after inoculation but the time of their appearance varies according to the environmental conditions and susceptibility of the wheat cultivar. Under favourable conditions initial spots turn into necrotic lesions, surrounded by a yellow margin and covered with the pycnidia of the fungus. Pycnidia are formed exclusively within substomatal cavities and are present on both sides of the leaf surface. They often emerge in rows, following the linear arrangement of the stomata across the leaves. Their size can fluctuate in proportion to the number of pycnidia present on the infected tissue and the cultivar response (Eyal *et al.,* 1987). In that respect, higher numbers of pycnidia and more tolerant wheat cultivars usually result in smaller in size pycnidia (Eyal and Brown, 1976). However, the percentage of the necrotic leaf area, or the virulence of a *S. tritici* isolate does not significantly affect the size of the pycnidia and pycnidiospores produced (Shearer and Wilcoxson, 1978). Heavily infected leaves can become completely necrotic resulting in poor root development and reduced grain production, or even plant death. Leaf sheaths, glumes, stems, and especially nodes may also become infected. Infected nodes appear dark in colour and sunken and can also develop pycnidia.

Epidemiology

The fungus overwinters as pycnidia, pseudothecia, or mycelium in plant debris, such as straw and stubble, volunteer wheat or alternative hosts (Djerbi, 1977; Eyal *et al.,* 1987). Primary inoculum consists of rain-splashed pycnidiospores released from pycnidia and airborne ascospores from mature pseudothecia (Figure 1). Although initially underestimated, the role of ascospores in epidemics of the disease is gradually given more attention. In the U.K. and U.S.A., ascospores are considered to be the primary source for initial infections during autumn in winter wheat crops (Shaw and Royle, 1989; Schuh, 1990). Ascospores can also be

released throughout the whole season and thus, significantly contribute to disease epidemics and the establishment of diverse genetic populations (Hunter *et al.,* 1999; Zhan *et al.,* 1998; Zhan *et al.,* 2001).

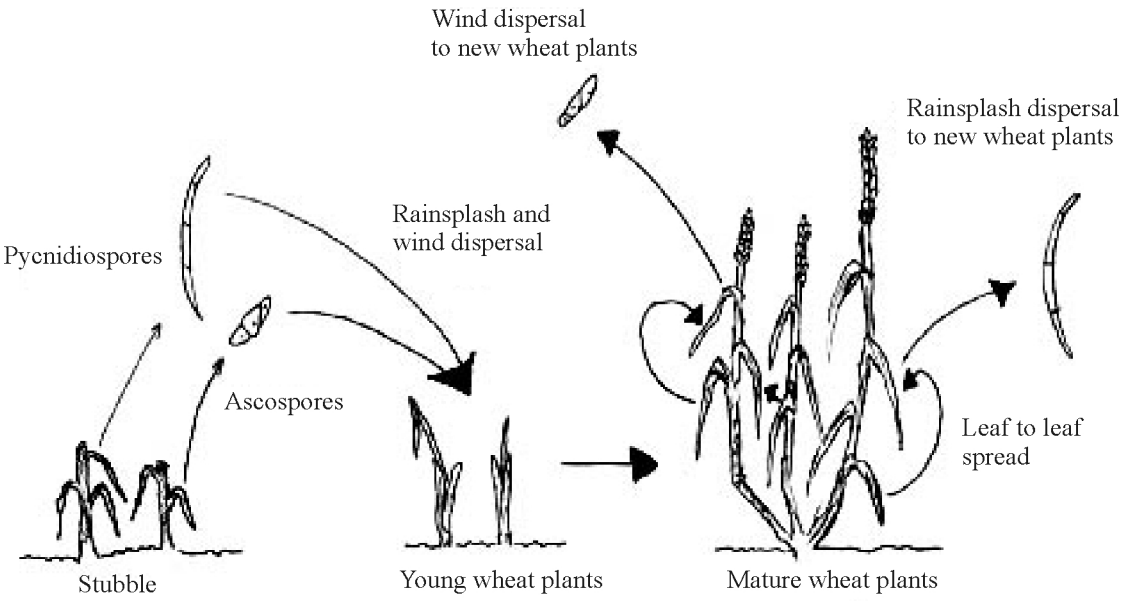


Figure 1. Life cycle of *Mycosphaerella graminicola* (source: Palmer and Skinner, 2002)

Both ascospores and pycnidiospores require conditions of high humidity that result in free water for their release (Eyal, 1971; Brown *et al.,* 1978; Sanderson and Hampton, 1978; Shaw, 1991). High humidity is also necessary for all stages of infection: spore germination, penetration and development within the host as well as pycnidia production (Hooker, 1957; Shaner and Finney, 1976). Temperatures required for disease development are a minimum of 2-3ºC and a maximum of 33-37ºC, with an optimum of 20-25ºC. Under optimum conditions the asexual cycle of the disease can be completed within fourteen to twenty-one days (Shipton *et al.,* 1971; Shaner and Finney, 1976) and a sexual cycle within thirty-five days in the Netherlands (Kema *et al.,* 1996a). However, environmental conditions such as moisture levels, temperature, and light intensity, the susceptibility of the wheat cultivar, and virulence of the isolates can influence the time needed for completion of the disease cycle (Shaw, 1991; Shaw and Royle, 1993). Pycnidiospores as well as ascospores can be dispersed to leaves by rain splash. This transport occurs both from the lower leaves of the plants or crop debris to leaves higher in the canopy (vertical transport) and to the surrounding leaves (horizontal transport). Horizontal transport may also occur via leaf contact between uninfected and

infected leaves and wind-transferred inoculum (Royle *et al.,* 1995). Therefore, the canopy structure can have an important role in determining development of the disease (Lovell *et al.,* 1997). In this respect, tall plants and late maturity often contribute to lower disease coverage of the upper leaves and can be considered as a source of resistance (Scott *et al.,* 1982; Eyal *et al.,* 1987; Van Beuningen and Kohli, 1990).

Infection process

Germinating pycnidiospores or ascospores develop germ tubes from both apical ends of the spore and as buddings from intermediate cells. Cytological analysis of the infection process showed that germ tubes enter the host almost exclusively through stomata (Kema *et al.,* 1996b; Duncan and Howard, 2000) (Figure 2), although direct penetration through the cuticle has also been reported (Dancer *et al.,* 1999).

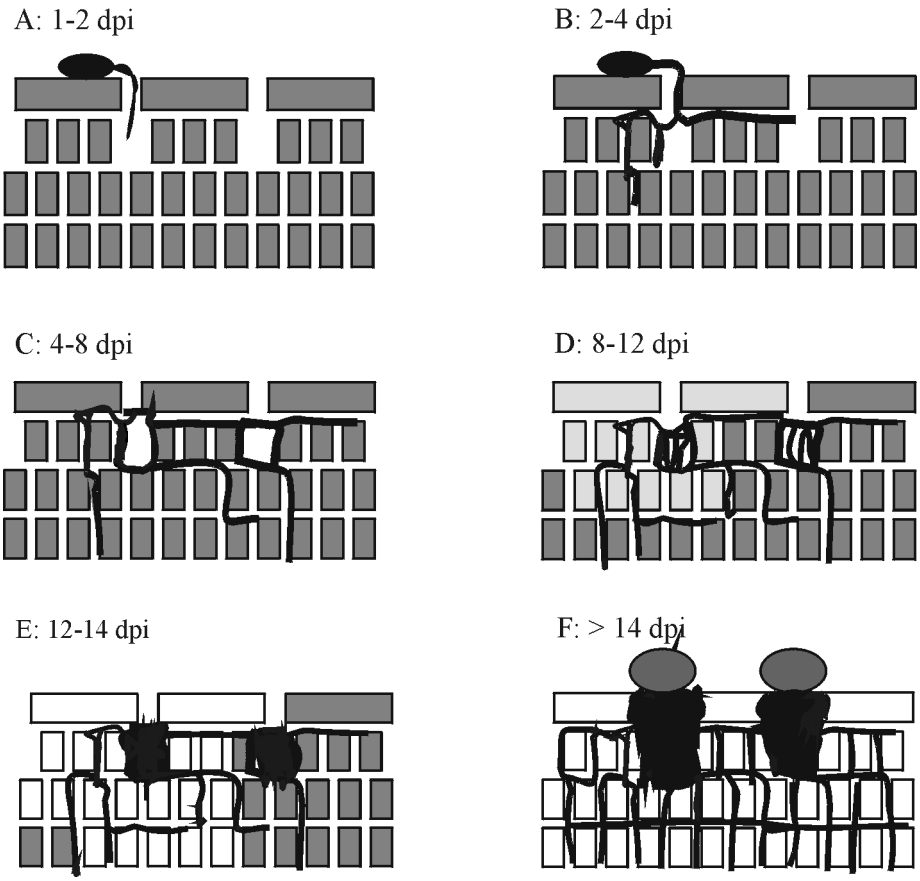


Figure 2. Infection process in time (days post inoculation; dpi) of *Mycosphaerella graminicola* in wheat leaves. A: penetration through stomata; B: infection hyphae expand to the mesophyll and to neighbouring substomatal cavities; C: formation of ring-like structures in substomatal cavities and further colonisation of mesophyll; D: chlorotic symptoms on infected leaves and beginning of the formation of mycelial baskets in substomatal cavities; E: cell collapse followed by necrotic symptoms on leaves and rapid mycelial proliferation; F: pycnidia in dead host tissue. (adopted from Duncan and Howard, 2000)

Penetration via stomata usually occurs within the first 24-48 hours after inoculation. No appressoria are formed, although the formation of appressoria-like structures (swellings) at the tip of the germ tubes has been reported, without their presence to be a prerequisite for stomatal penetrations (Duncan and Howard, 2000). From the substomatal cavities the fungus expands through the mesophyll and also towards neighbouring substomatal cavities thus, establishing multiple infections from a single penetration site. During pathogenesis, hyphae remain strictly intercellular and in close contact with mesophyll cells, but no haustoria or other specialised feeding structures are formed. At 8-10 days post inoculation (dpi), a massive increase of fungal biomass is present, stimulated by the release of nutrients after cell collapse. This increase is especially apparent around the substomatal cavities where the formation of the so-called baskets is observed. The rapid collapse of mesophyll cells and the presence of severely affected cells without the presence of mycelium in the vicinity led to the suggestion that soluble phytotoxic compounds are involved in the wheat - *M. graminicola* interaction (Zellikovitch *et al.,* 1992; Kema *et al.,* 1996b). However, no such specific compounds have been yet characterised and their role in pathogenesis remains to be established. Finally, at approximately 14 dpi the first pycnidia become visible, emerging from the stomata (Cohen and Eyal, 1993; Kema *et al.,* 1996b; Duncan and Howard, 2000).

Host specificity

Host specificity in *M. graminicola* has been reported by many authors but still remains controversial. Eyal *et al.,* (1973) indicated that host-response in the wheat - *M. graminicola* pathosystem is quantitative rather than qualitative and proposed the existence of physiological specialisation in the pathogen. Genetic variability reported for *M. graminicola* populations suggests that there is potential selection of isolates with increased virulence to resistant cultivars (McDonald and Martinez, 1990). Isolates of *M. graminicola* specifically adapted to bread or durum wheat (Eyal *et al.,* 1973; Kema *et al.,* 1996a) as well as specific cultivar-isolate interactions have been identified (Eyal *et al.,* 1985; Ahmed *et al.,* 1996; Kema and Van Silfhout, 1997; Cowger *et al.,* 2000; Brown *et al.,* 2001). These observations raise the possibility for a gene-for-gene relationship in the wheat - *M. graminicola* pathosystem. Kema *et al.,* (2000) reported that avirulence in the pathogen is controlled by a single locus, which is consistent with the idea of a gene-for-gene relationship. Till now, no specific avirulence genes have been characterised from this pathogen (Brading *et al.,* 2002).

Economic importance

M. graminicola is considered as the major threat to wheat crops in the Western Europe and around the Mediterranean basin, causing serious losses on both bread and durum wheat (Eyal and Ziv, 1974; Kema and Verstappen, 1999). The economic importance of the disease is rapidly increasing in areas with a mild climate and heavy infections from this pathogen have been reported in almost all wheat-growing areas of the world: North and South America, Africa, Asia, Australia, and New Zealand (Shipton *et al.,* 1971). In the U.K., wheat fields with infections by *M. graminicola* were detected at a frequency of 2% by 1976 and increased to 86% by 1988 (Polley and Thomas, 1991). This enormous rise in disease frequency was attributed partially to the introduction of more susceptible early maturing semidwarf wheat cultivars as well as to changes in cultural practices. The total losses from septoria diseases (*S. tritici* and *Stagonospora nodorum*) are estimated world-wide over 9 million metric tons (Kolomiets, 1999) as both diseases are capable of reducing yields as much as 30-40% (Eyal *et al.,* 1987).

Disease management

Disease management of *M. graminicola* is predominantly based on the use of resistant wheat cultivars, chemical control, and cultural practices.

Breeding for resistance. Host resistance to *M. graminicola* is complex but understanding the inheritance of resistance to this pathogen is essential for designing effective breeding programs. Wheat cultivars can vary significantly in their response to infections by *M. graminicola*. In general, no complete immunity to *M. graminicola* exists and thus, necrosis and/or pycnidia are generally present. Therefore, any delay or restriction in pathogen development is considered as a form of resistance (Nelson and Marshall, 1990). Several studies indicated that resistance to *M. graminicola* may be controlled by one or two dominant genes or partially dominant genes (Wilson, 1979; Lee and Gough, 1984; Somasco *et al.,* 1996), two or three recessive genes (Rosielle and Brown, 1979; Wilson, 1985), and by the additive effects of multiple genes (Van Ginkel and Scharen, 1987; Van Ginkel and Scharen, 1988; Jlibene *et al.,* 1994; Simon and Cordo, 1998). Physiological specialisation of the pathogen suggests that cultivars can provide protection against a particular race of *M. graminicola* but not to others (Kema *et al.,* 1996a; Eyal, 1999). In addition, frequent genetic recombination within the pathogen population may imply that resistance of cultivars can

easily be lost by changes in virulence of the pathogen (Chen and McDonald, 1996; Kema *et al.,* 1996a; Hunter *et al.,* 1999). Cultivars with high resistance to *M. graminicola* exist but their yields are significantly less than those of susceptible cultivars treated with fungicides (Hollomon *et al.,* 1999). In general, plant height and maturity level negatively correlate with pycnidia coverage by *M. graminicola* and in that respect, tall plant stature and late maturity are often linked with low infection levels (Tavella, 1978; Brokenshire, 1976; Danon *et al.,* 1982; Van Beuningen and Kohli, 1990). Short varieties are more prone to damage because transfer of rain-splashed pycnidiospores in the canopy is more efficient. Therefore, the widespread use of dwarf and semi-dwarf varieties from the 1970s onwards may have increased damage by septoria tritici blotch (Baltazar *et al.,* 1990). However, these factors may be less important in areas where the epidemic is generated by wind-born ascospores. Wheat cultivars that express a consistent host response over time, which is not associated with plant height and maturity and is combined with low pycnidial coverage, are likely to possess a more stable type of resistance (Eyal and Talpaz, 1990).

Chemical control. A large number of compounds that belong to different classes of fungicides are known to be active against *M. graminicola*. Protective fungicides, such as the dithiocarbamates maneb, mancozeb, and zineb and the aromatic fungicide chlorothalonil, have been used in the past in control of septoria diseases (Eyal and Wahl, 1975; Hims and Cook, 1992). However, protective fungicides require repeated applications every ten to fourteen days during the growing season for effective control of the pathogen, which significantly increases the cost of disease management. In that respect, systemic fungicides with curative activity and long protective action may be more beneficial than protective fungicides. Methyl-benzimidazole carbamate (MBC) fungicides, such as benomyl, have been extensively used for control of *M. graminicola* in the fields (Sanderson and Gaunt, 1980). However, isolates of *M. graminicola* resistant to benomyl have been reported (Fisher and Griffin, 1984; Zelikovitch *et al.,* 1986). Such isolates are cross-resistant to other benzimidazole fungicides, such as carbendazim and thiabendazole, but not to fungicides from other classes (Fisher and Griffin, 1984; Metcalf *et al.,* 1985). Sterol demethylation inhibitors (DMIs), such as the azoles cyproconazole, epoxiconazole, propiconazole, tebuconazole, and triadimefon, have been used extensively for the control of *M. graminicola* over the past 20 years. These are systemic fungicides with both protective and curative activity in disease control (Kuck and Scheinpflug, 1986). Although more effective when sprayed during the leaf emergence stage of

wheat development, they offer flexibility in time of application (Jordan *et al.,* 1986; Cook and Thomas, 1990; Loughman and Thomas, 1992; Duczek and Jones-Flory, 1994; Milus, 1994). In addition, they are broad-spectrum fungicides active also against other diseases, such as rusts and powdery mildew. Despite their extensive use, no resistance development of *M. graminicola* against these compounds has been detected although, populations of this pathogen with a broad-range in base-line sensitivity are present (Suty and Kuck, 1996; Gisi *et al.,* 2000). Recently, a new class of a broad-spectrum systemic fungicides derived from natural products, the strobilurins (azoxystrobin, kresoxim-methyl, trifloxystrobin), has been introduced. Their mode of action is based on inhibition of mitochondrial respiration by blocking electron transfer between cytochrome b and cytochrome c_1 at the QoI site of cytochrome b. For this reason, strobilurins are also described as QoI fungicides (QoIs) (Clough, 1993; Baldwin *et al.,* 1996; Bartlett *et al.,* 2002). QoIs may be used both in protective and curative applications against *M. graminicola* and initial trials indicate that they can provide efficient control of septoria diseases under field conditions (Godwin *et al.,* 1999; Rohel *et al.,* 2001; Rohel *et al.,* 2002).

Cultural practices. Despite the use of disease-tolerant wheat varieties and agrochemicals, cultural practices should remain the main pillar of control of septoria diseases. Cultural practices are of high importance, especially in low crop productivity systems where low yields do not justify additional expenses for disease control. Such cultural practices aim to reduce the amount of inoculum available for infections and the time that the crop is exposed to the pathogen. Removal of volunteer wheat and other alternative hosts in fields before planting, ploughing, burning of stubble, and rotation with other crops that are non-hosts to the pathogen, can drastically reduce the amount of primary inoculum available for infections (Bannon and Cooke, 1998). Narrow row spacing and high plant density tend to increase disease severity, enabling easier dispersal of the inoculum within the crop and creating high humidity conditions in the canopy. Therefore, such conditions that favor the disease should be avoided (Tompkins *et al.,* 1993). Overhead irrigation also enhances disease severity as compared to other methods of irrigation. Finally, crops planted later in the growing season are less prone to infections as they are exposed to infections for a shorter period of time (Murray *et al.,* 1990; Shaw and Royle, 1993).

Molecular mechanisms of azole resistance in fungi

General characteristics of azole fungicides

Azoles represent a large group of fungicides that has been available since the mid-1970s and currently constitute one quarter of the world's market for fungicides. Most azoles are imidazole (*e.g.* imazalil and prochloraz) or triazole derivatives (*e.g.* epoxiconazole, cyproconazole, propiconazole, tebuconazole, and triadimefon). They are systemic fungicides with both a protective and curative activity in disease control and active against a wide range of plant pathogenic fungi. *In vitro* studies have shown that most members of the Ascomycetes, Basidiomycetes, and Fungi Imperfecti are more or less sensitive to these compounds (Kuck and Scheinpflug, 1986; Scheinpflug and Kuck, 1987). Azoles have a low toxicity to mammals and therefore, several imidazoles (*e.g.* ketoconazole and miconazole) or triazoles (*e.g.* fluconazole and itraconazole) are also extensively used as antimycotics in the treatment of human and animal infections by fungi and yeasts. Their spectrum of antifungal activity includes *Aspergillus*, *Candida*, *Saccharomyces cerevisiae,* and other invasive opportunistic fungal pathogens such as *Mucor, Fusarium, Rhizomucor*, and *Absidia* species (Sheehan *et al.,* 1999).

Mode of action

Azoles act by inhibition of the activity of cytochrome P450 sterol 14α-demethylase ($P450_{14DM}$)-dependent oxidative demethylation of eburicol (filamentous fungi) or lanosterol (yeasts) in the ergosterol biosynthesis pathway. The heterocyclic ring of azoles can bind to the sixth ligand position of the central iron atom in the haem group of $P450_{14DM}$ and thus, prevent substrate binding and oxygen activation (Buchenauer, 1977; Sisler and Ragsdale, 1984; Vanden Bossche *et al.,* 1987; Yoshida and Aoyama, 1987; Yoshida, 1988; Yoshida and Aoyama, 1990; Yoshida and Aoyama, 1991; Vanden Bossche and Marichal, 1991; Vanden Bossche and Marichal, 1992; Vanden Bossche, 1998). For this reason azoles are also described as sterol demethylation inhibitors (DMIs). Inhibition of $P450_{14DM}$ activity induces the depletion of ergosterol in cell membranes as well as accumulation of C14-methylated precursors. This results in disturbance of membrane fluidity and structure, and can alter the activity of membrane-bound enzymes, such as chitinase (Sancholle *et al.,* 1984; Vanden Bossche *et al.,* 1984; Marichal *et al.,* 1985; Weete, 1986; Sancholle *et al.,* 1988; Weete, 1989;

Vanden Bossche, 1990; Kelly *et al.,* 1995). Consequently, morphological alterations in fungi, such as extensively branched and swollen cells with severe thickening of cell walls induced by azoles are frequently observed and have been described in germ tubes and hyphae of many fungal species, such as *Cladosporium cucumurinum* (Sherald *et al.,* 1973), *Monilinia fructigena* (Kato *et al.,* 1975), *Botrytis cinerea* (Kato *et al.,* 1980)*, Ustilago avenae* (Hippe 1984), *Sclerotium rolfsii* (Roberson *et al.,* 1989; Fuller *et al.,* 1990), and *Fusarium culmorum* (Kang *et al.,* 2001).

Resistance

Resistance is the genetic adaptation of a fungus to a certain fungicide or group of fungicides that results in reduced sensitivity to the compounds. This phenomenon, which is also described as *genotypic* or *acquired* resistance, is found in many fungal species that were sensitive to fungicides prior to their exposure. Fungicide resistance can be achieved by single mutations in genes of the pathogen or from the increase in frequency of naturally occurring, less-sensitive sub-populations. Resistance differs from *natural* or *intrinsic* insensitivity of species that are not included in the antifungal spectrum of a certain compound (Delp and Dekker, 1985; Brent, 1995).

Because of their site-specific mode of action, azoles are more prone to resistance development than multisite inhibitors. Under laboratory conditions, azole-resistant mutants can easily be obtained, but such mutants often display reduced fitness with respect to spore germination, mycelial growth, and virulence and their levels of resistance are relatively low. Therefore, resistance development to azoles under field conditions was considered unlikely (Dekker, 1981; De Waard and Fuchs, 1982; Fuchs and De Waard, 1982). However, in course of time, azole resistance emerged in several fungal populations, although relatively slowly as compared to other classes of fungicides (De Waard, 1994). The first case of field resistance development to azoles was reported for *Sphaerotheca fuliginea,* the causal agent of cucumber powdery mildew (Schepers, 1985). Since then, azole resistance is also reported for other fungal pathogens, such as *Erysiphe graminis* f. sp. *tritici* (Kendall *et al.,* 1993), *Penicillium digitatum* (Eckert, 1987), *Venturia inaequalis* (Hildebrand *et al.,* 1988), and *Rhynchosporium secalis* (Kendall *et al.,* 1993).

Resistance mechanisms

Studies on the genetics of resistance to the azole imazalil in *Aspergillus nidulans* identified eight loci allocated to six different linkage groups conferring resistance to this compound (Van Tuyl, 1977). Polygenic control of resistance to azole fungicides has been reported for other fungi as well and may imply the involvement of several mechanisms (Kalamarakis *et al.,* 1991; Brent and Hollomon, 1998). In filamentous fungi four general mechanisms have been described to confer resistance to azoles: 1) reduced intracellular concentration of the antifungal compound, so that it can no longer reach or saturate its target, 2) target site alterations that result in reduced affinity of the antifungal compound for its target, 3) overexpression of the target site that results in a titration effect of the compound 4) compensation of the toxic effects of azoles in cells by alterations in the sterol-biosynthesis pathway.

Reduced intracellular accumulation. The way azoles enter fungal cells is not yet fully understood but current models support the idea of passive diffusion through cell walls and membranes. Therefore, altering drug penetration through changes in cell wall and/or membrane composition can reduce the intracellular accumulation of antifungal compounds in cells. Lower ergosterol levels or a decreased ratio between phosphatidyl-choline and phosphatidyl-ethanolamine in the membranes can reduce permeability to azoles and such changes have been reported in some azole-resistant strains of the pathogenic yeast *C. albicans* (Hitchcock *et al.,* 1986; Loeffler *et al.,* 2000). Other studies indicate that modifications in cell wall structure caused by altered glycosylation of surface proteins can also result in changes in azole sensitivity in this pathogen (Timpel *et al.,* 1998). Yet, the vast majority of findings support the idea that reduced intracellular levels of azoles can be achieved by active excretion of drugs from cells (De Waard and Van Nistelrooy, 1979; 1980), mediated by transporters of the ATP-binding cassette (ABC) and major facilitator superfamily (MFS) (Stergiopoulos *et al.,* 2002). These systems are extensively reviewed in Chapter 2 of this thesis.

Target site alterations. Point mutations in the *CYP51* or *ERG11* encoding $P450_{14DM}$ gene of filamentous fungi or yeasts, respectively, can also confer resistance to azoles. In *C. albicans* two mutations in *ERG11*, namely G464S (Loeffler *et al.,* 1997; Franz *et al.,* 1998; Sanglard *et al.,* 1998; Kelly *et al.,* 1999) and R467K (White, 1997a) were frequently detected in fluconazole-resistant isolates of this pathogen. Both amino acids are located near the haem-binding domain of $P450_{14DM}$ and the mutations result in structural or functional alterations

that cause reduced affinity of $P450_{14DM}$ to fluconazole but without the enzyme entirely loosing its catalytic activity. Additional mutations in *ERG11* of *C. albicans*, such as T315A (Lamb *et al.,* 1997), Y132H (Sanglard *et al.,* 1998), S405F (Sanglard *et al.,* 1998), and I471T (Kakeya *et al.,* 2000) along with several other mutations, have also been described in azole-resistant isolates of this pathogen (Sanglard *et al.,* 1998; Asai *et al.,* 1999; Favre *et al.,* 1999; Marichal *et al.,* 1999; Perea *et al.,* 2001). Point mutations in *CYP51* associated with decreased azole-sensitivity have also been described in pathogens of agricultural importance, such as laboratory-generated mutants of *Ustilago maydis* (Joseph Horne *et al.,* 1995a; Butters *et al.,* 2000) and *P. italicum* (Van Nistelrooy *et al.,* 1996), and in field isolates of *Uncinula necator* (Dèlye *et al.,* 1997), and *E. graminis* f. sp. *hordei* (Dèlye *et al.,* 1998). In all instances the mutation resulted in a Y136F substitution of $P450_{14DM}$, except for *U. maydis*. In the latter case, the G464D mutation was detected.

Increase in target site concentration. Increased levels of $P450_{14DM}$, caused by overexpression of *CYP51* (or *ERG11*) is also described as a resistance mechanism to azoles. An increase in $P450_{14DM}$ production requires higher intracellular concentrations of azoles to achieve full inhibition of $P450_{14DM}$ activity. Overexpression of *ERG11* has been reported in several azole-resistant isolates of *C. albicans* and *S. cerevisiae* (Vanden Bossche *et al.,* 1994; White, 1997b; Kontoyiannis *et al.,* 1999; Perea *et al.,* 2001). Levels of resistance achieved by overexpression of $P450_{14DM}$ vary considerably and are generally lower as compared to other resistance mechanisms. In a clinical azole-resistant isolate of *Candida glabrata* overproduction of $P450_{14DM}$ was closely linked with *ERG11* gene amplification, caused by duplication of the entire chromosome containing the *ERG11* gene (Vanden Bossche *et al.,* 1992; Marichal *et al.,* 1997). In the agricultural important pathogens *P. digitatum* and *V. inaequalis*, tandem repeats in the promoter region of *CYP51* enhanced transcription of this gene and the copy number of these repeats positively correlated with *CYP51* expression and decreased sensitivity to azoles (Hamamoto *et al.,* 2000; Schnabel and Jones, 2001).

Alterations in the sterol biosynthesis pathway. Azoles exert their toxic effects in fungi by depletion of ergosterol as well as the accumulation of toxic C14-methylated precursors of sterols, especially C14α-methylergosta-8,24(28)-dien-3β,6α-diol (diol) (Kelly *et al.,* 1995). Accumulation of diol is known to cause growth arrest in *C. albicans* (Kelly *et al.,* 1997) and *S. cerevisiae* (Vanden Bossche, 1991; Kelly *et al.,* 1995). The toxic effects of this precursor in *S. cerevisiae* can be abolished by mutations in the *ERG3* gene, which encodes $\Delta^{5,6}$ desaturase

(ERG3) (Watson *et al.,* 1989; Bard *et al.,* 1993; Kelly *et al.,* 1995). This enzyme acts at an earlier step in the sterol biosynthesis pathway than $P450_{14DM}$ and inhibition of ERG3 results in the absence of diol from cells as well as in higher C14α-methyl fecosterol levels. This sterol precursor allows fungal growth and can compensate for ergosterol in cells when present in sufficient amounts (Watson *et al.,* 1989; Kelly *et al.,* 1996; Kelly *et al.,* 1997; Nolte *et al.,* 1997). Recently it was also shown that deletion of *ERG3* from *C. albicans* results in reduced sensitivity of the mutants to fluconazole (Miyazaki *et al.,* 2001). Altered $\Delta^{5,6}$ desaturase activity has also been reported in azole-resistant mutants of *U. maydis* (Joseph-Horne *et al.,* 1995a; Joseph-Horne *et al.,* 1995b).

REFERENCES

Ahmed HU, Mundt CC, Hoffer ME and Coakley SM (1996) Selective influence of wheat cultivars on pathogenicity of *Mycosphaerella graminicola.* Phytopathology **86:** 454-458

Asai K, Tsuchimori N, Okonogi K, Perfect JR, Gotoh O and Yoshida Y (1999) Formation of azole-resistant *Candida albicans* by mutation of sterol 14-demethylase P450. Antimicrob Agents Chemother **43:** 1163-1169

Baldwin BC, Clough JM, Godfrey CRA, Godwin JR and Wiggins TE (1996) The discovery and mode of action of ICIA5504. *In* Modern fungicides and antifungal compounds (Eds) Russell PE and Sisler HD, Andover, UK, pp. 105-109

Baltazar BM, Scharen AL and Kronstad WE (1990) Association between dwarfing genes 'Rht' and 'Rht' and resistance to *Septoria tritici* blotch in winter wheat (*Triticum aestivum* L. em Thell). Theoret Appl Genet **79:** 422-426

Bannon FJ and Cooke BM (1998) Studies on dispersal of *Septoria tritici* pycnidiospores in wheat-clover intercrops. Plant Pathol **47:** 49-56

Bard M, Lees ND, Turi T, Craft D, Cofrin L, Barbuch R, Koegel C and Loper JC (1993) Sterol synthesis and viability of erg11 (cytochrome P450 lanosterol demethylase) mutations in *Saccharomyces cerevisiae* and *Candida albicans.* Lipids **28:** 963-967

Bartlett DW, Clough JM, Godwin JR, Hall AA, Hamer M and Parr-Dobrzanski B (2002) The strobilurin fungicides. Pest Manag Sci **58:** 649-662

Brading PA, Verstappen ECP, Kema GHJ and Brown JKM (2002) A gene-for-gene relationship between wheat and *Mycosphaerella graminicola*, the *Septoria tritici* blotch pathogen. Phytopathology **92:** 439-445

Brent KJ (1995) Fungicide resistance in crop pathogens: How can it be managed? FRAC Monograph **No 1**. Publ. by GCPF: Global crop protection Federation, Brussels

Brent KJ and Hollomon D (1998) Fungicide resistance: The assesment of risk FRAC Monograph **No 2**. Publ. by GCPF: Global crop Protection Federation, Brussels

Brokenshire T (1975) The role of *Graminaceous* species in the epidemiology of *Septoria tritici* on wheat. Plant Pathol **24:** 33-38

Brokenshire T (1976) The reaction of wheat genotypes to *Septoria tritici.* Ann Appl Biol **82:** 415-423

Brown JS, Kellock AW and Paddick RG (1978) Distribution and dissemination of *Mycosphaerella graminicola* (Fückel) Schroeter in relation to the epidemiology of speckled leaf blotch of wheat. Austral J Agric Res **29:** 1139-1145

Brown JKM, Kema GHJ, Forrer HR, Verstappen ECP, Arraiano LS, Brading PA, Foster EM, Fried PM and Jenny E (2001) Resistance of wheat cultivars and breeding lines to *Septoria tritici* blotch caused by isolates of *Mycosphaerella graminicola* in field trials. Plant Pathol **50:** 325-338

Buchenauer H (1977) Mode of action and selectivity of (systemic) fungicides which interfere

with ergosterol biosynthesis in *Ustilago avenae*, (comparison studies). British Crop Protection Conference: Pests and Diseases, Brighton, UK, pp. 699-711

Butters JA, Zhou MC and Hollomon DW (2000) The mechanism of resistance to sterol 14α-demethylation inhibitors in a mutant (Erg 40) of *Ustilago maydis*. Pest Manag Sci **56:** 257-263

Chen RS and McDonald BA (1996) Sexual reproduction plays a major role in the genetic structure of populations of the fungus *Mycosphaerella graminicola.* Genetics **142:** 1119-1127

Clough JM (1993) The strobilurins, oudemansins, and myxothiazols, fungicidal derivatives of beta-methoxyacrylic acid. Nat Prod Rep **10:** 565-567

Cohen L and Eyal Z (1993) The histology of processes associated with the infection of resistant and susceptible wheat cultivars with *Septoria tritici*. Plant Pathol **42:** 737-743

Cook R and Thomas M (1990) Influence of site factors on yield response of winter wheat to fungicide programmes in England and Wales, 1979-1987. Plant Pathol **39:** 548-557

Cowger C, Hoffer ME and Mundt CC (2000) Specific adaptation by *Mycosphaerella graminicola* to a resistant wheat cultivar. Plant Pathol **49:** 445-451

Danon T, Sacks JM and Eyal Z (1982) The relationships among plant stature, maturity class, and susceptibility to Septoria leaf blotch of wheat. Phytopathology **72:** 1037-1042

De Waard MA (1994) Resistance to fungicides which inhibit sterol 14α-demethylation, an historical perspective. *In* Fungicide Resistance, (Eds) Heaney S, Slawson D, Hollomon DW, Smith M, Russell PE, Parry DW, pp. 3-10

De Waard MA and Van Nistelrooy JGM (1979) Mechanism of resistance to fenarimol in *Aspergillus nidulans.* Pestic Biochem Physiol **10:** 219-229

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* Pestic Biochem Physiol **13:** 255-266

De Waard MA and Fuchs A (1982) Resistance to ergosterol biosynthesis inhibitors, II. Genetic and physiological aspects. *In* Fungicide resistance in Crop Protection (Eds) Dekker J and Georgopoulos SG, Pudoc, Wageningen, The Netherlands, pp. 87-100

Dancer J, Daniels A, Cooley N and Foster S (1999) *Septoria tritici* and *Stagonospora nodorum* as model pathogens for fungicide discovery. *In Septoria* on cereals: a study of pathosysems (Eds) Lucas JA, Bowyer P and Anderson HM. CABI publishing, Wallingford, UK, pp. 316-331

Dekker J (1981) Impact of fungicide resistance of disease control toxicity. British Crop Protection Conference: Pests and Diseases, Brighton, UK, pp. 857-863

Delp CJ and Dekker J (1985) Fungicide resistance: definitions and use of terms. EPPO Bul **15:** 333-335

Dèlye C, Laigret F and Corio Costet MF (1997) A mutation in the 14α-demethylase gene of *Uncinula necator* that correlates with resistance to a sterol biosynthesis inhibitor. Appl Environ Microbiol **63:** 2966-2970

Dèlye C, Bousset L and Corio Costet MF (1998) PCR cloning and detection of point mutations in the eburicol 14α-demethylase (*CYP51*) gene from *Erysiphe graminis* f. sp. *hordei*, a "recalcitrant" fungus. Curr Genet **34:** 399-403

Desmazieres JBHJ (1842) Cryptogames nouvelles. Annales Sciences Naturelles **17:** 91-118

Djerbi M (1977) Epidemiology of *Septoria tritici* Rob. & Desm. Conservation and mode of formation of the primary inoculum. Travaux dedies a Georges Viennot-Bourgin Societe Francaise de Phytopathologie Paris France, pp. 91-101

Duczek LJ and Jones-Flory LL (1994) Effect of timing of propiconazole application on foliar disease and yield of irrigated spring wheat in Saskatchewan from 1990 to1992. Can J Plant Sci **74:** 205-207

Duncan KE and Howard RJ (2000) Cytological analysis of wheat infection by the leaf blotch pathogen *Mycosphaerella graminicola*. Mycol Res **104:** 1074-1082

Eckert JW (1987) *Penicillium digitatum* biotypes with reduced sensitivity to imazalil. (Abstr). Phytopathology **77:** 1728

Eyal Z (1971) The kinetics of pycnidiospore liberation in *Septoria tritici*. Can J Bot **49:** 1095-1099

Eayl Z (1999) Breeding for resistance to septoria and stagonospora diseases of wheat. *In Septoria* on cereals: A Study of Pathosystems (Eds) Lucas JA, Bowyer P and Anderson HM. CABI publishing, Wallingford, UK, pp. 1-25

Eyal Z, Amiri Z and Wahl I (1973) Physiological specialisation of *Septoria tritici.* Phytopathology **63:** 1087-1091

Eyal Z and Ziv O (1974) The relationship between epidemics of Septoria leaf blotch and yield losses in spring wheat. Phytopathology **64:** 1385-1389

Eyal Z and Wahl I (1975) Chemical control of septoria leaf blotch disease of wheat in Israel. Phytoparasitica **3:** 76-77

Eyal Z and Brown MB (1976) A quatitative

method for estimating density of *Septoria tritici* pycnidia on wheat leaves. Phytopathology **63:** 1087-1091

Eyal Z, Scharen AL, Huffman MD and Prescott JM (1985) Global insights into frequencies of *Mycosphaerella graminicola.* Phytopathology **75:** 1456-1462

Eyal Z, Scharen AL, Prescott JM and Van Ginkel M (1987) The Septoria diseases of wheat: Concepts and methods of disease management CIMMYT, Mexico, pp. 51

Eyal Z and Talpaz H (1990) The combined effect of plant stature and maturity on the responses of wheat and triticale accessions to *Septoria tritici.* Euphytica **46:** 133-141

Favre B, Didmon M and Ryder NS (1999) Multiple amino acid substitutions in lanosterol 14α-demethylase contribute to azole resistance in *Candida albicans*. Microbiology **145:** 2715-25

Fisher N and Griffin M (1984) Bendimidazole (MBC) resistance in *Septoria tritici.* ISPP Chem Control News Lett **5:** 8-9

Fournet J (1969) Properties et role du cirrhe du *Septoria nodorum* Berk. Ann Phytopathol **1:** 87-94

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. Antimicrob Agents Chemother **42:** 3065-3072

Fuchs A and De Waard MA (1982) Resistance to ergosterol biosyntesis inhibitors, I. Chemistry and Phenomenological aspects. *In* Fungicide resistance in Crop protection (Eds) Dekker J and Georgopoulos SG, Pudoc, Wageningen, The Netherlands, pp. 71-86

Fuller MS, Roberson RW and Gisi U (1990) Effects of the sterol demethylase inhibitor, cyproconazole, on hyphal tip cells of *Sclerotium rolfsii*. II. Cell wall cytochemistry. Pestic Biochem Physiol **36:** 115-126

Gisi U, Chin KM, Knapova G, Farber RK, Mohr U, Parisi S, Sierotzki H and Steinfeld U (2000) Recent developments in elucidating modes of resistance to phenylamide, DMI and strobilurin fungicides. Crop Prot **19:** 863-872

Godwin JR, Barlett DW and Heaney SP (1999) Azoxystrobin: Implications of biological mode of action, pharmacokinetics and resistance management for spray programmes against Septoria diseases of wheat. *In Septoria* on cereals: A study of pathosystrems (Eds) Lucas JA Bowyer P and Anderson HM, CABI publishing, Wallingford, UK

Hamamoto H, Hasegawa K, Nakaune R, Lee YJ, Makizumi Y, Akutsu K and Hibi T (2000) Tandem repeat of a transcription enhancer upstream of the sterol 14α-demethylase gene (*CYP51*) in *Penicillium digitatum*. Appl Environ Microbiol **66:** 3421-3426

Hildebrand PD, Lockhart CL, Newberry RJ and Ross RG (1988) Resistance of *Venturia inaequalis* to bitertanol and other demethylation-inhibiting fungicides. Can J Plant Pathol **10:** 311-316

Hims MJ and Cook RJ (1992) Disease epidemiology and fungicide activity in winter wheat. British Crop Protection Conference: Pests and Disease, Brighton, UK, pp. 615-620

Hippe S (1984) Electron microscopic investigations on the cytology of *Ustilago avanae* as influenced by the systemic fungicides triadimefon, nuarimol and imazalil-nitrate: a thin section and freeze-fracture study. Pest Biochem Physiol **21:** 170-183

Hitchcock CA, Barrett-Bee KJ and Russell NJ (1986) The lipid composition of azole-sensitive and azole-resistant strains of *Candida albicans*. J Gen Microbiol **132:** 2421-2431

Hollomon DW, Fraaije B, Rohel E, Butters J and Kendall S (1999) Detection and diagnosis of septoria diseases: the problem in practice. *In Septoria* on cereals: A Study of Pathosystems, CABI Publishing, Wallingford, UK, pp. 273-285

Hooker AL (1957) Methods of inoculation and determining varietal reactions in the Septoria disease of oats. Plant Discov Rep **41:** 592-597

Hunter T, Coker RR and Royle DJ (1999) The teleomorph stage, *Mycosphaerella graminicola*, in epidemics of *Septoria tritici* blotch on winter wheat in the UK. Plant Pathol **48:** 51-57

Jlibene M, Gustafson JP and Rajaram S (1994) Inheritance of resistance to *Mycosphaerella graminicola* in hexaploid wheat. Plant Breed **112:** 301-310

Jordan VWL, Hunter T and Fielding EC (1986) Biological properties of fungicides for the control of *Septoria tritici*. 1986 British Crop Protection Conference: Pests and Diseases, Brighton, UK, pp. 1063-1069

Joseph-Horne T, Hollomon D, Loeffler RST and Kelly SL (1995a) Altered P450 activity associated with direct selection for fungal azole resistance. FEBS Lett **374:** 174-178

Joseph-Horne T, Manning NJ, Hollomon D and Kelly SL (1995b) Defective sterol Δ5(6)desaturase as a cause of azole resistance in *Ustilago maydis*. FEMS Microbiol Lett **127:** 29-34

Kakeya H, Miyazaki Y, Miyazaki H, Nyswaner K, Grimberg B and Bennett JE (2000) Genetic analysis of azole resistance in the Darlington strain of *Candida albicans*. Antimicrob Agents Chemother **44:** 2985-2990

Kalamarakis AE, De Waard MA, Ziogas BN and Georgopoulos SG (1991) Resistance to fenarimol in *Nectria haematococca* var. *cucurbitae*. Pestic Biochem Physiol **40:** 212-220

Kang Z, Huang L, Krieg U, Mauler-Machnik A and Buchenauer H (2001) Effects of tebuconazole on morphology, structure, cell wall components and trichothecene production of *Fusarium culmorum in vitro*. Pest Manag Sci **57:** 491-500

Kato T, Yamamoto S, Kawase Y and Ueda M (1975) Fungitoxic properties of a N-3-pyridylimidadithiocarbonate derivate. Ann Phytopathol Soc of Japan **41:** 1-8

Kato T, Shoami M and Kawase Y (1980) Comparison of tridemorph with buthioate in antifungal mode of action. J Pestic Sci **5:** 69-79

Kelly SL, Lamb DC, Corran AJ, Baldwin BC and Kelly D (1995) Mode of action and resistance to azole antifungals associated with the formation of 14α-methylergosta-8,24(28)-dien-3β,6α-diol. Biochem Biophys Res Commun **207:** 910-915

Kelly SL, Lamb DC, Kelly DE, Loeffler J and Einsele H (1996) Resistance to fluconazole and amphotericin in *Candida albicans* from AIDS patients. Lancet **348:** 1523-1524

Kelly SL, Lamb DC, Kelly DE, Manning NJ, Loeffler J, Hebart H, Schumacher U and Einsele H (1997) Resistance to fluconazole and cross-resistance to amphotericin B in *Candida albicans* from AIDS patients caused by defective sterol delta5,6-desaturation. FEBS Lett **400:** 80-82

Kelly SL, Lamb DC, Loeffler J, Einsele H and Kelly DE (1999) The G464S amino acid substitution in *Candida albicans* sterol 14α-demethylase causes fluconazole resistance in the clinic through reduced affinity. Biochem Biophys Res Commun **262:** 174-179

Kema GHJ, Annone JG, Sayoud R, Van Silfhout CH, Van Ginkel M and De Bree J (1996a) Genetic variation for virulence and resistance in the wheat - *Mycosphaerella graminicola* pathosystem: I. Interactions between pathogen isolates and host cultivars. Phytopathology **86:** 200-212

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

Kema GHJ and Van Silfhout CH (1997) Genetic variation for virulence and resistance in the wheat-*Mycosphaerella graminicola* pathosystem. III. Comparative seedling and adult plant experiments. Phytopathology **87:** 266-272

Kema GHJ and Verstappen ECP (1999) Genetic control of avirulence in *Mycosphaerella graminicola* (anamorph *Septoria tritici*). Septoria and Stagonospora diseases of cereals: a compilation of global research, CIMMYT, Mexico, pp. 51-52

Kema GHJ, Verstappen ECP and Waalwijk C (2000) Avirulence in the wheat *Septoria tritici* leaf blotch fungus *Mycosphaerella graminicola* is controlled by a single locus. Mol Plant Microb Interact **13:** 1375-1379

Kendall SJ, Hollomon DW, Cooke LR and Jones DR (1993) Changes in sensitivity to DMI fungicides in *Rhynchosporium secalis.* Crop Protect **12:** 357-362

Kolomiets S (1999) Populations of *Septoria* spp. affecting winter wheat in the forest-steppe zone of the Ukraine. *In* Septoria and Stagonospora diseases of cereals: a compilation of global research, CIMMYT, Mexico, pp. 32-33

Kontoyiannis DP, Sagar N and Hirschi KD (1999) Overexpression of Erg11p by the regulatable GAL1 promoter confers fluconazole resistance in *Saccharomyces cerevisiae.* Antimicrob Agents Chemother **43:** 2798-2800

Kuck KH and Scheinpflug H (1986) Biology of sterol-biosynthesis inhibiting fungicides. *In* Chemistry of Plant Protection (Eds) Haug G and Hoffman H, Springer-Verlag, Berlin, Germany

Lamb DC, Kelly DE, Schunck WH, Shyadehi AZ, Akhtar M, Lowe DJ, Baldwin BC and Kelly SL (1997) The mutation T315A in *Candida albicans* sterol 14α-demethylase causes reduced enzyme activity and fluconazole resistance through reduced affinity. J Biol Chem **272:** 5682-5688

Lee TS and Gough FJ (1984) Inheritance of Septoria leaf blotch (*S. tritici*) and Pyrenophora tan spot (*P. tritici-repentis*) resistance in *Triticum aestivum* cv. Carifen 12. Plant Dis **68:** 848-851

Loeffler J, Kelly SL, Hebart H, Schumacher U, Lass-Flörl C and Einsele H (1997) Molecular analysis of cyp51 from fluconazole-resistant *Candida albicans* strains. FEMS Microbiol Let **151:** 263-268

Loeffler J, Einsele H, Hebart H, Schumacher U, Hrastnik C and Daum G (2000) Phospholipid and sterol analysis of plasma membranes of azole-resistant *Candida albicans* strains. FEMS Microbiol Let **185:** 59-63

Loughman R and Thomas G (1992) Fungicide and cultivar control of Septoria diseases of wheat. Crop Protect **11:** 349-354

Lovell DJ, Parker SR, Hunter T, Royle DJ and Coker RR (1997) Influence of crop growth and structure on the risk of epidemics by *Mycosphaerella graminicola* (*Septoria tritici*) in winter wheat. Plant Pathol **46:** 126-138

Marichal P, Gorrens J and Vanden Bossche H (1985) The action of itraconazole and

ketoconazole on growth and sterol synthesis in *Aspergillus fumigatus* and *Aspergillus niger.* Sabouraudia **22:** 13-21

Marichal P, Vanden Bossche H, Odds FC, Nobels G, Warnock DW, Timmerman V, Van Broeckhoven C, Fay S and Mose LP (1997) Molecular biological characterization of an azole-resistant *Candida glabrata* isolate. Antimicrob Agents Chemoth **41:** 2229-2237

Marichal P, Koymans L, Willemsens S, Bellens D, Verhasselt P, Luyten W, Borgers M, Ramaekers FC, Odds FC and Vanden Bossche H (1999) Contribution of mutations in the cytochrome P450 14α-demethylase (Erg11p, Cyp51p) to azole resistance in *Candida albicans.* Microbiology **145:** 2701-2713

McDonald BA and Martinez JP (1990) Restriction fragment length polymorphisms in *Septoria tritici* occur at a high frequency. Cur Genet **17:** 133-138

Metcalf NDS, Sanderson RA and Griffin MJ (1985) Comparison of carbendazim and propiconazole for control of *Septoria tritici* at sites with different levels of MBC resistance. ISPP Chem Control Newsl **6:** 9-11

Milus EA (1994) Effect of foliar fungicides on disease control, yield and test weight of soft red winter wheat. Crop Protect **13:** 291-295

Miyazaki T, Miyazaki Y, Kakeya H, Miyazaki H, Nakamura K and Kohno S (2001) Abstracts to the 41st Interscience Conference on Antimicrobial Agents and Chemotherapy, Chicago, IL, USA, Abstr J-1840

Murray GM, Martin RH and Cullis BR (1990) Relationship of the severity of *Septoria tritici* blotch of wheat to sowing time, rainfall at heading and average susceptibility of wheat cultivars in the area. Austral J Agric Res **41:** 307-315

Nelson LR and Marshall D (1990) Breeding wheat for resistance to *Septoria nodorum* and *Septoria tritici*. Adv Agron **44:** 257-277

Nolte FS, Parkinson T, Falconer DJ, Dix S, Williams J, Gilmore C, Geller R and Wingard JR (1997) Isolation and characterization of fluconazole- and amphotericin B-resistant *Candida albicans* from blood of two patients with leukemia. Antimicrob Agents Chemother **41:** 196-199

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. Antimicrob Agents Chemother **45:** 2676-2684

Polley RW and Thomas MR (1991) Surveys of diseases of winter wheat in England and Wales, 1976-1988. Ann App Biol **119:** 1-20

Roberson RW, Fuller MS and Grabski C (1989) Effects of the demethylase inhibitor, cyproconazole, on hyphal tip cells of *Sclerotium rolfsii*. I. A light-microscope study. Pestic Biochem Physiol **34:** 130-142

Rohel EA, Cavelier N and Hollomon DW (2001) Microscopic analysis of the effect of azoxystrobin treatments on *Mycosphaerella graminicola* infection using green fluorescent protein (GFP)-expressing transformants. Pest Manag Sci **57:** 1017-1022

Rohel EA, Laurent P, Fraaije BA, Cavelier N and Hollomon DW (2002) Quantitative PCR monitoring of the effect of azoxystrobin treatments on *Mycosphaerella graminicola* epidemics in the field. Pest Manag Sci **58:** 248-254

Rosielle AA and Brown AGP (1979) Inheritance, heritability and breeding behaviour of three sources of resistance to *Septoria tritici* in wheat. Euphytica **28:** 385-392

Royele DJ, Parker SR, Lovell DJ and Hunter T (1995) Interpreting trends and risks for better control of *Septoria* in winter wheat. *In* A vital role for fungicides in cereal production (Eds) Hewitt HG, Tyson D, Hollomon DW, Smith JM, Davies WP and Dixon KR. BIOS Scientific Publishers, Oxford, UK, pp.105-115

Sancholle M, Weete JD and Montant C (1984) Effects of triazoles on fungi. 1. Growth and cellular permeability (Fungicides). Pestic Biochem Physiol **21:** 31-44

Sancholle M, Dargent R, Weete JD, Rushing AE, Miller KS and Montant C (1988) Effects of triazoles on fungi. VI. Ultrastructure of *Taphrina deformans*. Mycologia **80:** 162-175

Sanderson FR (1972) A *Mycosphaerella* species as the ascogenous state of *Septoria tritici* Rob. and Desm. New Zeal J Bot **10:** 707-709

Sanderson FR (1976) *Mycosphaerella graminicola* (Fückel) Sanderson comb. nov., the ascogenous state of *Septoria tritici* Rob. apud Desm. New Zeal J Bot **14:** 359-360

Sanderson FR and Hampton JG (1978) Role of the perfect states in the epidimiology of the common *Septoria* diseases of wheat. New Zeal J Agric **21:** 277-281

Sanderson FR and Gaunt RE (1980) Commercial control of speckled leaf blotch (*Mycosphaerella graminicola*, imperfect stage *Septoria tritici*) on wheat using fungicides. 3rd International Wheat Conference, Madrid, Spain, pp. 554-557

Sanglard D, Ischer F, Koymans L and Bille J (1998) Amino acid substitutions in the cytochrome

P-450 lanosterol 14α-demethylase (CYP51A1) from azole-resistant *Candida albicans* clinical isolates contribute to resistance to azole antifungal agents. Antimicrob Agents Chemother **42:** 241-253

Scheinpflug H and Kuck KH (1987) Sterol biosynthesis inhibiting piperazine, pyridine, pyrimidine and azole fungicides. *In* Modern selective fungicides: properties, applications, mechanisms of action (Eds) Lyr H in cooperation with H Buchenhauer *et al.,* Harlow, Essex, England: Longman Scientific & Technical, pp. 173-204

Schepers HTAM (1985) Fitness of isolates of *Sphaerotheca fuliginea* resistant or sensitive to fungicides which inhibit ergosterol biosynthesis. Neth J Pl Path **91:** 65-76

Schnabel G and Jones AL (2001) The 14α-demethylase (*CYP51A1*) gene is overexpressed in *Venturia inaequalis* strains resistant to myclobutanil. Phytopathology **91:** 102-110

Schuh W (1990) Influence of tillage systems on disease intensity and spatial pattern of Septoria leaf blotch. Phytopathology **80:** 1337-1340

Scott PR, Benedikz PW and Cox CJ (1982) A genetic study of the relation between height, time of ear emergence and resistance to *Septoria nodorum* in wheat. Plant Pathol **31:** 45-60

Shaner G and Finney RE (1976) Weather and epidemics of septoria leaf blotch of wheat. Phytopathology **66:** 781-785

Shaw MW and Royle DJ (1989) Airborne inoculum as a major source of *Septoria tritici* (*Mycosphaerella graminicola*) infections in winter wheat crops in the UK. Plant Pathol **38:** 35-43

Shaw MW (1991) Interacting effects of interrupted humid periods and light on infection of wheat leaves by *Mycosphaerella graminicola* (*Septoria tritici*). Plant Pathol **40:** 595-607

Shaw MW and Royle DJ (1993) Factors determining the severity of epidemics of *Mycosphaerella graminicola* (*Septoria tritici*) on winter wheat in the UK. Plant Pathol **42:** 882-899

Shearer BL and Wilcoxson RD (1978) Variation in the size of macropycnidiospores and pycnidia of *Septoria tritici* on wheat. Can J Bot **56:** 742-746

Sheehan DJ, Hitchcock CA and Sibley CM (1999) Current and emerging azole antifungal agents. Clin Microbiol Rev **12:** 40-79

Sherald JL, Ragsdale NN and Sisler HD (1973) Similarities between the systemic fungicides triforine and triarimol. Pestic Sci **4:** 719-727

Shipton WA, Boyd WRJ, Rosielle AA and Shearer BI (1971) The common Septoria diseases of wheat. Bot Rev **37:** 231-262

Simon MR and Cordo CA (1998) Diallel analysis of four resistance components to *Septoria tritici* in six crosses of wheat (*Triticum aestivum*). Plant Breed **117:** 123-126

Sisler HD and Ragsdale NN (1984) Biochemical and cellular aspects of the antifungal action of ergosterol biosynthesis inhibitors. Symp. Ser. Br. Mycol. Soc., Cambridge, pp. 257-282

Sivanesan A (1990) *Mycosphaerella graminicola.* CMI descriptions of pathogenic fungi and bacteria No. 986. Mycopathologia **109:** 51-53

Somasco OA, Qualset CO and Gilchrist DG (1996) Single-gene resistance to *Septoria tritici* blotch in six crossess of wheat (*Triticum aestivum*). Plant Breed **115:** 261-267

Sprague R (1950) Some leafspot fungi on Western Gramineae. Mycologia **42:** 758-777

Stergiopoulos I, Zwiers L-H and De Waard MA (2002) Secretion of natural and synthetic toxic compounds from filamentous fungi by membrane transporters of the ATP-binding cassette and major facilitator superfamily. Eur J Plant Pathol **108:** 719-734

Suty A and Kuck KH (1996) Sensitivity of wheat leaf spot *Septoria tritici* to tebuconazole. British Crop Prot Conference: Pests and Diseases, Brighton, UK, pp. 689-694

Tavella CM (1978) Date of heading and plant height of wheat varities as related to Septoria leaf blotch damage. Euphytica **27:** 577-580

Timpel C, Strahl-Bolsinger S, Ziegelbauer K and Ernst JF (1998) Multiple functions of Pmt1p-mediated protein O-mannosylation in the fungal pathogen *Candida albicans.* J Biol Chem **273:** 20837-20846

Tompkins DK, Fowler DB and Wright AT (1993) Influence of agronomic practices on canopy microclimate and *Septoria* development in no-till winter wheat produced in the Parkland region of Saskatchewan. Canad J Plant Sci **73:** 331-344

Van Beuningen LT and Kohli MM (1990) Deviation from the regression of infection on heading and height as a measure of resistance to *Septoria tritici* blotch in wheat. Plant Dis **74:** 488-493

Van Ginkel M and Scharen AL (1987) Generation mean analysis and heritabilities of resistance to *Septoria tritici* in durum wheat. Phytopathology **77:** 1629-1633

Van Ginkel M and Scharen AL (1988) Diallel analysis of resistance to *Septoria tritici* isolates in durum wheat. Euphytica **38:**

Van Nistelrooy JGM, Van den Brink JM, Van Kan JA, Van Gorcom RF and De Waard MA (1996) Isolation and molecular characterisation of the gene encoding eburicol 14α-demethylase

(*CYP51*) from *Penicillium italicum*. Mol Gen Genet **250:** 725-733

Van Tuyl JM (1977) Genetics of fungal resistance to systemic fungicides. Mededelingen Landbouwhogescholl Wageningen, Publish. Veenman H and Zonen BV, Wageningen, The Netherlands.

Vanden Bossche H (1990) Importance and role of sterols in fungi. *In* Biochemistry of cell walls and membranes in fungi (Eds) Kuhn PJ, Trinci APJ, Jung MJ, Goosey MW and Copping LG, Springer-Verlag, Berlin, Germany, pp. 135-157

Vanden Bossche H (1991) Ergosterol biosyntesis inhibitors. *In Candida albicans* (Eds) Prasad R, Spriger-Verlag, Berlin, Germany, pp. 239-257

Vanden Bossche H (1998) Mode of action of pyridine, pyrimidine and azole antifungals. *In* Sterol biosynthesis inhibitors: pharmaceutical and agrochemical aspects (Eds) Chichester D, Berg D and Plempel M, Ellis Horwood, West Sussex, England, pp. 79-119

Vanden Bossche H, Lauwers W, Willemsens G, Marichal P and Cornelissen F (1984) Molecular basis for the antimycotic and antibacterial activity of N-substituted imidazoles and triazoles: the inhibition of isoprenoid biosynthesis. Pestic Sci **15:** 188-198

Vanden Bossche H, Marichal P, Gorrens J, Bellens D, Verhoeven H, Coene MC, Lauwers W and Janssen PAJ (1987) Interaction of azole derivatives with cytochrome P-450 isozymes in yeast, fungi, plants and mammalian cells. Pestic Sci **21:** 289-306

Vanden Bossche H and Marichal P (1991) Mode of action of anti-Candida drugs: focus on terconazole and other ergosterol biosynthesis inhibitors. Am J Obstet Gynecol **165:** 1193-1199

Vanden Bossche H and Marichal P (1992) Azole antifungals: mode of action. *In* Recent progress in antifungal chemotherapy (Eds) Yamaguchi H, Kobayashi GS and Takahashi H, Marcel Dekker, New York, USA, pp. 177-190

Vanden Bossche H, Marichal P, Odds Frank C, Le Jeune L and Coene Marie C (1992) Characterization of an azole-resistant *Candida glabrata* isolate. Antimicrob Agents Chemoth **36:** 2602-2610

Vanden Bossche H, Marichal P and Odds Frank C (1994) Molecular mechanisms of drug resistance in fungi. Trends Microbiol **2:** 393-400

Watson PF, Rose ME, Ellis SW, England H and Kelly SL (1989) Defective sterol C5-6 desaturation and azole resistance: a new hypothesis for the mode of action of azole antifungals. Biochem Biophys Res Commun **164:** 1170-1175

Weber GF (1922) Septoria diseases of cereals. II. Septoria diseases of wheat. Speckled leaf blotch of wheat. Phytopathology **12:** 558-585

Weete JD (1986) Mechanism of fungal growth suppression by inhibitors of ergosterol biosynthesis. ACS Symp Ser Am Chem Soc: Ecology and Metabolism of Plant Lipids, Miami Beach, pp. 268-285

Weete JD (1989) Structure and function of sterols in fungi. Adv Lipid Res **24:** 115-167

White TC (1997a) The presence of an R467K amino acid substitution and loss of allelic variation correlate with an azole-resistant lanosterol 14α-demethylase in *Candida albicans.* Antimicrob Agents Chemother **41:** 1488-1494

White TC (1997b) 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. Antimicrob Agents Chemother **41:** 1482-1487

Williams JR and Jones DG (1973) Infection of grasses by *Septoria nodorum* and *S. tritici*. Trans Brit Mycol Soc **60:** 355-358

Wilson RE (1979) Resistance to *Septoria tritici* in two wheat cultivars, determined by independent single domininant genes. Australas Plant Pathol **8:** 16-18

Wilson RE (1985) Inheritance of resistance to *Septoria tritici* in wheat. Montana State University, Bozeman, pp. 33-35

Yoshida Y (1988) Cytochrome P-450 of fungi: Primary target for azole antifungal agents. *In* Current topics in medical mycology (Eds) McGinnis MR, Springer-Verlag, Berlin Germany, 2, pp. 388-418

Yoshida Y and Aoyama Y (1987) Interaction of azole antifungals agents with cytochrome P-45014DM purified from *Saccharomyces cerevisiae* microsomes. Biochem Pharmacol **36:** 229-235

Yoshida Y and Aoyama Y (1990) Stereoselective interaction of an azole antifungal agent with its target, lanosterol 14-α-demethylase (cytochrome P-450-14DM): A model study with stereoisomers of triadimenol and purified cytochrome P-450-14DM from yeast. Chirality **2:** 10-15

Yoshida Y and Aoyama Y (1991) Sterol 14α-demethylase and its inhibition: structural considerations on interaction of azole antifungal agents with lanosterol 14α-demethylase (P-450(14DM)) of yeast. Trans Biochem Soc **19:** 778-782

Zelikovitch N, Levy E and Eyal Z (1986) The effects of mixtures of *Mycosphaerella graminicola* isolates on the expression of

symptoms on wheat seedlings. Phytopathology **76:** 1061

Zellikovitch N, Eyal Z and Kashman Y (1992) Isolation, purification, and bilogical activity of an inhibitor from *Septoria tritici.* Phytopathology **82:** 275-278

Zhan J, Mundt CC and McDonald BA (1998) Measuring immigration and sexual reproduction in field populations of *Mycosphaerella graminicola.* Phytopathology **88:** 1330-1337

Zhan J, Mundt CC and McDonald BA (2001) Using Restriction Fragment Length Polymorphisms to assess temporal variation and estimate the number of ascospores that initiate epidemics in field populations of *Mycosphaerella graminicola.* Phytopathology **88**: 1330-1337

CHAPTER 2

SECRETION OF NATURAL AND SYNTHETIC TOXIC COMPOUNDS FROM FILAMENTOUS FUNGI BY MEMBRANE TRANSPORTERS OF THE ATP-BINDING CASSETTE AND MAJOR FACILITATOR SUPERFAMILY

Ioannis Stergiopoulos, Lute-Harm Zwiers and Maarten A. De Waard

European Journal of Plant Pathology
(2002) ***108:*** *719-734*

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. This has been observed in a variety of organisms and can impose a serious threat to the effective control of pathogenic fungi.

Keywords: antibiotic compounds; fungicides; mycotoxin; pathogenicity; plant defence compounds; resistance; toxin

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 upon 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 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 remarkable 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 minimize their toxic action (Nelissen *et al.,* 1997; Pao *et al.,* 1998; Bauer *et al.,* 1999). 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).

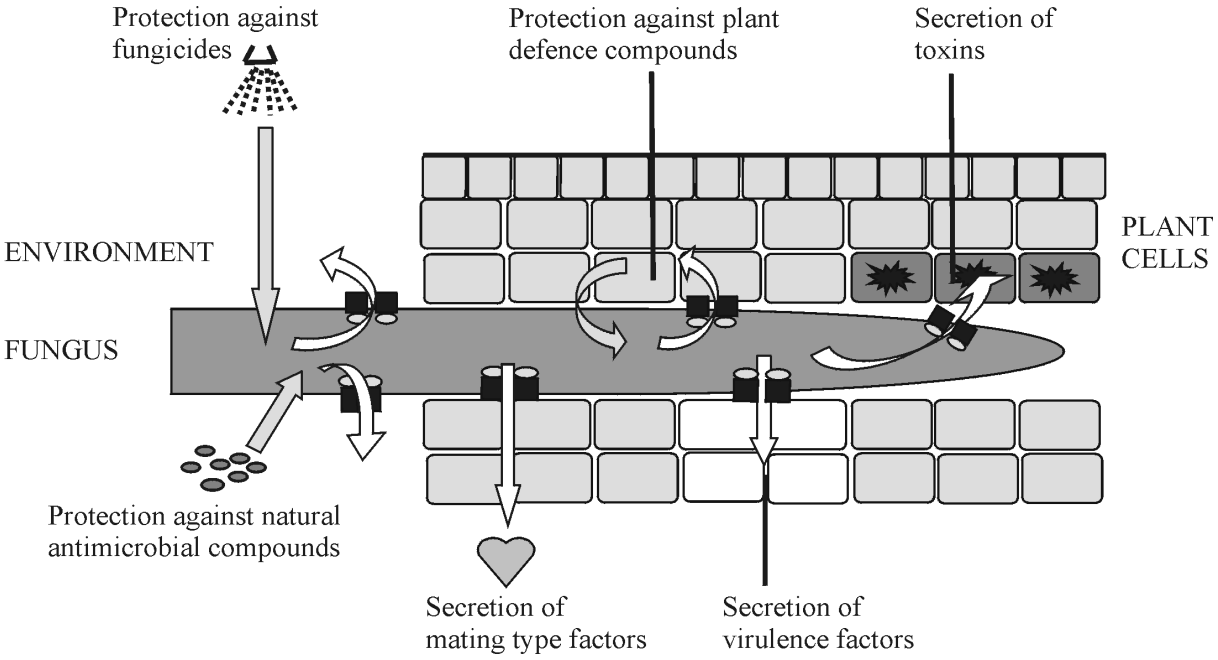


Figure 1. Functions of ABC and MFS transporters in filamentous fungi

The physiological functions of ABC and MFS transporters in fungi are also highly significant. These transporters may 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 wide-spread 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 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.,* 1997; Sanglard *et al.,* 1995; 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 in literature 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 (TMD) 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).

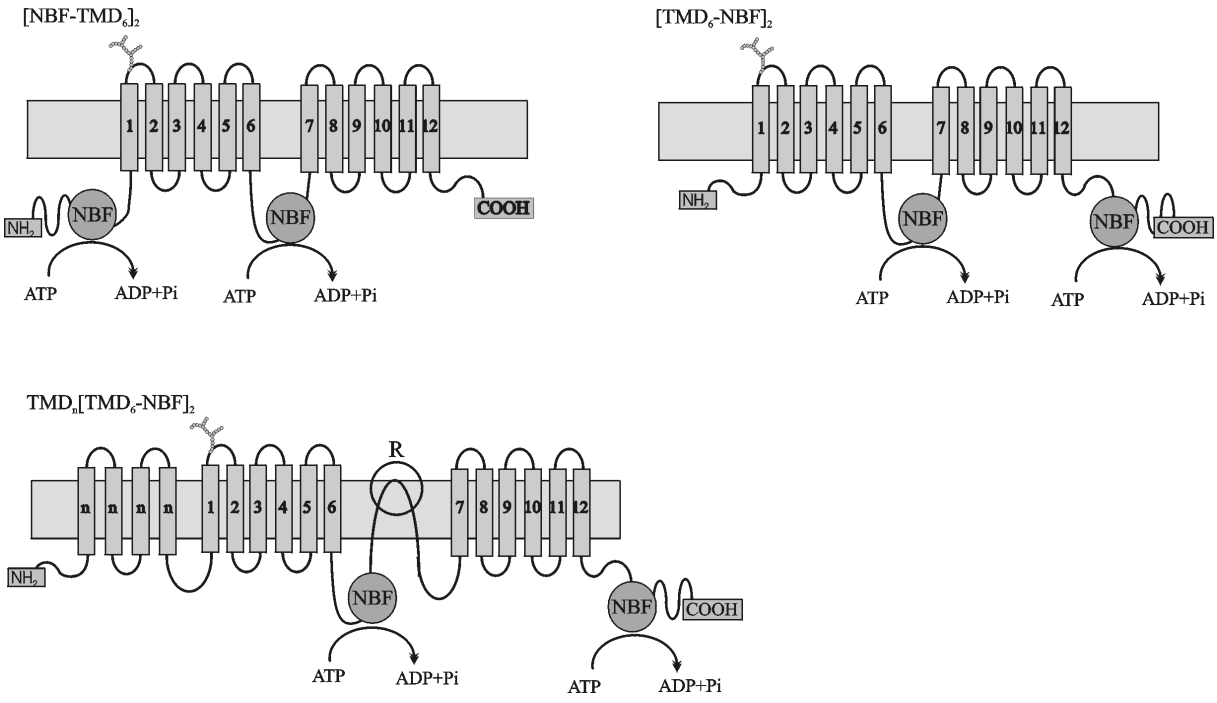


Figure 2. Schematic representation of ATP-Binding Cassette (ABC) transporters with the $[NBF\text{-}TMD_6]_2$ and $[TMD_6\text{-}NBF]_2$ topology. Multidrug Resistance-related Proteins (MRP) are ABC transporters with a $TMD_n[TMD_6\text{-}NBF]_2$ topology. Trans-Membrane Domains (TMDs) are indicated as boxes and are numbered. The Nucleotide-Binding Folds (NBFs) are indicated as circles. "R" is the "Regulatory" or "connector" domain of the MRP-like proteins.

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 homologues 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 of hydrophobic nature, and enter the cells through passive diffusion (Gottesman and Pastan, 1993). The way that these compounds are transported is not yet 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 are characterised 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 proteins with a $[NBF\text{-}TMD_6]_2$ topology, while *atrC* and *atrD* encode proteins with a $[TMD_6\text{-}NBF]_2$ 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, 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 at least some of the *A. nidulans* ABC transporters play a role in defence against a wide range of natural toxic products and fungicides.

Table 1. ABC transporter genes from *Aspergillus nidulans*

ABC gene	Accession number	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, 2000
atrF	AJ309281	Andrade, 2000
atrG	AJ309282	Andrade, 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

Since ABC transporters can play a role in protection of fungi against the activity of DMI fungicides, ample 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 $[TMD_6\text{-}NBF]_2$ predicted topology but no function for this protein has been reported yet. ABC transporter genes cloned from *A. fumigatus* are listed in Table 2.

Table 2. ABC transporter genes from *Aspergillus fumigatus*

ABC gene	Accession number	Reference
AfuMDR1	U62934	Tobin *et al.,* 1997
AfuMDR2	U62936	Tobin *et al.,* 1997
ADR1	-	Slaven *et al.,* 1999
abcA	AJ417501	-
atrF	AJ311940	-

AfuMDR1 encodes a protein with a predicted $[TMD_6\text{-}NBF]_2$ topology while *abcA* and *atrF* encode proteins with a $[NBF\text{-}TMD_6]_2$ topology. Interestingly, *AfuMDR2* encodes a protein with only four putative TMDs and a single NBF. Heterologous expression of *AfuMDR1* in *S. cerevisiae* resulted in increased 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).

Table 3. ABC transporter genes from *Botrytis cinerea*

ABC gene	Accession number	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

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 $[NBF\text{-}TMD_6]_2$ 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 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 $[NBF\text{-}TMD_6]_2$ 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 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 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 $[NBF\text{-}TMD_6]_2$ 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*) is able of infecting potato tubers through open wounds causing the dry rot disease. Recently, the ABC transporter gene *Gpabc1* (Acc. AJ306607), encoding a protein with a $[NBF\text{-}TMD_6]_2$ 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 colonize 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 $[NBF\text{-}TMD_6]_2$ 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 die 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 still 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 the plant pathogenic fungus *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 can 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, and natural compounds, such as the plant metabolites berberine and camptothecin, and the mycotoxin diacetoxyscirpenol (DAS).

Table 4. ABC transporter genes from *Mycosphaerella graminicola*

ABC gene	Accession number	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

The function of *MgAtr1-MgAtr5* in virulence of *M. graminicola* on wheat was investigated with knockout mutants. Analysis of the tranformants showed that Δ*MgAtr5* mutants have a small increase in sensitivity to the putative wheat defence compound resorcinol and to the grape phytoalexin resveratrol, 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 *et al.,* submitted). Thus, the possibility that some of these transporters are involved in protection against natural and synthetic toxic compounds can not be excluded. All transformants were also 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, shows 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.

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.

Table 5. ABC transporter genes from *Penicillium digitatum*

ABC gene	Acc. number	Reference
PMR1	AB010442	Nakaune *et al.,* 1998
PMR3	-	Nakaune *et al.,* 2001
PMR4	-	Nakaune *et al.,* 2001

Treatment of the fungus with the DMI fungicide triflumizole induced *PMR1* transcription levels in both DMI-sensitive and resistant strains. Disruption of *PMR1* in DMI-sensitive and resistant isolates resulted in increased sensitivity to DMIs as well as to camptothecin, phloretin, and oligomycin (Nakaune *et al.,* 1998; Nakaune *et al.,* 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 camptothecin and resveratrol. The results demonstrate that PMR5 and PMR1 possess distinct substrate specificity and can 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 the plant pathogenic fungus *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. cerevis*iae 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 multidrug resistance, thereby protecting the fungus against natural and synthetic toxic compounds (Taylor and Condie, 1999).

Four ABC transporter genes have been cloned from the plant pathogenic fungus *Venturia inaequalis*, the causal agent of the apple scab disease (Table 6).

Table 6. ABC transporter genes from *Venturia inaequalis*

ABC gene	Accession number	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

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* are less sensitive to the trichothecene 4,15-diacetoxyscirpenol (Muhitch *et al.,* 2000).

Preliminary data are 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 diacetoxyscirpenol (DAS), a mycotoxin produced by *Fusarium graminearum*. Kema *et al.,* (1996) proposed that formation of necrotic lesions on infected wheat leaves caused with *M. graminicola* may be 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

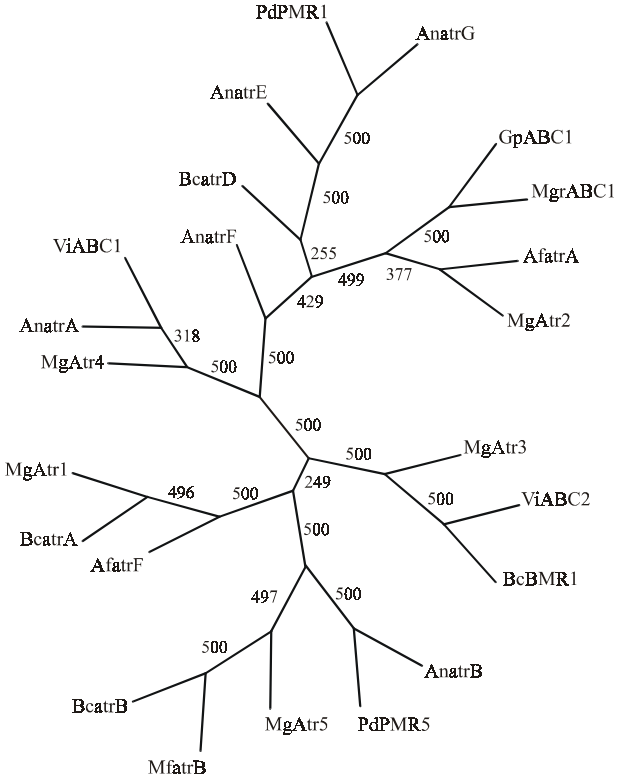


Figure 3. Phylogenetic unrooted tree of 23 ATP-Binding Cassette (ABC) transporters with the $[NBF\text{-}TMD_6]_2$ topology from filamentous fungi. The tree was based on parsimony as calculated using PROTPARS. Alignments were performed using the ClustalW alignment programme. The code of the proteins mentioned in the tree are preceded 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*

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.

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 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).

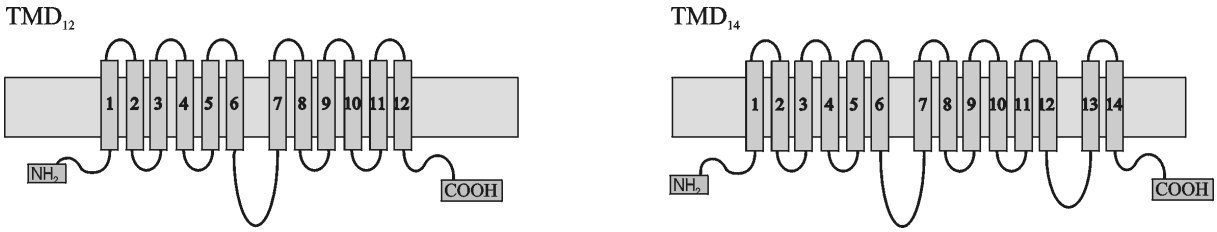


Figure 4. Schematic representation of Major Facilitator Superfamily (MFS) of transporters with 12 and 14 Trans-Membrane Domains (TMDs). The TMDs are indicated in boxes and are numbered.

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 trafficking of sugars, drugs, polyols, vitamines, neurotransmitters, Krebs-cycle metabolites, phosphorylated glycolytic intermediates, amino acids, peptides, osmolites, iron-siderophores, nucleosides, organic and inorganic anions, and cations. In this way, they facilitate various 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 *HTS*1 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 self-protection against this toxin and virulence (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), a 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 so far, 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 myo-inositol (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 during evolution 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).

ABC transporters characterised so far 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 can 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 synthesized 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 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 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 can 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 may 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 programs aimed at the discovery of new fungitoxic lead compounds. The reason is that test organisms may 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 may 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 can 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 optimization 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 can increase 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 available so far 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 may 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 and De Waard, 2000; Zwiers *et al.,* submitted). 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 result in the discovery of new disease control agents.

ACKNOWLEDGEMENTS

We kindly acknowledge Prof. Dr P.J.G.M. De Wit for critical reading of the manuscript. I. Stergiopoulos is financially supported by the Training and Mobility of Researchers (TMR) Programme – Marie Curie Research Training Grants, The European Commission (Contract No. ERBFMBICT983558) and L-H. Zwiers by the Graduate School of Experimental Plant Sciences (EPS), Wageningen University, The Netherlands.

REFERENCES

al Shawi MK and Senior AE (1993) Characterization of the adenosine triphosphatase activity of Chinese hamster P-glycoprotein. J Biol Chem **268**: 4197-206

Albertson G, Niimi M, Cannon R and Jenkinson H (1996) Multiple efflux mechanisms are involved in *Candida albicans* fluconazole resistance. Antimicrob Agents Chemother **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. Mol Gen Genet **261**: 977-84

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. Proc Natl Acad Sci USA **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. Annu Rev Pharmacol Toxicol **39**: 39361-98

Ames GFL, Nikaido K, Groarke J and Petithory J (1989) Reconstitution of periplasmic transport in inside-out membrane vesicles: Energization by ATP. J Biol Chem **264**:3998-4002

Andrade AC (2000) ABC transporters and multidrug resistance in *Aspergillus nidulans.* Thesis Wageningen University, Wageningen.

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. Microbiol UK **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. Mol Gen Genet **263**: 966-977

Avendano C and Menendez JC (2002) Inhibitors of multidrug resistance (MDR) to antitumor agents. Curr Med Chem **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. Mol Cell Biol **9**: 5289-97

Bauer BE, Wolfger H and Kuchler K (1999) Inventory and function of yeast ABC proteins: about sex, stress, pleiotropic drug and heavy metal resistance. Biochim Biophys Acta **1461:** 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. J Biol Chem **269**: 4180-4186

Bosch I and Croop J (1996) P-glycoprotein multidrug resistance and cancer. Biochim Biophys Acta **1288**: F37-54

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. Mol Plant-Microbe In **12**: 901-10

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*. Appl Environ Microbiol **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. Mol Gen Genet **255**: 226-236

De Waard MA (1997) Significance of ABC transporters in fungicide sensitivity and resistance. Pestic Sci **51**: 271-275

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

De Waard MA, Groeneweg H and Van Nistelrooy JGM (1982) Laboratory resistance to fungicides which inhibit ergosterol biosynthesis in *Penicillium italicum*. Neth J Plant Pathol: 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* fungicides. Pestic Sci **13**: 279-286

De Waard MA and Van Nistelrooy JGM (1984a) Differential accumulation of fenarimol by a wild-type isolate and fenarimol-resistant isolates of *Penicillium italicum*. Neth J Plant Pathol **90**: 143-153

De Waard MA and Van Nistelrooy JGM (1984b) Effects of phthalimide fungicides on the accumulation of fenarimol by *Aspergillus nidulans*. Pestic Sci **15**: 56-62

De Waard MA and Van Nistelrooy JGM (1988) Accumulation of SBI fungicides in wild-type and

fenarimol-resistant isolates of *Penicillium italicum*. Pestic Sci **22**: 371-382

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

Decottignies A and Goffeau A (1997) Complete inventory of the yeast ABC proteins. Nat Genet **15**:137-145

Dekker J (1981) Impact of fungicide resistance of disease control toxicity. The British Crop Protection Council (BCPC) Conference: Pests and Diseases, Brighton, UK, **3**: 857-863

Del Sorbo G, Andrade AC, Van Nistelrooy JGM, Van Kan JA, Balzi E and De Waard MA (1997) Multidrug resistance in *Aspergillus nidulans* involves novel ATP-binding cassette transporters. Mol Gen Genet **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 Genet Biol **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*. Antimicrob Agents Chemother **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. Mol Plant-Microbe In **9**: 775-781

Do Nascimiento AM, Terenzi MF, Goldman MMH and Goldman GH (1999) A novel ATP-binding cassette transporter is involved in multidrug resistance in the filamentous fungus *Aspergillus nidulans*. Fun Gent News Let **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. Mol Plant-Microbe In **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. Mol Gen Genet **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 s*trains. Antimicrob Agents Chemother **42:** 3065-3072

Gottesman MM and Pastan I (1988) The multidrug transporter, a double-edged sword. J Biol Chem **263**: 12163-6

Gottesman MM and Pastan I (1993) Biochemistry of multidrug resistance mediated by the multidrug transporter. Annu Rev Biochem **62**: 385-427

Greenberger LM (1993) Major photoaffinity drug labelling sites for iodoaryl azidoprazosin in P-glycoprotein are within, or immediately C-terminal to, transmembrane domains 6 and 12. J Biol Chem **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. Curr Opin Cell Biol **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 14α-demethylase gene (*CYP51*) in *Penicillium digitatum*. Appl Environ Microbiol **66**: 3421 - 3426

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.* Pestic Biochem Physiol **70**: 168-179

Hayashi K, Schoonbeek H and De Waard MA (2002) *Bcmfs1*, a novel major facilitator superfamily transporter from *Botrytis cinerea*, provides tolerance to the natural toxic compounds camptothecin and cercosporin and towards fungicdes. Appl Environ Microbiol **68:** 4996-5004

Henderson P (1993) The 12-transmembrane helix transporters. Curr Opin Cell Biol **5**: 708-721

Higgins CF (1992) ABC transporters: from microorganisms to man. Annu Rev Cell Biol **8**: 67-113

Higgins CF and Gottesman M (1992) Is the multidrug transporter a flippase? Trends Biochem Sci **17**: 18-21

Ishikawa T (1992) The ATP-dependent glutathione S-conjugate export pump. Trends Biochem Sci **17**: 463-8

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. Biosci Rep **17**: 189-207

Juliano RL and Ling V (1976) A surface glycoprotein modulating drug permeability in Chinese hamster ovary cell mutants. Biochim Biophys 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. Curr Opin Biotech **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. Embo J **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. Mol Pharmacol **51**: 1034-41

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. J Biol Chem **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. J Biol Chem **270**: 22957-22961

Marger MD and Saier MHJ (1993) A major superfamily of transmembrane facilitators that catalyse uniport, symport and antiport. Trends Biochem Sci **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 Sci **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. J Gen Plant Pathol **67**: 212-214

Nakaune R, (2001) ABC transporter genes involved in multidrug resistance in Penicillium digitatum. J Gen Plan Pathol **67:** 251

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*. Appl Environ Microbiol **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*. Mol Genet Genomics **267:** 179-85

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 Microbiol Rev **21:** 113-134

Pao SS, Paulsen IT and Saier MH Jr (1998) Major facilitator superfamily. Microbiol Mol Biol Rev **62**: 1-34

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 **124**: 1-11

Paulsen IT, Brown MH and Skurray RA (1996) Proton-dependent multidrug efflux systems. Microbiol Rev **60**: 575-608

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. Antimicrob Agents Chemother **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. J Biol Chem **265**: 3975-3980

Roepe P (1994) Indirect mechanism of drug transport by P-glycoprotein. Trends Pharmacol Sci **15**: 445-446R

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. J Biol Chem **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---Val-185 substitution in P-glycoprotein. Proc Natl Acad Sci USA **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. FASEB J **12**: 265-74

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. Antimicrob Agents Chemother **39**: 2378-2386

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

Schnabel G and Jones LA (2001) Fungicide resistance genes in *Venturia inaequalis*. *In:* Modern Fungicides and Antifungal Compounds, Proceedings of 13th International Reinhardsbrunn Symposium, 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. Mol Plant-Microbe In **14**: 562-571.

Slaven JW, Anderson MJ, Sanglard D, Dixon GK, Bille J, Roberts IA and Denning DW (1999) Fungal Genet News Lett **46**: 64

St Georgiev V (2000) Membrane transporters and antifungal drug resistance. Curr Drug Targets **1**: 261-284

Stergiopoulos I, Gielkens MMC, Goodall SD, Venema K and De Waard MA (2002a) Molecular cloning and characterisation of three new ATP-binding cassette 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, Pisa, Italy, pp. 250

Taglicht D and Michaelis S (1998) *Saccharomyces cerevisiae* ABC proteins and their relevance to human health and disease. Methods Enzymol **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 1999, Amsterdam, The Netherlands, pp. L71

Theodoulou FL (2000) Plant ABC transporters. Biochim Biophys Acta **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 Lett **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 Microbiol Lett **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. EMBO J **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. Med Mycol **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 Manag Sci **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. Curr Genet **39**: 377-383

Wadkins RM and Roepe PD (1997) Biophysical aspects of P-glycoprotein-mediated multidrug resistance. Int Rev Cytol **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. EMBO J **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. Antimicrob Agents Chemother **41**: 1482-1487

Wolfger H, Mamnun YM and Kuchler K (2001) Fungal ABC proteins: pleiotropic drug resistance, stress response and cellular detoxification. Res Microbiol **152**: 375-389

Yoder OC and Turgeon BG (2001) Fungal genomics and pathogenicity. Curr Opin Plant Biol **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. J Biol Chem **270**: 5441-5448

Zwiers L-H and De Waard MA (2000) Characterization of the ABC transporter genes *MgAtr1* and *MgAtr2* from the wheat pathogen *Mycosphaerella graminicola*. Fungal Genet Biol **30**: 115-125

Zwiers L-H, Stergiopoulos I, Gielkens MC, Goodall SD and De Waard MA ABC transporters of the wheat pathogen *Mycosphaerella graminicola* function as protectants against biotic and xenobiotic toxic compounds. *Submitted*

CHAPTER 3

MOLECULAR CLONING AND CHARACTERISATION OF THREE NEW ATP-BINDING CASSETTE TRANSPORTER GENES FROM THE WHEAT PATHOGEN *MYCOSPHAERELLA GRAMINICOLA*

Ioannis Stergiopoulos, Marco M.C. Gielkens, Stephen D. Goodall, Koen Venema and Maarten A. De Waard

Gene

(2002) ***289:*** *141-149*

ABSTRACT

Three single copy ATP-binding cassette (ABC) transporter encoding genes, designated *MgAtr3*, *MgAtr4,* and *MgAtr5,* were cloned and sequenced from the plant pathogenic fungus *Mycosphaerella graminicola*. The encoded ABC proteins all exhibit the [NBD-TMS_6]$_2$ configuration and can be classified as novel members of the pleiotropic drug resistance (PDR) class of ABC transporters. The three proteins are highly homologous to other fungal and yeast, ABC proteins involved in multidrug resistance or plant pathogenesis. MgAtr4 and MgAtr5 possess a conserved ABC motif at both the N- and C-terminal domain of the protein. In contrast, the Walker A motif in the N-terminal and the ABC signature in the C-terminal domain of MgAtr3, deviate significantly from the consensus sequence found in other members of the PDR class of ABC transporters. Expression of *MgAtr3* could not be detected under any of the conditions tested. However, *MgAtr4* and *MgAtr5* displayed distinct expression profiles when treated with a range of compounds known to be either substrates or inducers of ABC transporters. These included synthetic fungitoxic compounds, such as imazalil and cyproconazole, natural toxic compounds, such as the plant defence compounds eugenol and psoralen, and the antibiotics cycloheximide and neomycin. The expression pattern of the genes was also dependent on the morphological state of the fungus. The findings suggest a role for MgAtr4 and MgAtr5 during plant pathogenesis and in protection against toxic compounds.

Keywords: ATP-binding cassette transporters; multidrug resistance; plant pathogenesis; *Septoria tritici*

INTRODUCTION

ATP-binding cassette (ABC) transporters are membrane proteins that utilise the energy derived from the hydrolysis of ATP to drive the transport of compounds over biological membranes. They are members of one of the largest protein families to date, present in both pro- and eukaryotic organisms (Higgins, 1992). Genome analysis of *Saccharomyces cerevisiae* identified 31 putative ABC proteins, which can be classified into six clusters (Decottignies and Goffeau, 1997) or classes (Taglicht and Michaelis, 1998) based on their structure and topology. Most ABC transporters are composed of two similar halves, each consisting of a cytoplasmatic nucleotide-binding domain (NBD) and six trans-membrane segments (TMS_6). The majority of these proteins have a $[TMS_6\text{-NBD}]_2$ or $[\text{NBD-}TMS_6]_2$ topology, but half size transporters with a single TMS_6-NBD or NBD-TMS_6 configuration have also been described. The NBDs of ABC transporters contain conserved amino acid sequences which are called the Walker A and Walker B motif (Walker *et al.,* 1982) and the ABC signature (Ames *et al.*, 1990).

ABC transporters are known to play an essential role in multidrug resistance (MDR) of cells to chemically unrelated compounds (Juliano and Ling, 1976). ABC transporters involved in drug resistance have also been described in filamentous fungi (De Waard, 1997). In plant pathogenic fungi ABC transporters may act as virulence factors if they mediate secretion of pathogenicity factors or provide protection against plant defence compounds during pathogenesis (Del Sorbo *et al.,* 2000). This hypothesis has been validated in an insertional mutagenesis screen for pathogenicity mutants of the rice blast fungus *Magnaporthe grisea,* which identified the ABC transporter ABC1 as a novel pathogenicity factor, probably protecting the fungus against plant defence compounds produced by the host (Urban *et al.,* 1999).

In our laboratory ABC transporters from *Mycosphaerella graminicola* (Fückel) Schroeter (anamorph state: *Septoria tritici* Rob.ex.Desm.), the causal agent of septoria leaf blotch of wheat, are studied. This disease can cause a significant reduction in yield. Typical disease symptoms are necrotic spots filled with the asexual pycnidia and sexual pseudothecia of the fungus (Eyal *et al.,* 1987). Formation of the necrotic lesions may be associated with secretion of phytotoxic compounds by the pathogen (Kema *et al.,* 1996). Wheat is known to produce plant defence compounds, such as 2,4-dihydroxy-7-methoxy-2*H*-1,4-benzoxazin-3(4*H*)-one

(DIMBOA) (Weibull and Niemeyer, 1995). Therefore, the fungus may have evolved specific ABC transporters that secrete toxins, or reduce the intracellular accumulation of plant defence compounds.

Recently, Zwiers and De Waard (2000) cloned and characterised the ABC transporter genes *MgAtr1* and *MgAtr2* from *M. graminicola*. Both genes are differentially expressed when mycelium and yeast-like cells of this dimorphic fungus are treated *in vitro* with antibiotics, plant secondary metabolites or azole fungicides. This paper extends these studies by reporting the cloning and characterisation of the ABC transporter encoding genes *MgAtr3*, *MgAtr4,* and *MgAtr5*.

MATERIALS AND METHODS

Fungal culture conditions

M. graminicola isolate IPO323 (Kema and Van Silfhout, 1997) was used for the construction of a genomic library and for expression studies. Genomic DNA was isolated from 5-day-old yeast-like cells (Raeder and Broda, 1985). Yeast-like cells and mycelium of *M. graminicola* were grown as described by Zwiers and De Waard (2000).

Isolation and characterisation of *MgAtr3, MgAtr4,* and *MgAtr5*

A polymerase chain reaction (PCR) based approach was followed using *M. graminicola* IPO323 genomic DNA as template. Degenerate oligonucleotide primers were designed on the conserved amino acid sequences of the Walker A and Walker B motif and the ABC signature of the pleiotropic drug resistance (PDR) and multidrug resistance-related protein (MRP) class of ABC transporters, based on a phylogenetic analysis of the yeast ABC proteins (Taglicht and Michaelis, 1998) (Table 1). The PCR reaction mix contained 200 µM of dNTPs, 30 ng μl^{-1} of each primer, 1x PCR buffer (Boehringer Mannheim B.V.), 2 U *Taq* polymerase, and approximately 100 ng DNA template. PCR-cycles consisting of 5 min denaturation at 94ºC followed by an additional step of 1 min at the same temperature, 45 sec of annealing at 48ºC, and 45 sec extension at 68ºC were performed. A final extension step of 3 min at 68ºC completed the reaction. Specific products were reamplified with *Pwo* polymerase and cloned into pCR-Blunt (Invitrogen) plasmid vector.

Table 1. Degenerate primers used for PCR amplification of ABC transporter domains from *Mycosphaerella graminicola*[a]

Primer name	Sequence (5′ - 3′)	Motif	Region
PDR class			
KV1	GGCCGHCCNGGHTSNGGNTG	Walker A	N–terminal
KV2	CGCTCDCCDCCSSWVACCNCC	ABC signature	N–terminal
KV3	CCNCKVGTVSWRTTRTCCCA	Walker B	N–terminal
KV4	GGNGCNGGHMGCACBACBCT	Walker A	C–terminal
KV5	SAGDCCVSAGGTNGGYTCRTC	Walker B	C–terminal
MRP class			
KV7	NGARATRCCYTTYTCNCC	ABC signature	N–terminal
KV9	GGCCGMACNGGHGCNGGNAAR	Walker A	C–terminal
KV11	GMVGTVGCYTCRTCNAR	Walker B	C–terminal

[a] Primers were designed based on the conserved amino acid sequences of the Walker A and Walker B motif, and ABC signature of the PDR and MRP class of ABC transporters from *Saccharomyces cerevisiae*.

Cloned PCR fragments were sequenced and used to probe a phage-library of genomic DNA from *M. graminicola* IPO323 (Zwiers and De Waard, 2000). Hybridising clones from phages with inserts of interest were subcloned into either pBluescript (Stratagene) or pZErO^{tm}-2 (Invitrogen) plasmid vectors. Sequencing was performed by the dideoxy chain-termination method using ampli-Taq polymerase and fluorescent dideoxy dNTPs. BLAST (Basic Local Alignment Search Tool) homology searches of databases were performed using the BLAST 2.0 version package at NCBI (http://www.ncbi.nlm.nih.gov/BLAST; Altschul *et al.,* 1997). Sequences were analysed using the DNAstar 4.2 version package (DNASTAR Inc., Madison, U.S.A.).

Reverse transcription (RT)-PCR was performed on *M. graminicola* RNA to determine intron positions. Total RNA was extracted as described by Zwiers and De Waard (2000) and PCR amplifications were performed using the 'Superscript One-step RT-PCR system' (GIBCO BRL) according to the manufacturer's instructions. 5′ and 3′ rapid amplification of DNA complementary to RNA (cDNA) ends (RACE) experiments were performed using the Boehringer Mannheim RACE kit according to the manufacturer's instructions.

Southern blot analysis

The copy number of *MgAtr3*, *MgAtr4,* and *MgAtr5* in the genome of isolate IPO323 was studied by Southern blot analysis. Gene-specific probes were prepared and genomic DNA (3 µg) was digested with specific restriction enzymes (20 units). Blotting was performed onto Hybond-N$^+$ membranes (Amersham Pharmacia Biotech) according to the manufacturer's instructions. Hybridisations were done at 65ºC in Modified Church buffer (0.36 M Na_2HPO_4, 0.14 M NaH_2PO_4, 1 mM EDTA, 2% SDS, pH. 7.2). Washings were performed twice at 56ºC in 2x SSC and 0.5x SDS.

RNA isolation and northern blot analysis

Total RNA was isolated from yeast-like cells and from mycelium of isolate IPO323 after treatment with plant defence compounds, xenobiotics, lipids or fatty acids. The final concentration of the chemical compounds were as follows: acriflavin (50 μg ml^{-1}, Fluka), cycloheximide (50 μg ml^{-1}, Merck), the cyclosporin derivative PSC833 (10 μg ml^{-1}, Syngenta), cyproconazole (10 μg ml^{-1}, Syngenta), eugenol (0.01% (v/v), Sigma-Aldrich), imazalil nitrate (10 μg ml^{-1}, Janssen Pharmaceutica), lanosterol (100 μg ml^{-1}, Sigma-Aldrich), linoleic acid (1 mg ml^{-1} Sigma-Aldrich), neomycin (100 μg ml^{-1}, Sigma-Aldrich), nystatin (5 μg ml^{-1}, Duchefa), oleic acid (1 mg ml^{-1}; Sigma-Aldrich), palmitic acid (1 mg ml^{-1}, Sigma-Aldrich), phosphatidyl choline (100 μg ml^{-1}, Sigma-Aldrich), progesterone (100 μg ml^{-1}, Sigma-Aldrich), psoralen (50 μg ml^{-1}, Sigma-Aldrich), quercetin (100 μg ml^{-1}, Sigma-Aldrich), reserpine (100 μg ml^{-1}, Sigma-Aldrich), resveratrol (50 μg ml^{-1}, Sigma-Aldrich), tomatine (10 μg ml^{-1}, Sigma-Aldrich), and triflupromazine (20 μg ml^{-1}, Sigma-Aldrich). Most of the compounds were added from a thousand-fold concentrated stock solution in water or methanol. Eugenol, linoleic acid, neomycin, oleic acid, palmitic acid, and phosphatidyl choline were added to the cultures as pure products. Solvents (0.1%) were added to control treatments. RNA isolations were performed according to Zwiers and De Waard (2000). Homologous hybridisation of the blots was performed overnight at 65ºC in Nasmyths solution buffer (18.5% Dextran sulphate, 1.85% sarcosyl, 0.011 M EDTA, 0.3 M Na_2HPO_4, 1.1 M NaCl, pH 6.2). The solution (5.4 ml) was mixed with distilled water (4.6 ml) just before use to obtain the hybridisation buffer (10 ml). Sheared herring sperm DNA (100 μg ml^{-1}) was included as a blocking agent. Blots were washed twice in 2x SSC, 0.1x SDS and 0.1x SSC, 0.1x SDS, each for 15 min at 65ºC.

Probes

Gene-specific probes used in Southern and northern blot analysis were: a 0.85 kb *Sal*I fragment down-stream of the ABC signature sequence in the N-terminal domain of MgAtr3, a 1.14 kb *BamH*I/*Pst*I fragment between the NBDs of MgAtr4, and a 0.6 kb *Eco*RI fragment down-stream the Walker A sequence of the C-terminal domain of MgAtr5 (Figure 2). Equal loading of the samples on the blots was examined with a probe derived from the *gpd1* gene of *M. graminicola,* encoding glyceraldehyde 3-phosphate dehydrogenase.

Randomly primed DNA isotopic probes were prepared by enzymatic incorporation of [α-^{32}P]-labelled dATP. In each labelling reaction, 50 ng of a probe template was used. Probes were purified using the QIAquick Nucleotide Removal kit (QIAGEN) before adding to the hybridisation solution.

RESULTS

Cloning of *MgAtr3, MgAtr4,* and *MgAtr5*

PCR performed on genomic DNA from *M. graminicola* isolate IPO323 using degenerate primers derived from conserved sequences within the NBDs of ABC transporters, resulted in the amplification of several fragments of the expected size. Sequence analysis of the cloned

fragments revealed that only the amplification products of primer combinations KV1 with KV2, KV1 with KV3, and KV4 with KV5, showed homology to ABC transporter domains. These primers were designed on consensus sequences of the PDR class of ABC transporters (Table 1). Two of the amplified fragments represented parts of *MgAtr1* and *MgAtr2,* previously described by Zwiers and De Waard (2000). PCR products with high homology to other ABC transporter genes were used to screen a *M. graminicola* genomic library and clone the corresponding genes. This screen resulted in the identification of three new genes encoding ABC transporters. These genes were named *MgAtr3, MgAtr4,* and *MgAtr5.* Alignment of MgAtr3, MgAtr4, and MgAtr5 showed that the deduced proteins have homology with ABC transporters from other filamentous fungi or yeasts (Table 2, Figure 1). Southern analysis revealed that all three genes have a single copy in the genome of *M. graminicola* (data not shown). The genes are deposited in the GenBank under the Accession numbers AF364105, AF329852, and AF364104, respectively.

Table 2. Homology of the ABC transporters MgAtr3, MgAtr4, and MgAtr5 from *Mycosphaerella graminicola* with ABC transporters from filamentous fungi and yeasts.

ABC transporters from *Mycosphaerella graminicola*	ABC transporters from other filamentous fungi and yeasts			Homology		
	Protein	Source organism	Accession Number	e-value	Identity (%)	Similarity (%)
MgAtr3	BMR1	*Botrytis cinerea*	AB028872	0.0	32	48
	MgAtr5	*Mycosphaerella graminicola*	AF364104	e-117	28	43
	atrB	*Aspergillus nidulans*	Z68905	e-112	28	42
	BFR1	*Schizosaccharomyces pombe*	P41820	e-122	26	41
MgAtr4	atrA	*Aspergillus nidulans*	Z68904	0.0	57	70
	BcatrD	*Botrytis cinerea*	AJ272521	0.0	48	63
	atrE	*Aspergillus nidulans*	AJ276241	0.0	46	60
	MgAtr2	*Mycosphaerella graminicola*	AJ243113	0.0	45	60
MgAtr5	BcatrB	*Botrytis cinerea*	AJ006217	0.0	67	77
	atrB	*Aspergillus nidulans*	Z68905	0.0	62	72
	MgAtr1	*Mycosphaerella graminicola*	AJ243112	0.0	38	55
	BMR1	*Botrytis cinerea*	AB028872	0.0	37	54

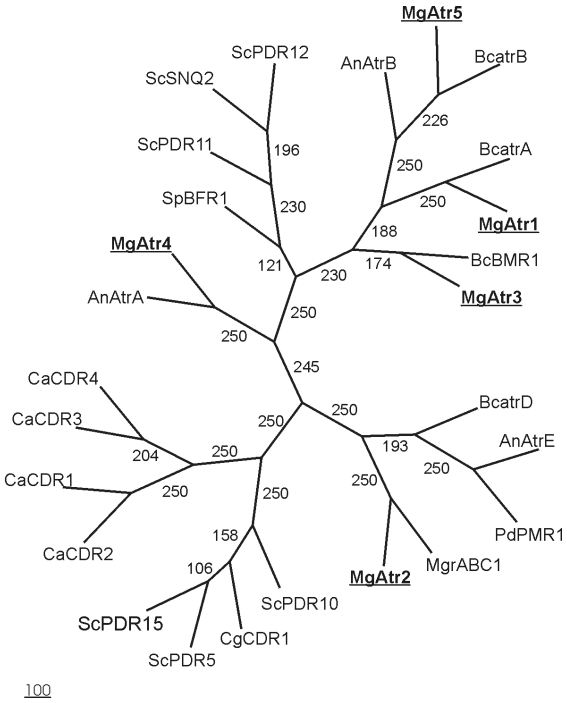


Figure 1. Phylogenetic unrooted tree of 26 fungal ABC transporters with the $[NBD\text{-}TMS_6]_2$ configuration. The tree was based on parsimony as calculated using PROTPARS. Alignments were performed using the CLUSTALX alignment programme. The *Mycosphaerella graminicola* ABC proteins are printed in bold and underlined. The code name of the genes mentioned in the tree are preceded by two letters, indicating the source organism: An: *Aspergillus nidulans*, Bc: *Botrytis cinerea*, Ca: *Candida albicans*, Cg: *Candida glabrata*, Mg: *Mycosphaerella graminicola*, Mgr: *Magnaporthe grisea*, Pd: *Penicillium digitatum*, Sc*: Saccharomyces cerevisiae*, Sp: S*chizosaccharomyces pombe*

Sequence analysis

A 6.954 kb *Xba*I-*Not*I fragment containing the *MgAtr3* gene was cloned into pZERO-2. The entire sequence of *MgAtr3* was determined along with 2.23 kb of promoter and 584 bp of the 3′ flanking region. The open reading frame (ORF) of *MgAtr3* is 3.966 kb long and encodes a putative protein of 1321 amino acids with a calculated mass of 145 kDa (Figure 2). The ORF is interrupted by three introns of 66, 62, and 54 bp, respectively. All three introns fit the features generally found in genes from filamentous fungi (Unkles, 1992). The introns and their positions were confirmed by sequencing of cDNA generated by RT-PCR. Analysis of RACE products revealed that the 5′ untranslated region (UTR) is at least 65 bp long and the 3′ UTR 432 bp. At the 5′ flanking region of *MgAtr3* a 7/8 conserved Kozak sequence (***C***CGCCATG) spans the putative translation start and a putative GC rich box (CCGCCC) is located at position -113 bp. Three TATAAA-like sequences enabling the TFIID transcription factor to bind to the TATA box in yeast are located at positions -466, -558, and -605 bp, respectively. At the 3′ flanking region of *MgAtr3*, a typical polyadenylation signal (AATAAA) is located at 256 bp downstream of the putative translation stop codon.

Sequencing of *MgAtr4* clones revealed a 4.908 kb ORF encoding a protein of 1635 amino acids with a predicted mass of 182 kDa. Additional DNA up- (2.37 kb) and downstream (2.7 kb) of *MgAtr4* was also sequenced. The ORF of *MgAtr4* is probably not interrupted by introns

(Figure 2), since searches for putative splicing junctions were negative. The high homology of this gene with other ABC transporter genes confirmed this conclusion. Repeated attempts to determine the transcriptional start and end of the gene were not successful. A 7/8 conserved putative Kozak sequence (C***A***GCCATG) was detected around the putative translation start of *MgAtr4.*

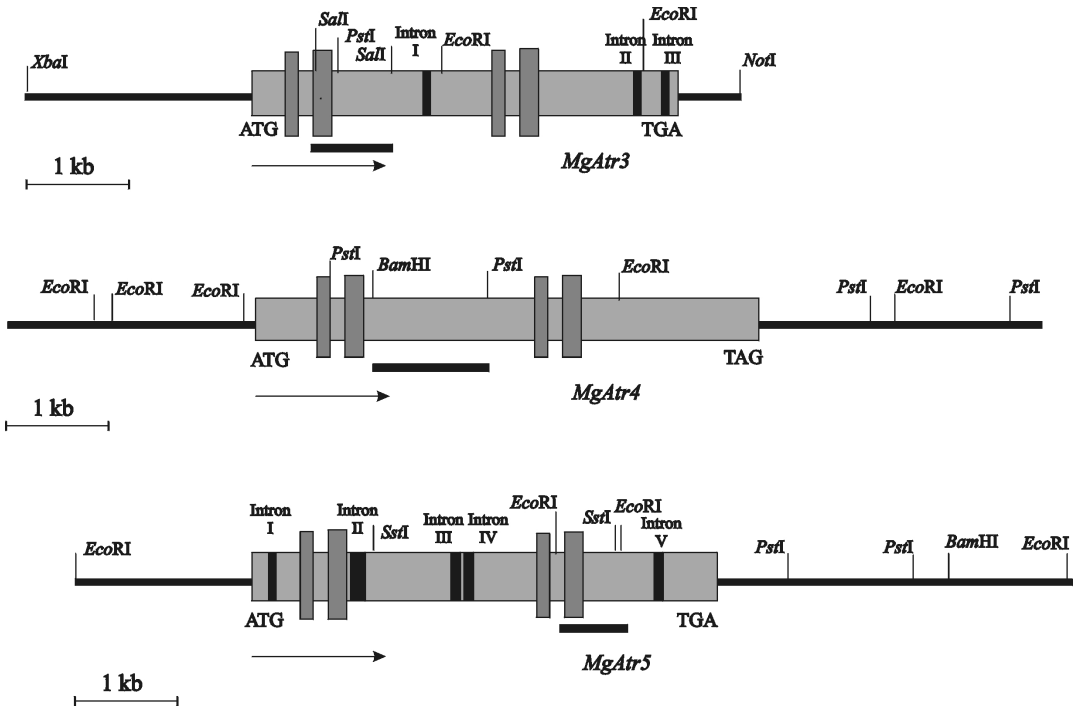


Figure 2. Physical map of *MgAtr3, MgAtr4*, and *MgAtr5* from *Mycosphaerella graminicola*. The positions of the ATP-binding cassette domains are indicated as dark gray boxes. Introns are depicted as black boxes and numbered with Latin numbers. Black horizontal boxes indicate the probes used in Southern and northern blot analysis. Direction of transcription is given by arrows.

The sequence determined for *MgAtr5* was 9.747 kb long and contained 1.78 and 3.34 kb of the 5′ and 3′ non-coding flanking regions, respectively. The ORF of *MgAtr5* is 4.281 kb long and encodes a protein of 1426 amino acids with a calculated mass of 159 kDa (Figure 2). The ORF is interrupted by five introns of 56, 118, 57, 51, and 54 bp, respectively, which all fit the features reported for introns in genes from filamentous fungi. The introns and their positions were confirmed by sequencing a full-length cDNA clone of *MgAtr5,* generated by RT-PCR. Analysis of RACE products revealed that the 5′ UTR is at least 59 bp long. Two putative transcriptional stops were also found 151 and 180 bp downstream of the putative stop codon. At the 5′ flanking region of *MgAtr5,* a 7/8 conserved Kozak sequence (C***G***ACCATG) is

spanning the putative translation start. At position -247 bp, a 5/6 conserved putative PDR1/PDR3 binding-site (CCGCG***C***) is found.

Secondary structure

Hydropathy analysis predicts that MgAtr3, MgAtr4, and MgAtr5 contain a polypeptide sequence of two homologous halves each comprising of six putative TMSs and one NBD, the major diagnostic features of the ABC superfamily. The proteins have a [NBD-TMS_6]$_2$ predicted topology. Hence, they can be classified as novel members of the PDR class of ABC transporters (Taglicht and Michaelis, 1998).

Table 3. Conserved amino acid sequences of the Walker A and Walker B motif, and the ABC signature, in the PDR class of fungal ABC transporters[a].

Protein	Source	Walker A	ABC signature	Walker B
N-terminal				
MgAtr1	Mg[b]	ML**VLG**R**PG**S**GC**STF**LK**	V**RGVSGGERKR**VS**I**A**E**TLASKSTVVC**WDN**ST**RGLD**A
MgAtr2	Mg	LV**VLG**P**PG**S**GC**STF**LK**	L**RGVSGGERKR**VT**I**A**E**ASLSGAALQA**WDN**ST**RGLD**S
MgAtr3	Mg	VL**V**ASAEPAE**C**TQL**L**R	V**RGI**S**GGER**RRLSVA**E**ALLSGANVLCF**D**DLT**RGLD**A
MgAtr4	Mg	LI**VLG**R**PG**S**GC**STL**LK**	V**RGVSGGERKR**VS**I**A**E**MALAGSALAA**WDN**ST**RGLD**S
MgAtr5	Mg	LL**VLG**R**PG**A**GC**TSL**LK**	V**RGVSGGERKR**VS**I**L**E**TMAARATVVC**WDN**ST**RGLD**A
AtrB	An	LL**VLG**R**PG**S**GC**TTL**LK**	I**RGVSGGERKR**VS**II**E**C**LGTRASVFC**WDN**ST**RGLD**A
BcatrB	Bc	LL**VLG**R**PG**A**GC**TTL**LK**	V**RGVSGGERKR**VS**II**E**M**LASRGSVMC**WDN**ST**RGLD**A
BMR1	Bc	VL**VLG**R**PG**S**GC**TTF**LK**	V**RGVSGGERKR**VS**I**A**E**MMITSGTVCA**WDN**ST**RGLD**A
ABC1	Mgr	LV**VLG**P**PG**S**GC**STF**LK**	I**RGVSGGERKR**VT**I**A**E**AALSGAPLQC**WDN**ST**RGLD**S
PMR1	Pd	LI**VLG**R**PG**S**GC**STF**LK**	I**RGVSGGERKR**VS**I**A**E**ATLCGSPLQC**WDN**ST**RGLD**S
PDR5	Sc	LV**VLG**R**PG**S**GC**TTL**LK**	V**RGVSGGERKR**VS**I**A**E**VSICGSKFQC**WDN**AT**RGLD**S
C-terminal				
MgAtr1	Mg	V**ALMG**A**SGAGKTTLL**N	**LGVEQR--K**RLT**IGVEL**AAK**P**SL**LL**F**LDE**PT**SGLD**S
MgAtr2	Mg	T**ALMG**V**SGAGKTTLL**D	**LNVEQR--K**RLTV**GVEL**AAK**P**QL**LL**F**LDE**PT**SGLD**S
MgAtr3	Mg	L**A**V**MG**A**SGAGKTTLL**N	SELSA**R**DR**K**RTTIA**VEL**AAK**P**D-IL**F**L**DE**P**TTGL**GS
MgAtr4	Mg	T**ALMG**V**SGAGKTTLL**D	**LNVEQR--K**LLT**IGVEL**AAK**P**KL**LL**F**LDE**PT**SGLD**S
MgAtr5	Mg	G**ALMG**S**SGAGKTTLL**D	**LSVEQR--K**RLT**IGVEL**VSK**P**SI**L**I**F**L**DE**P**TSGLD**G
AtrB	An	G**ALMG**S**SGAGKTTLL**D	**LSVEQR--K**RVT**IGVEL**VSK**P**SI**L**I**F**L**DE**P**TSGLD**G
BcatrB	Bc	G**ALMG**S**SGAGKTTLL**D	**LSVEQR--K**RLT**IGVEL**VSK**P**SI**L**I**F**L**DE**P**TSGLD**G
BMR1	Bc	T**ALMG**S**SGAGKTTLL**D	**LAVEQR--K**RVT**IGVEL**AAK**P**EL**LL**F**LDE**PT**SGLD**S
ABC1	Mgr	T**ALMG**V**SGAGKTTLL**D	**LNVEQR--K**RLT**IGVEL**AAK**P**PL**LL**F**V**DEPTSGLD**S
PMR1	Pd	T**ALMG**V**SGAGKTTLL**D	**LNVEQR--K**RLT**IGVEL**AAK**P**QL**LL**F**LDE**PT**SGLD**S
PDR5	Sc	T**ALMG**A**SGAGKTTLL**D	**LNVEQR--K**RLT**IGVEL**TAK**P**KL**LV**F**LDE**PT**SGLD**S

[a] Identical sequences in fungal PDR-like ABC transporters, except for MgAtr3, in bold
[b] Mg: *Mycosphaerella graminicola*, An: *Aspergillus nidulans*, Bc: *Botrytis cinerea*, Mgr: *Magnaporthe grisea*, Pd: *Penicillium digitatum*, Sc: *Saccharomyces cerevisiae*.

The NBDs of MgAtr3, MgAtr4, and MgAtr5 are characterised by the presence of an ATP binding cassette, containing the conserved amino acid sequences of the Walker A and Walker B motif (Walker *et al.,* 1982) and the ABC signature (Ames *et al.,* 1990). MgAtr4 and

MgAtr5 possess a conserved ABC motif at both the N- and C-terminal domain of the protein. In contrast, the Walker A motif in the N-terminal domain and the ABC signature in the C-terminal domain of MgAtr3, deviate significantly from the consensus sequence described for PDR-like ABC transporters (Table 3).

Genes in flanking regions of *MgAtr3, MgAtr4,* and *MgAtr5*

Flanking regions of *MgAtr3*, *MgAtr4*, and *MgAtr5* were (partially) sequenced to determine the presence of other genes. A gene encoding a trichodiene oxygenase related cytochrome P450 enzyme is located at 1.7 kb downstream of *MgAtr3*. This gene is homologous to other cytochrome P450 monooxygenase (*TRI4*) encoding genes, involved in the initial oxygenation step of trichothecene biosynthesis. BLAST results show highest homology to the *TRI4* genes from *Myrothecium roridum* (Acc. AF009417, 27% ident., 44% simil.), *Fusarium sporotrichioides* (Acc. AAB72032, 29% ident. 44% simil.), and *Neurospora crassa* (Acc. CAB99378, 27% ident. 41% simil.). Striking direct repeats (DR) are present between *MgAtr3* and the cytochrome P450 gene. They consist of three repeats of 30 nt (ATCGCACA*G*CA*G*TGACACCTCTGTAGGGAACG) and an additional imperfect fourth copy, which could function in the regulation of the putative *M. graminicola TRI4* gene. In the 5′ flanking region of *MgAtr3,* three genes were identified. They encode an epoxide hydrolase, an oxidoreductase, and a dioxygenase. All these enzymes could be involved in detoxification processes or breakdown routes. The epoxide hydrolase exhibits highest homology to a soluble human analogue (Acc. AF233336, 22% ident., 35% simil.), the oxidoreductase (partially sequenced) to a β-keto-acyl-reductase from *Pseudomonas aeruginosa* (Acc. AF052586, 27% ident., 42% simil.), and the dioxygenase (only partially cloned) to dioxygenase from *E. coli,* (Acc. P37610) which is involved in taurine catabolism.

In the 3′ flanking region of *MgAtr4,* a gene encoding a putative protein with highest homology to an abnormal spindle (*asp*) gene from *Drosophila melanogaster* (Acc. AE003749, 29% ident., 39% simil.), was identified. This gene encodes a microtubule binding protein putatively involved in microtubule-based processes in cells. No additional ORFs with significant homology to any gene in the GenBank were identified in the 5′ and 3′ flanking regions of *MgAtr5*.

Expression of *MgAtr3, MgAtr4,* and *MgAtr5* after treatment with different compounds

In northern analysis experiments no signal of *MgAtr3* could be detected neither in mycelium nor in yeast-like cells, treated with a range of compounds as listed in materials and methods (data not shown). However, spliced messenger RNA (mRNA) of *MgAtr3* was obtained in 5′ RACE experiments using RT-PCR, indicating that low levels of transcripts, undetected in the northern blot analysis experiments, are present. RT-PCR analysis also revealed that the population of *MgAtr3* transcripts was not properly processed. The pool of *MgAtr3* mRNA, still contained transcripts with unspliced sequences from intron 1 and/or intron 2 (data not shown).

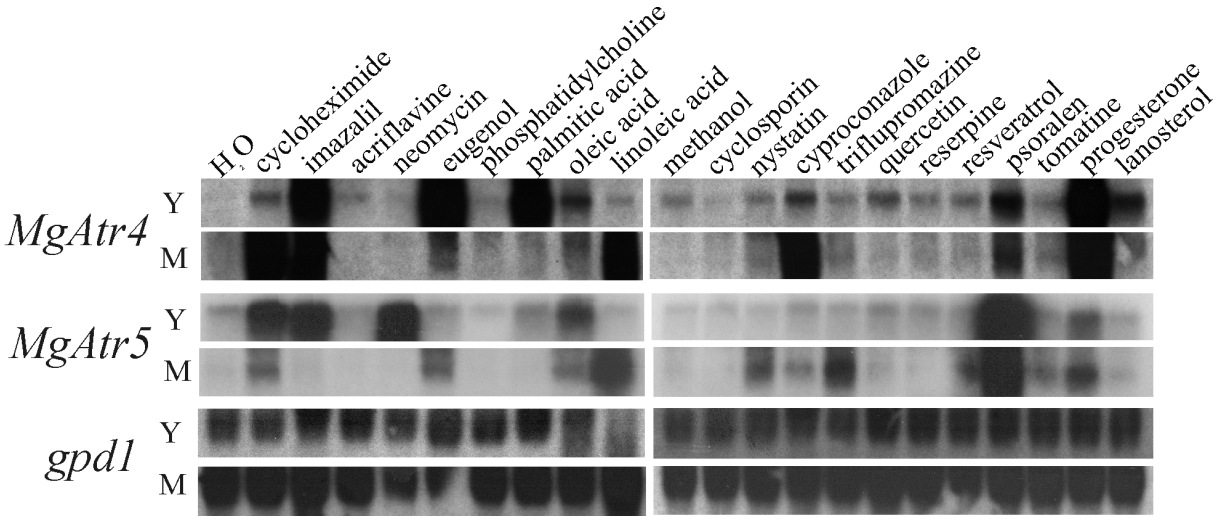


Figure 3. Northern blot analysis of *MgAtr4, MgAtr5,* and *gpd1* expression in yeast-like cells (Y) and mycelium (M) of *Mycosphaerella graminicola* after treatment with test compounds.

A low basal level of *MgAtr4* expression was observed in untreated yeast-like cells and mycelium of the fungus. Transcripts of *MgAtr5* were also detected in these samples, but signals were weaker than for *MgAtr4*. Treatment of the fungus with a range of compounds strongly induced transcription of both *MgAtr4* and *MgAtr5* (Figure 3). However, the induction of both genes depended significantly on the morphological state of this dimorphic fungus. For example, treatment with psoralen and progesterone resulted in increased transcript levels of *MgAtr4* and *MgAtr5* in both yeast-like cells and mycelium, while treatment with neomycin only induced *MgAtr5* expression in yeast-like cells. Linoleic acid strongly induced expression of *MgAtr4* in mycelium but hardly any induction was noticed in yeast-like cells. Imazalil induced an increase in transcript levels of *MgAtr4* in both morphological states of the fungus but only induced transcripts of *MgAtr5* in yeast-like cells.

DISCUSSION

A PCR-based approach using degenerate primers directed against conserved amino acid sequences in the NBDs of ABC transporters from the PDR and MRP class of *S. cerevisiae*, resulted in the cloning of three new ABC transporter genes (*MgAtr3, MgAtr4,* and *MgAtr5*) from the plant pathogenic fungus *M. graminicola*. The approach also identified the ABC transporter genes *MgAtr1* and *MgAtr2* previously reported by Zwiers and De Waard (2000). Based on their predicted topology, ABC transporters from *S. cerevisiae* can be divided into six classes (Taglicht and Michaelis, 1998). The proteins encoded by *MgAtr3, MgAtr4,* and *MgAtr5* all exhibit the same $[NBD\text{-}TMS_6]_2$ topology, which classifies them as putative members of the PDR class of ABC transporters. ABC transporter genes from other classes were not detected. Primers designed on ABC domains of the MRP class of yeast ABC proteins, resulted in the amplification of only unspecific products. Combinations of primers derived from the two classes of ABC transporters gave the same outcome. Hence, the results indicate that the homology of ABC proteins in the MRP class is probably lower than that of the PDR class or that the primers used were too degenerate to allow efficient amplification of specific products of the MRP class.

ABC transporter proteins are characterised by the presence of highly conserved amino acid sequences in their NBDs. Consensus sequences comprise 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 (Walker *et al.,* 1982), and the ABC signature [L-S-G-G-(X)3-R-hydrophobic-X-hydrophobic-A] (Ames *et al.,* 1990). Members of the PDR class of ABC proteins differ from the general consensus sequence of other ABC transporters by the presence of a cysteine residue instead of a lysine downstream the third conserved glycine residue (VLGxPGxGC) in the Walker A motif of the N-terminal domain of the protein. The N-terminal ABC signature of the PDR class (SGGERKR) of ABC transporters also differs from the other five predicted classes of the yeast ABC proteins. In addition, the SXGQ/E motif, present in ABC signature of the C-terminal domain of most ABC transporters is replaced by the LXVEQ motif in fungal PDR-like sequences (Decottignies and Goffeau, 1997). MgAtr4 and MgAtr5 match these consensus sequences for members of the PDR class. In contrast, particularly the N-terminal Walker A motif and the C-terminal ABC signature of MgAtr3 significantly deviate from the above patterns (Table 3). These domains are important for ATPase activity of the transporter and

introduced mutations over these regions usually result in an altered protein function (Loo and Clarke, 1995). Therefore, MgAtr3 may be an exceptional member of the PDR-like class of ABC transporters with different substrate specificity. This conclusion is strengthened by the fact that no circumstances could be found that induce expression of this gene.

The physiological function of the ABC proteins encoded by *MgAtr3, MgAtr4,* and *MgAtr5* is not yet known. However, members of the PDR class of ABC transporters with high homology to MgAtr4 include atrA from *Aspergillus nidulans*, PMR1 from *Penicillium digitatum,* ABC1 from *M. grisea* (Figure 1). PMR1 is associated with resistance to sterol demethylation inhibitors (DMIs) in *P. digitatum* (Nakaune *et al.,* 1998) while ABC1 is important for pathogenicity of *M. grisea* on rice (Urban *et al.,* 1999). MgAtr5 shows high homology with the multidrug transporters atrB and BcatrB from *A. nidulans* and *Botrytis cinerea,* respectively. AtrB is known to be involved in the transport of compounds such as camptothecin, fludioxonil, and rhodamine 6G (Andrade *et al.,* 2000). Substrates of BcatrB include the phenylpyrrole fungicide fenpiclonil and the grapevine phytoalexin resveratrol (Schoonbeek *et al.,* 2001). Other well characterised fungal or yeast ABC proteins of the PDR class include, Pdr5p, Snq2p, and Pdr12p from *S. cerevisiae* (Bissinger and Kuchler, 1994), CDR1 (Prasad *et al.,* 1995) and CDR2 (Sanglard *et al.,* 1997), from *Candida albicans* and others. Most of these transporters function in multidrug or pleiotropic drug resistance (MDR or PDR). The high level of homology displayed by MgAtr4 and MgAtr5 with the proteins mentioned above might imply that these transporters can have similar substrate profiles. The observed expression profile of the genes after treatment with a range of compounds also points in this direction. Hence, MgAtr4 and MgAtr5 may play a role in providing protection of *M. graminicola* against a wide range of natural toxic compounds present in its host plants and against fungicides used in disease control.

Expression studies in the wild type isolate IPO323 of *M. graminicola*, revealed that both *MgAtr4* and *MgAtr5* exhibit a relatively low basal level of expression, although somewhat higher for *MgAtr4* than for *MgAtr5*. *MgAtr4* also exhibited higher levels of basal expression when compared with *MgAtr2*, a previously cloned ABC transporter from the same pathogen (Zwiers and De Waard, 2000). The constitutive expression of *MgAtr4* may reflect an intrinsic metabolic activity of the deduced protein. No basal level of expression was detected for *MgAtr3* in northern blot experiments. Expression of *MgAtr3* was also not observed after treatment of the fungus with a range of chemically different compounds. However, the

presence of partially spliced transcripts obtained by RT-PCR analysis exclude the possibility that *MgAtr3* is a pseudogene. Partial splicing of transcripts has also been observed for *MgAtr2* (Zwiers and De Waard, 2000). This mechanism has been implicated as a mean of cells to regulate expression of genes and the specificity of their encoded proteins (Birch *et al.,* 1995; Fossa *et al.,* 1995).

Treatment of *M. graminicola* with various compounds increased transcription of *MgAtr4* and *MgAtr5*. In general, the two genes display distinct expression profiles for some compounds but overlapping expression profiles for others. Such variations suggest that the genes are probably under different regulatory control and may also reflect the ability of the encoded proteins to transport similar but also different compounds. This is also supported by the observations that different transcriptional regulatory elements are found in the promoter region of each gene. Expression of *MgAtr4* and *MgAtr5* was upregulated by either synthetic fungitoxic compounds, such as imazalil and cyproconazole or natural occurring compounds, such as the plant secondary metabolites eugenol and psoralen, and the antibiotics cycloheximide and neomycin. These findings also support the hypothesis that the encoded ABC transporters provide protection against toxic compounds present in the environment of the fungus.

The expression profile of *MgAtr4* and *MgAtr5* differs significantly in yeast-like cells and mycelium, the two dimorphic forms of *M. graminicola*. This could be the result of differences in cell membrane or cell wall composition of the two morphological stages. Changes in lipid, sugar, and protein composition of cell membranes leads to an altered solubility profile and, consequently, influence the perception of compounds by cells (Pallares-Trujillo *et al.,* 2000; Ouar *et al.,* 1999). Moreover, it has been shown that differences in the membrane environment or membrane composition can significantly affect substrate specificity and ATPase activity of ABC transporters. Sinicrope *et al.,* (1992) demonstrated that alterations in membrane lipid fluidity of canalicular membrane vesicles modulate the ABC transporter-mediated accumulation of MDR-type drugs. Moreover, a reduced ability of Pdr5p to confer resistance to different drugs was observed in *S. cerevisiae* mutants, in which genes involved in the ergosterol biosynthesis pathway had been disrupted (Kaur and Bachhawat, 1999).

Recently, an *Agrobacterium tumefaciens* mediated transformation method has been developed for *M. graminicola* (Zwiers and De Waard, 2001) to disrupt or replace genes by homologous recombination events. In future work, this technology will be used to generate

knockout mutants of *MgAtr3, MgAtr4,* and *MgAtr5* in order to elucidate their function in drug resistance and virulence.

ACKNOWLEDGEMENTS

We kindly acknowledge Dr G.H.J. Kema, E.C.P. Verstappen, and Dr C. Waalwijk (Plant Research International, Wageningen, The Netherlands) for providing the IPO323 isolate of *Mycosphaerella graminicola* and for helpful suggestions and discussions during this work within the Wageningen Mycosphaerella group. We also acknowledge Dr L-H Zwiers and Prof. Dr P.J.G.M. De Wit for critical reading of the manuscript. I. Stergiopoulos was financially supported by the Training and Mobility of Researchers (TMR) Programme – Marie Curie Research Training Grants, The European Commission. (Contract No. ERBFMBICT983558). M.M.C. Gielkens, S.D. Goodall, and K. Venema were financially supported by the EU-BIOTECH 2 programme (Reference No. BIO4CT960352).

REFERENCES

Altschul SF, Madden TL, Schaffer AA, Zhang J, Zhang Z, Miller W and Lipman DJ (1997) Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. Nucleic Acids Res **25:** 3389-3402

Ames GF-L, Mimura C and Shyamala V (1990) Bacterial periplasmic permeases belong to a family of transport proteins operating from Escherichia coli to human traffic ATPases. FEMS Microbiol Rev **75:** 429-446

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

Birch PR, Sims PF and Broda P (1995) Substrate-dependent differential splicing of introns in the regions encoding the cellulose binding domains of two exocellobiohydrolase I-like genes in *Phanerochaete chrysosporium*. Appl Environ Microbiol **61**: 3741-3744.

Bissinger PH and Kuchler K (1994) Molecular cloning and expression of the *Saccharomycescerevisiae* Sts1 gene product - A yeast ABC transporter conferring mycotoxin resistance. J Biol Chem **269:** 4180-4186

De Waard MA (1997) Significance of ABC transporters in fungicide sensitivity and resistance. Pestic Sci **51**: 271-275.

Decottignies A and Goffeau A (1997) Complete inventory of the yeast ABC proteins. Nat Genet **15:** 137-145

Del Sorbo G, Schoonbeek H and De Waard MA (2000) Fungal transporters involved in efflux of natural toxic compounds and fungicides. Fun Genet Biol **30:** 1-15

Eyal Z, Scharen AL, Prescott JM and Van Ginkel M (1987) The Septoria diseases of wheat: Concepts and methods of disease managment CIMMYT, Mexico, pp. 51

Fossa A, Beyer A, Pfitzner E, Wenzel B and Kunau WH (1995) Molecular cloning, sequencing and sequence analysis of the *fox*-2 gene of *Neurospora crassa* encoding the multifunctional beta-oxidation protein. Mol Gen Genet **247**: 95-104

Higgins CF (1992) ABC transporters: from microorganisms to man. Annu Rev Cell Biol **8:** 67-113

Juliano RL and Ling V (1976) A surface glycoprotein modulating drug permeability in Chinese hamster ovary cell mutants. Biochim Biophys Acta **455:** 152-162

Kaur R and Bachhawat AK (1999) The yeast multidrug resistance pump, Pdr5p, confers reduced drug resistance in erg mutants of *Saccharomyces cerevisiae*. Microbiology **145:** 809-818

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

Kema GHJ and Van Silfhout CH (1997) Genetic variation for virulence and resistance in the wheat-*Mycosphaerella graminicola* pathosystem. III. Comparative seedling and adult plant experiments. Phytopathology **87**: 266-272

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. J Biol Chem **270**: 22957-22961

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*. Appl Environ Microbiol **64:** 3983-3988

Ouar Z, Lacave R, Bens M and Vandewalle A (1999) Mechanisms of altered sequestration and efflux of chemotherapeutic drugs by multidrug-resistant cells. Cell Biol Toxicol **15**: 91-100

Pallares-Trujillo J, Lopez-Soriano FJ and Argiles JM (2000) Lipids: A key role in multidrug resistance? Int J Oncol **16:** 783-98.

Prasad R, De Wergifosse P, Goffeau A and Balzi E (1995) Molecular cloning and characterization of a novel gene of *Candida albicans*, CDR1, conferring multiple resistance to drugs and antifungals. Curr Genet **27:** 320-9

Raeder U and Broda P (1985) Rapid preparation of DNA from filamentous fungi. Lett Appl Microbiol **1:** 17-20

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

Schoonbeek W, 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. Mol Plant-Microbe In **14:** 562-571

Sinicrope FA, Dudeja PK, Bissonnette BM, Safa AR and Brasitus TA (1992) Modulation of P-glycoprotein-mediated drug transport by alterations in lipid fluidity of rat liver canalicular membrane vesicles. J Biol Chem **267:** 24995-5002

Taglicht D and Michaelis S (1998) *Saccharomyces cerevisiae* ABC proteins and their relevance to human health and disease. Method Enzymol **29:** 130-162

Unkles SE (1992) Gene organization in industrial filamentous fungi. *In* Applied molecular genetics of filamentous fungi (Eds) Kinghorn JR and Turner G, Blackie Academic & Proffessional, Glasgow, pp. 28-53

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

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. EMBO J **1:** 945-951

Weibull J and Niemeyer HM (1995) Changes in dihydroxymethoxybenzoxazinone glycoside content in wheat plants infected by three plant pathogenic fungi. Physiol Mol Plant Pathol **47:** 201-212

Zwiers L-H and De Waard MA (2000) Characterization of the ABC transporter genes *MgAtr1* and *MgAtr2* from the wheat pathogen *Mycosphaerella graminicola*. Fun Genet Biol **30:** 115-125

Zwiers L-H and De Waard MA (2001) Efficient *Agrobacterium tumefaciens*-mediated gene disruption in the phytopathogen *Mycosphaerella graminicola*. Curr Genet **39:** 388-393.

CHAPTER 4

ABC TRANSPORTERS OF THE WHEAT PATHOGEN *MYCOSPHAERELLA GRAMINICOLA* FUNCTION AS PROTECTANTS AGAINST BIOTIC AND XENOBIOTIC TOXIC COMPOUNDS

Lute-Harm Zwiers, Ioannis Stergiopoulos, Marco M.C. Gielkens, Stephen D. Goodall, and Maarten A. De Waard

Submitted

ABSTRACT

The role in multidrug resistance (MDR) of five ABC transporter genes (*MgAtr1-MgAtr5*) from the wheat pathogen *Mycosphaerella graminicola* was studied. This was done by complementation of *Saccharomyces cerevisiae* with the *M. graminicola* ABC transporter genes and by analysis of ABC transporter knockout mutants of *M. graminicola* with respect to sensitivity to natural toxic compounds, xenobiotics, and antagonistic bacteria. The *S. cerevisiae* complementation experiments showed that all ABC transporter genes tested encode proteins that provide protection against chemically unrelated compounds, indicating that they function as multidrug transporters with distinct but overlapping substrate specificity. Their substrate range in yeast includes fungicides, plant metabolites, antibiotics, and a mycotoxin from *Fusarium graminearum* (diacetoxyscirpenol). Transformants of *M. graminicola* with disrupted or deleted ABC transporter genes did not exhibit clear phenotypes, probably due to the redundancy of transporters with an overlap in substrate specificity. *MgAtr5* deletion mutants of *M. graminicola* showed an increase in sensitivity to the putative wheat defence compound resorcinol and to the grape phytoalexin resveratrol, suggesting a role for this transporter in protection against plant defence compounds. Bioassays with antagonistic bacteria indicated that MgAtr2 provides protection against phenylpyrrole antibiotics produced by *Pseudomonas fluorescens* and *Burkholderia cepacia*. In summary, the results show that ABC transporters from *M. graminicola* play a role in protection against toxic compounds from natural and artificial origin.

Keywords: ATP-binding (ABC) cassette transporters; antibiotic; *Mycosphaerella graminicola*; multidrug resistance;

INTRODUCTION

ATP-binding cassette (ABC) transporters or traffic ATPases are membrane-bound proteins present in both prokaryotic and eukaryotic organisms that drive the uptake or efflux of compounds by hydrolysis of ATP (Higgins, 2001). ABC transporters are able to prevent intracellular accumulation of toxic compounds by extrusion and thus, provide cells with protection against deleterious effects of toxicants. ABC transporters can also be involved in the establishment of resistance in drug-based treatment of infectious diseases, such as candidiasis and malaria, and in chemotherapy of cancers (Foote *et al.*, 1989; Sanglard *et al.*, 1995). This may result in the simultaneous development of resistance to a wide range of chemically unrelated compounds, a phenomenon described as multidrug resistance (MDR). MDR can be caused by overproduction of ABC transporters and has also been described for the yeasts *Candida albicans*, *Saccharomyces cerevisiae* and *Schizosaccharomyces pombe* (Nishi *et al.*, 1992; Kolaczkowski *et al.*, 1998). The development of MDR also threatens the successful control of filamentous fungi that are pathogenic to plants and mammals with fungicides and antimycotics. ABC transporters from *Aspergillus nidulans*, *Aspergillus fumigatus*, *Aspergillus flavus*, *Botrytis cinerea*, *Penicillium digitatum* and *Mycosphaerella graminicola* have been implicated in resistance against antifungal compounds (Stergiopoulos *et al.*, 2002c).

Besides acting as a general detoxification mechanism against hydrophobic toxicants, ABC transporters can be involved in more specific functions. For instance, the fungal ABC transporters STE6 and mam1 from *S. cerevisiae* and *S. pombe*, respectively, are mating peptide transporters (Christensen *et al.,* 1997; McGrath and Varshavsky, 1989). Another proposed function is the maintenance of membrane integrity by transport of membrane constituents to the outer leaflet of the lipid bilayer and extrusion of hydrophobic compounds that might disturb the membrane (Gottesman and Pastan, 1993; Mahe *et al.,* 1996). In plant pathogenic fungi, ABC transporters have been implicated to act as virulence factors by providing protection against plant defence compounds or by secretion of pathogenicity factors (De Waard, 1997; Del Sorbo *et al.,* 2000). These functions have been demonstrated for *Magnaporte grisea* (Urban *et al.,* 1999)*, B. cinerea* (Schoonbeek *et al.,* 2001), *Gibberella pulicaris* (Fleissner *et al.,* 2002) and *M. graminicola* (Stergiopoulos *et al.,* 2002b).

We are interested in the role of ABC transporters in the wheat-*M. graminicola* interaction. *M. graminicola*, the causal agent of septoria tritici blotch of wheat, is a major pathogen on this crop. Despite its economic importance not much is known about the molecular mechanisms underlying this disease. In this paper we describe the functional complementation of *S. cerevisiae* mutants with the ABC transporter genes *MgAtr1-MgAtr5* from *M. graminicola* in order to identify potential natural substrates of these transporters. Furthermore, the sensitivity of individual *M. graminicola* knockout mutants to natural toxic compounds, xenobiotics, and antibiotic producing bacteria was tested.

MATERIALS AND METHODS

Fungal material and culture

M. graminicola IPO323 was used as standard isolate (Kema and Van Silfhout, 1997). Yeast-like cells were either grown in liquid yeast-sucrose medium (YSM; yeast extract 10 g l^{-1}, sucrose 10 g l^{-1}) at 18°C and 140 rpm, on solid V8 plates (50% V8 vegetable juice, 50% water, 2.5% agar) at 18°C, or on potato dextrose agar (PDA; 39 g l^{-1}) (Merck) at 18°C.

Complementation of *S. cerevisiae* was performed using strain AD12345678 (Δ*yor1*, Δ*snq2*, Δ*pdr5*, Δ*pdr10*, Δ*pdr11*, Δ*ycf1*, Δ*pdr3*, Δ*pdr15, Δura3*) (Decottignies *et al.,* 1998)

Molecular biological techniques

Basic DNA and RNA manipulations were performed according to standard procedures (Sambrook *et al.,* 1989). *Escherichia coli* strain DH5α was used for propagation of constructs. Fungal genomic DNA was isolated from freeze-dried material using DNAzol™ reagent (Life-Technologies). Induction experiments were performed as described previously (Zwiers and De Waard, 2000). Total RNA of *M. graminicola* was isolated using the TRIzol® reagent (Life Technologies). RNA (10 µg) was separated on a 1.2% agarose gel containing glyoxal and transferred to Hybond-N membranes (Amersham). Equal loading and transfer of RNA was determined by staining northern blots with methylene-blue followed by hybridisation with the 18S rRNA subunit of *Aspergillus niger* (Melchers *et al.,* 1994). Hybridisation was performed at 65°C in Nasmyth's hybridisation solution (1.1 M NaCl, 0.3 M Na_2HPO_4, 0.011 M Na_2EDTA, 1.85% sarcosyl, 18.5% dextran sulphate, pH 6.2, 100 µg denatured herring sperm DNA ml^{-1}). Probes used in northern analysis were an 840 bp *Eco*RI fragment from *MgAtr1*, a 750 bp *Sal*I fragment from *MgAtr2*, an 800 bp *Sal*I fragment from *MgAtr3*, an 480 bp *Bam*HI/ *Pst*I fragment from *MgAtr4* and a 600 bp *Eco*RI fragment from *MgAtr5* (Figure 1).

Full length cDNA clones of *MgAtr1*, *MgAtr2*, and *MgAtr5* were made from poly A^+ RNA isolated from yeast-like cells of *M. graminicola*. Amplification of full-length cDNA was performed with the Advantage Klentaq polymerase mix (Clontech) according to the manufacturer's instructions. cDNA clones were cloned in the yeast

expression vector pYes2 (Invitrogen) and transformed to *S. cerevisiae* strain AD12345678. As *MgAtr4* does not contain any introns a full-length genomic fragment was cloned in pYes2. Yeast transformants containing the empty vector pYes2 were used as controls.

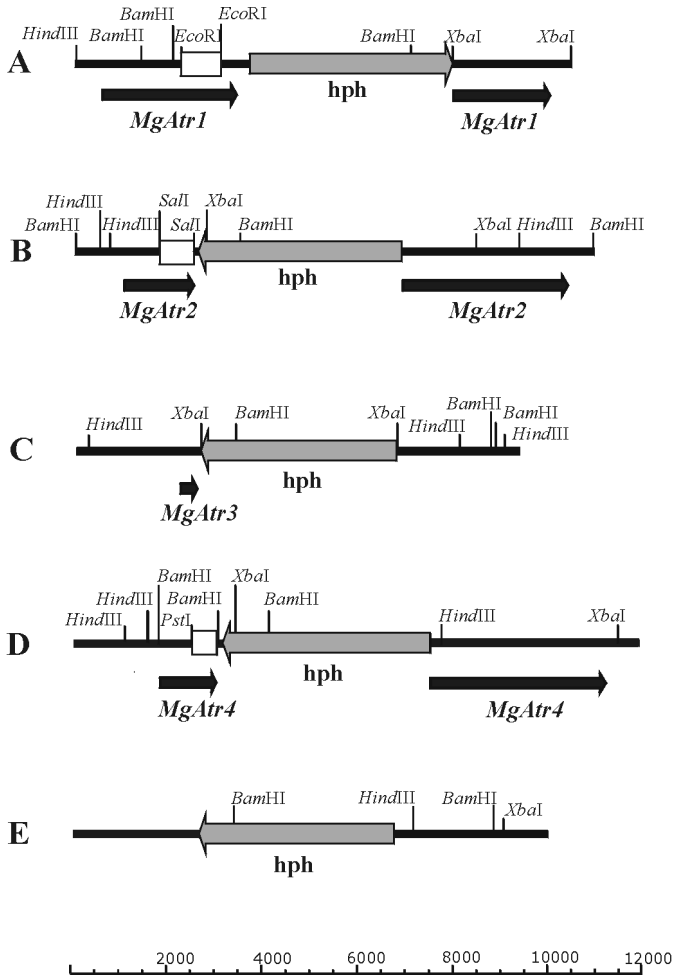


Figure 1. Schematic representation of *MgAtr1-MgAtr5* loci in *Mycosphaerella graminicola* transformants. Knockout mutants of *MgAtr1* (A), *MgAtr2* (B), and *MgAtr4* (D) were generated by gene disruption and of *MgAtr3* (C) and *MgAtr5* (E) by gene replacement. Remaining parts of *MgAtr1-MgAtr5* coding regions are indicated with black arrows. Fragments used as probe in analysis of disruptants are indicated as open boxes. All genes were disrupted or replaced by the hygromycin resistance cassette (hph) which is indicated by a grey arrow.

Plasmid construction

Disruption constructs of *MgAtr1* and *MgAtr4* were made by means of the GPS™ Mutagenesis system (New England Biolabs) using the approach previously described for the construction of a *MgAtr2* disruption construct (Zwiers and De Waard, 2001).

Target plasmid pCGNAtr1 was constructed by the insertion of a 5.9 kb *Hind*III/*Xba*I fragment containing the entire *MgAtr1* into pCGN1589 digested with *Hind*III/*Xba*I. The target plasmid pCGNAtr4 was generated by the ligation of a 7.5 kb *Kpn*I/*Spe*I insert in pCGN1589 digested with *Kpn*I/*Xba*I. Both target plasmids pCGNAtr1 and pCGNAtr4 were used in a transposition reaction together with the transprimer donor vector pGPS3Hyg. Screening and analysis of plasmids containing an insertion was performed as described before (Zwiers and De

Waard, 2001). The disruption constructs pCGNΔAtr1 with 3.5 and 2.4 kb of flanking homologous DNA and pCGNΔAtr4 with 3.1 and 4.4 kb of flanking homologous DNA were subsequently transformed into *A. tumefaciens* LBA1100 by electroporation.

The plasmid used for the targeted replacement of *MgAtr3* was constructed in such a way that a 4.4 kb *Sma*I/*Not*I fragment containing almost the entire ORF of *MgAtr3* was replaced by a 4.1 kb fragment containing the hygromycin-resistance cassette. The final replacement construct pBΔAtr3 contained 2.6 kb of the 5' flanking region and 2.5 kb of the 3' flanking region. Prior to transformation the construct was digested with *Apa*I/*Not*I and the 9.2 kb fragment was used for the transformation.

The construct used to disrupt *MgAtr5* was generated by amplifying a 2.4 kb region immediately upstream of the *MgAtr5* ORF and a 3.2 kb region immediately downstream of the *MgAtr5* ORF. These PCR products were cloned to either side of the hygromycin B resistance cassette in pBluescript (Stratagene) such that the entire construct could be excised with a *Apa*I digestion prior to transformation.

***M. graminicola* transformation**

A. tumefaciens-mediated transformation was used to disrupt *MgAtr1* and *MgAtr4* in IPO323 as described for *MgAtr2* (Zwiers and De Waard, 2001). *MgAtr3* and *MgAtr5* replacement mutants were generated according to the method of Payne *et al.,* (1998) with the following exceptions. Instead of dimethylsulfoxide (DMSO), 28 µl of PTC solution (40% (w/v) PEG3350, 50 mM Tris-HCl, pH 8.0, 50 mM $CaCl_2$) was added with 5 µl of linearized DNA (1 µg μl^{-1}).

Sensitivity assays

Minimum inhibitory concentrations (MICs) of fungitoxic compounds were determined for yeast-like growing cells of *M. graminicola* on agar plates. Toxicity tests were performed by spotting cells (5 µl at a density of 4 x 10^5 cells ml^{-1}), harvested from 3-day-old liquid medium, on 9-ml Petri dishes containing PDA amended with different concentrations of toxicants. Compounds tested are indicated in Table 1. Experiments were performed in duplicate and repeated at least three times. MIC values were assessed after 10 days of incubation at 18°C in the dark.

Sensitivity assays of *S. cerevisiae* were performed on solid synthetic media containing bacto-yeast nitrogen base without amino acids (Difco; 6.7 g l^{-1}), drop-out mix (2 g l^{-1} containing amino acids without uracil), galactose (20 g l^{-1}), noble agar (Difco; 20 g l^{-1}), and toxicants at different concentrations. Cultures of *S. cerevisiae* grown in liquid synthetic medium were grown overnight at 30°C. The overnight culture was diluted to an optical density of 0.5 at 600 nm (OD_{600}), and subsequently 5 µl were spotted on plates. Drug sensitivity was scored visually after incubation for 3 days at 30°C in the dark.

Chemicals

Chemicals used in toxicity assays were; carbendazim (DuPont De Nemours), cyproconazole, diacetoxyscirpenol (DAS), fludioxonil, and propiconazole (Syngenta), fluazinam (ISK Biosciences), gramine (Fluka), imazalil nitrate, and miconazole (Janssen Pharmaceutica), kresoxim-methyl (BASF), tebuconazole (Bayer AG), thiram (AAgrunol), amphotericin B, berberine, camptothecin, cholesterol, colchicine, corticosterone, cycloheximide,

ergocryptine, ergosterol, estradiol, eugenol, hygromycin, lanosterol, linoleic acid, neomycin, 4-nitroquinoline-N-oxide, oligomycin, phosphatidyl-choline, progesterone, psoralen, quercetine, reserpine, resorcinol, resveratrol, rhodamine 6G, sitosterol, stigmasterol, testosterone, tomatine, triflupromazine, vinblastine, and vincamine (Sigma-Aldrich).

Table 1. Compounds used in toxicity assays with *Saccharomyces cerevisiae* and *Mycosphaerella graminicola*.

Antibiotics[a]	**Fungicides**	**Plant metabolites**	**Sterols / Fatty acids**	**Miscellaneous**
Amphotericin B	**Carbendazim**	**Berberine**	Cholesterol	**Diacetoxyscirpenol**
Cycloheximide	**Cyproconazole**	**Camptothecin**	Corticosterone	Ergocryptine
Hygromycin	**Fluazinam**	Colchicine	**Ergosterol**	**4-NQO**
Neomycin	**Fludioxonil**	**Eugenol**	Estradiol	**Rhodamine 6G**
Oligomycin	**Imazalil**	**Gramine**	**Lanosterol**	Triflupromazine
	Kresoxim-methyl	Pisatin	Linoleic acid	
	Miconazole	**Psoralen**	Phosphatidylcholine	
	Propiconazole	Quercetine	**Progesterone**	
	Tebuconazole	**Reserpine**	Sitosterol	
	Thiram	**Resorcinol**	Stigmasterol	
		Resveratrol	Testosterone	
		Tomatine		
		Vinblastine		
		Vincamine		

[a] All compounds were tested against *S. cerevisiae*, compounds in bold only against *M. graminicola*

Agar diffusion tests

The antagonistic activity of *Pseudomonas fluorescens* Pf5 and *Burkholderia cepacia* B37W on growth of *M. graminicola* was examined in agar diffusion tests. Bacteria were cultured on King's medium B (King *et al.,* 1954) (KMB) at 25°C in the dark. A single colony was transferred from KMB-agar to 3 ml KMB-broth and incubated in an orbital shaker at 180 rpm and 28°C for 16 h. Bacterial suspensions were mixed with 1/3 PDA (13 g l^{-1}) of 42°C, poured in Petri-dishes (14 cm diameter, 50 ml), and subsequently incubated at 25°C in the dark for 10 days. *M. graminicola* yeast-like cells (5 x 10^8) were mixed with 50 ml ½ PDA (19.5 g l^{-1}) of 42°C and poured in Petri-dishes (14 cm diameter). Agar plugs (13 mm diameter) were transferred from the plates containing the bacteria and placed on the plates seeded with fungal cells. Plates were subsequently incubated at 18°C in the dark and fungal inhibition zones were measured after 1 week.

RESULTS

Complementation of *S. cerevisiae*

To identify potential substrates of ABC transporters from *M. graminicola*, *S. cerevisiae* was complemented with *MgAtr1* (AJ243112), *MgAtr2* (AJ243113), *MgAtr4* (AF329852), and *MgAtr5* (AF364104). Full-length cDNA clones were constructed and cloned in the yeast

expression vector pYes2. The generation of a full-length cDNA from *MgAtr3* (AF364105) was not successful. Clones were transformed to the *S. cerevisiae* strain AD12345678, in which several ABC transporter genes have been deleted (Decottignies *et al.,* 1998). The sensitivity of the yeast transformants was tested against forty-five compounds of either natural or synthetic origin (Table 1). Most compounds tested were non-toxic to *S. cerevisiae* or displayed no difference in toxicity between the control recipient strains and the ABC transporter gene-expressing yeast transformants. However, ten compounds showed a differential activity to some of the transformants tested (Table 2). These compounds included fungicides, plant metabolites, the antibiotic cycloheximide, and the fungal mycotoxin diacetoxyscirpenol (DAS). All ABC transporters were capable of providing protection against some compounds. MgAtr1 had the broadest substrate range whereas the substrate range of MgAtr5 was limited to only a few of the differentiating compounds.

Table 2. Compounds with differential activity to *Saccharomyces cerevisiae* AD12345678 expressing the *Mycosphaerella graminicola* ABC transporters MgAtr1, MgAtr2, MgAtr4, or MgAtr5.

	MgAtr1	**MgAtr2**	**MgAtr4**	**MgAtr5**
Fungicides				
Cyproconazole	+[a]	+	+	-[b]
Propiconazole	+	+	+	-
Tebuconazole	+	+	+	-
Sterols/fatty acids				
Ergosterol	+	+	+	-
Progesterone	+	+	+	-
Plant metabolites				
Berberine	+	-	-	+
Camptothecin	+	-	-	+
Antibiotics				
Cycloheximide	+	-	-	-
Other				
Diacetoxyscirpenol	+	-	+	-
Rhodamine 6G	-	+	+	-

[a] Increase in resistance as compared to pYes2 transformed control strain;
[b] No difference in sensitivity as compared to pYes2 transformed control strain;

Decreased sensitivity to cycloheximide was only provided by MgAtr1. In contrast, protection against the other compounds listed could be provided by at least two ABC transporters. However, the transporters did differ in their capacity to provide protection as reflected by differences in resistance levels between the transformants. For instance, MgAtr1, MgAtr2, and MgAtr4 were all capable of providing protection against azole fungicides, but

yeast transformed with *MgAtr1* and *MgAtr4* survived higher concentrations of the azole fungicide cyproconazole as compared to yeast transformed with *MgAtr2.* The same phenomenon was observed with the mycotoxin DAS. The presence of both MgAtr1 and MgAtr4 increased resistance to this compound, but resistance was highest in yeast transformants containing MgAtr1. As an example of these distinct but overlapping effects of MgAtr1, MgAtr2, MgAtr4, and MgAtr5 on the sensitivity of *S. cerevisiae* to toxicants, the activities of the plant metabolite camptothecin, the antibiotic cycloheximide, the azole fungicide cyproconazole, and the mycotoxin DAS are shown in Figure 2.

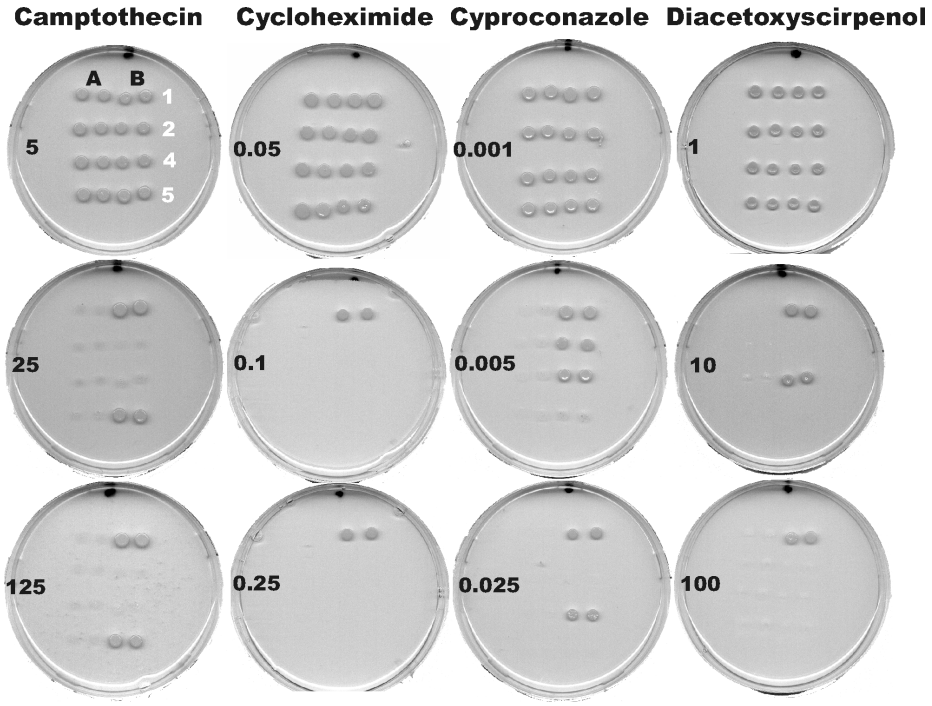


Figure 2. Effect of heterologous expression of *MgAtr1, MgAtr2, MgAtr4,* or *MgAtr5* from *Mycosphaerella graminicola* in *Saccharomyces cerevisiae* AD12345678 on sensitivity to camptothecin, cycloheximide, cyproconazole, or diacetoxyscirpenol. Each row (1, 2, 4, 5) displays two independent *S. cerevisiae* control transformants with the empty vector pYes2 (A) and two independent *S. cerevisiae* transformants (B) containing the full length clone of *MgAtr1* (1), *MgAtr2* (2), *MgAtr4* (4), or *MgAtr5* (5). Concentrations of compounds in the agar are indicated in mg l^{-1}.

Disruption and replacement in *M. graminicola*

Five ABC transporter genes of *M. graminicola* were subjected to either disruption or replacement. The replacement of *MgAtr3* and *MgAtr5* was performed through protoplast-PEG-mediated transformation. However, replacement through this procedure was not very efficient. Only two out of eighty-six (2.3%) *MgAtr3* transformants lacked the *MgAtr3* coding sequence and did not contain ectopically integrated copies. Similar results were obtained for

MgAtr5 where in two out of thirty-six transformants (5.6%) the coding region was replaced. Therefore, disruption of *MgAtr1*, *MgAtr2*, and *MgAtr4* was performed using *Agrobacterium tumefaciens*-mediated transformation. Analysis of eight putative *MgAtr1* disruptants revealed that two transformants were disruptants containing a single copy of the transforming DNA. This efficiency is in the same order as described for the *A. tumefaciens*-mediated disruption of *MgAtr2* (Zwiers and De Waard, 2001). The disruption of *MgAtr4* was even more effective as nine out of twelve (75%) transformants analysed were disruptants containing a single copy of the introduced DNA.

For all genes two independent knockout mutants were used for phenotypical characterisation. A transformant containing a single copy of the hygromycin-resistance cassette was used as a control. Southern blot analysis showed that all knockout mutants used contained a single copy of the transforming DNA (data not shown). Schematic representations of the *MgAtr1-MgAtr5* loci in the *M. graminicola* knockout mutants are shown in Figure 1. None of the transformants showed an altered growth rate in yeast sucrose medium (YSM) as compared to the wild-type recipient isolate IPO323 or the control transformant Sp2. Under the conditions tested, no morphological differences could be observed.

Expression analysis

The expression of all five ABC transporter genes was analysed in the *M. graminicola* knockout mutants and the recipient wild-type isolate. RNA was isolated from cells either untreated or treated with the azole fungicide cyproconazole, the antibiotic cycloheximide, the steroid hormone progesterone, or the plant secondary metabolite psoralen, all known inducers of one or more *M. graminicola* ABC transporter genes (Stergiopoulos *et al.,* 2002a; Zwiers and De Waard, 2000). Northern blot analyses showed that *MgAtr5* expression in the *MgAtr5* deletion mutants was completely abolished whereas the *MgAtr1*, *MgAtr2*, and *MgAtr4* disruptants did not produce full-length mRNA's (Figure 3A). The analyses could not confirm the deletion of *MgAtr3* as in the wild-type isolate *MgAtr3* expression was already undetectable under all conditions tested (Stergiopoulos *et al.,* 2002a).

The expression analysis demonstrated that the basal expression level of *MgAtr1* was upregulated in *MgAtr4* disruptants (Figure 3B). Such an alteration in expression was not observed in any of the other knockout mutants.

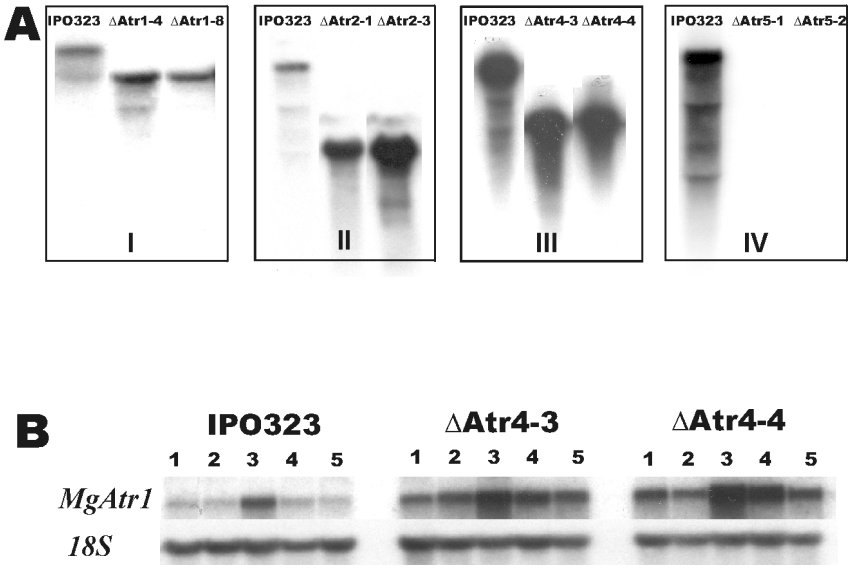


Figure 3: A-B. Expression analyses of *Mycosphaerella graminicola* transformants. **A:** *MgAtr1*, *MgAtr2*, *MgAtr4*, and *MgAtr5* expression in transformants containing a disrupted *MgAtr1* (I), *MgAtr2* (II) or *MgAtr4* (III) allele or a deleted *MgAtr5* allele (IV), respectively. In all panels, expression in the wild-type recipient isolate IPO323 and in two independent transformants is shown. Expression is shown after treatment of cells with 100 mg progesterone l^{-1} (I, II, and III) or 100 mg psoralen l^{-1} (IV). **B:** Expression of *MgAtr1* in *MgAtr4* disruption mutants of *M. graminicola*. IPO323 is the wild-type recipient isolate and ΔAtr4-3 and ΔAtr4-4 are two independent *MgAtr4* disruption mutants. The 18S ribosomal RNA gene is used as a loading control. RNA was isolated from cells treated with 0.1% methanol (1), 10 mg cyproconazole l^{-1} (2), 100 mg progesterone l^{-1} (3), 100 mg cycloheximide l^{-1} (4) or water (5).

Toxicity assays with knockout mutants

Based upon the results obtained with the yeast-complementation assay twenty-five compounds were tested for their activity against growth of *M. graminicola* transformants containing a disrupted or deleted allele of *MgAtr1-MgAtr5.* Compounds included are listed in Table 1. An alteration in sensitivity was only observed for the *MgAtr5* deletion mutants, which exhibited an increase in sensitivity to resorcinol (1,3-benzenediol) and to the phytoalexin resveratrol (trans-3,5,4'-trihydroxystilbene) as compared to the wild-type isolate or a hygromycin-resistant control transformant (Table 3). These data suggest that MgAtr5 can protect *M. graminicola* against the toxic activity of these compounds. Interestingly, growth of the *MgAtr5* deletion mutants was completely inhibited at 200 μg ml^{-1} resveratrol but restored at higher concentrations of resveratrol (Figure 4).

Table 3. Minimum Inhibitory Concentrations (µg ml^{-1}) of resorcinol and resveratrol to the wild-type *Mycosphaerella graminicola* strain IPO323, the control transformant Sp2 and two independent *MgAtr5* knockout mutants.

	Resorcinol	Resveratrol
IPO323	800	> 800
Sp2	800	> 800
ΔMgAtr5-1	600	200
ΔMgAtr5-2	600	200

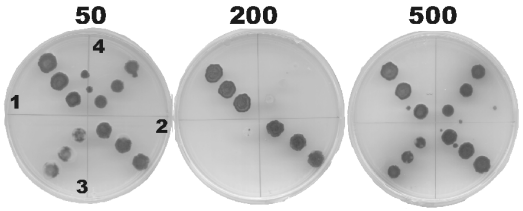


Figure 4. Effect of deletion of *MgAtr5* in *Mycosphaerella graminicola* on sensitivity to resveratrol. 1, represents the recipient wild-type isolate IPO323; 2, the control transformant Sp2; 3, and 4, represent two independent *MgAtr5* deletion mutants. Cells were spotted on PDA amended with 50, 200 and 500 µg resveratrol ml^{-1}.

Antagonistic activity of bacteria

B. cepacia B37W and *P. fluorescens* Pf5, known to produce the phenylpyrrole antibiotics pyrrolnitrin and pyoluteorin (Burkhead *et al.,* 1994; Howell and Stipanovic 1979), were tested for their antagonistic activity against *M. graminicola* transformants in agar diffusion tests. Both bacterial strains exhibited a clear antagonistic activity, as distinct inhibition zones were present. In addition, the antagonistic activity of both bacterial strains was significantly higher to *MgAtr2* disruptants than to the other *M. graminicola* transformants tested (Figure 5). These results indicate that ABC transporters from *M. graminicola* can provide protection against antibiotic compounds produced by bacteria.

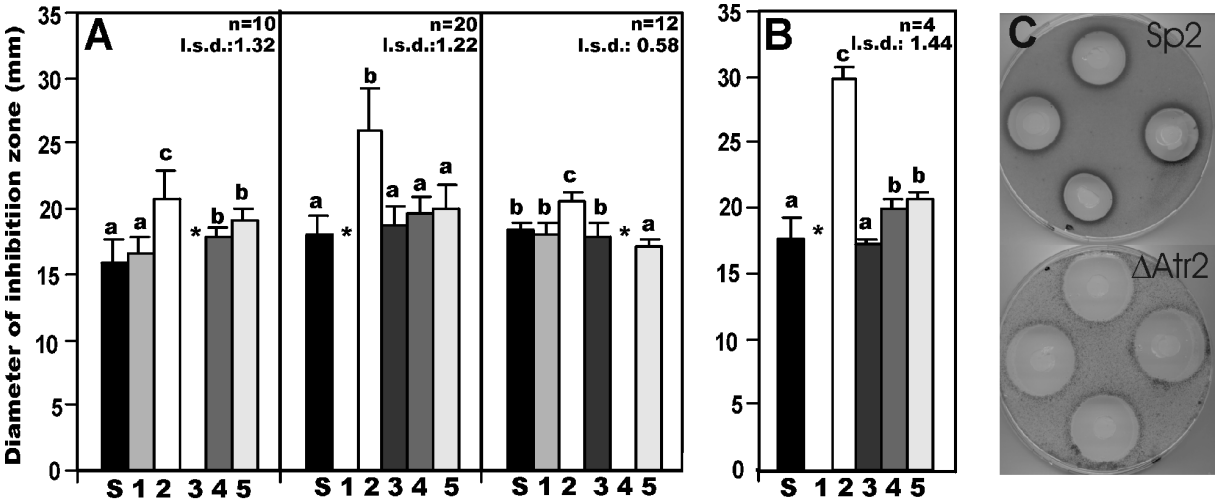


Figure 5. Antagonistic activity of *Burkholderia cepacia* B37W (**A**) and *Pseudomonas fluorescens* Pf5 (**B**) on growth of *Mycosphaerella graminicola* transformants with disrupted or deleted alleles of *MgAtr1-MgAtr5* in agar diffusion tests. S, represents Sp2, a control transformant containing a single copy of the hygromycin-resistance cassette and 1, 2, 3, 4, and 5 represent transformants of *M. graminicola* with disrupted or deleted alleles of *MgAtr1-MgAtr5*, respectively. Bars represent average results within a single experiment. Letters above bars indicate significantly different inhibition zones as determined by ANOVA using Genstat 5 (fourth edition). Least significant differences of means (l.s.d.) were determined at the 0.05 level. Asterisks indicate samples not determined due to lack of growth of *M. graminicola* pre-cultures. (**C**) Example of the antagonistic effect of *B. cepacia* B37W on growth of the control transformant Sp2 and the *MgAtr2* disruption mutant ΔAtr2 in an agar diffusion test.

DISCUSSION

This study describes the effect of disruption or deletion of five different ABC transporter genes from the wheat pathogen *M. graminicola* on the sensitivity to natural toxic compounds, xenobiotics, and antibiotics. As far as we know this is the most extensive analysis of the protective role of ABC transporters from a plant pathogenic fungus.

Heterologous expression of the ABC transporters in *S. cerevisiae* indicates that *MgAtr1-MgAtr5* from *M. graminicola* can protect *S. cerevisiae* against the toxic activity of a wide variety of chemically unrelated toxicants. We assume that this protection is based upon active transport of the compounds by the encoded proteins. MgAtr1 provides protection against nine out of ten substrates identified in the *S. cerevisiae* complementation assay. Therefore, MgAtr1 can be considered as a true multidrug transporter. This contrasts with MgAtr5 that has a more limited substrate range in yeast, as only the plant metabolites berberine and camptothecin are accepted as substrates. The substrate range of MgAtr2 and MgAtr4 is very similar. This might be a consequence of the high homology between MgAtr2 and MgAtr4 (45% identity, 60% similarity), which is the highest amongst the five ABC transporters cloned from *M. graminicola.*

In the yeast complementation assay, three of the *M. graminicola* transporters (MgAtr1, MgAtr2, and MgAtr4) provide protection against azole fungicides that are currently used to control septoria tritici leaf blotch of wheat. The results suggest that these ABC transporters can play a role in determining the intrinsic sensitivity of *M. graminicola* populations to azoles and can be involved in the development of resistance against these compounds. This has recently been demonstrated for MgAtr1 in azole-resistant laboratory-generated strains (Zwiers *et al.*, 2002). Ergosterol, the main sterol constituent of fungal membranes, is a substrate for MgAtr1, MgAtr2, and MgAtr4. This could imply that these ABC transporters are not only

involved in protection against toxicants but also in maintenance of membrane integrity as proposed for *S. cerevisiae* (Mahe *et al.,* 1996). MgAtr1 and MgAtr5 can transport the plant alkaloids berberine and camptothecin as substrates. This suggests that they are involved in protection of the fungus against fungitoxic plant defence compounds. However, these two plant alkaloids are not present in wheat but are produced by *Berberis vulgaris* and *Camptotheca acuminata*, respectively (Rothenberg, 1997; Stermitz *et al.,* 2000). Whether the capacity to transport these compounds is relevant in pathogenesis of *M. graminicola* on wheat is unclear. Possibly wheat produces, as yet unknown, alkaloids. In that case MgAtr1 and MgAtr5 could be regarded as virulence factors that act in protection of the fungus against such compounds. Plant metabolites known to be produced by wheat and implicated in protection against pests and pathogens include hydroxamic acids such as 2,4-dihydroxy-7-methoxy-2*H*-1,4-benzoxazin-3(4*H*)-one (DIMBOA) and (alkyl)resorcinols (Seitz, 1992; Wilkes *et al.,* 1999). These compounds were either not available (DIMBOA) or not toxic to *S. cerevisiae* (resorcinol). The mycotoxin DAS produced by *F. graminearum* (Muhitch *et al.,* 2000) is a substrate for both MgAtr1 and MgAtr4. *F. graminearum* is the causal agent of wheat head blight and might compete with *M. graminicola* for the same ecological niche on wheat. Hence, protection against DAS and other mycotoxins by ABC transporters can act as a mechanism of survival in its natural niche.

The yeast complementation assays indicated that several chemically unrelated compounds are potential substrates of *M. graminicola* ABC transporters. However, none of the ABC transporter knockout mutants of the pathogen did display an alteration in sensitivity to these compounds. Vice versa, resorcinol and resveratrol, which were identified as substrates in the phenotypic analysis of *M. graminicola* knockout mutants were no substrates in the yeast assays. This discrepancy in substrate range found between the two assays can be explained by a redundancy of ABC transporters. It is known that ABC transporters are members of a large protein superfamily and can have an overlap in substrate specificity (Kolaczkowski *et al.,* 1998). This is also clearly demonstrated by the results of our yeast-complementation assays. Hence, the presence of other ABC transporter genes can mask the function of disrupted or deleted ABC transporter genes. The observed upregulation of *MgAtr1* expression in a *MgAtr4* disruption background is suggestive in this respect. Another explanation could be that the yeast assay does not necessarily reflect the situation in *M. graminicola* itself, as it is known

that the membrane environment influences drug resistance conferred by ABC transporters (Krishnamurthy and Prasad, 1999; Zwiers *et al.,* 2002).

The *MgAtr5* deletion mutants of *M. graminicola* exhibited an increased sensitivity to the phenolic compounds resorcinol and resveratrol. Resorcinol is used in the treatment of acne and other skin disorders as eczema and psoriasis. Besides antibacterial action, antifungal activity of (alkyl)resorcinol has been described (Suzuki *et al.,* 1996). Resveratrol is a stilbene phytoalexin especially produced in the skin and seeds of grapes. The susceptibility of grapevine to the fungal pathogen *B. cinerea* is inversely correlated with the resveratrol content (Jeandet *et al.,* 1995). The closest homologues of MgAtr5, are the ABC transporters BcatrB and atrB from *B. cinerea* and *A. nidulans*, respectively (Stergiopoulos *et al.,* 2002a). The replacement of both *BcatrB* and *atrB* has rendered transformants hypersensitive to resveratrol (Andrade *et al.,* 2000; Schoonbeek *et al.,* 2001). This suggests that these three orthologs are involved in protection against plant derived compounds and that MgAtr5 might play a role in protection against wheat defence compounds.

The *MgAtr5* deletion mutants display increased sensitivity to low concentrations of resveratrol but upon exposure to high concentrations they are as sensitive to this compound as control strains. This implies that besides ABC transporter activity additional mechanisms of resistance to resveratrol, *e.g.* detoxification by laccases, can function in *M. graminicola* and that ABC transporter mediated resistance acts as the first line of defence in protection against toxic compounds.

The agar diffusion tests show that *B. cepacia* B37W and *P. fluorescens* Pf5 exhibit an increased antagonistic activity to *MgAtr2* disruptants. Both bacterial strains produce phenylpyrrole antibiotics (Burkhead *et al.,* 1994; Howell and Stipanovic, 1979). This suggests that phenylpyrroles are substrates for MgAtr2 and that ABC transporters can have a natural function in protecting *M. graminicola* against antibiotics present in its environment. The sensitivity to the fungicide fludioxonil, which is derived from pyrrolnitrin (Nyfeler and Ackermann, 1974), is not affected in *MgAtr2* disruptants, suggesting that the same transporter does not necessarily transport structurally closely related molecules. ABC transporters that transport antibiotics can play an important role in ecology and epidemiology of plant pathogenic fungi (Schoonbeek *et al.,* 2002). This may also apply to *M. graminicola*, since during the saprophytic phase of its lifecycle the fungus needs to cope with antagonistic effects exerted by bacteria and fungi. Moreover, several bacterial strains with antagonistic activity

against *M. graminicola* have been reported as potential biocontrol agents (Levy *et al.,* 1989). The presence of an ABC transporter-mediated resistance mechanism against compounds produced by antagonistic bacteria may impose severe limitations to the use of these bacteria as biocontrol agents for *M. graminicola*.

In summary, we show that *M. graminicola* contains multidrug transporters with overlapping substrate specificity. Substrates include fungicides, plant metabolites, antibiotics, and a mycotoxin. Moreover, the analysis of *M. graminicola* transformants with disrupted or deleted alleles of ABC transporter genes indicates phenotypes for antibiotics and plant metabolites. Therefore, ABC transporters of *M. graminicola* can contribute significantly to the competitive ability of the fungus as a successful pathogen.

ACKNOWLEDGMENTS

Dr G.H.J. Kema and Dr C. Waalwijk are acknowledged for discussions within the Wageningen Mycosphaerella group, Prof. Dr P.J.G.M. De Wit for critically reading the manuscript, Dr T. Hohn (Syngenta) for the generous gift of diacetoxyscirpenol, Dr J. Raaijmakers for providing *Pseudomonas fluorescens* Pf5 and B. cepacia B37W and H. Schoonbeek for help and discussions on the bacterial bioassay. L-H. Zwiers was financially supported by Syngenta, Switzerland, I. Stergiopoulos by the Training and Mobility of Researchers (TMR) Programme – Marie Curie Research Grants, The European Commission (Contract No. ERBFMBICT983558), and M.M.C. Gielkens and S.D. Goodall by the EU-BIOTECH 2 programme (Reference No. BIO4CT960352).

REFERENCES

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

Burkhead K, Schisler D and Slininger P (1994) Pyrrolnitrin production by biological control agent *Pseudomonas cepacia* B37w in culture and in colonized wounds of potatoes. Appl Environ Microbiol **60:** 2031-2039

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

De Waard MA (1997) Significance of ABC transporters in fungicide sensitivity and resistance. Pestic Sci **51:** 271-275

Decottignies A, Grant AM, Nichols JW, De Wet H, McIntosh DB and Goffeau A (1998) ATPase and multidrug transport activities of the overexpressed yeast ABC protein Yor1p. J Biol Chem **273:** 12612-12622

Del Sorbo G, Schoonbeek H and De Waard MA (2000) Fungal transporters involved in efflux of

natural toxic compounds and fungicides. Fun Genet Biol **30:** 1-15

Fleissner A, Copalla C and Weltring K-M (2002) An ABC Multidrug-Resistance transporter is necessary for tolerance of *Gibberella pulicaris* to phytoalexins and virulence on potato tubers. Mol Plant-Microbe In **15:** 102-108

Foote SJ, Thompson JK, Cowman AF and Kemp DJ (1989) Amplification of the multidrug resistance gene in some chloroquine-resistant isolates of *P. falciparum*. Cell **57:** 921-930

Gottesman MM and Pastan I (1993) Biochemistry of multidrug resistance mediated by the multidrug transporter. Annu Rev Biochem **62:** 385-427

Higgins CF (2001) ABC transporters: physiology, structure and mechanism: an overview. Res Microbiol **152:** 205-210

Howell CR and Stipanovic RD (1979) Control of *Rhizoctonia solani* on cotton seedlings with *Pseudomonas fluorescens* and with an antibiotic produced by the bacterium. Phytopathology**:** 480-482

Jeandet P, Bessis R, Sbaghi M and Meunier P (1995) Production of the phytoalexin resveratrol by grapes as a response to *Botrytis* attack under natural conditions. J Phytopathol **143:** 135-139

Kema GHJ and Van Silfhout CH (1997) Genetic variation for virulence and resistance in the wheat-*Mycosphaerella graminicola* pathosystem. III. Comparative seedling and adult plant experiments. Phytopathology **87:** 266-272

King EO, Ward MK and Raney DE (1954) Two simple media for the demonstration of pyocyanin and fluorescein. J Lab Clin Med **44:** 301-307

Kolaczkowski M, Kolaczkowska A, Luczynski J, Witek S and Goffeau A (1998) In vivo characterization of the drug resistance profile of the major ABC transporters and other components of the yeast pleiotropic drug resistance network. Microb Drug Resist **4:** 143-158

Krishnamurthy SS and Prasad RU (1999) Membrane fluidity affects functions of Cdr1p, a multidrug ABC transporter of *Candida albicans*. FEMS Microbiol Lett **173:** 475-481

Levy E, Eyal Z, Carmely S, Kashman Y and Chet I (1989) Suppression of *Septoria tritici* and *Puccinia recondita* of wheat by an antibiotic-producing fluorescent pseudomonad. Plant Pathol **38:** 564-570

Mahe Y, Lemoine Y and Kuchler K (1996) The ATP binding cassette transporters Pdr5 and Snq5 of *Saccharomyces cerevisiae* can mediate transport of steroids in vivo. J Biol Chem **271:** 25167-25172

McGrath JP and Varshavsky A (1989) The yeast STE6 gene encodes a homologue of the mammalian multidrug resistance P-glycoprotein. Nature **340:** 400-404

Melchers WJ, Verweij PE, Van den Hurk P, Van Belkum A, De Pauw BE, Hoogkamp Korstanje JA and Meis JF (1994) General primer-mediated PCR for detection of *Aspergillus* species. J Clin Microbiol **32:** 1710-1717

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 Sci **157:** 201-207

Nishi K, Yoshida M, Nishimura M, Nishikawa M, Nishiyama M, Horinouchi S and Beppu T (1992) A leptomycin B resistance gene of *Schizosaccharomyces pombe* encodes a protein similar to the mammalian P-glycoproteins. Mol Microbiol **6:** 761-769

Nyfeler R and Ackermann P (1974) Phenylpyrroles, a new class of agricultural fungicides related to the natural antibiotic pyrrolnitrin. ACS symp ser Washington, DC: American Chemical Society **504:** 395-404

Payne AC, Grosjean-Cournoyer MC and Hollomon DW (1998) Transformation of the phytopathogen *Mycosphaerella graminicola* to carbendazim and hygromycin B resistance. Curr Genet **34:** 100-104

Rothenberg ML (1997) Topoisomerase I inhibitors: review and update. Ann Oncol **8:** 837-855

Sambrook J, Fritsch EF and Maniatis T (1989) Molecular cloning: A laboratory Manual, Cold Spring Harbor Laboratory Press, New York

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. Antimicrob Agents Chemother **39:** 2378-2386

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. Mol Plant-Microbe In **14:** 562-571

Schoonbeek H, Raaijmakers JM and De Waard MA (2002) Fungal ABC transporters and microbial interactions in natural environments. Mol Plant-Microbe In **15:** 1165-1172

Seitz LM (1992) Identification of 5-(2-oxoalkyl)resorcinols and 5-(2-oxoalkenyl)resorcinols in wheat and rye grains. J Agr Food Chem **40:** 1541-1546

Stergiopoulos I, Gielkens MMC, Goodall SD, Venema K and De Waard MA (2002a) Molecular cloning and characterisation of three

new ABC transporter encoding 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 on Fungal Genetics, Pisa, pp. 250

Stergiopoulos I, Zwiers L-H and De Waard MA (2002c) Secretion of natural and synthetic toxic compounds from filamentous fungi by membrane transporters of the ATP-binding cassette and major facilitator superfamily. Eur J Plant Pathol **108:** 719-734

Stermitz FR, Lorenz P, Tawara JN, Zenewicz LA and Lewis K (2000) Synergy in a medicinal plant: Antimicrobial action of berberine potentiated by 5 '-methoxyhydnocarpin, a multidrug pump inhibitor. Proc Natl Acad Sci USA **97:** 1433-1437

Suzuki Y, Esumi Y, Hyakutake H, Kono Y and Sakurai A (1996) Isolation of 5-(8'Z-heptadecenyl)-resorcinol from etiolated rice seedlings as an antifungal agent. Phytochemistry **41:** 1485-1489

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

Wilkes MA, Marshall DR and Copeland L (1999) Hydroxamic acids in cereal roots inhibit the growth of take-all. Soil Biol Biochem **31:** 1831-1836.

Zwiers L-H and De Waard MA (2000) Characterization of the ABC transporter genes *MgAtr1* and *MgAtr2* from the wheat pathogen *Mycosphaerella graminicola*. Fun Genet Biol **30:** 115-125

Zwiers L-H and De Waard MA (2001) Efficient *Agrobacterium tumefaciens*-mediated gene disruption in the phytopathogen *Mycosphaerella graminicola*. Curr Genet **39:** 388-393

Zwiers L-H, Stergiopoulos I, Van Nistelrooy JGM and De Waard MA (2002) ABC transporters and azole susceptibility in laboratory strains of the wheat pathogen *Mycosphaerella graminicola*. Antimicrob Agents Chemother **46:** 3900-3906

CHAPTER 5

THE ABC TRANSPORTER MGATR4 IS A VIRULENCE FACTOR OF *MYCOSPHAERELLA GRAMINICOLA* THAT AFFECTS COLONISATION OF SUBSTOMATAL CAVITIES IN WHEAT LEAVES

Ioannis Stergiopoulos, Lute-Harm Zwiers and Maarten A. De Waard

Submitted

ABSTRACT

The role in virulence of the ATP-binding cassette (ABC) transporters *MgAtr1-MgAtr5* from *Mycosphaerella graminicola* was analysed in disruption or replacement strains, on seedlings of the susceptible wheat cultivar Obelisk. Disruption strains of *MgAtr1* and *MgAtr2*, and replacement strains of *MgAtr3* and *MgAtr5* displayed an unaltered phenotype in comparison to control strains, while virulence of the *MgAtr4* disruption strains was significantly reduced. This reduction in virulence was independent of the wheat cultivar used. Histopathological analysis of the infection process revealed that *MgAtr4* disruption strains colonise substomatal cavities less efficiently and display reduced intercellular growth in the apoplast of wheat leaves. *In vitro* growth experiments in different media showed no fitness penalty associated with the disruption of *MgAtr4*. Expression analysis demonstrated that transcripts of the constitutively expressed gene *CYP51,* encoding the fungal specific cytochrome P450 sterol 14α-demethylase from *M. graminicola,* were not detectable in interaction RNA from wheat infected with *MgAtr4* disruption strains thus, confirming the reduced intercellular growth of these strains. The results indicate that MgAtr4 is a virulence factor of *M. graminicola* during pathogenesis on wheat and may function in protection against fungitoxic compounds present around the substomatal cavities of wheat leaves. MgAtr4 is the first virulence factor cloned from this important plant pathogen.

Keywords: compatible interaction, incompatible interaction, pathogenicity, plant defence compounds, *Septoria tritici,* toxins

INTRODUCTION

The ascomycete fungus *Mycosphaerella graminicola* (Fuckel) J. Schroeter in Cohn (anamorph state: *Septoria tritici* Roborge in Desmaz.) is the causal agent of septoria tritici blotch on wheat. This is one of the most important economic diseases of wheat with potential yield losses of up to 30-40% (Eyal *et al.,* 1987). Typical symptoms of the disease are irregular chlorotic areas on the infected leaves that develop into necrotic lesions covered with dark brown to black pycnidia or pseudothecia of the fungus (Eyal *et al.,* 1987). Conditions of high humidity and moderate temperatures are known to favour the disease.

Histopathological analysis of the infection process showed that the fungus primarily enters its host directly through stomata. Penetration of the substomatal cavities usually occurs within 24-48 hours (h) after inoculation and subsequently growth extends to the leaf apoplast and to neighbouring substomatal cavities thus, establishing multiple stomatal infections from a single penetration site. During pathogenesis, infection hyphae remain strictly intercellular and in close contact with mesophyll cells, but no haustoria or other specialised feeding structures are formed. The first symptoms on infected plants become visible at 8-10 days post inoculation (dpi) as chlorotic areas on the leaf surface. From this time point onwards there is a massive increase of fungal biomass, induced by the release of nutrients after cell collapse. This increase is especially apparent around the substomatal cavities where the formation of the so-called baskets is observed. At approximately 14 dpi chlorotic areas turn into necrotic lesions and the first pycnidia become visible emerging from stomata with mycelial baskets (Cohen and Eyal, 1993; Duncan and Howard, 2000; Kema *et al.,* 1996).

Incompatible responses in the wheat - *M. graminicola* interaction are described by the inability of the fungus to grow within its host. Although no differences are observed during the first stages of the infection process, only very little increase in fungal biomass is observed during the later stages of pathogenesis as the fungus is unable to colonise mesophyll cells and remains restricted to the substomatal cavities (Kema *et al.,* 1996). Incompatible interactions can still result in necrosis and production of pycnidia, but considerably less as compared to compatible interactions. The quantitative differences in response between compatible and incompatible interactions led to the suggestion that no complete immunity to *M. graminicola* exists. In such cases, any restriction or delay in pathogen development can be regarded as a form of resistance (Nelson and Marshall, 1990).

Although a considerable amount of data is present regarding epidemiology of infections caused by *M. graminicola*, the biochemical and molecular basis of virulence of the fungus is poorly understood, and little is known about actual genes involved in host resistance and virulence of the pathogen. Recent data suggest a gene-for-gene relation in the wheat - *M. graminicola* interaction and studies on the segregation of avirulence led to the identification of a single avirulent locus in *M. graminicola* (Brading *et al.,* 2002; Kema *et al.,* 2000). However, specific virulence genes have not yet been identified from this pathogen.

ATP-Binding Cassette (ABC) transporters comprise one of the largest protein families known to date. They are present in a wide variety of organisms ranging from bacteria to man (Higgins, 1992). ABC transporters are located in the outer plasma membrane or in membranes of intracellular compartments and are capable of transporting a wide variety of hydrophobic compounds against a concentration gradient. The energy needed for transport is generated by ATP hydrolysis and for this reason they are considered as primary active transport systems. In filamentous fungi, ABC transporters can function in protection against synthetic toxic compounds or antibiotics produced by competing microorganisms present in the environment of the fungus. Overexpression of ABC transporters can have pleiotropic effects resulting in simultaneous resistance to a number of chemically unrelated compounds, a phenomenon described as MultiDrug Resistance (MDR). In plant pathogenic fungi ABC transporters can also act as virulence factors if they provide protection against plant defence compounds during pathogenesis or mediate secretion of pathogenicity factors, such as host specific toxins (Stergiopoulos *et al.,* 2002b). So far, a role of ABC transporters in pathogenesis has been demonstrated for the plant pathogens *Magnaporthe grisea* (Urban *et al.,* 1999), *Botrytis cinerea* (Schoonbeek *et al.,* 2001), and *Gibberella pulicaris* (Fleissner *et al.,* 2002).

We study the role of ABC transporters in the wheat - *M. graminicola* interaction. Five ABC transporter genes, coded *MgAtr1-MgAtr5,* have been cloned and characterised (Stergiopoulos *et al.,* 2002a; Zwiers and De Waard, 2000). All genes are highly homologous to other fungal ABC transporter genes involved in MDR or pathogenesis. Heterologous expression in *Saccharomyces cerevisiae* demonstrated that the encoding proteins can transport a wide variety of natural and synthetic toxic compounds. Functional analysis of *MgAtr1-MgAtr5* disruption or replacement strains showed that they may also provide protection against fungicides and secondary plant metabolites in *M. graminicola* itself (Zwiers *et al.,* submitted).

In this paper, we report the characterisation of the disruption or replacement strains of *MgAtr1-MgAtr5* with respect to virulence on wheat. We demonstrate that *MgAtr4* disruption strains are significantly less virulent on wheat seedlings as compared to control strains. The reduction in virulence was observed on various cultivars tested. Histopathological studies revealed that the *MgAtr4* disruption strains have a defect in colonising substomatal cavities and display reduced intercellular growth in the apoplast of infected wheat leaves. Northern analysis of interaction RNA confirmed the reduced *in planta* growth of the *MgAtr4* disruption mutants, while i*n vitro* growth experiments showed no fitness penalty associated with the disruption of *MgAtr4*. The results show that MgAtr4 is essential for virulence of *M. graminicola* on wheat.

MATERIALS AND METHODS

Strains, media, and growth conditions

M. graminicola isolate IPO323 (Kema and Van Silfhout, 1997) was used as a wild-type control and as recipient strain for the generation of disruption or replacement strains. Single gene disruption strains of *MgAtr1, MgAtr2,* and *MgAtr4* were generated using *Agrobacterium tumefaciens*-mediated transformation (Zwiers and De Waard, 2001; Zwiers *et al.,* submitted). Gene replacement strains of *MgAtr3* and *MgAtr5* were made according to Payne *et al.,* (1998) and Zwiers *et al.,* (submitted). Strain Sp2 is a control transformant containing a single-copy of the hygromycin-resistance cassette, generated by *A. tumefaciens*-mediated transformation (Table 1).

Table 1. Strains of *Mycosphaerella graminicola* tested in virulence assays

Strain name	Gene disrupted or replaced	Transformation method
IPO323[a]	-	-
Sp2[b]	-	*A. tumefaciens*[c]
O4	*MgAtr1*	*A. tumefaciens*
O8	*MgAtr1*	*A. tumefaciens*
T1	*MgAtr2*	*A. tumefaciens*
T2	*MgAtr2*	*A. tumefaciens*
58	*MgAtr3*	PEG[d]
85	*MgAtr3*	PEG
ΔMgAtr4.3	*MgAtr4*	*A. tumefaciens*
ΔMgAtr4.4	*MgAtr4*	*A. tumefaciens*
141	*MgAtr5*	PEG
237	*MgAtr5*	PEG

[a] Wild type isolate;
[b] Transformation control with an ectopic integration of the hygromycin-resistance cassette;
[c] *Agrobacterium tumefaciens*-mediated transformation (Zwiers and De Waard, 2001). Transformation strains are gene-disruption mutants;
[d] Polyethylene glycol (PEG)-mediated transformation (Payne *et al.,* 1998; Zwiers *et al.,* submitted). Transformation strains are gene-replacement mutants;

Yeast-like cells were grown in liquid Yeast-Sucrose Medium (YSM, yeast extract 10 g l^{-1}, sucrose 10 g l^{-1}) in the dark at 18°C and 140 rpm. Cultures (20 ml) were inoculated with yeast-like cells derived from stocks stored at –80°C. The optical density of cultures was determined at 600 nm (OD_{600}) and new starting cultures (20 ml) were prepared with an OD_{600} of 0.05. Three days later, the OD_{600} of these cultures was adjusted to 0.5 in fresh medium (20 ml) and the cultures were incubated again overnight under similar conditions as described above. In all cases the antibiotic streptomycin sulfate (100 mg l^{-1}) was added to the cultures to prevent bacterial growth.

Virulence Assays

Virulence of *M. graminicola* strains was tested on the wheat cultivar (cv) Obelisk, susceptible to isolate IPO323. For each *MgAtr* gene two independent disruption or deletion strains were tested (Table 1) and virulence assays were repeated at least three times. *M. graminicola* yeast-like cells grown in liquid YSM were harvested by centrifugation at 3,000 *g* for 10 min at 10°C. Pellets were washed once in sterile Millique water and resuspended in 0.15% Tween-20 at a density of 10^7 cells per ml. Suspensions were sprayed onto 7-9-day-old wheat seedlings till run-off. Control treatments were performed with a solution of 0.15% Tween-20. Inoculated plants were placed in sealed perspex-lidded containers at 18°C, and a 16-h-daylight period. Relative Humidity (RH) in the containers was kept above 80% by placing a water-soaked cloth in the bottom of the boxes. Emerging second leaves were clipped every 4-5 days to facilitate disease assessment and light penetration to the inoculated first emerging leaf. Virulence was assessed visually by evaluation of the leaf area covered with symptoms and the abundance of pycnidia in necrotic lesions.

In addition to cv Obelisk, virulence of the *MgAtr4* disruption strains was also assessed on cultivars Taichung 29, Bussard, Clement, Shafir, and Toropi. The response of these cultivars to infections with IPO323 varied from susceptible (Taichung 29, Bussard), to semi-susceptible (Clement), and resistant (Shafir, Toropi).

Histopathological analysis

Wheat seedlings of cv Obelisk were inoculated with strains Sp2 and ΔMgAtr4.3 as previously described. Entire leaves (n=5) were collected from inoculated plants at 3, 6, 10, 14, 17, and 21 dpi and stained with trypan-blue (Heath, 1971; Wilson and Coffey, 1980) in order to monitor fungal growth inside leaf tissue. Stained leaves were examined with a Zeiss Axiovert 10 (Zeiss, Jena, Germany) microscope and photographs were made on a 35 mm Kodak Ektachrome 64T Colour Reversal Film (Kodak, Herts, U.K.).

Quantitative assessment of disease caused by strains Sp2 and ΔMgAtr4.3 was made at 21 dpi by counting the number of colonised substomatal cavities present in the two parallel rows adjacent to the central vein on one side of the leaves. Counting started from the tip of the leaves and the first 500 substomatal cavities from each row were used for calculations (in total 5000 for each interaction). Assessment of fungal biomass in colonised substomatal cavities was performed by scoring the presence of limited mycelial growth (level 1) or the presence of a mycelial basket or a mature pycnidium at the substomatal cavities (level 2). The systemic nature of substomatal colonisation by strains Sp2 and ΔMgAtr4.3 was calculated by counting the number of colonised substomatal cavities flanking a colonised substomatal cavity.

***In vitro* growth experiments**

Growth of strain Sp2 was compared to growth of the *MgAtr4* disruption strains ΔMgAtr4.3 and ΔMgAtr4.4 in liquid culture media. These were YSM, M-1-D, and Intercellular Washing Fluid (IWF) isolated from un-inoculated and IPO323-inoculated (IWF-IPO323) wheat seedlings. M-1-D is a defined minimal medium containing sucrose (87.6 mM), $MgSO_4$ (30 mM), ammonium tartrate (27.1 mM), $Ca(NO_3)_2$ (1.2 mM), KNO_3 (0.79 mM), KCl (0.87 mM), NaH_2PO_4 (0.14 mM), KI (45 µM), H_3BO_3 (22 µM), $MnSO_4$ (30 µM), $ZnSO_4$ (8.7 µM), $FeCl_2 \cdot 6H_2O$ (7.4 µM) (Pinkerton and Strobel, 1976). IWF was isolated from wheat seedlings grown for 10-12 days at 18°C and RH above 80%, by water infiltration *in vacuo*, followed by centrifugation at 3,000 *g* (De Wit and Spikman, 1982). IWF from wheat seedlings inoculated with IPO323 was isolated according to the same procedure from leaves inoculated 8 days post sowing and harvested at 4 dpi. The IWFs were concentrated by freeze-drying to half of their original volume before use.

Yeast-like cells of the strains used in growth test experiments were pre-cultured for 3 days in liquid YSM as described previously. Growth was assessed by measuring the OD_{600} in cuvettes with a 1 cm optical path length, with a BioPhotometer (Eppendorf, Hamburg, Germany). New cultures (25 ml) with an OD_{600} of 0.01 were initiated in the media described above and incubated in the dark at 18°C and 140 rpm. Samples (n=5) were taken daily for a period of 10 (YSM, IWF, IWF-IPO323) or 14 days (M-1-D). The OD_{600} of these samples was corrected with the OD_{600} values of the culture filtrates obtained after centrifugation at 10,000 *g* for 5 min.

Interaction RNA and northern blot analysis

Northern analysis was performed with total RNA isolated from infected wheat leaves in time course experiments. Seedlings of cv Obelisk (9-day-old) were inoculated with isolate IPO323 and strains ΔMgAtr4.3 and ΔMgAtr4.4. Control inoculations were performed with a solution of 0.15% Tween-20. First emerging leaves (n=5) were collected over a period of 22 days at 6, 8, 12, 14, 16, 18, and 22 dpi, instantly frozen in liquid nitrogen, and stored at –80°C until RNA isolation.

Interaction RNA was isolated from frozen biomass using the TRIzol reagent (Life Technologies Inc., Maryland, U.S.A.) according to the manufacturer's instructions. Isolated RNA (15 µg, 4.5 µl) was denatured in a solution of 6 M glyoxal (4.5 µl), 0.1 M sodium phosphate (3.0 µl), and dimethyl sulfoxide (13.3 µl) at 50°C for 1 h. RNA was subjected to electrophoresis on 1.6% agarose gel in a 10 mM sodium phosphate buffer, pH 7.0. Blotting was carried out on Hybond-N^+ membranes (Amersham Pharmacia Biotech, Buckinghamshire, U.K.) by capillary transfer with 10x SSC solution. RNA was cross-linked to membranes by irradiation under UV light (0.6 J per square centimeter). Homologous hybridisations were performed overnight at 65ºC in Nasmyth's solution buffer (18.5% dextran sulphate, 1.85% sarcosyl, 0.011 M EDTA, 0.3 M Na_2HPO_4, 1.1 M NaCl, pH 6.2). This solution (5.4 ml) was mixed with distilled water (4.6 ml) just before use to obtain the hybridisation buffer (10 ml). Blots were washed twice in 2x SSC, 0.1x SDS and twice in 0.1x SSC, 0.1x SDS at 65ºC, for 15 min.

Probes

Probes used in northern blot analysis were a 0.84 kb *Eco*RI fragment of *MgAtr1*, a 0.75 kb *Sal*I fragment of *MgAtr2*, a 0.85 kb *Sal*I fragment of *MgAtr3,* a 0.48 kb *Bam*HI/*Pst*I fragment of *MgAtr4*, a 0.6 kb *Eco*RI fragment of *MgAtr5,* and a 0.65 kb DNA fragment of *CYP51* from *M. graminicola*. This last fragment was obtained by

PCR using genomic DNA from isolate IPO323 as template. Equal loading of samples on blots was examined with a 0.6 kb *Eco*RI fragment of the *18S* rRNA gene from *Aspergillus niger*.

Randomly primed DNA isotopic probes were prepared by enzymatic incorporation of [α^{32}P]-dATP. In each labelling reaction 50 ng of a probe template was used. Probes were purified using the QIAquick Nucleotide Removal kit (QIAGEN, Leusden, The Netherlands) before addition to the hybridisation solution.

Production of phytotoxic compounds

Production of phytotoxic compounds by *M. graminicola* was tested in liquid M-1-D medium according to Perrone *et al.,* (1999). Cultures (100 ml) of strains IPO323, Sp2, ΔMgAtr4.3, and ΔMgAtr4.4 were initiated at a starting OD_{600} of 0.1 and grown for a period of 14 days in the dark at 22°C and 150 rpm. Cell free culture filtrates were obtained by centrifugation at 10,000 *g* at 4°C for 10 min. The supernatant was concentrated to half of the original volume by freeze-drying and filter-sterilised through a 0.2 µm filter (FP 30/0.2 CA-S, Schleicher and Schuell, Dassel, Germany). Culture filtrates were supplemented with 0.2% v/v Tween-80 and phytotoxic activity was examined on 10-day-old wheat seedlings of cultivars Obelisk, Bussard, and Shafir by spraying till run-off. Control treatments were performed with M-1-D medium supplemented with 0.2% v/v Tween-80. Plants were incubated in sealed Perspex-lidded containers at 18°C and a 16-h-daylight period for 3 weeks. Phytotoxicity was assessed at the end of the 3-week period.

RESULTS

Virulence assays

Virulence of all disruption or replacement strains of *MgAtr1-MgAtr5* from *M. graminicola* (Table 1) was compared with control strains IPO323 and Sp2 on wheat seedlings of the susceptible cultivar (cv) Obelisk. For all strains, first symptoms became visible at 8-10 dpi. These emerged as small chlorotic spots, most frequently starting from the tip of the leaves. These symptoms expanded longitudinally across the leaf surface and developed into necrotic lesions covered with pycnidia of the fungus. No difference in symptom development and necrotic leaf area was observed for strains IPO323, Sp2, and the disruption or replacement strains of *MgAtr1*, *MgAtr2*, *MgAtr3*, and *MgAtr5* at 21 dpi. However, for the *MgAtr4* disruption strains (ΔMgAtr4.3 and ΔMgAtr4.4) symptom development was delayed and lesions remained limited in size. At 21 dpi, 50% of the leaf area from plants infected by the *MgAtr4* disruption strains did not show any symptoms, while leaves infected with the control strains were completely necrotic (Figure 1). Lesions caused by the *MgAtr4* disruption strains slowly continued to expand in time and plants became completely necrotic at 30-35 dpi.

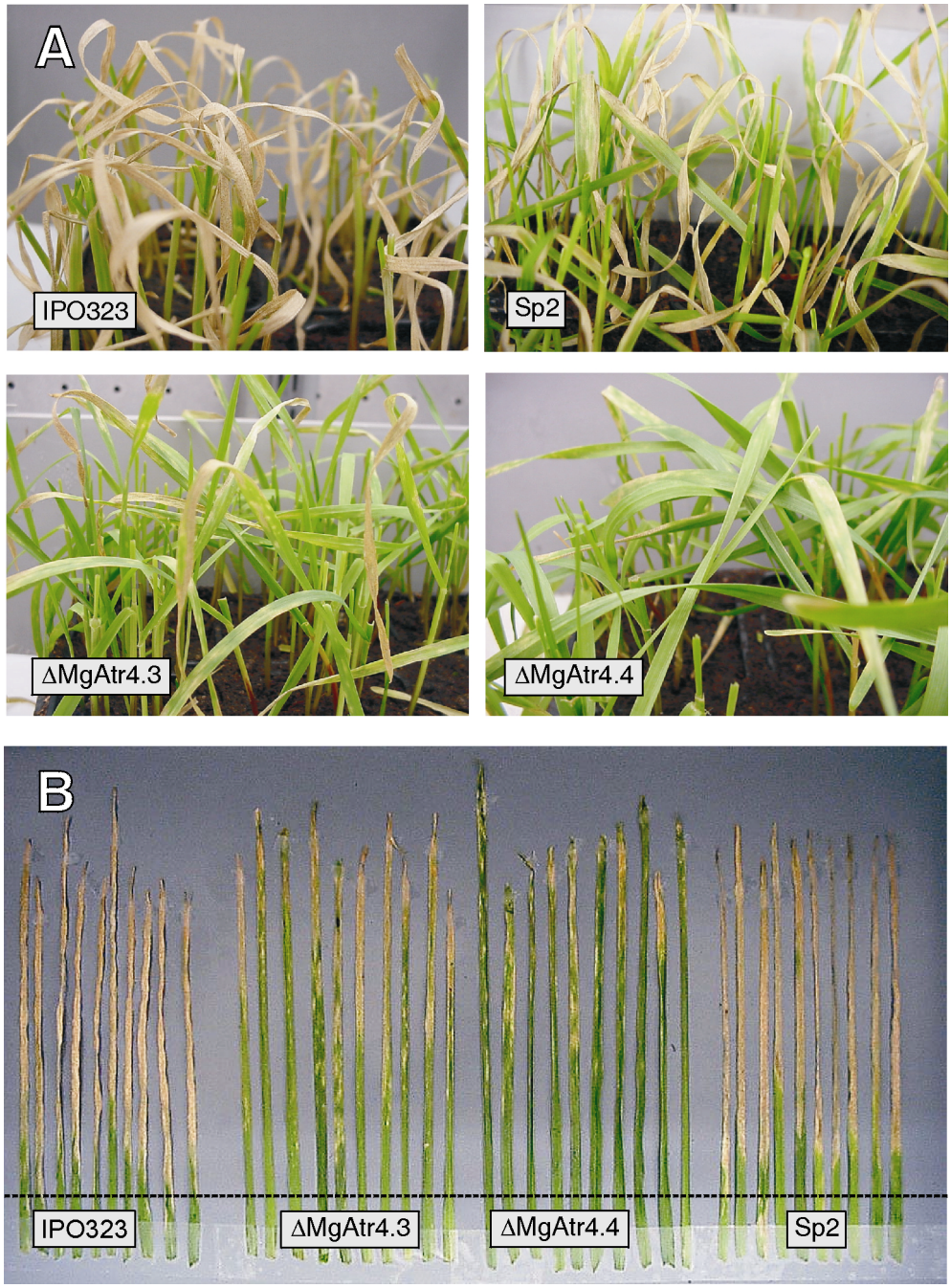


Figure 1. Virulence of *Mycosphaerella graminicola* strains on wheat seedlings of cultivar Obelisk at 21 days post inoculation. Strains tested are IPO323 and Sp2 (controls) and ΔMgAtr4.3 and ΔMgAtr4.4 (*MgAtr4* disruption strains). A: symptoms of whole plants. B: symptoms on first emerging leaves

Both *MgAtr4* disruption strains tested showed the same phenotype.

None of the strains tested was impaired in pycnidia formation. Small necrotic lesions caused by the *MgAtr4* disruption strains showed abundant pycnidia. Pycnidiospores isolated from plants inoculated with the *MgAtr4* disruption strains were still able to grow on PDA plates amended with hygromycin (100 mg l^{-1}). Plants inoculated with these pycnidiospores

showed the same reduction in virulence as plants inoculated with *in vitro*-grown yeast-like cells of the strains.

Virulence of strains ΔMgAtr4.3 and ΔMgAtr4.4 was also assessed on the wheat cultivars Taichung 29 and Bussard (susceptible to IPO323), Clement (semi-susceptible to IPO323), and Shafir and Toropi (resistant to IPO323). Both *MgAtr4* disruption strains showed reduced virulence on all cultivars tested. The reduction in virulence was most clear on the susceptible and semi-susceptible cultivars (data not shown). Here, disease symptoms caused by control strains and the *MgAtr4* disruption strains started at 8-10 dpi, but developed slower for the disruption strains as compared to the control strains. In case of the resistant cultivars differences between control and *MgAtr4* disruption strains were less pronounced due to the low infection levels incited by all strains. Production of pycnidia within necrotic areas was similar for all strains on all cultivars tested.

In planta growth monitoring

Histopathological analysis of the infection process was examined by staining wheat leaves inoculated with control strain Sp2 and the *MgAtr4* disruption strain ΔMgAtr4.3 with trypan-blue (Table 2). Germinated yeast-like cells showed germ tubes from both apical ends and no signs of growth inhibition on the leaf surface were present for any of the strains tested. Chemo- or thigmotropism towards stomata was not observed, as in many cases germ tubes by-passed guard cells of stomata without establishing infections. Infections appeared to occur only through stomata, as direct penetration of epidermal cells was not observed.

The control strain Sp2 had successfully entered and colonised the substomatal cavities by 6 dpi. Infection hyphae extended longitudinally towards neighbouring substomatal cavities establishing multiple infections from a single penetration site. At 10 dpi, the first macroscopical symptoms became visible on infected plants as chlorotic spots at the tip of the leaves (Figure 2Aa). At this time point, microscopical observations showed strict intercellular growth of the fungus (Figure 2Ab) between mesophyll cells (Figure 2Ac). Colonisation of the leaf apoplast and increase of fungal biomass progressed rapidly from 10 dpi onwards and mycelial proliferation around substomatal cavities became apparent by 14 dpi. Collapse of leaf tissue and necrosis described the macroscopical symptoms at 17 dpi (Figure 2Ba). This coincided with abundant hyphal growth around mesophyll cells (Figure 2Bb) and the formation of mycelial baskets in the substomatal cavities (Figure 2Bc), followed by the

Table 2. Histopathological analysis of pathogenesis by control strain Sp2 and the *MgAtr4* disruption strain ΔMgAtr4.3 of *Mycosphaerella graminicola*. Table depicts differences between the two strains at the different stages of infection.

	Stages in pathogenesis								
	Leaf surface		Stomatal infections		Colonisation of apoplast		Symptoms		
Strains	Germination 1[a]	Growth 1-2	Entry 1-2	Substomatal colonisation 3-6	Longitudinal growth 6-10	Biomass build-up 10-21	Necrosis 21	Pycnidia 21
Sp2	++[b]	++	++	++	++	++	++	++
ΔMgAtr4.3	++	++	++	+	+	+	+	+

[a] Days post inoculation (dpi)
[b] Relative to control: reduced (+) and similar (++)

development of pycnidia. At 21 dpi, leaves were almost completely necrotic (Figure 2Ca). In dead leaf tissue pycnidia were commonly present and coincided with extensive colonisation of the apoplast (Figure 2Cb, c).

Germ tubes of ΔMgAtr4.3 penetrated the substomatal cavities at the same rate as Sp2, but colonised substomatal cavities significantly slower. Growth of ΔMgAtr4.3 towards the apoplast was also restricted. However, as for the control strain, chlorotic symptoms at the tip of the leaves became visible at 10 dpi (Figure 2Ad). At this time point, fungal growth was still restricted to the substomatal cavities (Figure 2Ae, f). To a limited extent the fungus escaped infected substomatal cavities and grew longitudinally towards neighbouring substomatal cavities but the majority of established infections did not expand (Figure 2Be). Increase in fungal biomass in infected substomatal cavities and the apoplast progressed only slowly in time (14 and 17 dpi) (Figure 2Bf). At 21 dpi, cell collapse was mainly observed in restricted necrotic lesions (Figure 2Cd, e, f), but in these lesions pycnidia formation progressed normally.

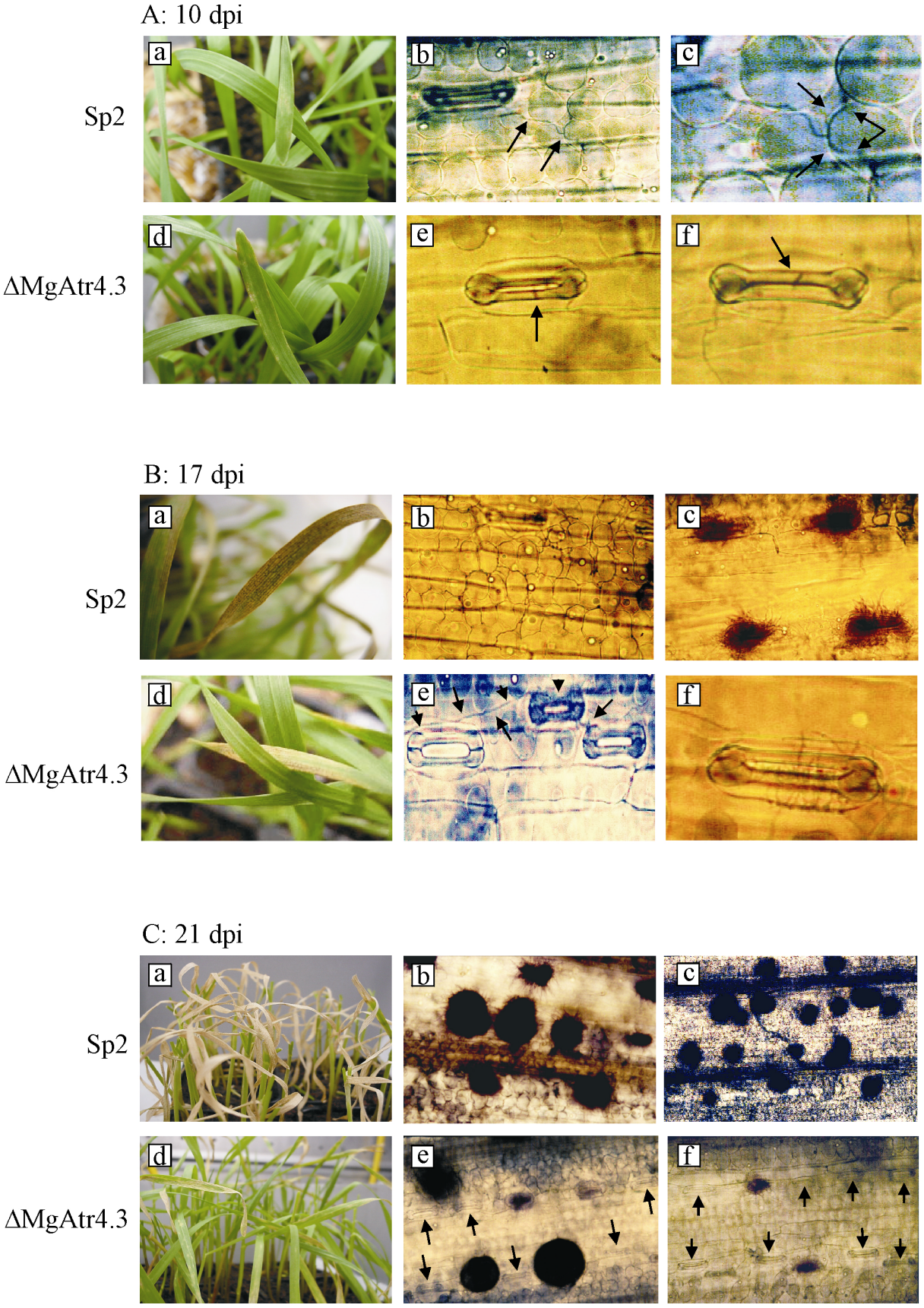

A: 10 dpi
Sp2
ΔMgAtr4.3
a
b
c
d
e
f
B: 17 dpi
Sp2
ΔMgAtr4.3
a
b
c
d
e
f
C: 21 dpi
Sp2
ΔMgAtr4.3
a
b
c
d
e
f

Figure 2. Histopathological analysis of infection of wheat leaves by *Mycosphaerella graminicola* strains Sp2 (control) and ΔMgAtr4.3 (*MgAtr4* disruption strain) in time.
Aa: macroscopical symptoms incited by Sp2 at 10 dpi; Ab: intercellular growth to the apoplast (pointed by arrows); Ac: infection hyphae growing around mesophyll cells (pointed by arrows) (detail of Ab); Ad: macroscopical symptoms incited by ΔMgAtr4.3 at 10 dpi; Ae: infection hyphae restricted at the substomatal cavity (pointed by arrow); Af: same as Ae; Ba: macroscopical symptoms incited by Sp2 at 17 dpi; Bb: extensive mycelial growth in the apoplast; Bc: mycelial baskets in substomatal cavities (level 2); Bd: macroscopical symptoms incited by ΔMgAtr4.3 at 17 dpi; Be: longitudinal growth to neighbouring substomatal cavities without basket formation in substomatal cavities (pointed by arrows); Bf: limited mycelial biomass in substomatal cavities (level 1); Ca: macroscopical symptoms incited by Sp2 at 21 dpi; Cb: Pycnidia in necrotic lesions; Cc: same as Cb; Cd: macroscopical symptoms incited by ΔMgAtr4.3 at 21 dpi; Ce: stomata with pycnidia and uninfected stomata (pointed by arrows); Cf; scattered infected stomata on the leaf surface (arrows indicate uninfected stomata).

Quantification of leaf colonisation

Colonisation of wheat leaves infected by control strain Sp2 and the *MgAtr4* disruption strain ΔMgAtr4.3 was quantified at 21 dpi by counting the number of colonised substomatal cavities, the abundance of biomass in the substomatal cavities, and the degree that adjacent substomatal cavities were colonised (systemic infection). In the latter case, the number of colonised substomatal cavities with either one or both of the two flanking substomatal cavities colonised was assessed (Table 3).

Table 3. Quantification of infection of *Mycosphaerella graminicola* strain Sp2 (control) and ΔMgAtr4.3 (*MgAtr4* disruption strain) on wheat leaves of cultivar Obelisk at 21 days post inoculation.

A. Number of colonised substomatal cavities				
	Sp2		ΔMgAtr4.3	
	Number	%	Number	%
Colonised	2205	44	1066	21
Non-colonised	2795	56	3934	79
B. Biomass in colonised substomatal cavities				
	Number	%	Number	%
Level 1[a]	1516	69	740	69
Level 2	689	31	326	31
C. Systemic colonisation of substomatal cavities				
	Number	%	Number	%
0-1-0[b]	298	13.5	423	40
0-1-1 or 1-1-0	674	30.5	332	31
1-1-1	1233	56.0	311	29

[a] Level 1: limited biomass; level 2: mycelial basket or pycnidium;
[b] Figures indicate patterns of colonisation of two substomatal cavities (first and third figure) flanking a colonised substomatal cavity (middle figure). Figure 0 represents un-colonised substomatal cavities and figure 1 colonised substomatal cavities;

The total number of colonised substomatal cavities was significantly higher for Sp2 than for ΔMgAtr4.3 (Table 3A). Within necrotic lesions biomass and pycnidia formation in colonised substomatal cavities was similar for the two strains tested (Table 3B). However, the systemic nature of substomatal colonisation varied significantly between the two strains, since the percentage of flanking substomatal cavities that were colonised was much higher for strain Sp2 than for strain ΔMgAtr4.3 (Table 3C). Thus, the results indicate that the *MgAtr4* disruption strains possess a reduced ability to colonise substomatal cavities and expand into mesophyll tissue.

***In vitro* growth**

The effect of disruption of *MgAtr4* on fitness of the fungus was studied in *in vitro* growth test experiments. Growth of strains ΔMgAtr4.3 and ΔMgAtr4.4 was compared with control strain Sp2 in different liquid culture media. Growth of ΔMgAtr4.3 and ΔMgAtr4.4 was similar to that of Sp2 in all media tested. An exponential increase in fungal growth was observed between 2 and 5 days of culturing in all media tested, except for M-1-D. Exponential growth in this medium was still observed after 14 days of culturing. Biomass levels at the stationary phase were highest in the rich YSM and significantly lower in IWF and IWF-IPO323 (Figure 3).

Expression analysis

Expression of the ABC transporter genes *MgAtr1, MgAtr2, MgAtr3, MgAtr4,* and *MgAtr5,* and the fungal specific *CYP51* gene, encoding cytochrome P450 sterol 14α-demethylase ($P450_{14DM}$) (Kalb *et al.,* 1987) from *M. graminicola,* was examined in interaction RNA isolated from wheat seedlings inoculated with the wild-type strain IPO323 and strains ΔMgAtr4.3 and ΔMgAtr4.4, in time course experiments (Figure 4). In all cases, expression of *MgAtr2* and *MgAtr3* was not detected. Low transcript levels of *MgAtr1* and *MgAtr5* were observed at 22 dpi in interaction RNA isolated from plants inoculated with IPO323 (data not shown). Transcripts of *MgAtr4* and *CYP51* became already visible at 12 dpi and their levels gradually increased in time until 18 dpi. At 22 dpi a small decrease in the transcript level of these genes was observed. Transcripts of *MgAtr4*, *CYP51,* and the other genes tested could not be detected at any time point after infection in interaction RNA isolated from plants inoculated with the *MgAtr4* disruption strains (Figure 4).

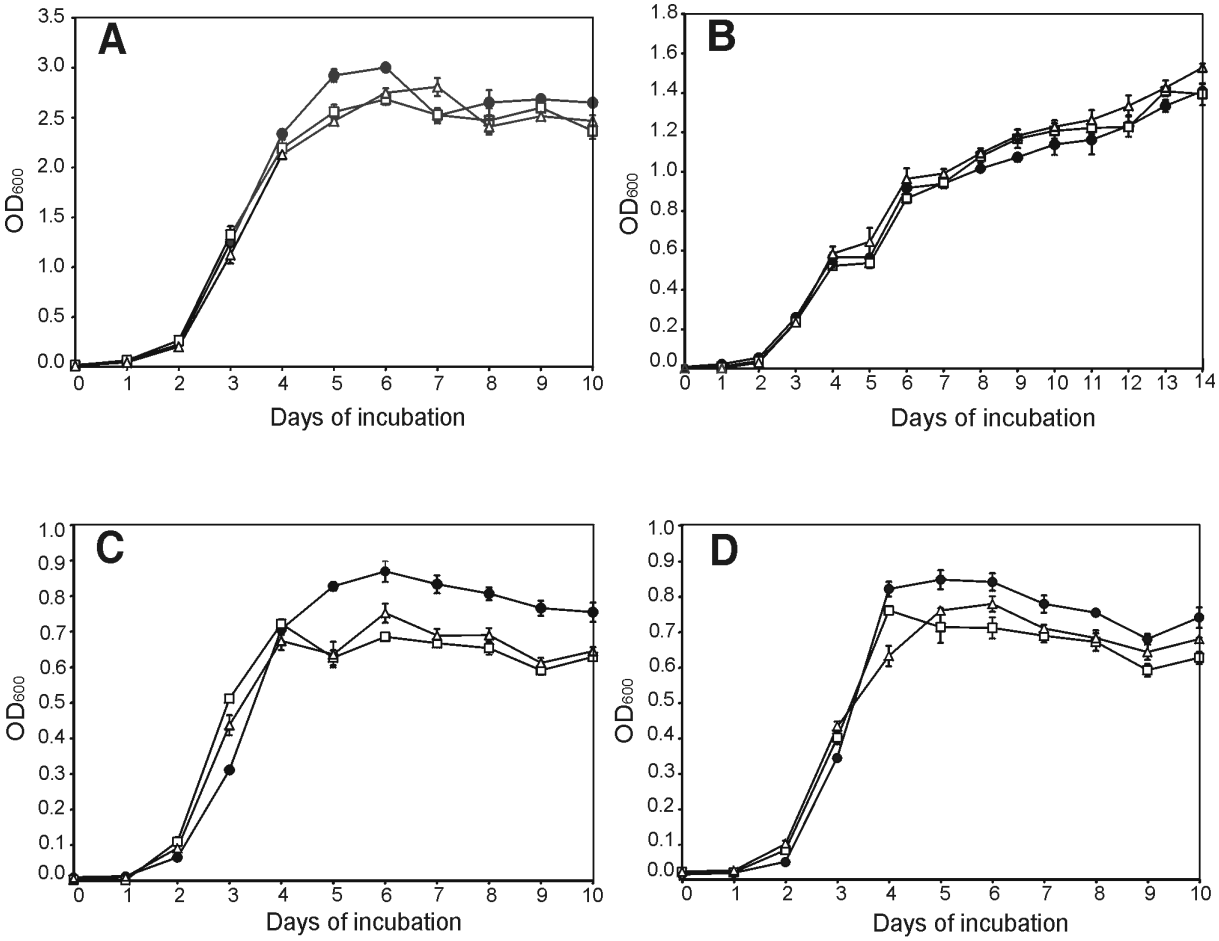


Figure 3. *In vitro* growth of *Mycosphaerella graminicola* strains Sp2 (•) (control) and ΔMgAtr4.3 (□) and ΔMgAtr4.4 (△) (*MgAtr4* disruption strains) in liquid growth media: A: Yeast Sucrose Medium, B: M-1-D minimal medium, C: Intercellular Washing Fluid, and D: Intercellular Washing Fluid isolated from plants infected with the wild-type *M. graminicola* isolate IPO323. Growth was assessed by measuring the optical density of the cultures at 600 nm (OD_{600}).

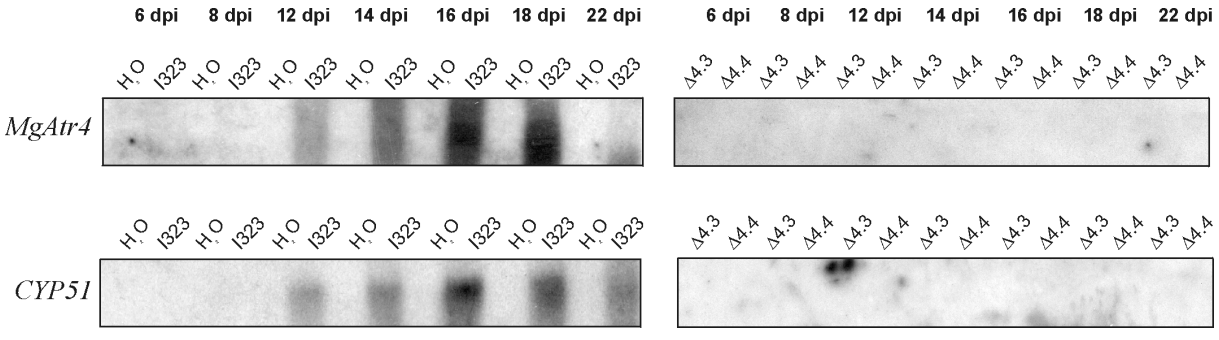


Figure 4. Expression analysis of *MgAtr4* and *CYP51* from *Mycosphaerella graminicola* in interaction RNA isolated from leaves of wheat seedlings inoculated with the wild-type strain IPO323 (I323), and the *MgAtr4* disruption strains ΔMgAtr4.3 (Δ4.3) and ΔMgAtr4.4 (Δ4.4) in a time-course experiment. Control treatments are indicated as H_2O.

Phytotoxic activity of culture filtrates

Histopathological studies suggested the involvement of toxins in the wheat – *M. graminicola* interaction (Kema *et al.,* 1996). Perrone *et al.,* (1999) reported the production of a non-cultivar-specific phytotoxic metabolite by *M. graminicola* in liquid M-1-D medium. Therefore, the phytotoxic activity of M-1-D culture filtrates from strains IPO323, Sp2, ΔMgAtr4.3, and ΔMgAtr4.4 was examined on wheat seedlings of cultivars Obelisk, Bussard (susceptible to IPO323), and Shafir (resistant to IPO323). 14-Day-old culture filtrates from these strains showed phytotoxic activity on all cultivars tested. Symptoms incited by all strains consisted of chlorotic spots that became visible at 6-8 days post spraying (data not shown). These symptoms resembled the initial chlorotic spots observed upon infection with the fungus. Phytotoxicity of all culture filtrates was slightly higher to cv Shafir than to cultivars Obelisk and Bussard, which suggests no relationship between phytotoxic activity of culture filtrates of the strains used and susceptibility of the wheat cultivars to *M. graminicola*. Nevertheless, with none of the cultivars a difference in phytotoxic activity between the culture filtrates from strains IPO323, Sp2, ΔMgAtr4.3, and ΔMgAtr4.4 was observed.

DISCUSSION

This study analyses the function of five ABC transporter genes from the wheat pathogen *M. graminicola* in virulence on wheat seedlings. The results clearly demonstrate that *MgAtr4* disruption strains have a reduced virulence in comparison to the wild-type control and the other *MgAtr* disruption or replacement strains tested. Thus, MgAtr4 can be regarded as a virulence factor of *M. graminicola* on wheat. This is the first virulence factor identified so far from this important plant pathogen.

MgAtr4 disruption strains have a normal *in vitro* growth and therefore, the delayed *in planta* growth of these strains can not be ascribed to a general fitness penalty. Histopathological analysis of the infection processes revealed that the reduced virulence of the *MgAtr4* disruption strains can be ascribed to unsuccessful colonisation of substomatal cavities. This early stage in pathogenesis is essential for establishment of the disease, further expansion of the fungus to the apoplast, and colonisation of neighbouring substomatal cavities, thereby allowing multiple stomatal infections from a single penetration site (Duncan

and Howard, 2000). Hence, retarded growth of the *MgAtr4* disruption strains in the apoplast is probably the result of hampered growth in substomatal cavities.

Expression analysis of interaction RNA isolated from wheat inoculated with strain IPO323 showed increased transcript levels of *MgAtr4* in time. A similar increase in transcript levels was observed for the constitutively expressed *CYP51* from *M. graminicola* (Gisi *et al.,* 2000). The increase in transcript levels of these two genes during infection correlated with an increase in fungal biomass, characteristic for compatible interactions (Kema *et al.,* 1996). In contrast, no transcripts of *CYP51* were detected in interaction RNA of the *MgAtr4* disruption strains indicating that biomass proliferation of these strains during infection was extremely low. Hence, results from the expression analysis corroborate the finding of reduced *in planta* growth of the *MgAtr4* disruption strains.

In plant pathogenic fungi ABC transporters could act as virulence factors if they provide protection against plant defence compounds during pathogenesis or mediate secretion of pathogenicity factors, such as host specific toxins or other virulence factors (Stergiopoulos *et al.,* 2002b). Pnini-Cohen *et. al.,* (1997) detected the presence of fluorescent compounds produced around stomata infected by *M. graminicola* that did not completely arrest infection, but rather affected the rate of *in planta* mycelial development at the onset of the infection. It is possible that MgAtr4 is involved in protection of the fungus against such fungitoxic compounds produced at the substomatal cavities. This would explain the decreased potency of the *MgAtr4* disruption strains to colonise substomatal cavities and the retarded growth of these strains in the apoplast. Wheat is also known to produce the plant defence compound 2,4-dihydroxy-7-methoxy-2*H*-1,4-benzoxazin-3(4*H*)-one (DIMBOA) (Weibull and Niemeyer, 1995). Currently, attempts to isolate DIMBOA and other plant defence compounds from wheat are made, to test their activity against *MgAtr4* disruption mutants.

MgAtr4 is highly homologous to ABC1, an ABC transporter from *M. grisea* that determines pathogenicity of this fungus on rice and barley (Urban *et al.,* 1999). *ABC1* deletion mutants show a dramatic growth arrest after penetration of the host and fail to produce extensive infection hyphae and to colonise the apoplast. Although the exact role of ABC1 during pathogenesis remains to be established, the most probable explanation for the loss of virulence of the deletion mutants is that ABC1 provides protection against antimicrobial compounds produced by the host. The high homology between MgAtr4 and ABC1 and the similarities in the observed phenotype in disruption mutants of both transporter genes, suggest

that the encoded proteins have a similar function during pathogenesis, possibly by providing protection against plant defence compounds produced by the host. Such a function in virulence has previously been demonstrated for the ABC transporter BcatrB from *B. cinerea* (Schoonbeek *et al.,* 2001) and GpABC1 from *G. pulicaris* (Fleissner *et al.,* 2002), which provide protection against the plant defence compounds resveratrol and rishitin, respectively. Heterologous expression of *MgAtr1, MgAtr2, MgAtr4,* and *MgAtr5* from *M. graminicola* in a multiple ABC transporter knockout strain of *S. cerevisiae,* showed that the encoded ABC transporters transport a wide variety of chemically-unrelated compounds and possess an overlap in substrate specificity (Zwiers *et al.,* submitted). Therefore, the presence of functional homologues of MgAtr4 in *M. graminicola* may explain why *MgAtr4* disruption strains do not show a complete loss of pathogenicity on host plants.

Formation of necrotic lesions caused by *M. graminicola* may be associated with secretion of phytotoxic compounds by the pathogen (Kema *et al.,* 1996). Therefore, the fungus may have evolved specific mechanisms for secretion of toxins during pathogenesis or self-protection against auto-toxicity of such products (Stergiopoulos *et al.,* 2002b). This phenomenon has been described for transporters of the major facilitator superfamily from *Cochliobulus carbonum* (Pitkin *et al.,* 1996), *Fusarium sporotrichioides* (Alexander *et al.,* 1999), and *Cercospora kikuchii* (Callahan *et al.,* 1999). Production of phytotoxic metabolites by *M. graminicola* in *in vitro*-grown cultures has been reported (Harrabi *et al.,* 1993; Perrone *et al.,* 1999; Voloshchuk *et al.,* 1994). Our results confirmed the potency of *M. graminicola* to secrete phytotoxic metabolites. However, a function of MgAtr4 in secretion of these phytotoxic metabolites could not be established since disruption of *MgAtr4* did not alter the phytotoxic activity of culture filtrates. A role of MgAtr4 in self-protection is also not likely since *in vitro* growth of *MgAtr4* disruption strains under conditions that promote the production of such metabolites was not impaired.

In summary, our results demonstrate that the ABC transporter MgAtr4 from *M. graminicola* plays a role in virulence of the fungus during colonisation of substomatal cavities of wheat seedlings. We suggest that *MgAtr4* disruption mutants become sensitive to fungitoxic plant compounds produced around the substomatal cavities. The nature of these compounds remains to be elucidated.

ACKNOWLEDGEMENTS

We kindly acknowledge Dr G.H.J. Kema, E.C.P. Verstappen, and Dr C. Waalwijk (Plant Research International, Wageningen, The Netherlands) for providing the *M. graminicola* isolate IPO323 and for helpful suggestions and discussions during this work within the Wageningen Mycosphaerella group. We also acknowledge Prof. Dr P.J.G.M. De Wit for critical reading of the manuscript. We thank M. Calivas and A.C. Prince for helpful contribution to the expression analysis and the *in vitro* growth test experiments. I. Stergiopoulos was financially supported by the Training and Mobility of Researchers (TMR) Programme – Marie Curie Research Training Grants of the European Commission. (Contract No. ERBFMBICT983558) and L-H. Zwiers by Syngenta, Switzerland.

REFERENCES

Alexander NJ, McCormick SP and Hohn TM (1999) TRI12, a trichothecene efflux pump from *Fusarium sporotrichioides*: gene isolation and expression in yeast. Mol Gen Genet **261:** 977-84

Brading PA, Verstappen ECP, Kema GHJ and Brown JKM (2002) A gene-for-gene relationship between wheat and *Mycosphaerella graminicola*, the Septoria tritici blotch pathogen. Phytopathology **92:** 439-445

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. Mol Plant-Microbe In **12:** 901-10

Cohen L and Eyal Z (1993) The histology of processes associated with the infection of resistant and susceptible wheat cultivars with *Septoria tritici*. Plant Pathol **42:** 737-743

De Wit PGM and Spikman G (1982) Evidence for the occurrence of race and cultivar-specific elicitors of necrosis in intercellular fluids of compatible interjections of *Cladosporium fulvum* and tomato Resistance genes, leaf mold. Physiol Plant Pathol **21:** 1-11

Duncan KE and Howard RJ (2000) Cytological analysis of wheat infection by the leaf blotch pathogen *Mycosphaerella graminicola*. Mycol Res **104:** 1074-1082

Eyal Z, Scharen AL, Prescott JM and van Ginkel M (1987) The Septoria diseases of wheat: Concepts and methods of disease managment CIMMYT, Mexico. pp. 51

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. Mol Plant-Microbe In **15:** 102-108

Gisi U, Chin KM, Knapova G, Farber RK, Mohr U, Parisi S, Sierotzki H and Steinfeld U (2000) Recent developments in elucidating modes of resistance to phenylamide, DMI and strobilurin fungicides. Crop Protect **19:** 863-872

Harrabi M, Cherif M, Amara H, Ennaiffer Z and Daaloul A (1993) *In vitro* selection for resistance to *Septoria tritici* in wheat. CIMMYT, Mexico. pp. 109-116

Heath MC (1971) Haustorial sheath formation in cowpea leaves immune to rust infection. Phytopathology **61:** 383-388

Higgins CF (1992) ABC transporters: from microorganisms to man. Annu Rev Cell Biol **8:** 67-113

Kalb VF, Woods CW, Turi TG, Dey CR, Sutter TR and Loper JC (1987) Primary structure of the P450 lanosterol demethylase gene from *Saccharomyces cerevisiae.* DNA **6:** 529-537

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

Kema GHJ and Van Silfhout CH (1997) Genetic variation for virulence and resistance in the wheat-*Mycosphaerella graminicola* pathosystem. III. Comparative seedling and adult plant experiments. Phytopathology **87:** 266-272

Kema GHJ, Verstappen ECP and Waalwijk C (2000) Avirulence in the wheat septoria tritici leaf blotch fungus *Mycosphaerella graminicola* is

controlled by a single locus. Mol Plant-Microbe In **13:** 1375-1379

Nelson LR and Marshall D (1990) Breeding wheat for resistance to *Septoria nodorum* and *Septoria tritici*. Adv Agron **44:** 257-277

Payne AC, Grosjean-Cournoyer MC and Hollomon DW (1998) Transformation of the phytopathogen *Mycosphaerella graminicola* to carbendazim and hygromycin B resistance. Curr Genet **34:** 100-104

Perrone G, Logrieco A, Kema GJH, Ritieni A and Bottalico A (1999) Phytotoxic activity of *Mycosphaerella graminicola* culture filtrates. Phytosfere '99, Rome, Italy, pp. 39-45

Pinkerton F and Strobel G (1976) Serinol as an activator of toxin production in attenuated cultures of *Helminthosporium sacchari* (Eyespot disease of sugarcane). Proc Natl Acad Sci USA **73:** 4007-4011

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

Pnini-Cohen S, Zilberstein A, Schuster S, Sharon A and Eyal Z (1997) 15th Long Ashton International Symposium, Understanding Pathosystems: A Focus on Septoria. Long Ashton, UK. pp. 20

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. Mol Plant-Microbe In **14:** 562-571.

Stergiopoulos I, Gielkens MM, Goodall SD, Venema K and De Waard MA (2002a) Molecular cloning and characterisation of three new ATP-binding cassette transporter genes from the wheat pathogen *Mycosphaerella graminicola*. Gene **289:** 141-149

Stergiopoulos I, Zwiers L-H and De Waard MA (2002b) Secretion of natural and synthetic toxic compounds from filamentous fungi by membrane transporters of the ATP-binding cassette and Major facilitator superfamily. Eur J Plant Pathol **108:** 719-734

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

Voloshchuk GD, Voloshchuk SI and Girko VS (1994) Effect of culture filtrates of some fungal pathogens on wheat suspension culture. Tritol i Genet **29:** 70-77

Weibull J and Niemeyer HM (1995) Changes in dihydroxymethoxybenzoxazinone glycoside content in wheat plants infected by three plant pathogenic fungi. Physiol Mol Plant Pathol **47:** 201-212

Wilson UE and Coffey MD (1980) Cytological evaluation of general resistance to *Phytophthora infestans* [late blight] in potato foliage. Ann Bot **45:** 81-90

Zwiers L-H, and De Waard MA (2000) Characterisation of the ABC transporter genes MgAtr1 and MgAtr2 from the wheat pathogen *Mycosphaerella graminicola.* Fung Gen Biol **30:** 115-125

Zwiers L-H and De Waard MA (2001) Efficient *Agrobacterium tumefaciens*-mediated gene disruption in the phytopathogen *Mycosphaerella graminicola*. Curr Genet **39:** 388-393

Zwiers L-H, Stergiopoulos I, Gielkens MMC, Goodall SD and De Waard MA ABC transporters of the wheat pathogen *Mycosphaerella graminicola* function as protectants against biotic and antibiotic toxic compounds. S*ubmitted*

CHAPTER 6

ABC TRANSPORTERS AND AZOLE SUSCEPTIBILITY IN LABORATORY STRAINS OF THE WHEAT PATHOGEN *MYCOSPHAERELLA GRAMINICOLA*

Lute-Harm Zwiers, Ioannis Stergiopoulos, Johannes G.M. Van Nistelrooy and Maarten A. De Waard

Antimicrobial Agents and Chemotherapy
(2002) ***46:*** *3900-3906*

ABSTRACT

Laboratory strains of *Mycosphaerella graminicola* with a decreased susceptibility to the azole antifungal agent cyproconazole showed a multidrug resistance phenotype by exhibiting cross-resistance to the unrelated chemicals cycloheximide and/or rhodamine 6G. Decreased azole susceptibility was found to be associated with either decreased or increased levels of accumulation of cyproconazole. No specific relationship could be observed between azole susceptibility and the expression levels of the ATP-binding cassette (ABC) transporter genes *MgAtr1-MgAtr5* and the sterol P450 14α-demethylase gene, *CYP51*. The ABC transporter MgAtr1 was identified as a determinant in azole susceptibility, since heterologous expression of the protein reduced the azole susceptibility of *Saccharomyces cerevisiae* and disruption of *MgAtr1* in one specific *M. graminicola* laboratory strain with constitutive *MgAtr1* overexpression, restored the level of susceptibility to cyproconazole to wild-type levels. However, the level of accumulation in the mutant with a *MgAtr1* disruption did not revert to the wild-type level. We propose that variations in azole susceptibility in laboratory strains of *M. graminicola* are mediated by multiple mechanisms

Keywords: ATP-binding cassette (ABC) transporters; azoles; multidrug resistance; mutant; resistance; *Septoria tritici*

INTRODUCTION

In wheat growing areas septoria tritici blotch, caused by the fungus *Mycosphaerella graminicola* (anamorph *Septoria tritici*) is recognised as a major disease. The resulting loss in yield is estimated in millions of metric tons of grain and billions of U.S. dollars each year (Eyal *et al.,* 1987). Therefore, control of this disease either by resistance breeding or through chemical control is of major importance.

An important group of antifungal agents used to control *M. graminicola* is the sterol demethylation inhibitors (DMIs), which interfere with ergosterol biosynthesis through inhibition of the sterol P450 14α-demethylase ($P450_{14DM}$). Azole antifungal agents in which the triazole ring acts as the active moiety are the most important group of DMIs. They have a broad-spectrum antifungal activity, protective and curative properties, and low phytotoxicity. Although mutants with resistance to DMIs could be easily obtained in the laboratory, the risk of resistance development in the field was initially considered low, as laboratory mutants generally suffer a fitness penalty (De Waard and Van Nistelrooy, 1990). At present, resistance to azoles is widespread in various foliar pathogens, such as powdery mildews and scab (Hollomon, 1993). However, these pathogens can still be controlled by a limited number of DMIs with relatively high intrinsic activity. This is also true for the control of septoria blotch of wheat, in which, despite the intensive use of cyproconazole and other azole antifungal agents, no indications of a decreased susceptibility to these compounds have been found (Gisi *et al.,* 1997).

Resistance to azole antifungal agents can be caused by alterations in sterol biosynthesis (Joseph-Horne *et al.,* 1995a), mutations at the $P450_{14DM}$ target site (Joseph-Horne *et al.,* 1995b; Sanglard *et al.,* 1998) or increased levels of expression of the $P450_{14DM}$-encoding gene, *CYP51* (Hamamoto *et al.,* 2000; Van den Brink *et al.,* 1996). Another important resistance mechanism is reduction of the intracellular concentration of the antifungal agent by means of an increased active efflux system. This mechanism operates in a broad variety of both plant and animal pathogens and is attributed to the increased activity of ATP-binding cassette (ABC) transporters (De Waard, 1997; Andrade *et al.,* 2000; Del Sorbo *et al.,* 2000). ABC transporters became known for their role in multidrug resistance (MDR) in human tumour cells (Juliano and Ling, 1976). They also function in MDR of filamentous fungi to

antifungal agents and unrelated chemicals (Andrade *et al.,* 2000; Balzi and Goffeau, 1995; Sanglard *et al.,* 1995).

We are interested in the role of ABC transporters from *M. graminicola* in pathogenesis and in the susceptibility of this fungus to antifungal agents. To assess the role of these transporters in azole susceptibility of *M. graminicola,* we selected laboratory strains with decreased susceptibility to the azole antifungal agent cyproconazole. All strains were analysed for their susceptibility to azoles and other chemically unrelated compounds, their levels of accumulation of [^{14}C]cyproconazole, and their levels of expression of five ABC-transporter genes (*MgAtr1-MgAtr5*) and the sterol P450 14α-demethylase gene (*CYP51)*. *MgAtr1* was disrupted by *Agrobacterium tumefaciens*-mediated transformation in strains with constitutive *MgAtr1* overexpression and the transformants were phenotypically characterised. Results presented indicate that in *M. graminicola*, multiple mechanisms may contribute to the variation in susceptibility to azole antifungal agents.

MATERIALS AND METHODS

Fungal material and culture conditions.

In this study two field isolates of *M. graminicola* were used, isolate I323 was isolated in The Netherlands in 1981 (Kema and Van Silfhout, 1997) and isolate M1 was collected in France in 1993 and was provided by Dr J.M. Seng (Biotransfer, Montreuil, France). Strains were grown yeast-like in liquid yeast-sucrose medium (yeast extract 10 g l^{-1}, sucrose 10 g l^{-1}) at 18°C and 140 rpm or on solid V8-agar plates (50% V8 vegetable juice, 50% water, 2.5% agar) at 18°C. Mycelium used in accumulation studies was obtained by inoculating 100 ml of Czapek Dox-mycological peptone (Czapek Dox 33.4 g l^{-1}, mycological peptone 5 g l^{-1}) with 3 x 10^4 cells per ml and incubation on a rotary shaker (25°C, 140 rpm) for an additional 3 days.

Complementation of *Saccharomyces cerevisiae* was performed using strain AD12345678 (Δ*yor1* Δ*snq2* Δ*pdr5* Δ*pdr10* Δ*pdr11* Δ*ycf1* Δ*pdr3* Δ*pdr15* Δ*ura3*) (Decottignies *et al.,* 1998).

Susceptibility assays and isolation of laboratory strains.

The antifungal agents tested were the triazoles cyproconazole (Syngenta), propiconazole (Syngenta), and tebuconazole (Bayer AG), the protein synthesis inhibitor cycloheximide (Sigma), and the dye rhodamine 6G (Sigma). Minimum inhibitory concentrations (MICs) were determined for yeast-like growing cells on V8-agar plates or potato dextrose agar plates (PDA; 39 g l^{-1}). Toxicity tests were performed by spotting 5 µl of a cell suspension with a density of 4 x 10^5 cells per ml, harvested from 3-day-old liquid medium, on 9-ml Petri dishes containing V8-agar or PDA amended with different concentrations of toxicants. The concentrations of compounds used in the susceptibility assays ranged from 0.025 to 1.5 mg l^{-1} for the triazoles, from 2.5 to 1500

mg l^{-1} for cycloheximide, and from 2.5 to 250 mg l^{-1} for rhodamine 6G. Experiments were performed three times in triplicate, and MIC values were assessed visually after 10 days of incubation at 18°C in the dark.

M. graminicola strains with decreased susceptibility to the azole antifungal agent cyproconazole were isolated by plating 1 x 10^5 yeast-like cells on V8-agar in 14-cm Petri dishes amended with cyproconazole at three times the MIC value of the wild-type parent strains. After 10 days of incubation colonies were isolated from these plates.

Susceptibility assays of *S. cerevisiae* were performed on solid synthetic media containing Bacto Yeast Nitrogen Base without amino acids (6.7 g l^{-1}), dropout mix (2 g l^{-1} containing amino acids without uracil), galactose (20 g l^{-1}), Noble agar (20 g l^{-1}), and cycloheximide or cyproconazole at various concentrations. Cycloheximide was used at 0.01, 0.05, 0.1, and 0.25 µg ml^{-1}, cyproconazole was used at 0.003, 0.01, and 0.025 µg ml^{-1}, and rhodamine 6G was used at 0.5, 1, and 5 µg ml^{-1}. Cultures of *S. cerevisiae* were grown overnight in liquid synthetic medium at 30°C. The overnight culture was diluted to an optical density at 600 nm (OD_{600}) of 0.5, and subsequently, 5 and 10 µl were spotted on plates. Drug susceptibility was scored visually after incubation for 3 days at 30°C in the dark.

Gene disruption.

Agrobacterium tumefaciens-mediated transformation was used to disrupt *MgAtr1* in strains I323C1 and M1C4. Generation of disruption constructs and selection of transformants with the disrupted *MgAtr1* gene were performed as described previously (Zwiers and De Waard, 2001).

Accumulation of [^{14}C]cyproconazole.

Mycelium was homogenised and harvested by filtering over 0.85-mm- and 55-µm-pore-size filters. Subsequently, mycelium was washed with 50 mM sodium-phosphate buffer (pH 6.0), resuspended in 50 mM sodium phosphate buffer (pH 6.0, 1% glucose) at a density of 6 mg wet weight per ml and incubated for 30 min at 25°C and 140 rpm. Subsequently, [^{14}C]cyproconazole (kindly provided by Syngenta) was added to an external concentration of 100 µM. Mycelium (5 ml) was harvested at intervals of 10 min by vacuum filtration, washed 5 times with 5 ml of phosphate buffer, and radioactivity in the biomass was measured with a Beckman LS6000TA liquid scintillation counter. Accumulation levels of [^{14}C]cyproconazole were calculated as nmol per mg of dry weight. Energy dependency of [^{14}C]cyproconazole accumulation was tested by the addition of carbonyl-cyanide m-chlorophenylhydrazone (CCCP, 20 µM) and subsequent measurement of the level of [^{14}C]cyproconazole accumulation.

DNA and RNA manipulations.

M. graminicola genomic DNA isolated from 5-day-old yeast-like cells (Raeder and Broda, 1985) was used to amplify the open reading frame (ORF) of the *M. graminicola CYP51* gene (GenBank accession no: AF263470). Amplification reaction volumes (50 µl) contained dATP, dCTP, dGTP and dTTP (200 µM), primers (1.2 µM), AmpliTaq DNA polymerase (0.5 U) (Perkin Elmer) and a reaction buffer (10 mM Tris-HCl pH 8.3, 50 mM KCl, 3 mM $MgCl_2$). DNA was denatured for 3 min at 94°C followed by 2 min at 50°C, and 2 min at 72°C. This initial cycle was followed by 29 cycles of 1 min at 94°C, 1 min at 58°C and 1 min at 72 °C. The amplification was

stopped with an extension of 10 min at 72°C. PCR primers used were CYP5' (GGTACCATGGGTCTCCTCCAGGAAG) and CYP3' (TCCCTCCTCTCCCACTTTAC). Amplification products were isolated from agarose gel and directly sequenced.

Northern blot analysis was performed with RNA isolated from wild-type and laboratory-generated strains. The levels of expression of the ABC transporter-encoding genes *MgAtr1* (GenBank accession no: AJ243112), *MgAtr2* (GenBank accession no: AJ243113) (Zwiers and De Waard, 2000), *MgAtr3* (GenBank accession no: AF364105), *MgAtr4* (GenBank accession no: AF329852), and *MgAtr5* (GenBank accession no: AF364104) (Stergiopoulos *et al.,* 2002) and the *CYP51* gene were examined. Total RNA was isolated using the TRIzol reagent (Life Technologies). RNA (10 µg) was separated on a 1.2% agarose gel containing glyoxal and transferred to Hybond-N nylon membranes (Amersham). Equal loading and transfer of RNA was determined by staining northern blots with methylene blue and hybridisation with the 18S rRNA subunit of *Aspergillus niger* (Melchers *et al.,* 1994). Hybridisations were performed at 65°C in Nasmyth's hybridisation solution (1.1 M NaCl, 0.3 M Na_2HPO_4, 0.011 M Na_2EDTA, 1.85% sarcosyl, 18.5% dextran sulphate, pH 6.2, and 100 µg of denatured herring sperm DNA ml^{-1}).

Complementation of *Saccharomyces cerevisiae*

Full-length cDNA clones of *MgAtr1* were made from poly-A^+ RNA isolated from yeast-like cells of *M. graminicola*. Amplification of full-length cDNA was performed with the Advantage Klentaq polymerase mix (Clontech) according to the manufacturer's instructions. cDNA clones were cloned in the yeast expression vector pYes2 (Invitrogen) and transformed to *S. cerevisiae* strain AD12345678. Yeast transformants containing the empty vector pYes2 were used as controls.

RESULTS

Susceptibility assays to azole antifungal agents.

The MIC values of cyproconazole for the field strains I323 and M1 were approximately 0.1 µg ml^{-1} on PDA and 0.3 µg ml^{-1} on V8-agar. Both strains showed a similar cross-susceptibility to the triazoles propiconazole and tebuconazole (data not shown).

To elucidate the potential role of ABC transporters in susceptibility to azoles, strains I323 and M1 were subjected to selection with cyproconazole. Strains with a decreased susceptibility to cyproconazole were isolated from V8-agar plates amended with cyproconazole at three times the MIC values of both parent strains. For both I323 and M1 the frequency of resistant colonies was around 10^{-4}. The relative decrease in susceptibility of several strains was determined and ranged between factors of 3 and 6 (Table 1). All strains showed cross-resistance to propiconazole and tebuconazole (data not shown). Repetitive

subculturing of the strains under nonselective conditions showed that all I323-derived strains were stable, whereas the M1-derived strains M1A1 and M1D1 lost their decreased susceptibility to cyproconazole. The susceptibility of the strains to several chemically unrelated chemicals was determined in order to study whether the strains would have an MDR phenotype. Indeed, all strains exhibited a low degree of cross-resistance to cycloheximide and/or rhodamine 6G (Table 1).

Table 1. Susceptibility of field isolates, laboratory-generated strains with decreased sensitivity to azoles, and *MgAtr1* disrupted mutants of *Mycosphaerella graminicola* to cyproconazole, cycloheximide, and rhodamine 6G.

[a] Strains I323A1, I323C1, I323C4, I323E1, and strains M1A1, M1B1, M1C1, M1C4, and M1D1 are laboratory-generated strains derived from the wild-type field isolates I323 and M1. Strains I323C1Δ1-1 and I323C1Δ1-2 and strains M1C4Δ1-1 and M1C4Δ1-2 are transformants of I323C1 and M1C4, respectively, with a disrupted allele of *MgAtr1*.

[b] The strains were initially resistant; resistance was lost upon subculturing.

[c] ND, not determined

	MIC (μg ml^{-1})		
Isolate[a]	Cyproconazole	Cycloheximide	Rhodamine 6G
I323	0.1	500	25
I323A1	0.4	1000	25
I323C1	0.75	1500	150
I323C4	0.5	1000	25
I323E1	0.75	1500	50
I323C1Δ1-1	0.1	25	25
I323C1Δ1-2	0.1	25	25
M1	0.1	500	15
M1A1	0.4[b]	ND[c]	30
M1B1	0.3	ND	30
M1C1	0.3	ND	30
M1C4	0.5	1500	100
M1D1	0.3[b]	ND	30
M1C4Δ1-1	0.5	1500	100
M1C4Δ1-2	0.5	1500	100

Accumulation of [^{14}C]cyproconazole.

The decreased susceptibility of the strains to cyproconazole could be due to reduced levels of intracellular accumulation of the antifungal agent in mycelial cells. Therefore, the levels of cyproconazole that accumulated in cells were measured over time (Figure 1A and B). The levels of [^{14}C]cyproconazole accumulation of the two wild-type strains I323 and M1 did not differ significantly and amounted, on average, 1 and 0.9 nmol cyproconazole per mg dry weight, respectively. In all strains derived from strain I323 the levels of accumulation of [^{14}C]cyproconazole decreased significantly by factors of 3 to 4 (Figure 1A). The levels of accumulation by the M1-derived strains M1B1 and M1C1 decreased by factors of 2 to 3. In contrast, the level of accumulation of [^{14}C]cyproconazole by strain M1C4 was higher than the level of accumulation by the wild-type and increased over time (Figure 1B). In all strains tested, accumulation appeared to be due to an energy-dependent efflux, as addition of CCCP increased the accumulation of cyproconazole (Figure 1C).

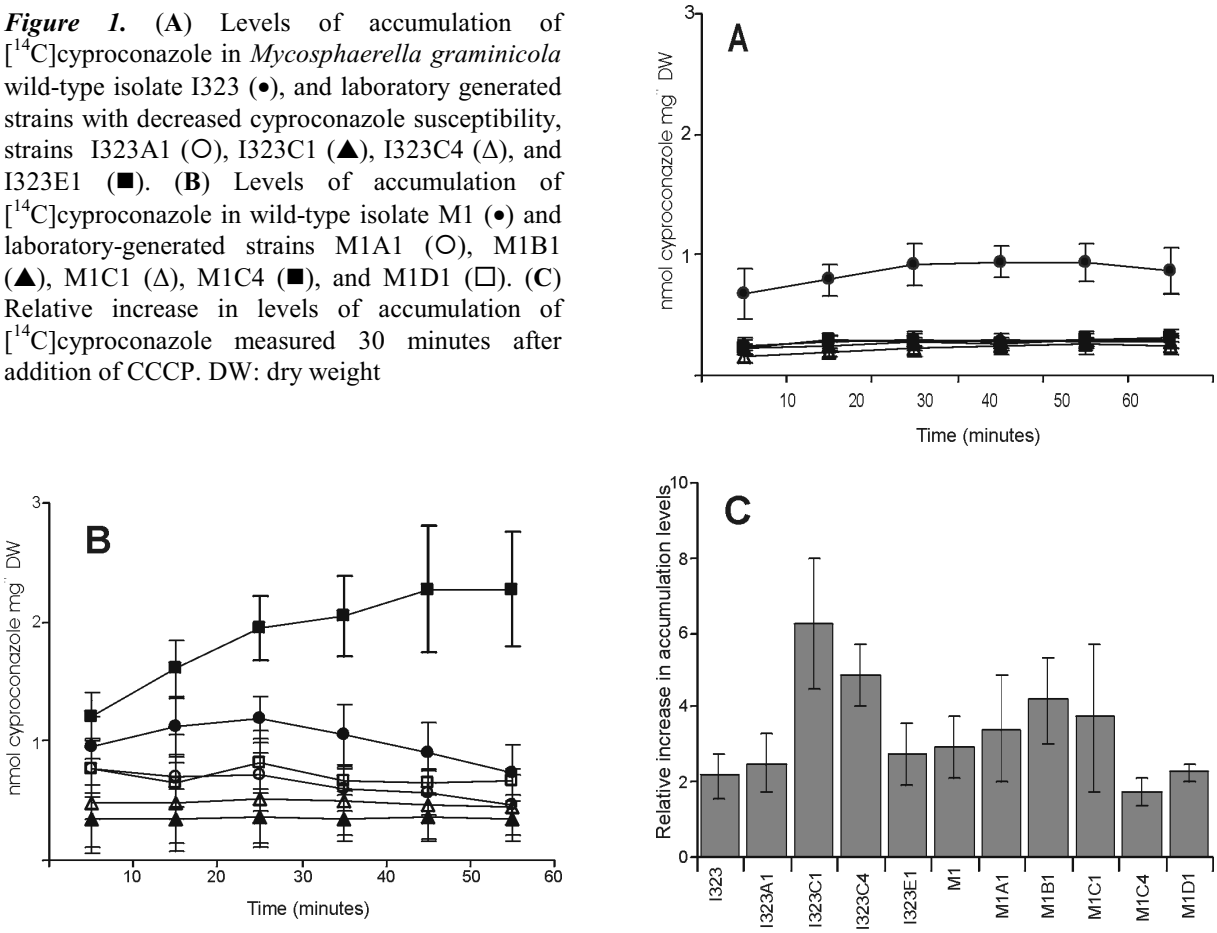


Figure 1. (**A**) Levels of accumulation of [^{14}C]cyproconazole in *Mycosphaerella graminicola* wild-type isolate I323 (•), and laboratory generated strains with decreased cyproconazole susceptibility, strains I323A1 (○), I323C1 (▲), I323C4 (Δ), and I323E1 (■). (**B**) Levels of accumulation of [^{14}C]cyproconazole in wild-type isolate M1 (•) and laboratory-generated strains M1A1 (○), M1B1 (▲), M1C1 (Δ), M1C4 (■), and M1D1 (□). (**C**) Relative increase in levels of accumulation of [^{14}C]cyproconazole measured 30 minutes after addition of CCCP. DW: dry weight

Northern analysis

The MDR phenotype of the laboratory-generated strains could indicate that ABC transporters are involved in the mechanism of resistance (Kolaczkowski *et al.,* 1998). Therefore, the levels of expression of the ABC transporter genes *MgAtr1-MgAtr5* were studied. Northern analysis in untreated yeast-like cells demonstrated that almost all laboratory-generated strains exhibit a change in the basal expression level of at least one of the ABC transporter genes whose levels of expression were tested (Figure 2). For instance, the levels of expression of *MgAtr1* were highly increased in strains I323C1 and M1C4 compared to those in their respective wild-type parent strains. In addition, both strains showed increased levels of expression of *MgAtr5*. Even strain M1A1, which lost its resistance to cyproconazole, still showed a clear overexpression of an ABC transporter gene, *e.g. MgAtr2*. However, all these expression data indicate that there is no consistent correlation between the observed susceptibility profile of a strain and the profile of expression of any of the ABC transporter genes tested. All strains were also tested for levels of expression of the *CYP51* gene. Only I323E1 showed, besides upregulation of *MgAtr4*, an increased level of *CYP51* expression.

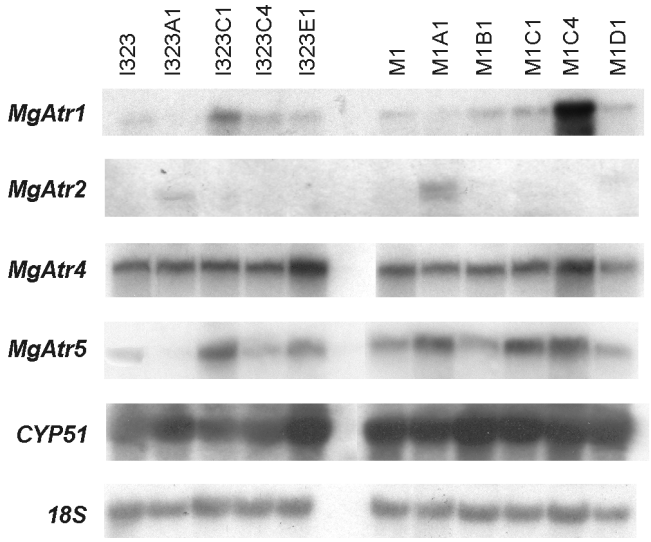


Figure 2. Expression levels of *MgAtr1*, *MgAtr2*, *MgAtr4*, *MgAtr5*, and *CYP51* in *Mycosphaerella graminicola* wild-type strains (I323 and M1) and laboratory-generated strains (I323A1, I323C1, I323C4, I323E1, M1A1, M1B1, M1C1, M1C4 and M1D1). The *18S* ribosomal RNA gene is used as loading control.

Analysis of *CYP51*

Using primers directed against the *CYP51* gene from *M. graminicola*, a 1903 bp fragment comprising the entire ORF was amplified from the wild-type strains I323 and M1 and from the laboratory-generated strains I323C1 and M1C4. The deduced amino acid sequence of the proteins encoded by the genes from wild-type strains and both laboratory-generated strains were identical (data not shown).

Analysis of *MgAtr1*

Strains I323C1 and M1C4 both constitutively overexpressed *MgAtr1*, but showed opposite accumulation of cyproconazole. The role of *MgAtr1* in the decreased susceptibility to cyproconazole was analysed in more detail by complementation of a *S. cerevisisae* mutant with *MgAtr1* and by disruption of *MgAtr1* in I323C1 and M1C4 by means of *A. tumefaciens*-mediated transformation.

The heterologous expression of *MgAtr1* in *S. cerevisiae* showed that the presence of MgAtr1 results in decreased susceptibility of the yeast to both cycloheximide and cyproconazole (Figure 3) Therefore, both cycloheximide and cyproconazole are potential substrates of MgAtr1.

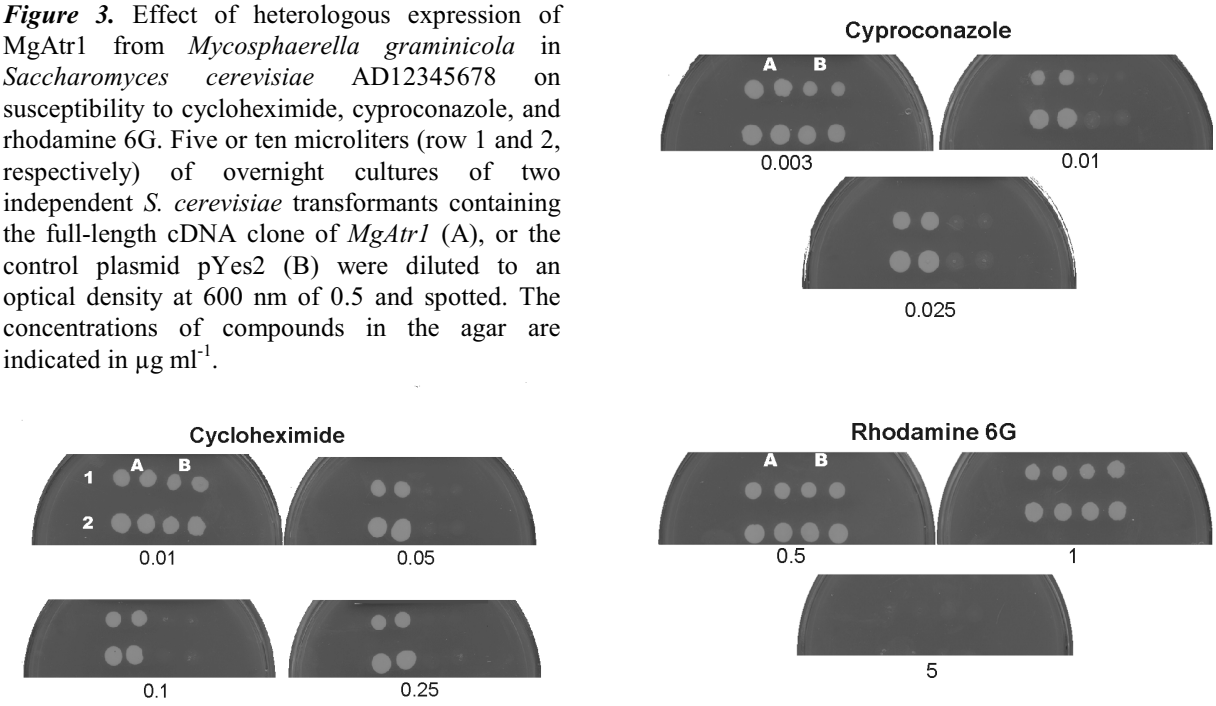


Figure 3. Effect of heterologous expression of MgAtr1 from *Mycosphaerella graminicola* in *Saccharomyces cerevisiae* AD12345678 on susceptibility to cycloheximide, cyproconazole, and rhodamine 6G. Five or ten microliters (row 1 and 2, respectively) of overnight cultures of two independent *S. cerevisiae* transformants containing the full-length cDNA clone of *MgAtr1* (A), or the control plasmid pYes2 (B) were diluted to an optical density at 600 nm of 0.5 and spotted. The concentrations of compounds in the agar are indicated in µg ml^{-1}.

After *A. tumefaciens*-mediated transformation of both strain I323C1 and strain M1C4, two disruptants containing a single copy of the transforming DNA were isolated and characterised with respect to *MgAtr1* expression, their azole susceptibility, and levels of cyproconazole accumulation. Northern analysis demonstrated that disruption of *MgAtr1* resulted in disappearance of full-length *MgAtr1* mRNA (Figure 4). The levels of accumulation of [^{14}C]cyproconazole by the *MgAtr1* disruptants of both I323C1 and M1C4 compared to those for their respective parent strains were similar (data not shown). The *MgAtr1* disruptants derived from I323C1 had wild-type (strain I323) susceptibility to cyproconazole and rhodamine 6G. Moreover, they became hypersensitive to cycloheximide (Figure 5A), demonstrating a role for *MgAtr1* in protection against these compounds in I323C1. In contrast, the *MgAtr1* disruptants derived from M1C4 remained less susceptible to these compounds (Figure 5B).

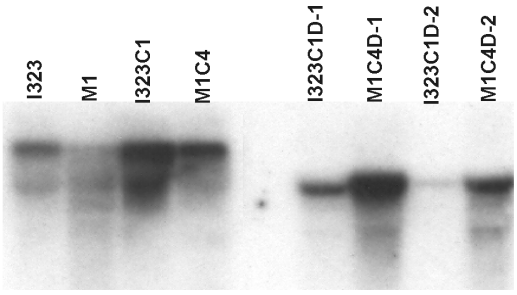


Figure 4. Expression of *MgAtr1* in *Mycosphaerella graminicola* wild-type strains (strains I323 and M1), laboratory-generated strains (strains I323C1 and M1C4) and independent transformants of I323C1 and M1C4 with a disrupted *MgAtr1* allele (I323C1Δ-1, I323C1Δ-2, M1C4Δ-1 and M1C4Δ-2).

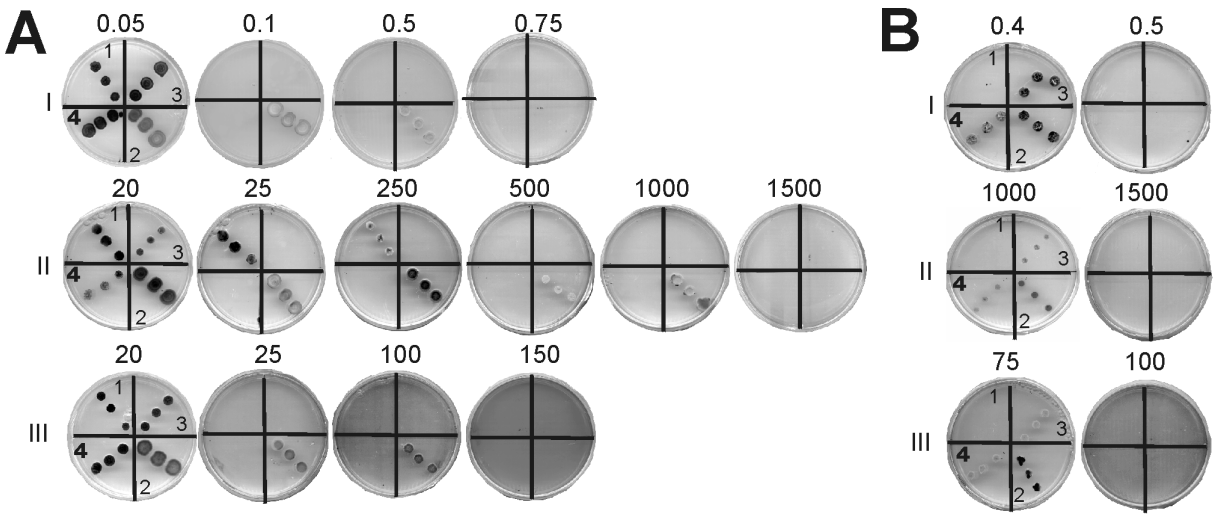


Figure 5. Susceptibility of *Mycosphaerella graminicola* wild-type strains, laboratory-generated azole-resistant strains, and *MgAtr1* knockout mutants of laboratory-generated azole-resistant strains to cyproconazole, cycloheximide, and rhodamine 6G. (A): wild-type isolate I323 (quadrant 1), laboratory-generated strain I323C1 (quadrant 2), and two independent transformants of I323C1 with the disrupted *MgAtr1* allele (quadrants 3 and 4); (B): wild-type isolated M1 (quadrant 1), laboratory-generated strain M1C4 (quadrant 2), and two independent transformants of M1C4 with the disrupted *MgAtr1* allele (quadrants 3 and 4). The concentration of cyproconazole (I), cycloheximide (II), and rhodamine 6G (III) used is indicated in µg ml^{-1}.

DISCUSSION

Laboratory-generated strains of *M. graminicola* with a decreased susceptibility to the azole antifungal agent cyproconazole were selected at a rate of around 10^{-4}. This frequency is not unique for *M. graminicola,* since similar frequencies have been observed for other filamentous fungi, *e.g*. *Aspergillus nidulans* and *Nectria haematococca*. Genetical analysis of azole-resistant strains of these organisms identified a polygenic system for azole resistance (Kalamarakis *et al.,* 1991; Van Tuyl, 1977). Recently, in *Candida glabrata* the development of so-called high-frequency azole resistance (HFAR), which occurred at frequencies comparable to those in *M. graminicola,* was described (Sanglard *et al.,* 2001). We propose that the decreased susceptibility of the laboratory-generated strains can be regarded as microbial resistance (Sanglard and Odds, 2002)

The susceptibility of the laboratory-generated strains is still within the range observed in field populations of *M. graminicola* (Gisi *et al.,* 1997). This suggests that the mechanisms underlying the microbial resistance to azoles in laboratory strains can also occur in natural

populations of the pathogen and, thus, contribute to the natural variation in baseline susceptibility.

Most strains of *M. graminicola* with decreased azole susceptibility exhibited cross-resistance to the chemically unrelated compounds cycloheximide and rhodamine 6G. Such an MDR phenotype in strains with decreased azole susceptibility has been described for the yeasts *Candida albicans* (Sanglard *et al.,* 1995), *C. glabrata* (Sanglard *et al.,* 2001), and *S. cerevisiae* (Balzi *et al.,* 1994) and the filamentous fungi *A. nidulans* (Del Sorbo *et al.,* 1997), *Botrytis cinerea* (Hayashi *et al.,* 2001), *Penicillium digitatum* (Nakaune *et al.,* 1998), and *P. italicum* (Guan *et al.,* 1992). Most laboratory strains of *M. graminicola* with an MDR phenotype exhibited decreased levels of accumulation of cyproconazole. This observation suggests that the MDR is associated with energy-dependent transport of drugs that results in a decreased cellular content of toxicants (De Waard, 1997; Del Sorbo *et al.,* 2000). However, the level of accumulation of [^{14}C]cyproconazole by strain M1C4 was higher than the level of accumulation by the wild-type and increased over time. This indicates that multiple mechanisms contribute to the variation in azole susceptibility in *M. graminicola*. The increased level of accumulation by M1C4 may be caused by cell wall changes that lead to an increased nonspecific binding of the compound to cell wall components. The relatively low increase in the level of accumulation of [^{14}C]cyproconazole in M1C4 after addition of the uncoupler CCCP might offer support for this mechanism. Increased levels of accumulation of azoles have also been described for triadimenol-resistant laboratory-strains of *Ustilago maydis* (Wellmann and Schauz, 1993). An alternative mechanism for the increased level of accumulation of cyproconazole by strain M1C4 might be active sequestration of the fungicide in vacuoles. This protection mechanism is well described in plants, in which sequestering of endotoxins, heavy metals, and natural pigments occurs through a specific subclass of ABC transporters (Rea, 1999). In line with this explanation is the increase in the level of [^{14}C]cyproconazole accumulation in M1C4 over time.

Differences in levels of drug accumulation can be mediated by changes in ABC transporter activity due to overexpression of ABC transporter genes. Therefore, the levels of expression of all ABC transporter genes cloned so far from *M. graminicola* (*MgAtr1-MgAtr5*) were analysed in all strains. Northern analysis indicated that the moderate changes in susceptibility to azoles are associated with profound changes in the levels of expression of the ABC transporter genes, suggesting that the regulation of the genes examined in the laboratory-

generated strains is quite different from that in the parent strains. However, it is not possible to associate the level of expression of a specific ABC transporter gene with the observed phenotype. This indicates that multiple transporters may be involved in azole susceptibility or that the transporter of prime importance for azole transport in *M. graminicola* is not yet identified.

We have studied the ABC transporter gene *MgAtr1* in more detail since this gene was overexpressed in strains I323C1 and M1C4, which displayed decreased and increased levels of accumulation of cyproconazole, respectively. Complementation of a *S. cerevisiae* mutant with *MgAtr1* resulted in a decrease in susceptibility of the yeast transformants to cyproconazole and cycloheximide. Disruption of *MgAtr1* in I323C1 restored the susceptibility to cyproconazole and rhodamine 6G to wild-type levels and even resulted in hyper-susceptibility to cycloheximide. Therefore, all three compounds are potential substrates of MgAtr1 in *M. graminicola*.

Disruption of *MgAtr1* in I323C1 did not cause the level of accumulation of cyproconazole to revert to the level found for the wild-type strain. Thus, our data prove that MgAtr1 can provide protection against azole antifungals in *S. cerevisiae* and *M. graminicola,* but also show that the overall level of accumulation is not affected by the disruption of *MgAtr1* in strain I323C1. These seemingly conflicting results are hard to explain but suggest that in I323C1 decreased influx and not increased efflux by MgAtr1 causes the reduced accumulation. Apparently, MgAtr1 can only act as a determinant of azole susceptibility when overexpressed and when azole influx is impaired. These results also imply that the overall level of accumulation of cyproconazole by laboratory strains of *M. graminicola* is probably not an indicator of either azole susceptibility or the exclusive involvement of ABC transporters. This contrasts with the situation in *A. nidulans* and *B. cinerea* (Andrade *et al.,* 2000; Stehmann and De Waard, 1995; Vermeulen *et al.,* 2001) and emphasises the complexity of mechanisms that contribute to azole accumulation in *M. graminicola*.

As *MgAtr1* is not upregulated in all selected laboratory strains selected and as disruption of *MgAtr1* in M1C4 did not alter the phenotype it is clear that besides overproduction of MgAtr1 other mechanisms act as determinants involved in the MDR phenotype. Disruption of other ABC transporter genes in the laboratory strains should indicate if additional ABC transporters are involved. This is well possible, since ABC transporters are members of a large protein

superfamily and are known to possess overlapping substrate specificities (Decottignies and Goffeau, 1997; Kolaczkowski *et al.,* 1998).

Sequencing of *CYP51* from *M. graminicola* strains I323C1 and M1C4 did not show any of the point mutations reported to confer resistance to azole antifungal agents in *C. albicans* (Gisi *et al.,* 2000; Sanglard *et al.,* 1998). This indicates that at least in these strains, mutations in the *CYP51* gene are not involved in decreased susceptibility to cyproconazole. However, overexpression of *CYP51*, as observed in strain I323E1, could contribute to decreased susceptibility (Hamamoto *et al.,* 2000; Van den Brink *et al.,* 1996).

In summary the data described suggest that in laboratory strains of *M. graminicola,* multiple mechanisms contribute to the variation in azole susceptibility. One mechanism involves the upregulation of *MgAtr1* and possibly more ABC transporter genes, which lead to an increased efflux of antifungal agents. Other mechanisms may involve changes in cell wall composition or sequestration of the antifungal agent in cellular compartments, resulting in increased levels of accumulation. Finally, reduced levels of passive influx may play a role. Multiple mechanisms probably operate in individual strains. Multiple mechanisms of azole resistance also function in *C. albicans* and *P. digitatum* (Hamamoto *et al.,* 2001; Kohli *et al.,* 2002; Perea *et al.,* 2001). This situation complicates investigations on mechanisms of resistance mechanisms to azoles in *M. graminicola*. At present, we are examining whether multiple mechanisms also account for the variation in azole susceptibility in field populations of *M. graminicola*.

ACKNOWLEDGEMENTS

The authors acknowledge Dr G.H.J. Kema and Dr C. Waalwijk (Plant Research International, Wageningen, The Netherlands) for discussions within the Wageningen Mycosphaerella group, Prof. Dr P.J.G.M. De Wit (WUR) and Dr D. Sanglard (University Hospital Lausanne, Switzerland) for critical reading of the manuscript. M. Collina, and J. Zilverentant are acknowledged for technical assistance. L-H. Zwiers was financially supported by Syngenta, Switzerland, and I. Stergiopoulos by the Training and Mobility of Researchers (TMR) Programme – Marie Curie Research Grants, The European Commission (Contract No. ERBFMBICT983558).

REFERENCES

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

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

Balzi E, Wang M, Leterme S, VanDyck L and Goffeau A (1994) PDR5, a novel yeast multidrug resistance conferring transporter controlled by the transcription regulator PDR1. J Biol Chem **269:** 2206-2214

Balzi E and Goffeau A (1995) Yeast multidrug resistance: the PDR network. J Bioenerg Biomembr **27:** 71-76

De Waard MA (1997) Significance of ABC transporters in fungicide sensitivity and resistance. Pestic Sci **51:** 271-275

De Waard MA and Van Nistelrooy JGM (1990) Stepwise development of laboratory resistance to DMI-fungicides in *Penicillium italicum*. Neth J Plant Pathol **96:** 321-329

Decottignies A and Goffeau A (1997) Complete inventory of the yeast ABC proteins. Nat Genet **15:** 137-145

Decottignies A, Grant AM, Nichols JW, De Wet H, McIntosh DB and Goffeau A (1998) ATPase and multidrug transport activities of the overexpressed yeast ABC protein Yor1p. J Biol Chem **273:** 12612-12622

Del Sorbo G, Andrade AC, Van Nistelrooy JGM, Van Kan JA, Balzi E and De Waard MA (1997) Multidrug resistance in *Aspergillus nidulans* involves novel ATP-binding cassette transporters. Mol Gen Genet **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 Genet Biol **30:** 1-15

Eyal Z, Scharen AL, Prescott JM and van Ginkel M (1987) The Septoria diseases of wheat: Concepts and methods of disease managment CIMMYT, Mexico, pp. 51

Gisi U, Hermann D, Ohl L and Steden C (1997) Sensitivity profiles of *Mycosphaerella graminicola* and *Phytophthora infestans* populations to different classes of fungicides. Pestic Sci **51:** 290-298

Gisi U, Chin KM, Knapova G, Farber RK, Mohr U, Parisi S, Sierotzki H and Steinfeld U (2000) Recent developments in elucidating modes of resistance to phenylamide, DMI and strobilurin fungicides. Crop Prot **19:** 863-872

Guan J, Kapteyn JC, Kerkenaar A and De Waard MA (1992) Characterisation of energy-dependent efflux of imazalil and fenarimol in isolates of *Penicillium italicum* with a low, medium and high degree of resistance to DMI-fungicides. Neth J Plant Pathol **98:** 313-324

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*. Appl Environ Microbiol **66:** 3421 - 3426

Hamamoto H, Nawata O, Hasegawa K, Nakaune R, Lee YJ, Makizumi K and Hibi T (2001) The role of the ABC transporter gene *PMR1* in demethylation inhibitor resistance in *Penicillium digitatum*. Pestic Biochem Physiol **70:** 19-26

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.* Pestic Biochem Physiol **70:** 168-179

Hollomon DW (1993) Resistance to azole fungicides in the field. Biochem Soc T **21:** 1047-1051

Joseph-Horne T, Manning NJ, Hollomon D and Kelly SL (1995a) Defective sterol $D^{5(6)}$desaturase as a cause of azole resistance in *Ustilago maydis*. FEMS Microbiol Lett **374:** 1-2

Joseph-Horne T, Hollomon D, Loeffler RST and Kelly SL (1995b) Altered P450 activity associated with direct selection for fungal azole resistance. FEBS Lett **374:** 174-178

Juliano RL and Ling V (1976) A surface glycoprotein modulating drug permeability in Chinese hamster ovary cell mutants. Biochim Biophys Acta **455:** 152-162

Kalamarakis AE, De Waard MA, Ziogas BN and Georgopoulos SG (1991) Resistance to fenarimol in *Nectria haematococca* var. *cucurbitae*. Pestic Biochem Physiol **40:** 212-220

Kema GHJ and Van Silfhout CH (1997) Genetic variation for virulence and resistance in the wheat-*Mycosphaerella graminicola* pathosystem. III. Comparative seedling and adult plant experiments. Phytopathology **87:** 266-272

Kohli A, Smriti NFN, Mukhopadhyay K, Rattan A and Prasad R (2002) *In vitro* low-level resistance to azoles in *Candida albicans* is associated with changes in membrane lipid fluidity and asymmetry. Antimicrob Agents Chemother **46:** 1046-1052

Kolaczkowski M, Kolaczkowska A, Luczynski J, Witek S and Goffeau A (1998) In vivo characterization of the drug resistance profile of the major ABC transporters and other components of the yeast pleiotropic drug resistance network. Microb Drug Resist **4:** 143-158

Melchers WJ, Verweij PE, Van den Hurk P, Van Belkum A, De Pauw BE, Hoogkamp Korstanje JA and Meis JF (1994) General primer-mediated PCR for detection of *Aspergillus* species. J Clin Microbiol **32:** 1710-1717

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*. Appl Environ Microbiol **64:** 3983-3988

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. Antimicrob Agents Chemother **45:** 2676-2684

Raeder U and Broda P (1985) Rapid preparation of DNA from filamentous fungi. Lett Appl Microbiol **1:** 17-20

Rea PA (1999) MRP subfamily ABC transporters from plants and yeast. J Exp Bot **50:** 895-913

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. Antimicrob Agents Chemother **39:** 2378-2386

Sanglard D, Ischer F, Koymans L and Bille J (1998) Amino acid substitutions in the cytochrome P-450 lanosterol 14alpha -demethylase (CYP51A1) from azole-resistant *Candida albicans* clinical isolates contribute to resistance to azole antifungal agents. Antimicrob Agents Chemother **42:** 241-253

Sanglard D, Ischer F and Bille J (2001) Role of ATP-binding-cassette transporter genes in High-Frequency Acquisition of Resistance to azole antifungals in *Candida glabrata*. Antimicrob Agents Chemother **45:** 1174-1183

Sanglard D and Odds FC (2002) Resistance of *Candida* species to antifungal agents: molecular mechanisms and clinical consequences. Lancet Infect Dis **2:** 73-85

Stehmann C and De Waard MA (1995) Accumulation of tebuconazole by isolates of *Botrytis cinerea* differing in sensitivity to sterol demethylation inhibiting fungicides. Pestic Sci **45:** 311-318

Stergiopoulos I, Gielkens MMC, Goodall SD, Venema K and De Waard MA (2002) Molecular cloning and characterisation of three new ABC transporter encoding genes from the wheat pathogen *Mycosphaerella graminicola*. Gene **289:** 141-149

Van den Brink HJ, Van Nistelrooy JGM, De Waard MA, Van den Hondel CA and Van Gorcom RF (1996) Increased resistance to 14 alpha-demethylase inhibitors (DMIs) in *Aspergillus niger* by coexpression of the *Penicillium italicum* eburicol 14 alpha-demethylase (cyp51) and the *A. niger* cytochrome P450 reductase (cprA) genes. J Biotechnol **49:** 13-8

Van Tuyl JM (1977) Genetics of fungal resistance to systemic fungicides. Meded Landbouwhogesch Wageningen, The Netherlands

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 Manag Sci **57:** 393-402

Wellmann H and Schauz K (1993) DMI-resistance in *Ustilago maydis*: II. Effect of triadimefon on regenerating protoplasts and analysis of fungicide uptake. Pestic Biochem Physiol **46:** 55-64

Zwiers L-H and De Waard MA (2000) Characterization of the ABC transporter genes *MgAtr1* and *MgAtr2* from the wheat pathogen *Mycosphaerella graminicola*. Fung Genet Biol **30:** 115-125

Zwiers L-H and De Waard MA (2001) Efficient *Agrobacterium tumefaciens*-mediated gene disruption in the phytopathogen *Mycosphaerella graminicola*. Curr Genet **39:** 388-393

CHAPTER 7

MULTIPLE MECHANISMS ACCOUNT FOR VARIATION IN BASE-LINE SENSITIVITY TO AZOLE FUNGICIDES IN FIELD ISOLATES OF *MYCOSPHAERELLA GRAMINICOLA*

Ioannis Stergiopoulos, Johannes G.M. Van Nistelrooy, Gert H.J. Kema and Maarten A. De Waard

Submitted

ABSTRACT

Molecular mechanisms that account for variation in base-line sensitivity to azole fungicides were examined in a collection of twenty field isolates collected in France and Germany, of the wheat pathogen *Mycosphaerella graminicola.* The isolates tested represent the wide base-line sensitivity to the azole fungicide tebuconazole described previously. The isolates were cross-sensitive to other azoles tested, such as cyproconazole and ketoconazole but not to unrelated chemicals like cycloheximide, kresoxim-methyl, or rhodamine 6G. Progenies from a genetic cross between an isolate with an intermediate and a high sensitivity to azoles displayed a continuous range of phenotypes with respect to cyproconazole sensitivity, indicating that variation in azole sensitivity in this haploid organism is polygenic. The basal level of expression of the ATP-binding cassette transporter genes *MgAtr1-MgAtr5* from *M. graminicola* significantly varied amongst the isolates tested, but no clear increase in the transcript level of a particular *MgAtr* gene was found in the less sensitive isolates. Cyproconazole strongly induced expression of *MgAtr4* but no correlation between expression levels of this gene and azole sensitivity was observed. One isolate with intermediate sensitivity to azoles overexpressed *CYP51,* encoding cytochrome P450 sterol 14α-demethylase from *M. graminicola*. Isolates with a low or high sensitivity to azoles were tested for accumulation of cyproconazole, but no clear correlation between reduced accumulation of the fungicide in mycelium and sensitivity to azoles was observed. Therefore, differences in accumulation can not exclusively account for the variation in base-line sensitivity of the isolates to azoles. The results indicate that multiple mechanisms account for differences in base-line sensitivity to azoles in field isolates of *M. graminicola*.

Keywords: ATP-binding cassette (ABC) transporters; azoles; base-line sensitivity; *CYP51*; sterol demethylation inhibitors (DMIs); fungicide resistance; *Septoria tritici*

INTRODUCTION

Azoles represent a major class of fungicides that has been extensively used over the past three decades in the control of fungal pathogens of medical and agricultural importance. They are systemic fungicides with both protective and curative activity in disease control (Kuck and Scheinpflug, 1986). Their mode of action is based on inhibition of cytochrome P450 sterol 14α-demethylase ($P450_{14DM}$) activity, a key enzyme involved in the biosynthesis of ergosterol in fungi. For this reason azole fungicides are also described as sterol demethylation inhibitors (DMIs) (Sisler and Ragsdale, 1984),

Because of their site-specific mode of action, the risk of resistance development to DMIs is significant. However, laboratory-generated azole-resistant mutants often display reduced fitness with respect to spore germination, mycelial growth, and virulence and their levels of resistance are low. For these reasons, resistance development under field-conditions was considered unlikely (De Waard and Fuchs, 1982; Fuchs and De Waard, 1982; Dekker, 1981). Nevertheless, DMI resistance emerged in several pathogen populations, although relatively slowly as compared to other classes of fungicides (De Waard, 1994). One of the first cases of field resistance was reported for *Sphaerotheca fuliginea*, the causal agent of cucumber powdery mildew (Schepers, 1985). Since then, DMI resistance is also reported in other fungal pathogens, such as *Penicillium digitatum* (Eckert, 1987), *Erysiphe graminis* f. sp. *hordei* (Heaney, 1988), *Venturia inaequalis* (Hildebrand *et al.,* 1988), and *Rhynchosporium secalis* (Kendall *et al.,* 1993).

Analysis of azole resistance in fungi revealed that different molecular mechanisms may operate in resistant isolates. In *Candida albicans,* several alterations in *CYP51* (also described as *ERG11*), that encodes $P450_{14DM}$, have been identified. These include point mutations in the coding region of *CYP51* that result in reduced affinity of azoles for the target enzyme (Marichal *et al.,* 1999) or overexpression of this gene that results in higher intracellular levels of $P450_{14DM}$ (Vanden Bossche *et al.,* 1994; White, 1997; Franz *et al.,* 1998; Kontoyiannis *et al.,* 1999; Perea *et al.,* 2001). Similar alterations in *CYP51* have been reported in fungal pathogens of agricultural importance. Point mutations in *CYP51* associated with decreased azole sensitivity have been described in laboratory-generated mutants of *Ustilago maydis* (Joseph-Horne *et al.,* 1995) and *P. digitatum* (Van Nistelrooy *et al.,* 1996), and in field isolates of *Uncinula necator* (Délye *et al.,* 1997) and *E. graminis* f. sp. *hordei* (Délye *et al.,*

1998). Resistance to azoles in *P. digitatum* and *V. inaequalis* positively correlates with increased expression of *CYP51*, caused by the presence of tandem repeats in the promoter region of this gene (Hamamoto *et al.,* 2000; Schnabel and Jones, 2001).

Reduced accumulation of azoles in cells as a result of active efflux mechanisms constitutes another well-characterised mechanism of resistance. Early reports indicated that fenarimol-resistant strains of *A. nidulans* accumulate less fungicide compared to the wild-type control (De Waard and Van Nistelrooy, 1979; De Waard and Van Nistelrooy, 1980). The reduced accumulation is ascribed to an increased energy-dependent efflux of the compounds from cells, mediated by ATP-binding cassette (ABC) transporters (Del Sorbo *et al.,* 1997; Andrade *et al.,* 2000). ABC transporters comprise one of the best-characterised protein families associated with active excretion of drugs. They utilise the energy derived from hydrolysis of ATP to drive the transport of a wide variety of cytotoxic agents over biological membranes (Higgins, 1992). In particular, ABC transporters of filamentous fungi are known to exhibit an important function in protection against synthetic toxic compounds such as antibiotics, fungicides, and other xenobiotics (Stergiopoulos *et al.,* 2002b). Overproduction of ABC transporters can result in pleiotropic effects such as simultaneous resistance to structurally unrelated compounds, a phenomenon described as multidrug resistance (MDR) (Gottesman and Pastan, 1993).

Despite the extensive knowledge on molecular mechanisms of azole resistance in laboratory-generated mutants, the relevance and frequency of such mechanisms in field populations of fungal pathogens is still unclear. In *P. digitatum* increased expression levels of ABC transporters genes such as *PMR1* and *PMR5* as well as increased expression of *CYP51* were detected in DMI-resistant isolates (Hamamoto *et al.,* 2000). In this paper we studied mechanisms that could potentially explain the wide variation in base-line sensitivity to azoles in field isolates of the fungus *Mycosphaerella graminicola* (Fuckel) J. Schroeter in Cohn (anamorph: *Septoria tritici* Roborge in Desmaz.), the causal agent of septoria tritici blotch of wheat. The economic importance of this disease has dramatically increased over the past few decades and control management of this pathogen has widely involved the use of azole fungicides. In laboratory-generated azole-resistant mutants of *M. graminicola,* different mechanisms of resistance to azoles may operate amongst others, overexpression of specific ABC transporter genes (Zwiers *et al.*, 2002). Resistance development in *M. graminicola* to azole fungicides under field conditions has not been reported (Gisi *et al.,* 1997). However,

field populations of *M. graminicola* consist of isolates with a broad range in sensitivity levels to these fungicides (Gisi and Hermann, 1994; Suty and Kuck, 1996).

Here we studied twenty field isolates of *M. graminicola* sampled in France and Germany that possess a significant difference in base-line sensitivity to the azole fungicide tebuconazole. Analysis of the progeny of a genetic cross between two isolates with a 100-fold difference in sensitivity to cyproconazole, indicated that azole sensitivity in this fungus is polygenic. Expression studies of ABC transporter genes and *CYP51* showed that isolates display large differences in basal level of expression of these genes. Accumulation of cyproconazole did not correlate with azole sensitivity. The results indicate that multiple mechanisms may operate in field isolates of *M. graminicola* that determine base-line sensitivity to azole fungicides.

MATERIALS AND METHODS

Fungal strains

Eleven field isolates of *M. graminicola* originating from France and nine field isolates derived from Germany (Table 1) were kindly provided by Dr J.M. Seng (Biotransfer, Montreuil Cedex, France) and Dr A. Suty (Bayer AG, Landwirtschaftszentrum Monheim, Leverkusen, Germany), respectively. The isolates were sampled from wheat fields treated with tebuconazole and selected for differential sensitivity to this fungicide. Single spore isolates were prepared from bulk samples and used throughout the whole study. A genetic cross between isolates M1 and M3 was made according to Kema *et al.,* (1996) and 40 progeny isolates (M1M3 1 - M1M3 40) were obtained. Stock-cultures of the isolates were kept as yeast-like cells at –80°C.

Culture conditions

Yeast-like cells of the isolates were grown in liquid Yeast Sucrose Medium (YSM, 10 g l^{-1} yeast extract, 10 g l^{-1} sucrose) at 18°C and 140 rpm. Mycelium was grown in liquid Czapek Dox-Mycological Peptone medium (CzD-MP, 33.4 g l^{-1} Czapek Dox, 5 g l^{-1} mycological peptone) at 25°C and 140 rpm. Toxicity assays were performed in Potato Dextrose Broth (PDB, 24 g l^{-1}) at 25°C.

Compounds

The chemicals used in this study were the azole fungicides cyproconazole (Syngenta, Basel, Switzerland), ketoconazole (Janssen Research Foundation, Beerse, Belgium), and tebuconazole (Bayer AG, Landwirtschaftszentrum Monheim, Leverkusen, Germany), the antibiotic cycloheximide (Sigma-Aldrich Chemie

BV, Zwijndrecht, The Netherlands), the QoI-inhibitor (QoI) kresoxim-methyl (BASF AG, Limburgerhof, Germany), and the dye rhodamine 6G (Sigma-Aldrich Chemie BV, Zwijndrecht, The Netherlands). All compounds were of technical grade and used as 100x concentrated stock solutions of 1000, 562, 316, 178, 100, 56.2, 31.6, 17.8, 10, 5.62, 3.16, 1.78, 1, 0.562, 0.316, 0.178 and 0.1 mg l^{-1} in methanol.

Fungicide activity test

Sensitivity of isolates to compounds was assessed in a microtitre plate test, adapted from Pijls *et al.,* (1994). Cell suspensions (10^5 ml^{-1}) of the isolates were prepared in PDB from three-day-old cultures. Wells of sterile flat-bottomed microtitre plates (Greiner BV, Alphen aan de Rijn, The Netherlands) were filled with cell suspension (50 µl) and mixed with PDB (150 µl) amended with fungicides at different concentrations. PDB with methanol (1%) was used as a control. All treatments were tested in four-fold per microtitre plate and tests were repeated at least once. Plates were sealed to prevent evaporation from wells and incubated for five days as still cultures at 25°C in the presence of light. Growth was assessed with a microtitre plate reader (Bio-Tek Instruments Inc. Vermont, U.S.A.) by measuring cell density at 630 nm. Sensitivity of the isolates to the compounds was expressed as Minimum Inhibitory Concentration (MIC) values. These values are the lowest concentration of compounds in the scale used that does not allow growth. The variation factor of isolates for sensitivity to compounds is defined as the ratio between the MIC value of the most and least sensitive isolate.

Expression analysis

RNA isolation and northern blot analysis.

YSM or CzD-MP (20 ml) was inoculated with yeast-like cells derived from stock cultures of the isolates tested. After three days of culturing at 18 or 25°C and 140 rpm, the optical density of the cultures was determined at 600 nm (OD_{600}) and new cultures (20 ml) with a starting density of OD_{600} 0.1 were initiated. Three days later, the OD_{600} of these cultures was adjusted to 0.5 in fresh medium (20 ml) and the cultures were incubated again overnight under similar conditions as described above. Cyproconazole treatments (10 mg l^{-1}, 0.1% methanol) were performed at 140 rpm for 1 h. Yeast-like cells or mycelium were grown in YSM at 18°C or CzD-MP at 25°C, respectively. Control treatments were performed with methanol (0.1%). Fungal biomass was harvested by centrifugation at 10.000 rpm and 0°C for 10 min, and instantly frozen in liquid nitrogen.

RNA was isolated from frozen biomass with the TRIzol® reagent (Life Technologies Inc. Maryland, U.S.A.) according to the manufacturer's instructions. RNA (10 µg, 4.5 µl) was denatured in a solution of 6 M glyoxal (4.5 µl), 0.1 M sodium phosphate (3.0 µl), and dimethyl sulfoxide (13.3 µl) at 50°C for 1 h and subjected to electrophoresis on 1.6% agarose gel in 10 mM sodium phosphate buffer, pH 7.0. Blotting was carried out on Hybond-N^+ membranes (Amersham Pharmacia Biotech, Little Chalfont Buckinghamshire, U.K.) by capillary transfer with 10x SSC solution. Homologous hybridisations were performed overnight at 65°C in Nasmyth's solution buffer (18.5% dextran sulphate, 1.85% sarcosyl, 0.011 M EDTA, 0.3 M Na_2HPO_4, 1.1 M NaCl, pH 6.2). This solution (5.4 ml) was mixed with distilled water (4.6 ml) just before use to obtain the hybridisation buffer (10 ml), while sheared herring sperm DNA (100 µg ml^{-1}) was included as blocking agent. Blots were washed twice in 2x SSC, 0.1x SDS and twice in 0.1x SSC, 0.1x SDS at 65°C for 15 min.

Probes.

Gene-specific probes of the ABC transporter genes *MgAtr1* (Acc. AJ243112), *MgAtr2* (Acc. AJ243113), *MgAtr3* (Acc. AF364105), *MgAtr4* (Acc. AF329852), and *MgAtr5* (Acc. AF364104) used in northern blot analysis represented a 0.84 kb *Eco*RI fragment of *MgAtr1*, a 0.75 kb *Sal*I fragment of *MgAtr2*, a 0.85 kb *Sal*I fragment of *MgAtr3*, an 1.14 kb *Bam*HI/*Pst*I fragment of *MgAtr4,* and a 0.6 kb *Eco*RI fragment of *MgAtr5*. A 0.65 kb DNA fragment of *CYP51* (Acc. AF263470), encoding $P450_{14DM}$ of *M. graminicola*, was obtained by PCR using genomic DNA from isolate IPO323 (Kema and Van Silfhout, 1997) as template. Primers used for amplification were CYPinternal4 (5′-CAGCACTCTTCATCTGCGAC-3′) and CYP3′anchor (5′-TCCCTCCTCTCCCACTTTAC-3′). PCR conditions consisted of 30 cycles of 1 min denaturation at 94°C, 1 min of annealing at 58°C, and 1 min of extension at 72°C with an additional extension step of 10 min at 72°C at the end of the reaction. Equal loading of samples on blots was examined with a 0.6 kb *Eco*RI fragment of the *18S* rRNA gene from *Aspergillus niger*.

Randomly primed DNA isotopic probes were prepared by enzymatic incorporation of [α^{32}P]-dATP. In each labelling reaction, 50 ng of a probe template was used. Probes were purified using the QIAquick Nucleotide Removal kit (QIAGEN, Westburg BV, Leusden, The Netherlands) before adding to the hybridisation solution.

Accumulation of [^{14}C]cyproconazole

Three-day-old mycelial cultures were harvested by filtering through a 0.85 mm pore sieve and by collection of the mycelium on a 0.055 mm pore sieve, washed with 50 mM phosphate buffer pH 6.0, and re-suspended in the same buffer at a net wet weight of 6 mg l^{-1}. Cultures were incubated at 25°C and 140 rpm for 30 min before addition of [^{14}C]cyproconazole, at an initial external concentration of 100 μM. Mycelium from cultures (5 ml) was collected by vacuum filtration at intervals of 5, 15, 25, 35, 45, 55, 95, and 105 min after addition of [^{14}C]cyproconazole, washed (5x) with phosphate buffer, pH 6.0 (5 ml) and stored in vials containing LUMASAFE™ PLUS (3 ml) (Groningen, The Netherlands). Energy-dependency of cyproconazole accumulation was examined by addition of carbonyl-cyanide m-chlorophenylhydrazone (CCCP, 20 μM), 55 min after addition of [^{14}C]cyproconazole. Radioactivity in the samples was measured with a Beckman LS6000TA liquid scintillation counter (Beckman Instruments Inc., Fullerton, U.S.A.). Accumulation of [^{14}C]cyproconazole was calculated as nmol of cyproconazole per mg dry weight of biomass.

Statistical analysis

Statistical analysis of the results was performed using SPSS for Windows release 10.0.5 (SPSS Inc. Chicago, Illinois, U.S.A.) and Microcal™Origin™ version 5.0 (Microcal Software Inc., Northampton, U.S.A.) statistical software. Linear association between sensitivity to different compounds or between sensitivity to azoles and accumulation of [^{14}C]cyproconazole was examined using the Pearson's correlation coefficient. Statistical significant differences between mean values of [^{14}C]cyproconazole accumulation were tested using the Student-Newman-Keuls (S-N-K) analysis at a significance level of 0.05 using 11 degrees of freedom.

RESULTS

Sensitivity assays

The sensitivity of the field isolates to the azole fungicides cyproconazole, ketoconazole, and tebuconazole varied significantly (Table 1). Mean MIC values of cyproconazole, ketoconazole, and tebuconazole were 44.8, 15.5, and 24.3 mg l^{-1}, respectively, indicating that ketoconazole was the most active azole tested (Table 2). MIC values of tebuconazole ranged between 0.316 and 56.2 mg l^{-1} indicating that the variation factor (defined as the ration between the MIC value of the most and least sensitive isolate) amounted 178. MIC values for cyproconazole and ketoconazole ranged between 0.0562 and 100 mg l^{-1} resulting in a variation factor of 1779. Box-and-Whiskers plots showed that the variation in MIC values of the isolates inside the boxes (50% of population) was lower for tebuconazole than for cyproconazole and ketoconazole (Figure 1).

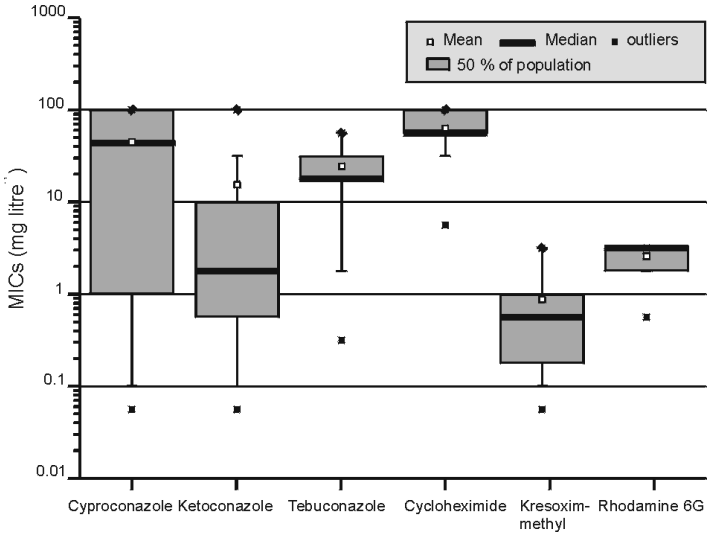


Figure 1. Box-and-Whiskers plots of MIC values of 20 field isolates of *Mycosphaerella graminicola* to the azole fungicides cyproconazole, ketoconazole, and tebuconazole, the antibiotic cycloheximide, the QoI-inhibitor kresoxim-methyl, and the dye rhodamine 6G.

Mean MIC values for the non-azole compounds cycloheximide, kresoxim-methyl, and rhodamine 6G were 63.0, 0.87, and 2.57 mg l^{-1}, respectively (Table 2). The data indicate that the QoI-inhibitor kresoxim-methyl was the most active compound of all chemicals tested. Sensitivity of the isolates to cycloheximide, kresoxim-methyl, and rhodamine 6G, showed a variation factor of 10, 56.2, and 5.62, respectively (Table 1). Hence, the variation in MIC values observed for non-azole compounds was much smaller in comparison to the variation observed for azoles.

Table 1. Sensitivity of *Mycosphaerella graminicola* field isolates to the azole fungicides cyproconazole, ketoconazole, and tebuconazole, the antibiotic cycloheximide, the QoI-inhibitor kresoxim-methyl, and the dye rhodamine 6G.

			Minimum Inhibitory Concentrations (mg litre^{-1})					
			Azole fungicides			Non-azole compounds		
Isolates	Year	Origin	Cyproconazole n=3 (M) / n=2 (S) [a]	Ketoconazole n=3 (M) / n=1 (S)	Tebuconazole n=2 (M) / n=1 (S)	Cycloheximide n=1	Kresoxim-methyl n=1	Rhodamine 6G n=1
M1	1993	France	0.1	0.178	1.78	100	3.16	3.16
M2	1993	France	1	10	17.8	5.62	0.1	3.16
M3	1993	France	17.8	5.62	31.6	100	0.0562	1.78
M4	1995	France	100	100	17.8	100	1	1.78
M5	1994	France	56.2	0.562	31.6	56.2	0.178	3.16
M6	1995	France	100	100	31.6	31.6	3.16	3.16
M7	1994	France	0.0562	0.1	0.316	56.2	0.562	1
M8	1995	France	0.178	0.1	0.562	56.2	3.16	3.16
M9	1995	France	56.2	10	17.8	56.2	0.178	3.16
M10	1994	France	56.2	31.6	31.6	56.2	0.0562	3.16
M11	1995	France	10	0.562	17.8	56.2	0.562	3.16
S006	1996	Germany	1	0.0562	1.78	10	1	0.562
S009	1996	Germany	31.6	5.62	31.6	100	3.16	3.16
S030	1998	Germany	56.2	1.78	17.8	100	1	3.16
S042	1998	Germany	100	1	31.6	100	0.562	1.78
S043	1998	Germany	0.1	0.1	17.8	56.2	0.178	3.16
S054	1996	Germany	100	0.562	56.2	31.6	1	3.16
S175	1997	Germany	10	0.562	17.8	100	0.178	1.78
S176	1997	Germany	100	31.6	56.2	56.2	0.562	1.78
S190	1997	Germany	100	10	56.2	31.6	0.562	3.16

[a] Number of repetitions (n) for French (M) and German (S) isolates

Table 2. Sensitivity of field isolates of *Mycosphaerella graminicola* to the azole fungicides cyproconazole, ketoconazole, and tebuconazole, the antibiotic cycloheximide, the QoI-inhibitor kresoxim-methyl, and the dye rhodamine 6G.

	Minimum Inhibitory Concentrations (mg l^{-1}) [a]			
Compounds	Minimum	Maximum	Mean	Median
Cyproconazole	0.0562	100	44.83	44.25
Ketoconazole	0.0562	100	15.50	11.66
Tebuconazole	0.316	56.2	24.36	17.80
Cycloheximide	5.62	100	63.00	56.20
Kresoxim-methyl	0.0562	3.16	0.87	0.79
Rhodamine 6G	0.562	3.16	2.57	3.16

[a] Average of 20 isolates tested

Cross sensitivity

Sensitivity of the isolates to cyproconazole and tebuconazole (r = 0.82, P < 0.0001), cyproconazole and ketoconazole (r = 0.71, P < 0.0001), and tebuconazole and ketoconazole (r = 0.64, P < 0.003) correlated positively, indicating cross sensitivity to the azoles tested. In contrast, no correlation was observed between sensitivity to azole fungicides and the non-azole compounds tested (Table 3, Figure 2). Similar results were obtained when cross sensitivity was examined for French and German isolates, separately (data not shown).

Table 3. Patterns of cross sensitivity of 20 field isolates of *Mycosphaerella graminicola* to the azole fungicides cyproconazole, ketoconazole, and tebuconazole, the antibiotic cycloheximide, the QoI-inhibitor kresoxim-methyl, and the dye rhodamine 6G.

Compounds	Correlation	Cyproco-nazole	Ketoco-nazole	Tebuco-nazole	Cyclohe-ximide	Kresoxim-methyl	Rhoda-mine 6G
Cyproconazole	r[a]		**0.71**	**0.82**	0.18	0.04	0.22
	P[b]		**< 0.001**	**< 0.001**	0.45	0.87	0.35
Ketoconazole	r	**0.71**		**0.64**	0.02	- 0.03	0.29
	P	**< 0.0001**		**0.003**	0.92	0.90	0.22
Tebuconazole	r	**0.82**	**0.64**		0.07	- 0.14	0.43
	P	**< 0.0001**	**0.003**		0.77	0.55	0.06
Cycloheximide	r	0.18	0.02	0.07		0.11	0.17
	P	0.45	0.92	0.77		0.65	0.49
Kresoxim-methyl	r	0.04	- 0.03	- 0.14	0.11		- 0.05
	P	0.87	0.90	0.55	0.65		0.85
Rhodamine 6G	r	0.22	0.29	0.43	0.17	- 0.05	
	P	0.35	0.22	0.06	0.49	0.85	

[a] Pearson correlation coefficient;
[b] Significant correlation at p=0.05 level (in bold);

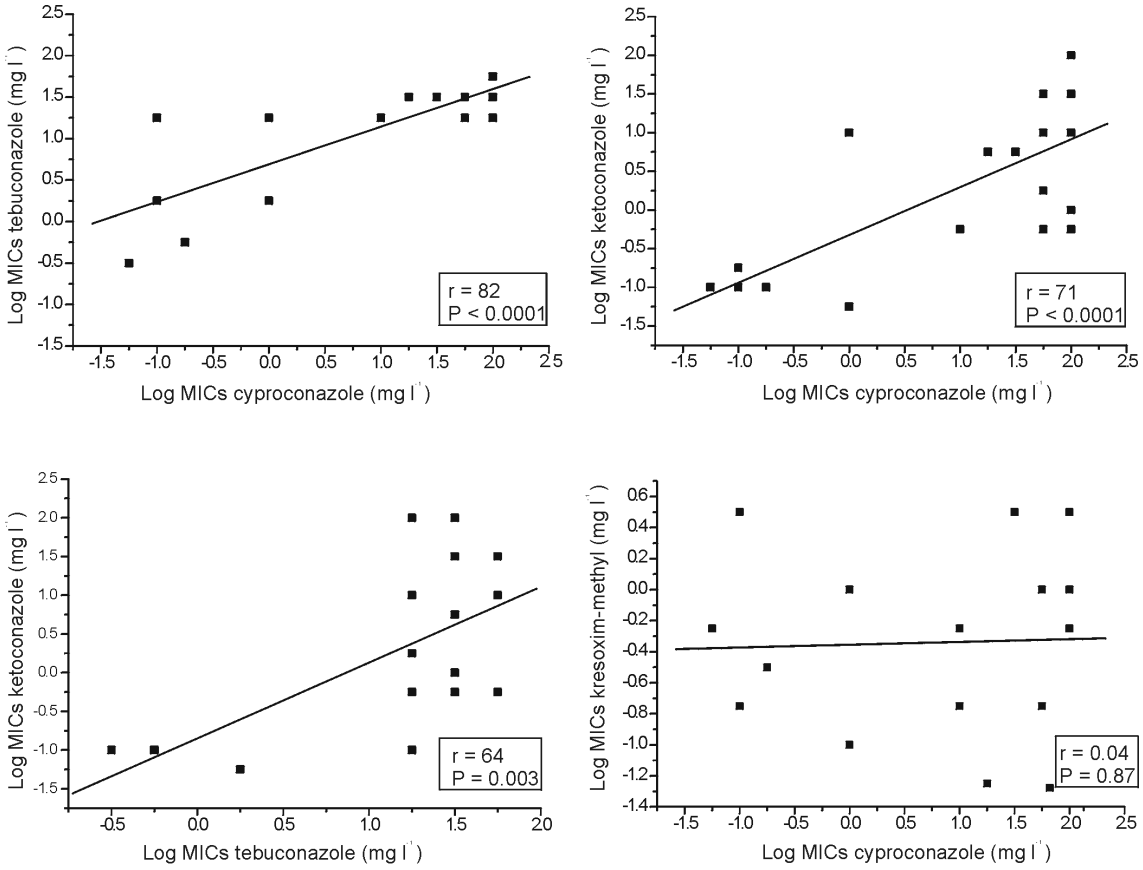
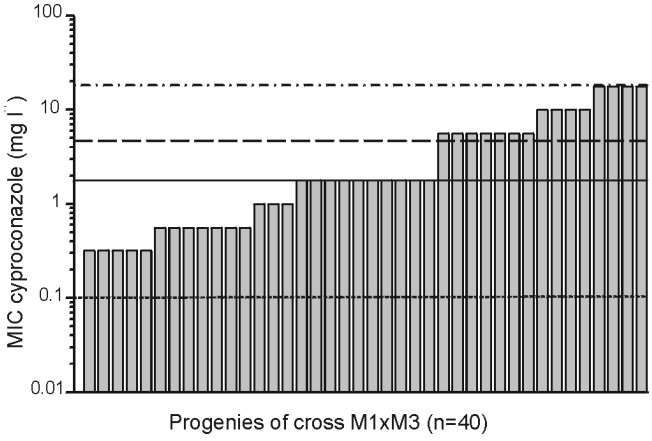


Figure 2. Correlation between sensitivity of 20 field isolates of *Mycosphaerella graminicola* to the azole fungicides cyproconazole, ketoconazole, and tebuconazole, and the QoI-inhibitor kresoxim-methyl.

Analysis of the progeny isolates

The sensitivity to cyproconazole of 40 progeny isolates obtained from a genetic cross between isolates M1 (MIC: 0.1 mg l^{-1}) and M3 (MIC: 17.8 mg l^{-1}) was tested. MIC values of the progeny isolates ranged between 0.316 and 17.8 mg l^{-1}, indicating a continuous distribution in sensitivity to cyproconazole between the MIC values of the parents (Figure 3).

Figure 3. Sensitivity of 40 progeny isolates of *Mycosphaerella graminicola* obtained from a cross between isolates M1 (MIC 0.1 mg l^{-1}) and M3 (MIC 17.8 mg l^{-1}) to cyproconazole. Median value is indicated as a continuous black line and mean value as a dashed line. Sensitivity of the parent isolates M1 and M3 are indicated as dotted and dash-dotted lines, respectively.



Expression analysis

Expression of the ABC transporter genes *MgAtr1-MgAtr5* (Zwiers and De Waard, 2000; Stergiopoulos *et al.,* 2002a) was examined in yeast-like cells and mycelium of isolates with high (M1, M7, M8, S043), medium (M3), and low (M4, M6, S054, S190) sensitivity to cyproconazole (Figure 4). Transcripts of *MgAtr2* and *MgAtr3* could not be detected in any of the isolates under the conditions tested (data not shown). In contrast, basal transcript levels of *MgAtr1*, *MgAtr4*, and *MgAtr5* varied significantly. Some isolates displayed a high basal level of expression for one specific gene only (*e.g. MgAtr1* in isolate S054) but not for the others. Such differences occurred in both yeast-like cells and the mycelium form of the fungus.

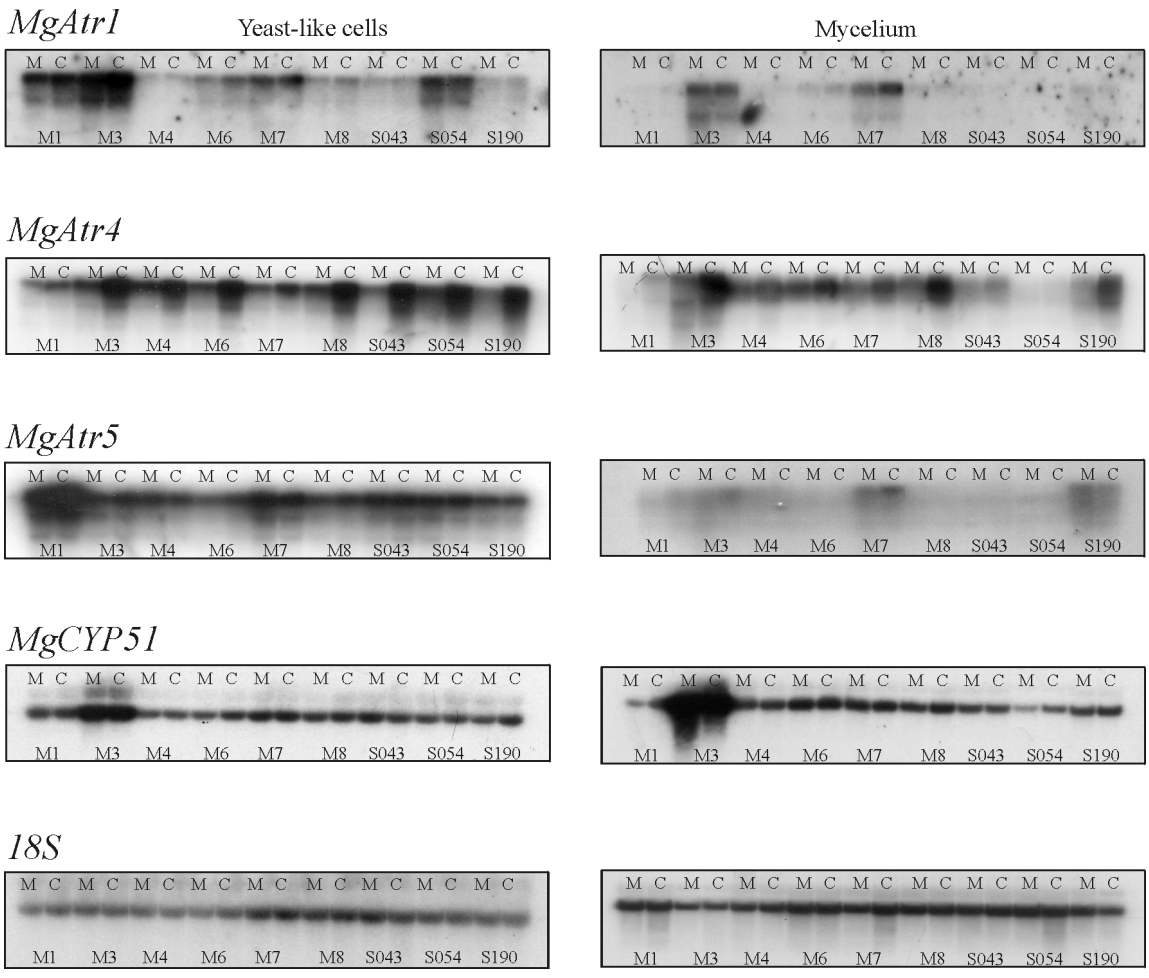


Figure 4. Northern blot analysis of the ABC transporter genes *MgAtr1, MgAtr4,* and *MgAtr5* and *CYP51* in yeast-like cells and mycelium of *Mycosphaerella graminicola* after treatment with cyproconazole (C). Control samples are treated with 0.1% methanol (M). Isolates tested had low (M1, M7, M8, S043), medium (M3), and high (M4, M6, S054, S190) MIC values of cyproconazole. Hybridisation of the blots with an *18S* rRNA probe from *Aspergillus niger* served as a loading control.

Obvious basal transcript levels of *MgAtr1* were found in yeast-like cells of isolates M1, M3, M7, and S054, and of *MgAtr4* and *MgAtr5* in all isolates tested, but for *MgAtr5* particularly in isolate M1. In mycelium, basal expression of *MgAtr1* was observed in isolates M3 and M7 and of *MgAtr4* in most of the isolates tested (except for M1 and S054). Basal expression of *MgAtr5* in mycelium was limited to isolates M3, M7, and S190. The basal expression level of *MgAtr5* significantly differed between yeast-like cells and mycelium for all the isolates tested.

Treatment with cyproconazole strongly induced transcription of *MgAtr4* in most of the isolates tested, especially in yeast-like cells. Induced levels of *MgAtr1* and *MgAtr5* were also observed but only for a limited number of isolates, such as M3 and M7 for *MgAtr1* and M1 for *MgAtr5*. Differences in induced level of expression among the isolates were also detected. For example, induction of *MgAtr4* in mycelium was much higher in isolates M3 and M8 than in isolates M6, M7, and S190. Nevertheless, a correlation between expression of a specific ABC gene and sensitivity of the isolates to cyproconazole was not clear.

CYP51 displayed basal transcripts in all isolates. However, in isolate M3 a relatively high basal level of expression was observed, especially in mycelium of the fungus. Treatment with cyproconazole did not effect the expression level of this gene (Figure 4).

Accumulation studies

Accumulation of [^{14}C]cyproconazole in thirteen field isolates tested proved to be constant in time. Mean accumulation levels measured ranged between 0.35 and 1 nmol of cyproconazole per mg dry weight (Table 4). Addition of CCCP induced higher cyproconazole accumulation levels in all isolates, indicating that accumulation was energy-dependent. No positive correlation between accumulation of [^{14}C]cyproconazole and sensitivity to cyproconazole (r = 0.39, P = 0.19), ketoconazole (r = 0.29, P = 0.34), or tebuconazole (r = 0.31, P = 0.31) was present. Statistical analysis for significant differences in cyproconazole accumulation identified four overlapping groups of isolates. Each group consisted of isolates with low and high sensitivity to azoles (Table 4). Isolates M4 and S190, both with a low sensitivity to cyproconazole (MIC 100 mg l^{-1}) displayed the highest (1.08 mg dry weight^{-1}) and lowest (0.36 mg dry weight^{-1}) accumulation level of cyproconazole as compared to the other isolates tested, respectively (Table 4; Figure 5).

Table 4. Accumulation of [^{14}C]cyproconazole by 13 field isolates of *Mycosphaerella graminicola* with different sensitivity levels to azole fungicides

Isolates	Sensitivity to cyproconazole (mg litre^{-1})	Number of replicates (n)	Mean Accumulation (nmol cyproconazole mg dry weight^{-1})	Statistical significance of accumulation[a]	Mean Accumulation after CCCP addition (nmol cyproconazole mg dry weight^{-1})
M7	0.0562	5	0.59 (0.14)[b]	a, b, c	2.08 (0.49)
M1	0.1	4	0.41 (0.09)	a, b	1.81 (0.42)
S043	0.1	8	0.51 (0.13)	a, b, c	1.76 (0.38)
M8	0.178	6	0.42 (0.06)	a, b	1.55 (0.50)
M2	1	5	0.80 (0.37)	b, c, d	2.11 (0.77)
S175	10	1	0.51 (-)	-	2.03 (-)
M3	17.8	6	0.58 (0.17)	a, b, c	2.08 (0.40)
M5	56.2	2	0.88 (0.01)	c, d	2.58 (0.05)
M4	100	9	1.08 (0.31)	d	2.59 (0.74)
M6	100	6	0.41 (0.09)	a, b	1.23 (0.28)
S054	100	6	0.88 (0.14)	c, d	2.40 (0.55)
S176	100	2	0.52 (0.12)	a, b, c	2.02 (0.37)
S190	100	3	0.36 (0.12)	a	1.43 (0.23)

[a] Statistical significant differences between mean values. Letters represent groups of homogeneous subsets calculated using the Student-Newman-Keuls (S-N-K) analysis. Sigma values for groups a, b, c, and d are 0.65, 0.09, 0.11, and 0.18, respectively. Differences were calculated at a significance level of p=0.05;
[b] Standard deviation;

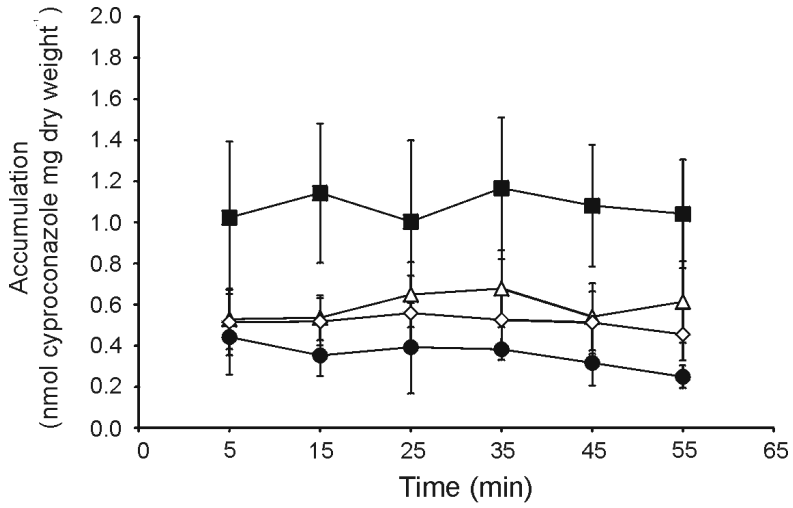


Figure 5. Accumulation of [^{14}C]cyproconazole by field isolates of *Mycosphaerella graminicola* with different levels of sensitivity to azole fungicides. MIC values of cyproconazole for isolates M4 (■), M7 (Δ), S043 (◊), and S190 (●) are 100, 0.0562, 0.1, and 100 mg l^{-1}, respectively.

DISCUSSION

In this study, we set out to investigate the physiological basis of variation in sensitivity to azole fungicides in field isolates of *M. graminicola*. The results obtained indicate that the field isolates of *M. graminicola* tested significantly vary in sensitivity to azole fungicides. The variation factor for sensitivity of the isolates for tebuconazole was 178. This value is in the same range as described by Suty and Kuck (1996), who reported a variation factor of 100 in a collection of more than 1500 isolates. The variation factors for the azole fungicides cyproconazole and ketoconazole amounted 1779 and this value is significantly higher than for tebuconazole. Such a broad variation in sensitivity for these compounds is intriguing, especially since the isolates were previously never exposed to ketoconazole, an azole antimycotic used in treatments of human fungal pathogens and to cyproconazole, which was not used in the fields from which the isolates were collected. Significant variation in base-line sensitivity to fungicides has been reported before and is ascribed to naturally tolerant genotypes that already exist in fungal populations prior to fungicide treatment (Brent, 1992). Studies monitoring the sensitivity of field populations of *M. graminicola* to azoles over the past decades have not yet detected the evolution of resistant phenotypes (Gisi *et al.,* 1997). Hence, the significant difference in sensitivity to the azoles tested is regarded as natural variation in base-line sensitivity to azole fungicides.

The field isolates tested showed cross-sensitivity to azole fungicides. Cross-sensitivity to azoles has been reported earlier for other fungal pathogens of both medical (White, 1997) and agricultural importance (Georgopoulos, 1982), including *M. graminicola* (Gisi *et al.,* 1997). A correlation in sensitivity between the azole fungicides and cycloheximide, kresoxim-methyl, or rhodamine 6G was not observed. These results indicate that field isolates with a relatively low sensitivity to azoles do not have a multidrug resistance phenotype, as described for laboratory-generated azole-resistant mutants of *M. graminicola* (Zwiers *et al.,* 2002). This discrepancy indicates that mechanisms of resistance that operate in laboratory-generated mutants and field isolates are different.

The sensitivity of progeny isolates obtained from a genetic cross between an isolate with high and intermediate sensitivity to cyproconazole ranged between the sensitivity of both parents. The frequency distribution of MIC values among the progeny was continuous and therefore, progeny isolates could not be classified in distinct sensitivity groups. We conclude

that sensitivity to cyproconazole in the haploid organism *M. graminicola* is polygenic. This observation may, at least in part, explain the extremely high variation factor in base-line sensitivity of the isolates to the azoles tested and may imply that different mechanisms are involved in azole tolerance in this fungus. This corroborates the observation that multiple resistance mechanisms may operate in laboratory-generated mutants of *M. graminicola* with decreased sensitivity to azoles (Zwiers *et al.,* 2002). Polygenic control of resistance to azole fungicides has been reported for *A. nidulans* (Van Tuyl, 1977) and *Nectria haematococca* var. *cucurbitae* (Kalamarakis *et al.,* 1991).

None of the *M. graminicola* progeny isolates were less sensitive to cyproconazole than parent isolate M3. This observation indicates that recombination of putative azole-resistance genes in the progeny isolates did not result in an additive effect that exceeded the tolerance level of the parent isolate M3. Therefore, genetic recombination in *M. graminicola* may not easily result in the generation of progeny isolates with azole sensitivity that exceeds the base-line sensitivity observed. Instead, a gradual shift in frequency of the least sensitive genotypes in the population may be expected as a result of the polygenic inheritance (Russell, 1995)

Expression of the ABC genes *MgAtr1-MgAtr5* was examined in northern blot experiments in both yeast-like cells and mycelium of the fungus. Differences in basal level of expression among the field isolates were observed, but no obvious increase in transcript levels of a specific *MgAtr* gene was found exclusively in the less sensitive isolates, except for *MgAtr1* in isolates M3 and S054. Cyproconazole-induced expression was observed in particular for *MgAtr4* but a correlation between induced transcript levels and azole sensitivity was not present. Increased expression of ABC transporter genes has been described as a resistance mechanism to azole antifungals in azole-resistant isolates of *P. digitatum* (Nakaune *et al.,* 1998), *A. nidulans* (Andrade *et al.,* 2000), and *B. cinerea* (Hayashi *et al.,* 2001). Hence, we do not exclude that increased transcript levels of *MgAtr1* might play a role in increased azole tolerance of isolates M3 and S054. This would corroborate the finding that overexpression of *MgAtr1* leads to azole resistance in some laboratory-generated mutants of *M. graminicola* (Zwiers *et al.,* 2002). It is also possible that not yet identified ABC transporter genes, play a role in azole-sensitivity of field isolates as well. This hypothesis is supported by the observation that from a single azole-sensitive cell line of *C. albicans,* multiple strains with different levels of resistance were obtained that displayed distinct overexpression patterns of at least four genes involved in azole resistance. These included the ABC transporter genes

CDR1 and *CDR2*, the major facilitator gene *MDR1*, and *ERG11*, the gene encoding the target enzyme of the azoles in the ergosterol biosynthetic pathway (Cowen *et al.,* 2000).

The expression studies demonstrate a high basal transcript level of *CYP51* in isolate M3. This isolate has an intermediate sensitivity to azoles as compared to other isolates tested. Increased expression of *CYP51* is described as a mechanism of resistance to azoles in many fungi (Joseph-Horne and Hollomon, 1997). In *C. albicans* and *S. cerevisiae* azole tolerance as a result of enhanced transcription or amplification of the *CYP51 (ERG11)* gene has also been observed (Vanden Bossche *et al.,* 1994; White, 1997; Kontoyiannis *et al.,* 1999; Perea *et al.,* 2001). In *P. digitatum* and *V. inaequalis*, tandem repeats in the promoter region of *CYP51* enhanced transcription of this gene and the copy number of these repeats correlated positively with *CYP51* expression and tolerance to azoles (Hamamoto *et al.,* 2000; Schnabel and Jones, 2001). It is possible that a similar mechanism is operating in isolate M3.

Uptake experiments demonstrated that accumulation levels of [^{14}C]cyproconazole by field isolates of *M. graminicola* did not correlate with sensitivity to azoles. Individual isolates with decreased sensitivity to azoles, such as S190, showed a relatively low accumulation level of [^{14}C]cyproconazole but other isolates with decreased sensitivity to azoles, such as M4, display a relatively high accumulation level of the fungicide. Hence, reduced accumulation of azoles from mycelium can not account exclusively for the differences in base-line sensitivity in the isolates tested. The absence of a correlation between accumulation of cyproconazole and azole sensitivity may reflect the high genetic diversity of *M. graminicola* field populations (Chen and McDonald, 1996) that accounts for morphological and physiological differences. These differences can have a significant effect on accumulation of [^{14}C]cyproconazole and may mask the variations in accumulation mediated by changes in ABC transporter activity. Studies on laboratory-generated azole-resistant mutants of *M. graminicola* showed that mutants occur with either a decreased or an increased accumulation of azoles as compared to their parent isolates (Zwiers *et al.,* 2002). Such a relation can not easily be made for field isolates since the genetic relationship between field isolates tested is virtually unknown. For these reasons it is still possible that the mechanism of resistance in the least sensitive field isolates is mediated by changes in ABC transporter activity resulting in either a decreased or increased accumulation of the fungicide. Active drug disposal in vacuoles or in lipid bodies could possibly explain the relatively high accumulation level observed for some of the least sensitive field isolates and might relate to overexpression of ABC transporters localised in

vacuolar membranes (Rea, 1999). This has been described for azole-resistant strains of *U. maydis* that accumulated triadimenol to higher levels as compared to sensitive strains (Wellmann and Schauz, 1993). Alternative mechanisms of resistance, such as alterations in the target-site of azoles can also not be excluded. For these reasons we anticipate that a high correlation between azole sensitivity and accumulation in field isolates of *M. graminicola* can not easily be established. However, previous literature data suggest that such a correlation is present (Joseph-Horne *et al.,* 1996; Gisi *et al.,* 2000). The discrepancy with the work of Joseph-Horne *et al.,* (1996) can be ascribed to the low number of isolates tested. The work of Gisi *et al.,* (2000) is difficult to judge since data that support their conclusion were not published.

In summary, our studies suggest that variation in sensitivity to azoles in field isolates of *M. graminicola* is based on multiple mechanisms. These may include decreased accumulation of the fungicide in the cytoplasm, increased accumulation of the fungicide as a result of sequestration of the fungicide in cellular compartments and overproduction of $P450_{14DM}$. These multiple mechanisms would corroborate the polygenic nature of azole sensitivity as shown by the analysis of a genetic cross of *M. graminicola* isolates.

ACKNOWLEDGEMENTS

We kindly acknowledge Dr J.M. Seng (Biotransfer, Montreuil Cedex, France) and Dr A. Suty (Bayer AG, Landwirtschaftszentrum Monheim, Leverkusen, Germany) for providing the field isolates of *Mycosphaerella graminicola* and E.C.P. Verstappen (Plant Research International, Wageningen, The Netherlands) for technical assistance with the crosses of *M. graminicola*. We are grateful to Prof. Dr P.J.G.M. De Wit (WUR) and Dr L-H. Zwiers for critical reading of the manuscript and the Wageningen Mycosphaerella group for key discussions and suggestions during this work. Ioannis Stergiopoulos was financially supported by the Training and Mobility of Researchers (TMR) Programme – Marie Curie Research Training Grants, The European Commission (Contract No. ERBFMBICT983558).

REFERENCES

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

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

Brent KJ (1992) Monitoring fungicide resistance: purposes, procedures and progress. *In* Resistance '91: Achievements and Developments in Compating Pesticide Resistance, (Eds) Denholm, I, Devonshire, AL and Hollomon, DW, Elsevier, London, pp. 1-18

Chen RS and McDonald BA (1996) Sexual reproduction plays a major role in the genetic structure of populations of the fungus *Mycosphaerella graminicola.* Genetics **142:** 1119-1127

Cowen LE, Sanglard D, Calabrese D, Sirjusingh C, Anderson JB and Kohn LM (2000) Evolution of drug resistance in experimental populations of *Candida albicans*. J Bacteriol **182:** 1515-1522

De Waard MA (1994) Resistance to fungicides which inhibit sterol 14a-demethylation, an historical perspective. *In* Fungicide Resistance, (Eds) Heaney S, Slawson D, Hollomon DW, Smith M, Russell PE, Parry DW, pp. 3-10

De Waard MA and Van Nistelrooy JGM (1979) Mechanism of resistance to fenarimol in *Aspergillus nidulans*. Pestic Biochem Physiol **10:** 219-229

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

De Waard MA and Fuchs A (1982) Resistance to ergosterol biosynthesis inhibitors, II. Genetic and physiological aspects. *In* Fungicide resistance in Crop Protection (Eds) Dekker J and Georgopoulos SG, Pudoc, Wageningen, The Netherlands, pp. 87-100

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

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

Délye C, Laigret F and Corio Costet MF (1997) A mutation in the 14alpha-demethylase gene of *Uncinula necator* that correlates with resistance to a sterol biosynthesis inhibitor. Appl Environ Microbiol **63:** 2966-2970

Délye C, Bousset L and Corio-Costet MF (1998) PCR cloning and detection of point mutations in the eburicol 14 alpha-demethylase (*CYP51*) gene from *Erysiphe graminis* f. sp. *hordei*, a "recalcitrant" fungus. Curr Genet **34:** 399-403

Eckert JK (1987) *Penicillium digitatum* biotypes with reduced sensitivity to imazalil. (Abstr). Phytopathology **77:** 1728

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. Antimicrob Agents Chemother **42**: 3065-3072

Fuchs A and De Waard MA (1982) Resistance to ergosterol biosyntesis inhibitors, I. Chemistry and phenomenological aspects. *In* Fungicide resistance in Crop protection, (Eds) Dekker J and Georgopoulos SG, Pudoc, Wageningen, The Netherlands, pp. 71-86

Georgopoulos SG (1982) Cross-resistance. *In* Fungicide Resistance in Crop Protection, (Eds) Dekker J and Georgopoulos SG, Pudoc, Wageningen, The Netherlands, pp. 53-59:

Gisi U and Hermann D (1994) Sensitivity behaviour of *Septoria tritici* populations on wheat to cyproconazole. *In Fungicide Resistance,* (Eds) Heaney S *et al.*, British Crop Protection Council (BCPC) Monograph, Farnham, UK, pp. 11-18

Gisi U, Hermann D, Ohl L and Steden C (1997) Sensitivity profiles of *Mycosphaerella graminicola* and *Phytophthora infestans* populations to different classes of fungicides. Pestic Sci **51:** 290-298

Gisi U, Chin KM, Knapova G, Farber RK, Mohr U, Parisi S, Sierotzki H and Steinfeld U (2000) Recent developments in elucidating modes of resistance to phenylamide, DMI and strobilurin fungicides. Crop Protect **19:** 863-872

Gottesman MM and Pastan I (1993) Biochemistry of multidrug resistance mediated by the multidrug transporter. Annu Rev Biochem **62:** 385-427

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*. Appl Environ Microbiol **66:** 3421 - 3426

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.* Pestic Biochem Physiol **70:** 168-179

Heaney SP (1988) Population dynamics of DMI fungicide sensitivity changes in barley powdery mildew. *In* Fungicide Resistance in North America, (Eds) Delp CJ, APS Press, St. Paul, Minnesota, pp. 89-92

Higgins CF (1992) ABC transporters: from microorganisms to man. Annu Rev Cell Biol **8:** 67-113

Hildebrand PD, Lockhart CL, Newberry RJ and Ross RG (1988) Resistance of *Venturia inaequalis* to bitertanol and other demethylation-inhibiting fungicides. Can J Plant Pathol **10:** 311-316

Joseph-Horne T, Manning NJ, Hollomon D and Kelly SL (1995) Defective sterol Δ5(6)desaturase as a cause of azole resistance in *Ustilago maydis*. FEMS Microbiol Lett: 1-2

Joseph-Horne T, Hollomon D, Manning N and Kelly SL (1996) Investigation of the sterol composition and azole resistance in field isolates of *Septoria tritici*. Appl Environ Microbiol: 184-190

Joseph-Horne T and Hollomon DW (1997) Molecular mechanims of azole resistance in fungi. FEMS Microbiol Lett **149:** 141-149

Kalamarakis AE, De Waard MA, Ziogas BN and Georgopoulos SG (1991) Resistance to fenarimol in *Nectria haematococca* var. *cucurbitae*. Pestic Biochem Physiol **40:** 212-220

Kema GHJ, Verstappen ECP, Todorova M and Waalwijk C (1996) Successful crosses and molecular tetrad and progeny analyses demonstrate heterothallism in *Mycosphaerella graminicola*. Curr Genet **30:** 251-8

Kema GHJ and Van Silfhout CH (1997) Genetic variation for virulence and resistance in the wheat-*Mycosphaerella graminicola* pathosystem. III. Comparative seedling and adult plant experiments. Phytopathology **87:** 266-272

Kendall SJ, Hollomon DW, Cooke LR and Jones DR (1993) Changes in sensitivity to DMI fungicides in *Rhynchosporium secalis.* Crop Protect **12:** 357-362

Kontoyiannis DP, Sagar N and Hirschi KD (1999) Overexpression of Erg11p by the regulatable GAL1 promoter confers fluconazole resistance in *Saccharomyces cerevisiae.* Antimicrob Agents Chemother **43:** 2798-2800

Kuck KH and Scheinpflug H (1986) Biology of sterol-biosynthesis inhibiting fungicides. *In* Chemistry of Plant Protection, (Eds) Haug, G and Hoffman, H, Springer-Verlag, Berlin, Germany

Marichal P, Koymans L, Willemsens S, Bellens D, Verhasselt P, Luyten W, Borgers M, Ramaekers FC, Odds FC and Bossche HV (1999) Contribution of mutations in the cytochrome P450 14alpha-demethylase (Erg11p, Cyp51p) to azole resistance in *Candida albicans.* Microbiology **145:** 2701-2713

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*. Appl Environ Microbiol **64:** 3983-3988

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. Antimicrob Agents Chemother **45:** 2676-2684

Pijls CFN, Shaw MW and Parker A (1994) A rapid test to evaluate *in vitro* sensitivity of *Septoria tritici* to flutriafol, using a microtitre plate reader. Plant Pathol **43:** 726-732

Rea PA (1999) MRP subfamily ABC transporters from plants and yeast. J Exper Bot **50:** 895-913

Russell PE (1995) Fungicide resistance: occurrence and management. J Agric Sci **124:** 317-323

Schepers HTAM (1985) Fitness of isolates of *Sphaerotheca fuliginea* resistant or sensitive to fungicides which inhibit ergosterol biosynthesis. Neth J Pl Path **91:** 65-76

Schnabel G and Jones AL (2001) The 14 alpha-demethylase (*CYP51A1*) gene is overexpressed in *Venturia inaequalis* strains resistant to myclobutanil. Phytopathology **91:** 102-110

Sisler HD and Ragsdale NN (1984) Biochemical and cellular aspects of the antifungal action of ergosterol biosynthesis inhibitors. Symp. Ser. Br. Mycol. Soc., Cambridge, pp. 257-282

Stergiopoulos I, Gielkens MM, Goodall SD, Venema K and De Waard MA (2002a) Molecular cloning and characterisation of three new ATP-binding cassette transporter genes from the wheat pathogen *Mycosphaerella graminicola*. Gene **289:** 141-149

Stergiopoulos I, Zwiers L-H and De Waard MA (2002b) Secretion of natural and synthetic toxic compounds from filamentous fungi by membrane transporters of the ATP-binding cassette and Major facilitator superfamily. Eur J Plant Pathol **108:** 719-734

Suty A and Kuck KH (1996) Sensitivity of wheat leaf spot *Septoria tritici* to tebuconazole. British Crop Protection Conference: Pests and Diseases, Brighton, UK, pp. 689-694

Van Nistelrooy JGM, Vanden Brink JM, Van Kan JA, Van Gorcom RFM and De Waard MA (1996) Isolation and molecular characterization of the gene encoding eburicol 14-alpha-demethylase (*CYP51*) from *Penicillium italicum*. Mol Gen Genet **250:** 725-733

Van Tuyl JM (1977) Genetics of fungal resistance to systemic fungicides. Mededelingen Landbouwhogescholl Wageninegn, Veenman H and Zonen BV, Wageningen, The Netherlands

Vanden Bossche H, Marichal P and Odds FC (1994) Molecular mechanisms of drug resistance in fungi. Trends Microbiol **2:** 393-400

Wellmann H and Schauz K (1993) DMI-resistance in *Ustilago maydis*: II. Effect of triadimefon on regenerating protoplasts and analysis of fungicide uptake. Pest Biochem Physiol **46:** 55-64

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. Antimicrob Agents Chemother **41:** 1482-1487

Zwiers L-H and De Waard MA (2000) Characterization of the ABC transporter genes *MgAtr1* and *MgAtr2* from the wheat pathogen *Mycosphaerella graminicola*. Fun Genet Biol **30**: 115-125

Zwiers L-H, Stergiopoulos I, Van Nistelrooy JGM and De Waard MA (2002) ABC transporters and azole susceptibility in laboratory strains of the wheat pathogen *Mycosphaerella graminicola.* Antimcrob Agents Chemother **46:** 3900-3906

CHAPTER 8

ACTIVITY OF AZOLE FUNGICIDES AND ABC TRANSPORTER MODULATORS ON *MYCOSPHAERELLA GRAMINICOLA*

Ioannis Stergiopoulos and Maarten A. De Waard

Journal of Phytopathology
(2002) ***150:*** *313-320*

ABSTRACT

The antimicrobial activity of the azole fungicides cyproconazole and propiconazole as single active ingredients and in mixtures with the ATP-binding cassette (ABC) transporter modulators rhodamine 6G, quercetin, quinidine, and verapamil, and the strobilurin kresoxim-methyl was assessed against the wheat pathogen *Mycosphaerella graminicola*. Interactions amongst these compounds were evaluated on germination and germ tube growth of pycnidiospores using the Colby and Wadley method. Water agar proved to be the best test medium since all pycnidiospores germinated within 24 h of incubation and apical germ tube growth dominated over bud formation by intermediate cells. Analysis with the Colby method revealed that interactions between the compounds in all mixtures tested on germination of pycnidiospores were additive. With regard to germ tube growth, mixtures of cyproconazole and verapamil or kresoxim-methyl displayed a synergistic interaction. Analysis of mixtures of cyproconazole and kresoxim-methyl with the Wadley method, revealed that the interaction between the two compounds was just additive. These results indicate that the Colby method overestimated the interaction between these two compounds in a mixture.

Keywords: ABC transporters; azoles; interaction in mixtures; modulators; *Mycosphaerella graminicola*

INTRODUCTION

The fungus *Mycosphaerella graminicola* (Fückel) Schroeter (anamorph state: *Septoria tritici* Rob.ex.Desm.) is the causal agent of septoria tritici leaf blotch of wheat, one of the most economically important diseases of wheat. Typical symptoms of the disease are irregular chlorotic lesions on infected leaves that under favourable conditions develop to necrotic spots, covered with dark brown to black pycnidia or pseudothecia of the fungus (Eyal *et al.,* 1987).

The economic importance of this disease has dramatically increased over the past few decades. Yield losses from this pathogen may amount up to 30-50% in areas without disease control. Disease control has widely involved the use of azole fungicides such as prochloraz, propiconazole, and cyproconazole. The mode of action of these fungicides is based on inhibition of cytochrome P450 sterol 14α-demethylase ($P450_{14DM}$), a key enzyme of the sterol biosynthetic pathway (Sisler *et al.,* 1984). Although the risk of resistance development against these compounds has been classified as moderate (Brent and Hollomon, 1998), extensive use of azoles over the past 20 years has lead to resistance development in several pathogens of both medical (Hitchcock, 1993) and agricultural importance (De Waard, 1994). One of the mechanisms proposed to operate in azole-resistant isolates of fungi is reduced accumulation of these fungicides in the mycelium (De Waard and Van Nistelrooy, 1980). This reduction in accumulation has been attributed to an energy-dependent efflux mechanism mediated by ATP-binding cassette (ABC) transporters (Del Sorbo *et al.,* 2000).

ABC transporters utilize the energy derived from the hydrolysis of ATP to drive the transport of endogenous metabolites and exogenous toxic compounds over biological membranes. Overproduction of ABC transporters can generate multidrug-resistant (MDR) phenotypes since ABC transporters can transport a variety of cytotoxic drugs (Gottesman and Pastan, 1993). Compounds able to modulate the activity of ABC transporters can reverse multidrug resistance, as they inhibit efflux of drugs from cells. Many compounds have been shown to directly interact with the multidrug transporter acting as competitive inhibitors of transport. Verapamil and quinidine are known to be members of this group (Ambudkar *et al.,* 1999). Compounds, such as respiration inhibitors that are able to block the generation of ATP in cells may also decrease the activity of ABC transporters. Indeed, synergistic activity between certain azole fungicides and respiration inhibitors like carbonyl-cyanide m-chlorophenylhydrazone (CCCP), oligomycin, and dicyclohexylcarbodiimide has been reported

(De Waard and Van Nistelrooy, 1982; 1984a, b). Strobilurins are natural antibiotics that inhibit mitochondrial respiration by blocking electron transfer at the cytochrome-bc1 complex (complex III) (Becker *et al.,* 1981). Derivatives of strobilurins such as kresoxim-methyl have been developed as commercial fungicides and used either alone or in mixtures with other fungicides (Sauter *et al.,* 1999).

This study describes the activity of the azole fungicides propiconazole and cyproconazole, either alone or in mixtures with the ABC transporter modulators rhodamine 6G, quercetin, quinidine, and verapamil and the strobilurin fungicide kresoxim-methyl. Interactions between the compounds were determined on activity against spore germination and germ tube growth of *M. graminicola* according to the methods of Colby (1967) and Wadley (1945, 1967).

MATERIALS AND METHODS

Fungus

Leaves displaying symptoms of septoria tritici leaf blotch were collected from an experimental wheat field in Wageningen, The Netherlands. Pycnidiospores of *M. graminicola* were isolated from pycnidia of infected leaf segments. These were attached to a glass slide and placed on the bottom of a Petri dish fitted with filter paper saturated with sterile tap water. After formation of oozing drops on top of the pycnidia, an aqueous suspension of pycnidiospores was prepared by vortexing the leaves in a test tube containing 5 ml of sterile tap water. The concentration of the spore suspension was determined with a "Coulter® Z Series" particle counter (Coulter Corporation, Florida U.S.A.) and adjusted to 10^5 spores ml^{-1}.

Chemicals

The chemicals used in this study were the azole fungicides propiconazole and cyproconazole (Syngenta Crop Protection AG, Basel, Switserland), the strobilurin fungicide kresoxim-methyl (BASF AG, Ludwigshafen, Germany) and the ABC transporter modulators rhodamine 6G, quercetin, quinidine, and verapamil (Sigma Chemical Company, St. Luis, Missouri, U.S.A.). All compounds used were of technical grade quality.

Media

Media used were, Potato-Dextrose Agar [PDA; 39 g l^{-1} (Merck)], Water Agar [WA; 15 g l^{-1} (Oxoid)], Yeast Malt Agar [YMA; yeast extract 4 g l^{-1} (Oxoid), malt extract 4 g L^{-1} (Difco laboratories), sucrose 4g l^{-1} (Merck), and

agar-agar 15 g l^{-1} (Oxoid)], Czapek-Dox [CzD; 45.5 g l^{-1} (Oxoid)], and Czapek-Dox + Peptone [CzD + P; Czapek Dox 45.5 g l^{-1} (Oxoid), peptone 5 g l^{-1} (Oxoid)].

Spore germination test

The activity of the compounds, as sole ingredients and in mixtures, on germination of pycnidiospores of *M. graminicola* was assessed in spore germination tests. Stocks of 100x concentrated solutions of the compounds were prepared in methanol. Media amended with these compounds were made by adding aliquots from the stock solutions. The final concentration of methanol in the medium was 1%. Agar with 1% methanol was used as a control. Spore suspensions (100 µl) were transferred to Petri dishes (9 cm) containing media (20 ml) amended with the test compounds at different concentrations. The antibiotic streptomycin sulfate (50 mg l^{-1}) was added to all plates to prevent bacterial growth. The spores were spread over the medium surface with a glass rod and the dishes were stored in an incubator at 18°C in the dark. At the end of the incubation period all dishes were stored at 4°C. Germination rates and length of germ tubes formed, both as an elongation of the apical cells and as buds from the intermediate cells (in between apical cells) of 100 counted spores, were assessed. Standard deviations of germination of pycnidiospores and germ tube growth were calculated and were less than 10 and 55% of the mean values, respectively.

Quantification of interactions

Interactions between fungicides in mixtures were analysed according to Colby (1967) using the formula $E= X_A Y_B/100$ in which X_A and Y_B represent growth as a percentage of the control with toxicant A used at dosage p and toxicant B used at dosage q, respectively. E is the expected growth as a percentage of the control for mixture A and B at dosages p and q. The observed response is obtained experimentally by comparing the activity of single compounds with mixtures containing the same rate of the constituents as applied singly. A deviation from the expected response as calculated from the level of interaction R between the expected and the observed response of the two compounds would indicate synergism or antagonism. The Wadley formula (1945, 1967) describes the theoretical response (EC_{50}(th)) of a two-component mixture as $EC_{50}(th)= (a + b)/(a/EC_{50}(A) + b/EC_{50}(B))$ where A and B represent the two components and a and b the ratio of the components in the mixture. The level of interaction R is calculated as $R= EC_{50}(th)/EC_{50}(obs)$ in which EC_{50}(obs) is the observed EC_{50} value of the specific mixture. By definition, in both formulas additive interactions are present if R = 1, synergism if R > 1 and antagonism if R < 1. Because of the biological variability of the test systems, synergism is considered significant if $R \geq 1.5$ and antagonism if $R \leq 0.5$. Additive interactions are present when $0.5 < R > 1.5$ (Gisi *et al.,* 1985).

RESULTS

Germination of pycnidiospores on different media

Germination of pycnidiospores of *M. graminicola* was assessed on WA, PDA, YMA, CzD, and CzD + P media. On all media germination became visible after 12-15 h of incubation and all pycnidiospores completed germination after 24 h of incubation (Table 1). By that time, the multicellular pycnidiospores displayed germ tubes at the apical cells and yeast-like buds at the intermediate cells (Table 2; Figure 1B). After 32 h of incubation, growth of the apical germ tubes and the yeast-like buds was still observed. After 24 h, bud formation on WA and to a lesser extend on CzD, was less frequent as compared to the other media tested. Germination on WA and CzD mainly proceeded with apical germ tubes and hardly any buds were observed. The germination of pycnidiospores on these media was accompanied by the formation of relatively long apical germ tubes as compared to germination on the complex media (Table 2).

Table 1. Time course of germination of pycnidiospores of *Mycosphaerella graminicola* on different media

	Time (h)				
Medium	12	15	18	21	24
PDA[a]	14[b](2)	15 (3)	32 (5)	83 (3)	100 (0)
WA	11 (3)	16 (6)	19 (3)	83 (5)	100 (1)
YMA	22 (4)	20 (2)	34 (6)	99 (1)	100 (0)
CzD	5 (2)	11 (2)	17 (6)	95 (2)	100 (0)
CzD + P	12 (4)	13 (3)	32 (2)	87 (2)	99 (0)

[a] PDA: potato dextrose agar, WA: water agar, YMA: yeast malt agar, CzD: Czapek-Dox, CzD-P: Czapek-Dox + peptone;

[b] Percentage of germination of 100 counted spores (standard deviation);

Activity of azole fungicides and test compounds.

The activity of the compounds on spore germination (Table 3) and apical germ tube growth (Table 4) was assessed after 32 h of incubation on WA. EC_{50} values of cyproconazole and propiconazole as calculated from dose-response curves, were 0.1 and 0.01 mg l^{-1}, respectively, for both inhibition of spore germination and germ tube growth (results not shown). The ABC transporter modulators, hardly affected germination but some of them inhibited germ tube

Table 2. Germination of pycnidiospores of *Mycosphaerella graminicola* after 24 h of incubation on different media.

	Medium									
	PDA[a]		WA		YMA		CzD		CzD + P	
Type of Germination	Percentage %	Length µm	Percentage %	Length µm	Percentage %	Length µm	Percentage %	Length µm	Percentage %	Length µm
Buds	90 [b](2)	32[c](14)	40 (4)	24 (13)	85 (3)	21 (9)	59 (7)	28 (16)	95 (1)	25 (11)
Apical germ tubes	83 (4)	47 (12)	96 (2)	67 (23)	74 (9)	41 (10)	100 (1)	84 (25)	95 (5)	50 (14)

[a] PDA: potato dextrose agar, WA: water agar, YMA: yeast malt agar, CzD: Czapek-Dox, CzD + P: Czapek-Dox + peptone;
[b] Percentage of germination (standard deviation); [c] Average length of 100 measured apical germ tubes or buds (standard deviation);

Table 3. Expected (Exp) and observed (Obs) germination (%) of pycnidiospores of *Mycosphaerella graminicola* on water agar amended with mixtures of test compounds after 32 h of incubation. The expected germination is calculated according to Colby (1967).

			Germination in presence of mixtures of compounds (%)[a]											
			Propiconazole						Cyproconazole					
	Concentration (mg l^{-1})	Germination in presence of single compounds (%)	0.01 mg l^{-1}			0.1 mg l^{-1}			0.01 mg l^{-1}			0.1 mg l^{-1}		
			Exp	Obs	**R**[b]	Exp	Obs	**R**	Exp	Obs	**R**	Exp	Obs	**R**
Quercetin	10	81	70	70	**1.00**	57	43	**1.32**	67	75	**0.89**	61	60	**1.02**
	100	85	73	72	**1.01**	60	54	**1.11**	71	73	**0.97**	64	62	**1.03**
Quinidine	10	95	82	83	**0.99**	67	50	**1.34**	79	71	**1.11**	71	77	**0.92**
	100	93	80	73	**1.09**	65	49	**1.33**	77	87	**0.88**	70	75	**0.93**
Rhodamine 6G	0.1	95	82	81	**1.01**	67	57	**1.17**	79	80	**0.99**	71	78	**0.91**
	1	86	74	73	**1.01**	60	62	**0.97**	71	79	**0.90**	65	61	**1.06**
Verapamil	10	94	81	75	**1.08**	66	53	**1.24**	78	66	**0.18**	70	65	**1.07**
	100	87	75	70	**1.07**	61	42	**1.45**	72	69	**1.04**	65	51	**1.27**
Kresoxim-methyl	0.0001	87	75	74	**1.01**	64	73	**0.88**	72	74	**0.97**	69	69	**1.00**
	0.003	87	75	78	**0.96**	64	48	**1.33**	72	72	**1.00**	69	68	**1.01**

[a] Figures represent germination of pycnidiospores, expressed as a percentage of control treatment. Germination percentages in the presence of 0.01 and 0.1 mg l^{-1} cyproconazole are 83 and 75% and in the presence of 0.01 and 0.1 mg l^{-1} propiconazole 86 and 70%, respectively;
[b] Ratio between expected and observed germination (R= Exp/Obs);

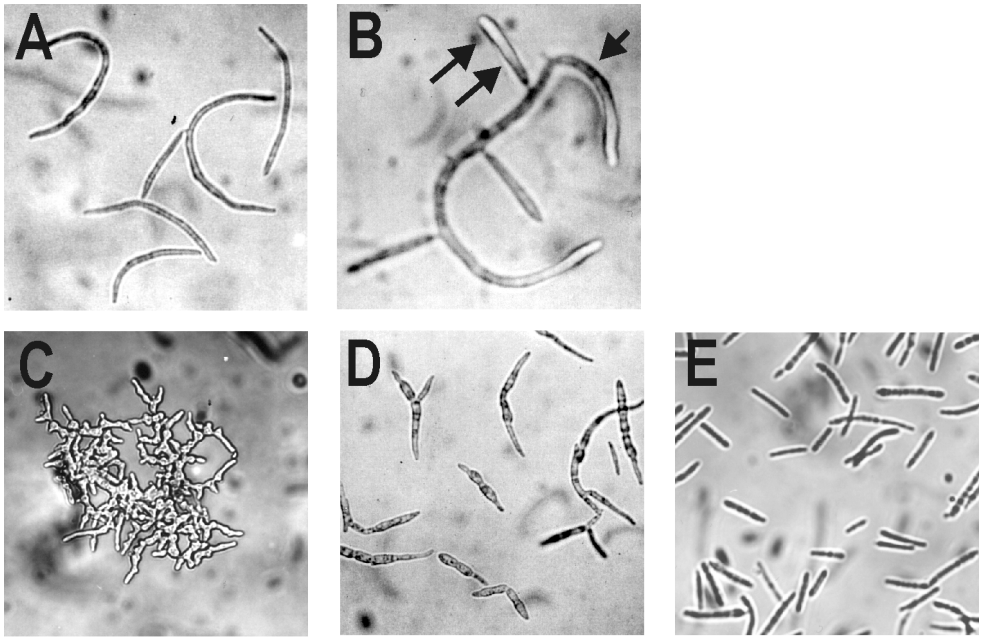


Figure 1. Morphological alterations of germinating pycnidiospores of *Mycosphaerella graminicola* after treatment with different compounds. **A:** control spores; **B:** detail of a spore displaying yeast-like buds (↑↑) and apical germ tube growth (↑); **C:** germinating spores after treatment with cyproconazole; **D:** germinating spores after treatment with rhodamine 6G; **E:** germinating spores after treatment with kresoxim-methyl.

growth at the concentrations used. EC_{50} values for quercetin, quinidine, rhodamine 6G, and verapamil for germ tube growth were about 100, 10, 1, and 10 mg l^{-1}, respectively (Table 4). The strobilurin fungicide kresoxim-methyl was very active with EC_{50} values for both germination and germ tube growth ranging between 0.01 and 0.003 mg l^{-1} (results not shown). This compound severely affected germination of pycnidiospores. Morphological alterations of the formed germ tubes caused by the activity of the compounds were markedly present. Upon treatment with the azole fungicides apical germ tubes become frequently branched and swollen. Kresoxim-methyl resulted in severely stunted germ tubes, while rhodamine 6G induced swollen spores with shorter germ tubes (Figure 1).

Interactions of the compounds in mixtures - Colby

Two-component mixtures of azole fungicides and test compounds were designed and their activity on spore germination was studied. The concentration of single compounds in these mixtures inhibited germination and germ tube growth less than 50% as compared to the controls. Expected interactions within the mixtures were calculated according to Colby.

Mixtures of propiconazole and cyproconazole with the ABC transporter modulators or kresoxim-methyl showed only additive effects with respect to germination of pycnidiospores (Table 3). With regard to germ tube growth, interactions with ratios higher than 1.5 were observed for combinations of cyproconazole with verapamil, and kresoxim-methyl (Table 4), suggesting a synergistic interaction in these mixtures.

Table 4. Expected (Exp) and observed (Obs) germ tube growth of pycnidiospores of *Mycosphaerella graminicola* on water agar amended with mixtures of test compounds after 32 h of incubation. The expected germ tube growth is calculated according to Colby (1967).

Test compound	Concentration (mg l^{-1})	Germ tube growth in presence of single compounds (%)	Germ tube growth in presence of mixtures of compounds (%)[a]					
			Propiconazole 0.01 mg l^{-1}			Cyproconazole 0.01 mg l^{-1}		
			Exp	Obs	**R**[b]	Exp	Obs	**R**
Quercetin	100	56	33	29	**1.14**	46	45	**1.02**
Quinidine	10	45	27	35	**0.77**	37	27	**1.37**
Rhodamine 6G	1	62	37	26	**1.42**	51	33	**1.38**
Verapamil	10	44	26	27	**0.96**	36	23	**1.56**
Kresoxim-methyl	0.0001	97	57	42	**1.36**	79	50	**1.58**
	0.003	81	48	41	**1.17**	67	38	**1.76**

[a] Figures represent the average length of apical germ tubes of 100 pycnidiospores, expressed as a percentage of control treatment. Germ-tube growth in the presence of 0.01 mg l^{-1} propiconazole and of 0.01 mg l^{-1} cyproconazole was 59 and 82%, respectively;
[b] Ratio between expected and observed growth (R= Exp/Obs);

Interactions between cyproconazole and kresoxim-methyl - Wadley

Interactions between cyproconazole and kresoxim-methyl on germ tube growth were analysed further according to Wadley. Three mixtures with different ratios of kresoxim-methyl were prepared. Dose-response curves of the single compounds and of the mixtures were constructed and used to calculate EC_{50} values (EC_{50}(obs)) (results not shown). The EC_{50} values of the single compounds were used to determine the theoretical EC_{50} values (EC_{50}(th)) of the mixtures. In all mixtures, EC_{50}(obs) were higher than EC_{50}(th). Interaction ratios ranged between 0.52 and 0.58 indicating additive effects between the compounds (Table 5).

Table 5. Expected and observed germ tube growth of pycnidiospores of *Mycosphaerella graminicola* on water agar amended with cyproconazole and kresoxim-methyl and with their mixtures in different ratios after 32 h of incubation. The expected germ tube growth is calculated according to Wadley (1945, 1967).

	Single products		Mixtures of compounds					
	Cyproconazole	Kresoxim-methyl	Mixture A (1: 0.01)[a]		Mixture B (1: 0.1)		Mixture C (1: 0.3)	
Concentration (mg l^{-1})	Growth %	Growth %	Concentration (mg l^{-1})	Growth %	Concentration (mg l^{-1})	Growth %	Concentration (mg l^{-1})	Growth %
0	100[b]	100	0	100	0	100	0	100
0.0001	100	98	0.00101	81	0.0011	85	0.0013	82
0.0003	100	92	0.00303	85	0.0033	85	0.0039	81
0.001	100	89	0.0101	84	0.011	80	0.013	68
0.003	100	46	0.0303	86	0.033	54	0.039	7
0.01	78	17	0.101	67	0.11	18	0.13	0
0.03	74	0	0.303	14	0.33	0	0.39	0
0.1	55	0	1.01	0	1.1	0	1.3	0
0.3	15	0						
1	15	0						
$EC_{50(obs)}$	0.12	0.0026	0.16 (0.158 + 0.002)[c]		0.04 (0.036 + 0.004)		0.018 (0.014 + 0.004)	
$EC_{50(th)}$			0.083		0.023		0.010	
R[d]			**0.52**		**0.58**		**0.58**	

[a] Ratio of cyproconazole to kresoxim-methyl in the mixtures;
[b] Apical germ tube growth relative to control treatment (%);
[c] Concentrations of individual compounds in the mixtures;
[d] R= EC_{50}(th)/EC_{50}(obs);

DISCUSSION

Water agar (WA) proved to be an appropriate medium for germination tests with pycnidiospores of *M. graminicola*. All pycnidiospores germinated within 24 h of incubation and had comparable growth vigor. Growth of the apical germ tubes was still observed after 32 h of incubation and was comparable to that of the other media tested. After this time period, growth rates on complex media were higher than on WA. Yet, we selected WA for the toxicity studies since this medium minimizes the possibility that components in the medium interact with the activity of the test compounds. In addition, spore germination on WA results in a preferential formation of apical germ tubes rather than the formation of branched hyphae as observed on rich media. Duncan and Howard (2000) have also reported similar observations previously.

The azole fungicides tested were potent inhibitors of germination of pycnidiospores. The ABC transporter modulators proved to be less active than the azole fungicides and affected only germ tube growth. Activity of azoles on spore germination is known to be species-dependent. For example, spore germination of *Cladosporium cucumerinum* is hardly affected by several azoles (Buchenauer, 1977), while spore germination of other fungal species, such as *Penicillium italicum* and *P. expansum* is significantly inhibited by imazalil or etaconazole (Kerkenaar and Barug, 1984). The morphological alterations caused by cyproconazole and propiconazole on germinating pycnidiospores resemble effects previously described for other fungal species such as *C. cucumurinum* (Sherald *et al.,* 1973), *Monilinia fructigena* (Kato *et al.,* 1975), and *Botrytis cinerea* (Kato *et al.,* 1980). These alterations are ascribed to depletion of ergosterol and accumulation of C14-methyl sterol intermediates. Kresoxim-methyl was found to be an extremely potent inhibitor of germination of pycnidiospores. This observation is in agreement with reports from literature, describing strobilurins as inhibitors of spore germination. For many fungi, inhibition of spore germination occurred at considerably lower concentrations, than those required for inhibition of mycelial growth (Sauter *et al.,* 1995).

Interactions of test compounds in mixtures were estimated according to Colby (1967). The Colby formula is a simple mathematical model, representing an easy and quick way to determine the interactions between two compounds with a different mode of action. With respect to spore germination, only additive interactions in mixtures of the azole fungicides and the ABC transporter modulators or kresoxim-methyl could be observed. With regard to germ

tube growth, combinations of cyproconazole and verapamil or kresoxim-methyl had a synergistic activity. Mixtures with kresoxim-methyl showed the clearest effect. Therefore, this combination was further examined according to the Wadley formula, which has been proposed as a more accurate way to investigate interactions in mixtures (Wadley, 1967). Our results indicate that mixtures containing different ratios of cyproconazole and kresoxim-methyl displayed only additive effects. Hence, calculations through the Colby and Wadley methods do not support each other. Probably, the Colby method overestimates the interactions between two compounds in a mixture. For this reason this method has been subject to serious criticism (Morse, 1978; Nash, 1981). Still this method is frequently used as a quick way to evaluate interactions between two components in a mixture.

Although our results indicate that, according to Wadley, no synergism occurred between the azole fungicides and the modulators tested, it does not mean that this phenomenon is absent in all cases. The occurrence of synergistic interactions between compounds can be highly dependent on the fungal strains and mixtures used. For instance, synergism between ABC transporter modulators and other compounds is more likely for MDR strains of a pathogen rather than for sensitive ones. Hence, strains that overexpress ABC transporters and accumulate less drugs than wild-type strains would be interesting for further studies (Ford, 1990). For example, the newly characterized immuno-suppressant tacrolimus strongly synergised the effect of azole antifungal agents against azole-resistant strains of *Candida albicans* (Maesaki *et al.,* 1998). However, in the same experiments synergistic effects were not observed with an azole-sensitive isolate. Other respiration inhibitors or MDR modulators such as verapamil, actinomycin A, oligomycin, sodium azide or carbonyl-cyanide m-chlorophenylhydrazone (CCCP) in combination with azoles did not show synergism either. In a similar way, transport of vinblastin by mammalian ABC transporters was inhibited by daunomycin, quinidine, and verapamil but not by other drugs able to reverse MDR, such as vanadate, vincristine, colchicine, and puromycin (Cornwell *et al.,* 1986; Cornwell *et al.,* 1987; Akiyama *et al.,* 1988). The inhibitory effect of the triazole fungicides propiconazole and tebuconazole on spore germination of *B. cinerea*, was strongly synergised by sub-lethal doses of ABC transporter modulators, such as bis-benzimide, rhodamine 6G, N-ethylmaleimide, and quercetin but not by daunomycin and quinidine (Del Sorbo *et al.,* 1998). Thus, the search for ABC transporter modulators that synergise the activity of fungicides to *M. graminicola* should continue as it could lead to new and innovative disease control methods. For example,

combined application of synthetic or natural fungicides with selected non-toxic ABC transporter modulators could result in reduced dose rates of the chemicals used for control of plant diseases.

ACKNOWLEDGEMENTS

We kindly acknowledge Dr G.H.J. Kema and E.C.P. Verstappen for providing wheat material infected with *Mycosphaerella graminicola* and for helpful suggestions and discussions during this work within the Wageningen Mycosphaerella group. We also acknowledge Dr G.H.J. Kema and Prof. Dr P.J.G.M. De Wit for critical reading of the manuscript. I. Stergiopoulos was financially supported by the Training and Mobility of Researchers (TMR) Programme – Marie Curie Research Training Grants, The European Commission (Contract No. ERBFMBICT983558).

REFERENCES

Akiyama S-I, Cornwell MM, Kuwano M, Pastan I and Gottesman MM (1988) Most drugs that reverse multidrug resistance can also inhibit photo affinity labeling of P-glycoprotein by a vinblastine analogue. Mol Pharmcol **33:** 144-147

Ambudkar S, Dey CS, Hrycyna A, Ramachandra M, Pastan I and Gottesman MM (1999) Biochemical, cellular, and pharmacological aspects of the multidrug transporter. Annu Rev Pharmacol Toxicol **39:** 361-398

Becker WF, von Jagow G, Anke T and Steglich W (1981) Oudemansin, strobilurin A, strobilurin B and myxothiazole: new inhibitors of the bc_1 segment of the respiratory chain with an E-β-methoxyacrylate system as common structural element. FEBS Lett **132:** 329-333

Brent KJ and Hollomon DW (1998) Fungicide Resistance: The assessment of risk. FRAC Monograph **No.2**, Global Crop Protection Federation, Brussels, pp. 48

Buchenauer H (1977) Mode of action and selectivity of fungicides which interfere with ergosterol biosynthesis. British Crop Protection Council Conference: Pests and Diseases, **3:** 699-711

Colby SR (1967) Calculating synergistic and antagonistic responses of herbicide combinations. Weeds **15:** 20-22

Cornwell MM, Safa AR, Felsted RL, Gottesman MM and Pastan I (1986) Membrane vesicles from multidrug-resistant human cancer cells contain a specific 150-170kDa protein detected by photoaffinity labeling. Proc Natl Acad Sci USA **83:** 3847-3850

Cornwell MM, Pastan I and Gottesman MM (1987) Certain calcium blockers bind specifically to multidrug resistant human KB carcinoma membrane vesicles and inhibit drug-binding to P-glycoproein. J Biol Chem **262:** 2166-2170

De Waard MA (1994) Resistance to fungicides which inhibit sterol 14α-demethylation, an historical perspective. *In* Fungicide resistance (Eds) Heaney S, Slawson D, Hollomon DW, Smith M, Russels PE and Parry DW, British Crop Protection Council, Surrey, UK, pp. 3-10

De Waard MA and Van Nistelrooy JGM (1980) An energy-dependent efflux for fenarimol in a wild-type strain and fenarimol-resistant mutants of *Aspergillus nidulans*. Pestic Biochem Physiol **10:** 219-229

De Waard MA and Van Nistelrooy JGM (1982) Antagonistic and synergistic activities of various chemicals on the toxicity of fenarimol to *Aspergillus nidulans*. Pestic Sci **13:** 279-286

De Waard MA and Van Nistelrooy JGM (1984a) Effects of phothalimide fungicides on the accumulation of the fenarimol by *Aspergillus nidulans*. Pestic Sci **15:** 56-62

De Waard MA, Van Nistelrooy JGM (1984b) Differential accumulation of fenarimol by a wild-type isolate and fenarimol-resistant isolates of *Penicillium italicum*. Neth J Pl Path **90:** 143-153

Del Sorbo G, Ruocco M, Lorito M, Scala F, Zoina A, Andrade AC and Waard MA (1998) Potential for exploitation of ATP-binding cassette (ABC) transporters in biological control. Mol Appr in Biol Con (IOBC wprs Bulletin), **21:** 241-246

Del Sorbo G, Schoonbeek H and De Waard MA (2000) Fungal transporters involved in efflux of natural toxic compounds and fungicides. Fung Gen Biol **30:** 1-15

Duncan EK and Howard RJ (2000) Cytological analysis of wheat infection by the leaf blotch pathogen *Mycosphaerella graminicola*. Mycol Res **104:** 1074-1082

Eyal Z, Scharen AL, Prescott JM and Van Ginkel M (1987) The Septoria diseases of wheat: Concepts and methods of disease management. (Eds) Hettel GP, CIMMYT, Mexico, pp. 51

Ford MJ (1990) Pharmacology of drugs that alter multidrug resistance in cancer. Pharmacol Rev **42:** 155-199

Gisi U, Binder H and Rimbach E (1985) Synergistic interactions of fungicides with different modes of action. Trans Br Mycol Soc **85:** 299-306

Gottesman MM and Pastan I (1993): Biochemistry of multidrug resistance mediated by multidrug transporter. Annu Rev Biochem **62:** 385-427

Hitchcok CA (1993): Resistance of *Candida albicans* to azole antifungal agents. Biochem Soc Trans **21:** 1039-1047

Kato T, Yamamoto S, Kawase Y and Ueda M (1975) Fungitoxic properties of a N-3-pyridylimidadithiocarbonate derivate. Ann Phytopathol Soc of Japan **41:** 1-8

Kato T, Shoami M and Kawase Y (1980) Comparison of tridemorph with buthioate in antifungal mode of action. J Pestic Sci **5:** 69-79

Kerkenaar A and Barug D (1984) Fluorescence microscope studies of *Ustilago maydis* and *Penicillium italicum* after treatment with imazalil or fenpropimorph. Pestic Sci **15:** 199-205

Maesaki S, Marichal P, Hossain MA, Sanglard D, Vanden Bossche H and Kohno S (1998) Synergistic effects of tacrolimus and azole antifungal agents against azole-resistant *Candida albicans* strains. J Antim Chem **42:** 747-753

Morse PM (1978) Some comments on the assessment of joint action in herbicide mixtures. Weed Sci **26:** 58-71

Nash RG (1981): Phytotoxic interaction studies-Techniques for evaluation and presentation of results. Weed Scien **29:** 147-155

Sauter H, Ammermann E, Benoit R, Brand S, Gold RE, Grammenos W, Kohle H, Lorenz G, Muller B, Rohl F, Schirmer U, Speakman JB, Wenderoth B and Wingert H (1995) Mitochodrial respiration as a target for antifungals: lessons from research on strobilurins. *In* Antifungal Agents-Discovery and Mode of Action (Eds) Dixon GK, Copping LG and Hollomon DW), BIOS Scientific Publishers, Oxford, UK, pp. 173-191

Sauter H, Steglich W and Anke T (1999) Strobilurins: Evolution of a new class of active substrates. Angew Chem Int Ed **38:** 1328-1349

Sherald JL, Ragsdale NN, Sisler HD (1973) Similarities between the systemic fungicides triforine and triarimol. Pestic Sci **4:** 719-727

Sisler HD, Ragsdale NN and Waterfield WW (1984) Biochemical aspects of the fungitoxic and growth regulatory action of fenarimol and other pyrimidin-5-ylmethanols. Pestic Sci **15:** 167-176

Wadley FM (1945) The evidence required to show synergistic action of insecticides and a short cut in analysis. *In* USDA, Washington DC, ET-223, US Department of Agriculture, pp. 1-8

Wadley FM (1967) Experimental Statistics in Entomology. Graduate School Press, *In* USDA, Washington DC, US Department of Agriculture, pp. 1-333

CHAPTER 9

GENERAL DISCUSSION

Cloning of ABC transporter genes from *M. graminicola*

The aim of this study was to clone ABC transporter genes from the wheat pathogen *M. graminicola* and establish the function of the encoded proteins in protection of the fungus against toxic compounds and pathogenesis. Two ABC transporter genes coded *MgAtr1* and *MgAtr2* were previously cloned from *M. graminicola* by heterologous screening of a genomic library of the fungus with a probe derived from *PDR5*, a well-characterised ABC transporter gene from *Saccharomyces cerevisiae* involved in Pleiotropic-Drug Resistance (PDR) (Zwiers and De Waard, 2000). Using a PCR-based approach with primers directed against conserved amino acid sequences of the Nucleotide-Binding Domains (NBDs) of ABC transporters we succeeded in cloning three additional ABC transporter genes, named *MgAtr3, MgAtr4,* and *MgAtr5* (Stergiopoulos *et al.,* 2002a; **Chapter 3**). Both approaches to clone ABC transporter genes from *M. graminicola* have been validated in cloning ABC genes from other filamentous fungi as well. These include *Aspergillus nidulans* (Del Sorbo *et al.,* 1997; Andrade, 2000), *Aspergillus flavus, Aspergillus fumigatus* (Tobin *et al.,* 1997), *Botrytis cinerea* (Schoonbeek *et al.,* 2001), and *Penicillium digitatum* (Nakaune *et al.,* 1998; Nakaune *et al.,* 2002).

Based on the topology of NBDs and Trans-Membrane Segments (TMSs), ABC transporters from *S. cerevisiae* can be subdivided into six clusters (Decottignies and Goffeau, 1997) or classes (Taglicht and Michaelis, 1998). MgAtr1-MgAtr5 from *M. graminicola* exhibit the $[NBD\text{-}TMS_6]_2$ topology, which classifies them as putative members of the PDR class of ABC transporters. ABC transporters from other classes were not identified (Zwiers and De Waard, 2000; Stergiopoulos *et al.,* 2002a; **Chapter 3**). Genome analysis programmes have reported the presence of 31 ABC proteins in *S. cerevisiae* (Taglicht and Michaelis, 1998) and a similarly high number in genomes of other fungi (Yoder and Turgeon, 2001). Hence, it is likely that *M. graminicola* also possesses more ABC proteins. Alternative approaches may be required to clone the corresponding genes. Screening of Expressed Sequence Tag (EST) libraries generated from RNA of *M. graminicola* grown *in vitro* under different cultural conditions or from wheat leaves infected by the pathogen would be an appropriate approach. EST libraries generated from interaction RNA may lead to identification of ABC proteins with a role in pathogenesis. Recently, such an approach identified several homologues of ABC proteins in *M. graminicola*. None of these proteins encoded PDR-like ABC transporters (Keon *et al.,* 2000).

A phylogenetic analysis of ABC transporters from filamentous fungi showed that ABC proteins with similar functions cluster in groups of orthologous proteins. An analysis with 23 ABC transporters with the [NBD-TMS_6]$_2$ topology from filamentous fungi identified a cluster involved in protection against DMI fungicides (Stergiopoulos *et al.,* 2002b; **Chapter 2**). Therefore, it might be possible to search for functional homologues of ABC proteins by screening orthologues within a cluster. Such an approach was validated in cloning ABC transporters from *A. nidulans* (Andrade, 2000) and *B. cinerea* (Hayashi *et al.,* 2001) involved in azole transport. Blast analysis of PMR1, an azole transporter from *P. digitatum,* with EST sequences from *A. nidulans* identified three ABC transporters from *A. nidulans,* highly homologous to azole efflux pumps from other organisms. Expression analysis demonstrated that the corresponding genes *(AnAtrE, AnAtrF,* and *AnAtrG)* are induced by azoles and display increased levels of expression in azole-resistant mutants of the fungus (Andrade, 2000).

Expression of ABC transporters from *M. graminicola* and substrate specificity

One of the major functions of ABC transporters in cells is to provide protection against toxic compounds present in the environment of the cell. Members of the PDR class of ABC transporters are known for their broad substrate specificity. Heterologous expression of *MgAtr1, MgAtr2, MgAtr4*, and *MgAtr5* from *M. graminicola* in *S. cerevisiae* mutants showed that the encoded proteins can transport a number of structurally unrelated compounds (**Chapter 4**). Therefore, MgAtr1, MgAtr2, MgAtr4, and MgAtr5 can be regarded as multidrug transporters from *M. graminicola*.

Expression analysis demonstrated that *MgAtr1-MgAtr5* are induced in *M. graminicola* by various chemical compounds (Zwiers and De Waard, 2000; Stergiopoulos *et al.,* 2002a; **Chapter 3**). However, heterologous expression of these genes in *S. cerevisiae* showed that the transformants had the same sensitivity to several inducing compounds as the wild-type strain. Disruption or replacement mutants of *MgAtr1-MgAtr5* in *M. graminicola* also displayed a similar sensitivity to most of the compounds tested as the wild-type strain (**Chapter 4**). Two laboratory-generated azole-resistant mutants of *M. graminicola* were detected (I323C1 and M1C4) that constitutively overexpressed *MgAtr1*. Disruption of *MgAtr1* in these mutants restored sensitivity to azoles only in I323C1 and not in M1C4 (**Chapter 6**). Finally, in field isolates of *M. graminicola* differences in basal level of expression of *MgAtr1-MgAtr5* did not

correlate with sensitivity to azoles (**Chapter 7**). Thus, compounds that induce expression of ABC transporter genes are not necessarily substrates of the encoded transporter proteins.

It has been demonstrated that expression of ABC transporter genes can be influenced by stress factors (Bauer *et al.,* 1999; Krishnamurthy *et al.,* 1998; Sukhai and Piquette-Miller, 2000). In that respect, it is not known whether the observed induction of ABC genes after treatment with compounds is due to the specific perception of the compound or an aspecific stress response caused by effects of the compound in cells. For example, a stress response might be the consequence of accumulation of azole fungicides in cell membranes, which can trigger a signalling pathway for activation of transcription of ABC transporter genes. However, transcription might also be activated indirectly by inhibition of $P450_{14DM}$ activity, which blocks sterol biosynthesis and results in the accumulation of abnormal sterol precursors that may have toxic activity (Kelly *et al.,* 1995; Vanden Bossche *et al.,* 1998). Such precursors may also act as stress factors and enhance transcription. The relation between overexpression of *MgAtr1-MgAtr5* and production of ABC protein in cells is also not known. Western blot analysis or functional assays measuring direct activity of ABC transporters could provide a more clear picture on the role of these proteins in transporting specific compounds.

Several compounds induce the expression of more than one ABC transporter gene. For example, cycloheximide, psoralen, and progesterone induce expression of *MgAtr1-MgAtr5* in at least one of the two dimorphic forms of the fungus (Zwiers and De Waard, 2000; Stergiopoulos *et al.,* 2002a; **Chapter 3**). Furthermore, several compounds induce the expression of both *MgAtr1* and *MgAtr4* and disruption of *MgAtr4* in *M. graminicola* leads to overexpression of *MgAtr1* (**Chapter 4**). Such observations suggest that some of the *M. graminicola* ABC genes might be under similar regulatory control. This would corroborate findings in other organisms where regulation of ABC proteins proceeds via a common transduction pathway (**Chapter 2**). The elucidation of such a pathway in *M. graminicola* would be important for a better understanding of the function of ABC transporters in this organism.

Redundancy of ABC transporters from *M. graminicola*

Heterologous expression of *MgAtr1-MgAtr5* in *S. cerevisiae* showed that the encoded proteins are able to transport a number of structurally unrelated compounds. However, *M. graminicola* mutants with disrupted or deleted ABC transporter genes showed increased sensitivity to only

a limited number of compounds tested (**Chapter 4**). ABC transporters constitute one of the largest protein families present in cells that can have broad and overlapping substrate specificity (Ambudkar *et al.,* 1999; Bauer *et al.,* 1999; Theodoulou, 2000; Stergiopoulos *et al.,* 2002b; **Chapter 2**). Hence, it is possible that upon deletion or replacement of *MgAtr1-MgAtr5* from *M. graminicola,* other efflux pumps with similar substrate specificity compensate for the non-functional transporter gene. Redundancy in ABC systems has been reported before (Ahmed *et al.,* 1995). In the pathogenic yeast *Candida albicans* at least two ABC proteins, CDR1 and CDR2, identified as azole-transporters in *S. cerevisiae,* were involved in azole resistance. However, although disruption of *CDR1* in *C. albicans* did result in increased sensitivity of this organism to azoles, mutants with disrupted *CDR2* showed increased sensitivity to azoles only in a *CDR1* disruption mutant and not in a wild-type isolate (Sanglard *et al.,* 1997). Disruption of *MgAtr4* in *M. graminicola* results in overexpression of *MgAtr1,* which might indicate that MgAtr1 compensates for the loss of MgAtr4. This idea is supported by the observation that MgAtr1 has a similar substrate specificity as MgAtr4 in the *S. cerevisiae* complementation experiments (**Chapter 4)** and a similar expression profile as determined by northern blot analysis (Zwiers and De Waard, 2000; Stergiopoulos *et al.,* 2002a; **Chapter 3**). The overlapping substrate specificity of MgAtr1 and MgAtr4 might also explain why the *MgAtr4* disruption mutants do not show complete loss of virulence on wheat seedlings (**Chapter 5**). This assumption would imply that MgAtr1 and MgAtr4 provide protection against similar compounds during pathogenesis.

ABC transporters as virulence factors in *M. graminicola*

Disruption of *MgAtr4* from *M. graminicola* resulted in reduced virulence of the fungus on wheat seedlings. Histopathological analysis of the infection process showed that *MgAtr4* disruption strains colonise substomatal cavities less efficiently than the wild-type strain and display reduced intercellular growth in the apoplast of infected wheat leaves. These results indicate that ABC transporters from *M. graminicola* can have an important function in determining virulence of the fungus on wheat plants (**Chapter 5**).

ABC transporters involved in virulence of phytopathogenic fungi have been described before (**Chapter 2**). In *Magnaporthe grisea* the ABC transporter ABC1 was identified as an important determinant of virulence of the fungus on barley and rice plants (Urban *et al.,* 1999). Expression analysis demonstrated that *ABC1* transcript levels are strongly induced by

multiple compounds, as for example the phytoalexin sakuranetin. However, *ABC1* disruption mutants do not show any increased sensitivity to these compounds and therefore, the exact role of this transporter in pathogenesis still needs to be established. Nevertheless, loss of virulence of *ABC1* disruption mutants on barley and rice plants was attributed to insufficient protection of the fungus against plant defence compounds (Urban *et al.,* 1999). In a similar way, the ABC transporters BcatrB and Gpabc1 were established as virulence factors of *B. cinerea* on grapevine leaves (Schoonbeek *et al.,* 2001) and *Gibberella pulicaris* on potato tubers (Fleissner *et al.,* 2002) by providing protection against resveratrol and rishitin, respectively. ABC transporters involved in the secretion of endogenously produced fungal toxins have not yet been identified. Instead, several transporters of the Major Facilitator Superfamily (MFS) with such a function have been reported (Stergiopoulos *et al.,* 2002b; **Chapter 2**).

The exact role of MgAtr4 during pathogenesis could not yet be established. We hypothesise that the transporter is involved in protection of the fungus against plant defence compounds produced around the substomatal cavities. The exact nature of such compounds is not yet known although Pnini-Cohen *et al.,* (1999) reported the production of fluorescent compounds in the vicinity of stomata infected by *M. graminicola*. Localised accumulation of autofluorescent compounds with antimicrobial activity has been reported in epidermal tissue in response to attack by *Erysiphe graminis* in barley (Kunoh *et al.,* 1982; Aist and Israel, 1986), oat (Kidger and Carver, 1981; Parry and Carver, 1986; Carver *et al.,* 1991), and wheat (Green *et al.,* 1975; Stadnik and Buchenauer, 1999). Production of the autofluorescent compounds was associated with cuticular contact of the pathogen with host cells and its intensity and extent was higher at appressorial than at germ-tube contact sites. Furthermore, accumulation of autofluorescent compounds at infection sites was much higher in resistant cultivars than in susceptible cultivars and the intensity of localised autofluorescence correlated with resistance to penetration by *E. graminis* (Mayama and Shishiyama, 1978; Carver *et al.,* 1991; 1992; 1995; Stadnik and Buchenauer, 1999). This autofluorescent response was independent of autofluorescence of epidermal and mesophyll cells undergoing hypersensitive cell death in race-specific resistance (Zeyen *et al.,* 1995). Characterisation of emission spectra of the compounds identified autofluorogens as phenolics, synthesised *de novo* in response to pathogen attack (Mayama and Shishiyama, 1978). Increased activity of phenylalanine ammonia-lyase (PAL) and tyrosine ammonia lyase activity as early as 4 hours after wheat

inoculation with *E. graminis* f. sp. *tritici* also suggests a role of phenols in plant defence (Green *et al.,* 1975). PAL is known to catalyse the first step in phenylpropanoid metabolism, by which it affects the synthesis of different phenolic compounds in plants (Dixon *et al.,* 1983; Jones, 1984).

The antimicrobial activity of phenolic compounds accumulating around the substomatal cavities may also explain the reduced virulence of the *MgAtr4* disruption mutants. This would imply a general response of wheat plants to attack by pathogens. To test this hypothesis, the intensity and extent of autofluorescence emitted from cells after inoculation with *M. graminicola* should be examined. Inhibitors of PAL activity, such as α-aminooxy-*β*-phenylpropionic acid (AOPP) and α-aminooxy acetic acid (AOA) can reduce biosynthesis of phenolic compounds in cells. Both inhibitors suppress accumulation of localised autofluorescent compounds and increase susceptibility of wheat, barley, and oat leaves to *E. graminis* (Carver *et al.,* 1991; 1992; 1994; 1996). Therefore, it would be interesting to investigate whether the use of these inhibitors may also restore virulence of the *MgAtr4* disruption mutants.

The isolation and characterisation of autofluorescent compounds is also important, since these defence compounds can be potentially useful in programmes to genetically engineer and select plants with a higher content of these compounds. Other options are to use autofluorescent compounds as lead structures in fungicide synthesis programmes or to search for activators of PAL activity that induce the production of phenolic compounds in plants (Nagai *et al.,* 1994; Dorey *et al.,* 1999).

Mechanisms of azole sensitivity in *M. graminicola*

Mechanisms of reduced sensitivity to azole fungicides were studied in laboratory-generated mutants as well as in field isolates of *M. graminicola*. Mechanisms studied involved decreased accumulation of azoles in cells, overexpression of the ABC transporter genes *MgAtr1-MgAtr5* and of *CYP51* encoding $P450_{14DM}$, and mutations in *CYP51*. Results from the studies indicated that multiple mechanisms may operate simultaneously (**Chapters 6** and **7**). These results corroborate findings with other organisms, such as *C. albicans*. Sub-populations of this pathogen generated from single clonal isolates did show distinct resistance mechanisms to azoles after treatment with these antifungal compounds, suggesting that the mechanism(s) of drug resistance is(are) selected rather randomly. In many cases, a rapid induction of

multidrug efflux pumps is observed after exposure to the antimicrobial agents, which suggests that this protective mechanism is utilised as a "first aid kit" by cells to oppose potential threats (De Waard, 1997; Franz *et al.,* 1998; Cowen *et al.,* 2000; Perea *et al.,* 2001; 2002; Martinez *et al.,* 2002; White *et al.,* 2002). This conclusion seems to corroborate the results with *M. graminicola.*

Decreased sensitivity to azoles in laboratory-generated mutants and field isolates of *M. graminicola* can coincide with either decreased or increased accumulation of the compounds in cells. In all isolates, addition of the uncoupler carbonyl-cyanide m-chlorophenylhydrazone (CCCP) induced an increased accumulation of cyproconazole in mycelial cells indicating that accumulation is energy-dependent (**Chapters 6** and **7**). This situation contrasts with that in other organisms, such as *A. nidulans* (De Waard and Van Nistelrooy, 1980), *B. cinerea* (Hayashi *et al.,* 2001), and *P. italicum* (De Waard and Van Nistelrooy, 1984) where decreased azole sensitivity always correlated with decreased accumulation of azoles in mycelium of the fungus. ABC and MFS transporters can account for the decreased accumulation of azoles in cells by efflux of these compounds into the extra-cellular space. However, these transporters may also be responsible for sequestration of azoles in vacuoles or other intracellular compartments that would result in increased intracellular accumulation of the compounds without affecting the cells. This mechanism could explain the higher accumulation of cyproconazole measured in some of the azole-tolerant field isolates and laboratory-generated mutants of *M. graminicola*. Intracellularly localised P-glycoprotein (P-gp), a well-characterised ABC protein of the MDR class of ABC transporters from human (Ambudkar *et al.,* 1999), plays a role in the sequestration of drugs within cytoplasmatic organelles (Shapiro *et al.,* 1998; Meschini *et al.,* 2000; Arancia *et al.,* 2001; Ferraoa *et al.,* 2001). This protection mechanism has also been described in plants where sequestering of endotoxins, heavy metals, and natural pigments occurs through a specific subclass of ABC transporters (Rea, 1999).

Accumulation of azoles by *M. graminicola* as measured in our uptake experiments is the net result of both influx and efflux of the compounds from cells. It is known that the cell wall and membrane composition can significantly reduce plasma membrane permeability and may contribute to azole resistance. The plasma membrane composition can also strongly influence substrate specificity and ATPase activity of ABC transporters (Hitchcock *et al.,* 1986; Sinicrope *et al.,* 1992; Baggetto, 1997; White *et al.,* 1998; Lavie *et al.,* 1999; Van Den Hazel *et al.,* 1999; Lavie and Liscovitch, 2000; Ferte, 2000; Pallares-Trujillo *et al.,* 2000). Our

studies seem to corroborate this observation, since differences in expression of ABC transporter genes in yeast-like cells and mycelium of *M. graminicola* (**Chapter 3**) could be attributed to differences in the membrane composition of the two dimorphic forms. Differences in substrate specificity of MgAtr1-MgAtr5 in *M. graminicola* and *S. cerevisiae* could also be the result of differences in the intrinsic membrane composition of the two organisms (**Chapter 4**). Therefore, the natural variation in the membrane composition of laboratory mutants and field isolates of *M. graminicola,* or alterations in membranes induced by the effect of azoles on ergosterol biosynthesis, can play a more important role in azole-sensitivity than initially anticipated.

Modulating the activity of ABC transporters

ABC transporters from *M. graminicola* can be involved as described before, in protection against antifungal compounds and in virulence of the fungus. Therefore, finding ways to modulate the activity of ABC proteins in the fungus might facilitate disease control.

Modulation of ABC transporter activity can occur with compounds known as ABC transporter modulators or chemosensitisers. By themselves such compounds generally have little or no toxic activity, but when used in combination with other antimicrobials they can improve their efficacy in disease control. Such compounds have already been synthesised and used in clinical trials for the treatment of MDR in tumor cells. Examples of modulators are anthracycline and *Vinca* alkaloid analogues, calcium channel blokers, calmodulin antagonists, cyclosporines, steroids and hormonal analogues, and other miscellaneous hydrophobic cationic compounds (Ford and Hait, 1990; 1993; Ford *et al.,* 1996; Scala *et al.,* 1997; Ambudkar *et al.,* 1999; Krishna and Mayer, 2000; Shabbits *et al.,* 2001). Modulators of ABC transporter activity can also improve the *in vitro* activity of azole fungicides against fungi, such as *A. nidulans* (De Waard and Van Nistelrooy, 1982)*, P. italicum* (De Waard and Van Nistelrooy, 1984) and *B. cinerea* (Hayashi *et al.,* in press).

We have tested the combined activity of known ABC transporter modulators in combination with azole fungicides to a randomly selected field isolate of *M. graminicola*. Only additive effects between the compounds tested were observed (**Chapters 8**). However, the isolate used in these experiments was relatively sensitive to azole fungicides and therefore, ABC transporters may have been less important for the sensitivity level of this isolate to azoles. As modulators may also have a selective activity on specific efflux pumps it is also possible that

we did not test the proper modulators. Therefore, we performed preliminary studies on the activity of modulators with field isolates of *M. graminicola* that varied in base-line sensitivity to azole fungicides and in cyproconazole accumulation levels (non-published results). The strains used were the field isolates M4, M7, S043, and S190 (**Chapter 7**). Compounds tested were the azole fungicide cyproconazole in combination with chlorpromazine (phenothiazine), cycloheximide (protein synthesis inhibitor), kresoxim-methyl (QoI-inhibitor), progesterone (steroid), quercetin (flavanoid), quinidine (alkaloid), reserpine (indole alkaloid), rhodamine 6G (dye), verapamil (phenylacetonitrile), and tacrolimus (macrolide antibiotic). The modulating activity of the compounds on fungicide activity of cyproconazole was qualitatively assessed in crossed-paper strip experiments (De Waard and Van Nistelrooy, 1982; Sugiura *et al.,* 1993). A synergistic interaction was only observed for chlorpromazine. The synergism was observed for all isolates tested and proved to be dependent of the concentration of both compounds. Results obtained with field isolate S190, which has a relatively low sensitivity to cyproconazole, are presented in Figure 1.

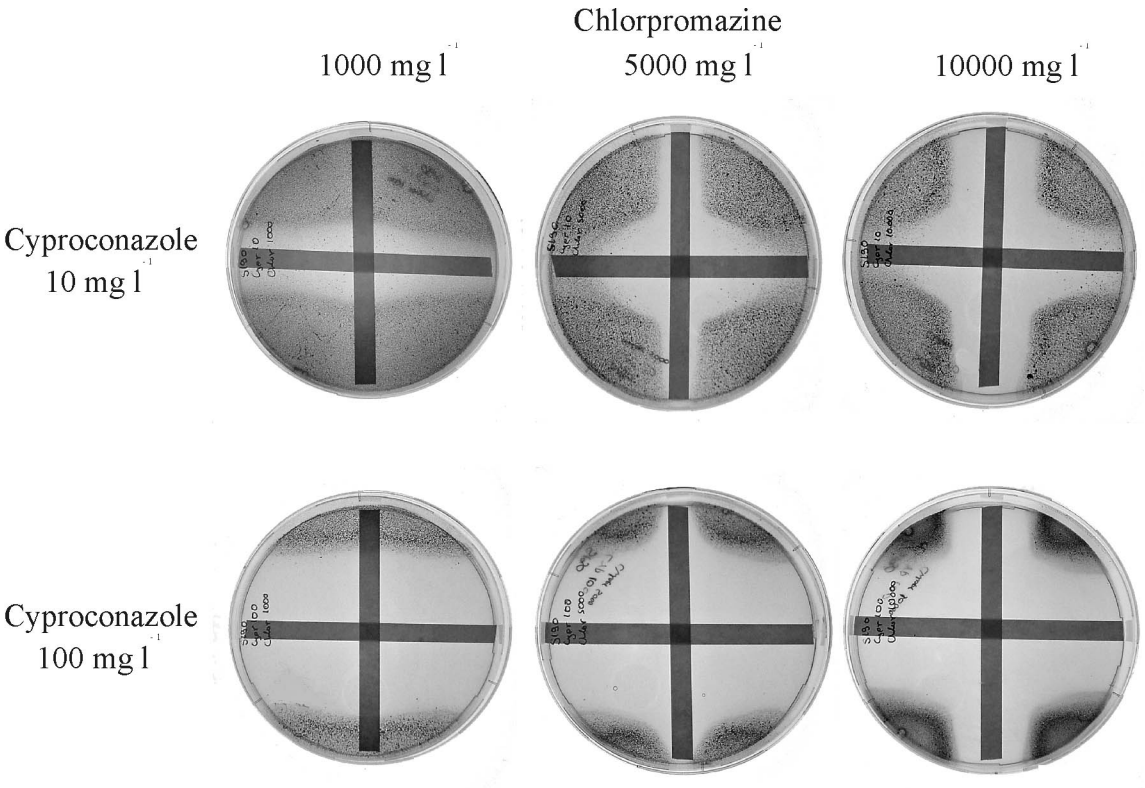


Figure 1. Synergistic activity between cyproconazole (horizontal paper-strips) and chlorpromazine (vertical paper-strips) to *Mycosphaerella graminicola* (field isolate S190). Figures indicate concentrations of solutions used to impregnate paper-strips with compounds, before application of the strips on agar seeded with yeast-like cells of the fungus.

These results suggest a role for ABC transporters in determining base-line sensitivity to azoles in these isolates. Similar concentrations of chlorpromazine induced a similar increase in sensitivity to cyproconazole in all isolates tested, suggesting that a specific azole-transporter is inhibited. Therefore, chlorpromazine-insensitive transporters or other mechanisms of resistance may account for decreased sensitivity to azoles of field isolates. This hypothesis corroborates our conclusion that multiple mechanisms are involved in the variation in base-line sensitivity of field isolates of *M. graminicola* to azoles (**Chapter 7**). Modulation of ABC transporter activity by chlorpromazine has been demonstrated before in mammalian tumor cells (Bebawy *et al.,* 2001), *C. albicans* (Krajewska-Kulak and Niczyporuk, 1993) and other yeast species (Ben-Gigi *et al.,* 1988), *Escherichia coli* (Molnar *et al.,* 1997), *Leishmania* spp. (Essodaigui *et al.,* 1999), and *B. cinerea* (Hayashi *et al.,* in press). The modulation is ascribed to competitive inhibition of transport with the test compounds (Syed *et al.,* 1996). An interesting question arises whether modulation of ABC transporter activity could also take place during pathogenesis. If so, modulators might improve efficacy of fungicides or increase activity of plant defence compounds (De Waard, 1997).

Modulation of the activity of plant ABC transporters can also be relevant for plant disease control. This hypothesis is based on the discovery of diverse compounds capable of protecting human cells against harmful drugs by modulating the P-gp-mediated efflux. These compounds can alter the P-gp substrate specificity either by enhancing the specificity of the transporter for some compounds and reducing it for others, or by enabling transport of compounds that were previously not a substrate of the protein. This phenomenon has been described as *Retargeting* of ABC transporters (Kondratov *et al.,* 2001). Pathogenesis of *M. graminicola* probably involves the secretion of toxins (Kema *et al.,* 1996). Therefore, it would be interesting to investigate whether such compounds could also be used to enhance wheat defence by retargeting of plant ABC proteins for enhanced transport of fungal toxins and wheat defence compounds. An important prerequisite of such modulators or *activators* would be selective action between target ABC proteins in the pathogen and the host. Kondratov *et al.,* (2001) speculated that P-gp can be “fine-tuned” with appropriate modulators to optimise substrate specificity. A similar approach might be possible for retargeting the substrate specificity of plant ABC transporters, without altering substrate specificity of fungal ABC proteins.

REFERENCES

Ahmed M, Lyass L, Markham PN, Taylor SS, Vazquez-Laslop N and Neyfakh AA (1995) Two highly similar multidrug transporters of *Bacillus subtilis* whose expression is differentially regulated. J Bacteriol 1995 **177:** 3904-3910

Aist JR and Israel HW (1986) Autofluorescence and ultraviolet-absorbing components in cell walls and papillae of barley coleoptiles and their relationship to disease resistance. Can J Bot **64:** 266-272

Ambudkar SV, Dey S, Hrycyna CA, Ramachandra M, Pastan I and Gottesman MM (1999) Biochemical, cellular, and pharmacological aspects of the multidrug transporter. Annu Rev Pharmacol Toxicol **39:** 361-98

Andrade AC (2000) ABC transporters and multidrug resistance in *Aspergillus nidulans.* Thesis Wageningen University, Wageningen, The Netherlands.

Arancia G, Molinari A, Calcabrini A, Meschini S and Chanfriglia M (2001) Intracellular P-glycoprotein in multidrug resistant tumor cells. Ital J Anat Embryol 2001 **106:** 59-68

Baggetto LG (1997) Biochemical, genetic, and metabolic adaptations of tumor cells that express the typical multidrug-resistance phenotype. Reversion by new therapies. J Bioenerg Biomembr **29:** 401-413

Bauer BE, Wolfger H and Kuchler K (1999) Inventory and function of yeast ABC proteins: about sex, stress, pleiotropic drug and heavy metal resistance. Biochim Biophys Acta **1461:** 217-236

Bebawy M, Morris MB and Roufogalis BD (2001) Selective modulation of P-glycoprotein-mediated drug resistance. Br J Cancer **85:** 1998-2003

Ben-Gigi G, Polacheck I and Eilam Y (1988) *In vitro* synergistic activity of ketoconazole with trifluoperazine and with chlorpromazine against medically important yeasts. Chemotherapy **34:** 96-100

Carver TLW, Robbins MP and Zeyen RJ (1991) Effects of two PAL inhibitors on the susceptibility and localised autofluorescent host cell response of oat leaves attacked by *Erysiphe graminis* DC. Physiol Mol Plant Pathol **39:** 269-287

Carver TLW, Zeyen RJ, Robbins MP and Dearne GA (1992) Effects of the PAL inhibitor AOPP, on oat, barley, and wheat cell responces to appropriate and inappropriate formae species of *Erysiphe graminis* DC. Physiol Mol Plant Pathol **41:** 149-163

Carver TLW, Zeyen RJ, Bushnell WR and Robbins MP (1994) Inhibition of phenylalanine ammonia-lyase and cinnamyl alcohol dehydrogenase increased quantitative susceptibility of barley to powdery mildew (*Erysiphe graminis* DC). Physiol Mol Plant Pathol **41:** 149-163

Carver TLW, Zeyen RJ and Lyngkjaer MF (1995) Plant cell defences to powdery mildew of Gramineae. Asp Appl Biol **42:** 257-266

Carver TLW, Zhang L, Zeyen RJ and Robbins MP (1996) Phenolic biosynthesis inhibitors supress adult plant resistance to *Erysiphe graminis* in oat at 20ºC and 10ºC. Physiol Mol Plant Pathol **49:** 121-141

Cowen LE, Sanglard D, Calabrese D, Sirjusingh C, Anderson JB and Kohn LM (2000) Evolution of drug resistance in experimental populations of *Candida albicans.* J Bacteriol **182:** 1515-1522

De Waard MA (1997) Significance of ABC transporters in fungicide sensitivity and resistance. Pestic Sci **51:** 271-275

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

De Waard MA and Van Nistelrooy JGM (1982) Antagonistic and synergistic activities of various chemicals on the toxicity of fenarimol to *Aspergillus nidulans*. Pestic Sci **13:** 279-286

De Waard MA and Van Nistelrooy JGM (1984) Effects of phthalimide fungicides on the accumulation of fenarimol by *Aspergillus nidulans*. Pestic Sci **15:** 56-62

Decottignies A and Goffeau A (1997) Complete inventory of the yeast ABC proteins. Nat Genet **15:** 137-145

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

Dixon RA, Dey MP and Lamp CJ (1983) Phytoalexins: enzymology and molecular biology. Adv Enzymol Rel Ar Mol Biol **55:** 1-135

Dorey S, Kopp M, Geoffroy P, Fritig B and Kauffmann S (1999) Hydrogen peroxide from the oxidative burst is neither necessary nor sufficient for hypersensitive cell death induction, phenylalanine ammonia lyase stimulation, salicylic acid accumulation, or scopoletin consumption in cultured tobacco cells treated with elicitin. Plant Physiol **121:** 163-172

Essodaigui M, Frezard F, Moreira ES, Dagger F and Garnier-Suillerot A (1999) Energy-

dependent efflux from *Leishmania* promastigotes of substrates of the mammalian multidrug resistance pumps. Mol Biochem Parasitol **100:** 73-84

Ferraoa P, Sincocka P, Colea S and Ashman L (2001) Intracellular P-gp contributes to functional drug efflux and resistance in acute myeloid leukaemia. Leukem Res **25:** 395-405

Ferte J (2000) Analysis of the tangled relationships between P-glycoprotein-mediated multidrug resistance and the lipid phase of the cell membrane. Eur J Biochem **267:** 277-294

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. Mol Plant-Microbe Interact **15:** 102-108

Ford JM and Hait WN (1990) Pharmacology of drugs that alter multidrug resistance in cancer. Pharmacol Rev **42:** 155-199

Ford JM and Hait WN (1993) Pharmacologic circumvention of multidrug resistance. Cytotechnology **12:** 171-212

Ford JM, Yang JM and Hait WN (1996) P-glycoprotein-mediated multidrug resistance: experimental and clinical strategies for its reversal. Cancer Treat Res **87:** 3-38

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. Antimicrob Agents Chemother **42:** 3065-3072

Green NE, Hadwiger LA and Shirl OG (1975) Phenylalanine ammonia-lyase, tyrosine ammonia-lyase, and lignin in wheat inoculated with *Erysiphe graminis* f. sp. *tritici*. Phytopathology **65:** 1071-1074

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.* Pestic Biochem Physiol **70:** 168-179

Hayashi K, Schoonbeek H-J and De Waard MA (in press) Modulators of membrane drug transporters potentiate the activity of the DMI fungicide oxpoconazole against *Botrytis cinerea*. Pest Bioch Physiol

Hitchcock CA, Barrett-Bee KJ and Russell NJ (1986) The lipid composition of azole-sensitive and azole-resistant strains of *Candida albicans*. J Gen Microbiol **132:** 2421-2431

Jones DH (1984) Phenylalanine ammonia-lyase: regulation of its induction, and its role in plant development. Phytochemistry **23:** 1349-1360

Kelly SL, Lamb DC, Corran AJ, Baldwin BC and Kelly D (1995) Mode of action and resistance to azole antifungals associated with the formation of 14 alpha-methylergosta-8,24(28)-dien-3 beta,6 alpha-diol. Biochem Biophys Res Commun **207:** 910-915

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

Keon J, Bailey A and Hargreaves J (2000) A group of expressed cDNA sequences from the wheat fungal leaf blotch pathogen, *Mycosphaerella graminicola* (*Septoria tritici*). Fun Gen Biol **29:** 118-133

Kidger AL and Carver TLW (1981) Autofluorescence in oats infected by powdery mildew. Trans Brit Mycol Soc **76:** 405-409

Kondratov RV, Komarov PG, Becker Y, Ewenson A and Gudkov AV (2001) Small molecules that dramatically alter multidrug resistance phenotype by modulating the substrate specificity of P-glycoprotein. Proc Natl Acad Sci USA **98:** 14078-14083

Krajewska-Kulak E and Niczyporuk W (1993) Effects of the combination of ketoconazole and calmodulin inhibitors against *Candida albicans in vitro.*Short communication. Arzneimittelforschung **43:** 1018-1019

Krishna R and Mayer LD (2000) Multidrug resistance (MDR) in cancer. Mechanisms, reversal using modulators of MDR and the role of MDR modulators in influencing the pharmacokinetics of anticancer drugs. Eur J Pharm Sci **11:** 265-283

Krishnamurthy S, Gupta V, Prasad R and Panwar SL (1998) Expression of CDR1, a multidrug resistance gene of *Candida albicans*: transcriptional activation by heat shock, drugs and human steroid hormones. FEMS Microbiol Lett **160:** 191-7

Kunoh H, Yamamori K and Ishizaki H (1982) Cytological studies of early stages of powdery mildew in barley and wheat. VIII. Autofluorescence at penetration sites of *Erysiphe graminis* f. sp. *hordei* on living barley coleoptiles. Physiol Plant Pathol **21:** 373-379

Lavie Y, Fiucci G, Czarny M and Liscovitch M (1999) Changes in membrane microdomains and caveolae constituents in multidrug-resistant cancer cells. Lipids **34 (Suppl):** S57-S63

Lavie Y and Liscovitch M (2000) Changes in lipid and protein constituents of rafts and caveolae in multidrug resistant cancer cells and their functional consequences. Glycoconj J **17:** 253-259

Martinez M, Lopez-Ribot JL, Kirkpatrick WR, Bachmann SP, Perea S, Ruesga MT and

Patterson TF (2002) Heterogeneous mechanisms of azole resistance in *Candida albicans* clinical isolates from an HIV-infected patient on continuous fluconazole therapy for oropharyngeal candidosis. J Antimicrob Chemother **49:** 515-524

Mayama S and Shishiyama J (1978) Localised accumulation of fluorescent and u.v.-absorbing compounds at penetration sites in barley leaves infected with *Erysiphe graminis* f. sp. *hordei*. Physiol Plant Pathol **13:** 347-354

Meschini S, Calcabrini A, Monti E, Del Bufalo D, Stringaro A, Dolfini E and Arancia G (2000) Intracellular P-glycoprotein expression is associated with the intrinsic multidrug resistance phenotype in human colon adenocarcinoma cells. Int J Cancer **87:** 615-628

Molnar J, Hever A, Fakla I, Fischer J, Ocsovski I and Aszalos A (1997) Inhibition of the transport function of membrane proteins by some substituted phenothiazines in *Escherichia coli* and multidrug resistant tumor cells. Anticancer Res **17:** 481-486

Nagai N, Kitauchi F, Okamoto K, Kanda T, Shimosaka M and Okazaki M (1994) A transient increase of phenylalanine ammonia-lyase transcript in kinetin-treated tobacco callus. Biosci Biotechnol Biochem **58:** 558-559

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*. Appl Environ Microbiol **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*. Mol Genet Genom **267:** 179-185

Pallares-Trujillo J, Lopez-Soriano FJ and Argiles JM (2000) Lipids: A key role in multidrug resistance? (Review). Int J Oncol **16:** 783-798

Parry AL and Carver TLW (1986) Relationships between colony development, resistance to penetration and autofluorescence in oats infected with powdery mildew. Trans Brit Mycol Soc **86:** 355-363

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. Antimicrob Agents Chemother **45:** 2676-2684

Perea S, Lopez-Ribot JL, Wickes BL, Kirkpatrick WR, Dib OP, Bachmann SP, Keller SM, Martinez M and Patterson TF (2002) Molecular mechanisms of fluconazole resistance in *Candida dubliniensis* isolates from Human immunodeficiency virus-infected patients with Oropharyngeal Candidiasis. Antimicrob Agents Chemother **46:** 1695-1703

Pnini-Cohen S, Zilberstein A, Schuster S, Sharon A and Eyal Z (1997) The expression of the uidA reporter gene and virulence in transformants of *Septoria tritici.* 15th Long Ashton International Symposium: Understanding Pathosystems: A Focus on Septoria. Long Ashton, UK, pp. 60

Rea PA (1999) MRP subfamily ABC transporters from plants and yeast. J Exp Bot **50 Special Iss:** 895-913

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. Microbiol **143:** 405-416

Scala S, Akhmed N, Rao US, Paull K, Lan LB, Dickstein B, Lee JS, Elgemeie GH, Stein WD and Bates SE (1997) P-glycoprotein substrates and antagonists cluster into two distinct groups. Mol Pharmacol **51:** 1024-1033

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. Mol Plant-Microbe Interact **14:** 562-571.

Shabbits JA, Krishna R and Mayer LD (2001) Molecular and pharmacological strategies to overcome multidrug resistance. Expert Rev Anticancer Ther **1:** 585-594

Shapiro AB, Fox K, Lee P, Yang YD and Ling V (1998) Functional intracellular P-glycoprotein. Int J Cancer **76:** 857-864

Sinicrope FA, Dudeja PK, Bissonnette BM, Safa AR and Brasitus TA (1992) Modulation of P-glycoprotein-mediated drug transport by alterations in lipid fluidity of rat liver canalicular membrane vesicles. J Biol Chem **267:** 24995-5002

Stadnik MJ and Buchenauer H (1999) Accumulation of autofluorogenic componds at the penetration site of *Blumeria graminis* f. sp. *tritici* is associated with both benzothiadiazole-induced and quantitative resistance of wheat. J Phytopath **147:** 615-622

Stergiopoulos I, Gielkens MM, Goodall SD, Venema K and De Waard MA (2002a) Molecular cloning and characterisation of three new ATP-binding cassette transporter genes from the wheat pathogen *Mycosphaerella graminicola*. Gene **289:** 141-149

Stergiopoulos I, Zwiers L-H and De Waard MA (2002b) Secretion of natural and synthetic toxic compounds from filamentous fungi by membrane

transporters of the ATP-binding cassette and Major facilitator superfamily. Eur J Plant Pathol **108:** 719-734

Sugiura H, Hayashi K, Tanaka T and Uesugi Y (1993) Mutual antagonism between sterol demethylation inhibitors and phosphorothiolate fungicides on *Pyricularia oryzae* and implications for their mode of action. Pestic Sci **39:** 193-198

Sukhai M and Piquette-Miller M (2000) Regulation of the multidrug resistance genes by stress signals. J Pharm Pharm Sci 2000 **3:** 268-280

Syed SK, Christopherson RI and Roufogalis BD (1996) Chlorpromazine transport in membrane vesicles from multidrug resistant CCRF-CEM cells. Biochem Mol Biol Int **39:** 687-696

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

Theodoulou FL (2000) Plant ABC transporters. Biochimica et Biophysica Acta (BBA) - 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

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

Van Den Hazel HB, Pichler H, do Valle Matta MA, Leitner E, Goffeau A and Daum G (1999) *PDR16* and *PDR17*, two homologous genes of *Saccharomyces cerevisiae*, affect lipid biosynthesis and resistance to multiple drugs. J Biol Chem **274:** 1934-1941

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

White TC, Marr KA and Bowden RA (1998) Clinical, cellular, and molecular factors that contribute to antifungal drug resistance. Clin Microbiol Rev **11:** 382-402

White TC, Holleman S, Dy F, Mirels LF and Stevens DA (2002) Resistance mechanisms in clinical isolates of *Candida albicans.* Antimicrob Agents Chemother **46:** 1704-1413

Yoder OC and Turgeon BG (2001) Fungal genomics and pathogenicity. Curr Opin Plant Biol **4:** 315-321

Zeyen RJ, Bushell WR, Carver TLW, Robbins MP, Clark TA, Boyles DA and Vance CP (1995) Inhibiting phenylalanine ammonia lyase and cinnamyl-alcohol dehydrogenase supresses Mla1 (HR) but not mlo5 (non-HR) barley powdery mildew resistances. Physiol Mol Plant Pathol **47:** 119-140

Zwiers L-H and De Waard MA (2000) Characterization of the ABC transporter genes *MgAtr1* and *MgAtr2* from the wheat pathogen *Mycosphaerella graminicola*. Fun Genet Biol **30:** 115-125

SUMMARY

The aim of this study was to clone ATP-binding cassette (ABC) transporter genes from the wheat pathogen *Mycosphaerella graminicola* and to establish the role of the encoded proteins for the fungus in pathogenesis on wheat and in protection against toxic compounds.

In ***Chapter 1*** a description of *M. graminicola* (anamorph state: *Septoria tritici*) is given. The pathogen is the causal agent of septoria tritici blotch of wheat. The disease has a worldwide distribution and is an increasingly important threat to wheat crops, especially in areas with high humidity and moderate temperatures. Therefore, understanding of the molecular mechanisms of pathogenesis and fungicide resistance development in this pathogen is important. Disease management of *M. graminicola* has widely involved the use of azole fungicides. The mode of action of these fungicides is based on inhibition of cytochrome P450 sterol 14α-demethylase ($P450_{14DM}$) activity, a key enzyme of the sterol biosynthetic pathway. In plant pathogenic fungi four major mechanisms of resistance to azoles have been reported. One of these is reduced accumulation of the fungicides in mycelium, attributed to an energy-dependant efflux mechanism mediated by ABC or major facilitator superfamily (MFS) transporters. These mechanisms are described in Chapter 1.

In ***Chapter 2*** ABC and MFS transporters from filamentous fungi are reviewed. These transporters comprise two of the largest protein families known to date. The proteins are located in the plasma membrane or in membranes of intracellular compartments and are capable of transporting a wide variety of cytotoxic agents against a concentration gradient. In case of ABC transporters, the energy needed for transport is generated by ATP-hydrolysis and therefore, ABC transporters are characterised as primary active transport systems. MFS transporters use the energy from the electrochemical gradient across membranes and for this reason they are classified as secondary transport systems. ABC and MFS transporters can play an essential role in multidrug resistance (MDR) of cells to chemically unrelated compounds. ABC and MFS transporters involved in resistance to fungitoxic compounds have also been described in filamentous fungi. In plant pathogenic fungi these transporters may act as virulence factors if they mediate secretion of virulence factors, such as host specific toxins, or provide protection against plant defence compounds during pathogenesis.

Chapter 3 describes the cloning and characterisation of three single copy ABC transporter genes from *M. graminicola*, using a PCR-based approach. The genes were named *MgAtr3*, *MgAtr4,* and *MgAtr5.* Based on the topology of the encoded proteins, MgAtr3-MgAtr5 can be classified as novel members of the PDR class of ABC transporters. Expression of *MgAtr3* could not be detected by northern blot analysis under the conditions tested. However, *MgAtr4* and *MgAtr5* displayed distinct expression profiles when treated with a range of compounds known to be either substrates or inducers of ABC transporters. These included synthetic fungitoxic compounds, such as imazalil and cyproconazole, natural toxic compounds, such as the plant defence compounds eugenol and psoralen, and the antibiotics cycloheximide and neomycin. The expression pattern of the genes was also dependent on the morphological state of the fungus.

In ***Chapter 4*** the function of *MgAtr1-MgAtr5* from *M. graminicola* in protection of the fungus against natural and synthetic toxic compounds is further analysed. Heterologous expression of *MgAtr1, MgAtr2, MgAtr4,* and *MgAtr5* in *Saccharomyces cerevisiae* showed that the encoded proteins can transport a number of chemically unrelated compounds and have distinct but overlapping substrate specificities. Therefore, MgAtr1, MgAtr2, MgAtr4, and MgAtr5 can be considered as multidrug transporters from *M. graminicola*. Analysis of *MgAtr1-MgAtr5* gene disruption or replacement mutants in *M. graminicola*, showed that deletion of *MgAtr5* slightly increases sensitivity of the fungus to the putative wheat defence compound resorcinol and the grape phytoalexin resveratrol. Bioassays with antagonistic bacteria indicated that MgAtr2 provides protection of *M. graminicola* against antibiotics produced by *Pseudomonas fluorescens* and *Burkholderia cepacia*.

Chapter 5 describes the function of *MgAtr1-MgAtr5* in virulence of *M. graminicola* on wheat. Gene disruption or replacement mutants of *MgAtr1, MgAtr2, MgAtr3,* and *MgAtr5* displayed an unaltered phenotype in comparison to the wild-type control strain. However, virulence of the *MgAtr4* disruption mutants was significantly reduced on seedlings of all wheat cultivars tested. Histopathological analysis of the infection process showed that *MgAtr4* disruption mutants have reduced capacity to colonise substomatal cavities and display reduced growth in the apoplast of infected wheat leaves. *In vitro* growth test experiments on different media showed no growth defects associated with the disruption of *MgAtr4*. Therefore, the results indicate that MgAtr4 is a virulence factor of *M. graminicola*. MgAtr4 is the first virulence factor identified so far from this important plant pathogen.

In ***Chapter 6*** studies on mechanisms of azole resistance in laboratory-generated azole-resistant mutants of *M. graminicola* are described. These mutants displayed an MDR phenotype by exhibiting cross-resistance to the unrelated chemicals cycloheximide and/or rhodamine 6G. Mechanisms studied involved increased efflux of azoles from cells mediated by ABC transporters, overexpression of *CYP51* encoding $P450_{14DM}$, and effects of mutations in the coding sequence of this gene. Reduced accumulation of azoles in mycelium of the fungus played a role in the resistance of some of the mutants to azoles. However, additional resistance mechanisms, such as sequestration of azoles in cellular compartments also seemed to operate.

In ***Chapter 7*** molecular mechanisms that account for variation in base-line sensitivity to azole fungicides in field isolates of *M. graminicola* are studied. The isolates tested showed a large variation in sensitivity to azoles. The isolates were cross-sensitive to the azoles cyproconazole, ketoconazole, and tebuconazole, but not to unrelated chemicals, such as cycloheximide, kresoxim-methyl, and rhodamine 6G. Genetic analysis showed that azole sensitivity in *M. graminicola* is a polygenic trait. The basal expression level of *MgAtr1-MgAtr5* significantly varied among the isolates but no correlation between expression of a specific ABC gene and azole sensitivity was observed. Cyproconazole particularly induced expression of *MgAtr4* but no correlation between expression of this gene and azole sensitivity was observed either. One isolate with intermediate sensitivity to azoles showed high levels of *CYP51* expression. Decreased accumulation of azoles in mycelium did not correlate with azole sensitivity. The results indicate that multiple mechanisms account for differences in base-line sensitivity to azoles in field isolates of *M. graminicola*. This conclusion is in line with the proposed mechanisms of resistance to azoles in laboratory-generated mutants of *M. graminicola*.

In ***Chapter 8*** the antifungal activity of the azole fungicides cyproconazole and propiconazole as single compounds and in combination with ABC transporter modulators is studied. Tests were performed with a wild-type field isolate of *M. graminicola* that possessed a moderate sensitivity to azole fungicides. The activity of the compounds was evaluated in spore germination and germ-tube growth tests. Analysis of interactions between the compounds with the Colby method showed additive effects in most mixtures tested. Some synergism in mixtures of cyproconazole with verapamil and kresoxim-methyl was also

observed. Analysis with the Wadley method revealed additive interactions in mixtures of cyproconazole and kresoxim-methyl.

Chapter 9 represents the general discussion of the thesis with special emphasis on selected topics, such as possible approaches to clone additional ABC transporter genes from *M. graminicola*, the relation between expression of ABC genes and substrate specificity of the encoded proteins, the redundancy in ABC transporters, the function of ABC transporters as virulence factors, the mechanisms of azole sensitivity and resistance in *M. graminicola*, and finally ways to modulate ABC transporter activity.

In conclusion, data presented in this thesis show that ABC transporters from *M. graminicola* have a number of important functions. They can act as virulence factors of plant pathogens. In addition, they may provide protection against natural and synthetic, toxic compounds and account for base-line sensitivity and fungicide resistance of fungi to azole fungicides.

SAMENVATTING

Het doel van dit onderzoek was de klonering van ATP-bindings cassette (ABC) transportgenen van het tarwepathogeen *Mycosphaerella graminicola* en de bestudering van hun functie tijdens de pathogenese van de schimmel op tarwe en bij bescherming tegen toxische verbindingen.

Hoofdstuk 1 geeft een beschrijving van *M. graminicola* (anamorf *Septoria tritici*). Het pathogeen is de veroorzaker van de septoria tritici bladvlekkenziekte op tarwe. De ziekte komt overal ter wereld voor en is in toenemende mate een bedreiging voor de tarweteelt, speciaal in gebieden met een hoge luchtvochtigheid en gematigde temperatuur. Om die redenen is een beter begrip van moleculaire mechanismen die tijdens de pathogenese een rol spelen en van de ontwikkeling van resistentie tegen fungiciden belangrijk. De bestrijding van *M. graminicola* berust in hoge mate op het gebruik van azoolfungiciden. Het werkingsmechanisme van deze fungiciden is gebaseerd op remming van de activiteit van cytochroom P450-afhankelijk sterol 14α-demethylase ($P450_{14DM}$), een sleutelenzym in de sterolbiosynthese. In plantenpathogene schimmels zijn vier belangrijke mechanismen van resistentie tegen azolen bekend. Eén van deze mechanismen is verminderde accumulatie van de fungiciden in mycelium, hetgeen wordt toegeschreven aan energie-afhankelijke efflux door ABC en major facilitator superfamily (MFS) transporteiwitten. Deze mechanismen worden eveneens in hoofdstuk 1 beschreven.

Hoofdstuk 2 geeft een overzicht van ABC en MFS transporteiwitten in filamenteuse schimmels. De transporteiwitten vormen twee van de grootste eiwitfamilies die momenteel bekend zijn. De eiwitten zijn gelokaliseerd in de plasmamembraan of in membranen van intracellulaire compartimenten en zijn in staat om uiteenlopende cytotoxische verbindingen tegen een concentratiegradiënt te transporteren. ABC transporteiwitten genereren de energie die nodig is voor transport door hydrolyse van ATP en daarom worden ABC transporteiwitten gekarakteriseerd als primair actieve transport systemen. MFS transporteiwitten benutten de energie van de elektrochemische gradiënt over membranen en daarom worden deze eiwitten beschreven als secundair actieve transport systemen. ABC en MFS transporteiwitten kunnen een essentiële rol vervullen in multidrug resistentie (MDR) van cellen tegen chemisch niet-verwante verbindingen. ABC en MFS transporteiwitten van schimmels kunnen betrokken zijn bij resistentie tegen fungiciden en andere fungitoxische verbindingen. In plantenpathogene

schimmels kunnen transporteiwitten ook fungeren als virulentiefactoren indien ze verantwoordelijk zijn voor de secretie van gastheer-specifieke toxinen of bescherming verschaffen tegen afweerstoffen van planten tijdens de pathogenese.

Hoofdstuk 3 beschrijft de klonering en karakterisering van drie ABC genen van *M. graminicola* door middel van PCR. De drie genen werden aangeduid als *MgAtr3*, *MgAtr4* en *MgAtr5*. De topologie van de gecodeerde eiwitten duidt erop dat MgAtr3-MgAtr5 kunnen worden geclassificeerd als nieuwe vertegenwoordigers van de PDR klasse van ABC transporteiwitten. In northern blot analyse experimenten kon onder de geteste omstandigheden geen expressie van *MgAtr3* worden aangetoond. *MgAtr4* en *MgAtr5* vertoonden echter duidelijke expressie profielen na behandeling met verbindingen die bekend zijn als substraat of inducer van ABC transporteiwitten. De geteste stoffen omvatten synthetisch fungitoxische verbindingen zoals imazalil en cyproconazool, natuurlijke toxische verbindingen zoals de plantenafweerstoffen eugenol en psoraleen en de antibiotica cycloheximide en neomycine. Het expressiepatroon van de genen was ook afhankelijk van de morfologie van de schimmel.

In ***Hoofdstuk 4*** wordt de functie van *MgAtr1-MgAtr5* van *M. graminicola* bij de bescherming van de schimmel tegen natuurlijke en synthetische, toxische verbindingen verder geanalyseerd. Heterologe expressie van *MgAtr1, MgAtr2, MgAtr4,* en *MgAtr5* in *Saccharomyces cerevisiae* toonde aan dat de gecodeerde eiwitten een aantal chemisch niet-verwante verbindingen kunnen transporteren. De eiwitten bezitten duidelijke overeenkomsten en verschillen in substraatspecificiteit. Op grond van deze waarnemingen kunnen MgAtr1, MgAtr2, MgAtr4, en MgAtr5 beschouwd worden als multidrug transporteiwitten van *M. graminicola*. Analyse van *MgAtr1-MgAtr5* gendisruptie- of genvervangings-mutanten van *M. graminicola* toonde aan dat deletie van *MgAtr5* een geringe toename in gevoeligheid van de schimmel voor de afweerstoffen resorcinol en resveratrol veroorzaakte. Biotoetsen met antagonistische bacteriën wezen er op dat MgAtr2 *M. graminicola* bescherming verschaft tegen antibiotica geproduceerd door *Pseudomonas fluorescens* en *Burkholderia cepacia*.

Hoofdstuk 5 beschrijft de functie van *MgAtr1-MgAtr5* in de virulentie van *M. graminicola* op tarwe. Gendisruptie- en genvervangings-mutanten van *MgAtr1*, *MgAtr2*, *MgAtr3* en *MgAtr5* vertoonden hetzelfde virulente fenotype als de wild-type controle stam. De virulentie van *MgAtr4* disruptie mutanten was echter significant verminderd op zaailingen van alle geteste tarwecultivars. Histopathologisch onderzoek van het infectieproces toonde aan dat *MgAtr4* disruptie mutanten substomatale ruimten slecht koloniseren en een verminderde groei

vertonen in de apoplast van tarweblad. *In vitro* groeiproeven op verschillende media toonden aan dat disruptie van *MgAtr4* het groeivermogen niet vermindert. Dit wijst er op dat MgAtr4 een virulentiefactor is van *M. graminicola*. MgAtr4 is de eerste virulentie factor die tot nu toe van dit belangrijk plantenpathogeen is geïdentificeerd.

In ***Hoofdstuk 6*** worden resistentie-mechanismen voor azoolfungiciden in azool-resisente laboratoriumstammen van *M. graminicola* beschreven. Deze mutanten vertoonden een MDR fenotype omdat ze kruisresistentie geven tegen de chemisch niet-verwante verbindingen cycloheximide en/of rhodamine 6G. De effecten die werden gevonden omvatten een toename in efflux van azolen door ABC transporteiwitten, overexpressie van *CYP51* dat codeert voor $P450_{14DM}$ en mutaties in het coderende gedeelte van het gen. Verminderde accumulatie van azolen in mycelium speelde een rol in resistentie van sommige mutanten tegen azolen. Additionele effecten zoals opslag van azolen in celcompartimenten zouden echter ook een rol kunnen spelen.

In ***Hoofdstuk 7*** worden moleculaire mechanismen die verantwoordelijk zijn voor variatie in basis-gevoeligheid voor azool fungiciden in veldisolaten van *M. graminicola* bestudeerd. De geteste isolaten vertonen een grote variatie in gevoeligheid voor azolen. De isolaten waren kruisgevoelig voor de azolen cyproconazool, ketoconazool, en tebuconazool maar niet voor niet-verwante verbindingen zoals cycloheximide, kresoxim-methyl en rhodamine 6G. Genetische analyse toonde aan dat azool-gevoeligheid in *M. graminicola* een polygene basis heeft. Het basale expressieniveau van *MgAtr1-MgAtr5* was voor de isolaten duidelijk verschillend, maar er kon geen correlatie tussen expressieniveau van een specifiek ABC gen en azool-gevoeligheid worden vastgesteld. Cyproconazool induceerde in het bijzonder de expressie van *MgAtr4,* maar een correlatie tussen expressie van dit gen en azool-gevoeligheid kon evenmin worden vastgesteld. Eén isolaat met intermediaire gevoeligheid voor azolen vertoonde een hoog *CYP51* expressieniveau. Verminderde accumulatie van azolen in mycelium correleerde niet met azool-gevoeligheid. De resultaten tonen aan dat meerdere mechanismen voor de verschillen in basis-gevoeligheid van veldisolaten van *M. graminicola* verantwoordelijk zijn. Een vergelijkbare conclusie werd in hoofdstuk 6 voor resistentiemechanismen in azool-resistente laboratoriummutanten van *M. graminicola* getrokken.

In ***Hoofdstuk 8*** wordt de fungitoxische activiteit van de azool-fungiciden cyproconazool en propiconazool alleen en in combinatie met ABC transportmodulators bestudeerd. De proeven

werden uitgevoerd met een veldisolaat van *M. graminicola* dat een matige gevoeligheid heeft voor azool fungiciden. De activiteit van de stoffen werd bepaald op de sporenkieming en kiembuisgroei. Analyse van de interacties tussen de stoffen met de Colby methode toonde aan dat de effecten in de meeste mengsels additief zijn. In mengsels van cyproconazool en verapamil of kresoxim-methyl werd enige synergistische activiteit waargenomen. Analyse van mengsels van cyproconazool en kresoxim-methyl met de Wadley methode toonde uitsluitend additieve interacties aan.

Hoofdstuk 9 geeft een algemene discussie van het proefschrift met speciale aandacht voor onderwerpen zoals: mogelijke strategieën om additionele ABC genen van *M. graminicola* te kloneren, de relatie tussen expressie van ABC genen en substraatspecificiteit van de gecodeerde eiwitten, de grote verscheidenheid aan ABC transporteiwitten, de functie van ABC transporteiwitten als virulentiefactor, de mechanismen van azool-gevoeligheid en resistentie in *M. graminicola*, en tenslotte mogelijkheden om ABC transportactiviteit te beïnvloeden.

Samenvattend, tonen de gegevens van dit proefschrift aan dat ABC transporteiwitten van *M. graminicola* een aantal belangrijke functies bezitten. Zij kunnen functioneren als virulentiefactor. Bovendien kunnen ze bescherming verschaffen tegen natuurlijke en synthetische, toxische stoffen en verantwoordelijk zijn voor basis-gevoeligheid en resistentie tegen azoolfungiciden.

ΠΕΡΙΛΗΨΗ

Σκοπός της μελέτης αυτής ήταν η κλωνοποίηση γονιδίων που κωδικοποιούν πρωτεΐνες επονομαζόμενες ως ATP–binding cassette (ABC) transporters από τον παθογόνο μύκητα *Mycosphaerella graminicola* και η ταυτοποίηση του ρόλου των πρωτεϊνών αυτών στον καθορισμό της παθογεννητικότητας του μύκητα στο σιτάρι και στην προστασία του ενάντια σε τοξικές ουσίες.

Στο ***Κεφάλαιο 1*** δίνεται η περιγραφή του μύκητα *M. graminicola* (ατελής μορφή *Septoria tritici).* Το παθογόνο αυτό προκαλεί την ασθένεια γνωστή και ως σεπτορίαση των σιτηρών. Η ασθένεια έχει παγκόσμια εξάπλωση και αποτελεί μία αυξανόμενης σημασίας απειλή σε καλλιέργειες σιταριού, ιδιαίτερα σε περιοχές με υψηλή σχετική υγρασία και μέτριες θερμοκρασίες. Για το λόγο αυτό, η κατανόηση των μοριακών μηχανισμών που σχετίζονται με την παθογεννητικότητα και ανθεκτικότητα του μύκητα σε μυκητοκτόνα σκευάσματα, είναι ιδιαίτερης σημασίας. Η καταπολέμηση της ασθένειας αυτής συμπεριλαμβάνει ευρέως τη χρήση αζολικών μυκητοκτόνων. Ο τρόπος δράσης των μυκητοκτόνων αυτών ουσιών βασίζεται στην αναστολή της αντίδρασης απομεθύλωσης του άνθρακα 14 κατά τη διάρκεια σύνθεσης των στερολών στα κύτταρα, παρεμβαίνοντας στη λειτουργία ενός ενζύμου εξαρτώμενου από το κυττόχρωμα $P450_{14DM.}$ Το ένζυμο αυτό παίζει ένα καθοριστικό ρόλο στη βιοσύνθεση όλων των στερολών. Σε παθογόνους μύκητες των φυτών, τέσσερις κυρίως μηχανισμοί ανθεκτικότητας στις αζόλες έχουν αναφερθεί. Ένας από αυτούς είναι και η μειωμένη συσσώρευση των ουσιών αυτών στο μυκήλιο του μύκητα, που αποδίδεται σε έναν ενεργειακά εξαρτώμενο εκκριτικό μηχανισμό κατευθυνόμενο από ABC transporters ή πρωτεΐνες της υπερ-οικογένειας των major facilitators (MFS). Αυτοί οι δύο, καθώς και άλλοι μηχανισμοί ανθεκτικότητας στις αζόλες, περιγράφονται επίσης στο Κεφάλαιο 1.

Στο ***Κεφάλαιο 2*** γίνεται μία αναθεώρηση των ABC και MFS transporters από μυκηλιακούς μύκητες. Οι πρωτεΐνες αυτές με μεταφορικό ρόλο για τα κύτταρα, αποτελούν δύο από τις μεγαλύτερες οικογένειες πρωτεϊνών που έχουν αναφερθεί έως τώρα. Εντοπίζονται κυρίως στην εξωπλασματική μεμβράνη ή στις μεμβράνες ενδοκυτταρικών διαμερισμάτων και είναι ικανές να μετακινούν ένα μεγάλο αριθμό κυτοτοξικών παραγόντων ενάντια στο βαθμό διαβάθμισης της πυκνότητάς τους εκατέρωθεν των κυτταρικών μεμβρανών. Στην περίπτωση των ABC transporters η ενέργεια που απαιτείται για την μετακίνηση αυτή προέρχεται από την υδρόλυση της τριφεσφορικής αδενοσύνης (ATP) και για τον λόγο αυτό οι πρωτεΐνες αυτές

χαρακτηρίζονται και ως ο κύριος ενεργός μηχανισμός μετακίνησης κυτοτοξικών παραγόντων στα κύτταρα. Από την άλλη πλευρά, οι MFS transporters, χρησιμοποιούν την ενέργεια της ηλεκτροχημικής διαβάθμισης εκατέρωθεν των μεμβρανών για τη μετακίνηση μορίων διαμέσου αυτών και γι' αυτόν το λόγο ταξινομούνται ως δευτερεύων μηχανισμός μετακίνησης κυτοτοξικών παραγόντων στα κύτταρα. Στο σύνολο τους οι ABC και MFS transporters μπορούν να παίξουν έναν καθοριστικό ρόλο στην ανάπτυξη διασταυρούμενης ανθεκτικότητας των κυττάρων σε πολλαπλούς κυτοτοξικούς και χημικά μη συσχετιζόμενους παράγοντες. ABC και MFS transporters συμμετέχοντες στην ανάπτυξη ανθεκτικότητας σε μυκητοξικές ενώσεις έχουν επίσης περιγραφεί σε μυκηλιακούς μύκητες. Σε παθογόνους μύκητες των φυτών, οι πρωτεΐνες αυτές μπορούν επίσης να δράσουν ως παράγοντες μολυσματικότητας των μυκήτων αν μεσολαβούν στην έκκριση μολυσματικών παραγόντων όπως ξενιστού–ειδικές τοξίνες, ή παρέχουν προστασία εναντίον φυτοπροστατευτικών ενώσεων κατά τη διάρκεια της παθογέννεσης.

Στο ***Κεφάλαιο 3*** περιγράφεται η κλωνοποίηση και χαρακτηρισμός τριών μονογονογονιδίων που κωδικοποιούν ως προς ABC transporters από το μύκητα *M. graminicola,,* χρησιμοποιώντας μία μέθοδο βασιζόμενη στην αλυσιδωτή αντίδρασης της πολυμεράσης (PCR). Τα γονίδια αυτά έχουν ονομαστεί *MgAtr3, MgAtr4* και *MgAtr5.* Βασιζόμενοι στην τοπολογική διάταξη των κωδικοποιημένων πρωτεΐνών, οι πρωτεΐνες MgAtr3-MgAtr5 μπορούν να ταξινομηθούν ως μέλη της πλειοτροπικής ανθεκτικότητας σε τοξικές ουσίες οικογένειας των ABC transporters. Έκφραση του γονιδίου *MgAtr3,* δεν ανιχνεύτηκε υπό τις συνθήκες χρησιμοποίησης της ανάλυσης Northern. Παρ' όλα αυτά, τα γονίδια *MgAtr4* και *MgAtr5* παρουσίασαν διάκριτη κατανομή έκφρασης μετά από έκθεση του μύκητα σε ένα φάσμα ενώσεων που αποτελούν είτε υποστρώματα για πρωτεΐνες ABC ή επάγουν την έκφραση των ανάλογων γονιδίων. Στις δοκιμαζόμενες ενώσεις συμπεριλαμβάνονται συνθετικά μυκητοτοξικά σκευάσματα, όπως imazalil και cyproconazole, τοξικές ενώσεις φυσικής προελεύσεως, όπως οι φυτοπροστατευτικές ενώσεις eugenol και psoralen, και τα αντιβιοτικά cycloheximide και neomycin. Η κατανομή έκφρασης των γονιδίων *MgAtr3-MgAtr5* ήταν επίσης εξαρτώμενη και από τη μορφολογική κατάσταση, σπόρια ή μυκήλιο, του μύκητα.

Στο ***Κεφάλαιο 4*** εξετάζεται περαιτέρω η λειτουργία των κωδικοποιούμενων από τα γονίδια *MgAtr1-MgAtr5* πρωτεϊνών του μύκητα *M. graminicola* στην προστασία αυτού ενάντια σε τοξικές ενώσεις φυσικής ή τεχνητής προελεύσεως. Ετερόλογη έκφραση των γονιδίων *MgAtr1, MgAtr2, MgAtr4,* και *MgAtr5* στη ζύμη *Saccharomyces cerevisiae,* έδειξε

ότι οι κωδικοποιούμενες πρωτεΐνες μπορούν να μεταφέρουν ένα μεγάλο αριθμό χημικά, μη συσχετιζόμενων ουσιών εκατέρωθεν των κυτταρικών μεμβρανών και κατέχουν διάκριτη αλλά επικαλυπτόμενη εξειδίκευση ως προς τα υποστρώματά τους. Για το λόγο αυτό, οι πρωτεΐνες MgAtr1, MgAtr2, MgAtr4, και MgAtr5 μπορούν να θεωρηθούν και ως πρωτεΐνες μεταφοράς πολλαπλών χημικά μη συσχετιζόμενων κυτοτοξικών σκευασμάτων (multidrug transporters*),* από το μύκητα *M. graminicola.* Ανάλυση στελεχών του μύκητα που φέρουν ρήξη ή αντικατάσταση ενός των γονιδίων *MgAtr1-MgAtr5* από γονίδια αναφοράς στον ίδιο το μύκητα *M. graminicola,* έδειξε ότι διαγραφή του γονιδίου *MgAtr5* προκαλεί μία μικρή αύξηση της ευαισθησίας του μύκητα στην ως φερόμενη φυτοπροστατευτική ένωση του σιταριού resorcinol και την φυτοαλεξίνη προερχόμενη από φυτά αμπελιού resveratrol. Πειράματα βιολογικής δοκιμασίας με ανταγωνιστικά βακτήρια, έδειξαν ότι η πρωτεΐνη MgAtr2 είναι ικανή να παρέχει προστασία του μύκητα *M. graminicola* ενάντια σε αντιβιοτικά παραγόμενα από τα βακτήρια *Pseudomonas fluorescens* και *Burkholderia cepacia.*

Στο ***Κεφάλαιο 5*** περιγράφεται η συμβολή των κωδικοποιούμενων από τα γονίδια *MgAtr1-MgAtr5* πρωτεϊνών στη μολυσματική ικανότητα του μύκητα *M. graminicola.* Στελέχη του μύκητα με ρήξη ή αντικατάσταση σε ένα από τα γονίδια *MgAtr1, MgAtr2, MgAtr3* και *MgAtr5,* έδειξαν παρόμοιο φαινότυπο ως προς τη μολυσματική τους ικανότητα, με το φυσικό στέλεχος του μύκητα που χρησιμοποιείτο ως μάρτυρας. Παρ΄ όλα αυτά η μολυσματική ικανότητα στελεχών του μύκητα με ρήξη στο γονίδιο *MgAtr4,* ήταν σημαντικά μειωμένη σε φυτάρια όλων των ποικιλιών σιταριού που δοκιμάστηκαν. Ιστοπαθολογική ανάλυση της διαδικασίας προσβολής φύλλων σιταριού από το μύκητα, έδειξε ότι στελέχη του μύκητα με ρήξη στο γονίδιο *MgAtr4* παρουσιάζουν μειωμένη ικανότητα αποικισμού της στοματικής κοιλότητας στομάτων του φύλλου και μειωμένη βλαστική ανάπτυξη του μυκηλίου στον αποπλάστη των προσβεβλημένων φυτών σιταριού. Παρ' όλα αυτά, πειράματα βλαστικής ανάπτυξης του μύκητα σε διαφορετικά θρεπτικά υποστρώματα δεν έδειξαν κάποια ελάττωση στην ανάπτυξη στελεχών του μύκητα με ρήξη στο γονίδιο *MgAtr4* υπό *in vitro* συνθήκες. Για το λόγο αυτό τα αποτελέσματα αποκαλείπτουν ότι η πρωτεΐνη MgAtr4 είναι ένας παράγοντας μολυσματικότητας για τον μύκητα *M. graminicola.* Η πρωτεΐνη αυτή (MgAtr4) αποτελεί τον πρώτο μολυσματικό παράγοντα που ταυτοποιείται από το σημαντικό αυτό μύκητα.

Στο ***Κεφάλαιο 6*** μελετούνται οι μηχανισμοί υπεύθυνοι για την ανθεκτικότητα σε αζόλες εργαστηριακά-παραγόμενων στελεχών του μύκητα *M. graminicola* ανθεκτικών στις ενώσεις αυτές. Τα στελέχη αυτά, εκτός των αζόλων παρουσίασαν ανθεκτικότητα στις χημικά μη σχετιζόμενες ενώσεις cycloheximide και/ή rhodamine 6G. Στους μελετηθέντες μηχανισμούς

συμπεριλαμβάνονται η αυξανόμενη έκκριση των αζόλων από τα κύτταρα δια μέσο των ABC transporters, η υπερέκφραση του γονιδίου *CYP51* που κωδικοποιεί ως προς το κυττόχρωμα $P450_{14DM}$, και η ύπαρξη τυχόν μεταλλάξεων στην κωδικοποιούσα αλληλουχία αυτού του γονιδίου. Τα αποτελέσματα δείχνουν ότι μειωμένη συσσώρευση των αζόλων στο μυκήλιο του μύκητα συνείσφερε στην ανθεκτικότητα ορισμένων στελεχών στις ενώσεις αυτές. Παρ' όλα αυτά, επιπλέον μηχανισμοί ανθεκτικότητας, όπως η μεσεγγύηση των αζόλων σε κυτταρικά διαμερίσματα φαίνονται επίσης να λειτουργούν.

Στο ***Κεφάλαιο 7*** μελετούνται οι μοριακοί μηχανισμοί που είναι υπεύθυνοι για την παραλλακτικότητα που παρουσιάζεται ως προς την ευαισθησία στις αζόλες φυσικών στελεχών του μύκητα *M. graminicola* προερχόμενων από τον αγρό. Τα στελέχη που εξετάστηκαν παρουσίασαν μία μεγάλη παραλλακτικότητα στην ευαισθησία τους ως προς τις αζόλες. Τα στελέχη αυτά κατείχαν διασταυρωμένη ανθεκτικότητα στις αζόλες cyproconazole, ketoconazole και tebuconazole, αλλά όχι σε άλλες χημικώς μη συσχετιζόμενες ουσίες όπως cycloheximide, kresoxim-methyl και rhodamine 6G. Γενετική ανάλυση έδειξε ότι η ευαισθησία στις αζόλες του μύκητα *M. graminicola* είναι πολυγονιδιακής φύσεως. Το βασικό επίπεδο έκφρασης των γονιδίων *MgAtr1-MgAtr5,* διαίφερε σημαντικά μεταξύ των στελεχών του μύκητα, αλλά καμιά συσχέτιση μεταξύ έκφρασης ενός συγκεκριμένου από αυτά τα γονίδια και ευαισθησία στις αζόλες δεν παρατηρήθηκε. Η ένωση cyproconazole προκάλεσε επαγωγή της έκφρασης ιδιαίτερα του γονιδίου *MgAtr4,* αλλά καμιά συσχέτιση μεταξύ έκφρασης αυτού του γονιδίου και ευαισθησία του μύκητα στις αζόλες δεν παρατηρήθηκε επίσης. Ένα στέλεχος του μύκητα με μέτρια ευαισθησία στις αζόλες παρουσίασε υψηλά επίπεδα έκφρασης του γονιδίου *CYP51.* Συσχέτιση μεταξύ μειωμένης συσσώρευσης των αζολών στο μυκήλιο του μύκητα και ευαισθησία αυτού στις ουσίες αυτές δεν παρατηρήθηκε επίσης. Τα αποτελέσματα δείχνουν ότι πολλαπλοί μηχανισμοί είναι υπεύθυνοι για την εμφανιζόμενη παραλλακτικότητα σε ευαισθησία στις αζόλες, φυσικών στελεχών του μύκητα *M. graminicola* προερχόμενων από τον αγρό. Το συμπέρασμα αυτό βρίσκεται σε συμφωνία με τους προτεινόμενους πολλαπλούς μηχανισμούς ανθεκτικότητας στις αζόλες, εργαστηριακά παραγόμενων στελεχών του μύκητα ανθεκτικών στις ουσίες αυτές.

Στο ***Κεφάλαιο 8*** μελετάται η τοξικολογική δράση των αζολικών μυκητοκτόνων cyproconazole και propiconazole ατομικά, ή σε συνδυασμό με ενώσεις που μπορούν να επηρεάσουν τη λειτουργία των ABC transporters. Η μελέτη αυτή πραγματοποιήθηκε χρησιμοποιώντας ένα φυσικό στέλεχος του μύκητα *M. graminicola,* προερχόμενο από τον αγρό, με σχετικά μέτρια ευαισθησία σε μυκητοκτόνα αζολικής φύσεως. Η τοξικολογική

δράση όλων των ενώσεων αξιολογήθηκε σε δοκιμές παρεμπόδισης βλάστησης σπορίων του μύκητα και βλαστικής ανάπτυξης του μυκηλίου αυτού. Ανάλυση της αλληλεπίδρασης μεταξύ των ενώσεων χρησιμοποιώντας τη μέθοδο Colby παρουσίασε μόνο αθροιστικά αποτελέσματα ως προς τη δράση τους στα περισσότερα από τα μείγματα που εξετάστηκαν. Συνεργειακή δράση παρατηρήθηκε με τη μέθοδο Colby σε μείγματα των ενώσεων cyproconazole και verapamil ή kresoxim-methyl. Παρ' όλα αυτά, ανάλυση με τη μέθοδο Wadley έδειξε μόνο αθροιστική αλληλεπίδραση μεταξύ των ενώσεων cyproconazole και kresoxim-methyl σε διάφορα μείγματα τους.

Στο ***Κεφάλαιο 9*** συζητούνται τα αποτελέσματα της συνολικής μελέτης με ιδιαίτερη έμφαση σε επιλεγμένα θέματα όπως τρόποι κλωνοποίησης επιπρόσθετων γονιδίων που κωδικοποιούν ως προς ABC transporters από το μύκητα *M. graminicola,,* η σχέση μεταξύ έκφρασης ABC γονιδίων και εξειδίκευσης των κωδικοποιουμένων πρωτεϊνών ως προς τα υποστρώματα τους, το φαινόμενο «εφεδρείας» στους ABC transporters, ο ρόλος των ABC transporters ως μολυσματικών παραγόντων, οι μηχανισμοί ανθεκτικότητας και ευαισθησίας του μύκητα *M. graminicola* στις αζόλες και τέλος, τρόποι επηρεασμού και διαμόρφωσης της δράσης των ABC transporters.

Ως τελικό συμπέρασμα, τα στοιχεία που παρουσιάζονται στη μελέτη αυτή δείχνουν ότι οι ABC transporters από το μύκητα *M. graminicola,* εκτελούν έναν αριθμό σημαντικών λειτουργιών για το μύκητα αυτό. Έτσι λοιπόν μπορούν να αποτελέσουν μολυσματικούς παράγοντες για το μύκητα ή επιπλέον να παρέχουν προστασία ενάντια σε φυσικής ή τεχνητής προελεύσεως τοξικές ουσίες και να αποτελέσουν έναν από τους παράγοντες καθορισμού της ευαισθησίας και ανθεκτικότητας του μύκητα σε αζολικά μυκητοκτόνα.

ACKNOWLEDGEMENTS

This thesis has been the result of a joint effort of several people without who it would have been difficult to be realised. Therefore, I take the opportunity to express my gratitude to all those that have directly or indirectly contributed to the completion of this work.

Maarten, as my co-promotor, I gratefully thank you for giving me the opportunity to join your group and the optimism, guidance, and trust you've showed to me all these years. You always provided with grate support and understanding, keeping yourself open to discuss new ideas or problems. That's really special! I admire the patients you've showed over my stubbornness in some of our "live" discussions and I apologise if I sometimes overdid it. I truly thank you!!! Pierre, as my promotor, you have provided with additional excellent scientific support and you were always willing to help when needed. I truly appreciate this! I thank you for the interest that you've showed not only in my work but also to other aspects of life.

Koen, you've guided my first steps into the field of molecular biology and you were a great daily supervisor. I really thank you for the efforts you took in me and I wish you all the best in your further carrier. You're truly a "big guy"! Marco and Stephen, you too have also contributed greatly at this work, each one of you on your own personal style. I learned a lot from both of you and for this you are always mostly appreciated! I'll have a bier for you two… Lute, our collaboration has been really magnificent! Not only you've been an excellent scientific advisor and colleague, but also a fun guy to work and share an office with. However, there is still one thing that I need to do before I go. Convince you over Macedonia…! Alan, Henk-jan, Keisuke, Tycho, Hans, Giovanni, Ciska, Ramin, Luc, Melanie, Paul, Kostas, Olavo, as part of the ABC transporters group you have all provided with great support and friendship and created a wonderful environment at the lab. That really makes a difference! Hans I also really appreciate your help in some of my experiments. To my students Manos and Anne I'm thankful for their assistance and their input in this thesis. Since both of you recently completed your studies, I wish you good luck in your further carriers. Anne, sorry I couldn't make it to your graduation.

To the rest of the staff members of the laboratory of Phytopathology, Francine, Jos, Jan, and Matthieu I thank them for their scientific advises and support.

I'm also thankful to colleagues from other groups for their assistance and friendship. Jorge, you've been one of my best and closest friends during my whole stay here! Although real friends don't say thanks to each other, I need to express my gratitude for all the support, help, and great moments of fun you have created. Good luck to you and your partner Flora. And jorge, you were right, twice…!!! Dear Maita, you are a person of special value and I thank you for your at times warm kindness and

understanding. I truly wish you all the best in all your further professional and personal affairs. Your music, still lives on. Maria, it was good to have another Greek around in the lab. I thank you for all your help and for all those great Greek dinners. Good luck in finishing your PhD too. Are we going to see a brother or a sister to Jonah soon? Special thanks to Loekas for his friendship. To the rest of the members of the laboratory of Phytopathology I express my gratitude for their support and the great working environment they have created. A special thanks to all the technicians, Willem, Hans, John, Paul, Rob, Esther, Grady, Corrie, Micke, Lia and the administration, Ali and Ria, of this laboratory for all their substantial help over these years.

Outside the laboratory of Phytopathology, I would like to thank several people that made my staying here certainly most enjoyable. Vetta, thank you for all the support and those great moments of fun during my first year here. I've certainly missed those days over the following years. We've been good friends and I hope it will continue like this in the future too! Your leaves are still on my wall... Sotiris, I'm maybe responsible for you coming to Wageningen, as you say, but I'm sure you haven't regretted it. You've been a very close and truthful friend and I truly appreciate all your support. I wish you all the luck with your further carrier. Manolis, "manolatsi manolatsi…", you are from the most trustworthy people I ever met in my life. You've been a great friend and willing to help in whatever situation. Really thank you! You've proved yourself a proud Cretan. Rika, I miss those walks through the woods. You were always an enjoyable company and I wish you all the luck in finishing your studies. To the rest of the members of the Greek community and other friends in Wageningen, who unfortunately I can not list all, a truthful thanks for all their support and friendship!

To the members of the Wageningen Mycosphaerella group at PRI, Gert, Cees, Els, Odette, Thamara, Theo, Rahim, Shara, I thank them for all their scientific and technical support.

To all the members of the Wageningen students fencing club *"De Schermutcelaers"* I really thank them for those great evenings…till late nights of fencing. Michiel, Michiel, Jouke, and Rolf you've been excellent friends and teaching assistants. "Maître" Ad thanks for the wonderful lessons. Victor and Andrian, great matches! I'll certainly miss all of you people there! Good luck to all and I certainly hope to see at least some of you in international competitions too!

Finally, to my family back in Greece, my father Spyros, my mother Eleni and my only brother Fotis, I thank them for the unlimited support, love, and care that I received from them over the years. I know it was hard for you to have me away from you but soon I will be back again!

Ioannis Sterziopoulos

ABOUT THE AUTHOR

Ioannis Stergiopoulos was born on the 3rd of May 1974 in Veria Imathias, in Macedonia, Greece. He completed his basic education in 1992, at the 2nd Public Lyceum in Alexandria Imathias, in Macedonia, Greece. In June 1992, he succeeded in a countrywide Panhellenic contest held by the Hellenic Ministry of Education and entered as a student at the Aristoteleian University of Thessaloniki, faculty of Geotechnical Sciences, department of Agriculture and Forestry. In July 1997, he was awarded the degree of the department of Agriculture, with specialisation in Crop Protection. During his study, he completed his diploma thesis in studying the fungal microflora of sugarbeet seeds, in collaboration with the Hellenic Sugar Industry. In October 1997 he started his Ph.D. program at the Wageningen University, in the Netherlands, at the department of Plant Sciences, laboratory of Phytopathology. His research was founded by the Training and Mobility of Researchers (T.M.R.) program - Marie Curie fellowships, of the European Union and was focused on cloning and characterisation of ABC transporter genes from the wheat pathogen *Mycosphaerella graminicola.* This research was curried out under the educational and supervision plan of the school of Experimental Plant Sciences (E.P.S.).

The address of Ioannis Stergiopoulos in Greece is:
Parodos Vezeenou 22
Alexandria Imathias T.K. 59 300
Macedonia,
Greece

Phone: 0030 2333 022846

LIST OF PUBLICATIONS

Stergiopoulos I, Zwiers L-H, Gielkens MMC, Goodall SD, Venema K and De Waard MA (2002) Functional analysis of transporters in the wheat pathogen *Mycosphaerella graminicola*. *In:* Modern Fungicides and Antifungal Compounds III. (Eds) Dehne H-W, Gisi U, Kuck K-H, Russell PE and Lyr H, AgroConcept, Bonn, Germany, **ISBN 3-7862-0144-7** pp. 177-186

Stergiopoulos I, Zwiers L-H and De Waard MA (2002) Secretion of natural and synthetic toxic compounds from filamentous fungi by membrane transporters of the ATP-binding cassette and major facilitator superfamily. (Review) Eur J Plant Pathol **108 (7):** 719-734

Stergiopoulos I, Gielkens MMC, Goodall SD, Venema K and De Waard MA (2002) Molecular cloning and characterisation of three new ATP-binding cassette transporter genes from the wheat pathogen *Mycosphaerella graminicola.* Gene **289 (1-2):** 141-149

Stergiopoulos I and De Waard MA (2002) Activity of azole fungicides and ABC transporter modulators on *Mycosphaerella graminicola.* J Phytopath **150 (6):** 313-320

Stergiopoulos I, Zwiers L-H and De Waard MA The ABC transporter MgAtr4 is a virulence factor of *Mycosphaerella graminicola* that affects colonisation of substomatal cavities in wheat leaves. *Submitted*

Stergiopoulos I, Van Nistelrooy JGM, Kema GHJ and De Waard MA Multiple mechanisms account for variation in base-line sensitivity to azole fungicides in field isolates of *Mycosphaerella graminicola. Submitted*

Zwiers L-H, Stergiopoulos I, Van Nistelrooy JGM and De Waard MA (2002) ABC transporters and azole susceptibility in laboratory strains of the wheat pathogen *Mycosphaerella graminicola.* Antimicrob Agents Chemother **46 (12):** 3900-3906

Zwiers L-H, Stergiopoulos I, Gielkens MMC, Goodall SD and De Waard MA ABC transporters of the wheat pathogen *Mycosphaerella graminicola* function as protectants against biotic and xenobiotic toxic compounds. *Submitted*