ABC TRANSPORTERS OF THE WHEAT PATHOGEN *MYCOSPHAERELLA GRAMINICOLA*

Lute-Harm Zwiers

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Voor Karin, Merit en Eline

Contents

CHAPTER 1

General Introduction and Outline

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MYCOSPHAERELLA GRAMINICOLA

Wheat is the staple food for 35% of the world population and occupies 17% of all cultivated land. As a consequence of world-wide population growth and the rise of living standards the demand for wheat in 2020 is estimated to be 40% higher than in 2000. To cope with this rising demand the global wheat productivity needs to increase (CIMMYT 2000). The use of higher-yield varieties and the intensified use of fertilisers and growth regulators have resulted in a world-wide increase in the average yield of wheat by 59% over the last 20 years (Oerke et al. 1994). However, the economic importance of plant pathogens, insects and weeds constraining wheat yield has also increased over the last 20 years. This is reflected by the annual increase in the use of crop protection compounds by 4% per year. The use of fungicides even increased with 8-10% per year, suggesting an increasing incidence of fungal pathogens. It is estimated that without any crop protection measures the world-wide wheat yield would be less than 50% of the attainable production (Oerke et al. 1994).

Septoria diseases

The potential losses due to diseases vary from 10% in Africa to 24% in South America. As a consequence of seed dressing and the introduction of resistant varieties the importance of smuts, bunts and stem rust has decreased. Over the last decades the importance of septoria diseases has increased in regions with a temperate climate, e.g. north-western Europe and North America (Daamen and Stol 1992; King et al. 1983; Polley and Thomas 1991). This increase is due to changes in cultural practices and the use of early-maturing semidwarf cultivars susceptible to the pathogens (Eyal et al. 1987).

The two main septoria diseases on wheat are septoria nodorum blotch caused by *Stagonospora nodorum* (teleomorph; *Phaeosphaeria nodorum*) and septoria tritici leaf blotch caused by *Septoria tritici* (teleomorph; *Mycosphaerella graminicola*). Both diseases can reduce yield by as much as 50%. In 1982, world-wide loss was estimated to be 9 million metric tons, which is approximately 10 times the yearly wheat production in the Netherlands (Eyal et al. 1987; Oerke et al. 1994). At the moment *M. graminicola* is considered to be the most important foliar pathogen of wheat (Cook et al. 1991).

Mycosphaerella graminicola

The ascomycetous fungus *Mycosphaerella graminicola* is the causal agent of septoria tritici leaf blotch. The disease is characterised by the development of irregular chlorotic lesions that

become necrotic in time. Inside the necrotic lesions the asexual pycnidia and / or the sexual pseudothecia develop. Moisture is required for all stages of infection and the optimum temperature for growth and infection is between 20–25 °C (Magboul et al. 1992). The penetration frequency of germinated conidia on wheat leaves is in general low, as germtube growth seems to be largely random. The mode of penetration might depend on the fungal isolate as different studies report either only stomatal penetration or both stomatal and direct penetration (Cohen and Eyal 1993; Kema et al. 1996; Rohel et al. 2001).

The importance of airborne ascospores in both the establishment of the disease and the progress of the epidemic during the growing season is now recognised. The teleomorphic pseudothecia can be found throughout the growing season and *M. graminicola* can complete a sexual cycle within five weeks of infection (Hunter et al. 1999; Kema et al. 1996; Scott et al. 1988). This implies that spread of the disease does not only depend on splash dispersal of asexual pycnidiospores but is also caused by a constant supply of ascospores (Shaw 1987; Shaw and Royle 1989). These data comply with population genetic studies showing that field populations of *M. graminicola* exhibit a vast genotypic diversity (Chen and McDonald 1996; McDonald and Martinez 1990; Schnieder et al. 2001). Moreover, gene-flow between geographically separated populations has been demonstrated as the amount and distribution of genetic variation among populations of *M. graminicola* is similar around the world (Boeger et al. 1993; McDonald et al. 1995). As a consequence of this continuous genetic exchange, the pathogen population is highly diverse and might easily adapt to selection exerted by changing conditions such as the use of fungicides or new wheat cultivars.

Since the first publication on the potential existence of a gene-for-gene relationship in the *M. graminicola*-wheat pathosystem, this race-cultivar specificity has been controversial (Eyal et al. 1973). However, by now the existence of specificity at host species (bread wheat and durum wheat) and cultivar level has been resolved (Kema et al. 1996; Kema and Van Silfhout 1997). The elucidation of the specificity was facilitated by the development of a crossing assay, which enabled classical genetic studies (Kema et al. 1996). The use of classical genetics and AFLP analysis has led to the identification in *M. graminicola* of a single locus controlling cultivar-specific avirulence (Kema et al. 2000). However, no specific avirulence gene from *M. graminicola* has been cloned yet.

As mentioned above, most research on *M. graminicola* has traditionally been focussed on epidemiology and population genetics. Molecular genetics has been hampered by the lack of adequate molecular tools. The first successful transformations of *M. graminicola* were first

described in 1998 and until now no gene disruption or gene replacement has been described (Payne et al. 1998; Pnini-Cohen et al. 1998). Therefore, characterisation of genes involved in the plant-pathogen interaction is limited. The number of cloned and fully sequenced genes present in the databases is still very small compared to the amount of genes described for several other phytopathogenic fungi (Table 1). Expressed sequence tags (EST) sequencing has only recently started and resulted in the partial sequencing of 704 unigenes of which 297 display no similarities with database entries (Keon et al. 2000). Considering the increasing importance of *M. graminicola* this lack of molecular knowledge is likely to change.

Table 1. Cloned and fully sequenced genes from *Mycosphaerella graminicola* present in public domain databases on September 30, 2001.

Gene	Accession number	Reference
ABC transporter (*MgAtr1*)	AJ243112	Zwiers and De Waard 2000 (This thesis)
ABC transporter (*MgAtr2*)	AJ243113	Zwiers and De Waard 2000 (This thesis)
ABC transporter (*MgAtr3*)	AF364105	Stergiopoulos et *al.* 2002
ABC transporter (*MgAtr4*)	AF329852	Stergiopoulos et *al.* 2002
ABC transporter (*MgAtr5*)	AF364104	Stergiopoulos et *al.* 2002
Acetyl-CoA-acetyl-transferase (*MgAcat1*)	AJ243195	Zwiers and De Waard 2000 (This thesis)
Actin	AJ300310	
Eburicol 14-α demethylase *(Cyp51)*	AF263470	Gisi et al. 2000
4-Hydroxyphenylpyruvate dioxygenase (*HPPD*)	AF038152	Keon and Hargreaves 1998
3-Isopropylmalate dehydrogenase (*LeuC*)	AF156181	
NAD-dependent formate dehydrogenase	AF123482	
Succinate dehydrogenase iron-sulphur protein	AF042062	Skinner et al. 1998
α-Tubulin	Y14509	Rohel et al. 1998
β-Tubulin	AJ310917	

ABC TRANSPORTERS

Transport is one of the most important and fascinating aspects of life and an essential requirement in all organisms. Unicellular organisms need to maintain their homeostatic balance with constant uptake and allocation of nutrients and the secretion of toxic (waste) products. They must also be able to sense changes in their biotic and abiotic environment. In addition, multicellular organisms need to transport metabolites and information to and from organs. Multicellular organisms even possess specialised organs (tissues) for transport functions, *e.g.*, the blood and nervous system in animals and the vascular tissue in plants.

The main barrier for any transport event is the plasma membrane. Compounds can passively cross this barrier by diffusion. Transport by diffusion is possible only down a

concentration gradient and is limited to solutes able to partition in hydrophobic membranes. Therefore, transport of most compounds over membranes is mediated by membrane bound proteins with specialised transport functions. With the unravelling of the genomes from different organisms the importance of membrane transporters becomes obvious. For instance, the complete genomic sequence of the gram-positive bacterium *Bacillus subtilis* possesses 2379 protein-encoding ORFs with a known function. Of these proteins, 381 are likely to be involved in transport (Kunst et al. 1997). This means that about 16% of the genes of this organism code for membrane transporters. Several types of membrane transporter systems can be distinguished.

Ion Channels

Ion channels are membrane complexes mediating the movement of ions across plasma membranes as well as membranes of cell organelles. These channels form a pore allowing the passive flux of ions down its electrochemical gradient. The opening of these channels is generally gated. This means that the opening is regulated by changes in membrane potential, membrane stretching or binding of a ligand. Ion channels play a role in diverse functions such as osmoregulation, cell growth, development, and nutrient uptake (Garrill et al. 1993).

Facilitators

In contrast to ion channels, facilitators or carriers bind molecules to be transported and undergo a reversible change in conformation during transport. Based on the energy source driving the transport, facilitators can be classified in primary and secondary active transport systems.

Primary Active Transport Systems. Transporters belonging to this system couple transport to ATP hydrolysis. This provides the energy to transport solutes against an electrochemical gradient. Besides proton translocating ATPases two other families of ATP utilising transporters are described. The P-type ATPases that make up a large superfamily of ATP-driven pumps involved in the transmembrane transport of charged substrates and the ATP-binding cassette (ABC) transporters (Andre 1995).

Secondary Active Transport Systems. Transporters belonging to this system derive the energy needed for transport from an electrochemical gradient over the membrane. Facilitated diffusion, the transport of solutes down its own electrochemical gradient, is generally mediated by uniporters. When transport of solutes takes place against an electrochemical

gradient, the energy to drive this process is supplied by the symport or antiport of H^+ or other ions down their electrochemical gradient. A well-characterised group is the **m**ajor facilitator (MF) superfamily of transporters. Members of this superfamily function as H^+-substrate antiporters that use the proton motive force to drive transport (Fling et al. 1991).

This review describes ABC transporters and presents an overview of their structural diversity and multifunctional character in a variety of biological systems. Emphasis will be on ABC transporters of (filamentous) fungi.

Significance of ABC transporters

ABC transporters are members of a large superfamily of transporters. Generally, they are located in plasma membranes and intracellular membranes and include both influx and efflux systems. ABC transporters are present from archae-bacteria to man but became especially known for their involvement in multidrug resistance (MDR) in tumour cells (Higgins 1992). The phenomenon of MDR is accompanied by a massive overproduction of ABC transporters (Beck 1991). Besides MDR, they are also involved in various diseases such as cystic fibrosis, diabetes, adrenoleukodystrophy and the Zellweger syndrome. Furthermore, they play a role as peptide transporters in antigen presentation and in chloroquine resistance in the malarial parasite *Plasmodium falciparum* (Foote et al. 1989; Lombard Platet et al. 1996; Neefjes et al. 1993).

Abundance of ABC Transporters

ABC transporter encoding genes are present in genomes of species representing all three domains of life *e.g.* archae, eubacteria and eukaryotae. In several of these classes of organisms, ABC transporters constitute the largest family of proteins (Table 2). Analysis of transport proteins in seven complete genomes of prokaryotic organisms shows that ABC-transporter and MF superfamilies account for an almost invariant fraction (0.38 to 0.53) of all transport systems per organism. The relative proportion of the two classes of transporters varies over a tenfold range, depending the organism (Paulsen et al. 1998).

In eukaryotes the number of ABC transporters reported in literature is steadily increasing. In *Saccharomyces cerevisiae*, to date the only eukaryotic organism with the complete genome sequenced, 29 ABC-transporter proteins have been identified. In ongoing genome sequencing projects, sequences homologous to ABC transporters have been detected as well.

Table 2. Number of ABC transporters in species representing different domains of life

Domain	Category	Species	Genome size (nt)	ABC transporters [a]	Ref.
Archae	Euryarchaeotae	*Archaeoglobus fulgidus*	2,178,400	40	Klenk et al. 1997
Eubacteria	Firmicutes	*Bacillus subtilis*	4,214,807	77	Kunst et al. 1997
Eubacteria	Proteobacteria	*Escherichia coli*	4,639,221	79	Blattner et al. 1997
Eukaryotae	Fungi	*Saccharomyces cerevisiae*	12,069,313	29	Decottignies and Goffeau 1997

[a] The figures do not give the number of transport systems, since these can be assembled from different polypeptides.

Molecular Architecture

ABC-transporter proteins are characterised by the presence of several highly conserved amino acid sequences in their ABC domain. Two of these motifs, 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], are found in any ABC transporter and in many other proteins which bind and hydrolyse nucleotides (Ames et al. 1990; Ames et al. 1989; Bishop et al. 1989; Walker et al. 1982). The Walker motifs are separated by 120-170 amino acids including a motif characteristic for ABC transporters. This so-called ABC signature, [L-S-G-G-$(X)_3$-R-hydrophobic-X-hydrophobic-A], is highly conserved among ABC transporters only (Croop 1993). The presence of multiple membrane spanning regions is also characteristic for ABC transporters.

All members of the ABC-transporter superfamily have a modular architecture. The majority of the ABC transporters in higher organisms consists of two transmembrane domains (TMD), each with six predicted membrane spanning regions, and two intracellular located nucleotide binding folds (NBF) in a two times two-domain configuration. The nucleotide-binding domain can be either located at the amino terminus or at the carboxy terminus of the polypeptide, yielding proteins with a $[TMD\text{-}NBF]_2$ or $[NBF\text{-}TMD]_2$ configuration. The best characterised examples of ABC transporters with the $[NBF\text{-}TMD_6]_2$ and $[TMD_6\text{-}NBF]_2$ configuration are the yeast multidrug transporter PDR5 and the human multidrug transporter P-glycoprotein (P-gp or MDR1), respectively (Balzi et al. 1994; Endicott and Ling 1989; Gottesman et al. 1995; Juliano and Ling 1976).

The domains can be formed as separate polypeptides or as a single polypeptide with one or more domains fused. Separate polypeptides subsequently aggregate to form functional transporters. In eukaryotic organisms the polypeptides are generally composed of at least two domains but usually contain all four domains (Blight and Holland 1990). The so-called "half-

sized" transporters with a [TMD-NBF] or [NBF-TMD] configuration are likely to function as dimers (Shani and Valle 1996).

Multidrug resistance associated proteins (MRP) form a subfamily of ABC transporters with a TMD_4-$[TMD_6\text{-}NBF]_2$ topology. The main difference with other ABC transporters resides in the presence of an additional transmembrane spanning region at the protein amino terminus. An additional difference is the presence of the so-called R-region located between the two homologous halves. The R-region is involved in regulation of the protein. MRPs act as glutathione-S-conjugate carriers and have been identified in a broad variety of organisms. The best-described example is the human MRP involved in broad-spectrum drug resistance (Broeks et al. 1996; Cole et al. 1992; Cui et al. 1996).

Substrates

Substrates of ABC transporters range from ions (*e.g.* Cl^-) to 107 kDa proteins (*e.g.* haemolysin) (Anderson et al. 1991; Blight and Holland 1990). Most of the mammalian MDR proteins are (by definition) able to transport a wide variety of compounds although substrate specific transporters also occur. For instance, the human ABC-transporter P-gp (MDR1) has 93 known substrates from various chemical classes either of natural or synthetic origin. The main denominator is their high hydrophobicity (Gottesman and Pastan 1993). Recently, a screening of the structures of these 93 substrates for potential spatial relationships between structural elements responsible for interaction with P-gp revealed that the presence of two or three electron donor groups with a spatial separation of 2.5 or 4.6 Å could be correlated with interaction with P-gp (Seelig 1998).

Eukaryotic organisms also contain ABC transporters with a specific substrate range. For example, STE6 from *S. cerevisiae* involved in secretion of the mating factor, and TAP1 and TAP2 involved in human antigen presentation (Kuchler et al. 1989; Shepherd et al. 1993). Bacterial ABC transporters involved in drug resistance have a very specific substrate specificity and are known as specific drug resistance transporters (SDR). Only one bacterial ABC transporter involved in MDR, LmrA from *Lactococcus lactis*, has been detected (Bolhuis et al. 1996; van Veen et al. 1998).

Although ABC transporters are generally described as transporters some can also act as channels and regulators of channels. The cystic fibrosis transmembrane conductance regulator (CFTR) is an ABC transporter with channel function. The associated chloride channel is time and voltage independent and requires ATP hydrolysis for opening (Bear et al.

1992; Riordan et al. 1989). The human P-gp seems to control an associated ATP-dependent volume regulated chloride channel activity (Hardy et al. 1995; Valverde et al. 1996).

P-GLYCOPROTEIN

The human P-gp (MDR1) is probably the best-characterised ABC transporter involved in multidrug resistance. Detailed structure-function relationship studies have been performed and its structure has been determined to 2.5 nm resolution (Rosenberg et al. 1997). P-gp was first described in hamster cell lines in which the MDR phenomenon correlated with the overexpression of a 170 kDa protein (Juliano and Ling 1976). In human, two P-gp homologues, MDR1 and MDR3, have been identified. MDR1 is involved in broad-spectrum drug resistance and MDR3 in the translocation of phosphatidylcholine (Van Helvoort et al. 1996). The overexpression of MDR1 in resistant cells with a low and high degree of resistance is due to elevated mRNA levels caused by regulatory mutations and gene amplification, respectively (Gudkov 1991).

Catalytic Sites

Biochemical evidence and amino acid sequence information suggest that P-gp has ATPase activity. Membrane bound or purified P-gp preparations show a basal ATPase activity, which can be stimulated by several drugs (Al Shawi and Senior 1993). Both nucleotide binding folds bind and hydrolyse ATP (Al Shawi et al. 1994). Synthetic half-sized P-gp molecules also display basal ATPase activity. However, interaction between both halves seems necessary for stimulation of ATPase activity by drugs (Loo and Clarke 1994). This is also demonstrated by mutating either of the two nucleotide-binding domains. Inactivation of NBF_1 results in a block of ATP hydrolysis in NBF_2 and abolishes the drug extrusion capacity of the cells, and *vice versa* (Loo and Clarke 1995). Interaction between nucleotide binding sites was also demonstrated by vanadate-trapping experiments. This inhibitor of ATPase activity traps ADP in a catalytic site and trapping of ADP at only one site is sufficient to block ATPase activity of the entire protein (Urbatsch et al. 1995; Urbatsch et al. 1995).

These results have lead to a model for the catalysis mediated by P-gp in which both catalytic sites alternately undergo ATP hydrolysis. ATP binding at one site promotes ATP hydrolysis at the other. This induces a conformational change preventing the hydrolysis of the new-bound ATP. This new conformation has a high energetic state and relaxation of this

conformation leads to the release of ADP and P_i, and transport of a substrate (Senior et al. 1995).

Substrate Binding and Transport

Photoaffinity labelling and mutant analyses indicate that both membrane-bound halves of ABC transporters are involved in substrate binding. The substrate binding sites are located at the cytoplasmic site of the membrane, especially in transmembrane loops 4, 5, 6, 10, 11 and 12 (Greenberger 1993; Loo and Clarke 1995; Safa et al. 1990; Zhang et al. 1995).

The way ABC transporters expel their substrates is not completely understood. ABC transporters probably act as "hydrophobic membrane cleaners" by detecting drugs which partition in membranes because of their hydrophobic nature. The possibility that transport out of the cytosol also contributes to the efflux can not be excluded (Bolhuis et al. 1996; Bolhuis et al. 1996; Gottesman and Pastan 1988). The result of both transport processes is reduced accumulation of toxic compounds at their intracellular target site. The recently determined structure of P-gp revealed a large central pore forming a chamber within the membrane (Rosenberg et al. 1997). Whether this pore is involved in the transport process or whether the transport occurs through conformational changes upon ATP binding and hydrolysis remains unclear.

PHYSIOLOGICAL FUNCTIONS

Prokaryotes

Bacterial ABC transporters can be functionally grouped in two major distinct subfamilies. The superfamily of importers is responsible for transport of nutritional substrates. These transporters are also called periplasmic permeases and have a multisubunit component system with similar structural organisation (Doige and Ames 1993). The presence of a periplasmic binding protein, and the synthesis of the import system subunits (NBF and TMD) as separate polypeptides are distinctive features of these prokaryotic ABC proteins. The histidine permease from *Salmonella typhimurium* is a well-characterised member of this subfamily. It is composed of the histidine-binding protein (HisJ) as the receptor, and the membrane-bound complex formed by two copies of HisP (NBF) plus the HisQ and HisM (TMD) (Kerppola et al. 1991).

The subfamily of ABC exporters is involved in secretion of proteins, peptides and non-protein compounds (Fath and Kolter 1993). In general, a basic functional structure for ABC

exporters is composed of dimeric molecules (Wandersman 1998). The ATP-binding motif of this subfamily shows a higher degree of similarity with the eukaryotic ABC proteins as compared to the above mentioned ABC importers. In addition, some ABC exporters have their NBF and TMD domains synthesised as a single polypeptide. In gram-negative bacteria, additional export proteins are required for transport to the extracellular medium, for instance HlyD and TolC that are involved in the secretion of haemolysin in *E. coli* (Wandersman and Delepelaire 1990). Other examples of prokaryotic ABC transporters are the export systems of proteases A, B and C in the phytopathogenic bacterium *Erwinia chrysanthemi* (Letoffe et al. 1990), the secretion machinery of peptide antibiotics (bacteriocins) from *Lactococcus lactis* (Stoddard et al. 1992), the β-1,2-glucans oligomers export systems of the plant pathogen *Agrobacterium tumefaciens* (CHVA) (Cangelosi et al. 1989), and the symbiont *Rhizobium meliloti* (NDVA) (Stanfield et al. 1988). β-1,2-glucans oligomers are involved in the attachment of the bacteria to plant cells. Therefore, CHVA can be regarded as a virulence and NDVA as a nodulation factor.

Table 3. ABC transporters from *Saccharomyces cerevisiae* with an identified physiological function

Gene	GenBank number	Size (aa)	TMD	Topology	Knockout	Function	Ref.
STE6	Z28209	1290	12	$[TMD + NBF]_2$	Viable	a-pheromone export	Kuchler et *al.* 1989
ATM1	Z49212	690	5	TMD + NBF	Restricted growth	Mitochondrial DNA maintenance	Leighton 1995
PXA1	L38491	870	5	TMD + NBF	Viable	VLCFA beta-oxidation	Hettema et *al.* 1996
PXA2	X74151	853	6	TMD + NBF	Viable	Interaction with PXA1	Hettema et *al.* 1996
GCN20	D50617	752	0	$[NBF]_2$	Viable	Interactions with tRNA and GCN2	Vazquez de Aldana et *al.* 1995
YEF3	U20865	1044	3	$[NBF]_2$	No growth	Aminoacyl-tRNA binding to ribosomes	Sandbaken et *al.* 1990

Eukaryotes

Yeasts. With the unravelling of the complete genome sequence of *S. cerevisiae* 29 ABC proteins were identified by sequence homology (Decottignies and Goffeau 1997). Only ten of these proteins have a known physiological function. The 29 encoded ABC polypeptide sequences could be divided in six subfamilies. The majority of the proteins have the tetra-

domain modular architecture comprising nine proteins with the $[NBF\text{-}TMD]_2$ and seven with the reverse $[TMD\text{-}NBF]_2$ topology. Furthermore, "half-sized" [TMD-NBF] proteins, which likely function as dimers, were detected. For instance, the peroxisomal ABC transporters PXA1 and PXA2 form heterodimers and are involved in long-chain fatty acid transport and β-oxidation (Shani and Valle 1996). The yeast ABC proteins with a known physiological function, different from a role in MDR, are listed in Table 3. The MDR proteins of *S. cerevisiae* are discussed below. In the fission yeast *Schizosaccharomyces pombe* and the human pathogen *Candida albicans*, ABC proteins have also functionally been described. All of them have an orthologue in the genome of *S. cerevisae* (Balan et al. 1997; Christensen et al. 1997; Ortiz et al. 1992; Ortiz et al. 1995; Raymond et al. 1998).

Filamentous Fungi. Members of the ABC-transporter superfamily have been described for at least seven fungal species (Table 4). The saprophyte *Aspergillus nidulans*, the human-pathogens *Aspergillus flavus* and *Aspergillus fumigatus* and the plant pathogens *Magnaporthe grisea* (rice blast), *Botryotinia fuckeliana* (grey mould), *Mycosphaerella graminicola* (wheat leaf blotch) and *Penicillium digitatum* (citrus green mould) (Del Sorbo et al. 1997; Schoonbeek et al. 2001; Tobin et al. 1997; Zwiers and De Waard 2000). In addition, ongoing fungal genome sequencing projects are expected to reveal many other members. We screened available expressed sequence tags (EST) data-bases of A. *nidulans* and *N. crassa* for potential homologues of ABC transporters with the conserved motifs listed in Table 4 (Nelson et al. 1997; Roe et al. 1998). The search was performed with the BLAST program of sequence alignment (Altschul et al. 1997) and yielded seven homologous sequences from *A. nidulans* and two from *N. crassa* (Table 5). EST clones identical to atrC and atrD, two previously characterised genes from *A. nidulans*, were also detected (Andrade et al. 1998).

The physiological relevance of ABC transporters in filamentous fungi is probably high (De Waard 1997). For instance, a number of them may be involved in secretion of secondary metabolites, which in the case of fungitoxic compounds, can act as a self-protection mechanism. Similarly, ABC transporters may provide protection against toxic metabolites produced by other micro-organisms present in particular ecosystems. Plant pathogenic fungi have to cope with a variety of plant defence compounds and they may possess ABC transporters that function in protection against the toxic action of such compounds as well. These hypotheses are supported by the observation that a wide variety of natural compounds such as isoflavonoids, plant alkaloids and antibiotics can act as substrates of ABC transporters (Gottesman and Pastan 1993; Seelig 1998). In addition, as shown for several yeast species, specific ABC transporters of

filamentous fungi may function in secretion of a mating factor. Therefore, ABC transporters can mediate processes important for survival of fungi in nature and hence, may function as significant parameters in the population dynamics of these organisms.

Table 4. Multiple alignment of conserved sequences from reported ABC-transporter proteins from filamentous fungi

Species	Gene	GenBank number	Domain		
			N terminal		
			Walker A	ABC signature	Walker B
A. nidulans	*atrA*	Z68904	LGRPGTGCSTFL	VSGGERKRVSIAE	AAWDNSSRGLD
A. nidulans	*atrB*	Z68905	LGRPGSGCTTLL	VSGGERKRVSIIE	FCWDNSTRGLD
B. fuckeliana	*pgp1*	Z68906	LGRPGSGCSTFL	VSGGERKRVSIAE	VSWDNSTRGLD
M. grisea	*abc1*	AF032443	LGPPGSGCSTFL	VSGGERKRVTIAE	QCWDNSTRGLD
A. fumigatus	*mdr1*	U62934	VGPSGSGKSTVV	LSGGQKQRIAIAR	LLLDEATSALD
A. flavus	*mdr1*	U62932	VGPSGSGKSTII	LSGGQKQRIAIAR	LLLDEATSALD
			* * * *	*** * *	* **
			C terminal		
			Walker A	ABC signature	Walker B
A. nidulans	*atrA*	Z68904	MGVSGAGKTTLL	LNVEQRKLLTIGV	LFLDEPTSGLD
A. nidulans	*atrB*	Z68905	MGSSGAGKTTLL	LSVEQRKRVTIGV	IFLDEPTSGLD
B. fuckeliana	*pgp1*	Z68906	MGASGAGKTTLL	LSVEQRKRVTIGV	LFLDEATSGLD
M. grisea	*abc1*	AF032443	MGVSGAGKTTLL	LNVEQRKRLTIGV	LFVDEPTSGLD
A. fumigatus	*mdr1*	U62934	VGPSGCGKSTTI	LSGGQKQRVAIAR	LLLDEATSALD
A. flavus	*mdr1*	U62932	VGASGSGKSTTI	LSGGQKQRIAIAR	LLLDEATSALD
A. fumigatus	*mdr2*	U62936	VGPSGGGKSTIA	LSGGQKQRIAIAR	LILDEATSALD
			* ** ** *	* * *	** ** **

Asterisks indicate identical amino acid residues.

Higher eukaryotes. Basically, the majority of the ABC transporters characterised in higher eukaryotes have an orthologue in the *S. cerevisae* genome or at least a very close homologue with similar substrate specificity. However, due to evolutionary speciation, physiological needs may be different and account for differences in ABC-transport proteins. This is well illustrated by the high number of MRP-like transporters already characterised in the genome of *Arabidopsis thaliana* (Tommasini et al. 1997). These transporters share with YCF1, the closest yeast homologue, glutathione-S-conjugate transport activity. In addition, plant MRP proteins have the property to transport chlorophyll catabolites (Lu et al. 1998; Tommasini et al. 1998). Other

physiological functions of ABC transporters in higher eukaryotes have been described as well (Anderson et al. 1991; Broeks et al. 1996; Luciani and Chimini 1996; Paulusma et al. 1996; Ruetz and Gros 1994; Van Helvoort et al. 1996).

Table 5 Partial sequences of putative ABC transporters detected in the Expressed Sequence Tags (EST) databases from *Aspergillus nidulans* (A.n.) and *Neurospora crassa* (N.c.)

Species	*EST clone*	*GenBank number*	*Walker A*	*ABC signature*	*Walker B*	*S. cerevisae homologue*	*BLAST score* [a]
A.n.	H8h04a1.r1	AA785885		NVEQRKRLTIGV	LFLDEPTSGLD	PDR10	e^{-47}
A.n.	C9e04a1.f1	AA783966		SGGQKQRLCIAR	LLLDEATSSLD	MDL1	e^{-17}
A.n.	O8f05a1.f1	AA787659			LLLDESTSALD	YCF1	e^{-09}
A.n.	E7d04a1.r1	AA784517	GPNGSGKTTLM			YEF3B	e^{-30}
A.n.	m7a02a1.r1	AA786886	GRNGAGKSTLM			YPL226	e^{-23}
A.n.	E4a06a1.r1	AA784449	GLNGCGKSTLI			YPL226	e^{-07}
A.n.	K5a05a1.f1	AA786673			SFLDEPTNTVD	YEF3B	e^{-44}
N.c.	NCM8C11T7	AA901957		SQGQRQLVGLGR	VIMDEATASID	YLL015	e^{-20}
N.c.	NCC3E5T7	AA901865		SDGQKSRIVFAL	LLLDEPTNGLD	YER036	e^{-65}

[a] Based on homology of the full EST clone.

MULTIDRUG RESISTANCE

The use of cytotoxic compounds such as drugs in clinical medicine and disease control agents in agriculture is an essential component of human life. However, the widespread and sometimes excessive use of these compounds has resulted in a high selection pressure resulting in drug resistant populations. This phenomenon is of major concern to society.

In general, the major mechanisms underlying resistance in prokaryotes and eukaryotes can be classified as follows: (a) enzymatic inactivation or degradation of drugs, (b) alterations of the drug target-site and, (c) decreased drug-accumulation caused by energy-dependent drug efflux. More than one mechanism may operate in concert and the sum of different alterations represents the final resistant phenotype.

In several cases the resistance mechanism not only confers decreased sensitivity to a specific drug (and analogues) used during the selection process, but also to several structurally and functionally unrelated compounds. This phenomenon, termed **multidrug resistance** (MDR) operates in a broad range of organisms. It relates to decreased accumulation of drugs via energy-

dependent drug efflux systems. The majority of the transport proteins involved in drug-extrusion as determinants of MDR belong either to the ABC transporter or the MF superfamilies.

ABC Transporters in MDR in Prokaryotes and Lower Eukaryotes

In prokaryotes most of the characterised efflux-systems involved in MDR utilise the proton motive force as energy source for transport and act via a drug/H^+ antiport mechanism. The first example of a prokaryotic ABC transporter involved in MDR is the LMRA protein from *Lactococcus lactis* (Van Veen et al. 1996). The gene encodes a 590 aa membrane protein with the TMD_6-NBF topology. The protein probably functions as a homodimer. Functional studies performed in *E. coli* indicate that its substrate specificity comprises a wide range of hydrophobic cationic compounds, very similar to the pattern displayed by the human MDR1. Surprisingly, when expressed in human lung fibroblast cells, LMRA was targeted to the plasma membrane and also conferred typical multidrug resistance, confirming the evolutionary relation of these two proteins (Van Veen et al. 1998).

Genes encoding ABC transporters in parasitic protozoa have been isolated and analysed from *Plasmodium, Leishmania*, and *Entamoeba spp.*, and variation in the copy number and/or levels of expression have been implicated in drug resistance (Ullman 1995).

From *S. cerevisiae*, at least four members of the ABC transporter superfamily are involved in MDR: PDR5, SNQ2, YCF1 and YOR1 (Balzi et al. 1994; Cui et al. 1996; Decottignies et al. 1995; Li et al. 1996). PDR5 and SNQ2 have the $[NBF\text{-}TMD]_2$ topology and a preferential substrate specificity for aromatic cationic compounds, whereas YCF1 and YOR1 have the $[TMD\text{-}NBF]_2$ orientation and substrate specificity for anionic compounds. Despite its inverted topology and low sequence similarity, PDR5 seems to be the yeast functional homologue of the human MDR1, if substrate specificity is considered (Kolaczkowski et al. 1996). The presence of several other ABC proteins from *S. cerevisiae* with high homology to the ones involved in MDR and with common regulatory mechanisms suggests that other ABC transporters may be involved in MDR of *S. cerevisiae* as well (Balzi and Goffeau 1995). In yeast species such as *S. pombe* and *C. albicans* multidrug-efflux systems based on overproduction of ABC transporters have also been identified. Examples are CDR1 and CDR2 from *C. albicans* and PMD1 and BFR^+ from *S. pombe* (Nagao et al. 1995; Nishi et al. 1992; Sanglard et al. 1996; Sanglard et al. 1997).

MDR in Filamentous Fungi

MDR in filamentous fungi has been reported for laboratory-generated mutants of *A. nidulans* selected for resistance to azole fungicides. In genetically defined mutants, resistance to azoles is based on an energy-dependent efflux mechanism that results in decreased accumulation of the compounds in fungal mycelium (De Waard et al. 1995). This mechanism also operates in other species such as *P. italicum*, *B. fuckeliana, Nectria haematoccoca* and probably *M. graminicola* (De Waard et al. 1995; Joseph Horne et al. 1996). In our laboratory, ABC transporter encoding genes from *A. nidulans*, *B. fuckeliana* and *M. graminicola*, have been isolated and are currently functionally characterised (Andrade et al. 1998; Del Sorbo et al. 1997; Schoonbeek and De Waard 1998; Zwiers and De Waard 1998). The isolated genes display a high degree of homology with PDR5 and PMD1, yeast ABC transporters involved in MDR. AtrB from *A. nidulans* complements a PDR5 null mutant of *S. cerevisiae*, suggesting a role in fungicide sensitivity and resistance. Another example is MDR1 from the human pathogen *A. fumigatus*, which confers decreased sensitivity to the antifungal compound cilofungin upon expression in yeast (Tobin et al. 1997).

Very recently, the involvement of the ABC transporter (PMR1) of the phytopathogenic fungus *P. digitatum*, the causal agent of citrus green mold has been established in azole resistance of field isolates (Nakaune et al. 1998).

EVOLUTIONARY ASPECTS OF ABC TRANSPORTERS

The ubiquitous occurrence of ABC transporters throughout the living world indicates the ancient character of this superfamily of proteins. They are believed to date back in evolutionary time for more than 3 billion years (Saier et al. 1998). Thus, the understanding of evolutionary relationships among these transporters might be helpful in elucidating the origins of multidrug efflux systems, their physiological functions, and more important, the nature of their substrate specificity.

Recently, two paradigms on the evolution of bacterial multidrug transporters have been proposed. The first one describes that the transporters have evolved to protect cells from structurally diverse environmental toxins. The second one states that the transporters initially functioned in transport of specific physiological compounds (or a group of structurally related natural compounds) with the ability to expel drugs being only a fortuitous side effect (Neyfakh 1997). Experimental evidence has been proposed for both hypotheses, but it is unlikely that

transport proteins have evolved numerous distinct binding sites for structurally dissimilar molecules and therefore, a physiological substrate is likely to exist (Poole 1997). Furthermore, the presence of accessory factors as determinants of substrate specificity, such as the periplasmic binding proteins of the prokaryotic uptake systems or the eukaryotic glutathione-S-conjugate export pumps, could explain, in part, the accommodation of structurally unrelated compounds by ABC proteins.

Comparison of multidrug transport systems from six complete genomes of bacteria (three pathogenic and three non-pathogenic), indicates that, with one exception (*Methanococcus jannaschii*), the number of multidrug efflux pumps is approximately proportional to the total number of encoded transport systems as well as the total genome size. Therefore, the similar numbers of chromosomally encoded multidrug efflux systems in pathogens and nonpathogens suggests that these transporters have not arisen recently in pathogenic isolates in response to antimicrobial chemotherapy (Saier et al. 1998). However, novel ABC transport proteins with modified substrate specificity might have evolved during speciation as a result of fusions, intragenic splicing, duplications and deletions, in order to accomplish the different needs of organisms occurring in distinct environments. This can be illustrated by the occurrence of bacterial ABC transporters as separate subunits (*e.g.*, NBF and TMD) and by the inverted topology of domains observed in eukaryotic proteins. In addition, neither homologous proteins nor a characteristic motif of the so-called cluster I of yeast ABC transporters have, as yet, been found in prokaryotes (Decottignies and Goffeau 1997).

The considerations mentioned above and the experimental data available suggest that a MDR phenotype is not primarily caused by the appearance of a novel transport protein with a modified substrate profile but rather by an increased expression level of a pre-existent transport system as a result of alterations in regulation of such proteins (PDR in yeast) or gene amplification (MDR in mammalian).

PERSPECTIVES

Since the early 1980's the significance of ABC transporters for drug sensitivity and resistance has been recognised in the medical field. A similar interest in the role of ABC transporters in agriculture only started recently. Now, there is a growing awareness that ABC transporters can be involved in mechanisms of natural insensitivity and acquired resistance in a wide range of

organisms. In this review, we provide evidence that this also holds true for (pathogenic) filamentous fungi.

In the treatment of MDR-cancer cells inhibitors of ABC-transporter activity are used as synergists of drugs to reduce the MDR phenotype. If MDR would be the main mechanism of resistance to azoles, similar inhibitors could be useful in mixtures with these fungicides to increase control of azole-resistant populations of (plant) pathogenic fungi. If ABC transporters also play a role in protection against plant defence compounds and/or secretion of pathogenicity factors, inhibition of ABC transporter activity would result in enhanced host resistance and/or reduced virulence of the pathogen. Both processes would reduce disease development. As described for *S. cerevisiae* and *S. pombe* specific ABC transporters can be responsible for the transport of a mating factor (Christensen et al. 1997; McGrath and Varshavsky 1989). Inhibition of the activity of such specific ABC transporters would prevent mating, reduce the genetic variation and retard the epidemiology of plant pathogenic fungi.

In *S. cerevisiae* and *C. albicans,* ABC transporters with a $[NBF]_2$ configuration have been described. These so-called cluster IV ABC transporters interact with tRNA and act as elongation factors. ABC proteins of this cluster are interesting target sites for antifungal compounds as they seem to be absent from mammals (Decottignies and Goffeau 1997; Vazquez de Aldana et al. 1995).

Although evidence is accumulating that fungal ABC transporters are involved in pathogenesis and (fungicide) resistance, more research is needed to assess the full significance of ABC transporters in these phenomena. Knockout mutants and mutants overexpressing ABC transporters will help gaining insight in the physiological functions of ABC transporters. Knockout mutants lacking the natural insensitivity provided by ABC transporters can also be used as tools to screen for compounds with intrinsic fungitoxic activity.

OUTLINE OF THE THESIS

The aim of this thesis is to establish the role of ABC transporters of the wheat pathogen *M. graminicola* in pathogenesis, fungicide sensitivity and multidrug resistance. Chapter 2 describes the cloning and characterisation of two ABC transporter genes that were isolated by a heterologous screening approach. Functional analysis of genes requires the generation of knockout mutants, which can only be achieved with the availability of a transformation system.

However, at the start of this thesis an efficient transformation protocol was not available for *M. graminicola*.

The bacterium *Agrobacterium tumefaciens* is a bacterial pathogen of plants that integrates part of its genetic material into the genome of a host. This feature has made *A. tumefaciens* an excellent tool for plant genetic engineering. Only recently, it has been shown that the DNA transfer mediated by *A. tumefaciens* is not limited to the plant kingdom but also extends to fungi and animals. Chapter 3 describes the development of a highly efficient transformation system using *A. tumefaciens* as DNA donor. This *A. tumefaciens*-mediated transformation enables targeted gene disruption at high frequencies.

Chapter 4 describes the disruption or deletion of five ABC-transporter genes (*MgAtr1-5*) of *M. graminicola* and the characterisation of these knockout mutants with respect to virulence on wheat. This chapter demonstrates that one of the encoded ABC transporters (MgAtr4) indeed acts as a virulence factor.

In Chapter 5 the substrate specificity of MgAtr1-5 is analysed by heterologous expression in *S. cerevisiae* and testing the sensitivity of these *S. cerevisiae* transformants to natural toxic compounds and xenobiotics. In addition, the sensitivity of ΔMgAtr1-5 mutants of *M. graminicola* was tested against a range of compounds and antagonistic bacteria. The results show that ABC transporters from *M. graminicola* play a role in protection of *M. graminicola* against abiotic and biotic toxic compounds.

Both expression data and yeast complementation data (Chapter 2 and 5) suggested that azole fungicides can act as substrates for ABC transporters of *M. graminicola*. Therefore, in Chapter 6 the role of ABC transporters in fungicide sensitivity is examined. Mutants with a decreased sensitivity to the azole fungicide cyproconazole were analysed and the data presented indicate that overexpression of an ABC transporter in *M. graminicola* is involved in resistance to this compound.

Finally the results presented in this thesis are discussed in Chapter 7. The *A. tumefaciens*-mediated transformation is reviewed in more detail. Furthermore, the role of ABC transporters in virulence and the use of homology analysis to predict function and substrate specificity of fungal ABC transporters are discussed.

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CHAPTER 2

Characterisation of the ABC transporter genes *MgAtr1* and *MgAtr2* from the wheat pathogen *Mycosphaerella graminicola*

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ABSTRACT

ATP-binding cassette (ABC) transporters are membrane-bound transporters involved in various physiological processes. In this paper we describe the cloning of the ABC-transporter encoding genes *MgAtr1* and *MgAtr2* from the wheat pathogen *Mycosphaerella graminicola* (anamorph *Septoria tritici*). Both deduced proteins MgAtr1 and MgAtr2 are highly homologous to other fungal ABC-transporters. RT-PCR revealed that the *MgAtr2* mRNA population consists of partially and fully spliced transcripts. Putative substrates of ABC transporters, modulators of ABC transporter activity and inducers of ABC-transporter gene transcription were analysed for their potential to induce expression of *MgAtr1* and *MgAtr2* in *M. graminicola*. The genes are differentially upregulated by compounds such as the plant secondary metabolites eugenol and reserpine. Similar results are obtained for several antibiotics and the azole fungicides cyproconazole and imazalil. Moreover, a different expression pattern between yeast-like cells and mycelium of this dimorphic fungus was observed. These results indicate that *MgAtr1* and *MgAtr2* play a role in protection of *M. graminicola* against natural toxic compounds and xenobiotics. A putative role in protection against plant defence compounds during pathogenesis is suggested.

INTRODUCTION

Like all organisms, filamentous fungi are constantly challenged by the presence of potential toxic compounds in the environment. During evolution organisms have developed several mechanisms to cope with this chemical threat. One mechanism to protect themselves against the cytotoxic effects of compounds is by reducing the accumulation of toxic compounds in cells. This can be accomplished by an energy- dependent efflux mechanism mediated through ATP-binding cassette (ABC) transporters. ABC transporters are members of a large protein superfamily of transporters present in both prokaryotic and eukaryotic organisms (Higgins 1992). They are membrane bound and include both influx and efflux systems. ABC transporters couple ATP hydrolysis to transport. ABC transporters became especially known for their role in multidrug resistance (MDR) in tumour cells caused by an overproduction of the human ABC transporter protein P-gp (MDR1) (Juliano and Ling 1976). Typical ABC-transporter dependent drug resistance has also been demonstrated in various ascomycetes such as *Aspergillus nidulans*, *Penicillium digitatum*, *Candida albicans* and *Saccharomyces*

cerevisiae (Balzi and Goffeau 1995; Del Sorbo et al. 1997; Nakaune et al. 1998; Sanglard et al. 1997).

In plant pathogenic fungi ABC transporters have been suggested to be involved in pathogenicity and fungal biology (De Waard 1997). Upon infection they may provide protection against fungitoxic compounds produced by plants such as phytoalexins and phytoncides. This is supported by observations that diverse natural compounds such as isoflavonoids and alkaloids can act as substrates of ABC transporters (Seelig 1998). Recently, an insertional mutagenesis screen for pathogenicity mutants of *Magnaporthe grisea* led to the identification of the ABC transporter ABC1 as a novel pathogenicity factor, probably protecting the fungus against anti-microbial compounds produced by the host (Urban et al. 1999). ABC transporters might also act as pathogenicity factors by secretion of (host-specific) toxins. The fungal ABC transporters STE6 and mam1 from *S. cerevisiae* and *Schizosaccharomyces pombe* respectively, have also been reported to be involved in the secretion of mating pheromones. (Christensen et al. 1997; McGrath and Varshavsky 1989).

We are interested in the role of ABC transporters in the wheat pathogen *Mycosphaerella graminicola* (anamorph *Septoria tritici)*, the causal agent of septoria tritici leaf blotch of wheat. At present, this dimorphic fungus is recognised as one of the most important pathogens of wheat in Europe. Typical disease symptoms are necrotic blotches filled with the asexual pycnidia and sexual pseudothecia. Formation of these lesions may be associated with the secretion of a phytotoxic compound by the pathogen (Kema et al. 1996). Wheat is also known to produce fungitoxic compounds (Weibull and Niemeyer 1995). The pathogen may have specific ABC transporters, which reduce their accumulation in mycelium, thereby favouring colonization of the host. *M. graminicola* is also an important target pathogen of azole fungicides, which interfere with fungal sterol biosynthesis. Resistance against these fungicides could be due to increased expression of ABC transporters (Nakaune et al. 1998). For these reasons ABC transporters of *M. graminicola* can be of high physiological significance.

In this paper the cloning and initial characterisation of two ABC-transporter- encoding genes, *MgAtr1* and *MgAtr2,* are described. Northern analysis proved that both genes are differentially expressed in mycelium and "yeast-like" cells of this dimorphic fungus. Furthermore, antibiotics, plant secondary metabolites and azole fungicides could induce expression of both genes. These results suggest that ABC transporters of *M. graminicola* can

play a role in protection against natural toxic compounds and xenobiotics and even can play a role during pathogenesis.

RESULTS

Cloning and characterisation of *MgAtr1* and *MgAtr2*

A genomic library of *M. graminicola* isolate IPO323 was screened with a probe comprising the entire N-terminal ATP-binding cassette domain of *PDR5*, a well characterised ABC-transporter gene from *S. cerevisiae*, involved in multidrug resistance (Balzi et al. 1994; Bissinger and Kuchler 1994). Sequencing of hybridising phage inserts revealed that two phages contained sequences highly homologous to ABC transporter genes. The cloned ABC transporter encoding genes were named *MgAtr1* and *MgAtr2* (***Mycosphaerella graminicola*** **ABC-tr**ansporter). Both sequences are available in the EMBL database under accession numbers AJ243112 and AJ243113, respectively. *MgAtr1* as well as *MgAtr2* are single-copy genes (data not shown). An 8.7 kb phage insert containing *MgAtr1* and a 6.7 kb phage insert containing *MgAtr2* were subcloned and sequenced.

The 8.7 kb fragment containing *MgAtr1* revealed an interrupted ORF of 4689 bp (Fig. 1). The presence of two introns was confirmed by sequencing of cDNA clones. The intron sizes (54 and 128 bp) and splice sites (Table 1) are typical for filamentous fungi (Unkles 1992). Within the 5'-flanking region no core-promoter elements such as TATA box or CAAT motif are present. At -92 and -172 relative to the deduced translational start, two CT-rich regions are present. These stretches could act as promoter elements as they are frequently found near the transcription initiation site of filamentous fungi. Other typical features found in the 5'-flanking region of *MgAtr1* are a putative Pdr3p/Pdr1p binding site (CCGCGG) (Hellauer et al. 1996) located at 597 bp upstream from the start codon, a sequence corresponding to a binding site for the ATTS/TEA class of transcription factors (CATTCT) (Gavrias et al. 1996) located at -491, and a core heat shock element (HSE) consensus sequence located at -362 (Sorger and Pelham 1987). In the 3'-flanking region of *MgAtr1* a putative polyadenylation site (AATAA) was found at +100 relative to the translational stop.

Sequencing of several cDNA clones revealed that the 4500 bp ORF of *MgAtr2* is interrupted with seven introns, ranging in size from 48 up to 122 bp (Fig. 1, Table 1). Within the 5'-flanking region a TATA-like sequence is present at -108 (TATAT) and two putative

CAAT motifs are present at -120 and -170 relative to the translational start. Other characteristic elements present at -630 and -651 (CATTCC) are cis-elements recognised by developmental regulators such as the *S. cerevisiae* TEC1 (Andrianopoulos and Timberlake 1994; Gavrias et al. 1996). Elements at -396 (TGAAAgA) and -608 (TGAAAtg) have high homology to the core sequence (TGAAACA) of *S. cerevisiae* pheromone response elements (PREs) recognised by PDR12 (Dolan et al. 1989). Within the 3' flanking region no obvious polyadenylation signals were found but a sequence block (TGTGTTGTT) with high homology to a consensus termination sequence (PyGTGTTPyPy) is present.

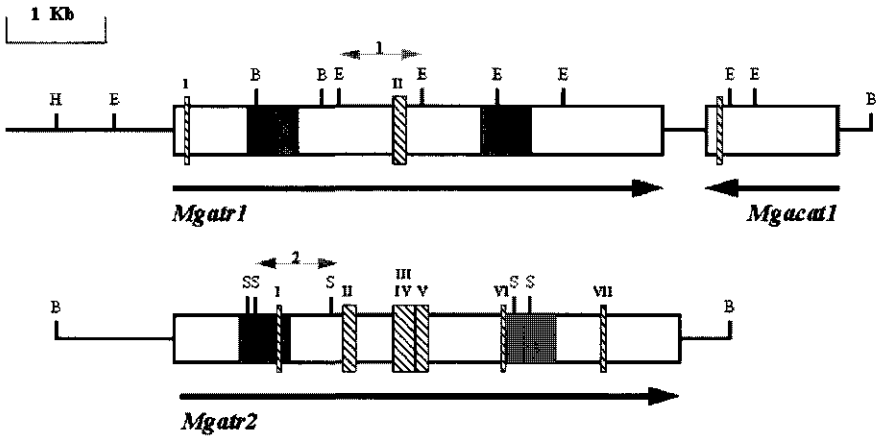

FIG. 1. Genomic organisation of *MgAtr1*, *MgAtr2* and *MgAcat1* from *Mycosphaerella graminicola*. Direction of transcription is indicated by arrows. Introns are numbered and hatched. Gray regions depict positions of ATP-binding cassettes. Regions used as probe are numbered 1 and 2 and indicated by double arrows. Restriction sites indicated are B: *Bam*HI, E: *Eco*RI, H: *Hind*III and S: *Sal*I.

The ORF of *MgAtr1* encodes a protein of 1562 amino acids with a calculated molecular weight of 174.7 kDa. The ORF of *MgAtr2* encodes a protein of 1499 amino acids with calculated molecular weight of 168 kDa. Hydropathy analyses predict that both MgAtr1 and MgAtr2 are composed of two similar halves, each with an N-terminal hydrophilic domain containing a nucleotide binding fold (NBF) and a hydrophobic domain including transmembrane regions (TM). The hydrophilic domains of both proteins contain an ATP binding cassette comprising the Walker A, Walker B and ABC signature (Walker et al. 1982). The N-terminal NBF of both proteins has a degenerated Walker A and Walker B motif and a well-conserved ABC-signature. The C-terminal NBF has conserved Walker motifs and a

degenerated ABC-signature (Fig. 2). BLAST homology searches of databases showed that both ABC transporters have the highest homology with ABC transporters from other plant pathogenic fungi. MgAtr1 shares the highest homology with BcAtrA (63% identity, 75% similarity; GenBank Z68906), an ABC transporter from *Botrytis cinerea*, and MgAtr2 with ABC1, an ABC transporter from *Magnaporthe grisea* (58% identity, 72% similarity; GenBank AF032443).

NH2-terminus

	Walker A	ABC signature	Walker B
MgAtr1	MLVLGRPGSG**C**STFLK	VRGVSGG**E**R**K**RVSIAETLASKSTVVCWDNSTRGLDA	
MgAtr2	LVVLGPPGSG**C**STFLK	LRGVSGG**E**R**K**RVTIAEASLSGAALQAWDNSTRGLDS	
BcAtrA	LLVLGRPGSG**C**STFLK	VRGVSGG**E**R**K**RVSIAETLPTKKTVVSWDNSTRGLDA	
MgABC1	LVVLGPPGSG**C**STFLK	IRGVSGG**E**R**K**RVTIAEAALSGAPLQCWDNSTRGLDS	
AnAtrA	LLVLGRPGTG**C**STFLK	VRGVSGG**E**R**K**RVSIAEMALAMTPFAAWDNSSRGLDS	
AnAtrB	LLVLGRPGSG**C**TTLLK	IRGVSGG**E**R**K**RVSIIECLGTRASVFCWDNSTRGLDA	
PMR1	LIVLGRPGSG**C**STFLK	IRGVSGG**E**R**K**RVSIAEATLCGSPLQCWDNSTRGLDS	
PDR5	LVVLGRPGSG**C**TTLLK	VRGVSGG**E**R**K**RVSIAEVSICGSKFQCWDNATRGLDS	
PDR10	LVVLGRPGAG**C**TTLLK	VRGVSGG**E**R**K**RVSIAEVSICGSKFQCWDNATRGLDS	
SNQ2	ILVLGRPGAG**C**SSFLK	VRGVSGG**E**R**K**RVSIAEALAAKGSIYCWDNATRGLDA	
CDR1	TVVLGRPGAG**C**STLLK	VRGVSGG**E**R**K**RVSIAEASLSGANIQCWDNATRGLDS	
CDR2	TVVLGRPGAG**C**STLLK	VRGVSGG**E**R**K**RVSIAEASLSGANIQCWDNATRGLDS	
CDR3	TVVLGRPGAG**C**STFLK	IRGISGG**E**R**K**RLSIAEVTLVQASIQCWDNSTRGLDA	
BFR1	VMVLGQPGSG**C**STFLR	VRGVSGG**E**R**K**RVTISEGFATRPTIACWDNSTRGLDS	
	*** ** ** *	** ******* * * *** ****	

COOH-terminus

	Walker A	ABC signature	Walker B
MgAtr1	VALMGASGAGKTTLLN	SSLG**V**E**Q**RKRLTIGVELAAKPSLLLFLDEPTSGLDS	
MgAtr2	TALMGVSGAGKTTLLD	EGL**N**VE**Q**RKRLTVGVELAAKPQLLLFLDEPTSGLDS	
BcAtrA	VALMGASGAGKTTLLN	RSLS**V**E**Q**RKRVTIGVELAAKPNLLLFLDEATSGLDS	
MgABC1	TALMGVSGAGKTTLLD	EGL**N**VE**Q**RKRLTIGVELAAKPPLLLFVDEPTSGLDS	
AnAtrA	TALMGVSGAGKTTLLD	EGL**N**VE**Q**RKLLTIGVELPPSPKLLLFLDEPTSGLDS	
AnAtrB	GALMGSSGAGKTTLLD	AGLS**V**E**Q**RKRVTIGVELVSKPSILIFLDEPTSGLDG	
PMR1	TALMGVSGAGKTTLLD	EGL**N**VE**Q**RKRLTIGVELAAKPQLLLFLDEPTSGLDS	
PDR5	TALMGASGAGKTTLLD	EGL**N**VE**Q**RKRLTIGVELTAKPKLLVFLDEPTSGLDS	
PDR10	TALIGASGAGKTTLLD	EGL**N**VE**Q**RKRLTIGVELAAKPKLLVFLDEPTSGLDS	
SNQ2	TALMGESGAGKTTLLN	CGL**N**VE**Q**RKKLSIGVELVAKPDLLLFLDEPTSGLDS	
CDR1	TALMGASGAGKTTLLN	EGL**N**VE**Q**RKRLTIGVELVAKPKLLLFLDEPTSGLDS	
CDR2	TALMGASGAGKTTLLN	EGL**N**VE**Q**RKRLTIGVELVAKPKLLLFLDEPTSGLDS	
CDR3	TALMGASGAGKTTLLN	EGL**N**VE**Q**RKRLTIAVELVARPKLLVFLDEPTSGLDS	
BFR1	TALMGESGAGKTTLLN	SGL**N**VE**Q**RKRATIGVELAAKPALLLFLDEPTSGLDS	
	** * *********	* ***** *** * * * ** *****	

FIG. 2. Alignment of ATP-binding cassette motifs from fungal ABC-transporters.
The multiple amino acid sequence alignment was generated using the CLUSTALW alignment program (Thompson *et al.*, 1994) and includes MgAtr1 and MgAtr2 from *Mycosphaerella graminicola*, BcAtrA from *Botrytis cinerea* (Z68906), ABC1 from *Magnaporthe grisea* (AF032443), AtrA and AtrB from *Aspergillus nidulans* (Z68904 and Z68905), PMR1 from *Penicillium digitatum* (AB010442), PDR5, PDR10 and SNQ2 from *Saccharomyces cerevisiae* (L19922, Z49821 and Z48008), CDR1, CDR2 and CDR3 from *Candida albicans* (P43071, P78595 and U89714), and BFR1 from *Schizosaccharomyces pombe* (P41820). Identical residues are marked with asterisks. Boldface residues are specific signatures for *S. cerevisiae* type I-1 ABC transporters (Decottignies and Goffeau, 1997).

Table 1

Intron characteristics of *MgAtr1* and *MgAtr2* from *Mycosphaerella graminicola*.

Gene	Intron	Size	5'-Splice site	Lariat sequence	3'-Splice site
MgAtr1	I	54	/GTATTT[2]	AGCTAAC	CAG/
	II	128	/GTAAGC	TGCTAAC	TAG/
MgAtr2	I	48	/GTATGC	-	CAG/
	II	119	/GTAAGT	AGCTAAC	CAG/
	III	122	/GTAAGT	TGCTAAC	TAG/
	IV	63	/GTGAGT	TG**T**GAAC	TAG/
	V	109	/GTAAGT	**G**AC**C**AAC	TAG/
	VI	55	/GTATGT	-	CAG/
	VII	51	/GTAAGC	**C**TCTAAC	CAG/
Consensus[1]			/GT$^{A}/_{G}$NG$^{C}/_{T}$	t$^{G}/_{A}$CTAAC	$^{C}/_{T}$AG/

[1] Consensus sequences based on (Gurr et al. 1988).
[2] Sequences deviating from consensus are indicated in boldface type.

Splicing

The results of the RT-PCR experiments, performed to determine the intron junctions, indicate that *MgAtr2* RNA is inefficiently spliced. PCR was performed on cDNA made from polyA^{+} RNA isolated from *M. graminicola* isolate IPO323 treated with 50 µg ml^{-1} cycloheximide. RT-PCR with primers designed to amplify the region from intron II until VI resulted in the amplification of three bands instead of the expected single band (Fig. 3, bands I, II and III). These three bands were subcloned and sequenced. Band I appeared to correspond with the genomic sequence indicating that none of the introns II, III, IV, V and VI were spliced. Although this band could be due to the presence of contaminating genomic DNA this is not likely as amplification on the same material using primers directed against intron I of *MgAtr2* and intron II of *MgAtr1* did only yield fragments corresponding with spliced transcripts (data not shown). Band II contains the sequence where introns II, III, IV and VI are spliced with intron V still present. Band III contains the RNA lacking introns II, III, IV and VI. This partial splicing could also be observed when RT-PCR was performed using primers directed against intron III up to V, this again showed that all introns but intron V were spliced. Partial splicing was not observed for introns in *MgAtr1*. These results indicate that the population of *MgAtr2* transcripts present consists of subpopulations with partially processed transcripts.

Flanking genes

Downstream of *MgAtr1* another ORF could be detected on the opposite DNA strand in a tail-to-tail orientation with *MgAtr1* (Fig. 1). The 3'-end of this ORF is only 438 bp downstream of

the translational stop of *MgAtr1*. This gene, coded *MgAcat* (EMBL database accession number AJ243195), consists of 1397 bp of coding region interrupted by one putative intron of 65 bp. Within the sequenced 168 bp upstream of the ATG no obvious promoter elements could be detected. The deduced protein is 444 amino acids long with high homology with mitochondrial acetyl-CoA-acetyltransferase precursors from human and rat (50 and 51% identity, respectively).

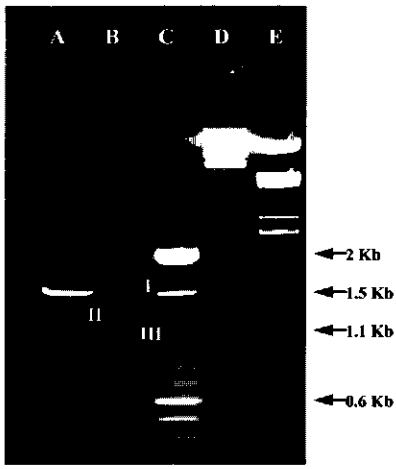

FIG. 3. Partial splicing of *MgAtr2* from *M. graminicola*. Fragments amplified from genomic DNA (lane A) and cDNA prepared from polyA$^+$ RNA isolated from isolate I323 treated with cycloheximide (50 μg ml^{-1}) (lane B). Primers were designed to amplify the region between introns II and VI. 100 bp marker (lane C), λ x *Hind*III (lane D), λ x *Pst*I (lane E). Band I corresponds to unspliced RNA. Band II corresponds to RNA still containing intron V, and band III corresponds to RNA lacking introns II, III, IV, V and VI.

Northern analysis

Both in yeast-like cells and mycelium (Fig. 4) a basal steady state level of *MgAtr1* mRNA was detected, and a low level of *MgAtr2*, as mRNA could only be detected by RT-PCR. Twenty compounds were tested as potential inducers of *MgAtr1* and *MgAtr2* expression. The compounds included antibiotics (cycloheximide, cyclosporin, neomycin and nystatin), drugs (triflupromazine), fungicides (cyproconazole and imazalil), lipids (lanosterol, linoleic acid, oleic acid, palmitic acid, phosphatidylcholine and progesterone), plant secondary metabolites (eugenol, psoralen, quercetin, reserpine, resveratrol and tomatine) and xenobiotics (acriflavine). The compounds used were chosen because they act, or based on structural similarities are expected to act, as (potential) substrates, modulators of ABC-transporter activity or inducers of transcription of ABC transporter genes.

The results (Figs 5 and 6) indicate that various compounds differentially influence the expression of *MgAtr1* and *MgAtr2*. For instance, in yeast-like cells cycloheximide, cyproconazole, and progesterone induce both genes, while eugenol and linoleic-acid induce

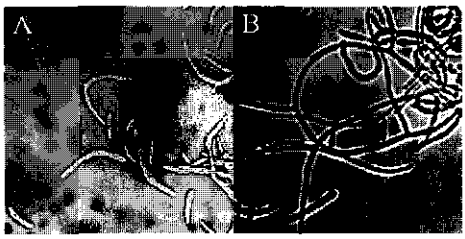

FIG. 4.
Dimorphic growth of *Mycosphaerella graminicola*.
(A) Yeast-like cells; (B) Mycelia

only MgAtr1. In mycelium both genes are induced by eugenol, while imazalil, palmitic acid and progesterone induce only MgAtr2. Differential expression is also found after treatment with the azole fungicide cyproconazole and the fatty acids oleic acid and linoleic acid. These compounds induce the expression of *MgAtr1* in cells one hour post treatment, but not in mycelium. On the other hand, palmitic acid, the plant secondary metabolite eugenol and the azole fungicide imazalil induce expression of *MgAtr2* in mycelium, whereas no upregulation of expression was found in yeast-like cells. These observations indicate that both genes are differentially expressed in yeast-like cells and mycelium after treatment with these compounds.

Finally, the regulation of both genes varies in time (Fig. 6). Whether induced or not, the level of *MgAtr1* transcripts remains more or less constant over the 24 hr period tested. However, expression of *MgAtr2* is strongly upregulated with a peak at one hour post addition of imazalil. These observations suggest that *MgAtr1* and *MgAtr2* are under different regulatory control.

DISCUSSION

Cloning and characterisation of *MgAtr1* and *MgAtr2*

Hybridisation of a genomic library of *M. graminicola* with a *PDR5* probe from *S. cerevisiae* led to the successful cloning of the ABC transporter genes *MgAtr1* and *MgAtr2*. A similar approach already led to the isolation of ABC transporter encoding genes from *A. nidulans* (Del Sorbo et al. 1997), *B. cinerea* (Schoonbeek, personal communication) and *P. digitatum* (Nakaune et al. 1998). This clearly illustrates the strong homology between different ABC transporters from different fungal species. ABC transporters constitute one of the largest protein superfamilies as illustrated by the 29 ABC transporter genes found in the genome of *S. cerevisiae* (Decottignies and Goffeau 1997) as well as the high numbers present in other

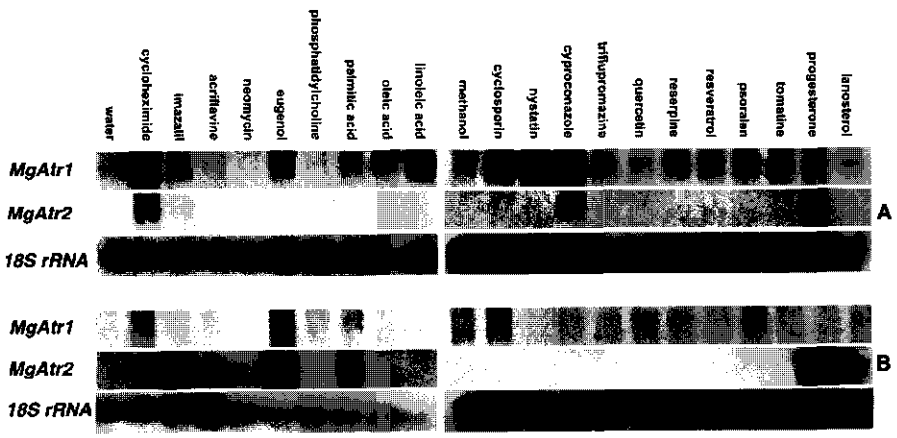

FIG. 5. Northern analysis of *MgAtr1*, *MgAtr2*, and *18S rRNA* expression in yeast-like cells (A) and mycelium (B) of *Mycosphaerella graminicola* after treatment with various compounds. Material was harvested 1 h after the compounds were added. Compounds tested are; antibiotics (cycloheximide, cyclosporin, neomycin and nystatin), drugs (triflupromazine), fungicides (cyproconazole and imazalil), lipids (lanosterol, linoleic acid, oleic acid, palmitic acid, phosphatidylcholine and progesterone), plant secondary metabolites (eugenol, psoralen, reserpine, resveratrol, tomatine and quercetine), and xenobiotics (acriflavine). Concentrations used are given under Materials and Methods.

organisms (Kunst et al. 1997). This holds true for *M. graminicola* as well, as a PCR-based approach (performed in our laboratory) using degenerate primers directed against ABC domains resulted in the cloning of three additional ABC transporter genes (Venema, personal communication).

Based on topology, amino acid sequence comparison and the presence of specific amino acid sequence motifs, ABC transporter proteins from *S. cerevisiae* can be divided into six clusters (Decottignies and Goffeau 1997). The deduced proteins MgAtr1 and MgAtr2 exhibit the $[NBF\text{-}TM]_2$ configuration which is characteristic for ABC transporters belonging to cluster I-1 of yeast ABC transporters. Representatives of this cluster are PDR5, SNQ2 and PDR12 involved in MDR and resistance to organic acids. The presence of a conserved cysteine in the N-terminal Walker A, glutamic acid and lysine in the N-terminal ABC signature as well as the valine-glutamic acid-glutamine motifs in the C-terminal ABC signatures is also characteristic for subcluster I-1 ABC transporters (Fig. 3). The classification of MgAtr1 and MgAtr2 in the yeast type I-1 cluster of ABC transporters may reflect functional homology, for instance related to substrate specificity.

MgAtr1 shows the highest similarity with BcAtrA from *B. cinerea* (63% identity, 75% similarity) and MgAtr2 with ABC1, an ABC transporter from *M. grisea* (58% identity, 72%

similarity). These are extremely high levels of identity considering that the homology between other fungal ABC transporters is usually around 40%. ABC1 of *M. grisea* has been identified as a pathogenicity factor. ABC1 mutants show an immediate growth arrest after penetrating rice or barley epidermal cells. (Urban et al. 1999). Whether the high structural homology between MgAtr2 and ABC1 reflects a similar function of MgAtr2 in pathogenesis of *M. graminicola* on wheat remains to be established.

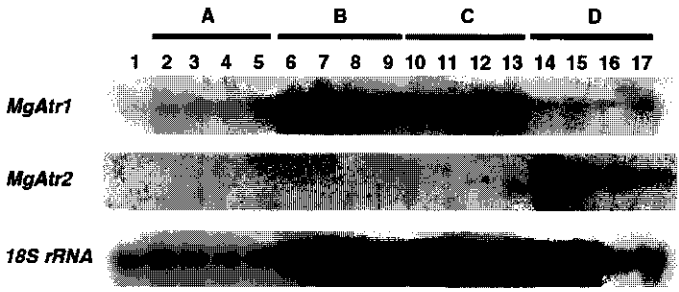

FIG. 6. Time-course of expression of *MgAtr1*, *MgAtr2*, and *18S rRNA* in mycelium of *Mycosphaerella graminicola* after treatment with various compounds. Mycelium was incubated with (A) water, (B) cycloheximide (50 µg ml^{-1}), (C) cycloheximide (100 µg ml^{-1}) and (D) imazalil (10 µg ml^{-1}). RNA was isolated at 1 h (lanes 2, 6, 10 and 14), 3 h (lanes 3, 7, 11 and 15), 6 h (lanes 4, 8, 12 and 16), and 24 h (lanes 5, 9, 13 and 17) after the compound was added. Lane 1 represents the situation at the start of the experiment.

Splicing

RT-PCR revealed that the population of *MgAtr2* transcripts contains partially processed transcripts. In addition to fully spliced transcripts, partially spliced messengers were detected which still contained intron II up to VI, or transcripts lacking all introns except intron V. This partial processing can not be due to splice site characteristics. For instance, both the 5' and 3' splice sites of intron V match the consensus splice sites. The putative branch site deviates from the consensus but this branch site seems to be dispensable for proper splicing as it is absent in the properly spliced introns I and VI (Table 1). The partial splicing of *MgAtr2* was observed in cells treated with cycloheximide. It can not be caused by the protein synthesis inhibitory effect itself as *MgAtr1* was properly spliced after induction with cycloheximide.

The phenomenon of partial splicing has been described before and seems to be a mechanism to regulate the expression of genes and the specificity of encoded proteins. For instance, in *Phanerochaete chrysosporium* it was demonstrated that the introns in the regions encoding the cellulose binding domains of two exocellobiohydrolase I-like genes (*cbhl1* and

cbhI2) were differentially spliced in a substrate-dependent manner (Birch et al. 1995). In *Neurospora crassa* the same phenomenon was observed for the *fox-2* gene, encoding the multifunctional beta-oxidation enzyme, in which one out of three introns was spliced inefficiently (Fossa et al. 1995).

The partially processed mRNA of *MgAtr2* leads to a truncated ABC transporter. A stop-codon present in intron V will result in a transcript encoding a protein of 684 instead of 1499 aa. Although not very likely, it can not be excluded that such a truncated ABC transporter is still functional in the cell with altered substrate specificity.

Flanking genes

MgAtr1 is located very closely to *MgAcat1*, a gene encoding an acetyl-CoA-acetyl transferase. This enzyme belongs to the family of thiolases, which can be divided into two classes. Type I thiolases are involved in degradative pathways such as fatty-acid-β-oxidation. Type II thiolases play a role in thiolysis of acetoacetyl-CoA and are involved in the biosynthesis of sterols and poly β-hydroxybutyrates. Both fatty acids and sterols are known to be substrates for ABC transporters (Shani and Valle 1996). Hence, the short distance between *MgAtr1* and *MgAcat1* suggests that both genes are members of a gene cluster and can be co-ordinately regulated. However, northern analysis with RNA isolated from yeast-like cells induced for *MgAtr1* expression by treatment with cycloheximide did not show any *MgAcat1* expression. This indicates that at least under this condition the genes do not show a coordinated regulation.

Expression studies

The expression studies clearly indicate that *MgAtr1* and *MgAtr2* are under different regulatory control. First, *MgAtr1* exhibits a clear basal level of expression whereas *MgAtr2* does not. Second, both genes are induced by different sets of compounds and the regulation of expression in time is different. Third, the expression patterns for both genes differ for the two morphological states tested. These results are not surprising, as the promoter regions of both genes are clearly distinct with different putative regulatory elements present for each gene.

The differential expression of *MgAtr1* and *MgAtr2* in yeast-like cells and mycelium is very interesting since the mycelial form of the fungus is responsible for penetration and colonisation of the host (Kema et al. 1996). In the 5' region of *MgAtr2* the putative

pheromone-response element (PRE) at -608 and the CATTCC element at -630 are only 16 bp apart. In *S. cerevisiae* the same elements, separated by 14 basepairs, form a composite DNA element described as a filamentation and invasion response element (FRE) (Madhani and Fink 1997). The presence of a FRE in the promoter region of *MgAtr2* suggests a role of *MgAtr2* in the switch to mycelial growth and possibly the pathogenic state of *M. graminicola*.

The differential expression of *MgAtr2* between yeast-like cells and mycelium upon eugenol treatment is also suggestive in this respect. Eugenol (2-methoxy-4-[2-propenyl]phenol), an essential-oil constituent of cloves with antifungal action, upregulates *MgAtr1* both in yeast-like cells and mycelium and *MgAtr2* in mycelium only. This may relate to observations that in *C. albicans* eugenol seems to interfere with the yeast-mycelium transition as germ tube formation is inhibited at lower concentrations than required to stop normal growth of yeast and mycelium (Boonchird and Flegel 1982). The mode of action of eugenol is unclear but there is some evidence that it might act as an uncoupler of oxidative phosphorylation and thus can inhibit ABC transporter activity by depleting ATP.

The differential expression of *MgAtr1* and *MgAtr2* in mycelium and yeast-like cells may also be a reflection of the cell membrane and/or cell wall composition. For several fungi exhibiting dimorphic growth, it has been demonstrated that the switch to either state involves quantitative changes in cell wall composition (San Blas and San Blas 1984). Changes in lipid, sugar and protein composition will probably lead to a changed solubility profile and, consequently, influence perception of compounds by the cell and alter expression of ABC transporter genes. Furthermore, the membrane environment plays an important role in determining the drug resistance conferred by ABC transporters (Kaur and Bachhawat 1999; Regev et al. 1999). This indicates that not only the expression of ABC transporter genes might change but also the activity of the ABC transporters can change as a result of changes in membrane composition.

MgAtr1 and / or *MgAtr2* expression is upregulated by plant secondary metabolites such as eugenol, psoralen and reserpine and by antibiotics such as cycloheximide, cyclosporine, neomycine and nystatin. Most of these unrelated compounds exhibit antifungal activity. Therefore, our findings suggest that the ABC transporters described may provide protection against natural toxic compounds present in the natural habitat of the fungus. This would explain why *M. graminicola* can survive for several months on host debris in soil and can protect itself against toxic compounds produced by fungi and bacteria. We did not test the

inducing activity of secondary metabolites of wheat, the natural host of *M. graminicola*. However, the chemical diversity of compounds capable of inducing *MgAtr1* / *MgAtr2* expression suggests that this also may be the case for antifungal compounds produced by wheat, such as hydroxamic acids (Weibull and Niemeyer 1995). If this is true, ABC transporters of *M. graminicola* may also play a role in virulence on wheat. Furthermore, the clear induction of *MgAtr1* expression by the azole fungicide cyproconazole and the induction of *MgAtr2* by imazalil suggest a role of these ABC transporters in fungicide activity.

Assessing the phenotype of knockout mutants for either gene can test these hypotheses. However, the construction of null-mutants by either gene-replacement or gene-disruption has not yet been accomplished as (i) transformation of *M. graminicola* is still inefficient and (ii) homologous recombination required to obtain null-mutants does not occur frequently as screening of 369 transformants only showed ectopic integrations. These results could imply that replacement or disruption of *MgAtr1* and *MgAtr2* is lethal. This is unlikely, as disruption of ABC transporter genes in general is not lethal. Further research on these topics is in progress.

MATERIALS AND METHODS

Fungal material and culture.

M. graminicola isolate IPO323 provided by Dr. G.H.J. Kema (IPO-DLO, Wageningen) was used for the construction of a genomic library and for expression studies. Yeast-like cells were grown in liquid yeast-sucrose medium (YSM; 10 g l^{-1} yeast extract, 10 g l^{-1} sucrose, 50 µg ml^{-1} streptomycin sulphate) on a lateral shaker (18°C, 140 rpm). Mycelium was obtained by diluting a three-day-old culture of yeast-like cells grown in YSM to an optical density of 0.05 at 600 nm in 20 ml of liquid Czapek Dox-mycological peptone (CDMP; 33.4 g l^{-1} Czapek Dox, 5 g l^{-1} mycological peptone, 50 µg ml^{-1} streptomycin sulphate) and incubation on a rotary shaker (25°C, 140 rpm) for an additional three days.

Molecular biological techniques and analyses

Basic DNA and RNA manipulations were done according to standard procedures (Sambrook et al. 1989). *E. coli* DH5α was used for propagation of constructs. For library construction, *M. graminicola* genomic DNA was isolated from five-day-old yeast-like cells essentially as described by (Raeder and Broda 1985). Genomic DNA was partially digested with *Sau*3A and size-fractionated on a 10-40% sucrose gradient. DNA in the size range of 9-20 kb was ligated into *Bam*HI-digested λEMBL3 and packaged according to the manufacturer's instructions (Promega EMBL3 *Bam*HI Arms cloning system plus packagene system).

The genomic library was screened with a partial *PDR5* probe from *S. cerevisiae* provided by Dr. A.C. Goffeau (Louvain-la-Neuve, Belgium). This probe consisted of a 1.5 kb *Bgl*II fragment digested with *Taq*I and comprised the entire N-terminal ATP-binding cassette domain of PDR5. Positive phages were subcloned in pBluescript II SK vectors (Stratagene) and sequenced by the dideoxy chain-termination method using ampli *Taq* polymerase and fluorescent dideoxy dNTPs.

DNA and deduced protein sequences were analysed with the GCG Package Version 8.0 (Genetics Computer Group (GCG), Madison, Wisc.) Multiple alignments were performed using the CLUSTALW algorithm at IBCP, France (http://pbil.icp.fr/) (Thompson et al. 1994). BLAST homology searches of databases were performed using Blast2.0 at NCBI (http://www.ncbi.nlm.nih.gov/blast) (Altschul et al. 1997).

Total RNA of *M. graminicola* was extracted using a guanidine hydrochloride procedure. RNA (15 µg) was separated on formaldehyde gels and transferred to HybondN nylon membranes (Amersham), according to the manufacturer's instructions. Equal loading and transfer of RNA was determined by staining gels and northern blots with ethidium bromide and by probing blots with the glyceraldehyde 3-phosphate dehydrogenase (*gpd*) gene of *M. graminicola*. An 840 bp *Eco*RI fragment and a 750 bp *Sal*I fragment were used as probes for *MgAtr1* and *MgAtr2*, respectively.

Homologous hybridisations were performed at 65°C and heterologous hybridisations at 56°C. Blots were hybridised with [α-^{32}P]dATP-labeled probes and treated as described by (Church and Gilbert 1984). Stringency of the final washings was 0.1 x SSC for homologous hybridisations and 1 x SSC for heterologous hybridisations.

cDNA amplification

Intron-exon junctions were determined by sequencing cDNA cloned in pCR-blunt (Invitrogen) maintained in *E. coli* strain Top10 (Invitrogen). Poly A$^+$ RNA was isolated from yeast-like cells of *M. graminicola* treated with 50 µg ml^{-1} of cycloheximide (Qiagen oligotex mini kit). First strand cDNA was synthesised using the Promega reverse transcription system. Amplification of the cDNA was performed with the Advantage Klentaq polymerase mix (Clontech) using a Perkin-Elmer DNA thermal cycler 480. PCR primers were designed to discriminate between contaminating genomic sequences and the desired cDNA.

Expression studies

Expression in yeast-like cells was studied by adding test chemicals to three-day-old cultures diluted to an optical density of 1 at 600 nm in fresh YSM. After addition of test chemicals cultures were incubated for 1 and 24 hours (18°C, 140 rpm). Cells were harvested by centrifugation (15 min, 1800 x g). The pellet was washed with 0.5 ml of demineralised water, frozen in liquid nitrogen and stored at -80°C until RNA isolation.

Induction of transcription in mycelium was studied by adding the test chemicals to three-day-old mycelial cultures. Mycelium was harvested 1 h after addition of test chemicals by centrifugation (15 min, 1800 x g). The pellet was briefly dried on filter paper, subsequently frozen in liquid nitrogen and stored at -80°C until RNA isolation.

The final concentrations of the chemicals were: acriflavine (50 µg ml^{-1}, Fluka), cycloheximide (50 µg ml^{-1}, Merck), the cyclosporin derivative PSC833 (10 µg ml^{-1}, Novartis), cyproconazole (10 µg ml^{-1}, Novartis),

eugenol (0.01% (v/v), Sigma), imazalil nitrate (10 μg ml^{-1}), lanosterol (100 μg ml^{-1}), linoleic acid (1 mg ml^{-1}), neomycin (100 μg ml^{-1}), nystatin (5 μg ml^{-1}), oleic acid (1 mg ml^{-1}), palmitic acid (1 mg ml^{-1}), phosphatidyl choline (100 μg ml^{-1}), progesterone (100 μg ml^{-1}), psoralen (50 μg ml^{-1}), quercetin (100 μg ml^{-1}), reserpine (100 μg ml^{-1}), resveratrol (50 μg ml^{-1}), tomatine (10 μg ml^{-1}), and triflupromazine (20 μg ml^{-1}). Most of the chemicals were added from thousand-fold stock solutions in water or methanol. The absolute amount of eugenol, linoleic acid, neomycin, oleic acid, palmitic acid and phosphatidyl choline was directly added to the cultures. The solvent methanol was added in control treatments to a final concentration of 0.1%.

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CHAPTER 3

Efficient *Agrobacterium tumefaciens*-mediated gene disruption in the phytopathogen *Mycosphaerella graminicola*

L.-H. Zwiers and M.A. De Waard. *Curr Genet* (2001) **39**: 388-393

ABSTRACT

Agrobacterium tumefaciens-mediated transformation has been successfully applied to the wheat pathogen *Mycosphaerella graminicola*. Both protoplasts and intact cells have been transformed to hygromycin B resistance. Furthermore, *A. tumefaciens*-mediated transformation using homologous DNA originating from the *M. graminicola* ABC transporter gene *MgAtr2* resulted in the efficient generation of disruption mutants. In 44% of the transformants, disruption of *MgAtr2* was achieved and transformants resulted from the integration of a single copy of the transforming DNA. These results indicate that *A. tumefaciens*-mediated transformation is a useful tool to generate targeted gene disruption in the phytopathogen *M. graminicola* where gene targeting by conventional methods is hardly possible.

INTRODUCTION

Mycosphaerella graminicola (anamorph *Septoria tritici*) is the causal agent of septoria tritici leaf blotch of wheat. The increasing significance of this pathogen in Europe and northern America has resulted in an enhanced interest in studying its molecular genetics and pathogenesis. Disruption or replacement of genes is a helpful tool to assess their role in pathogenesis. This method requires not only an efficient transformation system but also efficient gene-targeting by homologous recombination. Until now, both have been lacking for *M. graminicola*. Transformation of *M. graminicola* has been described using a protoplast/polyethylene glycol-based method (Payne et al. 1998; Pnini Cohen et al. 2000). However, depending on the isolate used, transformation is very inefficient and irreproducible. Transformation frequencies in our laboratory varied from 0 – 10 transformants μg^{-1} vector DNA, with an average of approximately 1 transformant μg^{-1} DNA. Furthermore, transformation is predominantly the result of multiple ectopic integrations and homologous recombination seems to be a rare event. Until now, no homologous recombination in *M. graminicola* has been reported.

We are interested in the role of ATP-binding cassette (ABC) transporters in the wheat-*M. graminicola* interaction. ABC transporters are membrane-bound proteins involved in a wide variety of physiological processes. In phytopathogenic fungi, they are implicated to act as virulence factors by providing protection against plant defence compounds produced by the host plants upon infection or by secretion of plant toxins (De Waard 1997; Del Sorbo et al. 2000). Recently an ABC transporter was identified in *Magnaporthe grisea* as a novel

pathogenicity factor (Urban et al. 1999). We have isolated and characterised two ABC transporter genes from *M. graminicola*, *MgAtr1* and *MgAtr2* (Zwiers and De Waard 2000). The generation of null mutants for either gene has not been successful, using protoplast-PEG-based transformation, lithium acetate-mediated transformation, or electroporation. Screening of more than 400 hygromycin resistant transformants showed only (multiple) ectopic integrations. In view of this situation, *Agrobacterium tumefaciens*-mediated transformation was investigated.

A. tumefaciens is used in the transformation of a broad variety of plant species. The use of *A. tumefaciens* in the transformation of the yeasts *Saccharomyces cerevisiae* and *Kluyveromyces lactis* has also been described (Bundock et al. 1995; Bundock et al. 1999). Recently, the *A. tumefaciens*-mediated transformation protocol was successfully applied to some filamentous fungi (Abuodeh et al. 2000; De Groot et al. 1998; Mullins et al. 2001). Apparently, this method has none of the disadvantages associated with the above-mentioned protocols. Transformation can be achieved using either protoplasts or intact cells. Transformation efficiencies can be up to 600 times higher, as compared with protoplast-PEG-based protocols and transformation is mainly the result of single copy integrations. Homologous recombination has been described, for *Aspergillus awamori, K. lactis* and *S. cerevisiae,* but not yet for any phytopathogenic fungus, such as *M. graminicola* (Bundock et al. 1995; Bundock et al. 1999; Gouka et al. 1999).

In the present paper, we report the successful *Agrobacterium. tumefaciens*-mediated transformation of both protoplasts and intact cells of *M. graminicola* to hygromycin resistance. In addition, transformation using a construct aimed at the disruption of the ABC transporter-encoding gene *MgAtr2* generates disruptants with high efficiency. Southern analysis indicates that the majority of the transformants contain a single copy of the transforming DNA. Therefore, this paper shows that *A. tumefaciens*-mediated transformation of *M. graminicola* is possible. Moreover, it also demonstrates the power of the system to obtain gene targeting by homologous recombination in a filamentous fungus, where gene targeting is otherwise inefficient.

RESULTS

Agrobacterium tumefaciens-mediated transformation

To test the *A. tumefaciens*-mediated transformation of *M. graminicola* both protoplasts and intact cells were co-cultivated with *A. tumefaciens* LBA1100 containing pUR5750 (De Groot

et al. 1998). Co-cultivation of both protoplasts and cells of *M. graminicola* with *A. tumefaciens* resulted in the formation of hygromycin-resistant colonies only when the co-cultivation was carried out in the presence of acetosyringone. After 18-21 days small colonies, visible to the naked eye, appeared. Most transformants were obtained using *M. graminicola* cells and protoplasts at a density of 10^8 ml^{-1}. Results of two independent experiments indicated no obvious difference in efficiency between protoplasts and whole cells. The average transformation frequency was seven transformants and five transformants per 10^7 yeast-like cells and protoplasts, respectively. Three hygromycin-resistant colonies derived from protoplasts and four hygromycin-resistant colonies derived from yeast-like cells co-cultivated with *A. tumefaciens* were analysed by Southern blot analysis. This analysis showed that six out of seven transformants contained a single copy of the hygromycin cassette. One transformant was the result of a tandem integration event (data not shown).

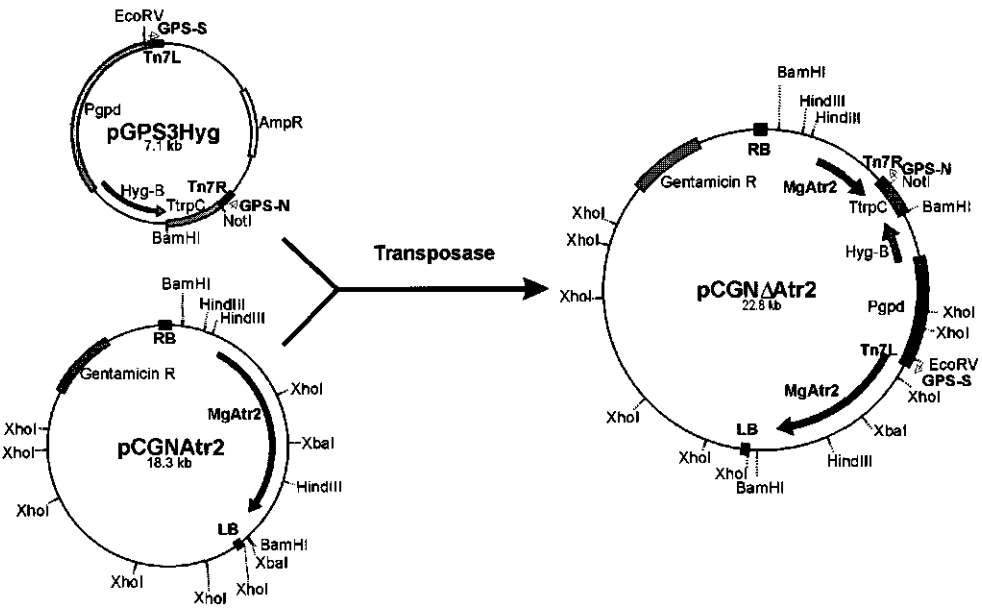

FIG. 1. Schematic representation of the generation of the disruption construct *pCGNΔAtr2* by means of a transposition reaction. *pGPS3Hyg* is the donor plasmid and *pCGNAtr2* is the target plasmid. *hyg-B* Hygromycin B phosphotransferase gene, *LB* T-DNA left border repeat, *Pgpd Aspergillus nidulans* glyceraldehyde-3-phosphate dehydrogenase promoter, *RB* T-DNA right border repeat, *Tn7L* Tn7 left transprimer end, *Tn7R* Tn7 right transprimer end and *TtrpC A. nidulans* trpC terminator

Plasmid construction by *in vitro* transposition

Disruption constructs were made using the GPS-M system. The transposition reaction was performed using pGPS3hyg as transposition donor and pCGNAtr2 as transposition target (Fig. 1). Transposition of the hygromycin resistance gene into pCGNAtr2 occurred in 2-3% of the plasmids, as checked by colony hybridisation. Restriction analysis of six plasmids containing an insertion indicated that five insertions occurred in the vector backbone and not in the coding region. One plasmid contained an insertion in the coding region of *MgAtr2*. The exact location of the insertion in the *MgAtr2* coding region was determined by sequencing using primers GPS-N and GPS-S. The insertion of the hygromycin cassette in pCGNAtr2 resulted in pCGNΔAtr2 with 2.6 kb and 4.1 kb of flanking homologous DNA. This construct was introduced into *A. tumefaciens* and subsequently used to transform *M. graminicola*.

Targeted integration through *A. tumefaciens*-mediated transformation

A. tumefaciens LBA1100 containing pCGNΔAtr2 was used for the generation of gene disruption mutants of *MgAtr2*. The experiment was performed by co-cultivation of 100 µl of *A. tumefaciens* with 100 µl of *M. graminicola* cells containing 10^7 cells. In two independent experiments, hygromycin-resistant colonies developed after 10-14 days at a rate of 50 colonies per 10^7 cells (Fig. 2).

FIG. 2. Example of *Agrobacterium tumefaciens*-mediated transformation of *Mycosphaerella graminicola*. *A. tumefaciens* strain LBA1100 containing pCGNΔAtr2 was co-cultivated with yeast-like cells of *M. graminicola* (10^8 ml^{-1}) either in the presence or absence of acetosyringone (AS).

Hygromycin-resistant colonies obtained with the *A. tumefaciens*-mediated transformation were transferred and 82 putative *MgAtr2* disruptants were screened by PCR. The PCR screen was performed with a mixture of three primers (GPS-n, 2y and 2z; Fig. 3A). The primers 2y and 2z amplify a product of approximately 900 bp with DNA from the wild type. This 900-bp band is also expected to be present in transformants with ectopic integrations of the transforming DNA. In disruptants, however, this 900-bp band should be absent. The primers 2y and GPS-n amplify a band of approximately 400 bp in transformants, whereas this band

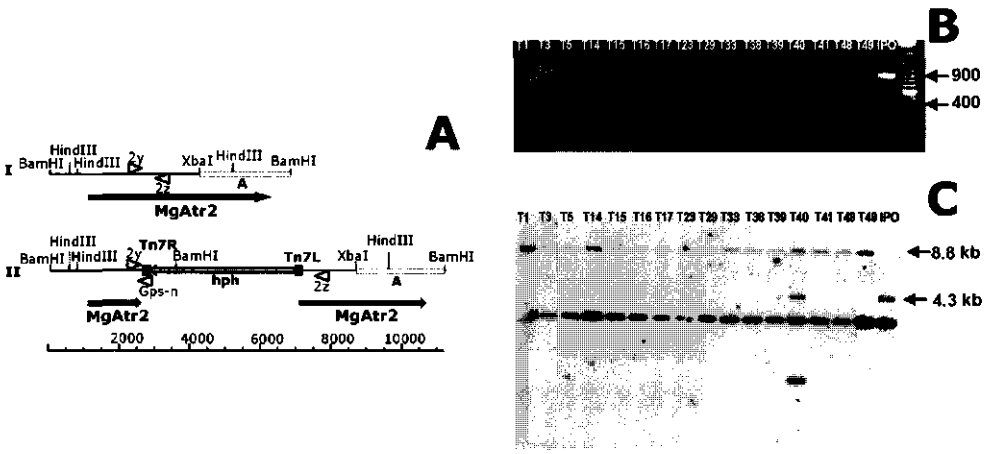

FIG. 3A-C. Analysis of *M. graminicola* transformants obtained after *A. tumefaciens*-mediated transformation. **A** Schematic representation of; *I* the wild type *MgAtr2* locus and *II* the disrupted locus of *MgAtr2*. *Box labelled A* indicates the probe used in Southern analysis. *Arrows labelled 2y, 2z and GPS-n* indicate the primers used in PCR-screening of transformants. **B** PCR screen of 16 selected transformants *(T1-T49)* and the recipient isolate IPO323 *(IPO)*, using primers 2y, 2z and GPS-n. *Arrows* indicate the wild-type 900-bp band and the 400-bp disruption band. **C** Southern blot analysis performed on the wild type strain IPO323 used for transformation and 16 independent transformants obtained after *A. tumefaciens*-mediated transformation. Genomic DNA was digested with *Hind*III and hybridised with probe A.

should be absent from the wild type. The PCR screen was designed to identify isolates lacking the 900-bp wild-type band, but containing the 400-bp "disruption" band. According to this PCR screen, 36 out of 82 transformants screened were disruptants (~44%). To analyse whether disruptants originated from integration of a single copy, a Southern blot analysis was performed. Genomic DNA was isolated, digested with *Hind*III and hybridised with probe A (Fig 3A). This digestion enables the discrimination between single copy disruptants and disruptants containing additional ectopic copies of the introduced construct (Fig. 3C). In the wild type the probe hybridises with a 4.3-kb fragment and a fragment of unknown size, of at least 1.6 kb. In disruptants the latter band is also present but the 4.3-kb fragment is shifted to 8.8 kb. Finally, in transformants containing an ectopic integration, hybridisation occurs with all three of these bands and with an additional band of unknown size. The analysis was performed on 16 transformants of which, based upon the PCR screen, 15 were putative disruptants and the remaining one (T40) contained an ectopic integration of the T-DNA (Fig 3B). The Southern blot analysis of these strains clearly demonstrates that all putative disruptants are true disruptants. Moreover, all transformants contain only a single copy of the introduced construct (Fig 3C).

Genomic DNA of several transformants, either *MgAtr2* disruptants or mutants containing ectopic integrations, was hybridised with a 980-bp *Xho*I probe from the binary

vector pCGN1589. This probe, containing the left border of the T-DNA, hybridised only to DNA of transformants with ectopic integrations (T2 and T40) and did not hybridise to DNA of *MgAtr2* disruptants (Fig. 4).

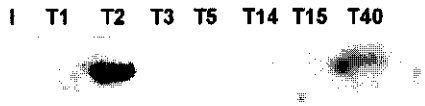

FIG. 4. Total genomic DNA of *M. graminicola* transformants obtained after *A. tumefaciens*-mediated transformation hybridised with a 980-bp *Xho*I probe containing the left border of the T-DNA. *I* Recipient wild-type strain IPO323, *T2* and *T40* contain ectopic integrations, *T1, T3 , T5, T14* and *T15* are *MgAtr2* disruptants.

DISCUSSION

We have shown that protoplasts and intact cells of *M. graminicola* can be transformed using *A. tumefaciens,* according to the protocol described by (De Groot et al. 1998) and that this transformation can result in highly efficient targeted integration. The efficiency of *A. tumefaciens*-mediated transformation of *M. graminicola* to hygromycin resistance using pUR5750 was about 5-7 transformants per 10^7 cells or protoplasts. This transformation frequency is considerably lower, as compared with *A. awamori* (300-7200 transformants per 10^7 protoplasts) but is in the same order as found for *Agaricus bisporus*, *Aspergillus niger* and *Fusarium venenatum* transformed to hygromycin resistance using *A. tumefaciens* (De Groot et al. 1998). Probably, the transformation efficiency can be optimised by adjusting the *A. tumefaciens* transformation system with respect to the bacterial strain used, the duration of co-cultivation, and the density of *Agrobacterium* cells (Chen et al. 2000; Mullins et al. 2001). We did not investigate these variables, since the efficiency of the system was already high enough to detect ample disruptants containing a single copy of the introduced DNA.

Two independent transformation experiments demonstrated that the *A. tumefaciens*-mediated transformation using pCGNΔAtr2 was about 50 transformants per 10^7 cells. Using this disruption construct, not only did the transformation efficiency increase, but the transformants also developed faster, as compared with pUR5750 (10-14 days vs 18-21 days). Similar differences between constructs with and without homologous DNA were found in ten protoplasts-PEG based transformations performed in our laboratory. In these experiments, transformation with pAN7-1 lacking homologous DNA, yielded 47 transformants with transformation frequencies of 0.5–2.0 transformants μg^{-1} DNA, while constructs containing homologous DNA yielded 199 transformants, with frequencies of 0–10 transformants μg^{-1} DNA. However, transformation never resulted in the generation of *MgAtr2* disruption mutants

(data not shown). These results suggest that the presence of homologous DNA-sequences in exogenous supplied DNA significantly influences the transformation frequency of *M. graminicola*. More research is needed to strengthen this hypothesis. The same phenomenon has been observed in *A. tumefaciens*-mediated transformation of *S. cerevisiae* and in protoplast-PEG based transformation of *Fusarium fujikori* (Bundock and Hooykaas 1996; Fernandez Martin et al. 2000).

Although in *M. graminicola* the mere presence of homologous DNA appears to have a positive effect on the transformation efficiency, it is not sufficient to achieve homologous recombination. In *M. graminicola* and other filamentous fungi, like *Aspergillus nidulans* (Bird and Bradshaw 1997), homologous recombination seems to be dependent on the size of the homologous sequences present. In the present study, the use of pCGNΔAtr2 with 2.6 kb and 4.1 kb of homologous DNA did result in homologous recombination. In previous studies, the use of constructs containing homologous flanking regions up to 2.2 kb never led to successful disruption of *MgAtr2*, neither with protoplast-PEG-based transformation (ten experiments) nor with *A. tumefaciens*-mediated transformation (two experiments; unpublished results). These results suggest that relatively long flanking regions are needed for successful homologous recombination. This is in contrast with the situation in *Candida albicans, F. fujikori* and *S. cerevisiae*, where constructs containing homologous DNA favour homologous recombination independent of the sequence, size and copy number of the fragments used (Fernandez Martin et al. 2000).

The DNA of disruptants and transformants containing an ectopic integration was hybridised with a probe containing the left border of the T-DNA. Hybridisation only occurred with transformants containing an ectopic integration. This indicates that, in those transformants, illegitimate recombination through the T-DNA border has occurred. Thus, the mechanism of recombination in *M. graminicola* resembles the mechanism in *S. cerevisiae* (Bundock and Hooykaas 1996).

Until now, homologous recombination using *A. tumefaciens*-mediated transformation has only been described for *A. awamori, K. lactis* and *S. cerevisiae*, but not for a filamentous fungus where targeted integration by conventional methods seems to be difficult. The transformation of *M. graminicola* with pCGNΔAtr2 resulted in the efficient isolation of hygromycin-resistant colonies. Moreover, the generation of ΔAtr2 mutants was fairly efficient, as 36 out of 82 transformants analysed were disruptants (44%). Southern blot analysis indicated that both *MgAtr2* disruptants and mutants with an ectopic integration

contained a single copy of the introduced construct. The high efficiency of targeted integration is not gene-specific, as *Agrobacterium. tumefaciens*-mediated transformation also resulted in the disruption of two additional *M. graminicola* ABC transporter genes (data not shown).

The construct used to obtain the *MgAtr2* disruptants was made using an *in vitro* transposition reaction. This strategy was chosen because, in principle, it allows an easy generation of insertion mutants without elaborate cloning steps. Despite the relatively low efficiency of transposition into *MgAtr2* (2-3%), recombinant plasmids were easily isolated, as large numbers of colonies can simply be screened for the presence of the introduced gene, in our case the hygromycin-resistance gene. The use of large transposon targets, like cosmids, should result in long homologous DNA stretches flanking the insert, thereby favouring homologous recombination. This has already been demonstrated in *Magnaporthe grisea*, where a similar *in vitro* transposition technique (TAG-KO™) was used to generate mutants (Tanzer and Adachi 1999).

In summary, *A. tumefaciens*-mediated transformation of *M. graminicola* is very feasible. Not only protoplasts but also intact cells can be transformed. Moreover, the generation of disruptants by targeted integration is efficient and the vast majority of the transformants originate from the integration of a single copy of the transforming DNA. These results not only make *A. tumefaciens*-mediated transformation an ideal tool for targeted gene disruption, but should also enable the development of insertional mutagenesis in *M. graminicola*.

MATERIALS AND METHODS

Fungal culture

In all experiments, *M. graminicola* isolate IPO323 was used. IPO323 yeast-like cells were grown in liquid yeast-sucrose medium (YSM; 10 g yeast extract l^{-1}, 10 g sucrose l^{-1}) on a lateral shaker (18 °C, 140 rpm). After 3 days of growth, cells were plated on solid V8 agar and incubated for an additional 4 days at 18°C and subsequently harvested.

Protoplast preparation

Cells grown on solid V8 agar were harvested in 10 ml sterile water and centrifuged (4000*g*, 10 min). After an additional wash in sterile water, protoplast preparation was performed as described by (Payne et al. 1998).

Plasmids and bacterial strains

DH5α was used for maintenance of plasmids in *Escherichia coli*. *A. tumefaciens* strain LBA1100 was used for maintenance of constructs and for the *A. tumefaciens*-mediated transformation (Beijersbergen et al. 1992). The vector pUR5750, (De Groot et al. 1998), containing the hygromycin resistance gene under the control of the *Aspergillus nidulans gpd* promoter and *trp*C terminator (Punt et al. 1987), was used to transform *M. graminicola* yeast-like cells and protoplasts to hygromycin resistance. The *MgAtr2* disruption construct was made in the binary vector pCGN1589 (McBride and Summerfelt 1990).

Plasmid construction

A disruption construct for *MgAtr2* was made using the GPS™-Mutagenesis system (New England Biolabs). This system enables the insertion of a transposable element into target (plasmid) DNA (Fig 1). The transprimer donor supplied with the kit, pGPS3, carries kanamycin resistance. A customised transprimer donor was made by replacing the kanamycin-resistance cassette by a hygromycin-resistance cassette. pAnH2-5, a pAN7.1 derivative (Van de Rhee et al. 1996), was digested with *Eco*RI/*Xba*I and the excised hygromycin resistance cassette was cloned in pBluescript II SK (Stratagene). Subsequently, the hygromycin-resistance cassette was excised with *Eco*RV/*Not*I and inserted into pGPS3 digested with *Eco*RV/*Not*I. The resulting transprimer donor vector was labelled pGPS3hyg.

The target plasmid pCGNAtr2 was constructed by the insertion of a 6.7-kb *Bam*HI fragment containing the entire *MgAtr2* into pCGN1589 digested with *Bam*HI. The transprimer donor vector, pGPS3hyg, and the target plasmid pCGNAtr2 were used in a transposition reaction, according to the manufacturer's instructions. pCGNAtr2 plasmids containing an insertion of the hygromycin cassette were screened by colony hybridisation, using the hygromycin resistance gene as probe. Hybridising plasmids were examined by restriction analysis to determine whether the insertion of the hygromycin cassette had occurred in the *MgAtr2* insert of pCGNAtr2 or in the vector backbone. The exact position of the insertion was determined by sequencing, using primers supplied with the GPS-M kit (GPS-N and GPS-S). The vector containing an insertion in *MgAtr2* was labelled pCGNΔAtr2. Finally, pCGNΔAtr2 was transformed into *A. tumefaciens* LBA1100 by electroporation.

DNA manipulations

Basic DNA manipulations were performed according to standard procedures (Sambrook et al. 1989). Fungal genomic DNA was isolated from freeze-dried material using the DNAzol™ reagent (Life-Technologies). DNA was digested with several restriction enzymes, separated on agarose gels and transferred to Hybond-N$^+$ nylon membranes (Amersham), according to the manufacturer's instructions. Hybridisations were performed at 65°C. Blots were hybridised with [α-^{32}P]dATP-labeled probes and treated as described by (Church and Gilbert 1984). Stringency of the final washings was 0.1 x SSC. Plasmid DNA was purified on Qiagen-columns.

A PCR assay was performed to select putative *MgAtr2* disruptants. Cells scraped from colonies growing on plates were transferred to a 1.5-ml Eppendorf tube and microwaved for 2 minutes at maximum power. After centrifugation, cells were resuspended in 50 µl of TE (10 mM Tris-HCl, pH 7.4, 1 mM EDTA, pH 8.0) and microwaved for an additional 2 minutes. Cell debris was spun down and 10 µl of supernatant was used in a PCR reaction. PCR was performed in a Cetus DNA Thermal Cycler 480 (Perkin Elmer). Amplification reaction volumes were 100 µl, containing 200 µM each of dATP, dCTP, dGTP and dTTP, 1 µM of each primer, 0.5 U of

AmpliTaq DNA polymerase (Perkin Elmer) and a reaction buffer (10 mM Tris-HCl, pH 8.3, 50 mM KCl, 4 mM $MgCl_2$). The DNA was initially denatured for 4 min at 94 °C followed by 30 cycles of 45 seconds at 94 °C, 1 minute at 56 °C and 1.5 minute at 72 °C. Primers used were GPS-N, Atr2y (GTGATTGTGCTCTACGAG) and Atr2z (GAAGATGAAAAAGAAGAAAGC).

Agrobacterium tumefaciens-mediated transformation

Transformation was performed as described (Bundock et al. 1995; De Groot et al. 1998). A. tumefaciens LBA1100 containing pUR5750 was cultured in A. tumefaciens minimal medium (10 mM K_2HPO_4, 10 mM KH_2PO_4, 2.5 mM NaCl, 2 mM $MgSO_4$, 0.7 mM $CaCl_2$, 9 µM $FeSO_4$, 4 mM $(NH_4)SO_4$, 10 mM glucose) supplemented with 250 µg spectinomycin ml^{-1} and 100 µg kanamycin ml^{-1}. A. tumefaciens LBA1100 containing pCGNΔAtr2 was grown overnight in minimal medium amended with 250 µg spectinomycin ml^{-1} and 10 µg gentamicin ml^{-1}. Prior to co-cultivation of A. tumefaciens with protoplasts or yeast-like cells of M. graminicola, A. tumefaciens cells were diluted to an optical density at 660 nm (OD_{660}) of 0.15 in induction-medium (minimal medium, 40 mM MES pH 5.3, 0.5% glycerol (w/v), 200 µM acetosyringone) and cultured until an OD_{660} of 0.25 was reached. Subsequently, 100 µl of fungal protoplasts or cells at a density of 10^5, 10^6, 10^7 or 10^8 ml^{-1} were mixed with 100 µl of A. tumefaciens culture. Mixtures were plated on nitrocellulose filters placed on induction medium with or without 200 µM acetosyringone and incubated for 2 days at room temperature. Filters were transferred to *Aspergillus* minimal medium amended with 200 µM cefotaxime and 100 µg hygromycin ml^{-1}. Colonies appearing after incubation at 18°C were transferred to potato dextrose agar (PDA) with 100 µg hygromycin ml^{-1} and incubated at 18°C. After subculturing twice on PDA amended with 100 µg hygromycin ml^{-1} the stability of the hygromycin resistance was tested by subculturing twice on non-selective PDA and twice in non selective liquid medium (YSM). Subsequently, transformants were again tested for hygromycin resistance.

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CHAPTER 4

ABC transporters of the wheat pathogen *Mycosphaerella graminicola* and virulence

To be submitted in modified form.

ABSTRACT

The role of the ABC transporter genes *MgAtr1-5* from *Mycosphaerella graminicola* in virulence on wheat was analysed with knockout mutants. Knockout mutants were generated either through protoplast-PEG or *Agrobacterium tumefaciens*-mediated transformation. Virulence was tested on the susceptible wheat cultivar Obelisk. All transformants, showed an unaltered phenotype with respect to the onset of symptom development. After prolonged incubation seedlings inoculated with *MgAtr4* disruptants displayed significantly less necrotic tissue than plants infected by control isolates or the other transformants. These results suggest that MgAtr4 acts as a virulence factor of *M. graminicola* during pathogenesis on wheat. However, the mechanism of action of the transporter and the nature of the transported substrate remains to be elucidated.

INTRODUCTION

Septoria tritici leaf blotch caused by the fungus *Mycosphaerella graminicola* (anamorph *Septoria tritici*) is generally recognised as one of the major wheat diseases. Despite its economic importance not much is known about the molecular mechanisms underlying this disease. Most research on *M. graminicola* has been focussed on the epidemiology and to a lesser extent on the histology of the infection process (Kema et al. 1996; McDonald et al. 1995; Rohel et al. 2001). The characterisation of genes involved in the establishment and progress of the disease has been hampered by the lack of molecular and physiological tools. Only recently tools for crosses and efficient transformation came available (Kema et al. 1996; Zwiers and De Waard 2001). Classical genetics and AFLP analysis has led to the mapping of a single locus controlling avirulence (Kema et al. 2000). So far, no specific avirulence or virulence gene has been cloned from *M. graminicola*.

We are interested in the role of ATP-binding cassette (ABC) transporters in the wheat-*M. graminicola* interaction. ABC transporters are membrane-bound transporters, known for their role in multidrug resistance (MDR) in human tumour cells (Juliano and Ling 1976). They are members of a large protein superfamily of transporters present in both prokaryotic and eukaryotic organisms (Higgins 2001). In plant pathogenic fungi ABC transporters have been implicated to act as virulence factors by providing protection against plant defence compounds produced by the host upon infection or by secretion of plant toxins (De Waard 1997; Del Sorbo et al. 2000). Recently, such a role of ABC transporters has been

demonstrated for the plant pathogenic fungi *Magnaporte grisea* and *Botrytis cinerea* (Schoonbeek et al. 2001; Urban et al. 1999). Furthermore, ABC transporters can be associated with more specific functions such as the secretion of mating factor in *Saccharomyces cerevisiae* and *Schizosaccharomyces pombe* (Christensen et al. 1997; McGrath and Varshavsky 1989).

In this chapter, the generation of knockout mutants for each of the five ABC transporter encoding genes (*MgAtr1-5*) cloned from *M. graminicola* (Zwiers and De Waard 2000; Stergiopoulos et al 2002) and the analysis of their role in virulence on wheat is described.

RESULTS

Disruption and replacement

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 2 out of 86 (2.3%) *MgAtr3* transformants lacked the *MgAtr3* coding sequence and did not contain ectopically-integrated copies. This was comparable for *MgAtr5* where in 2 out of 36 transformants (5.6%) the coding region was replaced. Therefore, disruption of *MgAtr1*, *MgAtr2* and *MgAtr4* was performed using *A. tumefaciens*-mediated transformation. Analysis of eight putative *MgAtr1* disruptants revealed that two transformants were indeed 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; Chapter 3). The disruption of *MgAtr4* was even more successful 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 *MgAtr1-5* loci in the *M. graminicola* knockout mutants are shown in figure 1. None of the transformants showed an altered growth rate in YSM as compared to the wild type

recipient isolate I323 or the control transformant Sp2. Under the conditions tested, no morphological differences could be observed.

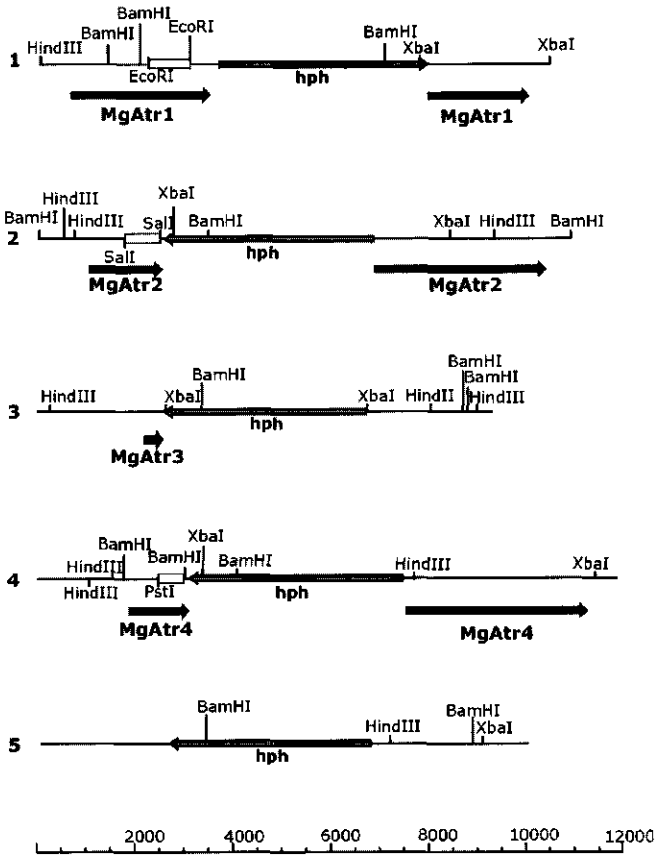

FIG. 1. Schematic representation of *MgAtr1-5* loci in *Mycosphaerella graminicola* transformants. Knockout mutants of *MgAtr1* (1), *MgAtr2* (2), and *MgAtr4* (4) were generated by gene disruption and of *MgAtr3* (3) and *MgAtr5* (5) by gene replacement. Remaining parts of *MgAtr1-5* coding regions 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.

Expression analysis

The expression of the 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 or the steroid hormone progesterone, all known inducers of *M. graminicola* ABC transporter genes (Zwiers and De Waard 2000; Chapter 2). As anticipated, 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 (Fig. 2A). The analyses could not confirm the deletion of *MgAtr3* as in the wild-type isolate *MgAtr3* expression was undetectable under all conditions tested.

The expression analysis demonstrated that the basal level expression of *MgAtr1* was upregulated in *MgAtr4* disruptants (Fig. 2B). Such an alteration in expression was not observed in any of the other knockout mutants.

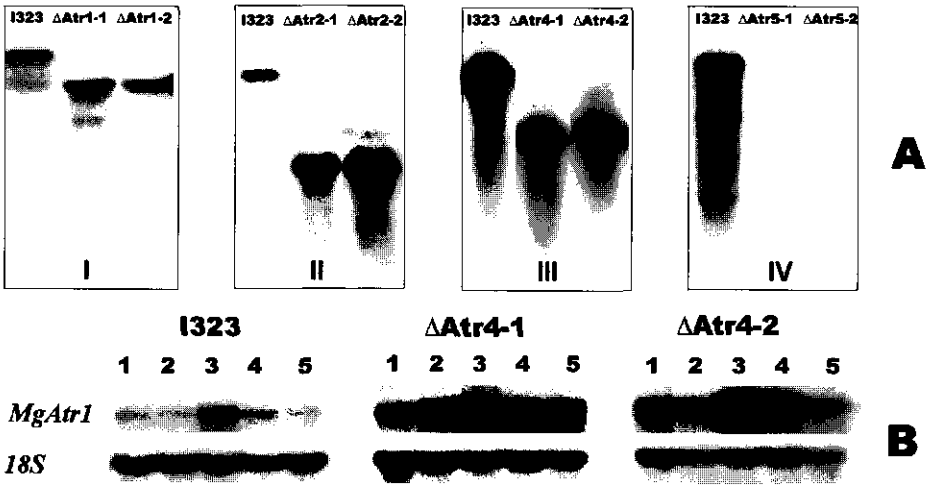

FIG 2.A-B Expression analyses of *M. graminicola* transformants.
A. *MgAtr1*, *MgAtr2*, *MgAtr4* and *MgAtr5* expression in transformants containing a disrupted *MgAtr1* (I), *MgAtr2* (II) or *MgAtr4* (III) allele or a deleted *MgAtr5* allele (IV), respectively. In all panels expression in the wild-type recipient isolate I323 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 *Mycosphaerella graminicola*. I323 is the wild-type recipient isolate and ΔAtr4-1 and ΔAtr4-2 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).

Virulence assay

All isolates tested incited the appearance of the first symptoms (small chlorotic spots) eight to nine days after inoculation. In time, these chlorotic lesions expanded longitudinally and became necrotic. Inside the necrotic tissue pycnidia developed. The spread of the lesions and the final necrotic leaf-area coverage was the same for all isolates (transformants and controls) except for the *MgAtr4* disruptants. For this mutant the spread of the necrotic spots was considerably delayed. Fifteen days after inoculation, lesions originating from control isolates

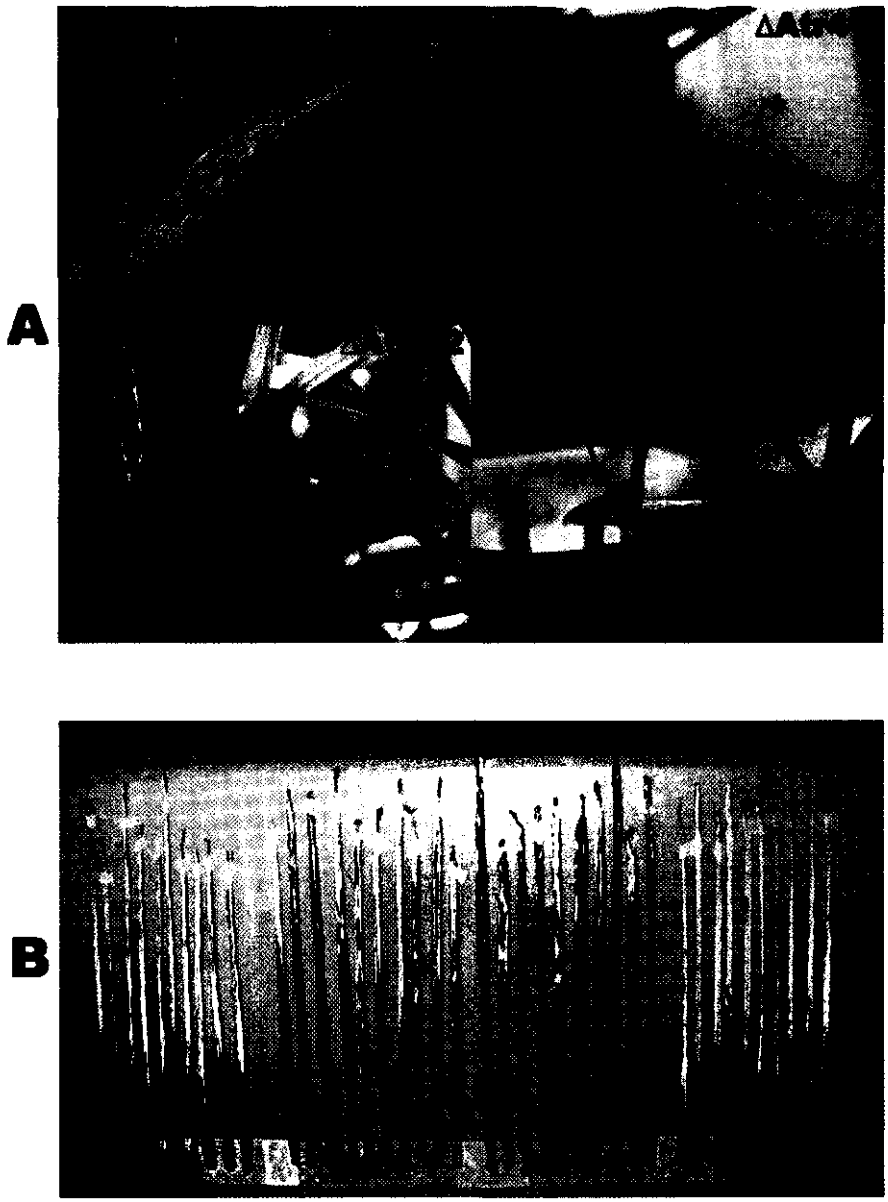

FIG. 3. Effect of disruption of *MgAtr4* on virulence of *Mycosphaerella graminicola* on wheat cultivar Obelisk. (A) Disease development 15 days post inoculation (dpi). (B) Disease development 21 dpi. I323 is the wild-type recipient isolate, ΔAtr4-1 and ΔAtr4-2 are two independent *MgAtr4* disruption mutants, Sp2 is a control transformant containing a single copy of the hygromycin-resistance cassette only. Leaf tissue above dotted line was inoculated.

still increased in size whereas the lesions caused by the *MgAtr4* disruptants remained restricted in size. Twenty-one days after inoculation leaves from plants inoculated with *MgAtr4* disruptants still showed a large unaffected area while control plants were completely necrotic (Fig. 3). None of the transformants was impaired with respect to pycnidia formation. Also the small necrotic lesions caused by the *MgAtr4*-disruptants showed ample pycnidia. Pycnidiospores of transformants were isolated and all grew well on PDA amended with hygromycin.

DISCUSSION

This study describes the effect of disruption or deletion of five different ABC transporter genes from the wheat pathogen *M. graminicola* on virulence on wheat. As far as we know this is the most extensive analysis of the potential role of ABC transporters from a plant pathogenic fungus in virulence. We show that mutants containing a disrupted allele of *MgAtr4* exhibit a significant delay in symptom development on wheat, suggesting that *MgAtr4* acts as a virulence factor in the wheat-*M. graminicola* interaction. So far, fungal ABC transporters acting as virulence factors have only been described for the plant pathogens *M. grisea* and *B. cinerea* (Schoonbeek et al. 2001; Urban et al. 1999).

The delay in symptom development observed for the *MgAtr4* disruptants is not caused by a defect in the infection process itself as the onset of symptom development was the same for all isolates tested. The delay can be explained by assuming that MgAtr4 protects the invading fungus against plant defence compounds or facilitates secretion of a virulence factor such as a host-specific toxin. The loss of virulence of *MgABC1* mutants from *M. grisea* is ascribed to the protective action of MgABC1 against a yet-unidentified plant compound (Urban et al. 1999). *B. cinerea BcAtrB* mutants show a reduction in virulence on grapevine leaves that correlates with an increased *in vitro* sensitivity to the grapevine phytoalexin resveratrol (Schoonbeek et al. 2001). Wheat is known to produce compounds with antifungal activity such as the hydroxamic acid 2,4-dihydroxy-7-methoxy-2*H*-1,4-benzoxazin-3(4*H*)-one (DIMBOA), and resorcinol derivatives (Seitz 1992; Weibull and Niemeyer 1995). The sensitivity of *MgAtr4* disruptants to these compounds should be tested to validate this hypothesis. This has been done for resorcinol but *MgAtr4* disruptants did not exhibit an alteration in sensitivity to this compound (Chapter 5). The observation that the initiation of

symptom development is not affected can be explained by assuming that production of plant defence compounds does not occur in early stages of infection.

Another explanation for the delay in symptom development might be that disruption of *MgAtr4* leads to a retardation of growth, which is not observable upon growth of the disruptants in media like YS and PDA. These media are relatively rich in nutrients. In contrast, the apoplast of wheat leaves, the site colonised by *M. graminicola*, is poor in nutrients.

ABC transporters are members of a large protein superfamily. Sequencing of the genome of *S. cerevisiae* revealed the presence of 29 ABC transporters whereas the recent sequencing of the genome of the corn pathogen *Cochliobolus heterostrophus* indicated the presence of 51 putative ABC transporters (Decottignies and Goffeau 1997; Yoder and Turgeon 2001). It is known that a large overlap in substrate specificity can exist between ABC transporters (Kolaczkowski et al. 1998). Moreover, disruption of one specific ABC transporter can lead to the activation of other ABC transporters with an overlap in substrate specificity (Decottignies et al. 1995). Therefore, we also determined the effect of disruption of an ABC transporter gene on expression of the other ABC transporter genes. Such an approach has not yet been described for any other plant pathogenic fungus. In mutants containing a disrupted *MgAtr4* allele, upregulation of *MgAtr1* expression was observed, while in none of the other knockout mutants an alteration in expression of any of the other ABC transporter genes was detected. Therefore, it might be argued that the observed decrease in virulence by the *MgAtr4* disruptant is not caused by the absence of *MgAtr4* but by the upregulation of *MgAtr1*. In this view, MgAtr1 might secrete a compound acting as an avirulence factor that triggers plant defence responses. However, we expect that the observed phenotype most likely is caused by the disruption of *MgAtr4* itself. MgAtr4 is homologous with MgABC1 (AF032443) (44% identity, 60% similarity) suggesting a similar function for MgAtr4 in the wheat-*M. graminicola* interaction. In addition, *MgAtr1* disruptants did not show any alterations with respect to virulence. Definite proof for the involvement of either gene in virulence might come from the disruption of *MgAtr1* in a *MgAtr4* deletion background, and vice versa.

In summary, our results indicate that the ABC transporter *MgAtr4* plays a role in the wheat-*M. graminicola* interaction. Its precise function and the nature of the transported substrate remain to be elucidated.

MATERIALS AND METHODS

Fungal culture

M. graminicola IPO323 was used as standard isolate in all experiments (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) at 18°C. The growth rate of isolates was monitored by counting cell density during one week of growth in 100 ml of YSM. Media were inoculated with 1 x 10^5 cells in duplo.

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 (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 for MgAtr1, a 750 bp *Sal*I fragment for *MgAtr2*, an 800 bp *Sal*I fragment for *MgAtr3*, an 480 bp *Bam*HI/ *Pst*I fragment from *MgAtr4* and a 600 bp *Eco*RI fragment from *MgAtr5* (Figure 1).

Plasmid construction

Disruption constructs of *MgAtr1* (AJ243112) and *MgAtr4* (AF329852) were made using the GPS™-Mutagenesis system (New England Biolabs) using the approach described for the construction of a *MgAtr2* (AJ243113) disruption construct (Zwiers and De Waard 2001).

Target plasmid pCGNAtr1 was constructed by the insertion of a 5.9 kb *Hin*dIII/ *Xba*I fragment containing the entire *MgAtr1* into pCGN1589 digested with *Hin*dIII/*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* (AF364105) 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* (AF364104) 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**

Agrobacterium 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 (Payne et al. 1998) with the following exceptions. Instead of dimethylsulfoxide (DMSO), 28 µl of PTC solution (40% (w/v) PEG3350, 50mM Tris-HCl, pH 8.0, 50 mM $CaCl_2$) was added with 5 µl of linearized DNA (1 µg μl^{-1}).

Virulence assay

M. graminicola strains used in the virulence assays were the wild-type recipient isolate I323, a control transformant containing a single copy of the hygromycin-resistance cassette, and the disruption and deletion transformants. For each gene two independent disruption or deletion transformants were used and virulence assays were performed at least three times. *M. graminicola* yeast-like cells grown in YSM were harvested by centrifugation at 3000 *g* for 10 minutes. Pellets were washed once and suspended in 0.15% Tween-20 at a density of 10^7 cells ml^{-1}. Suspensions were sprayed onto 7-9-day-old seedlings from the susceptible wheat variety Obelisk till run-off. Inoculated plants were placed in sealed perspex-lidded containers at 18 °C. Emerging second leaves were clipped in order to facilitate disease assessment and light penetration to the inoculated flag leaf. Virulence was visually assessed by scoring the development of necrotic spots and formation of pycnidia.

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CHAPTER 5

ABC transporters of *Mycosphaerella graminicola* function as protectants against biotic and xenobiotic toxic compounds

To be submitted in modified form.

ABSTRACT

The role in multidrug resistance (MDR) of five ABC transporter genes (*MgAtr1-5*) from the wheat pathogen *Mycosphaerella graminicola* was studied. This was done by complementation of *Saccharomyces cerevisiae* mutants 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 provide protection against chemically unrelated compounds indicating that these ABC transporters function as multidrug transporters with distinct but overlapping substrate specificity. Their substrate range includes fungicides (cyproconazole, propiconazole and tebuconazole), plant metabolites (berberine and camptothecin), and a mycotoxin from *Fusarium graminearum* (diacetoxyscirpenol). *MgAtr5* deletion mutants of *M. graminicola* showed a small increase in sensitivity to the putative wheat defence compound resorcinol, suggesting a role for this transporter in the wheat-*M. graminicola* interaction. Bioassays with antagonistic bacteria indicated that ABC transporters of *M. graminicola* can provide protection against phenylpyrrole antibiotics produced by *Pseudomonas fluorescens* and *Burkholderia cepacia*. Therefore, our results suggest that ABC transporters from *M. graminicola* play a role in protection against toxic compounds from natural and artificial origin.

INTRODUCTION

ATP-binding cassette (ABC) transporters or traffic ATPases are membrane-bound transporters present in both prokaryotic and eukaryotic organisms that drive the uptake or efflux of compounds by the hydrolysis of ATP (Higgins 2001). ABC transporters are able to prevent intracellular accumulation of toxic compounds by extrusion and thus provide cells protection against deleterious effects of toxicants. ABC transporters can also be involved in the establishment of resistance in drug-based treatment of infectious diseases (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 called multidrug resistance (MDR). MDR can be caused by overproduction of ABC transporters and has also been described for the yeasts *Candida albicans*, *Saccaromyces cerevisiae* and *Schizosaccharomyces pombe* (Kolaczkowski et al. 1998; Nishi et al. 1992). The successful control of filamentous fungi pathogenic to plants and mammals with

fungicides and antimycotics, is threatened by the development of MDR. ABC transporters from *Aspergillus nidulans*, *Aspergillus fumigatus*, *Aspergillus flavus*, *Botrytis cinerea* and *Penicillium digitatum* have been implicated in resistance against antifungal compounds (Andrade et al. 2000; Nakaune et al. 1998; Tobin et al. 1997; Vermeulen et al. 2001).

Besides acting in a general detoxification mechanism against hydrophobic toxicants, ABC transporters might 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 transporting membrane constituents to the outer leaflet of the lipid bilayer and extruding 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 of host plants or by secretion of plant toxins (De Waard 1997; Del Sorbo et al. 2000). These functions have now been demonstrated for *Magnaporte grisea*, *B. cinerea* and *Mycosphaerella graminicola* (Schoonbeek et al. 2001; Urban et al. 1999, Chapter 4).

We are interested in the role of ABC transporters in the wheat-*M. graminicola* interaction. *M. graminicola*, the causal agent of septoria tritici leaf blotch of wheat, is a major pathogen on this crop. In this paper we describe the functional complementation of *S. cerevisiae* mutants with the ABC transporter genes *MgAtr1-5* from *M. graminicola* in order to identify potential natural substrates of these transporters. Furthermore, the sensitivity of individual *M. graminicola* knockout mutants was tested to natural toxic compounds, xenobiotics and antibiotic producing bacteria.

RESULTS

Complementation of *S. cerevisiae*

Full length clones from *MgAtr1*, *MgAtr2*, *MgAtr4* and *MgAtr5* were constructed and cloned in the yeast expression vector pYES2. The generation of a full-length cDNA from *MgAtr3* was not successful. Clones were transformed to *S. cerevisiae* AD12345678 and the sensitivity of the yeast transformants was tested against 45 compounds of either natural or synthetic origin (Table 2). Most compounds tested were non-toxic to *S. cerevisiae* or displayed no difference in toxicity between the control recipient strains and the different ABC transporter gene-

expressing yeast transformants. However, ten compounds showed a differential activity to some of the transformants tested (Table 1). These compounds included fungicides, plant metabolites, 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 compounds.

Table 1. 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	+	-	+	-
Rhodamine6G	-	+	+	-

[a] Increase in resistance as compared to pYES2 transformed control strain.
[b] No difference in sensitivity as compared to pYES2 transformed control strain.

MgAtr1 was the only transporter that provided decreased sensitivity to cycloheximide. 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 (Fig.1). For instance, MgAtr1, MgAtr2 and MgAtr4 were all capable of providing protection against azole fungicides. Yeast transformed with *MgAtr1* and *MgAtr4* survived at 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 the 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 Fig. 1.

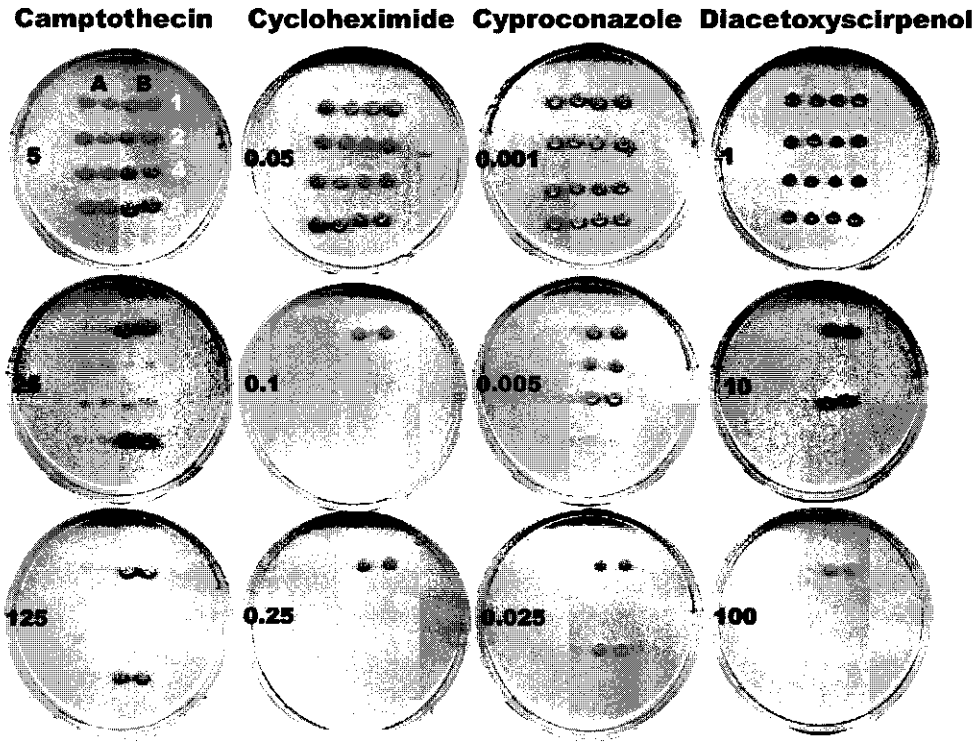

FIG. 1. Effect of heterologous expression of MgAtr1, MgAtr2, MgAtr4 or MgAtr5 from *Mycosphaerella graminicola* in *Saccharomyces cerevisiae* AD12345678 on sensitivity to camptothecin, cycloheximide, cyproconazole, or diacetoxyscirpenol. Each row (1, 2, 4, 5) displays two independent *S. cerevisiae* 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}.

Toxicity assays with *M. graminicola* knockout mutants

Nineteen compounds were tested for their activity against growth of *M. graminicola* transformants containing a disrupted or deleted allele of *MgAtr1-5*. Compounds included are listed in Table 2. The ΔMgAtr5 transformants exhibited a small increase in sensitivity to resorcinol as compared with the wild-type isolate or a hygromycin resistant control transformant (Fig. 2A), suggesting that MgAtr5 protects *M. graminicola* against the toxic activity of this compound. For none of the other tested compounds an increase in sensitivity of the ABC transporter knockout transformants was observed. Surprisingly, *MgAtr2* disruptants showed a decrease in sensitivity to the azole fungicide cyproconazole when plated on PDA amended with this compound (Fig. 2B).

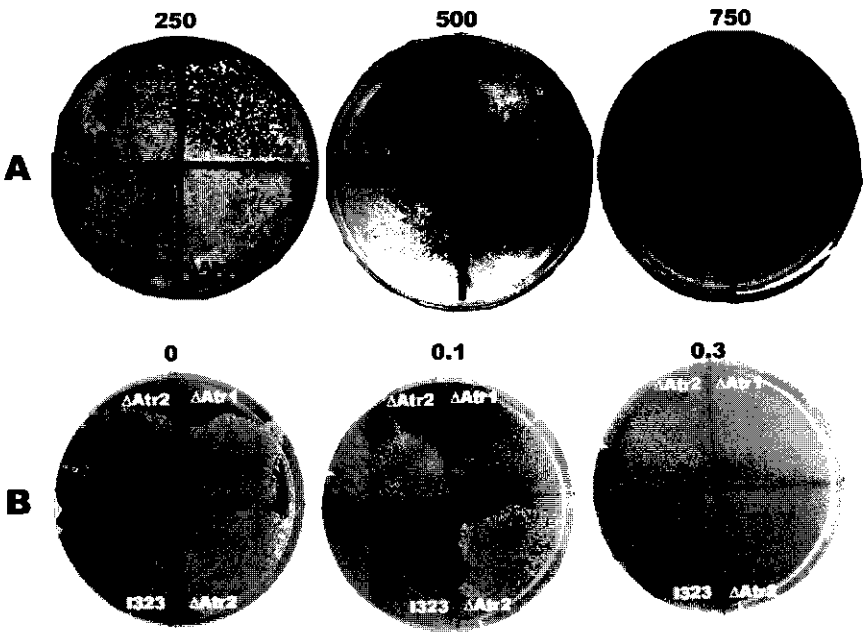

FIG.2 A-B. Toxicity assays with *Mycosphaerella graminicola* knockout mutants.
A. Effect of deletion of *MgAtr5* on the sensitivity of *M. graminicola* to resorcinol. I323 is the recipient wild-type isolate and Sp2 a control transformant containing a single copy of the hygromycin-resistance cassette, ΔAtr5-1 and ΔAtr5-2 represent two independent *MgAtr5* deletion mutants. Cells were plated on PDA containing 250, 500 and 750 mg resorcinol L^{-1}.
B. Effect of disruption of *MgAtr2* on the sensitivity of *M. graminicola* to cyproconazole. I323 is the recipient wild-type isolate, ΔAtr2-1 and ΔAtr2-2 represent two independent *MgAtr2* disruptants, and ΔAtr1 is a *MgAtr1* disruptant. Cells were plated on V8-medium containing 0, 0.1 and 0.3 mg cyproconazole L^{-1}

Antagonistic activity of bacteria

Burkholderia cepacia B37W and *Pseudomonas fluorescens* Pf5, known to produce the phenylpyrrole antibiotics pyrrolnitrin and pyoluteorin were tested for their antagonistic activity against *M. graminicola* transformants in agar diffusion tests. Both strains exhibited a clear antagonistic activity, as distinct inhibition zones were present. In addition, the antagonistic activity of both bacterial strains was higher to *MgAtr2* disruptants than to the other *M. graminicola* isolates tested (Fig. 3). These results indicate that ABC transporters from *M. graminicola* can provide protection against antibiotic compounds produced by bacteria.

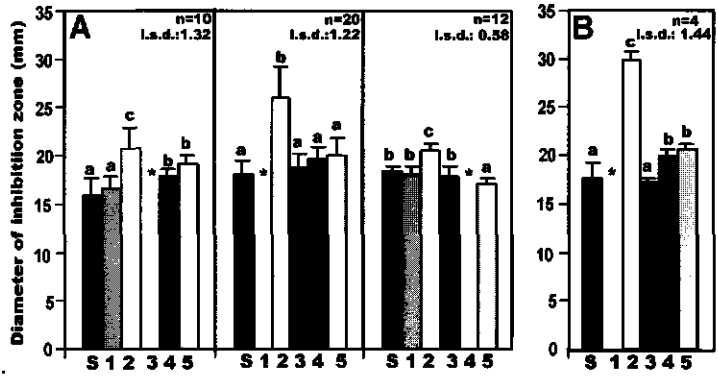

FIG. 3. Antagonistic activity of *Burkholderia cepacia* B37W (A) and *Pseudomonas fluorescens* Pf5 (B) on growth of *Mycosphaerella graminicola* transformants with disrupted or deleted alleles of *MgAtr1-5* in agar diffusion tests. S represents a control transformant containing a single copy of the hygromycin-resistance cassette, and 1, 2, 3, 4, and 5 transformants of *M. graminicola* with disrupted or deleted alleles of *MgAtr1-5*, 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.

DISCUSSION

Heterologous expression of *M. graminicola* ABC transporters in *S. cerevisiae* indicates that the presence of *MgAtr1-5* provides protection against a wide variety of chemically unrelated toxicants. We assume that this protection is based upon active transport of these compounds by the encoded proteins. 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 play a role in determining the intrinsic sensitivity of *M. graminicola* populations to azole fungicides and can be involved in the development of resistance against these compounds.

The *M. graminicola* ABC transporters MgAtr1 and MgAtr5 also accept the plant alkaloids berberine and camptothecin as substrates. This suggests that they might be involved in protection of the fungus against fungitoxic plant defence compounds. However, these two plant alkaloids are not present in wheat but 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 the protection of the fungus against such compounds. Plant metabolites known to be produced by wheat and implicated in protection

against pathogens include hydroxamic acids as 2,4-dihydroxy-7-methoxy-1,4-benzoxazin-3-one (DIMBOA) and (alkyl)resorcinols (Frey et al. 1997; Seitz 1992; Wilkes et al. 1999). These compounds were either not available (DIMBOA) or not toxic to *S. cerevisiae* (resorcinol). Resorcinol (1,3-benzenediol) was the only compound to which one of the ABC transporter knockout mutants (*ΔMgAtr5*) of *M. graminicola* exhibited a decreased sensitivity. Resorcinol is used in the treatment of acne and other skin disorders as eczema and psoriasis. Besides antibacterial action also antifungal activity of (alkyl)resorcinol has been described (Suzuki et al. 1996). Its activity is probably caused by perturbation of the cytoplasmic membrane due to interactions with membrane proteins (Kozubek and Demel 1981). We suggest that MgAtr5 can play a role in protection against this wheat defence compound.

The fact that *ΔMgAtr5* mutants are not impaired in virulence (Chapter 4) can be explained by the presence of other ABC transporters that are capable of transporting resorcinol *in planta*. It is known that ABC transporters are members of a large protein superfamily and can have overlaps in substrate specificity (Decottignies and Goffeau 1997; 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 ABC transporters. This could also explain why *MgAtr2* disruptants exhibit a decreased sensitivity to the azole fungicide cyproconazole. In chapter 4 we have studied the expression pattern of all the *M. graminicola* ABC transporter knockout mutants. In a *MgAtr4* disruption background *MgAtr1* is upregulated, indicating that redundancy of ABC transporters could be operating in *M. graminicola*.

The discrepancy between the substrate range found in the yeast complementation assays (ten substrates) and the sensitivity assays of the *M. graminicola* transformants (one substrate) can also be explained by a redundancy of ABC transporters. 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 (Kaur and Bachhawat 1999; Krishnamurthy and Prasad 1999).

In the yeast complementation assay ergosterol is a substrate for MgAtr1, MgAtr2 and MgAtr4. Ergosterol is the main sterol constituent of fungal membranes This could implicate 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 provides protection against nine out of ten substrates identified for *M. graminicola* ABC transporters in the *S. cerevisiae* complementation assay. Therefore, MgAtr1

Table 2. 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**	**Camptothecine**	Corticosterone	Ergocryptine
Hygromycin	**Fluazinam**	Colchicine	Ergosterol	**4-NQO**
Neomycin	**Fludioxonil**	**Eugenol**	Estradiol	**Rhodamine6G**
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 were tested against *M. graminicola*

Agar diffusion tests

The antagonistic activity of *Pseudomonas fluorescens* Pf5 (producing pyrrolnitrin and pyoluteorin) and *Burkholderia cepacia* B37W (producing pyrrolnitrin and pyoluteorin) (Burkhead et al. 1994; Howell and Stipanovic 1979) 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.

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) and amphotericin B, berberine, camptothecine, 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, rhodamine6G, sitosterol, stigmasterol, testosterone, tomatine, triflupromazine, vinblastine and vincamine (Sigma-Aldrich)

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despite the intensive use of cyproconazole and other azole fungicides, no indications of a decreased sensitivity have been found (Gisi et al. 1997).

Resistance against azole fungicides can be caused by alterations in sterol biosynthesis (Joseph Horne et al. 1995), mutations of the $P450_{14DM}$ target site (Joseph Horne et al. 1995; Sanglard et al. 1998) or increased expression of the $P450_{14DM}$ encoding *CYP51* gene (Brink van den et al. 1996; Hamamoto et al. 2000). Another important resistance mechanism is reduction of the intracellular concentration of the fungicide 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 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 fungicides and unrelated chemicals (Balzi and Goffeau 1995; Sanglard et al. 1995; Andrade et al. 2000).

We are interested in the role of ABC transporters from *M. graminicola* in pathogenesis and fungicide sensitivity. To assess the role of ABC transporters in azole sensitivity of *M. graminicola* we selected laboratory mutants with a decreased sensitivity to the azole fungicide cyproconazole from two genetically independent isolates. All mutants were analysed for their sensitivity to azole fungicides and other chemically unrelated compounds, the accumulation of [^{14}C]cyproconazole, the expression levels of five ABC-transporter genes (*MgAtr1-5*), and the sterol P450 14α-demethylase gene (*CYP51*). *MgAtr1*, which showed overexpression in some of the mutants, was disrupted by *Agrobacterium tumefaciens*-mediated transformation and the transformants were phenotypically characterised. Results presented indicate that in *M. graminicola* besides the upregulation of the ABC transporter gene *MgAtr1*, multiple mechanisms may operate in resistance to azole fungicides.

RESULTS

Sensitivity assays to azole fungicides.

The MIC values of cyproconazole for the field isolates IPO323 and M1 were approximately 0.3 μg ml^{-1}. Both isolates showed a similar cross-sensitivity to the triazoles propiconazole and tebuconazole (data not shown).

To elucidate the potential role of ABC transporters in fungicide sensitivity, mutants of I323 and M1 with a decreased sensitivity to the azole-fungicide cyproconazole were selected.

Mutants were isolated from V8-agar plates amended with cyproconazole at 3 times or 10 times its MIC value. For both isolates the mutation frequency was around 1 x 10^{-4}. The relative resistance of several mutants was determined and ranged between 3 and 6 (Table 1). All mutants showed cross-resistance to propiconazole and tebuconazole (data not shown). Repetitive subculturing of the mutants under non-selective conditions showed that all I323 derived mutants were stable whereas the M1-derived mutants M1A1 and M1D1 lost their resistance to cyproconazole. The sensitivity of the mutants to several chemically unrelated chemicals was determined in order to study whether the mutants would have a MDR phenotype. Indeed, all mutants exhibited a low degree of cross-resistance to cycloheximide and /or rhodamine 6G (Table 1).

Table 1. Relative resistance of laboratory mutants of *Mycosphaerella graminicola* to cyproconazole, cycloheximide and rhodamine 6G.

Isolate	Relative resistance level[a]		
	Cyproconazole	Cycloheximide	Rhodamine 6G
I323	1	1	1
I323A1	4	2	1
I323C1	6	3	2
I323C4	4	2	1
I323E1	6	3	2
M1	1	1	1
M1A1	4[b]	ND	2
M1B1	3	ND	2
M1C1	3	ND	2
M1C4	5	3	6
M1D1	3[b]	ND	2

[a] Ratio between MIC value of mutant and parent isolate. [b] Initial degree of resistance; resistance lost upon subculturing.

Accumulation of [^{14}C]cyproconazole.

The decreased sensitivity of the mutants to cyproconazole could be due to a reduced accumulation of the fungicide. Therefore, the cellular content of accumulated cyproconazole was measured in time (Fig. 1 A-B). [^{14}C]Cyproconazole accumulation of the two wild type isolates IPO323 and M1 did not differ significantly and amounted on average 1 and 0.9 nmol cyproconazole mg^{-1} dry weight, respectively. In all mutants derived from IPO323 the accumulation of [^{14}C]cyproconazole decreased significantly with a factor 3-4 (Fig. 1A). Accumulation by the mutants M1B1 and M1C1, derived from M1, decreased with a factor 2-3. In contrast, accumulation of [^{14}C]cyproconazole by mutant M1C4 was higher than that by the wild-type and increased in time (Fig. 1B). In all isolates tested the accumulation appeared

to be due to an energy-dependent efflux as addition of CCCP increased the accumulation of the fungicide (Fig. 1C).

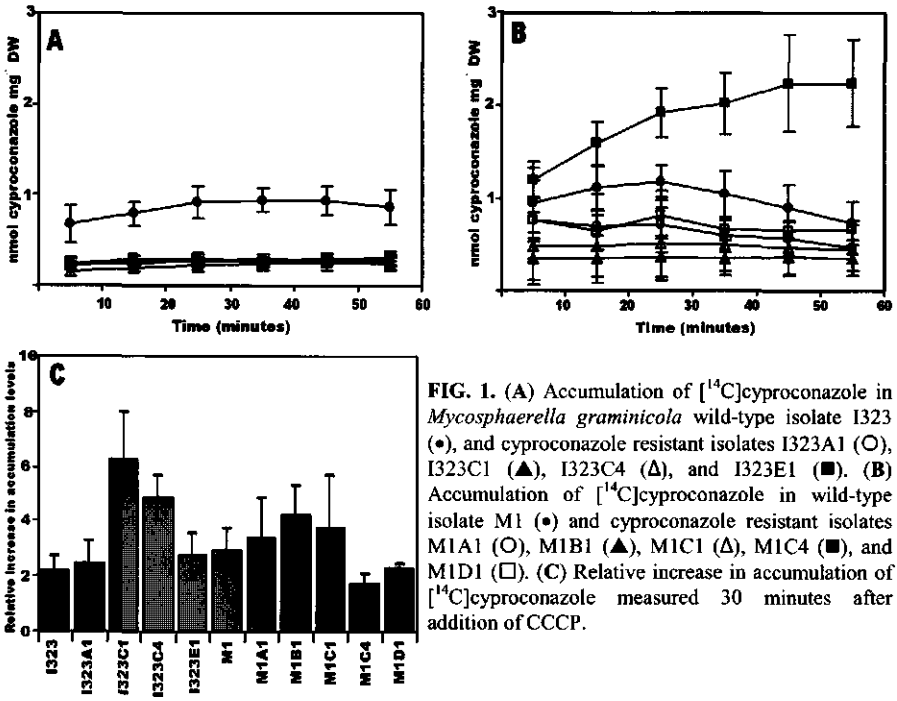

FIG. 1. (**A**) Accumulation of [^{14}C]cyproconazole in *Mycosphaerella graminicola* wild-type isolate I323 (•), and cyproconazole resistant isolates I323A1 (○), I323C1 (▲), I323C4 (Δ), and I323E1 (■). (**B**) Accumulation of [^{14}C]cyproconazole in wild-type isolate M1 (•) and cyproconazole resistant isolates M1A1 (○), M1B1 (▲), M1C1 (Δ), M1C4 (■), and M1D1 (□). (**C**) Relative increase in accumulation of [^{14}C]cyproconazole measured 30 minutes after addition of CCCP.

Northern analysis

The MDR phenotype of the laboratory mutants could indicate that ABC-transporters are involved in the mechanism of resistance (Kolaczkowski et al. 1998). Therefore, the expression of the ABC transporter encoding genes *MgAtr1-5* was studied. Northern analysis of these genes in untreated yeast-like cells demonstrated that almost all laboratory mutants exhibit a change in the basal expression level of one or more of the ABC transporter genes tested (Fig. 2). For instance, the expression level of *MgAtr1* was highly increased in I323C1 and M1C4 when compared to their respective wild-type parent isolates. In addition, both mutants showed increased expression of *MgAtr5*. Even mutant 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 no consistent correlation between the observed sensitivity profile of a mutant and the expression profile of any of the ABC transporter genes tested. All mutants were also tested for expression levels of the *CYP51* gene. Only I323E1 showed, besides upregulation of *MgAtr4*, an increased *CYP51* expression.

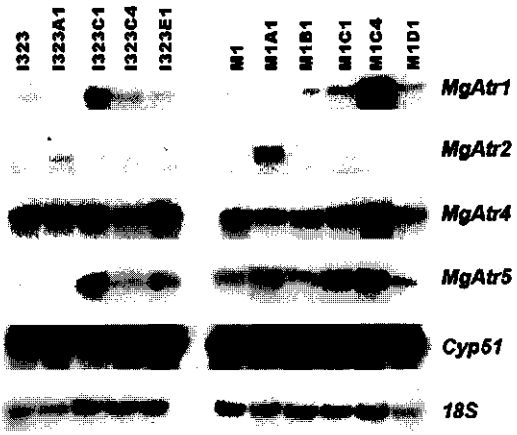

FIG. 2. Expression of *MgAtr1*, *MgAtr2*, *MgAtr4*, *MgAtr5*, and *CYP51* in *Mycosphaerella graminicola* wild-type isolates (I323 and M1) and laboratory mutants (I323A1, I323C1, I323C4, I323E1, M1A1, M1B1, M1C1, M1C4 and M1D1). The 18S ribosomal RNA gene is used as loading control.

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 isolates I323 and M1 and from the mutant isolates I323C1 and M1C4. The deduced amino acid sequence of the proteins encoded by the genes from wild-types and mutants were identical (results not shown).

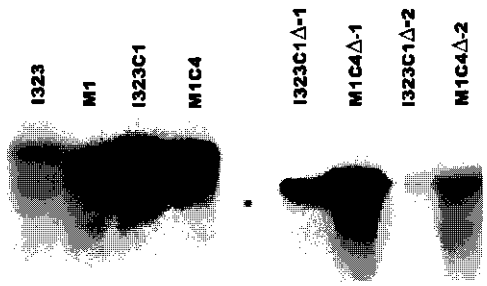

FIG. 3. Expression of *MgAtr1* in *Mycosphaerella graminicola* wild-type isolates (I323 and M1), laboratory mutants (I323C1 and M1C4) and independent transformants of I323C1 and M1C4 with a disrupted *MgAtr1* allele (I323C1Δ-1, I323C1Δ-2, M1C4Δ-1 and M1C4Δ-2).

Analysis of *MgAtr1* disruptants

Mutants I323C1 and M1C4, which both constitutively overexpress *MgAtr1*, were analysed in more detail by disruption of *MgAtr1* using *A. tumefaciens*-mediated transformation. Of both mutants two disruptants containing a single copy of the transforming DNA were isolated and characterised with respect to *MgAtr1* expression, azole sensitivity, and cyproconazole accumulation.

Northern analysis demonstrated that disruption of *MgAtr1* indeed resulted in the disappearance of full-length *MgAtr1* mRNA (Fig. 3). Accumulation of [^{14}C]cyproconazole by

the *MgAtr1* disruptants of both M1C4 and I323C1 was similar as compared to their parent isolates (results not shown). Surprisingly, both disruptants derived from I323C1 had a wild-type (I323) sensitivity to cyproconazole moreover, they became hypersensitive to cycloheximide (Fig. 4) demonstrating a role for *MgAtr1* in protection against these compounds in I323C1. In contrast, *MgAtr1* disruptants derived from M1C4, showed the same sensitivity to cyproconazole and cycloheximide as their parent isolate M1C4.

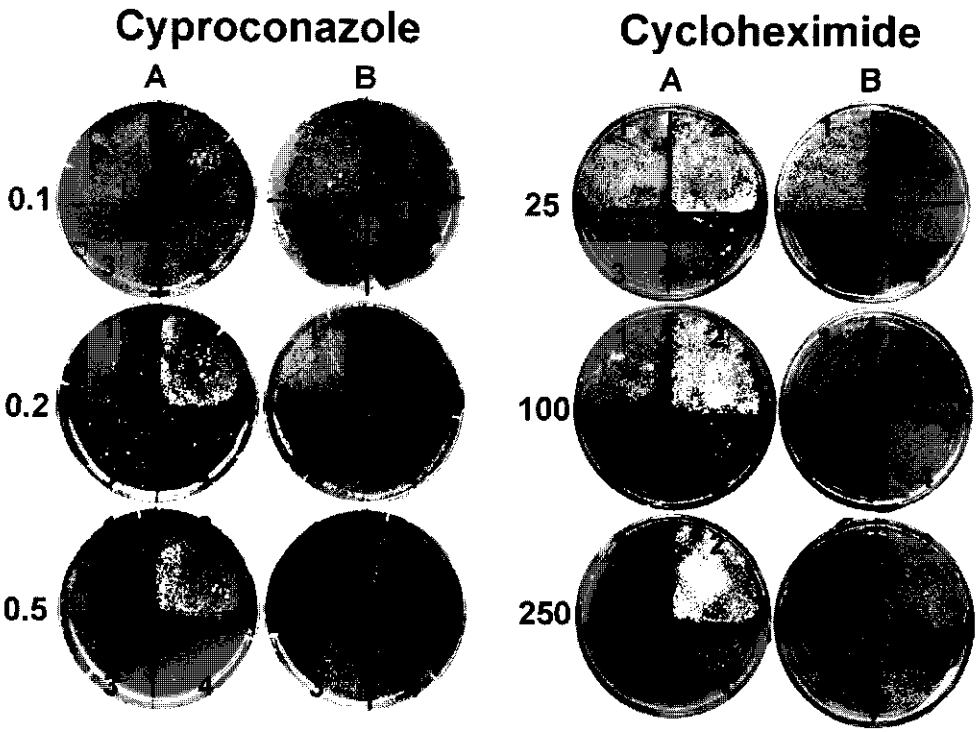

FIG.4. Effect of disruption of *MgAtr1* on sensitivity to cyproconazole and cycloheximide in *Mycosphaerella graminicola* isolates. (A): wild-type isolate I323 (1), cyproconazole-resistant isolate I323C1 (2) and two independent transformants of I323C1 with disrupted *MgAtr1* (3 and 4); (B): wild-type isolate M1 (1), cyproconazole-resistant isolate M1C4 (2) and two independent transformants of M1C4 with disrupted *MgAtr1* (3 and 4). The concentration of toxicants used is indicated in $\mu g\ ml^{-1}$.

DISCUSSION

Laboratory mutants of *M. graminicola* with a decreased sensitivity to the azole fungicide cyproconazole were selected at an unexpectedly high rate of around 1×10^{-4}. Recently, in *Candida glabrata* the development of so-called high-frequency azole resistance (HFAR) was described. These azole resistant mutants appeared at a comparable rate ($2\text{-}4 \times 10^{-4}$) and the

ABC transporter genes *CgCDR1* and *CgCDR2* were important determinants for the acquisition of this azole resistance phenotype (Sanglard et al. 2001). The *M. graminicola* laboratory mutants were selected without the use of a mutagenic agent and the sensitivity of the mutants to cyproconazole is within the range observed in field populations of *M. graminicola* (Gisi et al. 1997). Moreover, two mutants were tested for their virulence on wheat seedlings and did not show a change in virulence (results not shown). This suggests that HFAR can also occur in field populations of *M. graminicola* and can contribute to the natural variation in sensitivity of field populations.

It was hardly possible to generate any offspring in crosses between laboratory mutants and wild-type isolates (data not shown) which can easily be obtained with wild-type isolates (Kema et al. 1996). This suggests that the laboratory mutants have a fitness penalty associated with the resistance phenotype. This complies with reports describing the association of resistance to azole fungicides with a fitness penalty in other plant pathogenic fungi (De Waard and Nistelrooy 1990; Hollomon 1993) and might explain the slow development of resistance to azole fungicides under field conditions.

A resistance mechanism based on increased ABC transporter activity will usually result in an energy-dependent decreased cellular content of toxicants (Guan et al. 1992; Hayashi et al. 2001). All mutants derived from IPO323 as well as two mutants derived from M1 indeed showed a clear correlation between energy-dependent decrease in accumulation of [^{14}C]cyproconazole and decreased sensitivity to the fungicide. These results are in line with reports on an azole resistant isolate of *M. graminicola* with a decreased accumulation of the azole fungicide triadimenol (Joseph Horne et al. 1996). A test with 14 field isolates of *M. graminicola* also suggested a correlation between the sensitivity to cyproconazole and the accumulation levels of the fungicide (Gisi et al. 2000).

The accumulation of [^{14}C]cyproconazole by mutant M1C4 was higher than the accumulation by the wild-type and increased in time. This indicates that in this mutant a different resistance mechanism operates. The increased accumulation may be caused by cell wall changes, leading to an increased a-specific binding of fungicide to cell wall components. The relatively low increase in accumulation of [^{14}C]cyproconazole in M1C4 after addition of the uncoupler CCCP might support such a mechanism. This mechanism has been described for *Ustilago maydis* where triadimenol resistance in laboratory-mutants was associated with the presence of the cell wall and resistant cells accumulated 2-4 times more fungicide than sensitive strains (Wellmann and Schauz 1993). Alternatively, cyproconazole might be actively

sequestered in vacuoles. This protection mechanism is well described in plants where 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 of [^{14}C]cyproconazole accumulation in M1C4 in time. Both mechanisms might operate at the same time in M1C4.

The laboratory mutants exhibited cross-resistance to the unrelated chemicals cycloheximide and rhodamine 6G. This MDR phenotype also suggests that ABC transporters play a role in the mechanism of resistance. At present, overexpression of ABC transporters is recognised as a common resistance mechanism (Andrade et al. 2000; Nakaune et al. 1998; Sanglard et al. 1995). Therefore, expression of all ABC transporter genes cloned so far from *M. graminicola* (*MgAtr1-5*) was determined in all mutants. Northern analysis indicated that the moderate changes in sensitivity are associated with profound changes in expression levels of ABC transporter genes suggesting that the regulation of the examined genes in the mutants is quite different from that in the parent isolates. However, so far it is not possible to relate the expression of a specific ABC transporter gene with the observed phenotype, indicating that the MDR phenotype can be due to changes in the regulation of multiple ABC transporter genes. Overexpression of ABC transporter genes related to MDR has been demonstrated in many fungi including the yeasts *Candida albicans* (Sanglard et al. 1995), *C. glabrata* (Sanglard et al. 2001) and *Saccharomyces cerevisiae* (Balzi et al. 1994) and the filamentous fungi *Aspergillus nidulans* (Del Sorbo et al. 1997), *Penicillium digitatum* (Nakaune et al. 1998), and *Botrytis cinerea* (Hayashi et al. 2001).

Complementation of a *S. cerevisiae* mutant with *MgAtr1* resulted in a decrease in sensitivity of the yeast-transformant to cyproconazole and cycloheximide (Zwiers, in preparation). Therefore, both compounds are potential substrates for MgAtr1. The role of *MgAtr1* in cyproconazole sensitivity was examined in more detail by disrupting the gene in mutants with increased *MgAtr1* expression. Indeed, disruption of *MgAtr1* in I323C1 resulted in loss of resistance to cyproconazole and cycloheximide. These data prove that MgAtr1 indeed is capable of providing protection against azole fungicides in *M. graminicola* and thus might play a role in resistance development.

However, the situation seems to be more complex as disruption of *MgAtr1* in the parent isolate I323 did not alter the sensitivity to cyproconazole or cycloheximide (Zwiers in preparation). This suggests that in the wild-type isolate accumulation of [^{14}C]cyproconazole is not impaired by the activity of MgAtr1, possibly because in the wild type the protein is not

present in quantities to sufficiently transport these compounds. Only when the regulation of *MgAtr1* expression is disturbed, leading to overproduction of MgAtr1, MgAtr1 seems to have sufficient capacity to provide protection. In the *MgAtr1* disruptants of I323C1 the accumulation of cyproconazole did not revert to wild type levels. This suggests that in I323C1 the reduced accumulation is not caused by an increased energy-dependent efflux by MgAtr1 but might be the consequence of decreased influx. This result implies that the accumulation of cyproconazole by mutants of *M. graminicola* is probably not a proper indicator for the involvement of ABC transporters. This contrasts with the situation in *A. nidulans* and *B. cinerea* (Andrade et al. 2000; Stehmann and Waard 1995; Vermeulen et al. 2001).

As *MgAtr1* is not upregulated in all selected mutants and disruption of *MgAtr1* in M1C4 did not alter the sensitivity to cyproconazole and cycloheximide it is clear that overproduction of *MgAtr1* can not be the only determinant involved in cyproconazole / cycloheximide resistance. Disruption of other ABC transporter genes in the mutants should indicate if additional ABC-transporters are involved in fungicide sensitivity. This is well possible since ABC transporters are members of a large protein superfamily and are known to possess overlapping substrate specificity (Decottignies and Goffeau 1997; Kolaczkowski et al. 1998).

Sequencing of *CYP51* from *M. graminicola* isolates I323C1 and M1C4 did not show any of the point mutations reported to confer resistance to azole fungicides in *C. albicans* (Sanglard et al. 1998; Gisi et al. 2000). This indicates that at least in these mutants mutations in the *CYP51* gene are not involved in decreased fungicide sensitivity.

All data described here suggest that in laboratory mutants a variety of resistance mechanisms may operate. One mechanism involves the upregulation of one or more ABC transporter genes, presumably leading to a decreased accumulation of the fungicide. A second mechanism possibly involves changes in cell wall or cell membrane composition resulting in decreased influx. Finally, we have indications that a third mechanism might involve the sequestration of fungicide, resulting in an increased accumulation. Probably, in individual mutants multiple mechanisms may operate. This situation complicates the study on resistance mechanisms to azoles in *M. graminicola*. At present, we are examining whether resistance mechanisms found in laboratory mutants also occur in field isolates.

MATERIALS AND METHODS

Fungal material and culture conditions.

In this study two field isolates of *M. graminicola* were used, IPO323, isolated in the Netherlands in 1981 (Kema and Van Silfhout 1997) and M1 collected in France in 1993 and provided by Dr. J.M. Seng (Biotransfer, Montreuil Cedex, France). Isolates were grown yeast-like in liquid yeast-sucrose medium (YSM; 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 (CDMP; Czapek Dox 33.4 g L^{-1}, mycological peptone 5 g L^{-1}) with 3 x 10^4 cells ml^{-1} and incubation on a rotary shaker (25 °C, 140 rpm) for an additional 3 days.

Sensitivity assays and isolation of laboratory mutants.

Fungicides 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. Toxicity tests were performed by plating 1 x 10^4 cells, harvested from 3-day-old liquid medium, on 6-ml Petri dishes containing V8-agar amended with different concentrations of toxicants. Experiments were performed in triplicate and MIC values were assessed after 10 days of incubation at 18 °C in the dark.

M. graminicola mutants with a decreased sensitivity to the azole fungicide cyproconazole were isolated by plating 1 x 10^5 yeast-like cells on V8-agar in 14-cm Petri dishes amended with cyproconazole at 3 and 10 times the MIC value. After 10 days of incubation resistant colonies were isolated and MIC values of the different toxicants were assessed.

Gene disruption.

Agrobacterium tumefaciens-mediated transformation was used to disrupt *MgAtr1* in the laboratory mutants I323C1 and M1C4. Generation of disruption constructs and selection of transformants with the disrupted *MgAtr1* gene was performed as described previously (Zwiers and De Waard 2001, Chapter 3).

Accumulation of [^{14}C]cyproconazole.

Mycelium was homogenised and harvested by filtering over 0.85 mm and 55 µm 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 ml^{-1} and incubated for 30 min at 25 °C and 140 rpm. Then, [^{14}C]cyproconazole (kindly provided by Syngenta, Basel, Switzerland) 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 of [^{14}C]cyproconazole was calculated as nmol mg^{-1} of dry weight. Energy dependency of [^{14}C]cyproconazole accumulation was tested by the addition of carbonyl-cyanide3-chlorophenylhydrazone (CCCP, 20 µM) and subsequent measurement of the accumulation of [^{14}C]cyproconazole.

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 of the *M. graminicola CYP51* gene (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 isolates and laboratory-generated mutants. The expression levels of the ABC transporter encoding genes *MgAtr1* (accession no: AJ243112), *MgAtr2* (accession no: AJ243113) (Zwiers and De Waard 2000), *MgAtr3* (accession no: AF364105), *MgAtr4* (accession no: AF329852) and *MgAtr5* (accession no: AF364104) 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 HybondN 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). 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, and 100 µg ml^{-1} denatured herring sperm DNA).

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CHAPTER 7

General Discussion

The sequencing of several prokaryotic and eukaryotic genomes shows that the ATP-bindings cassette (ABC) transporter superfamily is one of the largest protein families. According to the latest estimations, the human genome contains 48 ABC transporter genes and the number of ABC transporter genes in other eukaryotic genomes such as *Caenorhabditis elegans* and *Drosophila melanogaster* are comparable (www.humanabc.org). The number of ABC transporter genes in plants is even higher as *Arabidopsis thaliana* contains an estimated 129 open reading frames (ORFs) potentially encoding ABC transporter proteins (Sanchez Fernandez et al. 2001). Based upon the few fully sequenced genomes of filamentous fungi, it can be expected that these organisms contain approximately 40-50 ABC transporter genes (Yoder and Turgeon 2001). According to their size, orientation, and domain organisation ABC transporters can be classified in distinct clusters. For instance, the yeast ABC transporters are divided in six clusters, which are subdivided in ten subclusters (Decottignies and Goffeau 1997). The human ABC transporters are divided in seven subfamilies, while those of *A. thaliana* can be divided in twelve subfamilies. This abundance of ABC transporters and the concomitant redundancy in function severely complicates the functional analysis of these genes.

Cloning of ABC transporter encoding genes

The aim of this thesis was to establish the function of ABC transporters from the wheat pathogen *Mycosphaerella graminicola* in pathogenesis, fungicide sensitivity and multidrug resistance (MDR). Chapter 2 of the thesis describes the cloning and characterisation of ABC transporter genes. The genes were isolated by means of heterologous hybridisation, as at the start of this research project sequence information on fungal ABC transporters was limited. Thus, other strategies to isolate ABC transporter genes from *M. graminicola*, such as PCR using degenerate primers or the screening of expressed sequence tag (EST) libraries, were not yet possible. Since *PDR5* from *Saccharomyces cerevisiae* is probably the best-characterised ABC transporter gene from a lower eukaryote, it was used as probe in the heterologous hybridisation (Balzi et al. 1994; Balzi and Goffeau 1995). Moreover, PDR5 possesses wide substrate specificity and is involved in MDR. In yeast this is called pleiotropic drug resistance (PDR) (Kolaczkowski et al. 1998; Kolaczkowski et al. 1996). PDR5 is considered to act as the functional homolog of the human MDR1 (Wolfger et al. 2001). This approach resulted in the cloning of *MgAtr1* and *MgAtr2*. Both ABC transporters are clear homologs of the yeast I-1 subcluster (or PDR cluster) of ABC transporters (Zwiers and De Waard 2000).

However, identification of ABC transporter genes by heterologous hybridisation with a probe derived from *PDR5* is biased and will predominantly lead to the isolation of *PDR5* homologs and not of ABC transporters belonging to any of the other subfamilies. An attempt to isolate *M. graminicola* homologs of the yeast cluster II ABC transporters by heterologous hybridisation with a probe derived from *STE6* was not successful. This thesis also describes the functional analysis of the *M. graminicola* ABC transporter genes *MgAtr3*, *MgAtr4* and *MgAtr5*, which were isolated by PCR using degenerate primers directed against various fungal ABC transporter subclusters (Stergiopoulos et al. 2002). This PCR approach did not result in the isolation of ABC transporter genes that belong to clusters other than the I-1 or PDR-cluster. Nevertheless, *M. graminicola* does contain ABC transporters belonging to other clusters. The screening of ESTs from *M. graminicola* for the presence of ABC transporter genes revealed the presence of several ABC transporter homologs (Keon et al. 2000). None of these genes were homologs of cluster I-1 ABC transporters.

Transformation of *M. graminicola*

Functional analysis of genes is commonly performed by assessing the phenotype of knock-out mutants. To generate such mutants an efficient transformation system enabling homologous integration should be available. Such a system was not available at the start of this research project. Several approaches were followed to obtain transformants with non-functional ABC transporter genes (Ito et al. 1983; Lemke and Peng 1995). Electroporation and lithium-acetate treatment of both yeast-like cells and mycelium did not result in the generation of any stable transformed colonies. Protoplast PEG-mediated transformation was more successful, but the transformation efficiency was very low and predominantly ectopic integrations occurred. Protoplast PEG-mediated transformation did not lead to the generation of *MgAtr1* and *MgAtr2* knock-out mutants but it did result in the formation of Δ*MgAtr3* and Δ*MgAtr5* mutants, albeit with a very low efficiency (Chapter 4). This suggests that the efficiency of targeted gene replacement is locus-dependent (Bird and Bradshaw 1997).

***Agrobacterium tumefaciens*-mediated transformation**

T-DNA mediated transfer by *Agrobacterium tumefaciens* is not limited to plants. By now the transfer of DNA to yeasts, filamentous fungi, and even human cells has been demonstrated (Bundock et al. 1995; De Groot et al. 1998; Kunik et al. 2001). Since the first publication describing the capacity of *A. tumefaciens* to transform filamentous fungi, many additional

papers describing this feature appeared (Abuodeh et al. 2000; Chen et al. 2000; Covert et al. 2001; De Groot et al. 1998; Malonek and Meinhardt 2001; Mikosch et al. 2001; Mullins et al. 2001). These papers show that *A. tumefaciens*-mediated transformation is a useful tool to transform fungi that are otherwise difficult to transform. *A. tumefaciens*-mediated transformation can be applied to conidia, mycelium, and even to fungal fruiting bodies. Moreover, transformants predominantly contain single-copy integrations of the transforming DNA, which makes this method also useful for insertional mutagenesis. Chapter 3 describes the establishment of an *A. tumefaciens*-based transformation protocol for *M. graminicola* and the use of this method to achieve efficient gene-disruption by homologous recombination (Zwiers and De Waard 2001). This is the first report describing gene disruption in *M. graminicola* as well as the first report describing targeted integration by *A. tumefaciens*-mediated transformation in a filamentous fungus, where targeted integration by conventional methods is difficult.

The efficiency of targeted integration achieved with *A. tumefaciens*-mediated transformation of *M. graminicola* is fairly high. Disruption of *MgAtr1*, *MgAtr2* and *MgAtr4* was successful in 25, 44 and 75% of the transformants tested, respectively (Chapter 3 and Chapter 4). This contrasts with the low efficiencies obtained for *MgAtr3* and *MgAtr5* mutants (2.3 and 5.6%, respectively) using the protoplast-PEG-mediated transformation (Chapter 4). Recently, an electro-transformation system was developed for *M. graminicola*. With the same 6.7 kb *MgAtr2* fragment as used in the *A. tumefaciens*-mediated transformation, disruption was achieved with an efficiency of only 5.3% (Adachi et al. 2001; Zwiers and De Waard 2001). This significant difference (5.3 vs. 43.9 %) can not be attributed to differences in the size of homologous DNA present in the transformation constructs, but is likely inherent to the transformation system used.

T-DNA is transferred as a single stranded (ss) copy of the coding strand of the T-DNA region. In *S. cerevisiae* homologous recombination is more efficient when the homologous DNA is present as ss-DNA (Simon and Moore 1987). This suggests that homologous recombination in *S. cerevisiae* has a preference for ss-DNA as a substrate. However, this preference for ss-DNA appears to be organism-dependent, as, for instance, in *Coprinus cinereus* ss-DNA does not improve the targeting efficiency compared to supercoiled circular DNA (Binninger et al. 1991). The T-DNA that enters the cell is coated with the proteins VirD2 and VirE2 (Citovsky et al. 1989; Young and Nester 1988). The association with these proteins protects the introduced DNA from degradation by cellular nucleases (Citovsky et al.

1989). Furthermore, both VirD2 and VirE2 participate in the nuclear import of the T-DNA to the nucleus. Both VirD2 and VirE2 contain nuclear localisation signals (NLSs) and localise in nuclei when expressed in plant cells. The emerging picture is that VirD2, through binding with a karyopherin α protein (in *Arabidopsis* AtKAPα) and probably the subsequent association with karyopherin β, promotes the nuclear import. VirE2 seems to be required for the nuclear import of large ssDNA molecules, possibly by making the conformation of the complex suitable for import through the nuclear pore complex. VirE2 does not bind to karyopherin α proteins but to VIP1 and VIP2. VIP1 might associate with AtKAPα and thus promote transport to the nucleus, but the association of VIP1 with VirE2 might also help in the establishment of the proper conformation for nuclear import. In contrast to the karyopherins no animal or yeast homologs of VIP1 are known. This suggests that VIP1 might be the plant-specific factor involved in nuclear uptake of VirE2 and that import of T-DNA in animal and yeast cells is mainly mediated through the conserved karyopherin pathway (Ballas and Citovsky 1997; Gelvin 1998; Rossi et al. 1993; Rossi et al. 1996;Tzfira et al. 2000; Ward et al. 2002; Ziemienowicz et al. 2001). Finally, besides protecting the ssDNA against degradation and facilitating nuclear import, VirD2 and VirE2 are also involved in the T-DNA integration itself. VIP2, as well as VIP1 are both implicated in the targeting of the T-DNA to active regions of chromatin thus promoting integration. VirD2 has *in vitro* ligase activity and possesses motifs involved in integration. (Mysore et al.1998; Ward et al. 2002). All these factors are likely to contribute to the observed differences in targeting efficiency between the *A. tumefaciens*-mediated transformation of *M. graminicola* compared to other transformation-protocols.

As described in Chapter 3, the presence of homologous DNA seems to have a positive effect on the transformation efficiency in *M. graminicola*. The same is found for *S. cerevisiae*, but in *Kluyveromyces lactis* the transformation efficiency is independent of the presence of homologous DNA between the T-DNA borders (Bundock and Hooykaas 1996; Bundock et al. 1999). These data suggest that in both *M. graminicola* and *S. cerevisiae* homologous recombination is more efficient than illegitimate recombination, whereas in *K. lactis* the fate of the transforming DNA is determined by the presence of homology of the T-DNA with the *K. lactis* genome. Thus, these data suggest that the mode of integration of the T-DNA, homologous or ectopic, is mainly determined by host cell-factors and not by the T-DNA.

ABC transporters and virulence

One of the proposed functions of ABC transporters in plant pathogenic fungi is to act as virulence factors (De Waard 1997). This function has now been established for several fungal ABC transporters. The first ABC transporter described with a function in pathogenesis is the *MgABC1* gene from the rice-blast fungus *Magnaporthe grisea*. Mutants lacking this transporter are non-pathogenic. In addition, an insertional mutation upstream of the *ABC1* start-codon results in lack of pathogenicity and in reduced up-regulation of *ABC1* expression after exposure to toxicants. This suggests that up-regulation of *MgABC1* expression is required for pathogenesis. The lack of pathogenicity might be explained by a presumed protective role of the transporter against antimicrobial compounds produced by the hosts. This idea is supported by the observation that antibiotics, antimycotics, and the rice phytoalexin sakuranetin induce *MgABC1* expression. However, *MgABC1* knock-out mutants do not show increased sensitivity to any of these compounds, indicating that the natural substrate(s) of MgABC1 still need(s) to be discovered (Urban et al. 1999). Similarly, the ABC transporter BcAtrB from *Botrytis cinerea* was described as a determinant in virulence of grapevine (Schoonbeek et al. 2001). Expression of *BcAtrB* is enhanced by exposure to the grapevine phytoalexin resveratrol. Moreover, BcAtrB replacement mutants exhibit increased sensitivity to resveratrol. In *Gibberella pulicaris*, the causal agent of potato dry rot, the ABC transporter GpABC1 acts as a virulence factor on potato by providing protection against the phytoalexin rishitin (Fleissner et al. 2002).

Also in *Cochliobolus heterostrophus* (G. Turgeon, personal communication) and in *M. graminicola* (Chapter 4) ABC transporters act as virulence factors. In these cases the function of the ABC transporters involved is still unknown. They might provide protection against plant defence compounds but they could also play a role in the secretion of fungal toxins. However, in filamentous fungi transporters involved in the secretion of endogenously produced toxins mainly belong to the major facilitator superfamily (MFS) of transporters and not to ABC transporters. The cyclic peptide HC-toxin from *Cochliobolus carbonum*, the polyketide cercosporin from *Cercospora kikuchii*, and the cyclic sesquiterpenoid trichothecenes from *Fusarium sporotrichioides* are all secreted by MFS transporters (Alexander et al. 1999; Callahan et al. 1999; Pitkin et al. 1996). Loss of the HC-toxin transporter TOXA in *C. carbonum* is lethal, disruption of the cercosporin transporter CFP results in increased sensitivity to cercosporin, and disruption of the trichothecene transporter TRI12 leads to decreased trichothecene production and increased sensitivity to trichothecenes.

These data indicate that MFS transporters are probably the major players in the secretion of toxins and function in self-protection to toxins.

Although, MFS transporters are most likely the principal transporters of fungal toxins, ABC transporters can transport these toxins as well. The *S. cerevisiae* ABC transporters SNQ2 and PDR5 confer resistance to the fungal toxins cercosporin and the trichothecene diacetoxyscirpenol (DAS), respectively (Muhitch et al. 2000; Ververidis et al. 2001). Also the *M. graminicola* ABC transporters MgAtr1 and MgAtr4 are able to transport DAS upon overexpression in *S. cerevisiae* (Chapter 5). The overlap in substrate specificity between ABC and MFS transporters might indicate that ABC transporters play a protective role against exogenous fungal toxins or play an additional role in self-protection against endogenously produced toxins.

During plant pathogenesis toxin production and secretion needs to be efficient over a relatively long time-period. Efficient secretion is even more important for toxins with auto-toxicity. MFS transporters drive transport by means of the proton-motive force and not by hydrolysis of ATP as is the case for ABC transporters. As cells continuously maintain a proton motive force, transport through MFS transporters may constitute a more reliable transport route for endogenous toxins than ABC transporters. ABC transporters might predominantly act as the first defence barrier against fungitoxic compounds produced by the plant during plant-pathogen interaction. Consequently, the ABC transporters are only active when needed, thereby reducing the energy costs related to their production and mode of action.

Substrate specificity

The determinants of substrate specificity of ABC transporters are still unknown. Both membrane-bound halves of ABC transporters are involved in substrate binding and substrate specificity is determined by the presence of binding sites located in transmembrane loops at the cytoplasmic site of the membrane. However, it is not yet feasible to locate any motifs in the primary sequence of ABC proteins that determine substrate specificity. Considering the broad substrate specificity of most ABC transporters and the fact that substrates are often chemically unrelated it is unlikely that specific binding-motifs occur.

To some extent, the overall homology of proteins, as reflected in phylogenetic analyses can be used to predict substrate specificity (Saurin et al. 1999). For example, the phylogenetic analysis of bacterial ABC2 permeases distinguished major clusters with different types of

substrates (Saier et al. 1998). Likewise, the amino acid sequence of the *Penicillium digitatum* ABC transporter PMR1, known to act as a transporter of azole fungicides, was used to identify additional ABC transporters (AnAtrE, AnAtrF and AnAtrG) involved in azole transport in *Aspergillus nidulans* (Nakaune et al.; 1998Andrade 2000). Expression of these genes was upregulated by the azole fungicide fenarimol. Analysis of knock-out mutants indicated that at least one of these ABC transporters is involved in azole transport (A.C. Andrade, personal communication). These transporters are also highly homologous to BcAtrD, an ABC transporter from *B. cinerea* involved in resistance to the azole fungicide oxpoconazole (Hayashi et al. 2001). Accordingly, a phylogenetic tree made from the alignment of 30 fungal ABC transporters distinguishes a branch containing azole "specific" transporters (Fig. 1). Thus, a functional search through homology analysis assuming that orthologous genes are functionally equivalent can lead to the isolation of ABC transporters with similar or even the same substrates. Since orthologs do not necessarily perform similar physiological functions and paralogs exhibiting a redundancy in function exist, homology analysis will not yield all functional homologs. This can be illustrated by the fact that the *A. nidulans* ABC transporter AnAtrB and the *M. graminicola* ABC transporters MgAtr1, MgAtr2 and MgAtr4 do not group with the putative azole branch, but still are capable of transporting azole fungicides (Andrade et al. 2000; Chapter 5 and Chapter 6).

Expression of the human multidrug transporter MDR1 is induced by physical stresses such as UV-light, X-ray irradiation, heat shock, and chemical stress-inducing agents (Chin et al. 1990; McClean et al. 1993; Uchiumi et al. 1993). These treatments cause DNA damage and sometimes generate nucleic acid fragments. It is postulated that these nucleic acid fragments are substrates or inducers of ABC transporters and that MDR1 would facilitate the removal of this "genetic waste material" (Seelig 1998). Similar mechanisms could also operate in filamentous fungi, a hypothesis supported by the existence of a putative azole branch of ABC transporters. Both azole fungicides and nucleotides are derivatives of heterocyclic compounds that may be the natural substrates for transporters belonging to this branch of ABC transporters.

The ABC transporters GpABC1 and MgABC1 from *G. pullicaris* and *M. grisea*, respectively, are both involved in pathogenesis. They are highly homologous and form a distinct branch in the phylogenetic tree (Fig 1). GpABC1 from *G. pullicaris* seems to exert its function by providing protection against the terpenoid rishitin (Fleissner et al. 2002). In rice,phytoalexins such as the flavanone sakuranetin and the terpenoid momilactones and

oryzalexins could be substrates for MgABC1 of *M. grisea* (Dillon et al. 1997; Kato et al. 1993). However, the phenotype of the MgABC1 mutant can not be attributed to an alteration in sensitivity to sakuranetin and the physiological substrate has not yet been determined (Urban et al. 1999). Therefore, it might be that the natural substrate of MgABC1 from *M. grisea* is one of the terpenoid phytoalexins from rice.

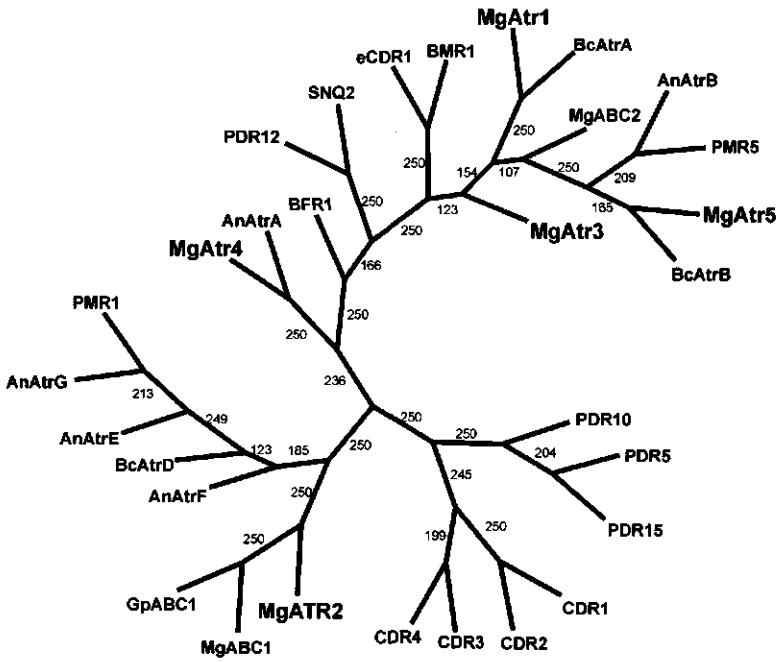

FIG. 1) Unrooted phylogenetic tree of 30 fungal ABC transporters with $[NBF\text{-}TM]_2$ configuration. Tree is based on maximum parsimony as calculated using PROTPARS from the Phylip software package. Numbers between branches indicate confidence of the consensus tree with a bootstrap option of 250. AnAtrA, AnAtrB, AnAtrE, AnAtrF, and AnAtrG are from *Aspergillus nidulans*, BcAtrA, BcAtrB, BcAtrD, and BMR1 from *Botrytis cinerea*, BFR1 from *Schizosaccharomyces pombe*, CDR1, CDR2, CDR3, and CDR4 from *Candida albicans*, eCDR1 from *Cryptococcus neoformans*, GpABC1 from *Gibberella pullicaris*, MgABC1 and MgABC2 from *Magnaporthe grisea*, MgAtr1, MgAtr2, MgAtr3, MgAtr4, and MgAtr5 from *Mycosphaerella graminicola*, PMR1 and PMR5 from *Penicillium digitatum*, and PDR5, PDR10, PDR12, PDR15, and SNQ2 from *Saccharomyces cerevisiae*.

Evolutionary relationships between ABC transporters

The phylogenetic tree depicted in figure 1 shows that all ABC transporters from true filamentous fungi are grouped in branches distinct from ABC transporters of *S. cerevisiae* and *Candida albicans*. The exceptions are BFR1 from *Schizosaccharomyces pombe* and PDR12 and SNQ2 from *S. cerevisiae* which group with the ABC transporters from filamentous fungi. This indicates that the yeasts *S. cerevisiae* and *C. albicans* are evolutionary quite distant from

the yeast *S. pombe* as well from filamentous fungi. This corresponds with other phylogenetic data, which show that *S. pombe* and *S. cerevisiae* are evolutionary distinct (Forsburg 1999; Paquin et al. 1997). This also suggests that *S. pombe* is a better model organism to study some aspects of fungal biology than *S. cerevisiae*.

Another phenomenon apparent from the phylogenetic analysis is the observation that paralogous fungal ABC transporters often show less homology than orthologs. This has also been observed for ABC transporters from archae, bacteria and eukaryotes where homologs are closely related. This could be explained by the occurrence of specialisation early in evolution suggesting that ABC transporters already existed before the separation of the three kingdoms (Dassa and Bouige 2001; Saurin et al. 1999). Alternatively, the high conservation between species and between kingdoms could be the consequence of horizontal gene transfer.

The phylogenetic analyses have led to a model for the evolution of ABC transporters (Saurin et al. 1999). The hypothetical ancestor presumably already contained differentiated ABC import and export systems composed of unfused ABC modules and transmembrane domains (TM). Eukaryotes may have acquired TM-ABC export systems through symbiotic bacteria. This would explain the fact that TM-ABC transporters are specifically located in the membranes of organelles. Duplication and fusion events could have led to the formation of $(TM\text{-}ABC)_2$ and $(ABC\text{-}TM)_2$ systems. In contrast to plants and fungi, humans do not contain ABC transporters with the $(ABC\text{-}TM)_2$ topology suggesting that plants and fungi share the same ancestor or that such a duplication event occurred independently in both the ancestors of fungi and plants. As a consequence the ancestor of filamentous fungi already contained a defined set of ABC transporters with particular substrate specificity and function. Additional gene duplications and modifications may have resulted in the formation of transporters with slightly altered substrate specificity. Subsequently, selection might have resulted in the establishment of paralogs. This would explain why besides orthologs with similar functions, *e.g.* the putative azole-transporter branch, also paralogs with overlap in function and substrate specificity do exist.

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SUMMARY

ATP-binding cassette (ABC) transporters belong to one of the largest protein families known. They play a role in numerous vital processes in the cell and are characterised by their capacity to transport a broad variety of substrates, ranging from simple ions to complex polypeptides. Many human diseases are correlated to malfunctioning of ABC transporters. Historically, ABC transporters became known because of their role in the development of multidrug resistance (MDR) during cancer therapy. MDR is the simultaneous development of resistance against chemically unrelated compounds and can be the consequence of overproduction of ABC transporters. MDR is not limited to human tumour cells but also occurs during chemical treatment of parasites, bacteria, and fungi. In plant pathogenic fungi ABC transporters can play a role in resistance against azole fungicides. Chapter 1 gives an overview of common functions and characteristics of ABC transporters.

This thesis describes the functional analysis of ABC transporters in *Mycosphaerella graminicola,* one of the most important pathogens on wheat. This fungus is the causal agent of septoria tritici leaf blotch. When not properly controlled, yield losses caused by this disease can be as high as 50%. Despite its importance, not much is known about the molecular biology of this fungus. Chapter 2 describes the cloning of the first two ABC transporter genes of this fungus, *MgAtr1* and *MgAtr2*. Both genes were isolated by means of heterologous hybridisation using a probe derived from the *Saccharomyces cerevisiae* ABC transporter *PDR5*. Exposure of *M. graminicola* to a broad variety of chemicals showed that several compounds enhanced expression of one or both genes. Compounds capable of increasing the expression include antibiotics, plant secondary metabolites, and fungicides used to control *M. graminicola*, suggesting a role for MgAtr1 and MgAtr2 in protection against these compounds. Another striking result is the differential expression of the two genes in yeast-like cells and mycelium, suggesting a morphology dependent regulation of expression.

Chapter 3 describes the development of an *Agrobacterium tumefaciens*-mediated transformation protocol. In contrast to several other methods, *A. tumefaciens*-mediated transformation is highly effective and results in the efficient generation of disruption mutants. The establishment of this transformation protocol enabled the functional analysis of ABC transporters of *M. graminicola* by gene disruption.

Independent knockout mutants were generated for five ABC transporter genes from *M. graminicola* and the virulence of these mutants was tested on wheat. As described in Chapter 4, Δ*MgAtr4* mutants were less virulent, while all the other transformants showed unaltered virulence. However, it is still unclear how MgAtr4 exerts its action. MgAtr4 is the first described virulence factor of *M. graminicola* and the fourth ABC transporter described as a virulence factor in plant pathogenic fungi.

The substrate specificity of the ABC transporters was studied by complementation of *S. cerevisiae* mutants with the *M. graminicola* ABC transporter genes and by analysis of the *M. graminicola* knockout mutants for sensitivity to a broad variety of compounds. The results of the yeast complementation assay presented in Chapter 5 clearly show that the ABC transporters tested function as multidrug transporters with overlap in substrate specificity. The substrate range in yeast includes antibiotics, fungicides, plant secondary metabolites, and a mycotoxin. The *M. graminicola* knockout mutants did not show altered sensitivity to any of these potential substrates. This can be explained by the fact that multiple ABC transporters exist with overlap in substrate specificity, which can take over the function of the distorted transporter. *MgAtr5* deletion mutants showed a small increase in sensitivity to the putative wheat defence compound resorcinol, suggesting a role for MgAtr5 in the *M. graminicola*-wheat pathosystem. Bioassays to test the effect of antagonistic bacteria on the growth of the ABC transporter knockout mutants, indicate that MgAtr2 can provide protection against antibiotics produced by these bacteria. Thus, the data presented in Chapter 5 indicate that ABC transporters from *M. graminicola* play a role in the protection against toxic compounds.

Both the expression analysis (Chapter 2) and the yeast complementation data (Chapter 5) suggest that azole fungicides can act as substrates for ABC transporters from *M. graminicola*. In Chapter 6 the potential role of ABC transporters in fungicide sensitivity is studied in more detail. *M. graminicola* mutants with decreased sensitivity to the azole fungicide cyproconazole were generated and shown to exhibit a MDR phenotype. Decreased azole sensitivity correlated with altered accumulation of cyproconazole, indicative for the involvement of ABC transporters. Expression of one or more of the ABC transporter genes studied was altered in all mutants. However, changes in fungicide sensitivity did not correlate

with alterations in expression of a specific ABC transporter gene. Disruption of *MgAtr1* in two mutants showing constitutive *MgAtr1* overexpression restored cyproconazole sensitivity to wild-type levels in only one of these mutants. These results show that overexpression of ABC transporters is one of the mechanisms leading to azole resistance in *M. graminicola*.

In conclusion, the data presented in this thesis show that *M. graminicola* possesses ABC transporters with overlapping substrate specificity. Substrates include xenobiotics and natural toxicants. This is confirmed by the findings that ABC transporters from *M. graminicola* act as virulence factor and can provide protection against mycotoxins, bacterial antibiotics, and azole fungicides. Therefore, ABC transporters of *M. graminicola* contribute to the success of this fungus as a pathogen.

SAMENVATTING

ATP-bindings cassette transporters (ABC) maken deel uit van één van de grootste in de natuur voorkomende eiwitfamilies en spelen een rol in vele essentiële cellulaire processen. Kenmerkend voor deze transporteiwitten is hun grote verscheidenheid aan substraten, variërend van ionen tot complexe eiwitten. Verschillende ziekten bij de mens worden veroorzaakt door het niet of niet goed functioneren van ABC transporters. ABC transporters zijn vooral bekend door hun rol bij het optreden van multidrug resistentie (MDR) in menselijke tumoren. MDR is de gelijktijdige ontwikkeling van resistentie tegen chemisch niet-verwante verbindingen en kan veroorzaakt worden door een overproductie van ABC transporter eiwitten. MDR is niet beperkt tot menselijke tumorcellen maar vormt ook een ernstige bedreiging voor de bestrijding van parasieten, bacteriën en schimmels. In plantenpathogene schimmels spelen ABC transporters een rol in de ontwikkeling van resistentie tegen fungiciden. Hoofdstuk 1 geeft een overzicht van de functies en eigenschappen van ABC transporters.

Dit proefschrift beschrijft de functionele analyse van ABC transporters uit één van de belangrijkste ziektenverwekkers van tarwe, *Mycosphaerella graminicola*, de veroorzaker van de septoria bladvlekkenziekte. Als deze ziekte niet afdoende wordt bestreden, kan *ca.* 50% van de opbrengst verloren gaan. Ondanks het belang van de ziekte is er niet veel bekend over de moleculaire biologie van de schimmel. In Hoofdstuk 2 wordt de klonering van de eerste twee ABC transportergenen van deze schimmel, *MgAtr1* en *MgAtr2*, beschreven. Beide genen werden geïsoleerd door middel van heterologe hybridisatie met behulp van een DNA-fragment afgeleid van het ABC transporter gen *PDR5* van de bakkersgist *Saccharomyces cerevisiae*. Chemische verbindingen welke de expressie van de twee geïsoleerde genen in *M. graminicola* induceerden zijn o.a. antibiotica, secundaire metabolieten van planten en fungiciden die gebruikt worden bij de bestrijding van deze schimmel. Deze resultaten suggereren dat MgAtr1 en MgAtr2 bescherming kunnen bieden tegen deze verbindingen. Een opvallend resultaat is het verschil in expressie van de twee genen tussen de dimorfe groeiwijzen van *M. graminicola*. Dit verschil duidt op een morfologie-afhankelijke regulatie van de genexpressie.

In Hoofdstuk 3 wordt de ontwikkeling van een transformatieprotocol, waarbij gebruik wordt gemaakt van *Agrobacterium tumefaciens*, beschreven. In tegenstelling tot andere transformatie protocollen, is de transformatie van *M. graminicola* met *A. tumefaciens* zeer effectief en kan de methode gebruikt worden voor het op een efficiënte manier verkrijgen van knock-out mutanten. De ontwikkeling van dit transformatieprotocol heeft de functionele analyse van ABC transporters in *M. graminicola* mogelijk gemaakt. De disruptie mutanten beschreven in Hoofdstuk 3 zijn de eerste knock-out mutanten beschreven voor *M. graminicola*.

Vijf ABC transporter genen van *M. graminicola* werden onafhankelijk van elkaar uitgeschakeld en de virulentie van deze mutanten werd getest op tarwe. Zoals beschreven in Hoofdstuk 4 waren alleen ΔMgAtr4 transformanten verminderd virulent. De rol van MgAtr4 in virulentie is nog onduidelijk. MgAtr4 is de eerste virulentie factor geïdentificeerd in *M. graminicola* en de vierde ABC transporter werkzaam als virulentie factor in een plantenpathogene schimmel.

De substraat-specificiteit van de ABC transporters werd onderzocht door de gevoeligheid van *S. cerevisiae* gecomplementeerd met de afzonderlijke *M. graminicola* ABC transporter genen en van de *M. graminicola* transformanten te testen op diverse chemische verbindingen. De resultaten van de complementatie experimenten met *S. cerevisiae*, zoals beschreven in Hoofdstuk 5, laten duidelijk zien dat de geteste ABC transporters kunnen functioneren als multidrug transporters en een overlap in substraat specificiteit bezitten. De resultaten tonen aan dat antibiotica, fungiciden, secundaire metabolieten van planten en een mycotoxine kunnen fungeren als substraat voor de transporters. Knock-out transformanten van *M. graminicola* vertoonden echter geen verandering in gevoeligheid voor één van deze potentiële substraten, hetgeen suggereert dat er in de transformanten nog andere ABC transporters aanwezig zijn die door een overlap in substraatspecificiteit de taak van de uitgeschakelde transporter kunnen overnemen. De *MgAtr5* deletie mutanten vertoonden een kleine toename in gevoeligheid voor resorcinol, een verbinding die mogelijkerwijs een functie heeft als afweerstof in tarwe. Dit suggereert dat MgAtr5 een rol kan spelen in de interactie tussen *M. graminicola* en tarwe. Biotoetsen waarin de invloed van antagonistische bacteriën op de groei van ABC transporter disruptanten werd getest, toonden aan dat MgAtr2 bescherming biedt tegen antibiotica die door deze bacteriën worden geproduceerd. Alle in Hoofdstuk 5 gepresenteerde resultaten duiden erop dat ABC transporters van *M. graminicola* een rol spelen bij de bescherming van deze schimmel tegen toxische verbindingen.

Zowel de expressie analyse (Hoofdstuk 2) als de gist-complementatie testen (Hoofdstuk 5) wijzen erop dat azool fungiciden substraat kunnen zijn voor ABC transporters van *M. graminicola*. Daarom wordt in Hoofdstuk 6 de potentiële rol van ABC transporters in de gevoeligheid voor fungiciden nader onderzocht. *M. graminicola* mutanten met een verminderde gevoeligheid voor het azool fungicide cyproconazool werden geselecteerd en deze bleken een MDR fenotype te vertonen. De afgenomen gevoeligheid voor azolen bleek gecorreleerd te zijn met veranderingen in de accumulatie van cyproconazool in de cel, hetgeen duidt op betrokkenheid van ABC transporters bij de resistentie. Alle mutanten vertoonden veranderingen in het expressie patroon van één of meerdere ABC transporters. De veranderingen in gevoeligheid voor cyproconazool konden echter niet worden gerelateerd aan veranderingen in expressie van één specifiek ABC transporter gen. Het uitschakelen van *MgAtr1* in twee mutanten met een constitutieve overexpressie van *MgAtr1* leidde in slechts één van deze mutanten tot het herstel van de wild-type gevoeligheid voor cyproconazool. Deze resultaten duiden erop dat overexpressie van ABC transporters één van de mechanismen is die kunnen leiden tot resistentie tegen azool fungiciden in *M. graminicola*.

De resultaten van dit proefschrift tonen aan dat *M. graminicola* ABC transporters bevat met een overlap in substraatspecificiteit. Toxische verbindingen van natuurlijke oorsprong en xenobiotica kunnen als substraat fungeren. Dit wordt bevestigd door de waarnemingen dat ABC transporters van *M. graminicola* kunnen functioneren als virulentie factor en bescherming kunnen bieden tegen mycotoxines, bacteriële antibiotica en azool fungiciden. ABC transporters leveren dus een duidelijke bijdrage aan het succes van deze schimmel als pathogeen.

CURRICULUM VITAE

Lute-Harm Zwiers werd 6 maart 1967 geboren te Rotterdam. In 1985 behaalde hij zijn Atheneum-B diploma aan het Ubbo Emmius Lyceum te Stadskanaal en begon hij met zijn studie Biologie aan de Rijksuniversiteit Groningen. In 1991 behaalde hij zijn doctoraalexamen Biologie, met als afstudeerrichtingen moleculaire biologie van planten en cel en planten genetica. Vervolgens vervulde hij zijn vervangende dienstplicht bij het ATO-DLO te Wageningen. Binnen de cluster bio-conversie werkte hij daar aan de biochemische en moleculair biologische analyse van koude verzoeting bij de aardappel. Na afloop van de 16 maanden vervangende dienstplicht vervolgde hij dit onderzoek nog gedurende 2 jaar als toegevoegd onderzoeker. Van mei 1995 tot januari 2001 was hij als AIO en "pre-postdoc" verbonden aan het laboratorium voor Fytopathologie van Wageningen Universiteit. Het aldaar uitgevoerde onderzoek heeft geleid tot dit proefschrift en werd gefinancierd door achtereenvolgens Sandoz AG en Novartis Crop Protection AG. Sinds juli 2001 is hij aangesteld als post-doc bij het laboratorium voor Fytopathologie, Wageningen Universiteit en is werkzaam binnen het door de onderzoeksschool Experimentele Plant Wetenschappen (EPW) gefinancierde project: "Functional genomics of transporter genes in the wheat pathogen *Mycosphaerella graminicola*".

Nawoord

En dan is het eindelijk zover, het "boekje" is af!
Het werken aan een "nieuw" organisme, waaraan nog vrijwel geen moleculair-biologisch werk wordt verricht heeft zeker zijn charmes. Maar het kan ook leiden tot de nodige frustraties. In mijn geval vooral toen bleek dat het transformeren van *Mycosphaerella* nou niet bepaald makkelijk ging. En nog meer frustratie toen bleek dat de transformanten die ik dan eindelijk had, niet de gehoopte knock-out mutanten waren. De voldoening toen het uiteindelijk na ruim 3 jaar wel lukte om disruptanten te krijgen was dan ook erg groot. Dat zijn de momenten die alle ellende weer snel doen vergeten.

Gelukkig zijn er in de afgelopen jaren ook een heleboel mensen geweest die hetzij direct hetzij indirect een belangrijke bijdrage hebben geleverd aan het tot stand komen van dit boekje. Maarten, allereerst wil ik jou bedanken. Ik heb jouw manier van begeleiden altijd als zeer prettig ervaren, niet sturend maar wel zeer betrokken. Je vertrouwen in een goede afloop van mijn onderzoek is erg belangrijk geweest. Ook in de laatste fase van het schrijven heb je een belangrijke rol gespeeld. Mijn eerste versies die na een lange incubatietijd dan eindelijk op je bureau belandden wist je altijd, vaak al binnen een dag, te ontdoen van vele bijzinnen en daardoor een stuk leesbaarder te maken. Ik ben blij dat ik de komende tijd nog in de ABC-groep verder kan werken. Pierre, jouw rol als promotor speelde zich meer op de achtergrond af maar misschien waren je "related to this" vragen en opmerkingen daarom wel zo ter zake.

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Dames: Ik houd van jullie.

Lute-Harm

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