ABC transporters from *Botrytis cinerea* in biotic and abiotic interactions

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List of abbreviations

2,4-DAPG	2,4-diacetylphloroglucinol
ABC transporter	ATP-binding cassette transporter
ATP	adenosine 5'-triphosphate
DHA 12/14	drug-H^+ antiporter with 12 or 14 transmembrane regions
DMI	sterol demethylation inhibitor
DNOC	dinitro-*o*-cresol (2-methyl-4,6-dinitrophenol)
EC_{50}	effective concentration for 50% inhibition of growth
EST	expressed sequence tag
MDR	multidrug resistance [$(TMD_6\text{-}NBF)_2$ topology]
MFS	major facilitator superfamily
MRP	multidrug resistance-related protein [$TMD_4\text{-}(TMD_6\text{-}NBF)_2$ topology]
NBF	nucleotide binding fold
PCA	phenazine-1-carboxylic acid
PCN	phenazine-1-carboxamide
PDR	pleiotropic drug resistance [$(NBF\text{-}TMD_6)_2$ topology]
SOPP	sodium *o*-phenylphenate
TMD_6	transmembrane domain with six transmembrane spans

Chapter 1

General introduction

THE IMPACT OF DISEASES AND PESTS OF PLANTS

Worldwide, agricultural production chains depend on the capacity of crops to use inorganic compounds, carbon dioxide, water, and sunlight to make feed, food, fuel and fibre available to humans and animals. Plant diseases and pests affect the quantity and quality of crop yield, which has severe consequences to maintain a sufficient food supply for the growing human population. It is estimated that more than one third of the world crop production is lost by competition of weeds, damage by pests such as insects, rodents and birds, and diseases caused by plant pathogens such as viruses, bacteria and fungi (Oerke *et al.* 1994). This situation strongly requires continuous efforts to improve pest management by resistance breeding, cultural practices and crop protection with chemical or biological agents.

The investments made to boost crop yields, such as the increased use of fertilisers and the selection of crop varieties with high yields, have made crop protection more cost effective. Therefore, intensive farming is accompanied by a higher demand for crop protection chemicals. For instance, the use of fungicides increased from $\$2.7 \times 10^9$ in 1967 (Cramer 1967) to $\$5.6 \times 10^9$ in 1990 (Oerke *et al.* 1994). In 1995, the estimated value of the worldwide agricultural production was \$1,200 to 1,300 $\times 10^9$, of which actual losses caused by various pests, diseases and weeds amounted $\$500 \times 10^9$. The potential loss of another $\$330 \times 10^9$ was prevented by crop protection practises. The value of agrochemicals involved in crop protection amounted up to $\$26 \times 10^9$. To improve existing crop protection measures, a better understanding of plant-microbe interactions is essential.

THE PLANT PATHOGEN *BOTRYTIS CINEREA*

The wide-spread plant pathogenic fungus *Botrytis cinerea* Pers.:Fr is the causal agent of grey mould and infects fruits, flowers and green tissues of at least 235 plant species (Jarvis 1977). This pathogen causes serious pre- and post-harvest diseases in a wide variety of plants including agronomically important crops such as grapevine, tomato, strawberry, cucumber, beans, bulb flowers and ornamental plants (Coley-Smith *et al.* 1980). The name *Botrytis* is derived from βοτρυς, the Greek word for grape, since the fungus produces spores like bunches of grapes. *B. cinerea* is the asexual stage or anamorph of *Botryotinia fuckeliana* (De Bary) Whetzel, which is the teleomorphic stage of the pathogen. Under natural conditions, ascocarps are rarely produced. Therefore, the teleomorphic name is not commonly used. The asexual stage of *B. cinerea* is classified in the genus *Botrytis*, which belongs to the family of the *Moniliaceae*. The teleomorph is a member of the genus *Botryotinia* and belongs to the family of the *Sclerotiniaceae*. All pathogenic *Botrytis* species are necrotrophic, since plant cells are actively killed during pathogenesis. Common symptoms of grey mould are soft rot, fire blight and damping-off of seedlings (Prins *et al.* 2000a). Many *Botrytis* species are rather specific with a limited range of host plants (Jarvis 1977). For example, the host range of *Botrytis aclada* is restricted to onion and leek, while *Botrytis fabae* is only pathogenic on broad bean. In contrast, *B. cinerea* has a very broad host range, including hosts of the more specialised species.

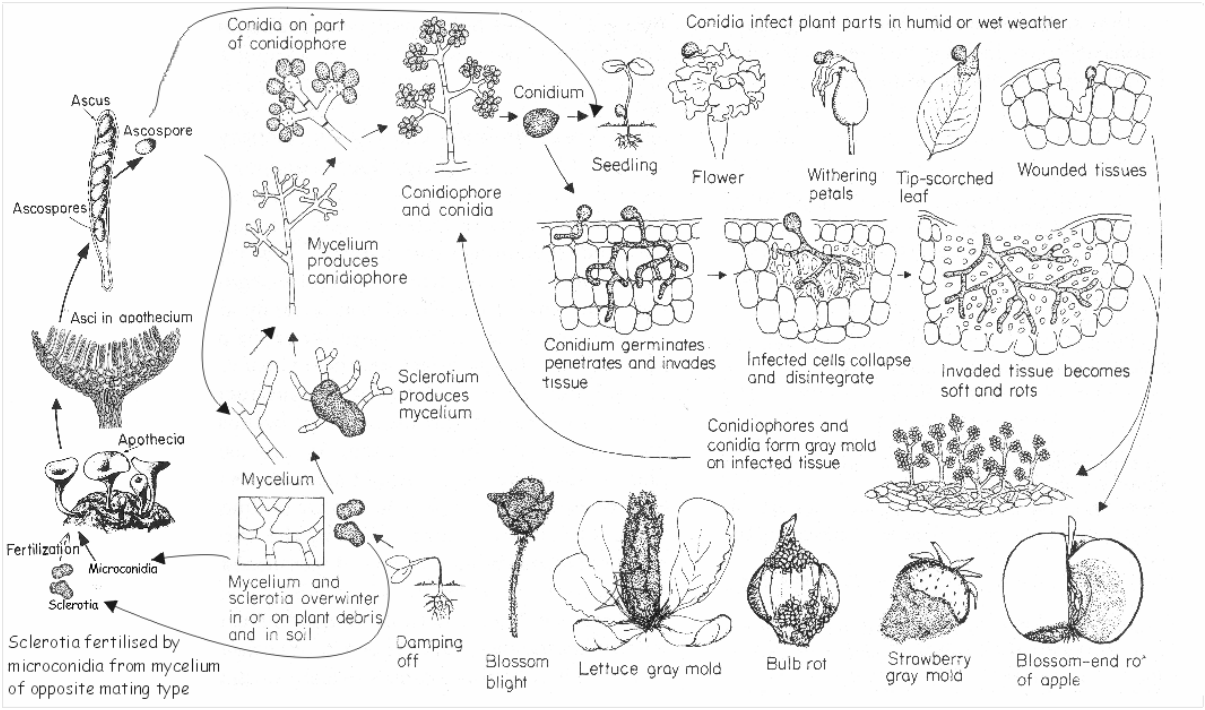

Figure 1. Overview of the lifecycle of *Botrytis cinerea*, adapted from Agrios (1997).

The life cycle of *B. cinerea* includes both a pathogenic and a saprophytic phase of growth (Fig. 1). In the saprophytic phase the fungus grows as mycelium on dead plant material and has to compete with other micro-organisms. The fungus can overcome unfavourable conditions as sclerotia. Damp and moderately cool weather (18-23 °C) favours growth, sporulation, spore release, spore germination, and infection. For this reason, the pathogen is important on crops grown in greenhouses where humidity is high. However, the fungus can also actively grow at low temperatures and even cause considerable losses of vegetables in cold storage (0-10 °C). Under moist conditions and in the presence of sufficient nutrients mycelium forms conidiophores, which bear the macroconidia organised as a bunch of grapes (Fig. 2).

B. cinerea is sometimes described as an opportunistic pathogen since infections commonly occur via wounds or previously colonised dead or senescent plant parts. Direct penetration of the intact plant cuticle is less common but may occur under high humidity conditions and in the presence of nutrients. In the latter case, penetration is often followed by a quiescent period during which disease progress is slow or even absent (Benito *et al.* 1998; Keller *et al.* 2003). Later, depending on the climate conditions and the developmental stage of the plant, primary lesions may expand into water-soaked or necrotic lesions. On the host surface white mycelium can be formed that turns grey with maturation of conidiophores bearing asexual conidia. The common name grey mould relates to these disease symptoms. Rapid spore formation yields abundant inoculum for a new infection cycle within a week.

VIRULENCE FACTORS OF *BOTRYTIS CINEREA*

A major issue in current research on *B. cinerea* is the identification of genes required for virulence. These genes may be important targets in resistance breeding and for the development of chemical or biological control agents. At the start of this project (1996), this type of research was still in its infancy. At that time, the number of virulence genes cloned from *B. cinerea* was limited. By 2003, the identified virulence genes include polygalacturonases, a MAP kinase, an ABC transporter, a G-protein, a laccase, and a pectin methylesterase (Table 1). The broad host range of *B. cinerea* most likely implies that the fungus possesses an arsenal of complementary virulence factors. These can include toxins, other hydrolytic enzymes and mechanisms to cope with plant defence mechanisms (Prins *et al.* 2000a). Phytotoxic compounds that play a role in pathogenesis are oxalic acid and botrydial (Colmenares *et al.* 2002; Germeier *et al.* 1994). Fungal enzymes that may be involved in infection and tissue colonization are cell wall degrading enzymes (CWDEs) such as endo- and exopolygalacturonases, endo- and exopectate lyases, pectin lyases, pectin methylesterases and, furthermore, proteases and phenoloxidases. The broad host specificity of *B. cinerea* may relate to the presence of multiple, degenerate gene families encoding some of these enzymes. The presence of multiple functional homologues could allow breakdown of cell wall tissues from various hosts under different conditions. This phenomenon has been validated for polygalacturonases (Ten Have *et al.* 1998). For instance, members of the endopolygalacturonase family are differentially expressed during pathogenesis on different types of tissue (Ten Have *et al.* 2001; Wubben *et al.* 2000). Enzymes involved in production of active oxygen species (AOS), such as glucose oxidase, may also play an important role in pathogenesis (Liu *et al.* 1998; Rolke *et al.* 2004; von Tiedemann 1997). AOS are produced in plant tissue away from the hyphae, where they may elicit a hypersensitive response (HR) (Muckenschnabel *et al.* 2001). In contrast to many (hemi)biotrophic pathogens, cell death incited by *B. cinerea* might facilitate growth of the pathogen, for example by release of nutrients (Govrin and Levine 2000). This strategy is especially effective since it operates in concert with enzymes involved in protection of the pathogen against oxidative stress such as peroxidases, laccases, catalases, gluthatione-S-transferase, glutathione peroxidase, and superoxide dismutase (Gil-ad *et al.* 2000; Schouten *et al.* 2002a).

The expression of genes required for pathogenicity on a certain host seems to be controlled by conditions encountered by *B. cinerea* in the invaded tissue. Specificity in expression patterns can, to a certain extent, be mimicked *in vitro*, by altering the pH and nutrient sources (Manteau *et al.* 2003; Ten Have *et al.* 2001; Wubben *et al.* 2000). Thus, the *in vitro* expression pattern can provide information about the functionality of genes during pathogenesis.

The ability to withstand toxic effects of plant defence compounds that act as constitutive or inducible chemical barriers (Osbourn 1999) can also contribute to the virulence of fungi (Delserone *et al.* 1999). *B. cinerea* possesses specific enzymes that degrade particular toxic plant compounds, such as α-tomatinase, that inactivates tomatin from tomato leaves (Quidde *et al.* 1998) and a laccase-like enzyme that inactivates resveratrol from grapevine (Adrian *et al.* 1998). The pathogen also possesses non-specific detoxification mechanisms such as glutathione-S-transferases (Marrs

1996; Prins *et al.* 2000b) and efflux pumps that can prevent the accumulation of multiple plant defence compounds in cells of the pathogen (Del Sorbo *et al.* 2000; Fleissner *et al.* 2002; Kolaczkowski *et al.* 1998; Schoonbeek *et al.* 2001). These non-specific mechanisms may be of particular relevance to *B. cinerea* since it has to cope with many, chemically unrelated, plant defence compounds in a broad host range.

Table 1. Genes involved in virulence of *B. cinerea*.

Gene	Encoded protein	Phenotype of mutants	Reference
Bcpg1	Endopolygalacturonase	Reduction in polygalacturonase activity and outgrowth of expanding lesions.	(Ten Have *et al.* 1998)
BMP1	MAP kinase	Reduction of growth rate on nutrient-rich medium; non-pathogenic on carnation flowers and tomato leaves.	(Zheng *et al.* 2000)
BcatrB	ABC transporter	Increased sensitivity to various fungitoxic compounds, including resveratrol; reduced virulence on grapevine.	(Schoonbeek *et al.* 2001)
Bcg1	G protein α-subunit	Altered colony morphology, no secretion of extracellular proteases; infection process stops after formation of primary lesions on bean and tomato.	(Schulte Gronover *et al.* 2001)
Bclcc2	Laccase	Resveratrol is not activated into a more fungitoxic metabolite.	(Schouten *et al.* 2002b)
Bcpme1	Pectin methylesterase	Reduced growth on pectin medium; reduced virulence on apple fruits, grapevine and *Arabidopsis thaliana* leaves.	(Valette-Collet *et al.* 2003)

DISEASE CONTROL OF *BOTRYTIS CINEREA*

This fungus is an ubiquitous pathogen present in many agricultural crops in subtropical and temperate regions (Coley-Smith *et al.* 1980). Infection of crops requires a minimal amount of inoculum potential and, therefore, stringent disease control is required. Several approaches can be employed to limit crop losses caused by *B. cinerea* and related pathogens. These include breeding of plant varieties with reduced susceptibility, cultural practices, biological control, and chemical control.

Figure 2. *Botrytis cinerea* conidiophores and conidia on infected tomato leaf.

Plant breeding

Susceptibility and resistance of crop plants to *B. cinerea* appears to be a polygenic trait (Diaz *et al.* 2002; Jarvis 1977). This implies a considerable variation in *Botrytis* susceptibility amongst different varieties, which cannot be attributed to a single characteristic. A common strategy to breed for resistance is to cross susceptible crop plants with resistant relatives, to introduce quantitative trait loci associated with *B. cinerea* resistance. Another approach is the generation of transgenic crops that overproduce phytoalexins with a known activity against *B. cinerea*. Transgenic tobacco expressing the grapevine stilbene synthase produced resveratrol and displayed increased resistance (Hain *et al.* 1993). It might be that this approach is also valid for other crops, although transgenic expression of this gene in tomato did increase resistance to *Phytophtora infestans* but not to *B. cinerea* (Thomzik *et al.* 1997).

Cultural methods

Good agricultural practice is very important in control of grey mould, of which only a limited account is given here. A common practice is sanitation to reduce sources of inoculum. This can be achieved by starting with clean material and keeping pruned plant material away from the crop. This practice is particularly useful in greenhouses. Another important practice is to reduce the length of leaf wetness periods, which is essential for spore germination and penetration. This can be realised by increasing plant distance, trimming of the canopy, ventilation, and control of temperature and relative humidity.

Biological control and induced resistance

Biocontrol is based on the application of competitive or parasitic micro-organisms (Buck 2002; Elad 1996; Kessel *et al.*; Paulitz and Belanger 2001). These may compete for space or nutrients (Buck 2002), produce antagonistic antibiotics (Janisiewicz and Roitman 1988) or hyperparasitize mycelium (Shearer 1995). In practice, successful results have, for example, been obtained with *Ulocladium atrum* which antagonised *B. cinerea* by competition for nutrients (Kessel *et al.* 2002).

Biocontrol agents can also exert their function indirectly by stimulation of plant responses. Some *Pseudomonas* species are able to induce resistance in crop plants against infection by multiple pathogens, including *B. cinerea* (Audenaert *et al.* 2002; Pieterse *et al.* 2003). Various signalling pathways are involved in the activation of induced resistance, such as systemic acquired resistance (SAR) or induced systemic resistance (ISR) (Van Loon *et al.* 1998). These pathways depend in different degrees on signalling molecules such as salicylic acid, ethylene, and jasmonic acid (Diaz *et al.* 2002; Elad 1996; Pieterse *et al.* 2000; Ton *et al.* 2002). The induction of systemic resistance can also be triggered by application of salicylic acid or its homologue benzothiadiazole (Gorlach *et al.* 1996) and by β-aminobutyric acid (Jakab *et al.* 2001).

Chemical control

B. cinerea is one of the first recorded targets of agrochemicals ever. The Romans already applied elemental sulphur to control grey mould and mildew diseases in grapes. Another old protection method is the application of “Bordeaux mixture”, a mixture of copper salts with lime, first applied in vineyards of the Bordeaux area in France around 1885. Non-systemic fungicides such as aromatic hydrocarbons (*e.g.* chlorothalonil and PNCB), dithiocarbamates (*e.g.* thiram and mancozeb), dinitrophenols and chlorophenyls, introduced from 1950 onwards, were also used for control of grey mould, but their specificity was low. The next generation of fungicides used for control of grey mould diseases comprised systemic fungicides such as benzimidazoles (*e.g.* benomyl), dicarboximides (*e.g.* vinclozolin), triazoles (*e.g.* tebuconazole) and *N*-phenylcarbamates (*e.g.* diethofencarb) (Lyr 1995). From 1995 onwards, novel classes of fungicides with specificity against *B. cinerea* were commercialised. These included the anilinopyrimidines cyprodinil, pyrimethanil and mepanipyrim, the phenylpyrroles fludioxonil and fenpiclonil, and the hydroxyanilide fenhexamid (Debieu *et al.* 2001; Gullino *et al.* 2000; Rosslenbroich and Stuebler

2000). Major problems in *B. cinerea* control are the low field performance of azole fungicides and related compounds (Stehmann and De Waard 1996) and the remarkable ability of the pathogen to develop resistance to fungicides (Leroux *et al.* 2002; Rosslenbroich and Stuebler 2000).

Resistance development to fungicides is due to the emergence of fungicide resistant mutants in wild-type populations upon selection pressure of fungicides in space and time. Resistance mechanisms are commonly based on modifications of the target site of the fungicides in the pathogen, or detoxification of the compound by enzymes from the pathogen. Mutants resistant to one compound from a chemical class of compounds usually display cross resistance to compounds that have the same mode of action or the same functional group. Resistance to chemically unrelated compounds can be based on multiple resistance or multidrug resistance. Multiple resistance occurs when an organism sequentially develops resistance to compounds from different classes via independent mechanisms (Brent 1995). Multidrug resistance (MDR) to compounds from different classes is mediated by a single mechanism (VandenBossche *et al.* 1998).

In *B. cinerea* several genes involved in different mechanisms of fungicide resistance have been described. Resistance to benzimidazole fungicides is conferred by mutations at the *benA* locus that result in reduced affinity of β-tubulin to these fungicides (Park *et al.* 1997). Genes that confer resistance to benzimidazole, dicarboximides, or aninilopyrimidines fungicides are described as *benA* and/or *Mbc1*, *Daf*, and *AniR1* genes, respectively (Faretra and Pollastro 1991; 1993; Kalamarakis *et al.* 2000; Leroux *et al.* 1999). Strains with multiple resistance to two or three classes of these compounds have been reported repeatedly (Baroffio *et al.* 2003; Faretra and Pollastro 1991; 1993; Kalamarakis *et al.* 2000; Leroux *et al.* 1999; Petsikos-Panayotarou *et al.* 2003; Pollastro *et al.* 1996; Steel and Nair 1993; Yourman and Jeffers 1999; Ziogas and Girgis 1993). *B. cinerea* strains with multidrug resistance to various classes of chemicals have only been identified in France (Chapeland *et al.* 1999). In a survey for anilinopyrimidine resistance (AniR), strains were found that showed also resistance to phenylpyrroles (AniR2) or sterol C14-demethylation inhibitors (DMIs, AniR3), encoded by a single major gene (Chapeland *et al.* 1999).

MULTIDRUG RESISTANCE

A well described mechanism of multidrug resistance is increased active efflux activity by transporters from the ATP-binding cassette (ABC) superfamily and major facilitator superfamily (MFS) (Balzi and Goffeau 1995; Higgins 1993; Paulsen *et al.* 1996). These membrane bound transporters can provide energy-dependent transport of compounds from diverse chemical classes against a concentration gradient over a membrane. ABC transporters derive the energy to drive transport from hydrolysis of ATP (Holland and Blight 1999), while MFS transporters are dependent on the proton motive force (Paulsen *et al.* 1996). Transported compounds include inorganic cations and anions, neutral and anionic organic compounds, sterols, sugars, amino acids, peptides, and enzymes. Physiological functions of ABC and MFS transporters include maintenance of cell membrane integrity and cellular iron homeostasis, import of nutrients, presentation of antigenic peptides, and secretion of mating factors and enzymes. A widely described function is efflux of

endogenous and exogenous toxic compounds (Dean *et al.* 2001; Fath and Kolter 1993; Paulsen *et al.* 1996; Theodoulou 2000; Wolfger *et al.* 2001).

Efflux of fungitoxic compounds from mycelium results in reduced accumulation in fungal cells and, thus, in a relatively low concentration of the fungicide at its target site. This can quickly provide sufficient protection for the fungus to become insensitive or bide the fungus time to express other, more structural, protection mechanisms (De Waard 1997; Del Sorbo *et al.* 2000).

In the case of efflux-mediated multidrug resistance, efflux of compounds can be increased by elevated expression and activity of transporter proteins or by changes in substrate specificity. Elevated expression can be the result of gene amplification, modifications in the promoter of the drug transporter genes or changes in regulatory proteins (Cole *et al.* 1992; Lyons and White 2000; Rogers *et al.* 2001; Sanglard *et al.* 1995). A reduced turnover rate of mRNA and transporter protein increase the quantity of active protein (Egner *et al.* 1995). The substrate specificity of transporters can be altered by point mutations or truncations of the primary protein sequence (Egner *et al.* 2000; Van Bambeke *et al.* 2000).

The pleiotropic drug resistance (PDR) network in *S. cerevisiae* constitutes a group of proteins containing ABC and MFS transporters and their transcriptional regulators like PDR1, PDR3, yAP1, and other factors (Balzi and Goffeau 1995; Zhang *et al.* 2000). Mutations in either the promoter of the transporter genes or in the regulatory genes have an effect on the expression levels of the transporters (DeRisi *et al.* 2000; Katzmann *et al.* 1996). Upregulation of transporter gene expression is a common phenomenon in azole resistant *Candida* isolates (Sanglard *et al.* 1998). Point mutations in PDR5 from *S. cerevisiae* affect both substrate specificity and inhibitor sensitivity (Egner *et al.* 2000). In animal and human pathology active efflux of antibiotics is considered an important mechanism in multidrug resistant clinical isolates of bacteria, fungi and protozoa (Alexander and Perfect 1997; Guilfoile and Hutchinson 1991; Moore *et al.* 2000; Nikaido 1994; Peel 2001; Sanglard *et al.* 1998; VandenBossche *et al.* 1998). However, MDR is thought to be of limited importance in plant pathogens (Del Sorbo *et al.* 2000; Leroux *et al.* 2002; Stergiopoulos *et al.* 2002). Extensive studies with the model fungus *Aspergillus nidulans*, demonstrate that ABC transporters can be responsible for efflux-mediated fungicide resistance of laboratory-generated mutants (Andrade *et al.* 2000; De Waard and Van Nistelrooy 1980). MDR mediated by active efflux has also been demonstrated in azole-resistant laboratory-generated mutants of *Penicillium italicum* and *B. cinerea* (De Waard and Van Nistelrooy 1988; Hayashi *et al.* 2001; Stehmann and De Waard 1995; Vermeulen *et al.* 2001). Recently, MDR has been reported in field strains of *Penicillium digitatum* and *B. cinerea* (Chapeland *et al.* 1999; Nakaune *et al.* 1998).

OUTLINE OF THIS THESIS

The aim of this thesis is to analyse the putative role of ABC and MFS transporters of *B. cinerea* in pathogenesis, multidrug resistance and ecological competitiveness. A structural and functional analysis of fungal ABC and MFS transporters is described in chapter 2.

Two ABC transporter genes from *B. cinerea*, *BcatrA* and *BcatrB*, were identified by heterologous screening of a genomic phage library with a probe from the ABC transporter gene PDR5 from *S. cerevisiae*. Chapter 3 describes the cloning of *BcatrB*, its characterisation, regulation and functional analysis. BcatrB proved to play a role in virulence on grapevine and sensitivity to plant defence compounds and fungicides.

The role of BcatrB and other membrane-bound transporters in fungicide resistance was studied further by expression studies in a wild-type *B. cinerea* strain, and in strains resistant to anilinopyrimidine, azole or phenylpyrrole fungicides. The sequence of twelve additional ABC transporter and three MFS transporter genes was retrieved from an EST library. Chapter 4 describes the cloning, sequencing and characterisation of the corresponding genomic fragments. Sensitivity, expression and accumulation studies were performed to identify *BcatrB* as the ABC transporter gene involved in phenylpyrrole sensitivity.

Chapters 5 and 6 describe *BcatrD* as the gene involved in reduced sensitivity to azole fungicides. The role of BcatrD in oxpoconazole sensitivity was confirmed in sensitivity and accumulation studies with wild-type and laboratory-generated mutant strains with increased and reduced sensitivity to azole fungicides.

A putative role of ABC and MFS transporters from *B. cinerea* in efflux of toxic compounds and in pathogenesis was studied by analysing gene-expression after treatment of germlings with a range of plant and microbial fungitoxic compounds. Plant defence compounds proved to be potent inducers of expression of some ABC transporter genes. The role of BcatrB in protection against the plant secondary metabolite eugenol was studied in more detail *in vitro* and on basil plants, which can contain eugenol (Chapter 7).

The broad host range of *B. cinerea* includes plants that produce a wide variety of plant defence compounds that can induce ABC transporter gene expression. This observation may reflect a role of the induced ABC transporter in virulence, and may indicate that gene-replacement of induced ABC transporters affects the virulence on host plants producing these compounds. However, results presented in Chapter 8 describe that gene-replacement mutants of single ABC transporter genes do not exhibit impaired virulence of *B. cinerea* on a number of *Solanaceaous* and *Leguminous* plant species.

Chapter 9 describes the role of ABC transporters in ecological competitiveness by testing the sensitivity of ABC transporter mutants of *B. cinerea* to antagonistic bacteria and the antibiotics they produce. Expression and accumulation studies indicate that BcatrB provides protection to

phenazine antibiotics and contributes to virulence of *B. cinerea* on tomato in the presence of *Pseudomonas* strains that produce phenazine antibiotics.

The results of the studies are summarised and discussed in Chapter 10. The relevance of expression studies and homology alignments to elucidate the physiological role of particular transporters are also discussed. Furthermore, the role of BcatrB as a broad-spectrum multidrug transporter is compared with that of homologous proteins from related fungi.

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Chapter 2

Fungal transporters involved in efflux of natural toxic compounds and fungicides

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ABSTRACT

Survival of microorganisms in natural environments is favoured by the capacity to produce compounds toxic to competing organisms, and the ability to resist the effects of such toxic compounds. Both factors contribute to a competitive advantage of organisms in ecosystems. All organisms have evolved active transport mechanisms by which endogenous and exogenous toxicants can be secreted. Two major classes of transporter proteins are the ATP-Binding Cassette (ABC) and the Major Facilitator Superfamily (MFS) transporters. Members of both classes can have broad and overlapping substrate specificities for natural toxic compounds, and can be regarded as a "first-line defence barrier" in survival mechanisms. In plant pathogens, these transporters can play an essential role in protection against plant defence compounds during pathogenesis. In addition, some transporters actively secrete host and non-host-specific toxins. Remarkably, ABC and MFS transporters can also play a major role in fungicide sensitivity and resistance. Their role in multidrug resistance of *Aspergillus nidulans, Candida albicans,* and *Saccharomyces cerevisiae* to azoles and other fungitoxic compounds is well established. Knowledge of ABC and MFS transporters opens possibilities to develop novel strategies to control plant diseases, either by modulation of transporter activity or by transgenic expression of transporter genes in plants.

INTRODUCTION

In the everlasting struggle for life, the ability of microorganisms to synthesize and secrete compounds toxic to competing organisms, and the ability to avoid noxious effects of such biotic toxicants in the environment, constitute precious weapons for successful survival and reproduction. These toxic compounds may have a qualitative or quantitative mechanism of action. A qualitative mechanism to cope with toxins is the absence of sensitive target sites. Quantitative mechanisms relate to a differential sensitivity of the target site in different organisms, and to differences in concentration of the toxin that can be build up at the target site. The toxin concentration at the target site is determined by factors such as uptake, transport, storage and metabolism of the toxicant. A most appealing discovery made in the last decades is that microorganisms, including the lower eukaryotes, have membrane efflux systems that can transport toxic compounds over plasma membrane or membranes that separate different cell compartments. The toxic substrates may be of endogenous or exogenous origin. In all instances toxin concentrations in certain cell compartments or the cytoplasm is lowered. This can result in reduced toxin concentrations at the target site and, hence, in protection of the organism. Remarkably, similar transporter systems can also play a role in sensitivity and multidrug resistance to fungicides in filamentous fungi (De Waard and Van Nistelrooy 1979; 1980; De Waard *et al.* 1996; Del Sorbo *et al.* 1997).

De Waard (1997) postulated that in plant pathogens the natural function of the transporters can be the secretion of endogenous pathogenicity factors (*e.g.* toxins) and exogenous plant defence compounds (Figure 1). This prediction has been validated during the last few years. A large amount of knowledge has been acquired on active secretion processes in lower eukaryotes which indicates

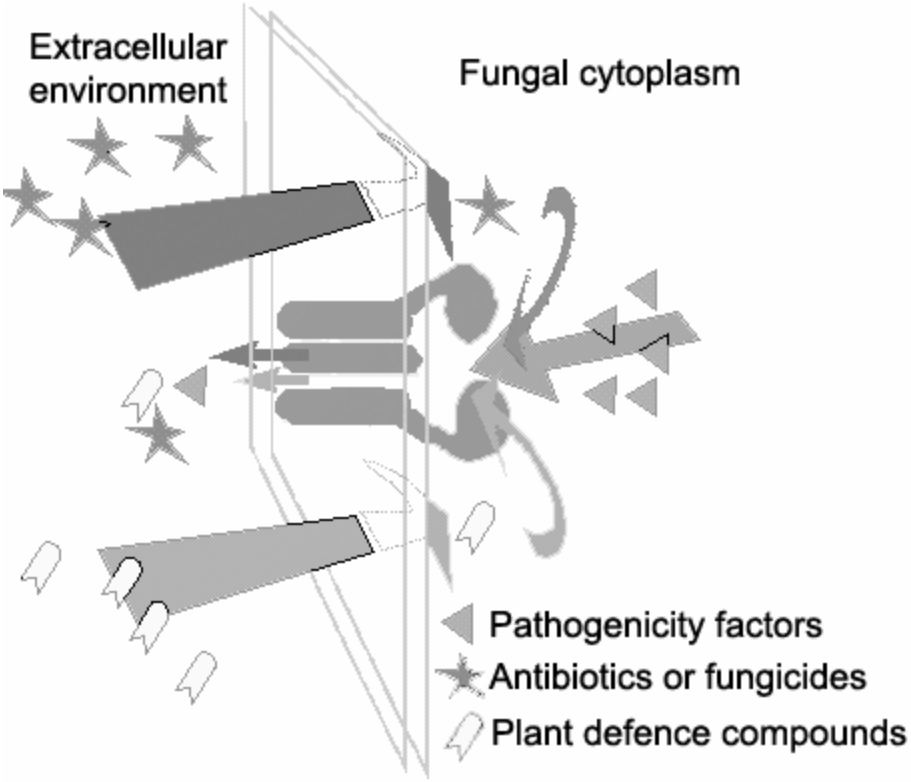

Figure 1. Cartoon demonstrating functions of fungal membrane transporters during plant pathogenesis.

that toxin transporters are involved in secretion of antibiotics, host-specific and non-host-specific toxins, mycotoxins, plant defence compounds and fungicides.

In fungi, as in all other organisms, various families of integral membrane proteins can mediate transport of natural toxic compounds over biological membranes. The two families that play a major role in these transport processes are the ATP-Binding Cassette (ABC) and the Major Facilitator Superfamily (MFS) of transporters (Figure 2). ABC transporters are able to bind and hydrolyse nucleotide triphosphates (mainly ATP) and use the energy generated to transport solutes across cell membranes. This can occur even against an electrochemical gradient. For this reason, ABC transporters are regarded as primary active transporter systems. They comprise the largest number of efflux pumps and account for transport of a great number of endogenous or exogenous toxicants (Higgins 1992). MFS transporters do not hydrolyse ATP. Transport of compounds by MFS transporters over membranes is driven by the proton-motive force, which is composed of the membrane potential and electrochemical proton gradient. For this reason MFS transporters are indicated as secondary active transport systems (Lewis 1994). More than 350 uni-, sym-, and anti-porters of sugars, peptides, drugs and organic and inorganic ions fall within this superfamily (Pao *et al.* 1998).

This review will focus on membrane transporters involved in transport of natural toxic compounds and fungicides in fungi, and their significance in biology, plant pathogenesis and fungicide resistance.

MODULAR ARCHITECTURE AND SEQUENCE HOMOLOGIES WITHIN FAMILIES OF TRANSPORTERS

Typical ABC transporters of fungi (Figure 2) contain two intracytoplasmic regions, which both harbour nucleotide binding domains (NBF) responsible for binding and hydrolysis of ATP, and two prevalently hydrophobic regions, each containing 6 transmembrane domains (TMD_6). These transporters are encoded by a single gene (Higgins 1992). This structure is typical for all eukaryotic ABC transporters. In the most common subfamilies of fungal ABC transporters, these four domains have a $(TMD_6\text{-}NBF)_2$ or a $(NBF\text{-}TMD_6)_2$ topology. These types of ABC transporter is composed of 1300-1600 amino acid residues, and its molecular mass is between 140 and 170 kDa. Other subfamilies of fungal ABC transporters are only made of two modules (*i.e.* $NBF\text{-}TMD_6$), and are described as "half-sized" transporters. These are thought to be functional after assembly into homodimers or heterodimers.

As for all other ATPases, the NBF domains of ABC transporters contain the conserved Walker A and Walker B motifs (Walker *et al.* 1982). In addition, a consensus sequence, (L,V)SGG-$(X)_3$-R-hydrophobic residue-X-hydrophobic residue-A, known as ABC signature or C motif, preceding the Walker B motif, is present. Unlike other ATPases, the Walker motifs of ABC transporters are separated by 90-120 amino acid residues (Hyde *et al.* 1990). Some additional regions of modest conservation are present in the "center region" of the NBF, *i.e.* about midway between Walker A and B motifs. There is only a low level of homology between the membrane domains of different ABC transporters, even between transporters that share an overlap in substrate specificity.

A MFS transporter is typically composed of 400-800 amino acid residues with 12 or 14 transmembrane spans and has a molecular mass of 45-90 kDa. Based on sequence homology and functional characteristics, Pao *et al*. (1998) described 17 MFS families. Only two families function as drug-H^+ antiporters. These are Drug H^+ Antiporter (DHA) 14 and DHA12 with 14 and 12 transmembrane spans, respectively, (Paulsen *et al.* 1996). Unlike ABC transporters, MFS transporters (Figure 2) have no characteristic signature. However, a 13-residue consensus motif present between the transmembrane spans 2 and 3 has been identified by Pao *et al*., (1998). Significant sequence homology occurs among all members of the superfamily, especially for members of the same family (Pao *et al.* 1998).

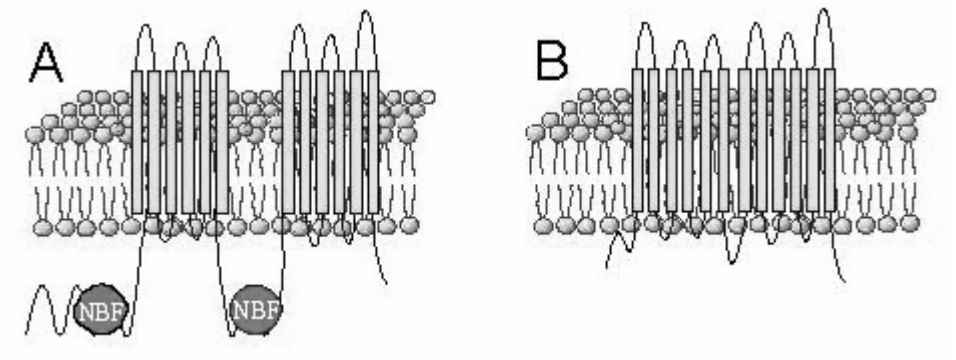

Figure 2. Representation of an ABC transporters with a (NBF-TMD6)2 topology (A) and a MFS transporter with 12 TMDs (B).

ABC TRANSPORTERS

Saccharomyces cerevisiae

Two independent analyses of the entire yeast genome revealed the presence of 30 (Taglicht and Michaelis 1998) or 29 (Decottignies and Goffeau 1997) open reading frames (ORFs) encoding putative ABC proteins. On basis of their topology they can be subdivided in six subfamilies or clusters. To date, the role of most of these transporters remains obscure, as their function has only been analyzed by examining viability of null mutants. We will therefore discuss only the ABC transporters for which a biological function has been found (Table 1).

The best characterized ABC transporters of *S. cerevisiae* are those involved in multidrug or pleiotropic drug resistance (MDR or PDR). This is the simultaneous resistance to a number of unrelated compounds. There are at least five plasma membrane-localized ABC transporters, which confer a PDR phenotype when overexpressed. These are Pdr5p, Snq2p, Pdr12p, Yor1p, and Ycf1p. Pdr5p, Snq2p and Pdr12p have the $(NBF\text{-}TMD_6)_2$ topology (Balzi *et al.* 1994; Bissinger and Kuchler 1994). The elucidation of the role of Pdr5p in MDR is a milestone in yeast transport biology. Overexpression of *PDR5* causes resistance to hundreds of chemically unrelated drugs (Kolaczkowski *et al.* 1996). These include many classes of clinical antimycotics and agricultural fungicides (*e.g.* anilinopyrimidines, benzimidazoles, dithiocarbamates, azoles, and strobilurin analogues). Resistance to antibiotics, mycotoxins, herbicides and anticancer drugs has also been described (Bissinger and Kuchler 1994; Kolaczkowski *et al.* 1996). Conversely, disruption of *PDR5* causes hypersensitivity to drugs and natural toxic compounds. Pdr5p has functional homology with the human P-glycoprotein, which is encoded by the *MDR1* gene. Overexpression of *PDR5* in mammals determines MDR to anticancer drugs, which can result in failure of tumor chemotherapy. Molecular characterization of *PDR5, MDR1*, and related genes has stimulated the analysis of structure-function relationships of multidrug transporter substrates.

Table 1. ABC transporter genes from fungi

Sub-family[1]	Gene	Acc. No.[2]	Size[3]	Protein topology[4]	Function	Key references
				Aspergillus flavus		
MDR	Aflmdr1	U62931	1307	$(TMD_6\text{-}NBF)_2$	Not known	(Tobin et al. 1997)
				Aspergillus fumigatus		
MDR	Afumdr1	U62934	1349	$(TMD_6\text{-}NBF)_2$	Resistance to cilofungin	(Tobin et al. 1997)
MDR	Afumdr2	U62936	791	TMD_6-NBF	Not involved in MDR	(Tobin et al. 1997)
-	ADR1	-	-	-	Up-regulated by itraconazole	(Slaven et al. 2002)
				Aspergillus nidulans		
PDR	AtrA	Z68904	1466	$(NBF\text{-}TMD_6)_2$	Not known	(Del Sorbo et al. 1997)
PDR	AtrB	Z68905	1426	$(NBF\text{-}TMD_6)_2$	Multidrug resistance	(Del Sorbo et al. 1997)
MDR	AtrC	AF071410	1284	$(TMD_6\text{-}NBF)_2$	Not known	(Andrade et al. 2000b)
MDR	AtrC2	AF082072	1293	$(TMD_6\text{-}NBF)_2$	Induced by cycloheximide	(Angermayr et al. 1999)
MDR	AtrD	AF071411	1348	$(TMD_6\text{-}NBF)_2$	Multidrug resistance	(Andrade et al. 2000b)
-	AbcA, AbcB, AbcC, AbcD			-	Not known	(Do Nascimiento et al. 1999)
				Botryotinia fuckeliana		
PDR	BcatrA	Z68906	1562	$(NBF\text{-}TMD_6)_2$	Up-regulated by cycloheximide	(Del Sorbo and De Waard 1996)
PDR	BcatrB	AJ006217	1439	$(NBF\text{-}TMD_6)_2$	Phenylpyrroles + stilbene resistance	(Schoonbeek et al. 2001)
				Candida albicans		
PDR	CDR1	X77589	1501	$(NBF\text{-}TMD_6)_2$	Multidrug resistance	(Prasad et al. 1995)
PDR	CDR2	U63812	1499	$(NBF\text{-}TMD_6)_2$	Multidrug resistance	(Sanglard et al. 1997)
PDR	CDR3	U89714	1501	$(NBF\text{-}TMD_6)_2$	Expressed in opaque-phase of growth	(Balan et al. 1997)
PDR	CDR4	AF044921	1490	$(NBF\text{-}TMD_6)_2$	Not involved in resistance to azoles	(Franz et al. 1998)
				Leptosphaeria maculans		
-	LmABC1	-	-		Multidrug resistance	(Taylor and Condie 1999)
-	LmABC2	-	-		Not involved in MDR	(Taylor and Condie 1999)
				Magnaporthe grisea		
PDR	ABC1	AF032443	1619	$(NBF\text{-}TMD_6)_2$	Essential for pathogenicity	(Urban et al. 1999)

			Mycosphaerella graminicola			
PDR	Mgatr1	AJ243112	1562	$(NBF\text{-}TMD_6)_2$	Up-regulated by cycloheximide and eugenol	(Zwiers and De Waard 2000)
PDR	Mgatr2	AJ243113	1499	$(NBF\text{-}TMD_6)_2$	Up-regulated by eugenol and imazalil	(Zwiers and De Waard 2000)
PDR	Mgatr3					(Zwiers et al. 1999)
PDR	Mgatr4					(Zwiers et al. 1999)
PDR	Mgatr5					(Zwiers et al. 1999)
			Penicillium digitatum			
PDR	PMR1	AB010442	1619	$(NBF\text{-}TMD_6)_2$	Resistance to azoles	(Nakaune et al. 1998)
			Saccharomyces cerevisiae[5]			
PDR	PDR5	L19922	1511	$(NBF\text{-}TMD_6)_2$	Multidrug resistance (=MDR)	(Balzi et al. 1994; Bissinger and Kuchler 1994)
PDR	PDR12	U39205	1511	$(NBF\text{-}TMD_6)_2$	C1-C7 organic acids resistance	(Piper et al. 1998)
PDR	PDR15	U32274	1529	$(NBF\text{-}TMD_6)_2$	Inducible upon stress	(Wolfger et al. 1999)
PDR	SNQ2	X66732	1501	$(NBF\text{-}TMD_6)_2$	Multidrug resistance	(Servos et al. 1993)
ALDP	PXA1	Z73503	758	$TMD_6\text{-}NBF$	Required for β-oxidation of fatty acids	(Shani and Valle 1996)
ALDP	PXA2	Z28188	853	$TMD_6\text{-}NBF$	Required for β-oxidation of fatty acids	(Shani and Valle 1996)
MRP[6]	BAT1	Z73153	1661	$(TMD_6\text{-}NBF)_2$	Bile acid transporter	(Ortiz et al. 1997)
MRP	YCF1	Z48179	1515	$(TMD_6\text{-}NBF)_2$	Multidrug and heavy metals resistance	(Szczypka et al. 1994)
MRP	YOR1	Z73066	1477	$(TMD_6\text{-}NBF)_2$	MDR	(Katzmann et al. 1995)
MDR	ATM1	Z49212	690	TMD6-NBF	Mitochondrial DNA mainteinance	(Leighton and Schatz 1995)
MDR	STE6	Z28209	1290	$(TMD_6\text{-}NBF)_2$	Secretion of the a mating factor	(McGrath and Varshavsky 1989)
YEF3	YEF3	U20865	1044	$(NBF)_2$	Interaction with aminoacyl-tRNA	(Sandbaken et al. 1990)
YEF3	GCN20	D50617	752	$(NBF)_2$	Interaction with tRNA and Gcn2p	(Vasquez de Aldana et al. 1995)
			Schizosaccharomyces pombe			
PDR	Abc1	Y09354	1427	$(TMD_6\text{-}NBF)_2$	unknown	(Christensen et al. 1997b)
MDR	Mam1	U66305	1336	$(TMD_6\text{-}NBF)_2$	secretion of mating factor	(Christensen et al. 1997a)
PDR	bfr1	S76267	1530	$(NBF\text{-}TMD_6)_2$	Multidrug resistance	(Nagao et al. 1995)
MDR	pmd1	D10695	1362	$(TMD_6\text{-}NBF)_2$	Multidrug resistance	(Nishi et al. 1992)

[1]Names of subfamilies based on sequence similarity with human (ALDP, MRP/CFTR, MDR) or yeast (PDR, YEF3) ABC transporters.

[2] Genbank accession number.

[3] Number of amino acid residues.

[4] NBF: nucleotide binding fold. TMD_6: transmembrane domain with six transmembrane spans.

[5] For *S. cerevisiae* only genes with an identified biological function have been listed.

[6] MRP: MRP/CFTR subfamily.

Snq2p confers resistance to 4-nitroquinoline-N-oxide and other toxicants (Servos *et al.* 1993). It has overlapping but distinct substrate specificity to Pdr5p. Pdr12p is specialized for transport of C1-C7 organic acids, such as sorbic, benzoic, and propionic acid, which are used as food preservatives (Holyoak *et al.* 1999; Piper *et al.* 1998). Yor1p and Ycf1p have the $(TMD_6\text{-}NBF)_2$ domain organization and are considered as analogues of the human ABC transporters CFTR (Cystic Fibrosis Transmembrane conductance Regulator) and MRP1 (Multidrug Resistance-related Protein 1). They were identified for their ability to confer resistance to oligomycin (Katzmann *et al.* 1995) and cadmium (Szczypka *et al.* 1994; Wemmie and Moye-Rowley 1997) respectively. Ycf1p is located on vacuolar membranes and able to transport glutathione-S-conjugates. Yor1p and Ycf1p have wide substrate specificities which partially overlap that of Pdr5p. Structure-function relationships of Ycf1p has contributed to understanding of cystic fibrosis, a serious human disease. *PDR5*, *SNQ2, YOR1* and other ABC transportes from yeast with unknown functions (*i.e. PDR10*, *PDR11* and *PDR15*) share common transcriptional activators, which are all zinc-finger binding proteins. These transcriptional activators are the products of the regulatory genes *PDR1* and *PDR3*, and constitute co-regulated circuits of the so-called PDR network (Balzi and Goffeau 1995). They also regulate transcription of additional genes not encoding ABC transporters, such as *HXT11* and *HXT9*, which are involved in glucose uptake (Nourani *et al.* 1997). Recently, genome microarray analysis was used to identify targets of Pdr1p and Pdr3p in *PDR1-3* and *PDR3-7* mutants with a PDR phenotype (DeRisi *et al.* 2000). In total, 49 targets with altered expression were identified. Most overexpressed targets are new and have an unknown function. Therefore, microarray analysis will certainly further unravel the network of MDR genes in yeasts and filamentous fungi. Other regulators of *PDR5* (Miyahara *et al.* 1996) and *YCF* (Wemmie *et al.* 1994) are y*AP-1* and *yAP-2*, encoding leucine zipper dimerization domains.

The debate on physiological functions of PDR type transporters, such as *PDR5* and *SNQ2*, is still open. Their null mutants are viable and show no phenotype in the absence of toxic compounds. It has been proposed that their products have a role in efflux of catabolites during stationary growth (Egner and Kuchler 1996). Alternatively, they could be involved in active secretion of endogenous aminophospholipids towards the outer layer of the plasma membrane, and thus contribute to maintaining the asymmetrical distribution of these compounds in the lipid bilayer (Pomorski *et al.* 1999).

A clear physiological function has been demonstrated for the product of the *STE6* gene. Ste6p, has a $(TMD_6\text{-}NBF)_2$ topology and is responsible for the secretion of the mature lipopeptide mating pheromone **a** (Kuchler *et al.* 1989; McGrath and Varshavsky 1989). *STE6* null mutants are unable to secrete the pheromone and are, therefore, sterile. Ste6p has a high similarity with mammalian P-glycoprotein and yeast PDR network members involved in MDR. *STE6* null mutants can be partially complemented by the mouse *MDR3* gene, encoding a P-glycoprotein involved in MDR (Raymond *et al.* 1992). However, Ste6p has no role in drug resistance. In fact, overexpression of *STE6* only confers resistance to FK520, a drug structurally related to the **a** pheromone (Raymond *et al.* 1994). Functional analogues of Ste6p involved in mating pheromones transport are likely to

occur in other species of filamentous fungi, and hence, may have important functions in epidemics of plant pathogens with a sexual cycle.

Another ABC transporter playing a role in yeast biology is Atm1p, a half-sized ABC transporter located in the inner mitochondrial membrane. Unlike most genes encoding ABC transporters, deletion of this gene has a clear phenotype. Strains deleted for Atm1p cannot grow on minimal medium, lack cytochromes in mitochondria, and possess instable mitochondrial DNA (Leighton and Schatz 1995). *PXA1* and *PXA2* also encode half-sized transporters with the TMD_6-NBF topology. These transporters are localized in the peroxisomal membranes and seem to be involved in β-oxidation of long chain fatty acids. *PXA1* and *PXA2* null mutants are unable to growth in media with oleate as the sole carbon source (Shani and Valle 1996). *PXA1* and *PXA2* are also of interest as they show similarity to the human genes *ALDP* and *PMP70*, which are associated with adrenoleukodistrophy and the Zellweger syndrome, respectively.

Schizosaccharomyces pombe

In the fission yeast *Schizosaccharomyces pombe* two genes encoding ABC transporters, *bfr1*+ and *pmd1*+, are responsible for PDR (Nagao *et al.* 1995; Nishi *et al.* 1992). *Bfr1*+ has a $(NBF\text{-}TMD_6)_2$ topology and, upon overexpression, confers resistance to a number of compounds including actinomycin, brefeldin A, cerulenin, and cytochalasin B. *Pmd1*+ has the $(TMD_6\text{-}NBF)_2$ topology and, upon overexpression, confers resistance to leptomycin B and other drugs. Recently, *mam1*, another gene encoding an ABC transporter with the $(TMD_6\text{-}NBF)_2$ topology has been characterized. The *Mam1* gene product is responsible for secretion of the mating pheromone M. Hence, this protein is the d functional counterpart of Ste6p (Christensen *et al.* 1997a). Several other genes or domains encoding ABC transporters from *S. pombe* have been cloned. However, their functions remain to be elucidated.

Candida albicans

Membrane efflux pumps of the dimorphic fungus *Candida albicans* have received much attention because of the increasing problem of drug resistance in treatment of candidiasis (VandenBossche *et al.* 1998). Five genes (*CDR1*, *CDR2*, *CDR3*, *CDR4* and *CDR5*), encoding ABC transporters, with the $(NBF\text{-}TMD_6)_2$ topology, have been described. *CDR1* seems to play a major role in resistance to fluconazole and miconazole, the two most common azole antimycotics used to treat candidiasis (Prasad *et al.* 1995; Sanglard *et al.* 1995). *CDR1* complements hypersensitivity to miconazole and other drugs in *S. cerevisiae PDR5* null mutants. Its level of expression is relatively high in most isolates resistant to fluconazole. Besides its role in resistance to azoles, *CDR1* also confers resistance to allylamines, morpholines, and several other drugs (Sanglard *et al.* 1997). *CDR2* is also involved in multidrug resistance. Like *CDR1*, *CDR2* is able to complement azole and allylamine hypersensitivity in *S. cerevisiae PDR5* null mutants. In contrast, disruption of *CDR2* in wild-type isolates of *C. albicans* does not increase drug sensitivity. Disruption of *CDR2* in Δ*CDR1* mutants, however, does increase drug sensitivity. This indicates that disruption of *CDR2* can be compensated

by expression of other multidrug transporter genes with a similar function. Cdr3p has a high level of identity with Cdr1p (56%) and Cdr2p (55%) but does not play a role in MDR. This gene is only expressed during the opaque phase in the life cycle of *C. albicans* but its function is still unknown (Balan *et al.* 1997).

Aspergillus spp.

The putative products of *atrA* and *atrB* from *A. nidulans* encode ABC transporters which share with yeast Pdr5p the $(NBF\text{-}TMD_6)_2$ topology. The role of *atrB* MDR was demonstrated by its ability to functionally complement a yeast *PDR5* null mutant for resistance to cycloheximide and other drugs (Del Sorbo *et al.* 1997). Transformation of a Δ*PDR5 S cerevisiae* strain with *atrA* did not alter the drug sensitivity profile. Expression of *atrB* was strongly enhanced after exposure of *A. nidulans* to several unrelated drugs, antibiotics, and plant defence compounds (Del Sorbo *et al.* 1997), suggesting a role in defence against natural toxic products. Increase in transcript levels of *atrA* coincides with azole efflux activity from germlings (De Waard and Van Nistelrooy 1979; 1980), suggesting a causal relationship between these processes. Currently, the substrate specificity of AtrB is studied by complementation of different knock-out mutants of *S. cerevisiae* (Ruocco *et al.* 1998), and by gene disruption and overexpression in *A. nidulans* (Andrade *et al.* 2000a). Recent results indicate that *atrB* is involved in sensitivity and resistance to all major classes of fungicides and some natural toxic compounds. Hence, *atrB* is probably a functional analogue of yeast *PDR5*. Imazalil induces transcription of *atrA* (Del Sorbo *et al.* 1997). However, in recent Northern analysis experiments no expression of *atrA* was detected in either unexposed or imazalil-treated germlings. *AtrC* and *atrD* encode ABC transporters with a $(TMD_6\text{-}NBF)_2$ topology (Andrade *et al.* 2000b). Functional analysis of these genes by gene disruption and overexpression revealed that *atrD* is involved in protection against a range of toxic compounds. An EST library of *A. nidulans* (Aramayo, dbEST Id:1703247) indicates that the genome of this fungus contains additional ABC genes. One is *atrC* (Angermayr *et al.* 1999), renamed as *atrC2*, which is strongly induced by cycloheximide.

Attempts have been undertaken to clone drug transporter genes from *A. flavus* and *A. fumigatus,* two important human pathogens, because increased expression of ABC transporters in these fungi might be associated with resistance to antimycotics. Two ABC transporter genes from *A. fumigatus*, *AfuMDR1* and *AfuMDR2,* and one ABC transporter gene from *A. flavus*, *AflMDR1*, have been cloned. Their encoded products all have the $(TMD_6\text{-}NBF)_2$ topology. A functional role has been found only for *AfuMDR1.* When expressed in *S. cerevisiae*, it confers resistance to cilofungin, an echinocandin analogue that inhibits the synthesis of cell wall components (1,3)-β-D glucans (Tobin *et al.* 1997). Recently, a third ABC gene from *A. fumigatus, ADR1,* has been discovered. Its expression is up regulated upon treatment with itraconazole. Itraconazole-resistant isolates show a high basal level of expression (Slaven *et al.* 1999). These results suggest that *ADR1* is involved in sensitivity and resistance to azole antimycotics.

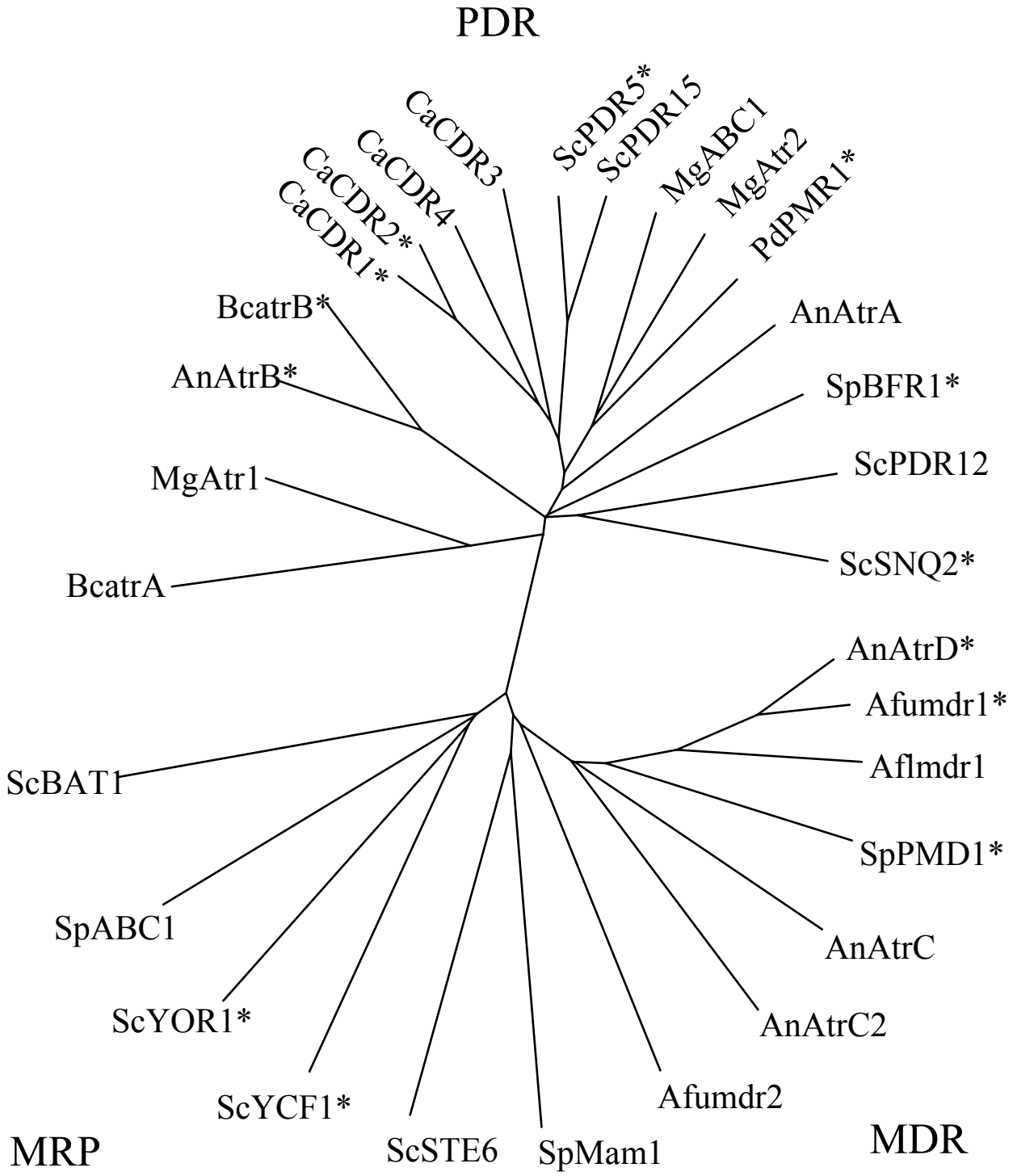

Figure 3. Dendrogram of 30 fungal ABC transporter proteins. The multiple alignment has been generated by the ClustalX program (Thompson et al. 1997). The multiple alignment mode was used with the gonnet 250 series as protein weight matrix, applying the following default parameters: gap opening penalty: 10, gap extension penalty: 0.20 and a 1000 times bootstrap. The code names of the proteins are composed of the gene name preceded by the initials of the species name (Table 1).

* Proteins with a role in protection against toxicants.

Plant pathogenic fungi

Studies on the function of ABC transporters in plant pathogens and plant-pathogen interactions are hot topics in phytopathology because they can function in the secretion of pathogenicity factors and in protection against plant defence compounds (Figure 2). Such a role has clearly been demonstrated for the transporter encoded by the gene *ABC*1 from *Magnaporthe grisea.* This gene was identified by insertional mutagenesis and screening of mutants for loss of pathogenicity. Abc1p has the $(NBF\text{-}TMD_6)_2$ topology and is strongly induced by azole fungicides and the rice phytoalexin sakuranetin. *ABC1* null mutants of *M. grisea* are non-pathogenic on rice and barley, thus indicating a necessary role of the gene during the infection process. The mutants penetrate epidermal cells normally, but die during initial colonization of host tissue. This suggests that the mutants have an increased sensitivity to a plant defence product. However, Δ*ABC1* mutants are not hypersensitive to any of the antifungal compounds (including sakuranetin) tested, and sensitivity to a number of fungicides also was unchanged. Therefore, the substrate specificity of ABC1 remains unsolved (Urban *et al.* 1999).

The ABC transporter genes *BcatrA* and *BcatrB* have been cloned from *Botryotinia fuckeliana*. The encoded proteins have a $(NBF\text{-}TMD_6)_2$ topology. Both genes show a low basal level of expression in germlings grown in liquid cultures. Treatment of germlings with cycloheximide, and to a minor extent with hydrogen peroxide, strongly induce transcription of *BcatrA* (Del Sorbo and De Waard 1996). The stilbene defence compound resveratrol from grapevine, as well as the phenylpyrrole fungicides fenpiclonil and fludioxonil, induce expression of *BcatrB* (Schoonbeek *et al.* 2001). Functional analysis of both genes has been carried out by targeted disruption. Disruptants of *BcatrA* do not display a clear phenotype with regard to fungicide sensitivity and virulence on some common host plants of the pathogen (Del Sorbo *et al*., unpublished data). However, disruption of *BcatrB* causes increased sensitivity to resveratrol and phenylpyrrole fungicides and a lower virulence on grapevine leaves (Schoonbeek *et al.* 2001). Hence, *BcatrB* seems to play a role in both pathogenesis and fungicide sensitivity. Additional genes encoding ABC transporters of *B. fuckeliana* have been identified in an EST library and are presently analyzed.

A search for ABC transporters from the wheat pathogen *Mycosphaerella graminicola*, yielded five genes, designated *Mgatr1-Mgatr5*. Expression studies revealed that cycloheximide and eugenol up-regulate *Mgatr1* in both yeast-like and mycelial growth stages. Increased transcript levels of *Mgatr2* were only observed after prolonged exposure to eugenol and imazalil. Up-regulation of *Mgatr2* was also noted upon exposure of mycelium to progesterone and palmitic acid (Zwiers and De Waard 2000). These results indicate that *Mgatr1* and *2* are differentially induced in the two morphological states of the fungus (Zwiers *et al.* 1999). The putative role of the five genes in virulence and fungicide resistance is under investigation.

LMABC1 and *LMABC2* are ABC transporter-encoding genes cloned from *Leptosphaeria maculans*, a pathogen on crucifers. The encoded proteins have the $(NBF\text{-}TMD_6)_2$ structure (Taylor and Condie 1999). *LMABC2* seems to be involved in MDR as it restores normal levels of sensitivity

towards cycloheximide and 4-nitroquinoline-N-oxide upon transfer to yeast *PDR5* and *SNQ2* null mutants. Unlike *LMABC2*, transfer of *LMABC1* to the same yeast strain does not cause any change in drug sensitivity. *LMABC1* could be involved in defence of *L. maculans* against *Brassica* phytoalexins since transcription of the gene is strongly up-regulated by methyl-4-chlorobenzyldithiocarbamate, an analogue of the *Brassica* phytoalexin brassinin. Although *LMABC1* is strongly up-regulated by miconazole in *L. maculans,* the gene does not restore normal levels of miconazole sensitivity upon expression in yeast PDR5 and SNQ2 null mutants. Functional analysis of both *LMABC1* and *LMABC2* in secretion of the phytotoxin sirodesmin has been hampered as so far strains disrupted in either of the two mentioned genes have not been obtained.

Transcription of the ABC transporter gene *PMR1* in the post-harvest fruit pathogen *Penicillium digitatum* is strongly induced by azole fungicides. Basal transcript levels of *PMR1* in azole-resistant isolates are relatively high and disruption of the gene in these strains restored azole sensitivity to almost wild-type level (Nakaune *et al.* 1998). These results prove that *PMR1* is important in resistance to azole fungicides in *P. digitatum*.

MFS TRANSPORTERS

Saccharomyces cerevisiae

Examination of the yeast genome database identified the presence of nine families of MFS transporters, including 28 ORFs of putative members of the MFS superfamily (Pao *et al.* 1998). Goffeau *et al*., (1997) grouped the 28 MFS transporters of *S. cerevisiae* in three clusters. Proteins classified in cluster I have12 putative TMDs (DHA12), whereas proteins classified in clusters II and III have 14 TMDs (DHA14). The only common conserved residues present in these three clusters of MFS transporters are the N-terminal motif N(E/D) $(X)_{2-4}$ GR and the W(R/S)W motif in the loop between TMD 5 and 6. In addition, each cluster has at least one typical conserved sequence. Members of cluster I have the PET motif in the loop between TMD 6 and 7, whereas members of cluster II have the GCILVPLTLA motif in TMD 9 (Goffeau *et al.* 1997). Members of cluster III contian the (I/L)GXX(I/L)(I/L)P motif in TMD 14. Functions of MFS transporters of cluster I remain largely unknown. They have sequence similarity with the MFS transporters Car1p from *Schizosaccharomyces pombe* (Jia *et al.* 1993), Bmrp or BenR from *C. albicans* (Fling *et al.* 1991), and Cyhrp from *Candida maltosa* (Sasnauskas *et al.* 1992). The latter transporters confer resistance to amiloride, benomyl and cycloheximide, and several other unrelated drugs (Ben-Yaacov *et al.* 1994). Thus it is expected that some members of cluster I MFS of *S. cerevisiae* have a function in MDR. This has been reported for *FLR1*, which confers resistance to fluconazole (Alarco *et al.* 1997), benomyl and methotrexate (Broco *et al.* 1999). Expression of *FLR1* and Flr1-mediated resistance is regulated by the PDR transcription regulators *yAP1* and *PDR3*. *ATR1* and *SGE1* from cluster II of *S. cerevisiae* also function in multidrug resistance. Atr1p confers resistance to 4-nitroquinoline-N-oxide and aminotriazole (Gömpel-Klein and Brendel 1990), whereas Sge1p confers resistance to crystal violet, ethidium bromide and methylmethane sulfonate (Ehrenhofer-

Murray *et al.* 1998). The function of the other members of cluster II remains unknown, which is also the case for MFS transporters of cluster III (Table 2).

Table 2. Fungal MFS transporter genes involved in multidrug resistance or toxin secretion.

TMD[1]	Gene	Acc.No.[2]	Size[3]	Function	Key references
			Aspergillus flavus		
-	AflT	-	-	Secretion of aflatoxin	(Chang *et al.* 1999)
			Candida albicans		
12	BenR/Ca MDR1	*X53823/ Y14703*	564	Multidrug resistance	(Fling *et al.* 1991)
			Candida maltosa		
12	Cyhr	*M64932*	552	Multidrug resistance	(Sasnauskas *et al.* 1992)
			Cercospora kikuchii		
14	CFP	*AAC78076*	607	Secretion of cercosporin	(Callahan *et al.* 1999)
			Cochliobolus carbonum		
14	TOXA	*AAB36607*	548	Secretion of HC toxin	(Pitkin *et al.* 1996)
			Fusarium sporotrichioides		
14	Tri12	*AF11355*	598	Secretion of trichotecenes	(Alexander *et al.* 1999)
			Gibberella pulicaris		
-	Rin6	*AJ132188*	528	Up-regulated by rishitin	(Weltring *et al.* 1998)
			Gibberella zeae (=Fusarium graminearum)		
14	Tri102	*AB024617*	589	Secretion of trichotecenes	(Wuchiyama *et al.* 2000)
			Saccharomyces cerevisiae[4]		
12	FLR1	*P38124*	548	Multidrug resistance	(Alarco *et al.* 1997)
14	ATR1	*M20319*	547	Multidrug resistance	(Gömpel-Klein and Brendel 1990)
14	*SGE1*	U02077	543	Multidrug resistance	(Ehrenhofer-Murray *et al.* 1998)
			Schizosaccharomyces pombe		
12	*Car1*	Z14035	526	Multidrug resistance	(Jia *et al.* 1993)

[1] TMD: number of transmembrane domains

[2] Genbank accession number

[3] Number of amino acid residues.

[4] For *S. cerevisiae* only the genes whose biological function has been demonstrated have been reported.

Candida albicans

BenR, also known as *CaMDR1*, encodes a MFS transporter with 12 TMDs. The protein is homologous to members of cluster I MFS transporters from *S. cerevisiae*. The gene was discovered for its ability to confer resistance to a number of compounds, including benomyl and metothrexate (Ben-Yaacov *et al.* 1994; Fling *et al.* 1991).

Plant pathogenic fungi

There are strong indications that MFS transporters play a role in secretion host-specific toxins by plant-pathogens. In the maize pathogen *Cochliobolus carbonum* TOXA, a MFS transporter-encoding gene possibly involved in secretion of the HC-toxin, has been identified. HC is a host-specific toxin essential for pathogenicity (Pitkin *et al.* 1996). Two linked copies of *TOXA* are present in the genome of *C. carbonum* isolates that produce HC-toxin. The genes flank the gene cluster required for synthesis of the toxin. All attempts to obtain knockout mutants for both copies of *TOXA* were unsuccessful. Hence, is has been postulated that the protein encoded by *TOXA* is involved in self-protection of *C. carbonum* against HC-toxin and is essential for virulence.

Recently, it has been demonstrated that the MFS transporter CFP from *Cercospora kikuchii* secretes the phytotoxic polyketide cercosporin. Mutants disrupted in *CFP* do not produce cercosporin, display a reduced virulence on soybean, and are more sensitive to this compound (Callahan *et al.* 1999). Interestingly, resistance to cercosporin can also be conferred by overexpression of the ABC transporter Snq2p in yeast (Ververidis *et al.* 2001). These results indicate that ABC and MFS transporters may have overlapping substrate specificities.

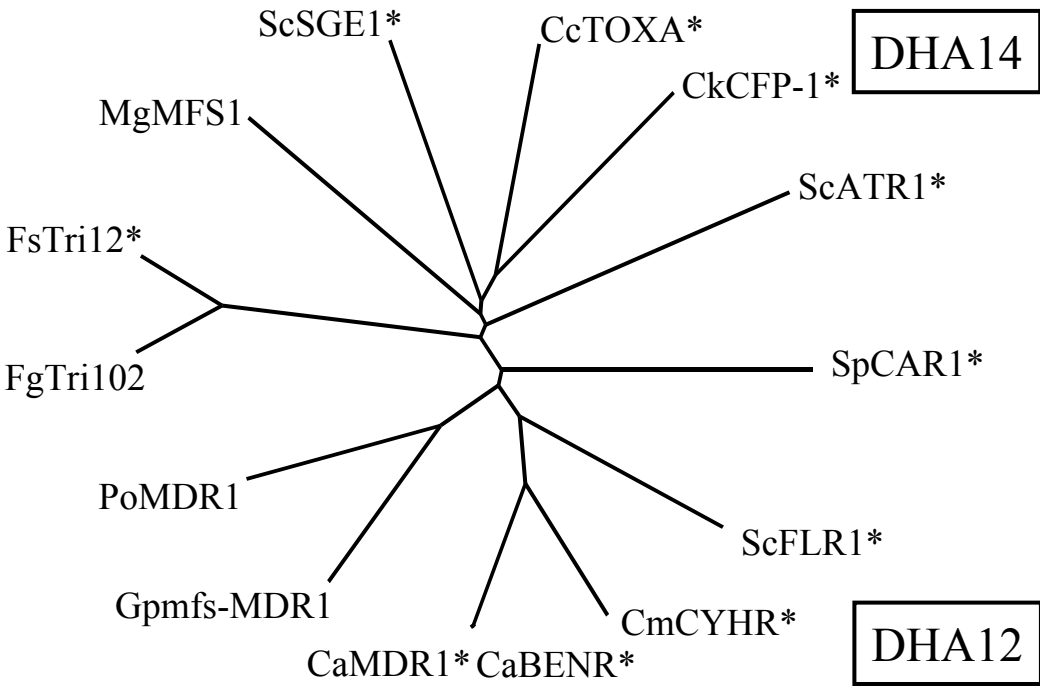

Figure 4. Dendrogram of fungal MFS transporter proteins. The multiple alignment has been generated by the ClustalX program (Thompson et al. 1997). The multiple alignment mode was used with the gonnet 250 series as protein weight matrix, applying the following default parameters: gap opening penalty: 10, gap-extension penalty: 0.20 and a 1000 times bootstrap. The code names of the proteins are composed of the gene name preceded by the initials of the species name (Table 2).

* Proteins with a role in protection against toxicants.

Fusarium sporotrichioides is a maize pathogen, which secretes trichotecene mycotoxins. Strains of *F. sporotrichioides* disrupted in *Tri12*, which encodes a MFS transporter, produce less trichotecene and are more sensitive to this compound. When expressed in *S. cerevisiae*, *Tri12* confers ability to secrete trichotecenes (Alexander *et al.* 1999). These results demonstrate that *Tri12* encodes a transporter involved in secretion of mycotoxins. Functional analysis of *Tri102*, a *Tri12* homologue in *F. graminearum* (*Gibberella zeae*) is in progress.

The function of other genes encoding MFS transporters, such as *aflT* from *A. flavus* and *rin6* from *Gibberella pulicaris* are being studied. A*flT* encodes a MFS transporter, which is highly homologous to TOXA of *C. carbonum*, and is located adjacent to *pksA*, encoding a polyketide synthase involved in aflatoxin biosynthesis (Chang *et al.* 1999). Transcription of *rin6* is strongly induced upon treatment of *G. pulicaris* mycelium with the potato sesquiterpenoid phytoalexin rishitin and its role in protection of the fungus against rishitin is being investigated (Weltring *et al.* 1998).

DISCUSSION AND OUTLOOK

ABC and, to a lesser extent, MFS transporters are highly relevant in MDR to fungicides or antimycotics. MDR phenotypes based on overexpression of ABC transporters readily develop, and can be selected upon exposure of wild-type populations to one single drug. At present, this phenomenon seriously compromises effective treatment of human diseases caused by microbes. MDR to fungicides in plant pathogens is less important than to antimycotics and antibiotics in mammalian pathogens and bacteria. However, MDR can play a major role in resistance to agricultural azole fungicides in plant pathogens (De Waard *et al.* 1996; De Waard 1997). For these reasons, characterization of drug transporters in plant pathogens has received much attention. A major goal of this type of research is to generate effective fungicide resistance management strategies.

The complete inventory of all ABC and MFS transporters of yeast indicates that microorganisms have a large network of transporters with overlapping specificities involved in drug/toxin efflux (Balzi and Goffeau 1995). The presence of such a network impairs the discovery of compounds with antifungal activity, especially when they have to be assayed at low concentrations. To overcome this problem, strains of microbes deleted in one or more drug transporter genes and a supersensitivity to toxic compounds are being used as tools in screening procedures (Rogers *et al.* 1999). The use of strains overexpressing specific ABC transporter genes in the same screening procedures can lead to the discovery of compounds, which circumvent the MDR resistance barrier. We anticipate that this is a most important issue for successful control of pathogenic microorganisms in future.

A most intriguing aspect of multidrug transporters is their capacity to bind a range of structurally unrelated drugs. Since many drugs have an amphipathic character, this may be explained by the presence in these proteins of a large amphipathic pocket, which could selectively bind drugs. Transcription of drug transporter genes can also be induced by a wide variety of

compounds. This raises the question how these drugs are being sensed. Recently, it has been demonstrated that some transcription regulators have drug sensor motifs, which can bind chemically unrelated drugs. Complexes of transcription regulator proteins and drugs can either act as activators or repressors of MFS genes in bacteria (Lewis 1999). We anticipate that gene regulation of ABC and MFS genes in filamentous fungi will be an important research topic in future. Inhibitors of transcription of these genes would act as strong synergists of drugs, which suffer from MDR.

Recent research has unequivocally demonstrated that MFS transporters are involved in secretion of host and non-host specific toxins in several species of plant pathogens. The presence of active toxin secretion mechanisms lowers intracytoplasmic toxin concentration, thus contributing to self-protection of a pathogen against its own toxin. At present, active toxin transport systems for fungal toxins and their encoding genes have been suggested for HC-toxin in *C. carbonum* (Pitkin *et al.* 1996), cercosporin in *C. kikuchii* (Callahan *et al.* 1999), and trichotecenes in *Fusarium spp* (Alexander *et al.* 1999). We propose that similar transporters are present in virtually all species of toxigenic fungi (*e.g*. *Fusarium* spp. *Alternaria* spp., *Aspergillus, Penicillium* spp.) and are likely to operate in secretion of a wide array of toxins. Hence, we hypothesize that active secretion of toxins by MFS transporters in fungi is likely to be a common virulence factor. It is not yet known whether ABC transporters may have a similar function.

Successful infection by pathogens of plants requires the ability to cope with plant defence compounds. This may be achieved by various mechanisms. A recently-discovered mechanism is the protection against plant defence compounds by active efflux. This process results in decreased accumulation of these plant compounds in the fungus, and hence, reduces their activity. For instance, in the pea pathogen *Nectria haematococca* tolerance to the phytoalexin pisatin can occur via an energy-dependent efflux system, which decreases pisatin accumulation in mycelium (Denny and Vanetten 1983; Denny *et al.* 1987). Such efflux mechanisms may be particularly important in polyphagous pathogens such as *B. cinerea* (Schoonbeek *et al.* 2001). In these instances, a limited number of ABC transporters with a broad substrate specificity could account for insensitivity to a variety of chemically unrelated defence compounds produced by their host plants. ABC transporters from fungi with a narrow host range may have a similar function in protection against a single plant defence compound. It is probably the case for the ABC transporter ABC1 from *M. grisea* (Urban *et al.* 1999). Results described so far suggest that transporters involved in protection against plant defence compounds are predominantly ABC transporters. This may be due to the relatively broad substrate range of most ABC transporters in comparison with MFS transporters. It might also suggest that ABC transporters are primarily involved in protection against exogenous toxic compounds while MFS transporters may have evolved primarily as a mechanism to secrete endogenous toxic metabolites.

The fact that transporters of natural toxic compounds function as virulence factors of plant pathogens, makes it conceivable to envision at least two novel disease control strategies. First, one could think of the development of modulators, which inhibit the activity of transporters involved in efflux of host-specific toxins or in protection against plant-defence compounds. Modulators are not

necessarily fungitoxic themselves, and therefore could act indirectly as disease control agents (De Waard 1997). For example, the combined use of azoles with plant-derived, non-toxic inhibitors of ABC transporters lowered the inhibitory dosage of these fungicides (Del Sorbo *et al.* 1998). Appropriate selective action between target and non-target organisms presents a major hurdle in the development of such modulators. A second new disease control strategy is molecular breeding by the construction of transgenic plants, which can resist phytotoxins or mycotoxins. This could be achieved by transformation of host plants with genes encoding fungal toxin transporters. This strategy could be particularly effective in pathosystems where a clear correlation between phytotoxin resistance and disease resistance exists. This may be the case in susceptibility of wheat to *F. graminearum* (Lemmens *et al.* 1994). Tobacco plants expressing the *PDR5* gene from *S. cerevisiae* did display an increased level of resistance to deoxynivalenol, a trichotecene toxin produced by *Fusarium graminearum* (Mitterbauer *et al.* 2000). Similarly, transformation of heavy metal transporter genes such as Ycf1p into plants could improve their heavy metal tolerance (Del Sorbo, unpublished results). A further step in exploitation of transgenic expression of toxin transporter genes in plants, and modulation of activity of efflux mechanisms in plant pathogens in control of plant and animal diseases, will be the utilization of efflux pumps with predetermined specificities. Their availability will be made possible by the rapid progress in studies on structure-function relationships of transporters (Egner *et al.* 1998).

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Chapter 3

The ABC transporter BcatrB affects the sensitivity of *Botrytis cinerea* to the phytoalexin resveratrol and the fungicide fenpiclonil

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The nucleotide sequence of *BcatrB* has been assigned EMBL accession number AJ006217.

ABSTRACT

During pathogenesis, fungal pathogens are exposed to a variety of fungitoxic compounds. This may be particularly relevant to *Botrytis cinerea*, a plant pathogen that has a broad host range and is consequently subjected to exposure to many plant defence compounds. In practice, the pathogen is controlled with fungicides belonging to different chemical groups. ATP-binding cassette (ABC) transporters might provide protection against both plant defence compounds and fungicides by ATP-driven efflux mechanisms. To test this hypothesis we have cloned *BcatrB*, an ABC transporter-encoding gene from *B. cinerea*. This gene encodes a 1439 amino acid protein with nucleotide binding fold (NBF) domains and transmembrane (TM) domains in a $[NBF\text{-}TM_6]_2$ topology. The amino acid sequence has 31 to 67% identity with ABC transporters from various fungi. The expression of *BcatrB* is upregulated by treatment of *B. cinerea* germlings with the grapevine phytoalexin resveratrol and the fungicide fenpiclonil. *BcatrB* replacement mutants are not affected in saprophytic growth on different media, but are more sensitive to resveratrol and fenpiclonil than the parental isolate. Furthermore, virulence of ΔBcatrB mutants on grapevine leaves was slightly reduced. These results indicate that BcatrB is a determinant in sensitivity of *B. cinerea* to both plant defence compounds and fungicides.

INTRODUCTION

The widely occurring plant pathogenic fungus *Botrytis cinerea* Pers.:Fr., anamorph of *Botryotinia fuckeliana* (De Bary) Whetzel, infects fruits, flowers or green tissues of at least 235 plant species (Jarvis 1977). Several of these hosts are known to produce defence compounds belonging to various chemical classes that act as constitutive or inducible chemical barriers (Osbourn 1999). Obviously, *B. cinerea* is able to withstand toxic effects of plant defence compounds with varying structures such as stilbenes, isoflavonoids, coumarines and sesquiterpenes. Pathogens can overcome the defence of their hosts by specific mechanisms such as enzymatic conversion of these compounds (Schafer *et al.* 1989). The specificity of such enzymes might delimit the host range of pathogens. More general mechanisms that provide protection against a broad range of toxicants, such as compartmentalisation and reduction of accumulation, would allow pathogens to cope with various defence compounds occurring in many different host species. Reduction of accumulation can be achieved with active efflux by ATP-binding cassette (ABC) transporters. These transporters are known to accept various classes of secondary plant metabolites as substrates (Kolaczkowski *et al.* 1998).

The superfamily of ABC transporters consists of membrane-bound proteins with an ATP-binding cassette (Higgins 1992). They use ATP to transport a wide spectrum of compounds over various membranes (Senior *et al.* 1995). Several subfamilies can be distinguished on the basis of the topology of hydrophobic domains, made up of trans membrane helices (TM), and hydrophilic domains, comprising the nucleotide binding fold (NBF). The latter is characterised by the common nucleotide binding motifs described by Walker (Walker *et al.* 1982) and the typical ABC signature.

Some subfamilies of ABC transporters are named after their first known member with a function in multidrug resistance (MDR). For instance, they can confer MDR to mammalian cancer cells, causing resistance to unrelated drugs used in chemotherapy. In mammals, MDR can be mediated by overexpression of two subfamilies of ABC transporters, the P-glycoprotein or MDR1-like ABC transporters with a $[TM_6\text{-}NBF]_2$ topology, and the multidrug-related proteins (MRP) with a $TM_n\text{-}[TM_6\text{-}NBF]_2$ topology (Ishikawa *et al.* 1997). In *Saccharomyces cerevisiae*, a pleiotropic drug resistance (PDR) network is involved in MDR (Balzi and Goffeau 1995). The PDR network is composed of transcriptional regulators, such as PDR1 and PDR3, that activate expression of many genes, including *PDR5* and *SNQ2*, PDR-like ABC transporters with a $[NBF\text{-}TM_6]_2$ topology. ABC transporters with additional topologies have been described in *S. cerevisiae* (Decottignies and Goffeau 1997). Transporters in the PDR network provide *S. cerevisiae* with tolerance to different toxic compounds. This becomes manifest as increased sensitivity upon disruption of one or more members of the network. ABC transporters present in *S. cerevisiae* can export a multitude of compounds comprising plant secondary metabolites (Kolaczkowski *et al.* 1998). For this reason ABC transporters of plant pathogens could play a significant role in pathogenesis, either by protection against plant defence compounds or by preventing suicidal effects of fungal toxins (De Waard 1997). Recently this hypothesis was confirmed by the finding that the ABC transporter *ABC1* from *Magnaporthe grisea* is involved in pathogenesis on rice since disruption of the *ABC1* gene results in strongly reduced virulence (Urban *et al.* 1999). Hitherto, no substrate has been identified for Abc1. It was proposed that this protein is involved in protection against unidentified plant defence compounds.

ABC transporters in filamentous fungi can also be involved in protection against fungicides. In *Aspergillus nidulans* strains selected for resistance to azole fungicides, cross-resistance to non-related compounds has been observed (Van Tuyl 1977). Resistance to azoles correlated with decreased accumulation due to increased energy-dependent efflux of these toxicants (De Waard and Van Nistelrooy 1979; 1980). It is hypothesised that ABC transporters account for this increased efflux activity (De Waard 1997). These azole-resistant mutants of *A. nidulans* indeed display increased expression of particular ABC transporter genes (Del Sorbo *et al.* 1997). Similarly, azole-resistant isolates of *Penicillium digitatum* overexpressed the ABC transporter PMR1 and disruption of the encoding gene reduced resistance of these mutant strains to these compounds (Nakaune *et al.* 1998). Furthermore, resistance of laboratory mutants of *B. cinerea* to azole fungicides (Ziogas and Girgis 1993) correlated with decreased accumulation of these compounds due to energy-dependent efflux (Stehmann and De Waard 1995). Recently, field isolates exhibiting cross-resistance to different fungicides accompanied by active efflux have been reported in France (Chapeland *et al.* 1999). These data confirm that ABC transporters might play a role in protection of *B. cinerea* to fungitoxic compounds.

This paper focuses on the function of ABC transporters from *B. cinerea* in plant pathogenesis and protection against fungicides. We have cloned and characterised the ABC transporter-encoding gene *BcatrB*. Replacement of the *BcatrB* gene resulted in increased sensitivity

to the grapevine phytoalexin resveratrol and a slight reduction in virulence on grapevine leaves. In addition, increased sensitivity to the phenylpyrrole fungicide fenpiclonil was observed. Hence, we have shown that BcatrB may be a virulence factor of B. cinerea, which also can play a role in sensitivity to commercial fungicides.

RESULTS

Cloning of genes encoding ABC transporters

Screening of a genomic library of *B. cinerea* in λ-EMBL3 with a 1.5 kb *Bgl*II-*Taq*I fragment from the yeast ABC transporter *PDR5* yielded several hybridising clones. Sequence analysis of two phages revealed open reading frames (ORFs) with homology to PDR5. The corresponding genes were assigned the names *BcatrA* and *BcatrB (B. cinerea* ATP-binding transporter A and B, respectively). Southern analysis with gene-specific probes derived from the less conserved regions of *BcatrA* and *BcatrB* demonstrated that they are single-copy genes that do not cross-hybridise. This paper describes the characterisation and functional analysis of *BcatrB*. *BcatrA* will be described in a separate paper.

A 5 kb *Eco*RV fragment and an overlapping 5.5 kb *Sal*I fragment were subcloned in pBluescript, a restriction map was constructed and the sequence was determined (Fig. 2). Assembly of sequences revealed a 4.3 kb ORF in which the presence of one intron was suggested based on sequence comparison. The presence of a 56 bp intron at position 1116-1171 was confirmed by RT-PCR. The 5'-flanking region (833 bp) of *BcatrB* contains a conserved Kozak sequence (CCAUCAUGG) around the deduced translation start (Kozak 1984) and a TATA box (Chen and Struhl 1988) at position -220. Besides these general promoter features a single putative Pdr1p/Pdr3p binding element (Katzmann *et al.* 1996) is present at position -555 and a Ste11 and a Mat1-Mc binding site at position -165 (Kjaerulff *et al.* 1997). In yeast, these elements are involved in regulation of expression of ABC transporters involved in MDR and mating. No polyadenylation signal consensus sequence (AATAAA) in the 3'-flanking region was found.

BcatrB encodes a 1439 amino acid protein with homology to ABC transporters of other fungi (Del Sorbo *et al.* 2000), such as PDR5 and SNQ2 from *S. cerevisiae* and AtrA and AtrB from *A. nidulans* (Table 1). The degree of homology between BcatrB and AtrB from *A. nidulans* is particularly high (67.5% identity). Alignment of the conserved amino acid stretches in the N- and C-terminal nucleotide binding folds of the genes (Table 2) demonstrates that the nucleotide binding domains in both halves are highly conserved in BcatrB and ABC transporters from other fungi and yeasts. The conserved sequence stretches in fungal ABC transporters are longer than the general consensus described for other organisms (Higgins 1992; Walker *et al.* 1982). Characteristics of fungal ABC transporters such as a degenerated Walker motif in the C-terminal NBF and the presence of a cysteine instead of a lysine residue in the N-terminal Walker A motif are also found in BcatrB. Prediction of transmembrane helices of BcatrB with the TMpred routine of the EMBL server (Hofmann 1993) yields a pattern that strongly resembles that of PDR5 and AtrB. The protein

consists of four domains, two hydrophobic TM_6s, and two hydrophilic NBFs, organised in a [NBF-TM_6]$_2$ topology.

Table 1. Homology of ABC transporters from *Botrytis cinerea (Bc), Aspergillus nidulans (An), Saccharomyces cerevisiae (Sc), Candida albicans (Ca), Magnaporthe grisea (Mg)* and *Penicillium digitatum (Pd)*, expressed as percentage identical amino acids.

Source	Acc. nr	Protein	BcatrA	AtrB	AtrA	Pdr5p	Snq2p	Cdr1p	Abc1	PMR1
Bc	AJ006217	BcatrB	36.5	67.5	32.7	31.3	33.3	32.3	33.1	32.2
Bc	Z68906	BcatrA	***	36.0	30.8	31.3	31.0	29.9	30.4	30.8
An	Z68905	AtrB		***	31.6	32.6	32.5	31.5	31.6	32.0
An	Z68904	AtrA			***	41.4	37.8	41.5	42.0	42.4
Sc	L19922	Pdr5p				***	35.9	53.6	43.3	45.5
Sc	X66732	Snq2p					***	36.5	33.9	35.8
Ca	X77589	Cdr1p						***	44.4	48.2
Mg	AF032443	Abc1							***	57.1
Pd	AB010442	PMR1								***

Expression of *BcatrB in vitro*

Northern blot analysis showed that *BcatrB* has a low level of basal expression in germlings grown in liquid shake culture. Transcription of *BcatrB* is specifically induced by the phytoalexins resveratrol (50 mg l^{-1}) and pisatin (20 mg l^{-1}) from grapevine and pea, respectively, and the phenylpyrrole fungicide fenpiclonil (10 mg l^{-1}) (Fig. 1). The fungicide tebuconazole (10 mg l^{-1}) (Fig. 1) and the antibiotic cycloheximide (50 mg l^{-1}) (data not shown) give only weak induction. Transcript levels of actin in lanes 4, 5 and 9 are relatively low. This may be because treatment of germlings with fenpiclonil and pisatin has a strong fungitoxic effect. The basal expression level of *BcatrB* in the azole-resistant isolate G25 and the benzimidazole-resistant isolate SAS405 did not differ significantly from that in the sensitive strains B3 and B05.10 (results not shown).

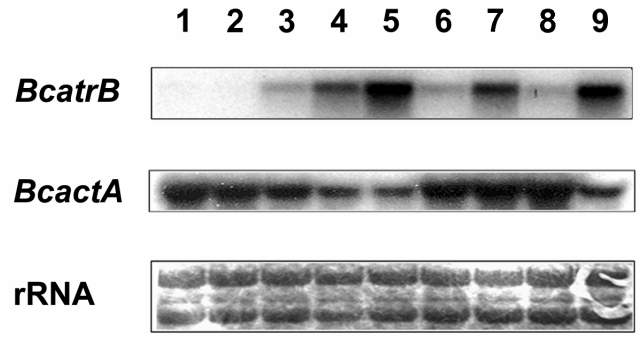

Figure 1. Expression of *BcatrB* in northern blot experiments. RNA was hybridised with the 0.7 kb *Hin*dIII fragment of *BcatrB* (Fig. 2). Transcript levels of *BcatrB* in germlings of strain B05.10 after 60 minutes of mock-treatment (lane 1) or treatment with methanol (0.1%, lane 2), fenpiclonil (1, 3 and 10 mg l^{-1}, lane 3, 4, and 5), resveratrol (20 and 50 mg l^{-1}, lane 6 and 7), tebuconazole (10 mg l^{-1}, lane 8), and pisatin (25 mg l^{-1}, lane 9). *BcactA* shows hybridisation with the *B. cinerea* actin gene and rRNA shows methylene blue staining of ribosomal RNA.

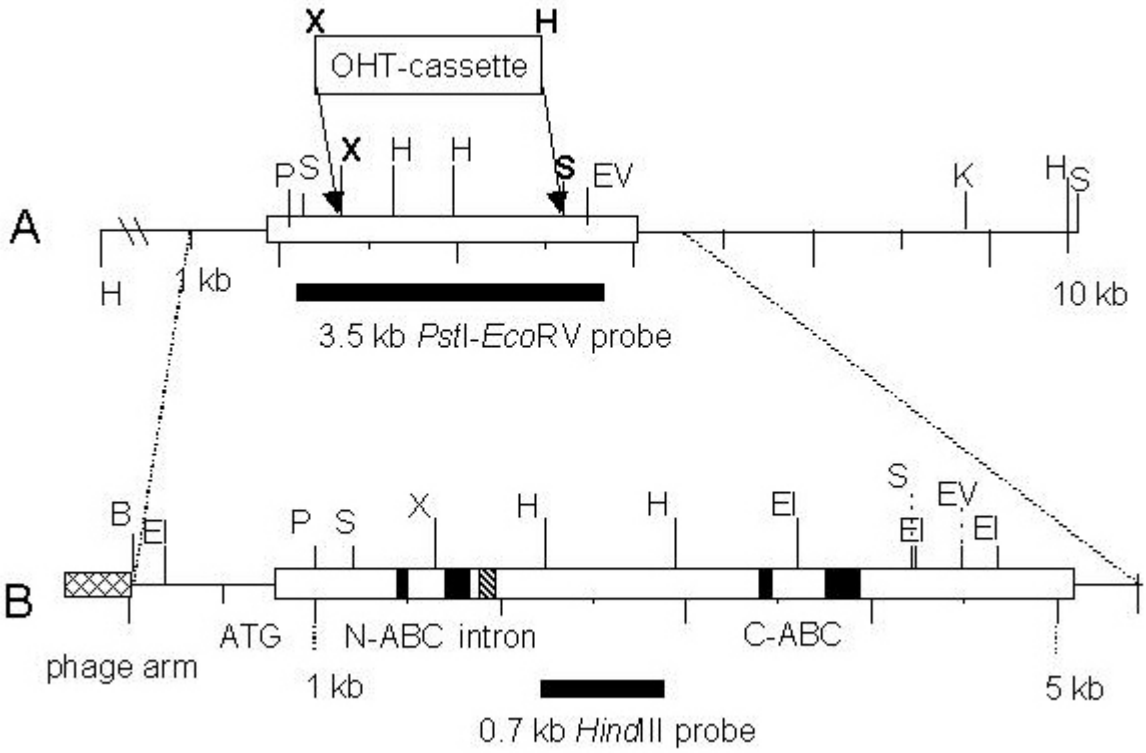

Figure 2. Physical map of the *BcatrB* locus from *Botrytis cinerea* in genomic DNA (A) and a phage clone (B). The conserved regions encoding the Walker A motif, the ABC-signature and the Walker B motif in the 5'- and 3'-half of the ORF are indicated by black boxes. In (B) the intron is given as a shaded box. Recognition sites for restriction enzymes are B= *Bst*XI, EI= *Eco*RI, EV= *Eco*RV, H= *Hin*dIII, S= *Sal*I, P= *Pst*I, X= *Xba*I. Sites used for construction of the disruption construct are given in bold case. Arrows indicate replacement of a 2.6 kb *Xba*I-*Sal*I fragment by the OHT cassette (a 2.7 kb *Xba*I-*Hin*dIII fragment) in the Δ*BcatrB* mutants. The black bars indicate the probes used for hybridisation. The 0.7 kb *Hin*dIII fragment was used as gene-specific probe in northern and Southern blotting. The 3.5 kb *Pst*I-*Eco*RV fragment was used as a probe in Southern blotting to determine the genomic organisation in the transformants.

Replacement of *BcatrB*

Functional analysis of BcatrB was studied in gene replacement mutants obtained with the construct pBABOHT in which a 2.6 kb *Xba*I-*Sal*I fragment of the *BcatrB* sequence is replaced by a 2.7 kb hygromycin resistance cassette (OHT) (Fig. 2). Transformation of the haploid *B. cinerea* strain B05.10 with pBABOHT yielded circa 60 hygromycin-resistant colonies. Homokaryotic transformants were purified by three rounds of monospore isolation and alternating growth on selective and non-selective media. To verify replacement of the *BcatrB* gene, DNA from the transformants was subjected to digestion with *Hin*dIII, Southern blotted and hybridised with the 3.5 kb *Sal*I-*Eco*RV probe (Fig. 2). Restriction of DNA from strains with a wild-type copy of *BcatrB* with *Hin*dIII results in three fragments containing BcatrB sequences: a 2.8 kb fragment from the 5'

side with flanking region, a 0.7 kb internal fragment and a 7 kb fragment from the 3' side with flanking region. Restriction with *Hin*dIII of DNA from mutants with *BcatrB* replaced by a double cross-over would result in two fragments containing *BcatrB* sequences, both approximately 5.2 kb in length, without an internal fragment. Genome analysis of transformants revealed three major types of integration events: 1) ectopic integrations, 2) heterokaryons in which only a fraction of all nuclei contained the mutant copy, and 3) homokaryons in which all nuclei contained the mutant copy (Fig. 3). From type 3, two independent transformants were selected: ΔBcatrB4 and ΔBcatrB5. Northern analysis of both mutants confirmed that wild-type mRNA of *BcatrB* was not present, not even after treatment with resveratrol or fenpiclonil at concentrations that induce elevated transcript levels in wild-type strains (Fig. 4). The absence of *BcatrB* in the replacement mutant strains was confirmed in three independent northern analysis experiments.

Sensitivity to toxicants

Vegetative growth of the mutants ΔBcatrB4 and ΔBcatrB5 on solid media (PDA, MEA, 1X Gamborg's B5) is similar to that of the wild-type strain B05.10 (data not shown). No difference in growth rate or timing and production of conidia was observed. In radial growth, the mutants showed increased sensitivity to the stilbene phytoalexin resveratrol when compared with the parental strain B05.10 (Table 3). All strains showed the characteristic brown discoloration associated with resveratrol conversion by laccase activity. No difference in sensitivity to the isoflavonoid phytoalexin pisatin from *Pisum sativum* was observed. The ΔBcatrB mutants did not show increased sensitivity to the antibiotic cycloheximide, the dicarboximide fungicide vinclozolin and the azole fungicide imazalil. The ΔBcatrB mutants exhibited a small but significant increase in sensitivity to the phenylpyrrole fungicide fenpiclonil (Table 3). The increase in sensitivity was found for spore germination, germ tube elongation (Fig. 5A) and colony formation (Fig. 5B) but was hardly detectable for mycelial growth (Fig. 6). The increase in sensitivity of ΔBcatrB mutant spores is most apparent at concentrations of fenpiclonil ranging from 0.05 to 0.1 mg l^{-1}.

Virulence assays

Virulence of the ΔBcatrB mutants was tested in wet chambers on detached grapevine leaves at 4 °C (Table 4). The size of lesions caused by the control transformant T132 was not significantly different from the parental strain (exp. 1 and 2). In two independent experiments (exp. 2 and 3) the spreading necrotic lesions caused by mutants ΔBcatrB4 and ΔBcatrB5 were significantly smaller ($P<0.05$ in Student's *t*-test) than those formed by the parental strain B05.10. In experiment 3 the determined growth rate of the mutants ΔBcatrB4 (1.1 ± 0.9 mm/day) and ΔBcatrB5 (1.1 ± 1.1 mm/day) was also significantly lower than that of B05.10 (2.2 ± 1.2 mm/day).

Table 2. Alignment of the ATP-binding domains of BcatrB with homologous sequences in ABC transporters[a]

Protein	Source	Walker A	ABC-signature	Walker B
N-terminal		GxSGxGKS[b]	SGGQ	LxxDExxSALD
BcatrB	Bc[c]	LL**VLG**R**PG**A**GC**TTL**LK**	**GVSGGER**K**RV**S**I**I**E**MLASRGSVMC**WDN**ST**RGLD**
BcatrA	Bc	LL**VLG**R**PG**S**GC**STF**LK**	**GVSGGER**K**RV**S**IA**E**TLPTKKTVVS**WDN**ST**RGLD**
AtrB	An	LL**VLG**R**PG**S**GC**TTL**LK**	**GVSGGER**K**RV**S**I**I**E**CLGTRASVFC**WDN**ST**RGLD**
AtrA	An	LL**VLG**R**PG**T**GC**STF**LK**	**GVSGGER**K**RV**S**IA**E**MALAMTPFAA**WDN**SS**RGLD**
PDR5	Sc	LV**VLG**R**PG**S**GC**TTL**LK**	**GVSGGER**K**RV**S**IA**E**VSICGSKFQC**WDN**AT**RGLD**
SNQ2	Sc	IL**VLG**R**PG**A**GC**SSF**LK**	**GVSGGER**K**RV**S**IA**E**ALAAKGSIYC**WDN**AT**RGLD**
CDR1	Ca	TV**VLG**R**PG**A**GC**STL**LK**	**GVSGGER**G**RV**D**IA**E**ASLSGANIQC**WDN**AT**RGLD**
Abc1	Mg	LV**VLG**P**PG**S**GC**STF**LK**	**GVSGGER**K**RV**T**IA**E**AALSGAPLQC**WDN**ST**RGLD**
PMR1	Pd	LI**VLG**P**PG**S**GC**STF**LK**	**GVSGGER**K**RV**S**IA**E**ATLCGSPLQC**WDN**ST**RGLD**
C-terminal		GxSGxGKS[b]	SGGQ	LxxDExxSALD
BcatrB	Bc	LG**ALMG**S**SGAGKTTLL**	**L**S**VEQRK**RLT**IGVEL**VSK**P**SI**L**I**F**L**DE**P**TSGLD**
BcatrA	Bc	MV**ALMG**A**SGAGKTTLL**	**L**S**VEQRK**RVT**IGVEL**AAK**P**N**LL**L**F**L**DEATSGLD**
AtrB	An	LG**ALMG**S**SGAGKTTLL**	**L**S**VEQRK**RVT**IGVEL**VSK**P**SI**L**I**F**L**DE**P**TSGLD**
AtrA	An	LT**ALMG**V**SGAGKTTLL**	**L**N**VEQRK**LLT**IGVEL**PPS**P**KL**LL**F**L**DE**P**TSGLD**
PDR5	Sc	LT**ALMG**A**SGAGKTTLL**	**L**N**VEQRK**RLT**IGVEL**TAK**P**KL**L**V**F**L**DE**P**TSGLD**
SNQ2	Sc	MT**ALMG**E**SGAGKTTLL**	**L**N**VEQRK**KLS**IGVEL**VAK**P**DL**LL**F**L**DE**P**TSGLD**
CDR1	Ac	IT**ALMG**A**SGAGKTTLL**	**L**N**VEQRK**RLT**IGVEL**VAK**P**KL**LL**F**L**DE**P**TSGLD**
Abc1	Mg	LT**ALMG**V**SGAGKTTLL**	**L**N**VEQRK**RLT**IGVEL**AAK**P**PL**LL**F**V**DE**P**TSGLD**
PMR1	Pd	CT**ALMG**V**SGAGKTTLL**	**L**N**VEQRK**RLT**IGVEL**AAK**P**QL**LL**F**L**DE**P**TSGLD**

[a] Identical sequences in fungal ABC-transporters in bold.

[b] General consensus sequence of ABC transporters (Higgins 1992; Walker *et al.* 1982).

[c] *Botrytis cinerea* (Bc), *Aspergillus nidulans* (An), *Saccharomyces cerevisiae* (Sc), *Candida albicans* (Ca), *Magnaporthe grisea* (Mg), and *Penicillium digitatum* (Pd).

DISCUSSION

The ABC transporter BcatrB from *B. cinerea* has high homology with ABC transporters from other fungi (Del Sorbo *et al.* 2000) such as the PDR-proteins PDR5 and SNQ2 from *S. cerevisiae* (Balzi and Goffeau 1995), AtrB from *A. nidulans* (Del Sorbo *et al.* 1997), Pmr1 from *P. digitatum* (Nakaune *et al.* 1998) and Abc1 from *M. grisea* (Urban *et al.* 1999). This suggests a similar role for

BcatrB in protection against exogenous toxic compounds or in plant pathogenesis. Indeed, our study shows that BcatrB is involved in protection against the grapevine phytoalexin resveratrol and the phenylpyrrole fungicide fenpiclonil. The finding that BcatrB protects against resveratrol is significant since it is known that susceptibility of grapevine to *B. cinerea* is inversely correlated with the resveratrol contents of the grapevine cultivar (Jeandet *et al.* 1995). The hypothesis that BcatrB can contribute to the virulence of *B. cinerea* on plants producing resveratrol was confirmed on grapevine leaves on which ΔBcatrB mutants showed slightly reduced virulence. Since virulence is not fully lost, we propose that BcatrB is only one of the many factors that contribute to virulence. Recently, it has been described that Abc1, an ABC transporter homologue from the rice blast fungus *M. grisea* is important for virulence on rice (Urban *et al.* 1999). The interaction between rice and *M. grisea* is accompanied by production of phytoalexins in the host (Kodama *et al.* 1992). Increased sensitivity to these plant defence compounds may be an explanation for loss of virulence of *ABC1* mutants. However, mutants do not show increased sensitivity to a number of phytoalexins *in vitro*, despite the ability of these compounds to induce expression of ABC1 in the wild type. Therefore, an explanation for the loss of virulence in the *ABC1* mutants of *M. grisea* is not yet known. It may be that Abc1 provides protection against a yet unidentified phytoalexin. Similar mechanisms may be involved in protection of other plant pathogens against phytoalexins. For instance, activity of ABC transporters could explain the non-degradative tolerance of pisatin, a phytoalexin from *P. sativum*, in *Nectria haematococca*, based on energy-dependent efflux (Denny *et al.* 1987).

Although pisatin induces higher transcript levels of *BcatrB* in the wild-type isolate of *B. cinerea,* the ΔBcatrB mutants have the same sensitivity to pisatin as the parental isolate. The fungus may cope with this phytoalexin by means of additional transporters or degradative enzymes, as described for other pathogens, for example pisatin demethylase (Schafer *et al.* 1989). Enzymatic degradation of resveratrol by *B. cinerea* can be accomplished by laccase-like activity (Sbaghi *et al.* 1996), which would explain why the increased sensitivity of ΔBcatrB mutants to resveratrol is only moderate. We propose that *BcatrB* serves as a "first aid" response to provide immediate protection against resveratrol and bridges the time required for breakdown of resveratrol by laccase activity, which was observed in both the parental strain B05.10 and the ΔBcatrB mutants.

For several ABC transporter genes a phenotype of single gene-replacements only becomes manifest in fungicide-resistant strains in which the ABC-transporter gene responsible for resistance is overexpressed. In *P. digitatum* disruption of the *PMR*1 gene in the azole-resistant strain LC2, which overexpresses PMR1, restores sensitivity to azole fungicides to wild type levels, but not beyond that level (Nakaune *et al.* 1998). The observation that replacement of *BcatrB* in a wild-type strain results in increased sensitivity to resveratrol and fenpiclonil indicates that BcatrB has a relatively high activity towards these compounds. Since the fungicide fenpiclonil is derived from the antibiotic pyrrolnitrin, the activity of BcatrB towards fenpiclonil may relate to the fact that *B. cinerea* needs to protect itself against natural toxins during saprophytic growth (Atlas and Bartha 1993). Amongst these are antibiotics such as pyrrolnitrin produced by *Pseudomonas* spp. (Ligon *et*

al. 2000). It is possible that BcatrB is involved in protection against pyrrolnitrin, as shown for the structurally related fenpiclonil. If ABC transporters provide protection against natural toxicants, they could be significant in determining the broad host range and the saprophytic ability of *B. cinerea*.

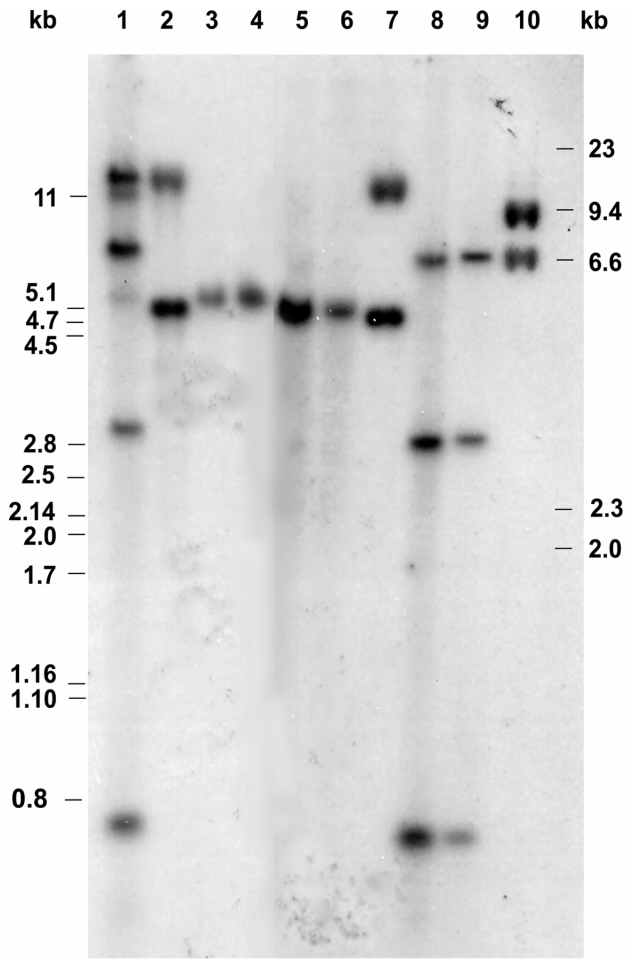

Figure 3 Southern blot with DNA from *Botrytis cinerea* strains SAS56 and B05.10 (wild type) and five putative ΔBcatrB mutants obtained by transformation with the construct pBABOHT. DNA was digested with *Hin*dIII and hybridised with a 3.5 kb *Pst*I-*Eco*RV probe from *BcatrB* (Fig. 2).

Heterokaryotic transformant (lane 1), λ*Pst*I marker (lane 2), homokaryotic transformants ΔBcatrB4, 5, 15 and 45 (lanes 3, 4, 5 and 6 respectively.), λ*Pst*I marker (lane 7), parental isolate B05.10 (lane 8), wild-type isolate SAS56 (lane 9), λ*Hin*dIII marker (lane 10). Molecular size markers (kb) are indicated in the margins.

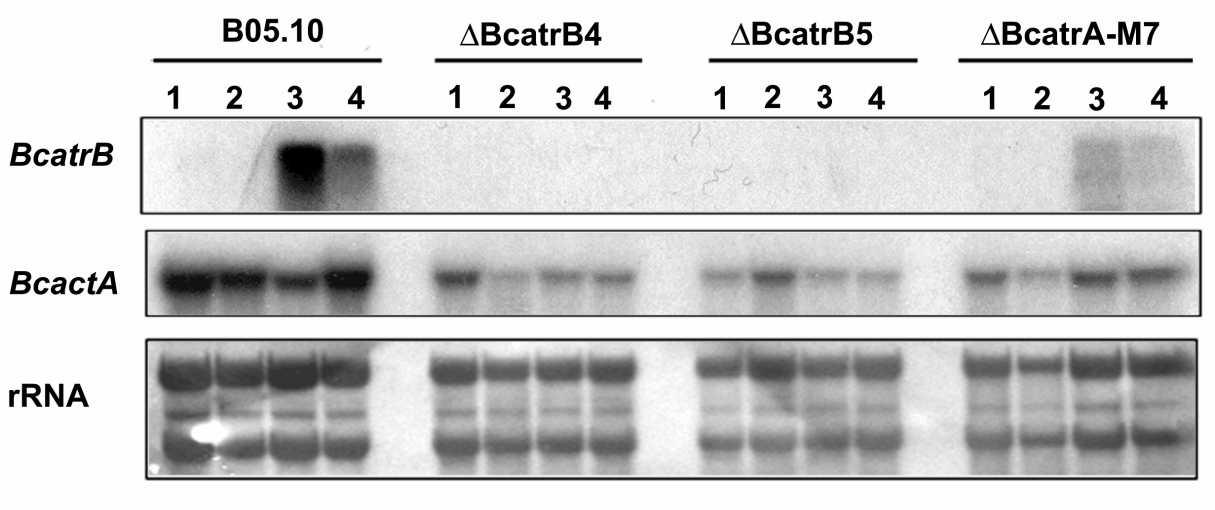

Figure 4 Expression of *BcatrB* in northern blot experiments. RNA was hybridised with the 0.7 kb *Hin*dIII fragment of *BcatrB* (Fig. 2). Transcript levels of *BcatrB* in parental strain B05.10, gene replacement mutants ΔBcatrB4 and ΔBcatrB5, and control strain ΔBcatrA-M7. Germlings were mock treated (lanes 1), treated with methanol (0.1%, lanes 2), fenpiclonil (10 mg l^{-1}, lanes 3) or resveratrol (50 mg l^{-1}, lanes 4). BcactA shows hybridisation with the *B. cinerea* actin gene and rRNA shows methylene blue staining of ribosomal RNA.

Transcription of *BcatrB* can be induced by fungitoxic compounds (resveratrol and cycloheximide) that also induce transcription of ABC transporter genes in other fungi (Del Sorbo *et al.* 1997; Miyahara *et al.* 1995). In fact, overlapping sets of compounds can induce several ABC transporters in *S. cerevisiae* and *C. albicans*. In *S. cerevisiae* induction of PDR5, SNQ2 and YOR1 is regulated by the transcription factors Pdr1p and Pdr3p (Mahé *et al.* 1996). The promoters of transporter genes in the PDR network all contain one or more PDR elements (**TCC**G(C/T)**GGA**A) (Katzmann *et al.* 1994). Binding of Pdr1p or Pdr3p to these boxes stimulates expression of these genes (Katzmann *et al.* 1996). Modification of Pdr1p, Pdr3p or the PDR elements leads to changes in expression of the PDR genes (Hallstrom and Moye-Rowley 1998), thereby altering the sensitivity to toxic compounds. The promoter of *BcatrB* contains a sequence stretch (TCCACGGAA, 551 bp upstream of the start codon) that strongly resembles the yeast PDR elements. Although no PDR1 or PDR3 homologue is known in *B. cinerea* to date, the presence of this box suggests that regulation of ABC transporters in *B. cinerea* may be similar to that in *S. cerevisiae*. Cycloheximide and pisatin induce expression of *BcatrB* but do not have an increased activity towards the ΔBcatrB mutants. This implies that inducers of transcription are not necessarily a substrate of that particular transporter. Furthermore, these compounds can be a substrate of other ABC transporters, which even may be upregulated in ΔBcatrB mutants, and mask the loss of BcatrB. Recently, the presence of at least 12 other ABC transporters has been demonstrated in an EST library of *B. cinerea* (Bitton F., Levis C., Fortini D., Pradier J.M., Brygoo Y. and Genoscope - Centre National de Séquençage, unpublished, 1999. Botrytis cinerea strain T4 cDNA library under conditions of nitrogen deprivation. EMBL AC# from AL110624 to AL117185). This situation resembles the functioning of the PDR network in *S. cerevisiae* (Balzi and Goffeau 1995) and the CDR gene family in *Candida*

albicans (Sanglard *et al.* 1997). There, concerted action of ABC transporters with overlapping substrate specificity provides the cell with a protection system against various toxic compounds (Carvajal *et al.* 1997; Kolaczkowski *et al.* 1998). For instance, *C. albicans* strains resistant to azole antimycotic drugs overexpress *CDR1*, *CDR2* or both genes (Sanglard *et al.* 1997). Disruption of *CDR1* results in an increase of sensitivity to azole drugs that can be overcome by overexpression of *CDR2*. Disruption of *CDR2* only increased sensitivity to azoles in an azole resistant Δ*CDR1* strain, which overexpresses *CDR2* and not in Δ*CDR1* strains with wild-type sensitivity. This illustrates that in many instances increased sensitivity to toxicants can only be observed in multiple knockout mutants.

In laboratory-generated mutants of *B. cinerea*, resistance to azole fungicides has been attributed to increased energy-dependent efflux of these compounds (Stehmann and De Waard 1995). A similar mechanism has been described for resistant field-isolates (Leroux *et al.* 1999). This increased efflux can be explained by enhanced activity of an ABC transporter. Resistance to azole fungicides accompanied by increased expression of a particular ABC transporter gene has been described before in *A. nidulans* (Andrade *et al.* 2000) and *P. digitatum* (Nakaune *et al.* 1998). However, the azole-resistant laboratory mutants of *B. cinerea* tested in our studies show the same low basal expression level of *BcatrB* as the parental strain. Treatment of these mutants with azoles gives a similar increase of *BcatrB* expression in mutants and wild type. These results indicate that a yet unidentified transporter is involved in resistance to azole fungicides.

In conclusion, our results suggest a mutual role for BcatrB from *B. cinerea* in protection against activity of plant defence compounds and fungicides. Further studies may elucidate the importance of BcatrB and other ABC transporters as virulence factors of the fungus on additional host plants and in protection against a wider variety of toxic compounds.

MATERIAL AND METHODS

Culturing of fungal strains

Strain SAS56, a monoascospore isolate (Van der Vlugt-Bergmans *et al.* 1993) and the haploid strain B05.10 derived from SAS56 (a gift from P. Büttner and Prof. Dr. P. Tudzynski, Institut für Botanik, Westfälische Wilhelms-Universität, Münster, Germany) were used as reference strains. T132 is a hygromycin resistant strain derived from B05.10 by transformation with the plasmid pOHT, containing the hygromycin resistance cassette OHT (Van Kan *et al.* 1997). ΔBcatrA-M7 is a gene-replacement mutant derived from B05.10 in which *BcatrA* is replaced by the OHT cassette (Del Sorbo, unpublished).

The monoascospore strain SAS405, provided by F. Faretra (University of Bari, Italy) contains the alleles *Mbc1Hr* and *Daf1LR*, which confer resistance to benzimidazoles and dicarboximides, respectively (Faretra and Pollastro 1991).

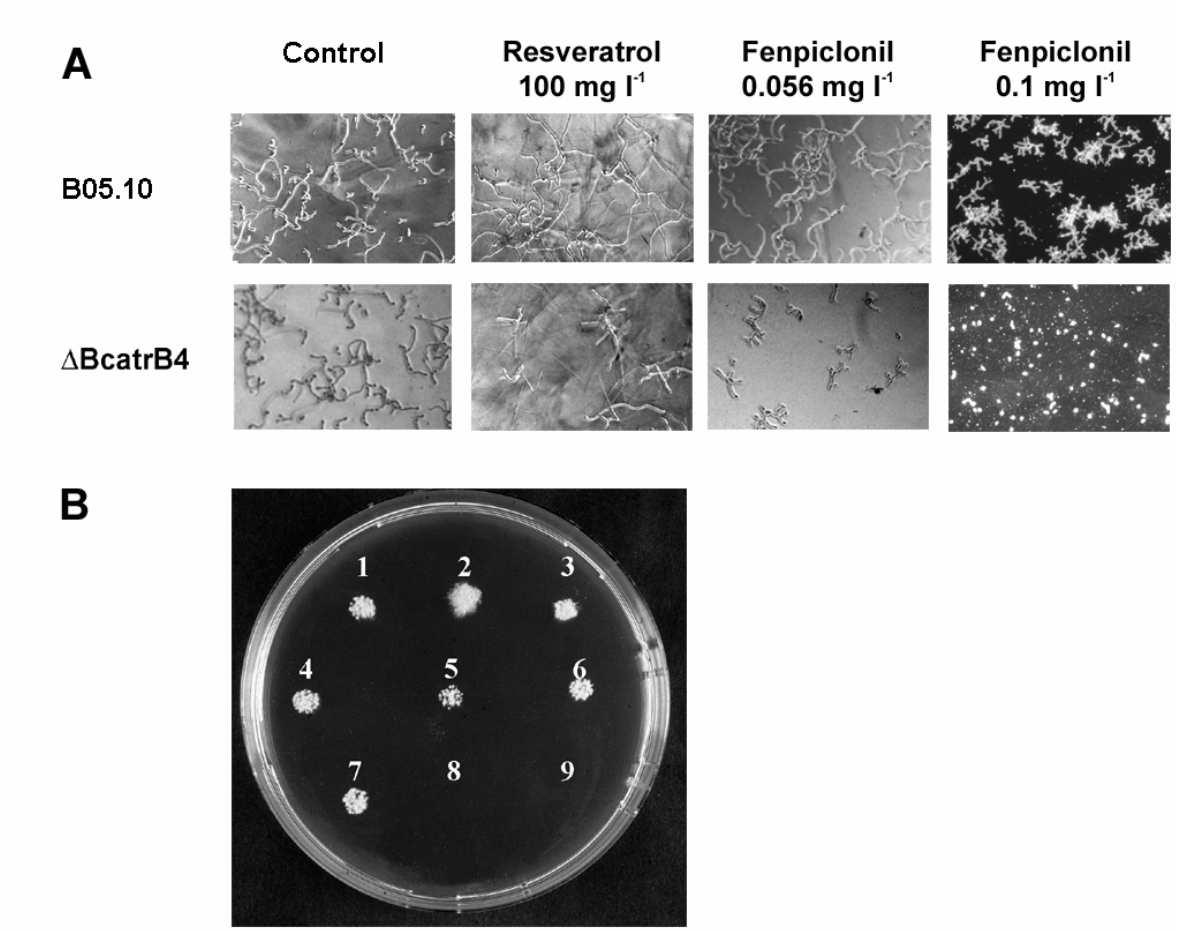

Figure 5. Effect of resveratrol and fenpiclonil on growth of *Botrytis cinerea*. A: spore germination and germtube growth of strains B05.10 (parental strain) and ΔBcatrB4 (BcatrB replacement mutant) in B5 medium after 22 hours incubation at 20°C. B: colony formation on B5-agar amended with 0.1 mg l^{-1} fenpiclonil after one week incubation at room temperature. Untransformed control strains (spots 1, 2 and 3), ΔBcatrA mutants (spots 4, 5 and 6), T132 control (B05.10 transformed with hygromycin resistance cassette) (spot 7), ΔBcatrB mutants (spots 8 and 9).

Isolate B3 is an azole-sensitive strain isolated from tomato in Greece. The monospore isolate G25 is a laboratory-generated mutant derived from B3 with reduced sensitivity to azole fungicides (Stehmann and De Waard 1996). They were kindly provided by Dr. B.N. Ziogas (University of Athens, Greece).

All strains were cultured at 20°C in the dark on tPDA (potato dextrose agar amended with 300 g of homogenised tomato leaves per litre). After 3 days, the cultures were exposed to near-UV-light for 16 hours and incubated for one additional week in the dark. Conidia were collected from sporulating cultures to inoculate liquid cultures in Gamborg's B5 medium (Duchefa Biochemie B.V., Haarlem, The Netherlands) supplemented with 1% sucrose and 10 mM ammonium phosphate (pH 6.5). Germlings from overnight cultures were used for induction experiments and DNA and RNA isolation. Hygromycin resistant transformants were maintained on MEA (malt extract agar,

Oxoid LTD., Basingstoke, Hampshire, England) plates amended with 100 mg l^{-1} hygromycin (Sigma) and transferred to tPDA to harvest spores for experiments.

B. cinerea strain B05.10 was transformed using protoplasts (Hamada *et al.* 1994) with modifications as described previously (Van Kan *et al.* 1997).

Molecular techniques

DNA manipulations were performed according to standard methods (Sambrook *et al.* 1989). *Escherichia coli* strain DH5α was used for propagation of constructs. The Promega λEMBL3 system was used to construct a library of *B. cinerea* strain SAS56 genomic DNA, partially digested with *Sau*IIIA.

The library was screened with a probe derived from the *S. cerevisiae* gene PDR5, provided by A. Goffeau (Louvain-la-Neuve, Belgium). This 1.5 kb *Bgl*II-*Taq*I fragment comprises the entire N-terminal ATP-binding cassette domain of PDR5. Membranes (Hybond-N^+, Amersham) were hybridised with random-primed (Gibco), [α-^{32}P]dATP labelled probes at 56°C in modified Church buffer (Church and Gilbert 1984) and washed at 56°C in 1x SSC. Fragments from positive phages were subcloned in pGEM3Z (+) (Promega) or pBluescript II SK (Stratagene). Sequencing was performed with the Thermo Sequenase II Cycle Sequencing kit (AP-biotech).

Genomic DNA of *B. cinerea* was isolated as described previously (Drenth *et al.* 1993), digested with restriction enzymes, fractionated on 1.0% agarose/TAE gels and transferred to Hybond-N^+-membranes (Amersham) by capillary blotting (Sambrook *et al.* 1989). Blots were hybridised with random-primed, [α-^{32}P]dATP labelled probes at 65°C in modified Church buffer, washed at 65°C in 0.2x SSC and exposed to Kodak X-OMAT AR films.

Gene expression

For RNA induction experiments, precultures were grown in 300 ml roundbottom flasks (100 ml B5 medium, 10^6 conidia per ml) at 20°C and 180 rpm for 13-15 hours. If necessary, mycelium of different cultures was pooled. Following distribution over fresh flasks, inducing agents were added from a 1000x concentrated stock solution in methanol. After additional incubation at 20°C and 180 rpm for 1 hr, mycelium was harvested on glassfibre filters, washed with ice-cold 10 mM sodium phosphate (pH 6.5) and sterile water, using a Millipore vacuum manifold.

Total RNA was extracted from mycelium frozen in liquid nitrogen using guanidine hydrochloride (Logemann *et al.* 1987). Samples of 10 µg RNA were denatured with glyoxal in DMSO and subjected to electrophoresis on a 1.4% agarose gel in 10 mM sodium phosphate. RNA was transferred to Hybond-N membranes (Amersham) by capillary blotting in 10x SSC (Sambrook *et al.* 1989). Northern blots were hybridised in modified Church buffer at 65°C using random primer [α-^{32}P]dATP labelled probes. The gene specific probes used were a 1.1 kb *Bam*HI-*Eco*RI fragment of *BcatrA*, a 0.7 kb *Hin*dIII fragment of *BcatrB* and a 1.2 kb *Hin*dIII fragment of the constitutively expressed actin gene of *B. cinerea*.

Construction of the gene replacement vector.

pBABOHT is the vector used for gene replacement of *BcatrB* (Fig.2). In a 8 kb *Bst*XI-*Kpn*I fragment a 2.6 kb *Xba*I-*Sal*I fragment from the coding region of *BcatrB*, containing both ABC motifs, was replaced by the OHT cassette from the plasmid pOHT, providing hygromycin resistance (Hilber *et al.* 1994) (provided by M. Ward, Genencor International, San Francisco, CA). The resistance marker was inserted as a 2.7 kb *Xba*I-*Hin*dIII fragment, retaining 0.8 kb of the 5'-flanking region and 1 kb of the coding region of *BcatrB*. Using *Hin*dIII from the multiple cloning sites and *Kpn*I, a 4.5 kb fragment from the 3'-end of the gene, was inserted. The plasmid was linearised with *Kpn*I prior to transformation to obtain double cross-over integration.

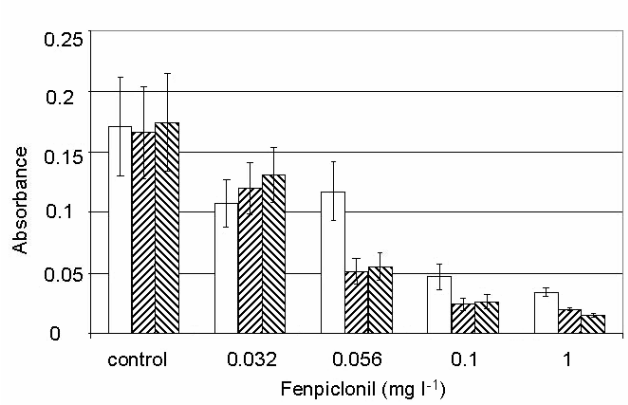

Figure 6. Effect of fenpiclonil on germling growth of *Botrytis cinerea* strains B05.10 (parental strain, white), ΔBatrB4 (dashed up) and ΔBatrB5 (dashed down, BcatrB replacement mutants) in liquid B5 medium in multiwell plates, 60 hours incubation at 20°C, measured as absorbance at 405 nm. Solvent concentrations never exceeded 0.1% methanol.

Toxicity assay.

For all toxicants EC_{50}-values for radial growth were determined as described previously (Stehmann and De Waard 1996) on PDA and solidified B5 medium. Test compounds were added in a range of concentrations, each concentration from 1000x stock solutions in methanol; resveratrol from freshly prepared 500x stock solutions. Experiments were carried out in triplicate and repeated two or three times. Student's *t*-test was used for statistical analysis.

Toxic activity of compounds in liquid B5 medium was determined in 96-well polystyrene plates (Greiner B.V. Alphen a/d Rijn, The Netherlands). Wells were inoculated with $5 \cdot 10^3$ spores in 100 µl medium and incubated at 20°C for 60 hours. Growth was measured as increase of absorbance at 405 nm in a BIO-TEK EL312 microplate reader (BIO-TEK instruments inc., Winoski, USA).

Virulence assay

Virulence assays were performed on detached leaves of grapevine placed in prewetted florist foam (OASIS) in humid chambers. Five (Exp. 1 and 2) or four (Exp. 3) leaves were inoculated with *B. cinerea* conidia ($2 \cdot 10^6$ ml^{-1}), preincubated in 1xB5 medium amended with 1% sucrose and 10 mM ammonium phosphate (pH 6.5) at 20°C for 2 hours to synchronise germination. Four sectors were

designated on the upper side of each leaf. Each sector was inoculated with 15 droplets (1 µl) of strains B05.10 (parental line), T132 (control transformant), ΔBcatrB4 or ΔBcatrB5 (BcatrB mutants). Lesion diameters were measured after incubation at 4°C for 6 days in experiment 1 and 2 and for 10 days in experiment 3. Mean values of lesion sizes were based on figures of spreading lesions (1>mm). Student's *t*-test was used for statistical analysis. In experiment 3, the growth rate of lesions is based on measurements on day 9, 10, and 11.

Table 4. Virulence of *Botrytis cinerea* on detached grapevine leaves at 4°C.

Exp.	Strain[1]			
	B05.10	T132	ΔBcatrB4	ΔBcatrB5
1	13.2 ± 2.9 a	12.7 ± 3.3 a		
2	8.4 ± 2.2[2] a	8.6 ± 3.8 a	5.9 ± 2.3 b	6.0 ± 1.5 b
3	5.7 ± 2.0 a		3.6 ± 1.6 b	4.0 ± 1.6 b

[1] B05.10 (parental wild-type strain), T132 (B05.10 transformed with the hygromycin resistance cassette), and ΔBcatrB4 and ΔBcatrB5 (BcatrB gene-replacement mutants).
[2] Average lesion size (diameter; mm); values followed by the same letter do not differ significantly ($P<0.05$, Student's *t*-test).

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Chapter 4

The ABC transporter BcatrB from *Botrytis cinerea* is a determinant of the activity of the phenylpyrrole fungicide fludioxonil

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ABSTRACT

This study demonstrates that the ATP-binding cassette (ABC) transporter BcatrB from *Botrytis cinerea* influences the activity of phenylpyrrole fungicides against the pathogen. This conclusion is based on toxicity assays and Northern analysis experiments which show that *BcatrB* replacement mutants, which do not express the *BcatrB* gene, show an increased sensitivity to the phenylpyrrole fungicides fludioxonil and fenpiclonil. Mutants overexpressing *BcatrB* exhibit a decreased sensitivity to these fungicides. In addition, accumulation of fludioxonil by *BcatrB* replacement mutants was higher than by wild-type isolates. For mutants overexpressing *BcatrB* the reverse was observed. Additional ABC and Major Facilitator Superfamily (MFS) transporter genes were identified in an Expressed Sequence Tag (EST) database, suggesting that *B. cinerea* has gene families of ABC and MFS transporters. Corresponding fragments of ten ABC (*BcatrC-BcatrN)* and three MFS transporter genes (*Bcmfs1-4*) were cloned and characterised. Fludioxonil affected the transcript level of some members of these gene families in germlings during a short treatment with the fungicide at sublethal concentrations. Hence, other ABC and MFS transporters may affect activity of phenylpyrrole fungicides as well. Other fungicides such as the anilinopyrimidine fungicide cyprodinil, the azole fungicide tebuconazole, the dicarboximide fungicide iprodione, and the strobilurin fungicide trifloxystrobin also induced transcription of some of the ABC and MFS transporter genes identified. Therefore, we propose that various ABC and MFS transporters function in protection of the fungus against fungicides and are involved in (multidrug) resistance development.

INTRODUCTION

The fungus *Botrytis cinerea* Pers.:Fr., anamorph of *Botryotinia fuckeliana* (De Bary) Whetzel is pathogenic on a wide variety of crop plants. The disease incited by this fungus is described as grey mould, causing serious economic losses (Jarvis 1980). The disease can be controlled by cultural practices. However, in many crops cultural practices can not provide sufficient disease control. Therefore, chemical control is also of utmost importance. Classes of fungicides available are multi-site inhibitors such as dithiocarbamates and single-site inhibitors such as benzimidazoles and *N*-phenylcarbamates, dicarboximides, anilinopyrimidines, and phenylpyrroles. Relatively new botryticides are fluazinam (a phenylpyridylamine) and fenhexamid (a hydroxyanilide). Application of the widely used azole and strobilurin fungicides for control of *B. cinerea* is very limited but highly effective compounds may be discovered in future.

Chemical control of grey mould in different crops heavily suffers from development of resistance to fungicides in *B. cinerea*. Widespread practical resistance to benzimidazole and phenylcarbamate fungicides was found within a few years after their introduction. Resistance to these fungicides results from mutations in the β-tubulin gene, which encodes the target site for these fungicides (Lyr 1995).

Isolates resistant to dicarboximide fungicides were also detected. These isolates also display cross resistance to chemically unrelated fungicides (Leroux *et al.* 1977; Lyr 1995). Laboratory-generated mutants resistant to dicarboximides are also resistant to phenylpyrrole fungicides such as fenpiclonil and fludioxonil (Hilber *et al.* 1995; Leroux *et al.* 1992). Interestingly, field isolates resistant to dicarboximides do not show this phenotype. The mode of action of dicarboximides in target organisms is not yet understood. Resistance may be conferred by mutations in a serine protein kinase (Orth *et al.* 1995).

Anilinopyrimidine fungicides interfere with methionine biosynthesis and inhibit secretion of hydrolytic enzymes, but their primary target is still unknown (Fritz *et al.* 1997; Milling and Richardson 1995). Isolates resistant to anilinopyrimidine fungicides were detected in the late 1990's. They were divided in phenotypic classes based on cross resistance to other classes of fungicides (Leroux *et al.* 1999). Isolates with phenotype AniR1 are highly resistant to anilinopyrimidines but not resistant to other compounds. Isolates with phenotypes AniR2 and AniR3 exhibit low resistance levels and can be controlled in practice. Isolates with these phenotypes show cross resistance to various unrelated fungicides such as azoles, dicarboximides, fenhexamid, and phenylpyrroles (Chapeland *et al.* 1999; Leroux *et al.* 1999).

Resistance to phenylpyrrole fungicides in field isolates of *B. cinerea* has not yet been detected. In the laboratory mutants resistance to these fungicides can easily be generated and show cross-resistance to dicarboximide fungicides (Faretra and Pollastro 1993; Hilber *et al.* 1995). The mechanism of action of phenylpyrroles may relate to transport-associated phosphorylation of glucose and protein kinase activity (Jespers and De Waard 1995; Pillonel and Meyer 1997).

A mechanism that may play a role in resistance to fungicides in *B. cinerea* is decreased accumulation of the compound in mycelial cells due to energy-dependent efflux. This mechanism has first been described for *imaB* mutants of *Aspergillus nidulans* resistant to azole fungicides and various unrelated chemicals (De Waard and Van Nistelrooy 1979; 1980). Later, a similar mechanism was reported to operate in laboratory-generated mutants of *B. cinerea* resistant to azole fungicides (Stehmann and De Waard 1995). The driving force behind the energy-dependent efflux of the azole fungicides can be ATP-binding cassette (ABC) transporters (De Waard 1997). Members of the Major Facilitator Superfamily (MFS) transporters may have similar functions. Genes encoding ABC and MFS transporters have now been identified in many yeasts and filamentous fungi (Del Sorbo *et al.* 2000). ABC transporter proteins from filamentous fungi known to function in transport of fungicides are atrB and atrD from *A. nidulans* (Andrade *et al.* 2000a; Andrade *et al.* 2000b; Del Sorbo *et al.* 1997), BcatrB from *B. cinerea* (Schoonbeek *et al.* 2001) and PMR1 from *Penicillium digitatum* (Nakaune *et al.* 1998). Increased expression of ABC transporter genes may result in overproduction of the encoded proteins and in increased pump capacity responsible for the energy-dependent efflux. Indeed, field isolates of *P. digitatum* resistant to azoles do show increased expression of *PMR1*, but data demonstrating that these mutants accumulate azoles to a lesser extent as compared to wild-type isolates are still not available. For azole-resistant mutants and azole-resistant field isolates of *B. cinerea* this situation is the opposite.

Accumulation of azoles by azole-resistant mutants and field isolates is low as compared to wild-type strains but the fungicide transporter responsible for the efflux of the compounds has yet to be identified (Chapeland *et al.* 1999; Stehmann and De Waard 1995).
Expression of the ABC transporter gene *BcatrB* from *B. cinerea* is strongly induced by treatment with the phenylpyrrole fungicide fenpiclonil (Schoonbeek *et al.* 2001). Strikingly, *BcatrB* replacement mutants show increased sensitivity to this fungicide. This observation prompted us to investigate the effect of the phenylpyrrole fungicide fludioxonil on basal and induced expression of *BcatrB* in fungicide-sensitive and resistant strains of *B. cinerea* and to correlate these effects with accumulation of the fungicide in various isolates of the fungus. In addition, the effect of fludioxonil and some other fungicides on transcription of additional ABC and MFS transporter genes have been investigated. The results suggest that *B. cinerea* possesses gene families of ABC and MFS transporters moderating the activity of several classes of toxic compounds. Some of these transporters can be regarded as fungicide pumps, which may account for multidrug resistance of *B. cinerea* to fungicides.

MATERIALS AND METHODS

Strains

The monospore isolate SAS56 and the haploid strain B05.10 (Buttner *et al.* 1994) derived from SAS56 were gifts from Dr. J.A.L. van Kan (Department of Phytopathology, Wageningen University, Wageningen, the Netherlands) and Prof. Dr. P. Tudzynski (Institut für Botanik, Westfälische Wilhelms-Universität, Münster, Germany), respectively. Isolates CH1.7 and CH1.8 are mono-ascospore isolates with decreased sensitivity to fludioxonil. They are progeny isolates from a cross between a wild-type isolate and a laboratory-generated isolate with decreased sensitivity to fludioxonil. The cross was made by Dr U. Hilber (Eidgenössische Forschungsanstalt, Wädenswil, Switzerland) and the isolates were provided by Dr. K.M. Chin (Novartis, Stein, Switzerland). The parent isolates of the cross were not available. Isolate AV5A/97 is a field isolate from grapevine resistant to pyrimethanil. It was kindly donated by Dr. M.L. Gullino (DI.VA.P.R.A., Università Degli Studi di Torino, Grugliasco, Italy). SAS56 was used as the wild-type reference strain for isolates CH1.7, CH1.8, and AV5A/97.

Strain B05.10 was used to generate replacement mutants ΔBcatrB4 and ΔBcatrB5 (Schoonbeek *et al.* 2001) and the laboratory mutant B05.10C highly resistant to fludioxonil. The latter mutant was selected from sectors of colonies on Leroux's mineral medium amended with fludioxonil at 10 mg l^{-1} and maintained on the same medium. Its ability to infect tomato leaves is similar to that of isolate B05.10 but the lesion expansion rate was about 30% less. Conidia of all strains were preserved as suspension of 10% glycerol in sterile water in Eppendorf vials and stored at –20 °C.

Chemicals

The fungicides (technical grade) used were cyprodinil, fenpiclonil, [^{14}C]fludioxonil, fludioxonil, trifloxistrobin, and cyproconazol (Novartis, Basel, Switzerland), tebuconazole (Bayer, Monnheim, Germany), prochloraz (Schering Agrochemicals, Saffron Walden, UK), fluazinam (ISK. Biosciences, Mentor, Ohio, USA), iprodione (Rhône-Poulenc, Lyon, France), and carbonyl cyanide m-chlorophenylhydrazone (CCCP; Sigma-Aldrich, Zwijndrecht, the Netherlands). Methanol stocks of these fungicides (10 g l^{-1}) were kept at 4 ºC in the dark for maximally 7 days. Fungicides were added to cultures or media from 1000x concentrated stock solutions unless stated otherwise.

Growth media and growth conditions

Strains were cultured on tPDA (potato dextrose agar amended with 300 g of homogenised tomato leaves per litre) and malt extract agar (50 g l^{-1}) containing 2 g l^{-1} yeast extract. Petri-dishes containing these media were inoculated in the centre with one drop of conidial suspension (containing 10^4 conidia) from stocks. Drops were streaked over the whole surface of a plate. Plates were incubated for 10 days at 20 ºC in the dark. Formation of conidia was induced by UV treatment for 24 h after 4 days of incubation. Conidia harvested from tPDA and malt agar plates were used to grow germlings for expression analysis and accumulation experiments, respectively. Radial growth experiments were carried out on Leroux's synthetic mineral medium amended with yeast extract (Leroux *et al.* 1977).

Fungicide activity tests

A drop (100 µl) of conidial suspension (10^8 conidia ml^{-1}) was inoculated on Petri-dishes with Leroux's medium and equally distributed over the surface. Plates were incubated overnight in the dark at 20 °C to allow germination of conidia. Agar plugs (5 mm diameter, 3 plugs per plate) from these plates were used to inoculate Petri-dishes with the same medium amended with fungicides at different concentration. In tests with cyprodinil, yeast extract was omitted from the medium. Diameters of mycelial colonies were measured after incubation in the dark at 20 °C for 48 hours. Average diameter of colonies on fungicide-amended agar was calculated as percentages of colony diameters in control treatment. EC_{50} values of fungicides were calculated from these figures by regression analysis of inhibitor response data. Experiments were performed in duplicate. Statistical analysis of the EC_{50} values was done after log transformation according to the one way Anova method using the LSD (T) comparison of means ($P < 0.05$). Analysis of data from the experiments involving iprodione was performed using Kruskall-Wallis mean rank analysis.

Cloning of genomic fragments from ABC and MFS genes

An EST library of *B. cinerea* (Bitton F., Levis C., Fortini D., Pradier J.M., Brygoo Y. and Genoscope - Centre National de Séquençage, unpublished, 1999. *Botrytis cinerea* strain T4 cDNA library under conditions of nitrogen deprivation. EMBL AC# from AL110624 to AL117185) was

screened in a batch blastx homology search with the keywords ABC, Atr, pleiotropic drug resistance, PDR, PDR*, multidrug resistance, MDR, MDR*, transporter, MF, MFS, major facilitator, drug, toxin, fungicide or resistance. Selected ESTs were used to design oligonucleotides with a length of 19-23 nucleotides and an average GC content of 65 %. Genomic DNA was isolated from strain B05.10 according to Drenth (1993). PCR amplification was performed with genomic DNA (30 ng) as template using Amplitaq polymerase in a touchdown protocol. Denaturation temperature 94 °C for 30 sec in each cycle; annealing temperatures 3x60 °C, 3x57 °C, 3x54 °C, and 30x52 °C or 3x60 °C, 3x58 °C, 3x56 °C, 3x54 °C, and 30x60° C for 30 sec per cycle; elongation temperature 72 °C for 1 min in each cycle. Amplified fragments were gel purified using Seakem agarose or the Qiaex II kit (Promega/Westburg, Leusden, The Netherlands) and subsequently cloned using the pGEM®-T Easy vector system (Promega, Madison, WI, USA). Sequencing was performed with the Thermo Sequenase II Cycle Sequencing kit (AP-biotech) using standard M13 derived primers and newly designed internal primers, if required.

Expression analysis

Expression analysis was performed on germlings grown overnight in Gamborg's B5 medium (Duchefa, Haarlem, The Netherlands) supplemented with 1% glucose and 10 mM ammonium phosphate (pH 6.5). Growth conditions, extraction of total RNA, electrophoresis of RNA on agarose, Northern blotting, and hybridisation of blots with random primer [α-^{32}P]dATP labelled DNA fragments from ABC and MFS genes were essentially the same as described previously (Schoonbeek *et al.* 2001). Gene-specific *Hin*dIII fragments of *BcatrA* (accession number Z68906; nucleotides 3411-4344) and *BcatrB* (accession number AJ006217; nucleotides 2246-2942) were also used. The RNA extraction procedure was modified by extraction from mycelium frozen in liquid nitrogen with TRIzol (Life Technologies, Breda, The Netherlands). Membranes were re-used in northern analysis experiments after stripping of blots with boiling SDS (1%). Hybridisation with 23S rRNA was used as a loading control.

Accumulation of fludioxonil

Accumulation experiments were performed according to slightly modified procedures as described by Stehmann and De Waard (1995). Standard germling suspensions were prepared with conidia collected from mycelium grown on malt-extract agar plates and had a density of 0.4 g wet weight mycelium per 100 ml buffer. Standard germling suspensions (60 ml in 300 ml Erlenmeyer flasks) were shaken on a reciprocal shaker at 20 °C for 20 min. Experiments were initiated by adding [^{14}C]fludioxonil [initial concentration in suspension 0.4 μM (1 mg l^{-1}); 250 Bq/nmol] from a 100x concentrated stock solution in methanol. Experiments were performed in triplicate, unless otherwise indicated.

Table 1. Activity of fungicides against various strains of *Botrytis cinerea* in radial growth tests

		EC_{50} values ± standard deviation (μg l^{-1})																	
Set	Strains[a]	Fludioxonil		Fenpiclonil		Cyprodinil		Iprodione		Cyproconazole		Prochloraz		Tebuconazole		Trifloxystrobin	
I	B05.10	3.5 ± 0.6	a[b]	13 ± 4	a	8.0 ± 0.8	a	154 ± 37	a	778	nt[c]	65 ± 25	a	279 ± 22	a	52 ± 13	a
	ΔBcatrB4	1.8 ± 0.6	b	8.8 ± 1.7	b	6.3 ± 1.1	ab	164 ± 18	a	333	nt	53 ± 15	a	140 ± 13	b	59 ± 40	a
	ΔBcatrB5	1.7 ± 0.9	b	7.7 ± 1.8	b	5.1 ± 0.7	b	139 ± 30	a	230	nt	36 ± 5	b	95 ± 6	c	87	
	B05.10C	> 10^3		> 10^3		7.5		> 10^3		nt		nt		320		40	
II	SAS 56	3.1 ± 1.0	a	10 ± 1	a	6.3 ± 1.9	a	154 ± 22	a	223	nt	39 ± 10	a	118 ± 35	a	51 ± 14	nt
	CH1.7	9.1 ± 1.1	b	28 ± 10	b	26 ± 5	b	1563 ± 880	b	325	nt	65 ± 13	b	140 ± 13	a	18 ± 5	nt
	CH1.8	8.0 ± 0.1	b	27 ± 10	ab	25 ± 7	b	1536 ± 734	b	330	nt	49 ± 16	ab	132 ± 18	a	21	nt
	AV5A	4.9	nt	nt		2599 ± 2	c	162 ± 23	a	nt		nt		nt		68	nt

[a] B05.10 (haploid wild-type strain), ΔBcatrB4 and ΔBcatrB5 (gene-replacement mutants of *BcatrB*) derived from B05.10, B05.10C (laboratory generated mutant with fludioxonil resistance), SAS 56 (wild-type strain), CH1.7 and CH1.8 (laboratory-generated mutants resistant to fludioxonil), and AV5A/97 (field isolate resistant to cyprodinil)

[b] Means in the same set of experiments followed by the same letter in the same column do not differ significantly (Anova, $P < 0.10$)

[c] Not tested

RESULTS

Sensitivity to fungicides

The sensitivity of wild-type strains, field isolates and laboratory-generated mutants of *B. cinerea* to the phenylpyrroles fenpiclonil and fludioxonil, the anilinopyrimidine cyprodinil, the dicarboximide iprodione, the azoles cyproconazole, prochloraz and tebuconazole, and the strobilurin trifloxystrobin was determined in radial growth tests (Table 1). Fludioxonil had the highest fungicidal activity towards wild-type isolates B05.10 and SAS56 of all compounds tested. *BcatrB* replacement mutants show a slight increase in sensitivity to fludioxonil and the related fungicide fenpiclonil (Isolate set I). The mutants also show a slight increase in sensitivity to tebuconazole and, possibly, to other azoles. The laboratory-generated mutant B05.10C was extremely insensitive to both phenylpyrrole fungicides and iprodione, indicating that the mutant may carry the *Daf1HR* mutation (Faretra and Pollastro 1991; 1993). The activity of phenylpyrrole fungicides to isolates CH1.7 and CH1.8 was low as compared to the reference isolate SAS56 (Isolate set II). These isolates also had a lower sensitivity to cyprodinil and iprodione, suggesting that they carry the *Daf1LR* mutation (Faretra and Pollastro 1991; 1993). Isolate AV5A/97 was highly resistant to cyprodinil only. The EC_{50} value of benomyl was higher than 100 mg l^{-1} for all isolates tested, except for isolate SAS56 (EC_{50} value < 1 mg l^{-1}).

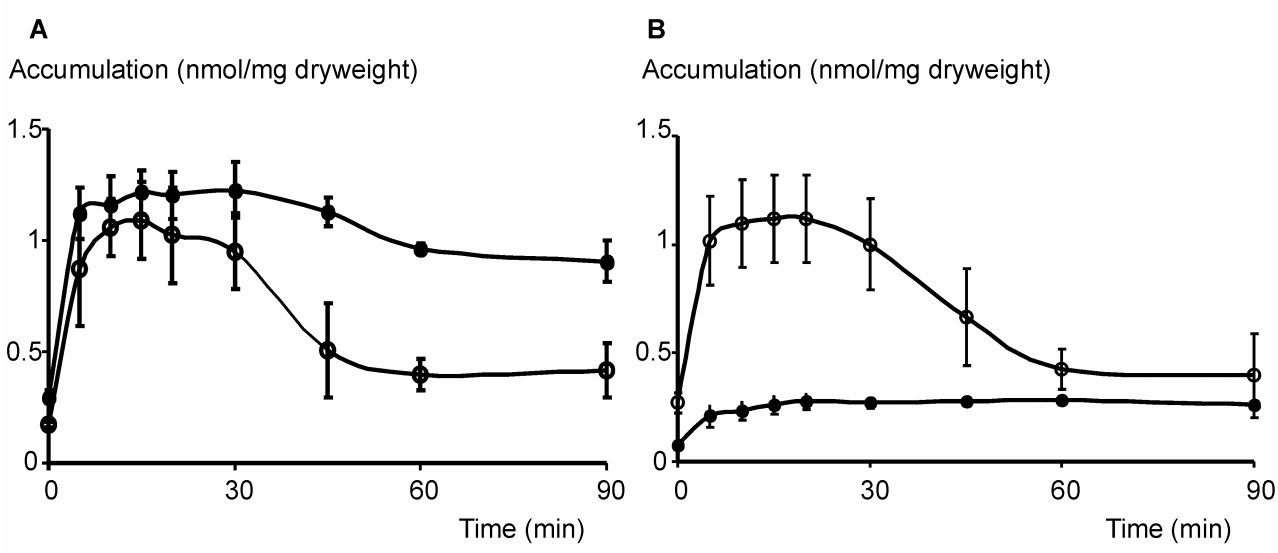

Figure 1. Time-course accumulation accumulation of [^{14}C]fludioxonil by germlings of *Botrytis cinerea*. Initial external concentration of fludioxonil at zero time 1 mg l^{-1}. A: accumulation by wild-type B05.10 (o) and ΔBcatrB4 replacement mutant (•). B: accumulation by wild-type SAS56 (o) and laboratory mutant CH 1.7 (•) with a low degree of resistance to fludioxonil.

Accumulation of [^{14}C]fludioxonil

Accumulation of [^{14}C]fludioxonil (initial external concentration 1 mg l^{-1}; 4 μM) by germlings of wild-type isolates and mutants was studied in time-course experiments. Accumulation of [^{14}C]fludioxonil by B05.10 and SAS56 was transient in time (Fig. 1). The maximum level of fludioxonil (1.1 nmol mg^{-1} dry mycelium) was found after 15 min of incubation. From 20 min onwards, accumulation decreased and reached a steady level (0.4 nmol mg^{-1} dry weight mycelium) after 60 min. Thereafter, accumulation levels remained unchanged for another 3 hours (data not shown). Addition of the respiratory inhibitor CCCP (10 μM) simultaneously with [^{14}C]fludioxonil induced a high and constant accumulation level (results not shown), as described previously for tebuconazole (Stehmann and De Waard 1995). Accumulation of [^{14}C]fludioxonil by mutant ΔBcatrB4 was relatively high (1.2 nmol mg^{-1} dry weight mycelium) compared to strain B05.10 and tends to remain constant in time (Fig. 1A). Similar results were observed for mutant ΔBcatrB5 (results not shown). In contrast, fludioxonil accumulation by the mutant B05.10C was transient although less prominent than accumulation by the wild-type (results not shown). Fludioxonil accumulation by CH1.7 was particularly low (0.3 nmol mg^{-1} dry weight mycelium) and constant in time (Fig. 1B). Similar results were obtained with isolate CH1.8 (results not shown). Treatment of germlings of isolate B05.10 with the fungicides cyprodinil, iprodione, fluazinam, and trifloxystrobin at 65 min after addition of [^{14}C]fludioxonil rapidly enhanced accumulation of [^{14}C]fludioxonil (Fig. 2).

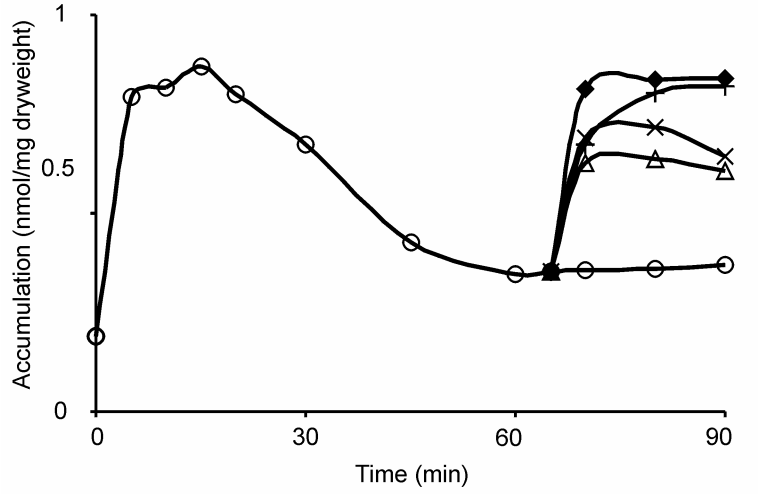

Figure 2. Effect of several several fungicides on accumulation of [14C]fludioxonil by germlings of *Botrytis cinerea* strain B05.10. Initial external concentration of fludioxonil at zero time (1 mg l^{-1}). Test fungicides were added 65 min after addition of [14C]fludioxonil. Control (o), 10 mg l^{-1} cyprodinil (x), 40 mg l^{-1} iprodione (Δ), 47 mg l^{-1} fluazinam (+), and 0.1 mg l^{-1} trifloxystrobin (♦).

Effect of fungicides on transcript levels of *BcatrB* in wild-type strain

The effect of fungicides on transcript levels of *BcatrB* in the wild-type strain B05.10 was studied by northern analysis (Fig. 3). Basal expression of *BcatrB* was hardly detectable under the test conditions used. Treatment of germlings with fludioxonil for one hour enhanced transcript levels of

the gene. Low levels of the *BcatrB* transcript could be detected when germlings were treated with 0.025 mg l^{-1}. The signal increased with increasing concentration of the fungicide. A strong signal was observed with 2.5 mg l^{-1} (Fig. 3A). A similar response was observed upon treatment of ungerminated conidia with fludioxonil (results not shown). Time-course experiments showed that the accumulation of transcripts was transient, with a maximum level after 15 min of treatment with fludioxonil at 2.5 mg l^{-1} (Fig. 3B). Under similar conditions, fludioxonil (2.5 mg l^{-1}) inhibited dry weight increase during a growth period of 8 h by 50% (results not shown), indicating that the 1-h fungicide treatment is not lethal.

Cyprodinil, tebuconazole, and trifloxystrobin also strongly enhanced transcript levels of *BcatrB* in strain B05.10, although the effective concentration varied for different compounds (Fig. 4). Similar results were observed with iprodione (results not shown). The dynamics of transcriptional induction was dependent of the compound. Time-course studies showed that the highest transcript levels of *BcatrB* were obtained for azoles, cyprodinil, and iprodione after 15 min of treatment and for trifloxystrobin after 60 min (Fig. 4).

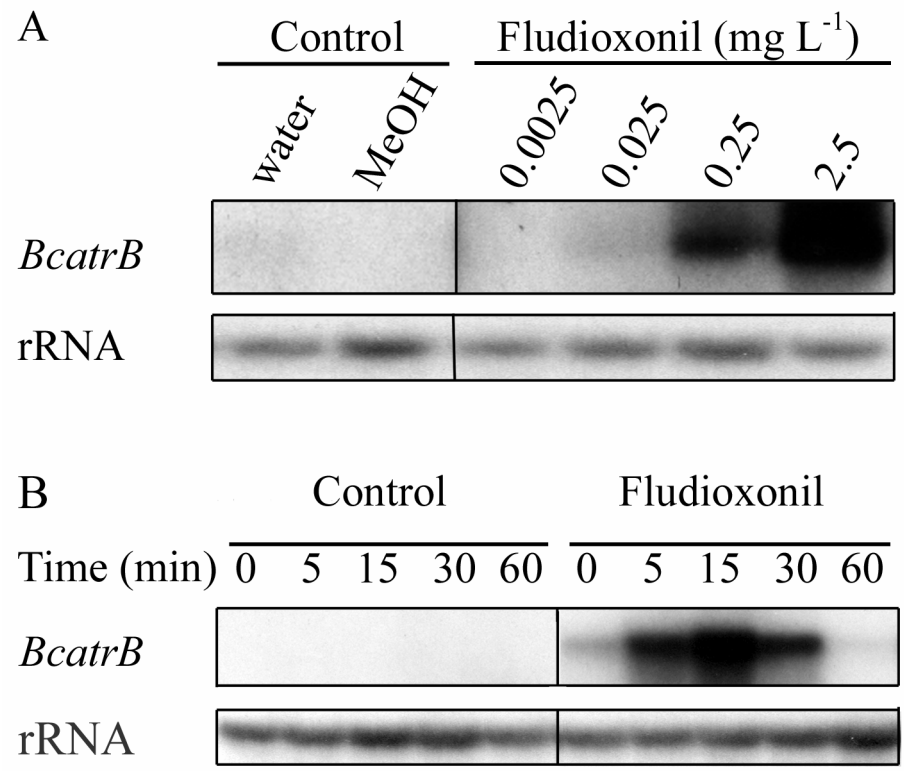

Figure 3. Effect of fludioxonil on expression of *BcatrB* in northern analysis experiments with RNA from germlings of *Botrytis cinerea* strain B05.10. A. Germlings treated with a water control, a solvent control (0.1% methanol) and fludioxonil (0.0025, 0.025, 0.25 and 2.5 mg l^{-1}) for 60 minutes. B. Time-course experiment with germlings mock-treated (control) or treated with fludioxonil (mg l^{-1}) for 0, 5, 15, 30 or 60 minutes. Loading control: ribosomal 23S RNA (rRNA). Exposure time of the autoradiogram shown in panel A is longer than in panel B.

Effect of fungicides on transcript levels of *BcatrB* in mutants

The effect of fludioxonil and other fungicides on *BcatrB* transcript levels has been studied in ΔBcatrB4 (replacement mutant), B05.10C (laboratory-generated mutant with a high degree of phenylpyrrole resistance), CH1.7 (laboratory-generated mutant with a low degree of phenylpyrrole resistance), and field isolate AV5A/97 (resistant to cyprodinil).

As expected, basal levels of expression of *BcatrB* in wild-type isolates B05.10 and SAS56 were not detectable under the test conditions used. This was also true for the mutants ΔBcatrB4 and B05.10C, and isolate AV5A/97. In contrast, isolate CH1.7 displayed a distinct basal expression level (Fig. 5). A similar result was obtained with isolate CH1.8 (results not shown).

Fludioxonil (2.5 mg l^{-1}) clearly increased transcript levels in all isolates, except for ΔBcatrB4 (no signal) and CH1.7 (similar signal as in control treatment (Fig. 5). Similar results were obtained for cyprodinil (2.5 mg l^{-1}) and iprodione (10 mg l^{-1}) (results not shown).

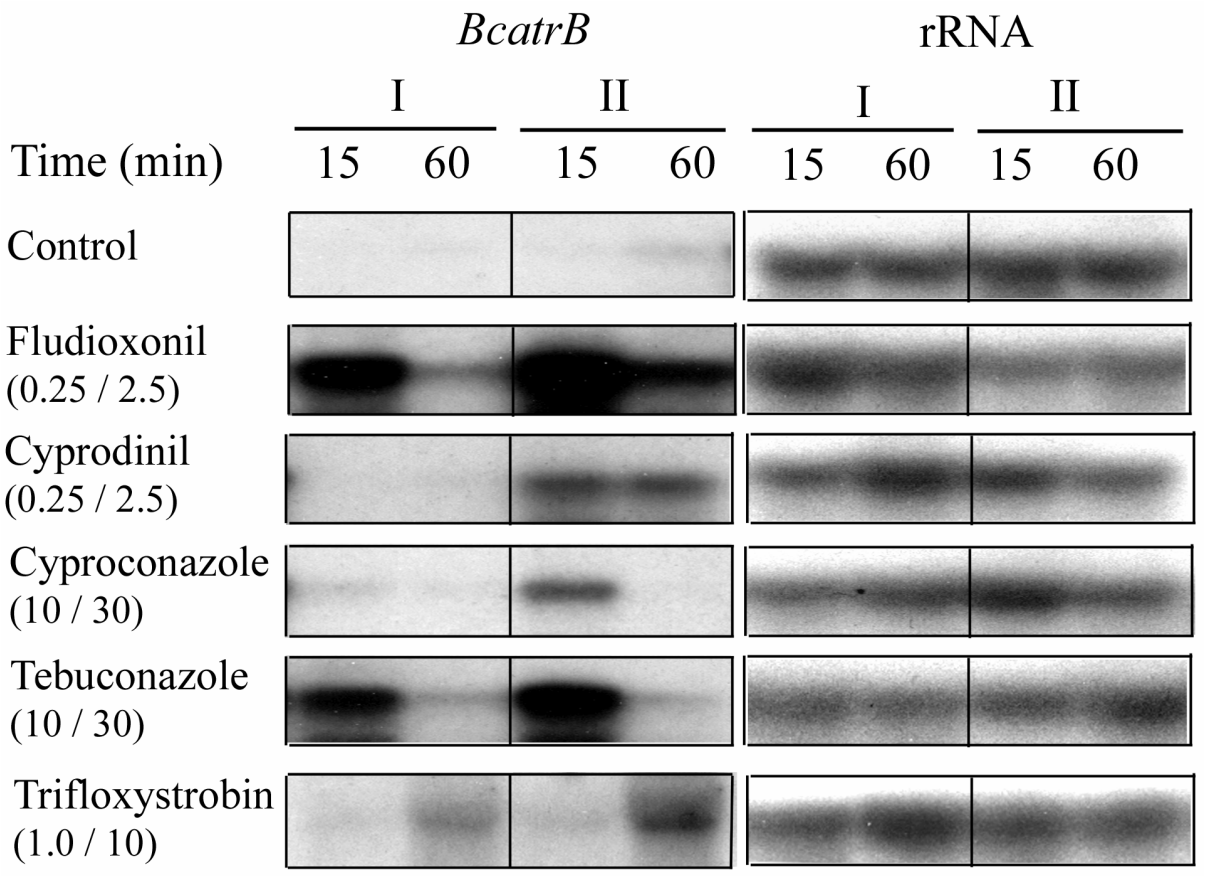

Figure 4. Effect of fungicides from different classes on expression of *BcatrB* in northern analysis experiments with RNA from germlings of *Botrytis cinerea* strain B05.10. For each fungicide two concentrations (mg l^{-1}) were used, indicated between brackets (low concentration in panel I / high concentration in panel II). Duration of treatment 15 or 60 minutes. Loading control: ribosomal 23S RNA (rRNA). Loading controls of all treatments are comparable to the ones presented.

Cloning of additional ABC and MFS genes

In an EST library of *B. cinerea* grown under nitrogen starvation conditions, sequences with homology to ABC and MFS genes were identified. Fragments of these sequences were amplified by PCR using genomic DNA of strain B05.10 as a template, cloned and sequenced. Sequence comparison and blast searches revealed that the amplified DNA fragments show identity with several ESTs of ABC and MFS genes in the GenBank database. The amplified sequences are coded *BcatrC* - *BcatrN* (*B. cinerea* ABC transporter genes) and *Bcmfs1 – Bcmfs4* (*B. cinerea* MFS transporter genes). They are deposited in the GenBank database and their accession numbers are listed in Table 2. The codes of the corresponding ESTs with the largest overlap with cloned DNA fragments are also given. *BcatrK* and *BcatrL* may be the same gene as the corresponding ESTs (W44C061 and W22C061, respectively) have only four different nucleotides. However, sequences in the non-coding region of the 3'-end diverge towards the polyA site. Gaps in the alignment of genomic and EST sequences could be attributed to the presence of introns in genomic DNA. The introns all showed minimal consensus sequence at the acceptor (5'GT) and donor sites (AG 3'). Fragments detected had homology with PDR, MDR, half-sized MDR, and MRP genes, encoding transporters with a [$(NBF\text{-}TMD_6)_2$], [$(TMD_6\text{-}NBF)_2$], [TMD_6-NBF], and [$TMD_n\text{-}(TMD_6\text{-}NBF)_2$] topology, respectively. *BcatrJ* and *BcatrM* have homology with prokaryotic ABC transporter genes of unknown function. *BcatrK* overlaps with *BMR1*, a sequence recently deposited in GenBank by Makizuma *et al.* (accession number AB028872). Cloning of genomic DNA (*BcatrM*) with homology to EST W43C091 was not successful. The three MFS fragments cloned show highest homology with genes belonging to the Drug H^+ Antiporters (DHA) with 12 or 14 transmembrane helices. On basis of the available sequence no further classification could be made. The sequence of *Bcmfs3* was not deposited since it appeared to be identical to *Bcmfs4*. Southern analysis indicated that all ABC and MFS identified are most likely single copy genes (results not shown).

Effect of fludioxonil on expression of ABC and MFS genes

The basal transcript levels of the newly identified ABC and MFS transporter genes in wild-type isolate B05.10 differed significantly in Northern experiments. Signals were either undetectable (*e.g. BcatrN*), as described previously for *BcatrA* and *BcatrB,* but could also be weak (*e.g. BcatrK*) or strong (*e.g. BcatrD* and *Bcmfs1)* (Table 3). Similar expression patterns were found in strain SAS56 (results not shown). Basal transcript levels in the mutants ΔBcatrB4 and B05.10C were more or less similar as compared to levels in isolate B05.10, but could also be weaker (*BcatrK* and *Bcmfs1*).

Treatment of germlings of isolate B05.10 with fludioxonil (2.5 mg l^{-1}) for 15 and 60 min not only elevated transcript levels of *BcatrB,* but also of *BcatrG, BcatrJ,* and *BcatrK.* Transcript levels of other genes either remained similar (*e.g. BcatrI* and *BcatrN*) or became down regulated (*e.g. BcatrD*, *BcatrH*, *Bcmfs1*, and *Bcmfs4*). For most of the ABC and MFS genes, the up or down regulating effect of the fungicide on transcript levels in mutants was comparable with that in the wild-type isolate B05.10. The exception to this was the absence of *BcatrB* transcripts in the mutant ΔBcatrB4. More remarkable exceptions were the enhanced expression of *BcatrK* in the laboratory-

generated mutant B05.10C and of *BcatrG* in the gene replacement mutant ΔBcatrB4 upon treatment with fludioxonil (2.5 mg l^{-1}) (Fig. 6).

Table 2. Characteristics of ABC and MFS gene fragments from *Botrytis cinerea* identified in the EST library.

Name	EST code	Accession number	Topology[a]	Length (bp)	Intron position[b]
BcatrC	W40G071	AF241315	PDR	598	116-184
BcatrD	W55C081	AJ272521	PDR	522	-[c]
BcatrE	W52D071	AF238224	MRP	364	-
BcatrF	W30H091	AF238230	MRP	315	-
BcatrG	W65E081	AJ278038	MRP	1043	358-468, 537-932
BcatrH	W05H121	AF241313	MDR[d]		392-446
BcatrI	W35A012	AF238229	MDR[d]	506	-
BcatrJ	W04E081	AF238228	Prokaryotic ABC transporter	515	-
BcatrK[e]	W44C061	AF238227	PDR	394	71-131
BcatrL	W22C061	-[f]	PDR	213	96-155
BcatrM	W43C091	-[g]	Prokaryotic ABC transporter		
BcatrN	W27A081	AF238226	MDR	201	-
Bcmfs1	W33C061	AF238225	DHA14	642	131-183, 239-294
Bcmfs2	W50H061	AF241312		753	298-474
Bcmfs4	W08H051	AF238231	DHA12	493	-

[a] PDR: Pleiotropic Drug Resistance [$(NBF\text{-}TMD_6)_2$ topology]; MDR: MultiDrug Resistance [$(TMD_6\text{-}NBF)_2$ topology]; MRP: Multidrug Resistance-related Protein [$TMD_4\text{-}(TMD_6\text{-}NBF)_2$ topology]; DHA 12/14: Drug-H^+ Antiporter with 12 or 14 transmembrane regions

[b] Relative to first nucleotide identified

[c] No intron detected in the DNA fragment identified

[d] Half sized MDR transporter [$(TMD_6\text{-}NBF)$ topology]

[e] Identical to *BMR1* (accession number AB 28872)

[f] Not deposited because of high similarity with *BcatrK*

[g] Not cloned

Effects of other fungicides on expression of ABC and MFS genes.

The effect of cyprodinil, iprodione, and trifloxystrobin on expression of all ABC and MFS genes was tested in strain B05.10. Cyprodinil (2.5 mg l^{-1}) enhanced transcript levels of *BcatrB*, *BcatrD*, *BcatrF*, *BcatrG*, *BcatrK* and *Bcmfs1*. The effect of the fungicide on expression of *BcatrK* and *Bcmfs1* was relatively strong (Fig. 7A). Iprodione treatment (10 mg l^{-1}) only induced expression of *BcatrB* (Fig. 7B). Trifloxystrobin (0.25 mg l^{-1}) induced expression of *BcatrG, BcatrN*, and *Bcmfs4* (Fig. 7C).

Table 3. Basal and induced transcript levels of ABC and MFS transporter genes from *Botrytis cinerea* B05.10 (wild type), ΔBcatrB4 (BcatrB replacement mutant), and B05.10C (laboratory-generated mutant with fludioxonil resistance) after treatment with fludioxonil (2.5 mg l^{-1}) for 15 or 60 min.

	B05.10				ΔBcatrB4				B05.10C			
	Control		Fludioxonil		Control		Fludioxonil		Control		Fludioxonil	
	15	60	15	60	15	60	15	60	15	60	15	60
BcatrA	0[a]	0	0	0	0	0	0	0	0	0	0	0
BcatrB	0	0	+++	+	0	0	0	0	0	0	+++	+
BcatrC	0	0	0	0	0	0	0	0	0	0	0	0
BcatrD	++	++	+	0	++	++	0	0	++	++	0	0
BcatrE	+	+	+	+	+	+	+	+	+	+	+	+
BcatrF	++	++	+	+	++	++	++	++	++	++	++	++
BcatrG	+	+	++	++	+	++	+++	+++	+	+	++	++
BcatrH	++	++	+	+	++	++	+	+	++	++	+	+
BcatrI	++	++	++	++	++	++	++	++	++	++	++	++
BcatrJ	0	0	++	++	0	0	+++	+++	0	0	+	+
BcatrK	+	+	++	+	0	0	++	++	0	0	+++	+++
BcatrN	0	0	0	0	0	0	0	0	0	0	0	0
Bcmfs1	++	++	0	0	+	+	0	0	++	++	+	+
Bcmfs2	0	0	0	0	0	0	0	0	0	0	0	0
Bcmfs4	++	++	0	0	++	++	0	0	++	++	+	+

[a] 0, +, ++, and +++: no, low, moderate, and high transcript levels, respectively

DISCUSSION

The ABC transporter BcatrB plays a significant role in the activity of the phenylpyrrole fungicide fludioxonil against *B. cinerea*. The following findings support this conclusion: (1) fludioxonil rapidly induces transcription of *BcatrB* at sublethal concentrations in wild-type isolates, (2) the basal level of expression of *BcatrB* in a laboratory-generated mutant CH1.7 with decreased sensitivity to fludioxonil is higher than in wild-type isolates, (3) accumulation of fludioxonil by Δ*BcatrB* mutants with increased sensitivity to the fungicide is significantly higher than in wild-type isolates, and (4) accumulation of fludioxonil by mutant CH1.7 is significantly lower than in wild-type isolates. We assume that the relatively high *BcatrB* transcript levels in mutant CH1.7 result in overproduction of the ABC transporter BcatrB, leading to increased capacity to secrete fludioxonil. In contrast, the absence of *BcatrB* transcripts in the gene replacement mutants will prevent the production of BcatrB, leading to decreased capacity to secrete fludioxonil. We propose that these differences in efflux activity result in a differential accumulation of the compound at its target site in mycelial cells. These results explain the different levels of fludioxonil sensitivity of the *B. cinerea* isolates tested. The observations support similar results described for fenpiclonil in *B. cinerea* (Schoonbeek *et al.* 2001) and for fludioxonil in *A. nidulans* (Andrade 2000). Differential accumulation of fungicides in sensitive and resistant isolates of filamentous fungi have also been reported as a mechanism of resistance to azoles in *A. nidulans* (De Waard and Van Nistelrooy 1979; 1980), *B. cinerea* (Chapeland *et al.* 1999; Stehmann and De Waard 1995), *Mycosphaerella graminicola* (Joseph-Horne *et al.* 1996) and *Nectria haematococca* var. *cucurbitae* (Kalamarakis *et al.* 1991). We hypothesise that in all cases elevated expression of ABC transporter genes may explain the resistance mechanisms. MFS transporters may also be involved since they can have similar functions (Del Sorbo *et al.* 2000).

Replacement mutants of *BcatrB* also displayed a slight increase in sensitivity to the anilinopyrimidine fungicide cyprodinil and the azole fungicide tebuconazole. This implies that BcatrB can also transport other types of fungicides. For this reason, the protein can be regarded as a multidrug transporter. Many ABC and MFS transporters in a wide variety of other organisms function in this way (Del Sorbo *et al.* 2000). The conclusion also corroborate the observation that BcatrB is a transporter of natural toxic products such as the grape vine phytoalexin resveratrol (Schoonbeek *et al.* 2001).

Analysis of the EST database from *B. cinerea* revealed the presence of many additional ABC and MFS genes. This observation indicates that the pathogen has families of transporter genes as has been described for *S. cerevisiae* (Decottignies and Goffeau 1997). To test the role of the newly identified genes in protection against exogenous toxic compounds we studied their expression after treatment with fungicides. Fludioxonil also induced transcript levels of *BcatrG*, *BcatrJ,* and *BcatrK*. Hence, the transporters encoded by these genes may contribute to the efflux capacity of *B. cinerea* for fludioxonil. This is not absolutely certain since compounds that induce expression of a fungicide transporter gene are not necessarily a substrate of the same transporter (Andrade *et al.* 2000a). Fludioxonil down-regulated transcript levels of *BcatrD*, *BcatrH*, *Bcmfs1*,

and *Bcmfs4*. These results suggest that fludioxonil interacts with a complex network of regulatory proteins, ABC transporters, and MFS transporters. This situation resembles the situation described for the pleiotropic drug resistance network in *S. cerevisiae* (Decottignies and Goffeau 1997).

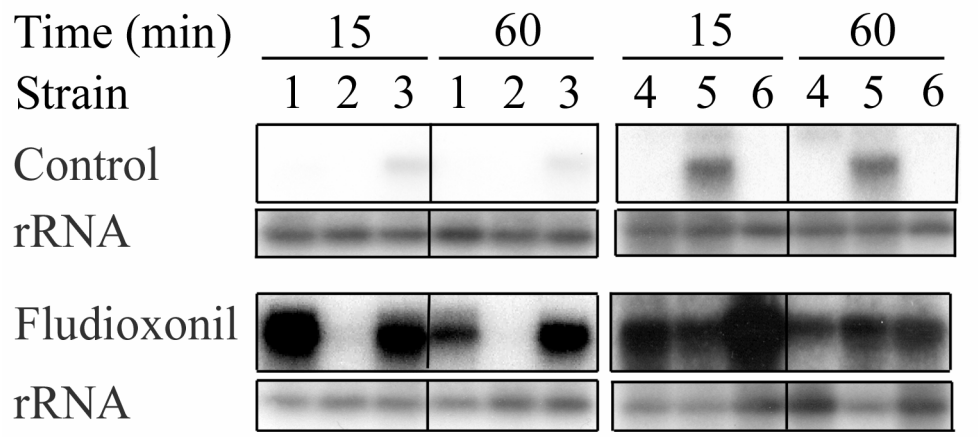

Figure 5. Effect of fludioxonil on transcript levels of *BcatrB* in northern analysis experiments with RNA from germlings of *Botrytis cinerea*. Control: basal expression in mock-treated germlings. Fludioxonil: expression after incubation with fludioxonil (2.5 mg l^{-1}) for 15 or 60 minutes. Strains tested: (1) wild-type B05.10, (2) ΔBcatrB4 replacement mutant, (3) laboratory mutant B05.10C with high degree of resistance to fludioxonil, (4) wild-type SAS56, (5) laboratory mutant CH 1.7 with a low degree of resistance to fludioxonil, and (6) field isolate AVA5/97 with resistance to cyprodinil. Loading control: ribosomal 23S RNA (rRNA).

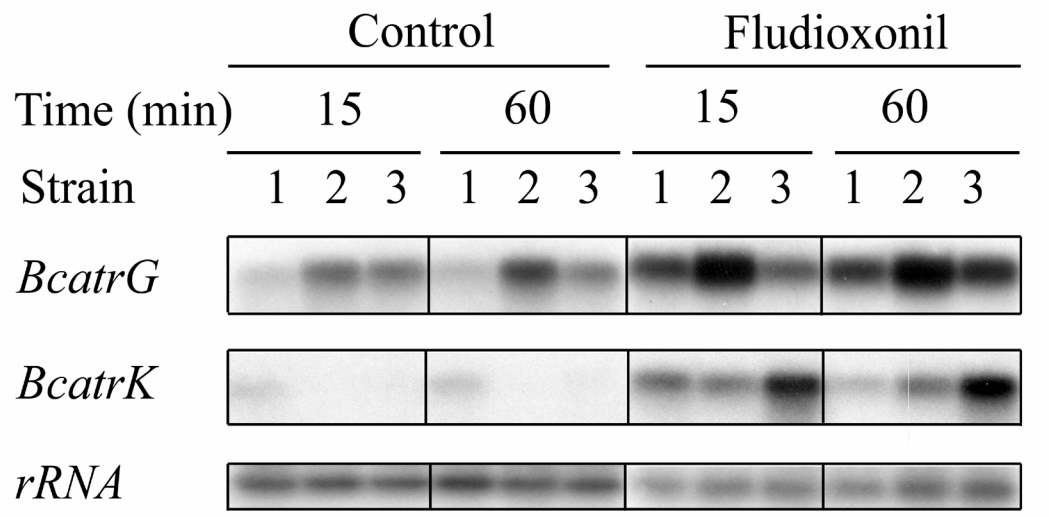

Figure 6. Effect of fludioxonil on transcript levels of *BcatrG* and *BcatrK* in northern analysis experiments with RNA from germlings of *Botrytis cinerea* strains (1) B05.10 wild-type, (2) ΔBcatrB4 replacement mutant, and (3) laboratory mutant B05.10C with a high degree of resistance to fludioxonil. Control: basal level of expression, Fludioxonil: expression after incubation with fludioxonil (2.5 mg l^{-1}) for 15 or 60 minutes. Loading control: ribosomal 23S RNA (rRNA).

The transient accumulation of [^{14}C]fludioxonil by *B. cinerea* strain B05.10 coincides with induction of a high transcript level of *BcatrB* at 15 min after addition of the fungicide. Accumulation by the gene-replacement mutants ΔBcatrB4 and ΔBcatrB5 is only slightly transient.

These observations indicate that BcatrB is the main determinant in efflux of fludioxonil in B05.10. Addition of the respiratory inhibitors fluazinam and trifloxystrobin to B05.10 after 60 min. of incubation with [^{14}C]fludioxonil strongly enhances accumulation, indicating that efflux of fludioxonil is energy-dependent. At this time the transcript level of *BcatrB* is lower than after 15 min. of incubation but still higher than in the control treatment. The half-life of the yeast homologue PDR5p is 60-90 minutes (Egner *et al.* 1995). Hence, we assume that activity of BcatrB accounts for the steady-state efflux activity 60 min. after addition of [^{14}C]fludioxonil.

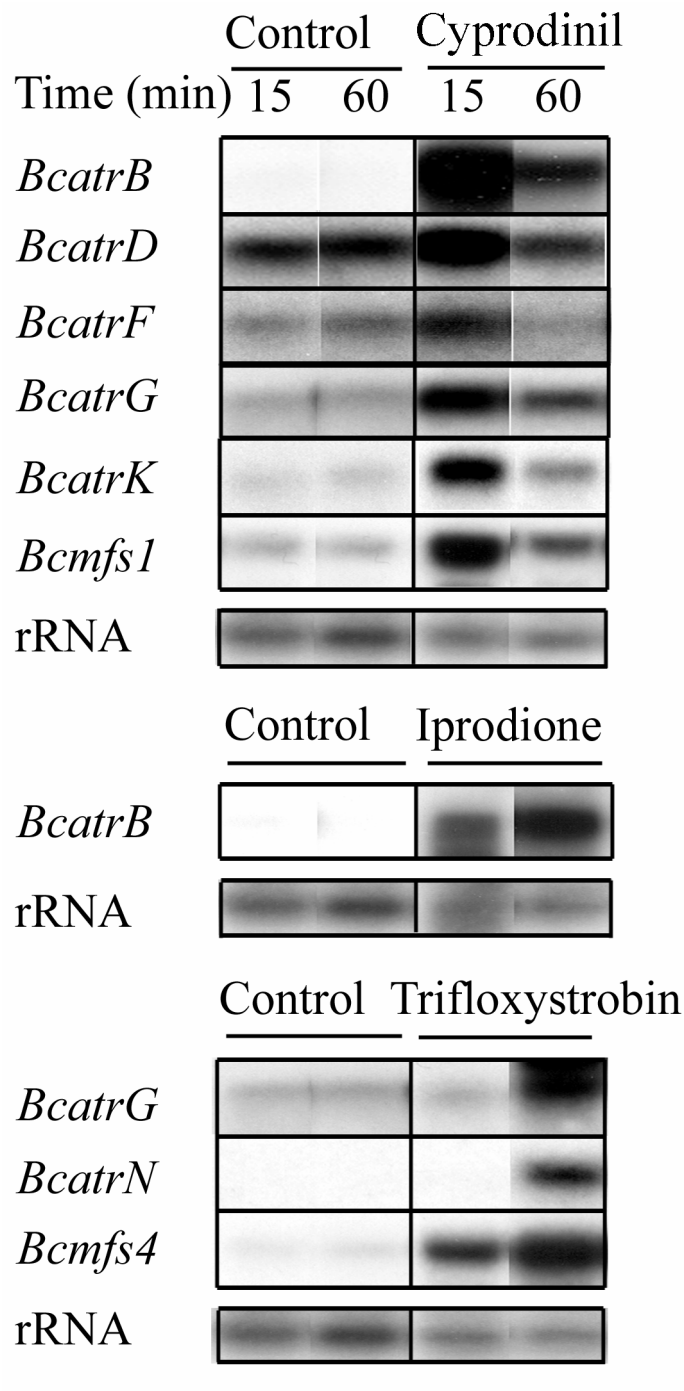

Figure 7. Effect of cyprodinil, iprodione, and trifloxystrobin on transcript levels of ABC and MFS genes in northern analysis with RNA from germlings of *Botrytis cinerea* strain B05.10. Control: basal level of expression. Treatment with cyprodinil (2.5 mg l^{-1}), iprodione (10 mg l^{-1}), and trifloxystrobin (0.25 mg l^{-1}) for 15 or 60 minutes. Loading control: ribosomal 23S RNA (rRNA).

The slightly transient accumulation curve of fludioxonil observed for replacement mutants ΔBcatrB4 and ΔBcatrB5 indicates that they still possess some inducible efflux activity. Northern analysis experiments demonstrated that fludioxonil treatment led to a stronger increase of transcripts of *BcatrG*, *BcatrJ*, and *BcatrK* in the replacement mutant ΔBcatrB4 than in the wild-type. This observation suggests that the transporters encoded by these genes may be responsible for remaining efflux activity and partly compensate for the loss in efflux activity mediated by BcatrB. The simultaneous up regulation of multiple genes in the replacement mutant also suggest that their expression is under control of the same transcription factors.

In contrast to fluazinam and trifloxystrobin, cyprodinil and iprodione are not known as inhibitors of mitochondrial respiration (Ellner 1994; Leroux *et al.* 1992). We hypothesise that their effect on accumulation of [^{14}C]fludioxonil (Fig. 2) can be ascribed to competitive inhibition of fludioxonil transport by BcatrB. This is supported by the observation that both compounds induce expression of the gene and are less active in growth inhibition of mutant CH1.7, which overexpresses *BcatrB*.

The mechanism of resistance to fludioxonil in the fludioxonil-resistant mutant B05.10C is probably different from that in CH1.7. Isolate B05.10C has a high degree of resistance to phenylpyrroles while the resistance level in isolate CH1.7 is low (Table 1). The two resistant isolates also differ in the dynamics of fludioxonil accumulation (Fig. 5). Accumulation by isolate B05.10C was transient as described for B05.10. On the other hand, accumulation by isolate CH1.7 was low and constant in time. Expression analysis showed that basal expression of *BcatrB* in isolate B05.10C could hardly be detected. Induction of *BcatrB* expression in B05.10C by fludioxonil was similar to the wild-type isolate B05.10. Induction of *BcatrK* was relatively strong after 15 and 60 min of treatment. Expression analysis of other transporter genes did not reveal any major differences compared to the wild-type isolate. These results suggest that BcatrK might play a role in resistance. However, since accumulation of fludioxonil by B05.10C was transient as described for B05.10, we hypothesise that in this laboratory mutant the resistance mechanism is not based on a change in efflux activity of BcatrK or any other fungicide transporters. This is rather surprising since the high level of cross resistance to fungicides from different chemical groups (dicarboximides) in this mutant suggests a multidrug resistance phenotype mediated by differential activities of fungicide transporters. As discussed earlier, the mechanism of resistance in isolate CH1.7 is ascribed to overproduction of BcatrB. However, an additional role of other fludioxonil transporters can not be excluded.

Isolate AV5A/97, can probably be classified as the AniR1 phenotype (Chapeland *et al.* 1999). This phenotype is characterised by high specific resistance levels to anilinopyrimidines. Cyprodinil resistance in AV5A/97 is not accompanied by increased basal or cyprodinil-induced transcript levels of *BcatrB*. Hence, this transporter does not account for the mechanism of resistance to anilinopyrimidines. Cyprodinil also induced expression of *BcatrD*, *BcatrF*, *BcatrG*, *BcatrK* and *Bcmfs1*, and so it could be that these transporters play a role in sensitivity and specific resistance to this group of fungicides. However, the mechanism of resistance involved can also be based on

changes in affinity of the target site of these fungicides, as there is an absence of a clear MDR phenotype in isolate AV5A/97.

Our results demonstrate that not only phenylpyrrole fungicides but also anilinopyrimidine, azole, dicarboximide and strobilurin fungicides can induce expression of genes encoding ABC or MFS transporters. Therefore, it may be that several fungicide transporters play a role in sensitivity and resistance to these groups of fungicides as well. Fungicide transporters may especially play a role in resistance if resistant phenotypes meet one of the following characteristics: a MDR phenotype, an initially low level of resistance, or a stepwise pattern of resistance development. These characteristics are well described in literature for various classes of fungicides (Lyr 1995).

Therefore, more research should be focused on the role of ABC and MFS transporters in resistance to fungicides. This applies not only to *B. cinerea,* but to other pathogens as well. The important role of ABC and MFS transporters in clinical resistance of *Candida albicans* to antimycotics reinforces this statement (VandenBossche *et al.* 1998). A better understanding of the role of these transporters in fungicide activity may result in the design of compounds, which are not substrates of fungicide transporters. This may result in the discovery of compounds with increased activity and compounds that are not subject to MDR.

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Chapter 5

Multidrug resistance in *Botrytis cinerea* associated with decreased accumulation of the azole fungicide oxpoconazole and increased transcription of the ABC transporter gene *BcatrD*

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ABSTRACT

Azole-resistant mutants of *Botrytis cinerea* have a multidrug resistance (MDR) phenotype since they exhibit cross resistance to unrelated chemicals. These mutants also display resistance to the new azole fungicide oxpoconazole. Resistance to oxpoconazole is associated with decreased accumulation of the fungicide, which is the result of energy-dependent efflux mediated by fungicide transporters. The ATP-binding cassette (ABC) transporter BcatrB (*B. cinerea* ABC transporter B), involved in efflux of phenylpyrrole fungicides, has no major role in efflux of oxpoconazole since accumulation of the fungicide by a replacement mutant of BcatrB showed a similar transient accumulation pattern as the wild-type isolate. The putative role of ten additional ABC and three Major facilitator superfamily (MFS) transporters in efflux of oxpoconazole was investigated by expression analysis of the corresponding genes. The basal transcription level of *BcatrD* in germlings of *B. cinerea* was correlated with the resistance level of two azole-resistant mutants. A short treatment of germlings with the azole fungicides oxpoconazole, prochloraz, and tebuconazole enhanced transcript levels of *BcatrD* in a wild-type isolate. Transcript levels induced by these fungicides in azole-resistant mutants correlated with resistance levels as well. We propose that BcatrD is the ABC transporter that plays a role in azole-sensitivity and azole-resistance of *B. cinerea*. Expression of *BcatrD* is also induced by treatment of germlings with the dicarboximide fungicide iprodione, the benzimidazole fungicide carbendazim, and the antibiotic cycloheximide suggesting that this gene indeed plays a role in multidrug resistance to fungicides.

INTRODUCTION

Recent genome sequence data revealed the presence of many membrane-bound transporter proteins in all living organisms. These transporters can be involved in secretion of a wide variety of compounds but became especially known for transport of drugs and other toxic products. Particular substrates identified for transporters from fungi are virulence factors (e.g. phytotoxins), plant defense compounds, and mating factors (Del Sorbo *et al.* 2000). Major groups of transporters involved in drug resistance can be divided into two superfamilies: the ATP-Binding-Cassette (ABC) superfamily (Dean and Allikmets 1995; Higgins 1992) and the Major Facilitator Superfamily (MFS) (Pao *et al.* 1998). ABC transporters directly utilize the energy generated by ATP hydrolysis to pump substrates across membranes against a concentration gradient (Driessen *et al.* 2000). On the other hand, MFS transporters are secondary transporters, driven by the proton motive force over membranes (Paulsen *et al.* 1996). Inhibitors of ABC and MFS transporter activity may act as synergists with fungicides that are substrates for these transporters (De Waard 1997). Inhibitors of transporter activity in plant pathogens may also result in decreased secretion of virulence factors or increased accumulation of plant defense compounds. Hence, such compounds can act as lead compounds in the discovery of disease control agents, which are not necessarily fungitoxic themselves.

Many fungi readily developed resistance to all major classes of fungicides with a site-specific mode of action. The most common mechanism of resistance is based on mutations in genes encoding the target protein of these fungicides by which affinity of the encoded protein to the fungicide is reduced. This mechanism applies to various antibiotics and fungicides such as azoles, benzimidazoles, and carboximides (Lamb et al. 1997; Lamb et al. 2000). Resistance to azole fungicides and antimycotics can also be due to decreased accumulation of the compounds in mycelium. This mechanism has been reported for *Aspergillus nidulans* (De Waard and Van Nistelrooy 1980; De Waard and van Nistelrooy 1981; De Waard and van Nistelrooy 1987; Siegel and Solel 1981), *Botrytis cinerea* (Chapeland et al. 1999; Stehmann and De Waard 1995), *Candida albicans* (Ryley et al. 1984), and *Penicillium italicum* (De Waard and van Nistelrooy 1984; De Waard and Van Nistelrooy 1988). Reduced accumulation can be mediated by ABC and MFS transporters. ABC transporters involved in energy-dependent efflux of azoles have been described for *A. nidulans* (Andrade et al. 2000a; Andrade et al. 2000b; Del Sorbo et al. 1997), *C. albicans* (Prasad et al. 1995; Sanglard et al. 1995), *Mycosphaerella graminicola* (Zwiers and De Waard 2000), and *P. digitatum* (Nakaune et al. 1998). A role of ABC transporters in resistance of *Saccharomyces cerevisiae* to dicarboximides was also reported (Nakaune et al. 1996).

A major research topic in the Ube Research Laboratory regards the discovery and development of fungicides. In this context, the company has introduced a new azole fungicide, oxpoconazole, with activity against *B. cinerea* under field conditions. The Department of Phytopathology of Wageningen University has strong interest in fungicide resistance and demonstrated previously that resistance to tebuconazole in azole-resistant laboratory mutants of *B. cinerea* is due to reduced accumulation of this fungicide in mycelium (Stehmann and De Waard 1995). This resistance mechanism was also found for tebuconazole in anilinopyrimidine-resistant field isolates of *B. cinerea* that show cross resistance to azole fungicides (Chapeland et al. 1999). Our current research focuses on resistance in *B. cinerea* to oxpoconazole and the potential mechanisms of resistance. We hypothesize that decreased accumulation of oxpoconazole in mycelium can be the major cause of resistance since this mechanism was also observed for tebuconazole. To test this hypothesis, we have studied the accumulation of oxpoconazole in a wild-type isolate and azole-resistant mutants of *B. cinerea*. Previously, we have reported on the ABC transporter genes of *BcatrA* (*B. cinerea* ABC transporter A) and *BcatrB* from *B. cinerea* (Schoonbeek et al. 2001). Furthermore, we reported the presence of ten additional ABC (*BcatrC-BcatrN*) and three MFS (*Bcmfs1-Bcmfs4*) genes, by analysis of an EST library from *B. cinerea* (Vermeulen et al. 2001). This study describes the expression analysis of all these ABC and MFS genes in a wild-type isolate and azole-resistant mutants of *B. cinerea*. We propose that BcatrD is the ABC transporter involved in azole resistance.

MATERIALS AND METHODS

Fungal Strains

B. cinerea strains used in this study were strain B3 (wild-type strain isolated from tomato in Greece), and strains G25 and G66 (laboratory-generated mutants selected from strain B3 on agar amended with 100 mg l^{-1} triadimefon) (Stehmann and De Waard 1996). These strains were kindly provided by Dr. B.N. Ziogas (University of Athens, Greece). Strain B05.10 is a haploid wild-type strain (Buttner *et al.* 1994) and ΔBcatrB4 is a *BcatrB* replacement mutant generated from strain B05.10 in our laboratory (Schoonbeek *et al.* 2001).

Compounds

Oxpoconazole, prochloraz, and iprodione (technical grade) were synthesized by Ube Industries Ltd. (Ube, Yamaguchi, Japan). Carbendazim, fluazinam, pyrimethanil, tebuconazole, and trifloxystrobin were kindly provided by Du Pont de Nemours & Co. (Wilmington, Delaware, USA), ISK Bioscience Co. (Mentor, Ohio, USA), Aventis (Lyon, France), Bayer AG (Leverkusen, Germany), and Syngenta (Stein, Switzerland), respectively. Cycloheximide was purchased from Sigma (St. Louis, Missouri, USA).

Fungicide activity test

B. cinerea was grown on PDAtom (20 g potato dextrose agar amended with 200 g of homogenized tomato leaves and 5 g agar per 0.7 l of water) at 20°C for 2-3 days. Then, plates were irradiated with near-UV light for 24 h to induce formation of conidia, and incubated for another 3-7 days at 20°C. Conidia were harvested in sterile distilled water with 0.1% Tween 20 and separated from mycelium by filtration through sterile glass wool. Concentrations of conidia in suspension were determined with a haematocytometer. Conidial suspensions (approximately 10^6 spores ml^{-1}) were spread on synthetic agar medium (Leroux *et al.* 1999) and incubated in the dark at 20°C for 1 day. Agar plugs (diameter 5 mm) from 1-day-old cultures were used to inoculate Petri dishes with synthetic agar amended with fungicides from 100x concentrated stock solutions in methanol. Fungicides concentrations used in the agar were below the solubility level of the compounds except for carbendazim (10 mg l^{-1}). The plates were inoculated with 3 agar plugs and incubated at 20°C for 2-3 days. EC_{50} values of fungicides were calculated from dose-response curves using Excel 97. Statistical analysis of the EC_{50} values was performed using the LSD (T) comparison of means. Experiments were performed in triplicate. Resistance levels Q, defined as the ratio between the EC_{50} value of a compound for radial growth of a mutant and the wild-type isolate, were calculated.

Accumulation of oxpoconazole by germlings

Conidial suspensions were prepared from cultures on malt extract agar (Oxoid Ltd., Basingstoke, Hampshire, England) amended with 0.2% yeast extract (Oxoid). After inoculation and incubation

for 3 days, formation of conidia was induced by irradiation with near-UV light for 24 h. The plates were incubated at 20°C for at least another 3 days.

Conidial suspensions were used to inoculate round bottom flasks (300 ml) with liquid synthetic medium (100 ml) (Fritz et al. 1977) to a final density of 2 x 10^6 conidia ml^{-1}. The flasks were incubated in a rotary shaker (180 rpm) in the dark at 20°C for 12 h. The cultures were filtered over a 0.85 mm pore sieve to remove clusters of mycelium. Germlings in the filtrate were collected on a 0.05 mm pore stainless steel sieve. Germlings were washed three times with 0.05 M potassium phosphate buffer (pH 6.0) containing 10 g l^{-1} D-glucose and resuspended in the same buffer (4 g wet weight l^{-1}).

Standard germling suspensions (50 ml in 300 ml Erlenmeyer flasks) were shaken on a reciprocal shaker at 20°C for 20 min (De Waard and Van Nistelrooy 1979; 1988). Accumulation experiments were initiated by adding [^{14}C]oxpoconazole (30 µM initial external concentration, 750 Bq $nmol^{-1}$) from a 100x concentrated stock solution in methanol. Accumulation of oxpoconazole was determined in germlings collected on glass microfiber filters (Whatman International Ltd., Maidstone, England) from samples (5 ml) at 0, 5, 10, 20, 30, 45, 60, 120, and 180 min after the addition of oxpoconazole. Collected germlings were washed 3 times in 30 sec with 5 ml of the same buffer. Radioactivity in mycelium was extracted with scintillation liquid (LUMASAFETM PLUS, LUMAC*LSC B.V., Groningen, The Netherlands) for 1 day and counted in a liquid scintillation spectrometer BECKMAN LS6000TA (Beckman Coulter Inc., California, USA).

Effects of compounds on uptake of [^{14}C]oxpoconazole were determined by addition from 1000x concentrated stock solution in methanol, 185 min after addition of [^{14}C]oxpoconazole, to standard germling suspensions. Samples (5 ml) were collected at 190, 200, 215, 245, 305, and 365 min after the addition of [^{14}C]oxpoconazole and assessed for accumulation of [^{14}C]oxpoconazole as described above.

Cloning of DNA fragments from ABC and MFS genes

From an EST library of *B. cinerea* (F. Bitton, C. Levis, D. Fortini, J. M. Pradier, and Y. Brygoo, Genoscope, Centre National de Sequençage, *B. cinerea* strain T4cDNA library under conditions of nitrogen deprivation. EMBL AC# fromAL110624 to AL117185, unpublished, 1999), ten ESTs with homology to ABC transporter genes and three ESTs with homology to MFS genes were selected. PCR amplifications of corresponding DNA fragments were done with primers based on the EST sequences. Genomic DNA from *B. cinerea* B05.10 was used as template. Amplified fragments were ligated in the pGEM®-T easy vector using pGEM®-T Vector Systems (Promega, Madison, Wisconsin, USA). The sequences of these inserts were determined with BigDye Terminator sequence-kits (Perkin-Elmer Corporation, Connecticut, USA). DNA manipulations were performed according to standard methods (Sambrook *et al.* 1989). *Escherichia coli* strain DH5α was used for propagation of constructs.

RNA isolation and northern blot analysis

Conidia of *B. cinerea* were added to 100 ml of B5 medium (1% sucrose, 10 mM $(NH_4)H_2PO_4$ and 0.31% Gamborg B5 medium elements (Duchefa, Haarlem, The Netherlands)) at a final density of 10^6 spores ml^{-1} in 300 ml round bottom flask and incubated at 20°C and 20 rpm for 2 h to synchronize germination. Then, flasks were shaken at 20°C and 180 rpm for 14-16 h. Cultures were divided into 20 ml aliquots in 50 ml Erlenmeyer flasks containing approximately 100 mg of wet weight (approximately 10 mg of dry weight) germlings. Fungicides were added to germlings from 1000x concentrated stock solution in methanol. Germlings were collected with a Millipore vacuum manifold on glassfiber filters. Harvested germlings were immersed immediately in liquid nitrogen. Frozen germlings were disintegrated with a dismembrator (B. Braum Biotech International GmbH, Melsungen, Germany), mixed with 1 ml TRIzol (Life Technologies Inc., Breda, The Netherlands), and incubated at room temperature for 1 h. Extracts were centrifuged at 12,000g at 4°C for 10 min to remove extracellular materials and polysaccharides. Supernatants were transferred, mixed with chloroform (0.2 ml), and centrifuged at 12,000g at 4°C for 15 min to separate the aqueous and organic phase. The water phase was transferred and isopropanol (0.5 ml) and 3 M sodium acetate (50 µl) were added. Mixtures were inverted several times and centrifuged at 12,000g at 4°C for 10 min. Supernatants were discarded. RNA pellets were washed twice with cold 75% ethanol, air dried at 55°C for 5 min, and dissolved in 50-200 µl of RNase free water. The concentration of RNA was determined by measuring the absorbance at 260 nm.

Northern blot analysis was performed by incubation of total RNA (10 µg in 9.2 µl of water) with 6 M glyoxal (4.5 µl), DMSO (13.3 µl), and 0.1 M sodium phosphate (3 µl) at 50°C for 1 h to denature RNA. Then, RNA was subjected to electrophoresis on a 1.6% agarose gel in 10 mM sodium phosphate using a SEA 2000 gel electrophoresis apparatus (Elchrom Scientific AG, Cham, Switzerland) for 2 h at 0.4 Amp. (3-4 V cm-1). RNA was blotted on to Hybond^{TM}-N^+ membranes (Amersham Pharmacia Biotech, Uppsala, Sweden) by capillary transfer (Sambrook et al. 1989) in 10x SSC overnight. RNA was cross-linked to membranes by irradiation with UV light (0.6 J cm^{-2}).

DNA probes from the EST library used in northern analysis were obtained by digestion of the plasmids described above with *Not*I. Gene specific *Hin*dIII fragments of *BcatrA* (accession number Z68906; nucleotides 3411-4344) and *BcatrB* (accession number AJ006217; nucleotides 2246-2942) were also used. The DNA fragments were purified using the QIAquick gel extraction kit (Qiagen GmbH, Hilden, Germany). Purified DNA fragments (30 ng) were radioactively labeled using the Prime-a-Gene® Labeling System (Promega) and 2 µl of [α-^{32}P]dATP (Amersham). Membranes were preincubated in Modified Church buffer (0.36 M Na_2HPO_4, 0.14 M NaH_2PO_4, 1 mM EDTA, 7% SDS, pH 7.2) at 65°C for 1 h and, next, hybridized with the probe in the same buffer at 65°C overnight. Blots were washed in 0.1% SDS with 2x SSC, 1x SSC, and 0.5x SSC at 65°C. The washed blots were autoradiographed at -80°C for 1-7 days using Kodak Scientific Imaging Film, X-OMAT^{TM} LS. Membranes were re-used in northern analysis experiment after stripping in boiling 1% SDS. Hybridization with 28S rRNA from *B. cinerea* (Prins et al. 2000) was

used as loading control. Ethidium bromide staining of agarose gels demonstrated that the quality of RNA was good (data not shown).

RESULTS

Fungicide activity tests

The sensitivity of the wild-type isolate B3 and the azole-resistant mutants G25 and G66 to various fungicides was tested in radial growth test (Table 1). Both mutants displayed cross resistance to all azole fungicides tested (oxpoconazole, prochloraz, and tebuconazole). The resistance ratio for all azoles tested was higher for strain G25 than for strain G66. A low degree of cross resistance was also found to the dicarboximide iprodione and the antibiotic cycloheximide. Sensitivity to the benzimidazole fungicide carbendazim was similar for all isolates tested.
The fungitoxic activity of azole fungicides tested to the BcatrB replacement mutant ΔBcatrB4 was slightly higher than to the parent isolate B05.10, although not significantly different. Activity of iprodione, carbendazim, and cycloheximide to strains B05.10 and ΔBcatrB4 was similar (Table 1). This was also found for the anilinopyrimidine fungicide pyrimethanil (data not shown).

Accumulation of oxpoconazole

Accumulation of oxpoconazole (initial external concentration 30 μM) by strain B3 was transient in time (Fig. 1). Initial accumulation by azole-resistant mutants G25 and G66 during the first 30 min of incubation was significantly lower than by strain B3. Accumulation by strain G25 was low and constant in time while that by strain G66 was still slightly transient.

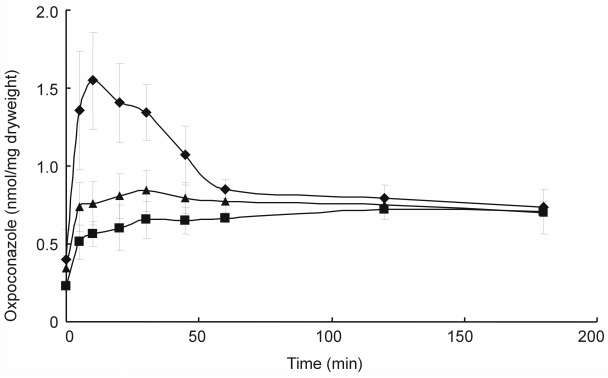

Figure 1. Accumulation of oxpoconazole (30 μM) by germlings of *Botrytis cinerea* wild-type strain B3 (◆) and azole-resistant mutants G25 (■) and G66 (▲).

Captan (100 μM), copper sulfate (10 μM), fluazinam (10 μM), and trifloxystrobin (10 μM) increased the accumulation of oxpoconazole by strains B3 and G25 when added 185 min after addition of oxpoconazole (Fig. 2). The strongest effect was found with the uncoupler fluazinam. This compound enhanced the accumulation levels of oxpoconazole from 0.8 to 4.0 nmol mg^{-1} dry weight of germlings of strain B3. No major differences between the effects of the compounds tested on accumulation by strains B3 and G25 were observed. Drops of germling suspension were sampled after the experiment and inoculated on malt-yeast extract agar plates. Even though there was some difference in growth rate, each sample of germlings readily formed a colony, indicating that the fungicide treatments were not lethal (data not shown).

Accumulation of oxpoconazole by the haploid wild-type strain B05.10 was also transient in time. Accumulation by mutant ΔBcatrB4 was slightly higher than by B05.10 but not significantly different (Fig. 3).

Table 1. Activity of fungicides against *Botrytis cinerea* in radial growth experiments.

		Oxpoconazole		Prochloraz		Tebuconazole		Iprodione		Carbendazim		Cycloheximide	
Exp.	Strain [a]	EC_{50} [b]	Q [c]	EC_{50}	Q	EC_{50}	Q	EC_{50}	Q	EC_{50}	Q	EC_{50}	Q
A	B3	0.056 ± 0.03 b [d]	-	0.027 ± 0.03 b	-	0.065 ± 0.03 b	-	0.050 ± 0.03 b	-	0.059 ± 0.03 a	-	0.718 ± 0.23 b	-
	G25	0.520 ± 0.18 a	(9.3)	0.173 ± 0.11 a	(6.4)	0.615 ± 0.22 a	(9.5)	0.126 ± 0.06 a	(2.5)	0.051 ± 0.04 a	(0.9)	1.340 ± 0.32 a	(1.9)
	G66	0.182 ± 0.10 a	(3.3)	0.136 ± 0.11 a	(5.0)	0.430 ± 0.25 a	(6.6)	0.127 ± 0.09 a	(2.5)	0.053 ± 0.03 a	(0.9)	1.370 ± 0.39 a	(1.9)
B	B05.10	0.081± 0.02 a	-	0.028± 0.02 a	-	0.161± 0.04 a	-	0.071 ± 0.03 a	-	>10 a	-	1.36 ± 0.56 a	-
	ΔBcatrB4	0.055± 0.01 a	(0.7)	0.025± 0.01 a	(0.9)	0.093± 0.04 a	(0.6)	0.068 ± 0.04 a	(1.0)	>10 a	(1.0)	1.75 ± 0.83 a	(1.3)

[a] B3 (wild-type strain), G25 and G66 (azole-resistant mutants selected in the laboratory from B3), B05.10 (haploid wild-type strain), and ΔBcatrB4 (*BcatrB* replacement mutant generated from B05.10).

[b] EC_{50} values and standard deviations (mg l^{-1}).

[c] Q-value is the ratio between EC_{50} values of azole-resistant mutant G25, G66 and wild-type isolate B3 (Experiment A) or between EC_{50} values of *BcatrB* replacement mutant ΔBcatrB4 and wild-type isolate B05.10 (Experiment B).

[d] Means followed by the same letters in the same column of panel A or B indicate that figures do not differ significantly ($P < 0.05$).

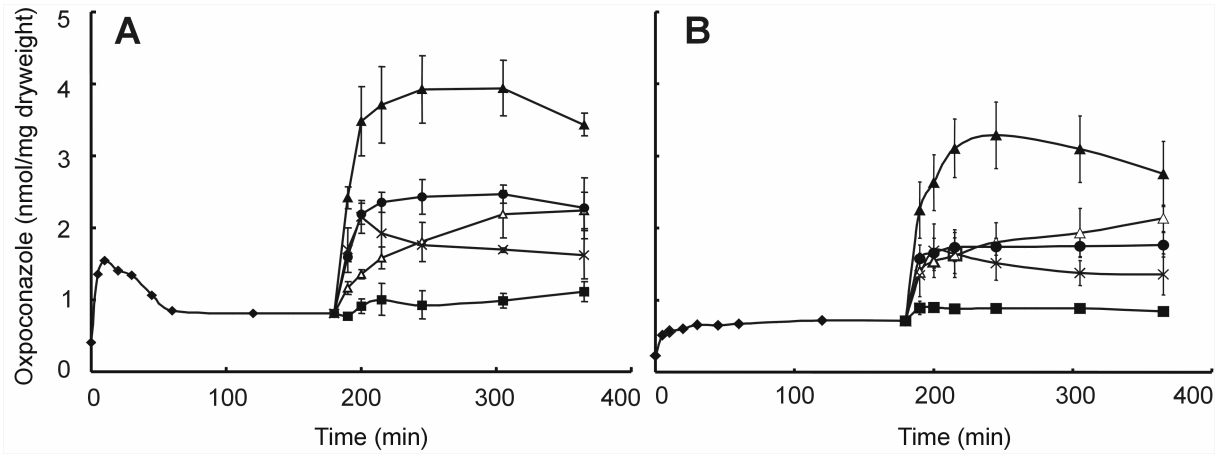

Figure 2. Effects of various compounds on the accumulation of oxpoconazole (30 µM) by germlings of *Botrytis cinerea* strains B3 (A) and G25 (B). No treatment: (◆). Treatments: methanol control (0.1%, ■), captan (100 µM, △), copper sulfate (10 µM, ●), fluazinam (10 µM, ▲), and trifloxystrobin (10 µM, ×).

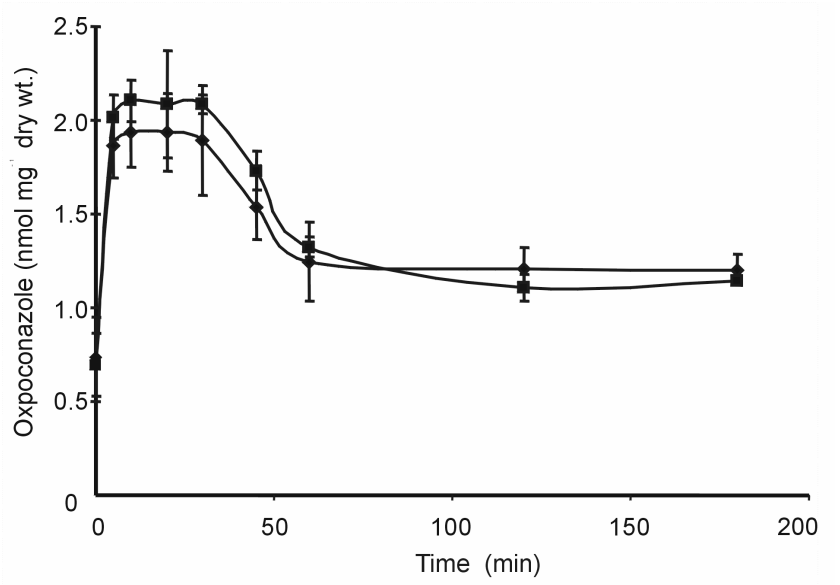

Figure 3. Accumulation of oxpoconazole (30 µM) by germlings of *Botrytis cinerea* strains B05.10 (◆) and ΔBcatrB4 (■).

Expression analysis

Expression of ABC and MFS transporter genes in the wild-type isolate B3 and azole-resistant mutants G25 and G66 was studied in northern analysis experiments using gene-specific fragments (Table 2) as probe (Vermeulen *et al.* 2001).

Basal transcript levels differed significantly for the various genes tested (Fig. 4). In wild-type isolate B3, no transcript signal was found for *BcatrA*, *BcatrC*, *BcatrE*, *BcatrF*, *BcatrL*, *BcatrM*, *BcatrN*, and *Bcmfs2* (results not shown), and *BcatrB* and *BcatrK* (Fig. 4). The other genes tested showed transcript signals (Fig. 4) which varied from low (*BcatrG*) to high (*Bcmfs4*), indicating that these genes are constitutively expressed. Basal expression of all genes was similar in wild-type isolate B3 and mutants G25 and G66, except for *BcatrD*. Transcript signals of *BcatrD*

were weak in the wild type and relatively strong in the mutants, especially in mutant G25. This differential expression of *BcatrD* in wild-type and mutant isolates has been demonstrated in three independent experiments.

Table 2. ABC and MFS genes from *Botrytis cinerea* used in northern analysis experiment.

Name	EST code	Accession number	Topology[a]
BcatrA	-[b]	Z68906	PDR
BcatrB	-	AJ006217	PDR
BcatrC	W40G071	AF241315	PDR
BcatrD	W55C081	AJ272521	PDR
BcatrE	W52D071	AF238224	MRP
BcatrF	W30H091	AF238230	MRP
BcatrG	W65E081	AJ278038	MRP
BcatrH	W5H121	AF241313	1/2MDR
BcatrI	W35A012	AF238229	1/2MDR
BcatrJ	W04E081	AF238228	Prokaryotic ABC transporter
BcatrK[c]	W44C061	AF238227	PDR
BcatrL	W22C061	-[d]	PDR
BcatrM	W43C091	-[e]	Prokaryotic ABC transporter
BcatrN	W27A081	AF238226	MDR
Bcmfs1	W33C061	AF238225	DHA14
Bcmfs2	W50H061	AF241312	
Bcmfs4	W08H051	AF238231	DHA12

[a] Topology of proteins to which the genes listed have highest homology. PDR: Pleiotropic Drug Resistance [(NBF-TMD_6)$_2$ topology]; MDR: MultiDrug Resistance [(TMD_6-NBF)$_2$ topology]; 1/2 MDR: [(TMD_6-NBF) topology]; MRP: Multidrug Resistance-related Protein [TMD_n-(TMD_6-NBF)$_2$ topology]; DHA 12/14: Drug-H^+ Antiporter with 12 or 14 transmembrane regions.

[b] *BcatrA* and *BcatrB* are not present in the EST library.

[c] 100% identity with BMR1 (accession number AB28872) (Nakajima *et al.* 2001)

[d] Similar to *BcatrK*.

[e] Not cloned.

Additional experiments on induction of expression of *BcatrD* in the wild-type isolate B3 by oxpoconazole, prochloraz, and tebuconazole (30 mg l^{-1}) showed that an increase of transcripts could already be observed after 5 min of treatment. Highest transcript levels were found after 15-30 min of treatment (Fig. 5). Treatment with the fungicides at a lower concentration (3 mg l^{-1}) delayed the rate of induction of transcription in time but induction in wild-type isolate and azole-resistant mutants became clearly differential (Fig. 4). For these reasons we studied the effect of all azole fungicides tested on transcription of ABC and MFS genes after 15 and 60 min of incubation at 3 mg l^{-1}.

None of the azole fungicides tested induced transcript levels of *BcatrA*, *BcatrC*, *BcatrE*, *BcatrF*, *BcatrL*, *BcatrM*, *BcatrN*, and *Bcmfs2* to detectable levels in any of the strain tested (data not shown). The treatments did not markedly influence the transcript signals of *BcatrH*, *BcatrI*, *BcatrJ*, and *Bcmfs4* (Fig. 4). In contrast, the treatments induced high transcript levels of *BcatrB*, *BcatrD*, *BcatrG*, *BcatrK*, and *Bcmfs1* in wild-type strain B3 (Fig. 4). Differential effects of azoles on transcript levels amongst the strains tested were found for *BcatrB* (relatively low signals in mutants) and *BcatrD* (relatively high signals in mutants). For *Bcmfs1* the correlation between resistance levels and expression was not clear (Fig. 4).

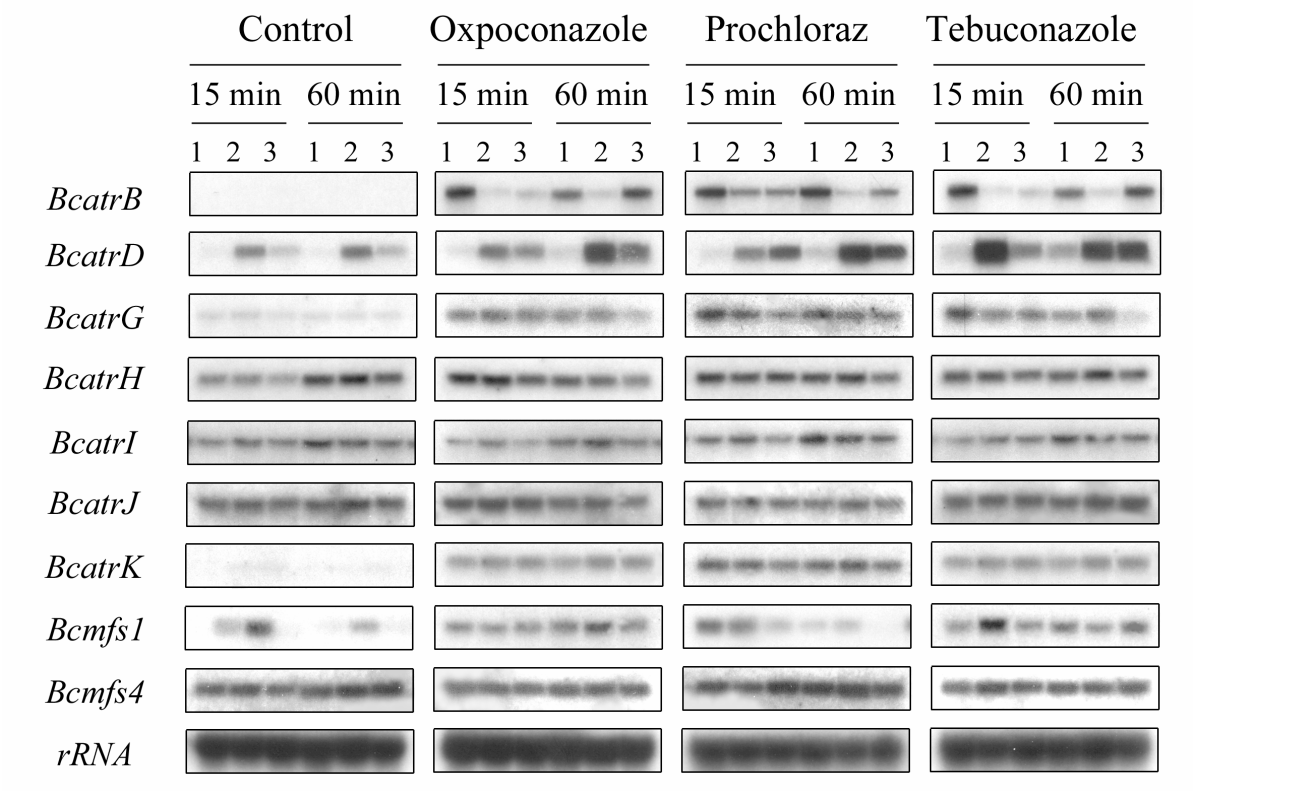

Figure 4. Effect of azole fungicides on transcript levels of ABC and MFS genes in northern blot analysis with RNA from germlings of *Botrytis cinerea*. Control (0.1% methanol): basal levels of expression. Treatments (3 mg l^{-1}): azole fungicides oxpoconazole, prochloraz and tebuconazole. Northern analysis after 15 and 60 min of treatment of wild-type isolate B3 (lanes 1) and azole-resistant mutants G25 (lanes 2) and G66 (lanes 3). Equal loading of lanes with RNA was checked by subsequent probing of the same blot with 28S RNA (rRNA).

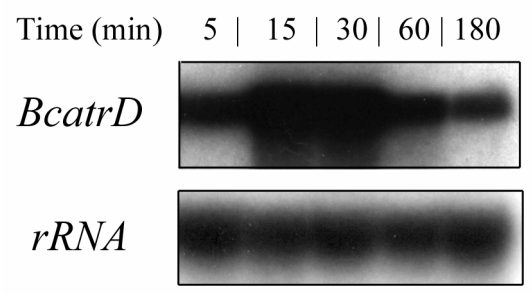

Figure 5. Time-course of of *BcatrD* transcription in wild-type strain B3 of *Botrytis cinerea* after treatment of germlings with oxpoconazole (30 mg l^{-1}). Treatment for 5, 15, 30, 60 and 180 min. Equal loading of lanes with RNA was checked by subsequent probing of the same blot with 28S RNA (rRNA).

The effect of compounds from other classes of fungicides was tested on expression of *BcatrB*, *BcatrD*, and *Bcmfs1* only (Fig. 6). Carbendazim enhanced transcript levels of *BcatrD* in mutants G25 and G66. The effect was strongest upon treatment for 60 min. Cycloheximide had a similar effect on transcript levels of *BcatrD* in both mutants as compared to carbendazim. In addition, the antibiotic also induced *BcatrB*, but relatively stronger in the wild type than in the mutants. This effect was only observed after 60 min of treatment. Cycloheximide also increased transcript levels of *Bcmfs1*, but no obvious differential effects among strains were observed. Iprodione enhanced transcript levels of *BcatrD*, especially in mutant G25. The effect on transcription of *BcatrB* was just the opposite since after 15 min of treatment signals were relatively strong in strain B3.

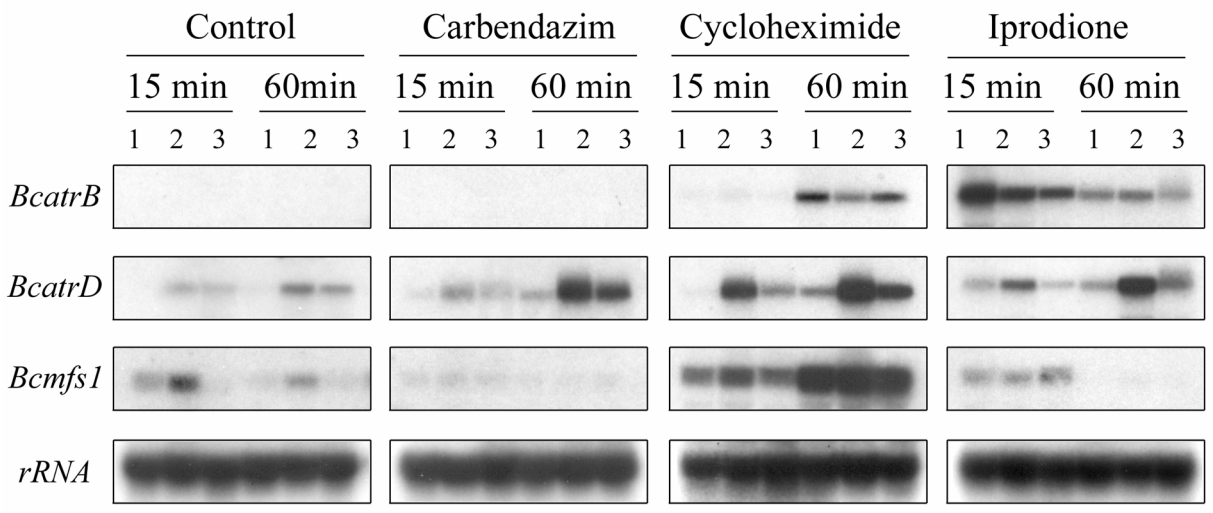

Figure 6. Effect of different fungicides on transcript levels of *BcatrB*, *BcatrD*, and Bcmfs1 in northern blot analysis with RNA from germlings of *Botrytis cinerea*. Control (water): basal levels of expression. Treatments: carbendazim (30 mg l^{-1}), cycloheximide (50 mg l^{-1}), and iprodione (30 mg l^{-1}). Northern analysis after 15 and 60 min of treatment of wild-type isolate B3 (lanes 1) and azole-resistant mutants G25 (lanes 2) and G66 (lanes 3). Equal loading of lanes with RNA was checked by subsequent probing of the same blots with 28S RNA (rRNA).

DISCUSSION

Mutants of *B. cinerea* selected for resistance to azoles have a low degree of cross resistance to non-related fungicides such as iprodione and cycloheximide. These results indicate that the mutants have a multidrug resistance phenotype (Table 1).

Multidrug resistance to unrelated drugs can be mediated by increased efflux activity of multidrug transporters (De Waard 1997). This also proved to be the case for oxpoconazole since the transient accumulation of the fungicide by wild-type strain B3 suggests the presence of inducible oxpoconazole efflux activity. Initial accumulation by azole-resistant mutants G25 and G66 was significantly lower than by strain B3 suggesting that oxpoconazole efflux activity in these isolates is high and constitutive. We suppose that the latter characteristics prevent that activity of the target enzyme of azole fungicides, sterol 14α-demethylase, is inhibited and, hence, explain the azole-resistance of the mutants (De Waard and Van Nistelrooy 1980; De Waard and van Nistelrooy 1984). The fact that various respiratory inhibitors (captan, copper sulfate, fluazinam, and trifloxystrobin) enhance the accumulation of oxpoconazole in both wild-type and azole-resistant strain indicates that efflux activity is energy-dependent. These observations corroborate that oxpoconazole resistance is mediated by increased energy-dependent efflux activity. A similar mechanism of resistance has been described before for the azole fungicide tebuconazole in the same mutants of *B. cinerea* (Stehmann and De Waard 1995) and in azole-resistant field isolates of *B. cinerea* (Chapeland et al. 1999).

Field isolates of *B. cinerea* with a reduced sensitivity to azoles are strains SD29 and D12 (Stehmann and De Waard 1995) and strains with an AniR3 phenotype (Chapeland et al. 1999; Leroux et al. 1999). The latter strains are resistant to anilinopyrimidine fungicides and display cross resistance to azoles. Accumulation of tebuconazole by strains SD29 and D12 was similar to the wild-type isolate (Stehmann and De Waard 1995). Remarkably, accumulation of the anilinopyrimidine fungicide pyrimethanil by wild-type and AniR3 strains was similar, while initial accumulation of the azole fungicide tebuconazole (100 μM) by AniR3 strains was significantly lower than by the wild-type strains (Chapeland et al. 1999). However, the transporter involved in efflux of azoles in AniR3 mutants is probably different from the one in G25 and G66 since the latter mutants do not exhibit cross-resistance to pyrimethanil.

Reduced initial accumulation of oxpoconazole by the mutants G25 and G66 can be caused by overproduction of drug transporters belonging to either the ABC or the MFS transporter family. Despite the fact that azoles induce expression of the ABC transporter *BcatrB*, the low accumulation of oxpoconazole in mutants G25 and G66 can not be due to overexpression of *BcatrB* because the gene replacement mutant ΔBcatrB4 shows similar accumulation of oxpoconazole as the wild-type isolate B05.10 (Fig. 3) and the sensitivity of ΔBcatrB4 and B05.10 to oxpoconazole is similar (Table 1).

We propose that BcatrD is the most probable drug transporter involved in azole resistance, since basal transcript levels of this transporter correlate with the resistance level of the mutants. Furthermore, all azole fungicides tested upregulate expression in mutants stronger than in the wild-

type strain. A similar basal and azole-induced expression pattern has been described in azole resistant strains of the plant pathogen *Penicillium digitatum* (Nakaune et al. 1998). *BcatrD* could also be induced with the non-azole fungicides iprodione and cycloheximide. In addition, mutants G25 and G66 overexpressing *BcatrD* were less sensitive to these compounds. Therefore, we also propose that BcatrD is a multidrug transporter.

Some fungicides induce expression of particular ABC genes while mutants with a changed basal level of expression of these genes do not show a phenotype. This is the case for *BcatrB* and *BcatrD* with respect to azoles and carbendazim, respectively. These results indicate that a fungitoxic compound may have the potency to induce transcription of an ABC gene, while it can not act as a substrate of the encoded transporter protein. This phenomenon has been described before (Andrade et al. 2000a; Schoonbeek et al. 2001).

Transcription of *BcatrG* and *BcatrK* was induced by azole fungicides, but induced transcript levels were similar in the wild-type isolates and both mutants (Fig. 4). It might be that the transporters encoded by these genes are also involved in transport of azoles. However, it is not likely that overproduction of *BcatrG* or *BcatrK* is responsible for the decreased accumulation of oxpoconazole observed in mutants G25 and G66. We suggest that *BcatrG* and *BcatrK* may be under the same regulatory control, which is similar in wild type and mutants.

Transcript levels of *BcatrB* in germlings after treatment with azoles for 15 min were lower in mutants G25 and G66 in comparison with wild-type isolate B3. This is just the opposite of what is seen for *BcatrD*. Relatively low transcript levels of various transporter genes have also been reported for azole-resistant mutants of *A. nidulans* after treatment with fenarimol (Andrade 2000) and this is ascribed to the relatively low initial accumulation of fenarimol in these mutants. This hypothesis could also be valid for the relatively low transcript levels of *BcatrB* in the azole-resistant mutants of *B. cinerea* after oxpoconazole treatment. However, this would suggest a similar response on expression of other ABC genes in *B. cinerea* as well. This is not the case. It might also be that *BcatrB* and *BcatrD* are under shared regulatory control and that the increased transcript levels of *BcatrD* are due to a mutation in a transcriptional regulator. Such a mutated transcriptional regulator might enhance transcription of one particular gene but reduce transcription of another. This may be the case if the transcriptional regulator of *BcatrB* and *BcatrD* is rather specific. Indeed, mutations in the transcriptional regulators PDR1 and PDR3 from S. cerevisiae lead to a concerted up- and down-regulation of a wide variety of genes (Balzi et al. 1994; DeRisi et al. 2000; Nourani et al. 1997). The different effects of carbendazim, cycloheximide, and iprodione on transcription of *BcatrB* and *BcatrD* also suggest that these genes are not under shared transcriptional control (Fig. 6). An alternative explanation for the increased expression of *BcatrD* in resistant mutants would be a mutation in a transcriptional regulatory element in the promoter of *BcatrD* (Hallstrom and Moye-Rowley 1998).

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Chapter 6

Expression of the ABC transporter BcatrD from *Botrytis cinerea* reduces sensitivity to sterol demethylation inhibitor fungicides

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ABSTRACT

The ATP-binding cassette (ABC) transporter gene *BcatrD* from *Botrytis cinerea* was cloned and characterized. The open reading frame of *BcatrD* contains seven introns and encodes a putative protein of 1502 amino acids. The function of *BcatrD* was analyzed by phenotypic characterization of gene replacement and overexpression mutants. Replacement mutants of *BcatrD* displayed a higher sensitivity to sterol demethylation inhibitor (DMI) fungicides as compared to the parental isolate. Gene replacement mutants also showed a relatively high accumulation of the DMI fungicide oxpoconazole. Overexpression mutants showed increased levels of basal and oxpoconazole-induced expression of *BcatrD*. Mutants with the highest expression level displayed the highest decrease in sensitivity to oxpoconazole and a relatively low accumulation of the compound. These results indicate a relation between oxpoconazole sensitivity, expression of *BcatrD,* and accumulation of oxpoconazole and demonstrate that the ABC transporter BcatrD is a determinant of the sensitivity of *B. cinerea* to DMI fungicides.

INTRODUCTION

The pathogenic fungus *Botrytis cinerea* Pers.:Fr., anamorph of *Botryotinia fuckeliana* (De Bary) is the causal agent of many diseases of worldwide importance. The pathogen has an extremely wide host range (Coley-Smith *et al.* 1980), suggesting that it developed mechanisms to cope with natural toxic compounds during evolution. *B. cinerea* is also known as a fungus that easily develops resistance to fungicides (Leroux *et al.* 1999). For these reasons, chemical control of diseases caused by *B. cinerea* is difficult and management of strategies to delay resistance development in the pathogen is important.

A group of fungicides commonly used in agriculture are azoles and related compounds. These fungicides inhibit P450-dependent 14α-demethylation ($P450_{14DM}$) of eburicol in fungal sterol biosynthesis. These fungicides are also described in literature as sterol demethylation inhibitors (DMIs) (Uesugi 1998). They comprise derivatives of imidazoles and triazoles (azole fungicides) and derivatives of pyridines, pyrimidines, and piperazines (azole-like fungicides).

Resistance to DMIs can be mediated by reduced affinity to the fungicides and overexpression of the target enzyme $P450_{14DM}$ (De Waard 1994). These mechanisms have been reported in *Candida albicans* (Lamb *et al.* 1997; Lamb *et al.* 2000) and *Penicillium digitatum* (Hamamoto *et al.* 2000). Resistance can also be due to decreased accumulation of DMIs in mycelium as a result of active efflux, as demonstrated in *Aspergillus nidulans* (De Waard and Van Nistelrooy 1979) and *P. italicum* (De Waard and Van Nistelrooy 1988). The efflux can be mediated by ATP-binding cassette (ABC) and major facilitator superfamily (MFS) transporters (Del Sorbo *et al.* 2000). ABC transporters use the energy of ATP to export compounds. MFS transporters use the proton-motive force of the transmembrane electrochemical proton gradient to drive transport of compounds (Driessen *et al.* 2000). ABC transporters involved in energy-dependent efflux of DMIs have been described for *A. nidulans* (Del Sorbo *et al.* 1997), *C. albicans* (Prasad *et al.* 1995),

Mycosphaerella graminicola (Zwiers and De Waard 2000), and *P. digitatum* (Nakaune *et al.* 1998). In *C. albicans* and *P. digitatum* the mechanism is involved in resistance to DMIs in clinical and agricultural situations, respectively. The significance of efflux mechanisms in resistance to DMIs in field isolates of *B. cinerea* (Chapeland *et al.* 1999) remains to be elucidated. A MFS transporter involved in resistance to DMIs was reported in *Saccharomyces cerevisiae* (Alarco *et al.* 1997). Both types of transporters are not only involved in efflux of DMIs but also in transport of many chemically unrelated compounds. For this reason, they can play a role in multidrug resistance (MDR) of fungi to a range of fungitoxic compounds (De Waard 1997). MDR mediated by ABC transporters in fungi has been described for *A. nidulans* (Andrade *et al.* 2000), *B. cinerea* (Schoonbeek *et al.* 2001) and *P. digitatum* (Nakaune *et al.* 1998).

Although DMI fungicides are used for control of a wide variety of plant diseases caused by *Ascomycetes*, *Basidiomycetes,* and *Fungi Imperfecti*, their field performance against diseases caused by *B. cinerea* is not satisfactory. Since the $P450_{14DM}$ target site in *B. cinerea* is very sensitive to DMIs (Stehmann *et al.* 1994), it is not clear what the reason for the poor field performance of DMIs is (Stehmann and De Waard 1996a). Possibly, their efficacy is reduced by activity of ABC and MFS transporters present in the pathogen.

Recently, Ube Industries, Ltd. developed the DMI fungicide oxpoconazole. This compound is effective against *B. cinerea* under field conditions. Since the efficacy of oxpoconazole might also be influenced by activity of transporters, we investigated the role of ABC and MFS transporters in sensitivity of *B. cinerea* to this fungicide. Previously, we identified 13 EST fragments with homology to ABC transporter (*BcatrC-N*) or MFS transporter (*Bcmfs1-4*) genes from *B. cinerea* (Hayashi *et al.* 2001; Vermeulen *et al.* 2001) and demonstrated that *BcatrD* and *Bcmfs1* showed higher transcript levels in DMI-resistant laboratory mutants as compared to the parental strain (Hayashi *et al.* 2001). These results suggest that BcatrD is a transporter of DMI fungicides. In this paper, we validate the role of *BcatrD* from *B. cinerea* in transport of DMI fungicides by studying the phenotype of gene replacement and overexpression mutants of *BcatrD* in relation to fungicide sensitivity, transcription of *BcatrD*, and oxpoconazole accumulation in germlings. The results indicate that BcatrD is a determinant in sensitivity of *B. cinerea* to DMI fungicides.

MATERIALS AND METHODS

Chemicals

Oxpoconazole, prochloraz, and procymidone (technical grade) were synthesized by Ube Industries, Ltd. (Ube, Yamaguchi, Japan). Captan, fenhexamid, and tebuconazole (Bayer AG, Leverkusen, Germany), epoxiconazole, tridemorph, and triforine (BASF AG, Limburgerhof, Germany), cyprodinil, fenpropimorph, fludioxonil, pyrifenox, and trifloxystrobin (Syngenta, Stein, Switzerland), iprodione and quintozene (Aventis, Lyon, France), fluazinam (ISK Bioscience Co., Mentor, OH), and fenarimol (Eli Lilly and Company, Indianapolis, IN) were kindly provided by

their producers. Camptothecin, CCCP (carbonyl cyanide *m*-chlorophenylhydrazone), cycloheximide, ergosterol, eugenol, 4-NQO (4-nitroquinoline-N-oxide), resveratrol, and rhodamine 6G were purchased from Sigma (St. Louis, MO). Progesterone was purchased from BDG Chemical Ltd. (Poole, England).

Fungal strains

B. cinerea strain B05.10, provided by Prof. Dr. P. Tudzynski (Institut für Botanik, Westfälische Wilhelms-Universität, Münster, Germany), is a haploid strain derived from SAS56 isolated by Dr. Faretra (Università degli studi di Bari, Bari, Italy). B05.10 was used as the parental isolate in all experiments. ΔBcatrB4 is a *BcatrB* gene-replacement mutant derived from B05.10 (Schoonbeek *et al.* 2001). These strains were maintained on MEA plates (malt extract agar; Oxoid Ltd., Basingstoke, Hampshire, England) amended with 0.2% yeast extract (Oxoid) at 20°C. Formation of conidia was induced by irradiation of cultures in Petri-dishes with near-UV light for 24 h after 3 days of incubation and continued incubation for 3-7 days.

Library screening

A genomic library of strain SAS56 in λEMBL3 was kindly provided by Dr. A. ten Have (Laboratory of Phytopathology, Wageningen University, Wageningen, The Netherlands) and screened with an EST gene fragment from *BcatrD* (Fig. 1) obtained by PCR amplification using genomic DNA as template. Positive and purified phages were digested with nine enzymes and a restriction map was constructed. Several overlapping fragments were subcloned in pBluescript II SK and used for sequencing. DNA manipulations were performed according to standard methods (Sambrook *et al.* 1989). *Escherichia coli* strain DH5α was used for propagation of the constructs. DNA was sequenced with the BigDye™ (Perkin-Elmer Corporation, CT) and DyEnamic™ET (AP-biotech) Terminator Cycle Sequencing kits. The sequence of fragments of *BcatrD* present in a purified phage was also determined by the primer-walk method. First primer's sequences were based on the EST.

cDNA synthesis

cDNA was amplified by RT-PCR using the SUPERSCRIPT™ One-Step RT-PCR with PLATINUM® *Taq* system (Life Technologies Inc., Breda, The Netherlands). Primers to amplify genomic DNA were based on the genomic *BcatrD* sequence. RNA isolated from germlings of *B. cinerea* strain B05.10 treated with 10 mg l^{-1} oxpoconazole was used as template. Amplified fragments were cloned in the pGEM®-T easy vector using the pGEM®-T Vector system (Promega, Madison, WI).

Multiple alignment of cDNA was performed by ClustalW analysis provided by the European Bioinformatics Institute (Thompson *et al.* 1994). Homology (as percentage identity) of the putative protein sequence derived from BcatrD cDNA with other ABC proteins was calculated using the clustal method by the program Megalign in DNAstar.

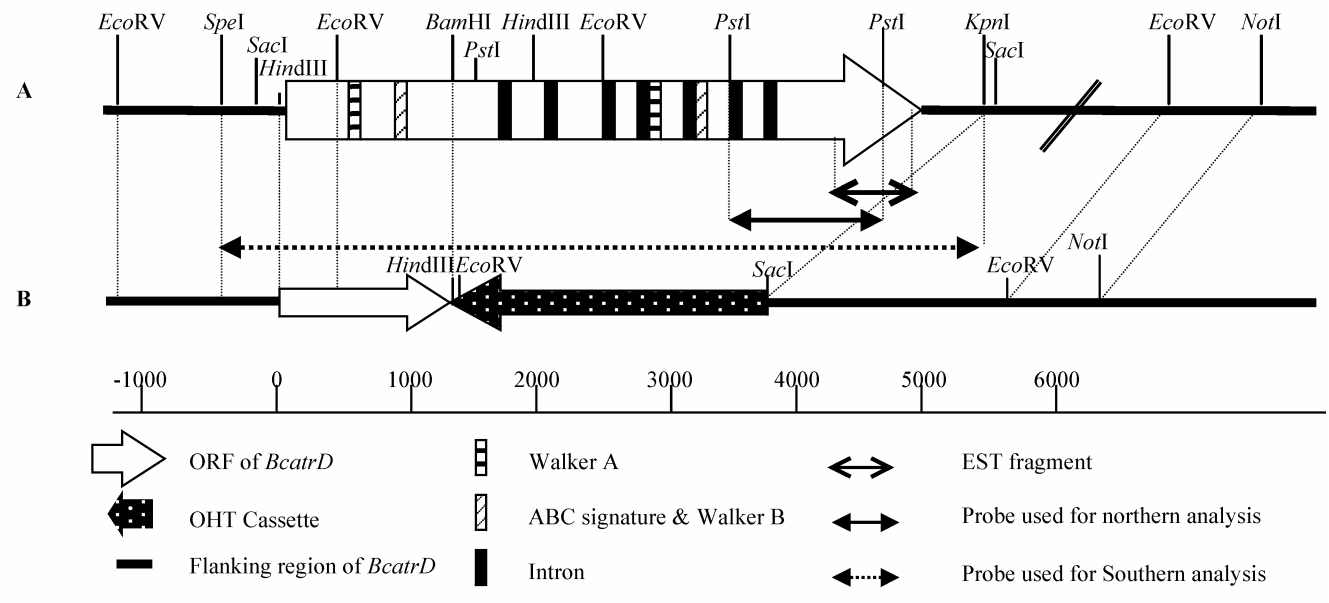

Figure 1. Physical map of *BcatrD* from *B. cinerea* wild-type strain B05.10 (A) and a replacement mutant of *BcatrD* (B)

Construction of replacement and overexpression mutants

The 4.2 kb *Bam*HI-*Sac*I fragment from the coding and terminator region of *BcatrD* was replaced by the 2.6 kb OHT cassette (Fig. 1) from pLOB1 to construct the replacement vector pΔBcatrD. pLOB1 is a pUC18 based vector carrying the hygromycin-resistance cassette (OHT) consisting of the *E. coli* hygromycin phosphotransferase gene under control of the *A. nidulans oliC* promoter and the *B. cinerea* tubulin terminator, kindly provided by Dr. J. A. L. van Kan and Dr. A. ten Have (Laboratory of Phytopathology, Wageningen University, Wageningen, The Netherlands). The replacement plasmid was constructed in two steps. First, a 2.5 kb fragment was excised from the 9.5 kb of *Bam*HI-*Not*I subclone using the 3' *Sac*I restriction site (Fig. 1) and a *Sac*I site from the polylinker of the plasmid, and ligated into pLOB1. Next, the *Hin*dIII fragment (1.3 kb) from the 4.5 kb *Sac*I-*Bam*HI subclone was ligated in this construct. The orientation of *Sac*I and *Hin*dIII fragments was checked by PCR using primers based on the sequence of the OHT cassette and *BcatrD*. Before transformation, the plasmid was linearised at the *Fsp*I restriction site in the backbone of pΔBcatrD to promote homologous integration.

A subclone containing the 6.1 kb *Spe*I-*Kpn*I fragment (Fig. 1) in pBluescript II SK was used to generate overexpression mutants. This plasmid was co-transformed with pLOB1.

Transformation of protoplasts was performed according to methods described previously (Van Kan *et al.* 1997). Protoplasts were obtained from 1-day-old germlings of *B. cinerea* treated with glucanex (5 g l^{-1}; Novo Nordisk, Kopenhagen, Denmark) in a solution containing 0.6 M KCl and 50 mM $CaCl_2$ at 20°C for 1 h.

Southern and Northern blot analysis

MEA plates with an overlay of a cellophane membrane were inoculated with mycelium discs. The plates were incubated at 20°C for 3 days. Then, mycelium mats were peeled from membranes, freeze dried overnight, and used for DNA isolation, according to methods described by Drenth *et al.* (Drenth *et al.* 1993). Genomic DNA (5 μg) was digested with *Eco*RV at 37°C for 5 h and loaded on a 0.7% agarose gel and capillary blotted to Hybond^{TM}-N^{+} membranes (Amersham Pharmacia Biotech, Uppsala, Sweden). The 6.1 kb *Spe*I-*Kpn*I fragment (Fig. 1) was labeled as described previously (Hayashi *et al.* 2001) and hybridized with the Southern blot at 65°C overnight.

Northern blot analysis was performed almost same as described previously (Hayashi *et al.* 2001). Total RNA was isolated from germlings of *B. cinerea* treated with oxpoconazole at 3, 10, and 30 mg l^{-1} for 15 min using TRIzol (Life Technologies Inc., Breda, The Netherlands). Denaturation of RNA was performed using the glyoxal method (Sambrook *et al.* 1989). The 1.1 kb *Pst*I fragment was used as a gene specific probe (Fig. 1).

Sensitivity assay

Sensitivity tests were performed as described previously (Chapeland *et al.* 1999). Drops of spore suspension (3 μl) of *B. cinerea* (10^6 conidia ml^{-1}) were inoculated on plates with synthetic medium amended with chemicals from 100x concentrated stock solutions in methanol. The plates were incubated at 20°C for 3 days. EC_{50} values of chemicals were calculated from dose-response curves using Excel 97. Experiments were performed three times and statistical analysis of the EC_{50} values was performed by the LSD (*t* test).

Accumulation of oxpoconazole

Accumulation experiments were performed as described previously (De Waard and Van Nistelrooy 1988). Germling suspensions in 0.05 M potassium phosphate buffer (pH 6.0) containing D-glucose (10 g l^{-1}) were preincubated on a reciprocal shaker at 20°C for 20 min. [^{14}C]oxpoconazole (initial external concentration 30 μM, 750 Bq nmol^{-1}) was added from a 100x concentrated stock solution in methanol. Samples (5 ml), taken from the suspensions at time intervals, were collected and washed three times with the same buffer on GF6 microglassfiber filter (Schleicher & Schuell, Dassel, Germany). Radioactivity in mycelium was extracted with scintillation liquid (LUMASAFE^{TM} PLUS, LUMAC*LSC B.V., Groningen, The Netherlands) for 1 day and counted in a liquid scintillation spectrometer BECKMAN LS6000TA (Beckman Coulter Inc., CA).

Virulence assay

Detached leaves of tomato (cv. Moneymaker Cf4) were placed in florist foam on wet paper in plastic chambers. Drops of spore suspensions (1 μl) of *B. cinerea* (2 x 10^6 conidia ml^{-1}) in B5 medium (1% sucrose, 10 mM $(NH_4)H_2PO_4$, and 0.31% Gamborg B5 medium elements (Duchefa, Haarlem, The Netherlands)) were inoculated on the surface of the tomato leaves. The reference isolate B05.10 and the mutants were inoculated on the same leaves. Inoculated leaves were

incubated in closed boxes at 20°C in the dark. Diameters of lesions were measured 3 days after inoculation. Experiments were performed twice.

RESULTS

Cloning of BcatrD

Screening of the genomic phage library with the EST fragment (Fig. 1) from *BcatrD* yielded six positive phages. One phage containing a 16 kb *Sal*I fragment was sequenced and carried the full-length *BcatrD* (4889 bp; acc. nr AJ272521). Comparison of this genomic sequence with the cDNA sequence of *BcatrD* obtained by RT-PCR revealed a 4506 bp ORF of *BcatrD* interrupted by seven introns. The EST fragment appeared to be located at the 3' terminal end of the ORF (Fig. 1). The size of the introns varied from 53 to 59 bp and 5'- and 3'-spliced sequences of the introns matched known intron sequences from filamentous fungi (Unkles 1992). The 5'-flanking region (1400 bp) contains typical promoter sequences such as a TATA box at -413 and a CAAT motif at -32 and -165 relative to the start codon. In addition, an ATTS/TEA binding site sequence (CATTCT) was present at -273 (Gavrias *et al.* 1996). A putative Pdr1p/Pdr3p-binding sequence, a HMG box sequence, and a heat-shock element sequence were absent. In the 3'-flanking region, a polyadenylation signal consensus sequence (AATAAA) was located at +730 from the end of the *BcatrD* ORF.

The ORF of *BcatrD* encodes a 1502 amino acids protein. BLAST database searches provided by the National Center for Biotechnology Information showed that *BcatrD* is highly homologous to other ABC transporters such as *AtrE* from *A. nidulans, PMR1* from *P. digitatum, MgAtr2* from *M. graminicola*, and *ABC1* from *Magnaporthe grisea* (Fig 2). Hydropathy analysis (Kyte and Doolittle 1982) indicated that BcatrD has two hydrophilic domains including the nucleotide-binding fold (NBF) and twelve hydrophobic domains including transmembrane regions (TM), which are typical for ABC transporters with a $[NBF\text{-}TM_6]_2$ topology (Walker *et al.* 1982). Walker A, B and ABC signatures were found in the hydrophilic regions of BcatrD (Table 1).

Replacement of BcatrD

Protoplasts of *B. cinerea* strain B05.10 were transformed with 1 μg linearised pΔBcatrD containing flanking region of *BcatrD* (1.3 kb at the 5' and 2.5 kb at the 3' end) and the OHT cassette. Selection with hygromycin (50 mg l^{-1}) yielded about 50 transformants. The majority of these transformants were purified by successive transfers to selective (100 mg l^{-1} hygromycin) and non-selective medium followed by single spore isolation on selective medium. Genomic DNA from 20 putative transformants was isolated, digested with *Eco*RV, and analyzed in Southern blots by hybridization with a 6.1 kb *Spe*I-*Kpn*I fragment (Fig. 1). Lanes with DNA from the parental strain B05.10 showed three bands (4.3, 2.0, and 1.8 kb) (Fig. 3). Homokaryotic transformants were expected to show two bands (1.8 and 0.7 kb) (Fig. 1). This was observed in lanes with DNA from two transformants (ΔBcatrD-8 and ΔBcatrD-12) (Fig. 3). The reference strain HR-9, obtained by transformation with

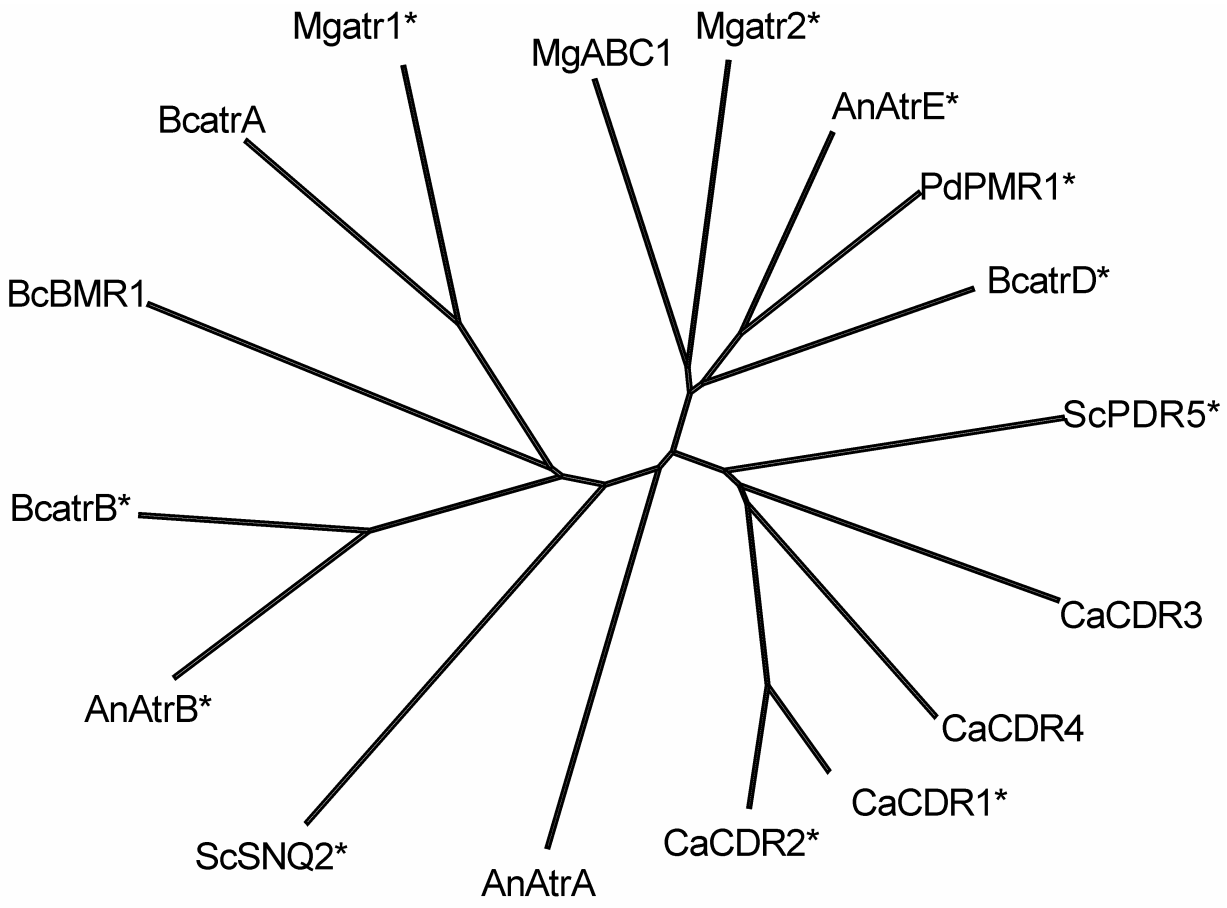

Figure 2. Dendrogram of 17 fungal and yeast ABC transporter proteins with the $[NBF\text{-}TMD_6]_2$ topology. AnAtrA (46.2%, Z68904), AnAtrB (33.0%, Z68905), and AnAtrE (60.5%, AJ276241) from *Aspergillus nidulans*, BcatrA (32.8%, Z68906), BcatrB (33.1%, AJ006217), BcatrD (100%, AJ272521), and BcBMR1 (33.2%, AB028872) from *Botrytis cinerea*, CaCDR1 (46.8%, X77589), CaCDR2 (45.4%, U63812), CaCDR3 (45.4%, U89714), and CaCDR4 (47.9%, AF044921) from *Candida albicans*, MgABC1 (54.5%, AF032443) from *Magnaporthe grisea,* Mgatr1 (34.2%, AJ243112) and Mgatr2 (53.2%, AJ243113) from *Mycosphaerella graminicola*, PdPMR1 (56.4%, AB010442) from *Penicillium digitatum,* and ScPDR5 (46.5%, L19922) and ScSNQ2 (37.2%, X66732) from *Saccharomyces cerevisiae.*Between brackets: % identity with BcatrD and EMBL database accession numbers. Asterisks indicate proteins involved in efflux of DMI fungicides.

pLOB1, and strain ΔBcatrB4 revealed the same hybridization pattern as the parental strain B05.10 (Fig. 3).

Transcript levels of *BcatrD* in the parental isolate B05.10 and the various mutants were studied with the *BcatrD* specific *Pst*I probe (Fig. 1). Basal transcript levels were not detectable in any of the gene replacement mutants tested (Fig. 4). After treatment of germlings with oxpoconazole (30 mg l^{-1}) for 15 min, high levels of transcripts were observed in the wild-type strain B05.10, and the control strains HR-9 and ΔBcatrB4. No transcripts were observed in the replacement mutants ΔBcatrD-8 and ΔBcatrD-12 (Fig. 4).

Table 1. Alignment of ATP-binding domains of BcatrD and other ABC transporters from filamentous fungi.

N-terminus

Protein	Walker A	ABC-signature	Walker B
BcatrD[a]	178-GEMLVVLGRPGSGCSTLLK	300-GVSGGERKRVSIAEAAVGGSPLQCWDNSTRGLD	
AtrA	163-GELLLVLGRPGTGCSTFLK	285-GVSGGERKRVSIAEMALAMTPFAAWDNSSRGLD	
AtrB	129-GEMLLVLGRPGSGCTTLLK	250-GVSGGERKRVSIIECLGTRASVFCWDNSTRGLD	
AtrE	191-GEMLVVLGRPGSGCSTFLK	313-GVSGGERKRVSIAEATLSQAPLQCWDNSTRGLD	
BcatrA	228-GEMLLVLGRPGSGCSTFLK	343-GVSGGERKRVSIAETLPTKKTVVSWDNSTRGLD	
BcatrB	138-GEMLLVLGRPGAGCTTLLK	258-GVSGGERKRVSIIEMLASRGSVMCWDNSTRGLD	
BMR1	188-GEMVLVLGRPGSGCTTFLK	309-GVSGGERKRVSIAEMMITSGTVCAWDNSTRGLD	
MgABC1	228-GEMLVVLGPPGSGCSTFLK	350-GVSGGERKRVTIAEAALSGAPLQCWDNSTRGLD	
Mgatr1	228-GEMMLVLGRPGSGCSTFLK	343-GVSGGERKRVSIAETLASKSTVVCWDNSTRGLD	
Mgatr2	173-GEMLVVLGPPGSGCSTFLK	295-GVSGGERKRVTIAEASLSGAALQAWDNSTRGLD	
PMR1	157-GEMLIVLGRPGSGCSTFLK	279-GVSGGERKRVSIAEATLCGSPLQCWDNSTRGLD	
	** *** ** ** * **	********** * *	**** ****

C-terminus

Protein	Walker A	ABC-signature	Walker B
BcatrD	875-LTALMGVSGAGKTTLLD	984-GLNVEQRKRLTIGVELAAKPALLLFLDEPTSGLD	
AtrA	861-LTALMGVSGAGKTTLLD	970-GLNVEQRKLLTIGVELPPSPKLLLFLDEPTSGLD	
AtrB	826-LGALMGSSGAGKTTLLD	935-GLSVEQRKRVTIGVELVSKPSILIFLDEPTSGLD	
AtrE	886-CTALMGVSGAGKTTLLD	995-GLNVEQRKRLTIGVELAAKPQLLLFLDEPTSGLD	
BcatrA	933-MVALMGASGAGKTTLLN	1038-SLSVEQRKRVTIGVELAAKPNLLLFLDEATSGLD	
BcatrB	842-LGALMGSSGAGKTTLLD	950-GLSVEQRKRLTIGVELVSKPSILIFLDEPTSGLD	
BMR1	879-LTALMGSSGAGKTTLLD	987-GLAVEQRKRVTIGVELAAKPELLLFLDEPTSGLD	
MgABC1	926-LTALMGVSGAGKTTLLD	1035-GLNVEQRKRLTIGVELAAKPPLLLFVDEPTSGLD	
Mgatr1	934-MVALMGASGAGKTTLLN	1039-SLGVEQRKRLTIGVELAAKPSLLLFLDEPTSGLD	
Mgatr2	870-LTALMGVSGAGKTTLLD	979-GLNVEQRKRLTVGVELAAKPQLLLFLDEPTSGLD	
PMR1	857-CTALMGVSGAGKTTLLD	966-GLNVEQRKRLTIGVELAAKPQLLLFLDEPTSGLD	
	**** *********	* ***** * **** * * * ** *****	

Identical sequences are marked with asterisks. Accession numbers are mentioned in Fig. 2.

[a] AtrA, AtrB, and AtrE are from *Aspergillus nidulans*, BcatrA, BcatrB, BcatrD, and BMR1 from *Botrytis cinerea*, MgABC1 from *Magnaporthe grisea*, Mgatr1 and Mgatr2 from *Mycosphaerella graminicola*, and PMR1 from *Penicillium digitatum*.

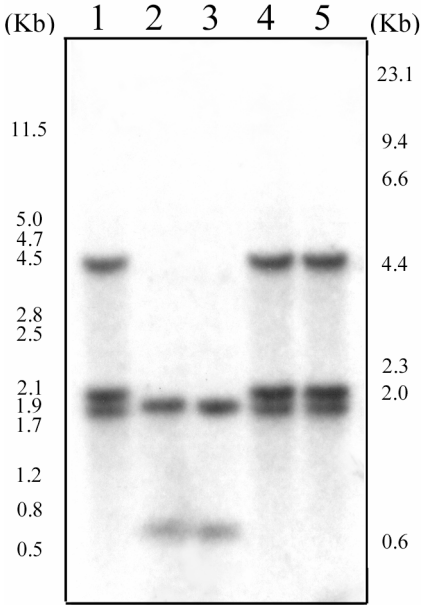

Figure 3. Southern blot analysis with DNA of *Botrytis cinerea* parental strain B05.10 (lane 1), two *BcatrD* replacement mutants ΔBcatrD-8 (lane 2) and ΔBcatrD-12 (lane 3), and two reference strains HR-9 (lane 4) and ΔBcatrB4 (lane 5). Genomic DNA (5 μg) was digested with *Eco*RV and hybridized with a 6.1 kb *Spe*I-*Kpn*I probe (Fig. 1).

Overexpression of BcatrD

Protoplasts from B05.10 were co-transformed with the plasmid containing the 6.1 kb *Spe*I-*Kpn*I fragment (1 μg) and pLOB1 (1 μg). Selection of transformants resistant to hygromycin (50 mg l^{-1}) was performed as described for the isolation of gene replacement mutants. Subsequently, hygromycin-resistant transformants were tested for sensitivity to oxpoconazole (0.5 mg l^{-1}) and eight strains with a lower sensitivity to oxpoconazole than the parental strain B05.10 were identified. Northern blot analysis of these isolates allowed an arbitrary classification of mutants with a low (OVD-15), medium (OVD-21), and high (OVD-2) basal levels of expression of *BcatrD* (Fig. 5). Codes of representative mutants are indicated between brackets. Transcript levels of

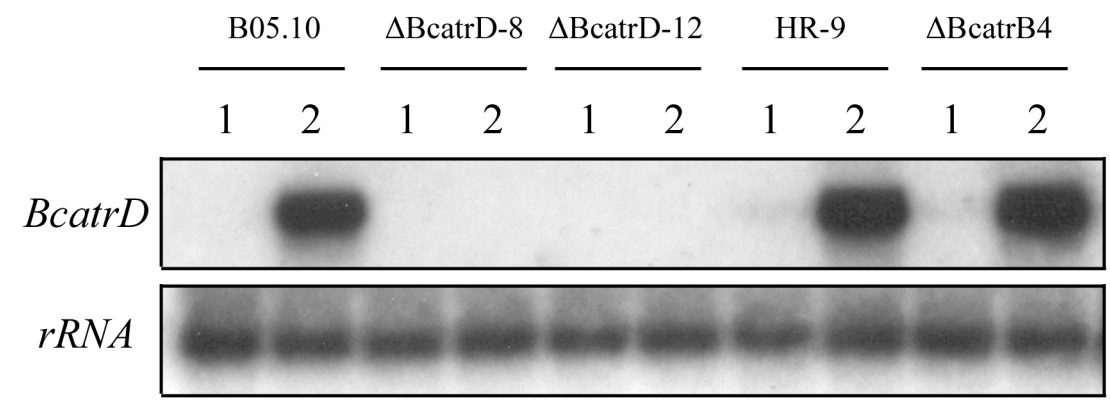

Figure 4. Northern analysis of replacement mutants of *BcatrD* with RNA from germlings of *Botrytis cinerea* parental strain B05.10, two *BcatrD* replacement mutants ΔBcatrD-8 and ΔBcatrD-12, and two reference strains HR-9 and ΔBcatrB4. Basal levels of expression (lanes1). Treatment with 30 mg l^{-1} oxpoconazole (lanes 2). RNA was hybridized with a 1.1 kb *Pst*I probe from *BcatrD* (Fig.1). Equal loading of lanes with RNA was checked by subsequent probing of the same blot with 28S rRNA.

BcatrD after treatment with oxpoconazole at 30 mg l^{-1} were similar for OVD-21 and OVD-2, but significantly higher than in the wild-type strain B05.10. Transcript levels of *BcatrB* induced by oxpoconazole (30 mg l^{-1}) are relatively low in the overexpression mutants as compared to the wild type.

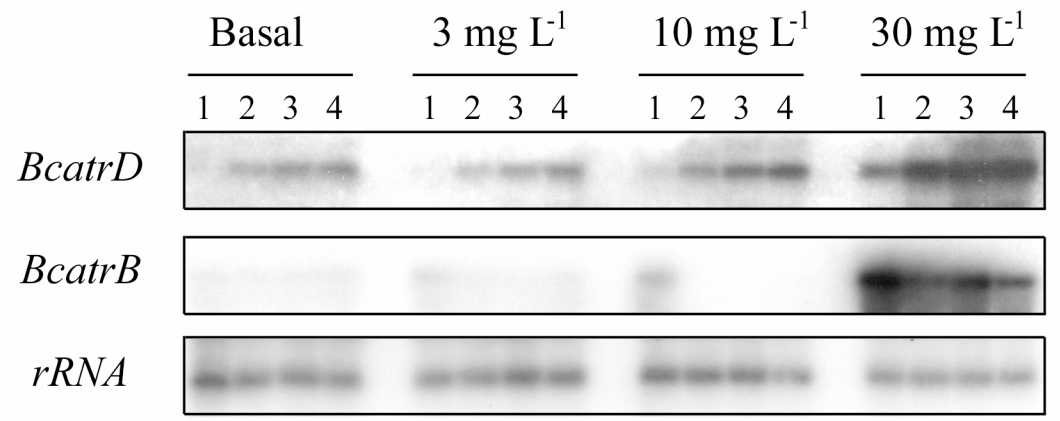

Figure 5. Northern analysis of overexpression mutants of *BcatrD* with RNA from germlings of *Botrytis cinerea* parental strain B05.10 (lanes 1), and the *BcatrD* overexpression mutants OVD-15 (lanes 2), OVD-21 (lanes 3), and OVD-2 (lanes 4). The figure shows basal levels of expression and induced expression after treatment with 3, 10, and 30 mg l^{-1} oxpoconazole. RNA was hybridized with a 1.1 kb *Pst*I probe from *BcatrD* (Fig.1). Equal loading of lanes with RNA was checked by subsequent probing of the same blot with 28S rRNA.

Phenotype assay

The sensitivity of the parental strain B05.10, the replacement mutants (ΔBcatrD-8 and ΔBcatrD-12), and the overexpression mutants (OVD-15, OVD-21, and OVD-2) to oxpoconazole and 27 other compounds was tested in radial growth experiments. The replacement mutants have an increased sensitivity to all DMI fungicides tested (Table 2). The overexpression mutants showed a decreased sensitivity to all DMIs tested (Table 2). This phenotype was most obvious for mutants OVD-2. Sensitivity of replacement and overexpression mutants to camptothecin, captan, CCCP, cycloheximide, cyprodinil, ergosterol, eugenol, fenhexamid, fenpropimorph, fluazinam, fludioxonil, iprodione, 4-NQO, procymidone, progesterone, quintozene, resveratrol, rhodamine 6G, tridemorph, and trifloxystrobin was similar as for B05.10 (results not shown). The sensitivity of B05.10 and HR-9 (control strain for transformation) was the same for all compounds tested (results not shown), including the DMI fungicides (Table 2).

Table 2. EC_{50} values of DMI fungicides against *Botrytis cinerea* in radial growth experiments.

Chemical class of DMIs	Compound	EC_{50} values (mg l^{-1})						
		B05.10[a]	ΔBcatrD-8	ΔBcatrD-12	OVD-15	OVD-21	OVD-2	HR-9
Imidazoles	Oxpoconazole	0.157 b[b]	0.043 a	0.039 a	0.198 bc	0.260 cd	0.325 d	0.160 b
	Prochloraz	0.036 b	0.025 a	0.025 a	0.046 c	0.052 cd	0.055 d	0.039 b
Triazoles	Tebuconazole	0.234 bc	0.065 a	0.065 a	0.267 bcd	0.322 cd	0.386 d	0.203 b
	Epoxiconazole	0.256 b	0.082 a	0.069 a	0.256 b	0.305 bc	0.377 c	0.237 b
Pyrimidine	Fenarimol	1.11 b	0.35 a	0.31 a	1.31 bc	2.12 c	2.63 c	1.05 b
Pyridine	Pyrifenox	0.078 b	0.048 a	0.048 a	0.099 bc	0.128 cd	0.136 c	0.079 b
Piperazine	Triforine	47.6 b	19.9 a	19.9 a	48.3 b	51.0 bc	63.5 c	41.4 b

[a] B05.10 (haploid wild-type strain), ΔBcatrD-8, ΔBcatrD-12 (*BcatrD* replacement mutants generated from B05.10), OVD-15, OVD-21, OVD-2 (*BcatrD* overexpression mutants generated from B05.10), and HR-9 (transformation reference strain).

[b] Means followed by the same letters in the same rows indicate that figures do not differ significantly (P = 0.05).

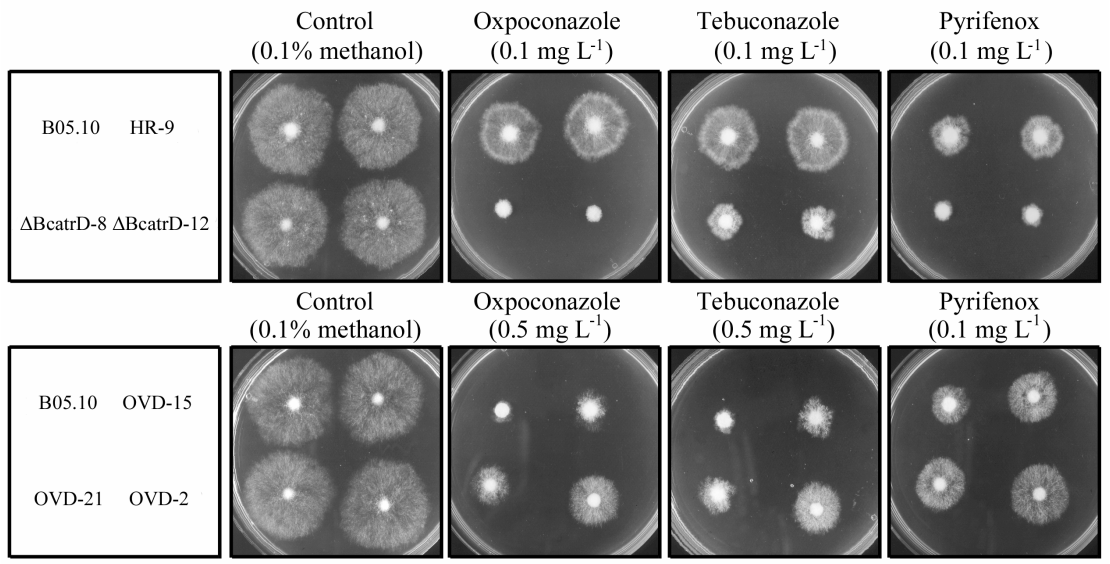

Figure 6. Activity of DMI fungicides from different chemical classes [oxpoconazole (an imidazole), tebuconazole (a triazole), and pyrifenox (a pyridine)] in radial growth experiments of wild-type strain B05.10, control transformant HR-9, gene replacement mutants (ΔBcatrD-8 and ΔBcatrD-12), and gene overexpression mutants (OVD-15, OVD-21, and OVD-2) of *Botrytis cinerea.*

Accumulation experiments

Accumulation of oxpoconazole (initial external concentration 30 μM) by the parental strain B05.10 and the control strain HR-9 was transient in time and did not differ significantly (Fig. 7). *BcatrD* replacement mutants (ΔBcatrD-8 and ΔBcatrD-12) accumulated more oxpoconazole than the

parental strain B05.10. Accumulation by the overexpression mutants (OVD-15, OVD-21, and OVD-2) was low as compared to the reference strains (B05.10 and HR-9). The accumulation levels in overexpression mutants OVD-21 and OVD-2 were particularly low and constant in time (Fig. 7).

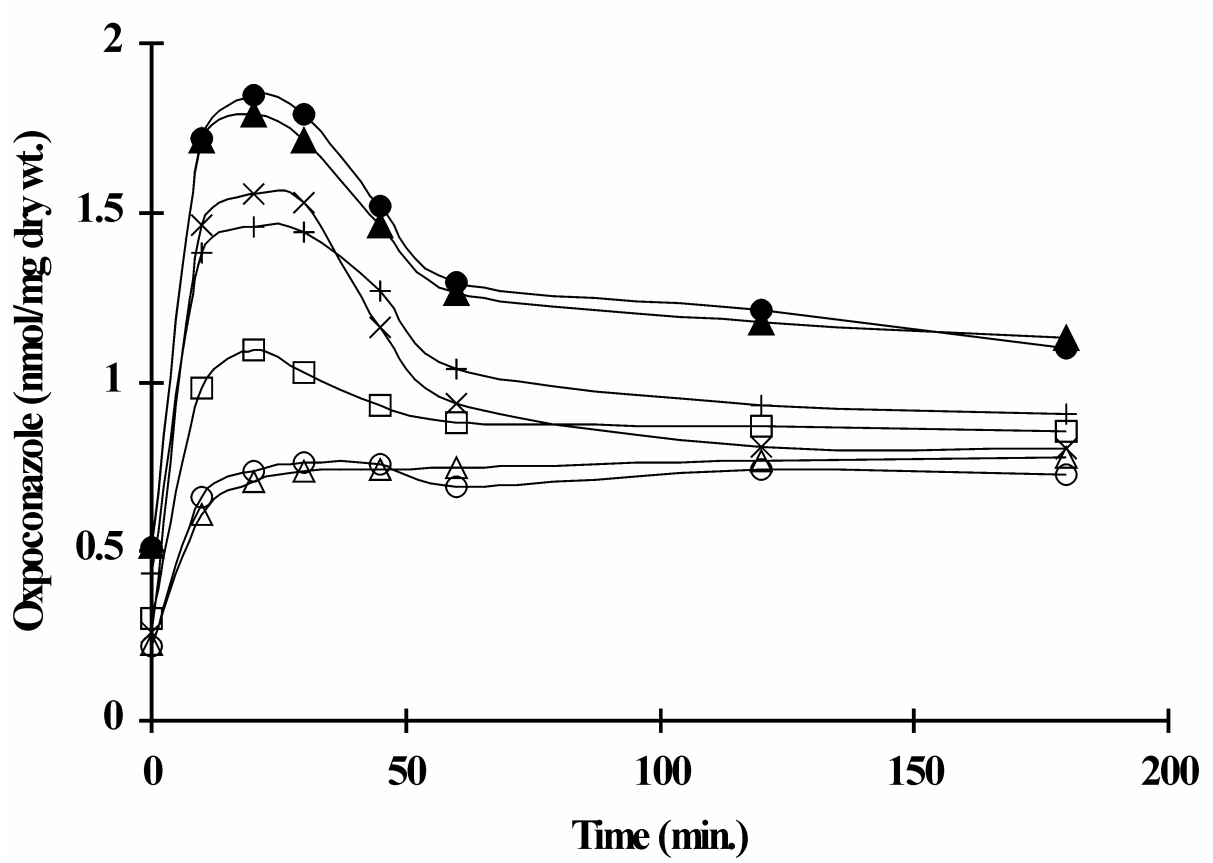

Figure 7. Accumulation of oxpoconazole (30 μM) by germlings of *Botrytis cinerea* wild-type strain B05.10 (×), control transformant HR-9 (+), replacement mutants ΔBcatrD-8 (●) and ΔBcatrD-12 (▲), and overexpression mutants OVD-15 (□), OVD-21 (○), and OVD-2 (△).

Virulence assay

Virulence of replacement (ΔBcatrD-8 and ΔBcatrD-12) and overexpression (OVD-15, OVD-21, and OVD-2) mutants was investigated on detached tomato leaves. The virulence of these mutants did not different significantly from B05.10 (results not shown).

DISCUSSION

BcatrD characterized in this study appears to be a member of the ABC gene family encoding transporters with a $[NBF\text{-}TM_6]_2$ topology. Results indicate that BcatrD functions as a transporter of DMI fungicides since we demonstrated a relation between sensitivity of replacement and overexpression mutants of *BcatrD* to DMI fungicides, *BcatrD* transcript levels, and accumulation levels of the DMI fungicide oxpoconazole.

A dendrogram of ABC transporters with the $[NBF\text{-}TM_6]_2$ topology (Fig. 2) shows three subclusters: one in which BcatrD is present (I), one, dominated by yeast ABC transporters such as PDR5 and CDR1-4 (II), and one with predominantly ABC transporters from filamentous fungi (III). The potency of the transporters to transport DMI fungicides (indicated by an asterisk in the dendrogram) seems to be evenly distributed over the transporters of the three subclusters. Hence, no obvious relation between homology of transporters to BcatrD and the ability to transport DMIs can be established. Examples of ABC transporters with a wide substrate range are AtrB from *A. nidulans*, BcatrB from *B. cinerea*, CDR1 and CDR2 from *C. albicans*, and PDR5 and SNQ2 from *S. cerevisiae*. The substrate specificity of ABC transporters from subcluster I seems to be less broad since no substrates have been identified for ABC1 from *M. grisea* yet (Urban *et al.* 1999), and the substrate specificity of Mgatr2 from *M. graminicola* and PMR1 from *P. digitatum* also seems to be limited (Nakaune *et al.* 1998; Zwiers 2002).

The conclusion that BcatrD is the major efflux pump of DMIs in *B. cinerea* corroborates our previous conclusion that BcatrB is of minor importance in this respect (Hayashi *et al.* 2001). However, *BcatrD* replacement mutants still display a transient accumulation of oxpoconazole in time (Fig. 7). These results suggest the existence of additional transporter(s) in *B. cinerea* involved in limited induced efflux of DMI fungicides. The transporter could be Bcmfs1 since expression of this gene was relatively high in DMI-resistant mutants of *B. cinerea* (Hayashi *et al.* 2001).

Besides DMIs, the anilinopyrimidine fungicide cyprodinil, the dicarboximide fungicide iprodione, and the antibiotic cycloheximide also induce transcription of *BcatrD* (Hayashi *et al.* 2001; Vermeulen *et al.* 2001). However, the sensitivity of *BcatrD* replacement and overexpression mutants to these fungicides is not significantly different from the parental isolate (results not shown). Similar phenomena were observed for replacement mutants of *BcatrB* with respect to sensitivity to cycloheximide and pisatin (Schoonbeek *et al.* 2001). Hence, we propose that fungitoxic compounds can induce expression of ABC genes, but do not necessarily act as a substrate for the encoded transporter protein (Hayashi *et al.* 2001). Another possibility is that activity of other ABC or MFS transporters compensates for the decrease in activity of BcatrD (Schoonbeek *et al.* 2001).

The overexpression mutants with increased transcript levels of *BcatrD* as compared to B05.10 have relatively low transcript levels of *BcatrB*. This observation can probably be ascribed to the fact that the mutants accumulate less oxpoconazole (Fig. 5). Hence, the potency to induce BcatrB (and possibly other transporter genes) in the overexpression mutants will be less. Similar observations have been described for *imaB* mutants of *A. nidulans* (Andrade 2000) which probably carry a mutation in a gene regulating expression of ABC transporter genes.

A common function transporters in pathogenic fungi could provide is protection against natural toxic compounds (e.g. plant defense compounds or antibiotics) (Del Sorbo *et al.* 2000). However, BcatrD seems to provide protection only against all classes of DMI fungicides but not against any of the natural toxic compounds tested. Furthermore, virulence of replacement and overexpression mutants of *BcatrD* on tomato leaves is similar to the parental strain B05.10

indicating that plant defense products in tomato leaves do not seem to act as substrates of BcatrD. These results also support the hypothesis that BcatrD has a narrow substrate specificity. Natural substrate(s) of BcatrD may be found by testing the sensitivity of *BcatrD* replacement mutants for a wider range of natural toxins or by studying the virulence of *BcatrD* replacement mutants on many hosts. The fact that the obviously high substrate specificity of BcatrD includes DMIs is difficult to explain. It may be due to the high hydrophobicity of these compounds due to the presence of aromatic rings, and a tendency of the compounds to be positively charged at neutral pH. These are two common conditions for substrates of ABC transporters (Gottesman and Pastan 1988). The spatial conformation of DMIs mimics fungal sterol intermediates. This might implicate that BcatrD functions as a sterol carrier. It is not understood why morpholine fungicides, which inhibit sterol synthesis at a site different from DMIs, are not transported by BcatrD. An explanation might be their completely different chemical structures and high hydrophilicity.

The baseline sensitivity of populations of *B. cinerea* to DMIs varies significantly (Stehmann and De Waard 1996b). The reason for this variation in sensitivity can probably be attributed to different factors, which have not been fully understood (Stehmann and De Waard 1996a). We propose that different levels of expression of *BcatrD* can contribute to the variation in sensitivity to DMIs of field isolates of *B. cinerea*. We also suggest that the evolution of isolates with a MDR phenotype in field populations of the pathogen (Chapeland *et al.* 1999; Leroux *et al.* 1999) can be attributed to overexpression of *BcatrD* or other transporter genes.

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Chapter 7

Functional analysis of ABC transporter genes from *Botrytis cinerea* identifies BcatrB as a transporter of eugenol

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ABSTRACT

The role of multiple ATP binding cassette (ABC) and major facilitator superfamily (MFS) transporter genes from the plant pathogenic fungus *Botrytis cinerea* in protection against natural fungitoxic compounds was studied by expression analysis and phenotyping of gene-replacement mutants. The expression of eleven ABC (*BcatrA-BcatrK)* and three MFS genes (*Bcmfs1, Bcmfs2* and *Bcmfs4*) was studied. All genes showed a low basal level of expression, but were differentially induced by treatment with cycloheximide and the plant defence compounds camptothecin, eugenol, resveratrol and rishitin. The latter compounds induced expression of *BcatrB* at a high level. Eugenol was more toxic to *BcatrB* gene-replacement mutants than to the control isolates. Eugenol also caused an instantaneous increase in mycelial accumulation of the fungicide fludioxonil, a known substrate of BcatrB. However, there was no difference in virulence between the wild-type and BcatrB gene-replacement mutants on *Ocimum basilicum*, a plant known to contain eugenol. The results indicate that BcatrB is a transporter of lipophilic compounds like eugenol but its role in virulence remains uncertain.

INTRODUCTION

The fungus *Botrytis cinerea*, anamorph of *Botryotinia fuckeliana* is pathogenic on a wide variety of crop plants. Diseases incited by this fungus are described as grey mould and cause serious economic losses (Jarvis 1977; Prins *et al.* 2000). *B. cinerea* can infect many plant species that produce defence compounds of various chemical classes (Dixon 2001). The pathogen appears to possess mechanisms to overcome their fungitoxic activity (Prins *et al.* 2000). Furthermore, *B. cinerea* can readily develop resistance to different fungicides (Chapeland *et al.* 1999; Rosslenbroich and Stuebler 2000). In some strains, resistance to azole and phenylpyrrole fungicides can be ascribed to reduced intracellulair accumulation of the toxicant (Chapeland *et al.* 1999; Stehmann and De Waard 1995). Reduced accumulation of plant defence compounds and fungicides, resulting in protection against these compounds, can be achieved through active efflux of the compounds by ATP-Binding Cassette (ABC) or Major Facilitator Superfamily (MFS) transporters that translocate them over the plasmamembrane to the outer environment (Hayashi *et al.* 2001; Hayashi *et al.* 2002; Vermeulen *et al.* 2001).

The substrate range of ABC transporters can vary from a single compound, as for Mam1, which exports the M-factor mating pheromone from *Schizosaccharomyces pombe* (Christensen *et al.* 1997), to a wide spectrum of compounds with no identified common feature, as for PDR5 from *Saccharomyces cerevisiae* (Kolaczkowski *et al.* 1998) and MDR1 from *Homo sapiens* (Chen and Simon 2000). Fungal ABC transporters with a role in protection against toxicants belong to two subfamilies of full-size transporters, the PDR subfamily with the nucleotide binding domain (NBF) and transmembrane domains (TMD) organised in a $(NBF\text{-}TMD_6)_2$ topology and the MDR subfamily with a $(TMD_6\text{-}NBF)_2$ topology (Del Sorbo *et al.* 2000). Information on the substrate range of ABC transporters from filamentous fungi is rather limited. AtrB and AtrD from

Aspergillus nidulans are transporters with a broad range of substrates, including antibiotics, fungicides and plant defence compounds (Andrade *et al.* 2000). BcatrB from *Botrytis cinerea* has a wide substrate range, comprising mainly aromatic compounds (Schoonbeek *et al.* 2001; Schoonbeek *et al.* 2002; Vermeulen *et al.* 2001), whereas sterol biosynthesis inhibiting (SBI) fungicides are the only substrates identified for BcatrD (Hayashi *et al.* 2001; Hayashi *et al.* 2002). Substrates of BMR1 from *B. cinerea* (also known as BcatrK (Vermeulen *et al.* 2001)) are polyoxin and iprobenfos (Nakajima *et al.* 2001). Multidrug resistance in *Penicillium digitatum* is provided by the ABC transporters PMR1 and PMR5 with a preference for SBI fungicides and resveratrol, respectively (Nakaune *et al.* 1998; Nakaune *et al.* 2002). Complementation studies with ABC transporter genes from *Mycosphaerella graminicola* in *S. cerevisiae* also suggest a redundancy in transporters of various natural and synthetic fungitoxic compounds (Stergiopoulos *et al.* 2002).

MFS proteins from filamentous fungi involved in transport of toxic products are drug-proton (H^+)-antiporters (DHA) with 12 or 14 membrane spanning domains (Del Sorbo *et al.* 2000; Paulsen *et al.* 1996). Their substrate specificity may be limited to endogenous toxins, thus providing self-protection to the producing organisms (Del Sorbo *et al.* 2000). This has been suggested for transport of aflatoxin, cercosporin, HC-toxin and trichothecene, by AflT from *Aspergillus flavus*, CFP1 from *Cercospora kikuchii*, TOXA from *Cochliobolus carbonum* and Tri12 from *Fusarium sporotrichioides*, respectively. However, some MFS transporters from yeasts, such as BenR and FLU1 from *Candida albicans* and FLR1 from *Saccharomyces cerevisiae* are involved in resistance to exogenous antifungal compounds (Del Sorbo *et al.* 2000).

To extend our knowledge on substrate specificity of ABC and MFS transporters from *B. cinerea*, we studied the effect of antibiotics, plant defence compounds and phytotoxic or mycotoxic fungal secondary metabolites and structurally related chemicals on expression of ABC and MFS genes, the sensitivity of gene-replacement strains to these compounds, and their effect on accumulation of [^{14}C]fludioxonil, a known substrate of the ABC transporter BcatrB. These studies identified eugenol, a secondary plant metabolite of basil (*Ocimum basilicum*) (Miele *et al.* 2001), as a substrate of BcatrB. However, *BcatrB* gene-replacement mutants displayed wild-type virulence on basil plants, which suggests that BcatrB is not an essential virulence factor on this host.

MATERIAL AND METHODS

B. cinerea strains

The haploid strain B05.10 (Buttner *et al.* 1994) was a gift from Prof. Dr. P. Tudzynski (Institut für Botanik, Westfälische Wilhelms-Universität, Münster, Germany). Strain B05.10 was used to generate the gene-replacement mutants ΔBcatrA-M7, ΔBcatrB4 and ΔBcatrB5 (Schoonbeek *et al.* 2001). Strain ΔBcatrA-M7 was kindly provided by G. Del Sorbo (ARBOVA, University of Naples, Naples, Italy). Isolate CH1.7 is a mono-ascospore isolate with decreased sensitivity to fludioxonil generated by Dr. U. Hilber (Eidgenössische Forschungsanstalt, Wädenswil, Switzerland). The isolate was kindly provided by Dr. K.M. Chin (Syngenta, Stein, Switzerland).

Compounds

The fungicides (technical grade) used were dinitro-*ortho*-cresol (DNOC; Luxan B.V., Elst, the Netherlands), fludioxonil and [^{14}C]fludioxonil (Syngenta, Basel, Switzerland) and sodium *ortho*-phenylphenate (SOPP; a kind gift from J.W. Eckert, University of California, Riverside, California, U.S.A.). Pisatin was isolated from pea pods (Fuchs *et al.* 1981). Rishitin was kindly provided by K.-M. Weltring (Institut für Botanik, Westfälische Wilhelms-Universität, Münster, Germany). Other compounds were purchased from Sigma-Aldrich (Zwijndrecht, the Netherlands). Compounds were added to cultures or media from 1000× concentrated stock solutions in methanol unless stated otherwise. Quercetin and reserpine were dissolved in DMSO.

Growth media and conditions

Conidia of all *B. cinerea* strains were preserved in 15% glycerol in Eppendorf vials and stored at -80 °C. The strains were cultured on malt extract agar (MEA; 50 g l^{-1}; Oxoid, Basingstoke, Hampshire, England). Gene-replacement mutants were grown on MEA amended with hygromycin (100 mg l^{-1}; Sigma). New cultures were started monthly from preserved spores. Plates with sporulating cultures were obtained by transfer of agar plugs from growing colonies to the centre of plates containing MEA with yeast extract (2 g l^{-1}; Oxoid), followed by incubation at 20 ºC in the dark for 10 days. Formation of conidia was induced by UV treatment after 3 days for 24 h. Conidia, harvested in 0.05% Tween 80, were used in sensitivity assays, expression analysis and accumulation experiments. The effective concentration of toxicants that inhibits radial growth by 50% (EC_{50}) was determined on potato dextrose agar (PDA; Oxoid) (Stehmann and De Waard 1996). Experiments were carried out in triplicate.

Expression analysis

The expression of the ABC genes *BcatrA-K* and the MFS genes *Bcmfs1, Bcmfs2* and *Bcmfs4* was studied in 16-hour-old germlings of *B. cinerea* strain B05.10 after treatment with test compounds for 20 or 60 min. Induction experiments, RNA extraction with TRIzol (Life Technologies, Breda, The Netherlands) and northern blot analysis with gene-specific fragments were performed as described by Vermeulen *et al.*(2001).

Accumulation of [^{14}C]fludioxonil

Accumulation of [^{14}C]fludioxonil by germlings of *B. cinerea* strains B05.10 and ΔBcatrB4 was determined as described before (Stehmann and De Waard 1995; Vermeulen *et al.* 2001) with minor modifications (Schoonbeek *et al.* 2002). Experiments were initiated by addition of [^{14}C]fludioxonil [final concentration 0.4 µM (1 mg l^{-1}); 250 Bq/nmol] from a 100× concentrated stock solution in methanol. The test compounds eugenol and resveratrol were added 65 min after the start of the incubation with the radiochemical to determine their effect on [^{14}C]fludioxonil accumulation.

Virulence assay

Virulence assays were performed on leaves of basil (*O. basilicum* cv. Genovese Gigante), grown under greenhouse conditions for eight weeks. Seven (Exp. 1) or five (Exp. 2) intact plants with three pairs of expanded leaves (15 cm high) were placed in humid chambers. The upperside of the leaves was inoculated with droplets (2 µl) of spore suspensions of *B. cinerea* ($5 \cdot 10^5$ ml^{-1}), preincubated in 1×B5 medium amended with 1% glucose and 10 mM ammonium phosphate pH 6.5 at 20°C for 2 h to synchronise germination. Each leaf was pairwise inoculated with three droplets of strain B05.10 (parental line) and three droplets of strain B05.10, ΔBcatrA-M7, ΔBcatrB4, or ΔBcatrB5. Lesion diameters were measured after incubation at 15°C for three days in the dark (Exp. 1) or in the light (Exp. 2). Statistical analysis with Duncan's *t*-test was based on average values of spreading lesions (with a diameter over 1 mm) of each pair of leaves.

RESULTS

Expression analysis

Northern blot analysis revealed that treatment of *B. cinerea* germlings with diverse natural toxic compounds induced expression of ABC and MFS genes. *BcatrB* was induced by a wide range of compounds. The best inducers of *BcatrB* were camptothecin, eugenol, psoralen, resveratrol and rishitin (Table 1). *BcatrA* and *BcatrD* were induced by eugenol and cycloheximide only. Expression of *BcatrF*, *BcatrG*, *BcatrK*, *Bcmfs2* and *Bcmfs4* was induced to a low level by a limited number of compounds (Table 1). Expression levels of *BcActA*, *BcatrC*, *BcatrE*, *BcatrH*, *BcatrI*, *BcatrJ* and *Bcmfs1* were not elevated by any of the compounds tested (data not shown). The fungal toxins AAL-toxin (1 and 5 mg l^{-1}) and HC-toxin (1 and 5 mg l^{-1}), and the antibiotics amphotericin B (5 and 25 mg l^{-1}), brefeldin A (2 and 10 mg l^{-1}), hygromycin B (10 and 50 mg l^{-1}), oligomycin (5 and 25 mg l^{-1}) and streptomycin (10 and 50 mg l^{-1}) did not induce the expression of any of the genes tested (data not shown).

The effects of compounds that affected the expression of any of the genes tested (Table 1) were studied in more detail. Germlings were treated with the test compounds at two concentrations for 20 and 60 min (Figure 1). The results indicate that induction of expression can be time- and concentration-dependent. Generally, induction of *BcatrB* expression was relatively high after 20 min, except after exposure to pisatin. *BcatrD* expression was relatively high after 60 min, especially after treatment with cycloheximide. Transcript levels correlated positively with the concentrations of pisatin, resveratrol and rishitin tested. However, negative correlations were also observed, noticeably in the case of eugenol and *BcatrB*.

Table 1. Expression levels of ABC and MFS transporter genes in *Botrytis cinerea* strain B05.10 after treatment with natural fungitoxic compounds for 20 min.

Treatment[a]	ABC transporter genes						MFS genes		Loading control	
	BcatrA	*BcatrB*	*BcatrD*	*BcatrF*	*BcatrG*	*BcatrK*	*Bcmfs2*	*Bcmfs4*	*rDNA*	*BcactA*
Controls										
Mock	+[b]	±	+	+	+	-	±	+	++	+++
Methanol (0.1%)	+	±	+	+	+	-	±	+	++	++
Antibiotics										
Cycloheximide (25)	++	+	+++	+	++	±	+	++	++	+++
Plant defence compounds										
Camptothecin (10)	+	+++	±	+	+	-	±	±	++	+++
Eugenol (100)	++	+++	++	+	+	+	±	+	++	+++
Lubimin (50)	+	±	±	±	±	-	±	+	++	+++
Pisatin (25)	+	+	+	+	+	-	±	+	++	+++
Psoralen (50)	+	+++	+	+	+	±	±	++	++	+++
Quercetin (50)	+	+	±	±	++	-	±	±	++	+++
Reserpine (50)	+	±	±	+	+	-	±	++	++	+++
Resveratrol (50)	+	++	+	++	++	±	±	+	++	+++
Rishitin (25)	+	++	+	+	±	-	±	+	++	+++
Tomatin (25)	+	±	+	+	++	-	+	+	++	+++

[a] Compounds added from 1000× concentrated stock solutions in methanol. Concentrations of compounds (mg l^{-1}) between brackets.

[b] Arbitrary quantification of expression: no expression detectable (-), very weak expression (±), weak expression (+), intermediate expression (++) and strong expression (+++).

Toxicity assays

The role of BcatrA and BcatrB in protection of *B. cinerea* against toxic compounds was studied in mycelial growth assays. The EC_{50} values for inhibition of radial growth was determined for the wild-type strain B05.10 and the gene-replacement mutants ΔBcatrA-M7, ΔBcatrB4 and ΔBcatrB5 . The EC_{50} for camptothecin, cycloheximide, pisatin, psoralen, quercetin, reserpine and rishitin did not differ significantly for the BcatrB gene-replacement mutants and the control strain (data not shown). However the EC_{50} values of eugenol for ΔBcatrB4 and ΔBcatrB5 were significantly lower than for B05.10 and ΔBcatrA-M7 (Table 2). EC_{50} values of SOPP and DNOC were similar for all strains tested (Table 2).

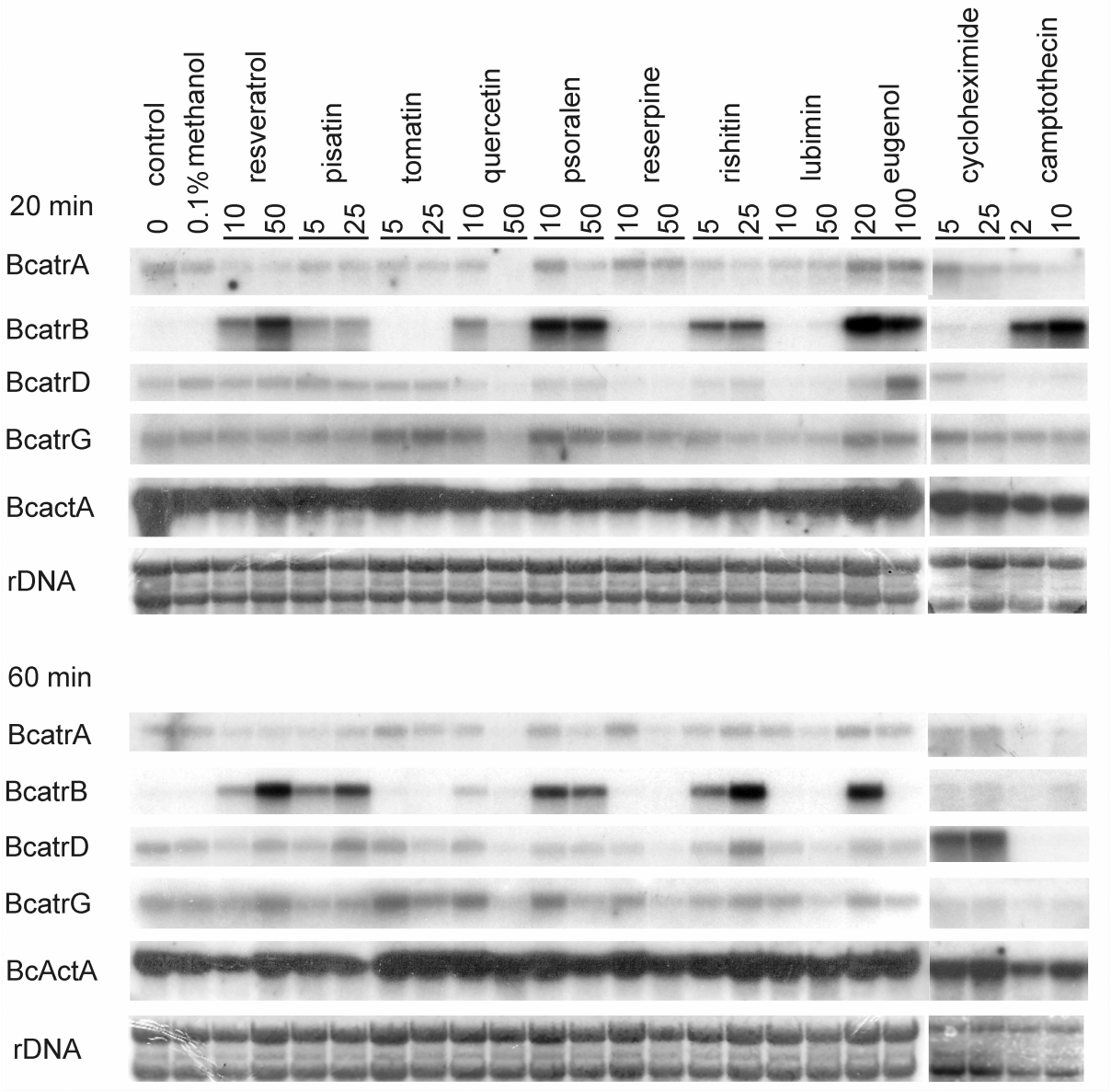

Figure 1. Expression analysis of the ABC transporter genes *BcatrA*, *BcatrB*, *BcatrD* and *BcatrG* after treatment of germlings from *Botrytis cinerea* strain B05.10 with natural fungitoxic compounds for 20 and 60 min. Figures indicate concentration of compounds in mg l^{-1}. Blots were hybridised with *BcactA* and rRNA probes from *Botrytis cinerea* as loading controls.

Table 2. Activity of phenolic compounds on radial growth of *Botrytis cinerea* strain B05.10, BcatrA gene-replacement mutant ΔBcatrA-M7, and BcatrB gene-replacement mutants ΔBcatrB4 and ΔBcatrB5.

	Eugenol		SOPP		DNOC	
B05.10	36[a]	a[b]	0.82	a	1.0	ab
ΔBcatrA-M7	31	a	0.73	a	1.2	a
ΔBcatrB4	23	b	0.88	a	0.97	ab
ΔBcatrB5	24	b	0.88	a	0.72	b

[a] EC_{50}: effective concentration (mg l^{-1}) that inhibits radial growth by 50%.

[b] Different letters within a column indicate significant differences between strains (Duncan's *t*-test, *P*=0.05).

Accumulation of [^{14}C]fludioxonil

The interference of eugenol with efflux activity of BcatrB was studied by measuring its effect on accumulation of [^{14}C]fludioxonil, a well characterized substrate of BcatrB (Vermeulen *et al.* 2001). Resveratrol, another substrate of BcatrB (Schoonbeek *et al.* 2001), was used as a refererence compound (Figure 2, Figure 3). The accumulation of [^{14}C]fludioxonil in control treatments proved to be as described previously (Vermeulen *et al.* 2001). In summary, accumulation by B05.10 was transient with a rapid initial increase followed by a low equilibrium level of circa 0.35 nmol mg^{-1} dry weight. Accumulation by ΔBcatrB4 rapidly increased and remained constant at circa 0.8 nmol mg^{-1} dry weight (data not shown). Addition of eugenol (100 mg l^{-1}) and resveratrol (100 mg l^{-1}) 65 min after addition of [^{14}C]fludioxonil resulted in a transient increase in accumulation of [^{14}C]fludioxonil by B05.10 (Figure 2). The maximal increase was observed after 15 min. Therefore the effect of eugenol and resveratrol on [^{14}C]fludioxonil accumulation by B05.10 and ΔBcatrB4 was measured 15 min after addition of the compounds (Figure 3). Addition of eugenol, but not of resveratrol, resulted in a significant increase of the accumulation of [^{14}C]fludioxonil by B05.10. In contrast, neither compound had a significant effect on accumulation of [^{14}C]fludioxonil by ΔBcatrB4. Pre-treatment of germlings of B05.10 with eugenol or resveratrol induced [^{14}C]fludioxonil efflux capacity (data not shown).

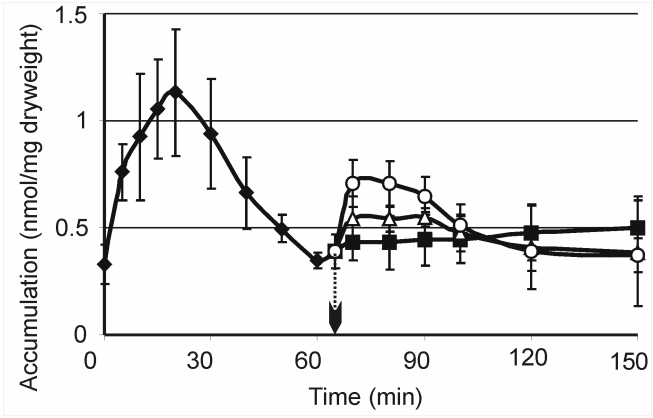

Figure 2. Effect of addition of eugenol and resveratrol on accumulation of [^{14}C]fludioxonil (1 mg l^{-1}) by germlings of *Botrytis cinerea* strain B05.10. Compounds were added 65 min after addition of [^{14}C]fludioxonil (arrow). No treatment (♦), solvent control (■; 0.1% DMSO), eugenol (○; 100 mg l^{-1}) and resveratrol (Δ; 100 mg l^{-1}).

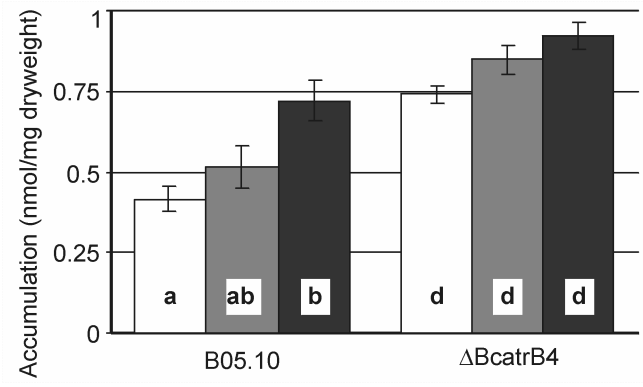

Figure 3. Effect of eugenol and resveratrol on accumulation of [^{14}C]fludioxonil (1 mg l^{-1}) by germlings of *Botrytis cinerea* strains B05.10 and ΔBcatrB4. Compounds were added 65 min after addition of [^{14}C]fludioxonil. Control (0.1% DMSO; white), resveratrol (100 mg l^{-1}; grey) and eugenol (100 mg l^{-1}; black). The height of columns represents mean accumulation levels of four samples taken after 15 minutes of treatment. Different letters in the columns indicate statistically significant differences between treatments (Duncan's *t*-test, P=0.05).

Virulence assays

Inoculation of basil with B05.10 resulted in expanding lesions with an infection frequency between 32 and 87%. The percentage and size of expanding lesions of B05.10 and any of the gene-replacement strains ΔBcatrA-M7, ΔBcatrB4, or ΔBcatrB5 did not differ significantly (Figure 4). Similar results were obtained in an experiment in which plants were incubated in light after infection (data not shown).

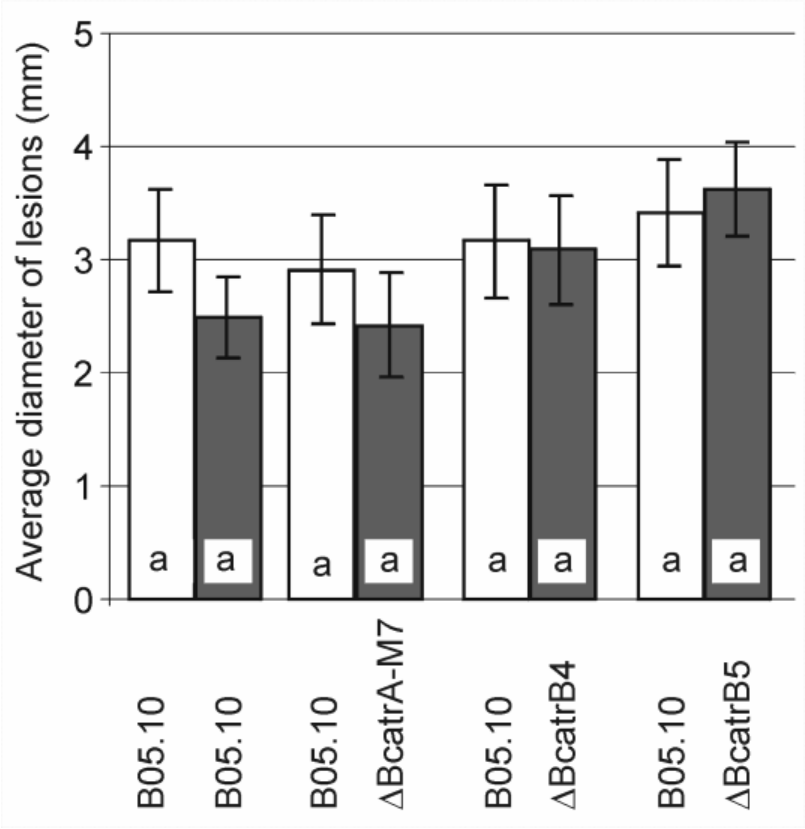

Figure 4. Virulence of *Botrytis cinerea* strains on leaves of basil (*Ocimum basilicum* cv Genovese Gigante) at 15 °C in the dark. Bars indicate the lesion size (mm) incited by pairwise inoculation with control strain B05.10 (white) and the test strains (black) B05.10, BcatrA gene-replacement mutant ΔBcatrA-M7, and BcatrB gene-replacement mutants ΔBcatrB4 or ΔBcatrB5. Bars marked with identical letters are not significantly different (Duncan's *t*-test, P=0.05).

DISCUSSION

The presence of multiple ABC and MFS genes in *B. cinerea* (Vermeulen *et al.* 2001) suggests that a complex network of transporters might protect the pathogen against natural fungitoxic compounds (Del Sorbo *et al.* 2000) and provide multidrug resistance to fungicides (De Waard 1997), similar to the PDR-network in *S. cerevisiae* (Bauer *et al.* 1999). Expression analysis indicated that plant defence compounds induce several transporter genes, especially *BcatrB* , suggesting that these transporters indeed might contribute to protection against these toxic products.

The inducer eugenol proved to be a substrate of BcatrB since gene-replacement mutants of *BcatrB* are more sensitive to eugenol than the parent isolate and eugenol interfered with BcatrB-mediated efflux of [^{14}C]fludioxonil, resulting in increased accumulation of this compound. This effect was much weaker in a BcatrB replacement mutant. These results suggest that BcatrB could function as a virulence factor on *O. basilicum* cv Genovese Gigante , a plant that has eugenol as the prevalent constituent of essential oils (200 to 2000 μg g^{-1} fresh weight) (Miele *et al.* 2001). However, BcatrB replacement mutants did not display reduced virulence on this basil cultivar. This result contrasts with the observation that BcatrB acts as a virulence factor for *B. cinerea* on grapevine leaves (Schoonbeek *et al.* 2001) and that ABC transporters related to BcatrB, such as GpABC1 from *Gibberella pulicaris* (Fleissner *et al.* 2002) and ABC1 from *Magnaporthe grisea* (Urban *et al.* 1999), act as virulence factors on potato and rice, respectively. BcatrB is also a transporter of resveratrol (Schoonbeek *et al.* 2001), phenylpyrrole fungicides (Schoonbeek *et al.* 2001; Vermeulen *et al.* 2001) and phenazine antibiotics (Schoonbeek *et al.* 2002). Hence, we confirm that BcatrB is a multidrug transporter of natural and synthetic fungitoxic compounds, but did not find evidence for the transporter to be an essential virulence factor on *O. basilicum*.

The remarkable difference in expression pattern of transporter genes from *B. cinerea* after treatment with a range of compounds (Table 1) suggest that the encoded transporters differ in substrate specificity. The increased sensitivity of BcatrB replacement mutants to eugenol indicates that the high capacity to induce *BcatrB* expression indeed correlates with the potency of BcatrB to accept this compound as a substrate. Previously we described that a similar phenomenon for resveratrol (Schoonbeek *et al.* 2001). However, the BcatrB replacement mutants did not display an increased sensitivity to other inducers tested such as pisatin and camptothecin. Therefore, BcatrB is not a general transporter of structurally unrelated plant defence compounds. Similar results have been observed for other compounds that induce BcatrB expression, including azole fungicides (Hayashi *et al.* 2001) and DAPG (Schoonbeek *et al.* 2002). Comparable observations have also been reported for AtrB from *A. nidulans* (Andrade *et al.* 2000). An explanation for unaltered sensitivity of ABC transporter mutants to some inducers is that these compounds are not a substrate of the induced transporter. However, this could also be due to redundancy of transporters that accept the same compound as a substrate (Kolaczkowski *et al.* 1998) and thereby mask the effect in single-gene replacement mutants. This may be true for cycloheximide and psoralen, since they not only induces *BcatrB* but also *BcatrA*, *BcatrG* and *Bcmfs4*. Other transporter genes have been found in the *B. cinerea* genome (Yoder and Turgeon 2001) and these might mask the loss of function of BcatrB as well. The same reasoning might explain why the increase in sensitivity of BcatrB gene-replacement mutants to eugenol and resveratrol is relatively low (Table 2) and why these mutants did not show decreased virulence on eugenol producing basil (Figure 4). Furthermore, the pathogen might possess a wide array of alternative mechanisms to cope with plant defences (Prins *et al.* 2000).

The instantaneous increase of [^{14}C]fludioxonil accumulation after addition of eugenol and resveratrol (Figure 2) suggests that these compounds inhibit [^{14}C]fludioxonil efflux activity by

competing for the fludioxonil binding site of the transporter protein. However, non-competitive inhibition via another binding site cannot be excluded. The transient nature of the elevated [^{14}C]fludioxonil accumulation suggests that eugenol and resveratrol induce additional efflux capacity, probably due to enhanced *de novo* synthesis of BcatrB. This conclusion is supported by the observation that eugenol and resveratrol exerted no significant effect on [^{14}C]fludioxonil accumulation by ΔBcatrB4 (Figure 3). Eugenol and resveratrol not only induce expression of *BcatrB* (Table 1), but also of *BcatrD, BcatrG* and *BcatrK*, that are also induced by fludioxonil (Vermeulen *et al.* 2001). Addition of eugenol or resveratrol to ΔBcatrB4 germlings did hardly affect the accumulation of [^{14}C]fludioxonil (Figure 3), suggesting that *BcatrD, BcatrG* and *BcatrK* do not significantly contribute to fludioxonil efflux activity. This is in agreement with the identification of BcatrB as the major efflux pump of fludioxonil in *B. cinerea* (Vermeulen *et al.* 2001).

Plant defence compounds identified as substrates for BcatrB are presented in Figure 5. Both eugenol and resveratrol are phenols with an aliphatic side chain (Langcake and Pryce 1976; Miele *et al.* 2001). However, this structural moiety is not crucial in determining the substrate specificity of BcatrB since structurally related synthetic phenols such as SOPP and DNOC were not apparent substrates of the transporter. Other substrates of BcatrB are the phenylpyrrole fungicides fenpiclonil and fludioxonil, and the phenazine antibiotics phenazine-1-carboxylic acid (PCA) and phenazine-1-carboxamide (PCN) (Schoonbeek *et al.* 2002). These are also aromatic compounds but lack a hydroxylated benzene ring (Figure 5). Hence, the only characteristic that known BcatrB substrates have in common is their aromatic character. This is in line with the hypothesis that substrates of multidrug ABC transporters only share a lipophilic character (Kolaczkowski *et al.* 1998).

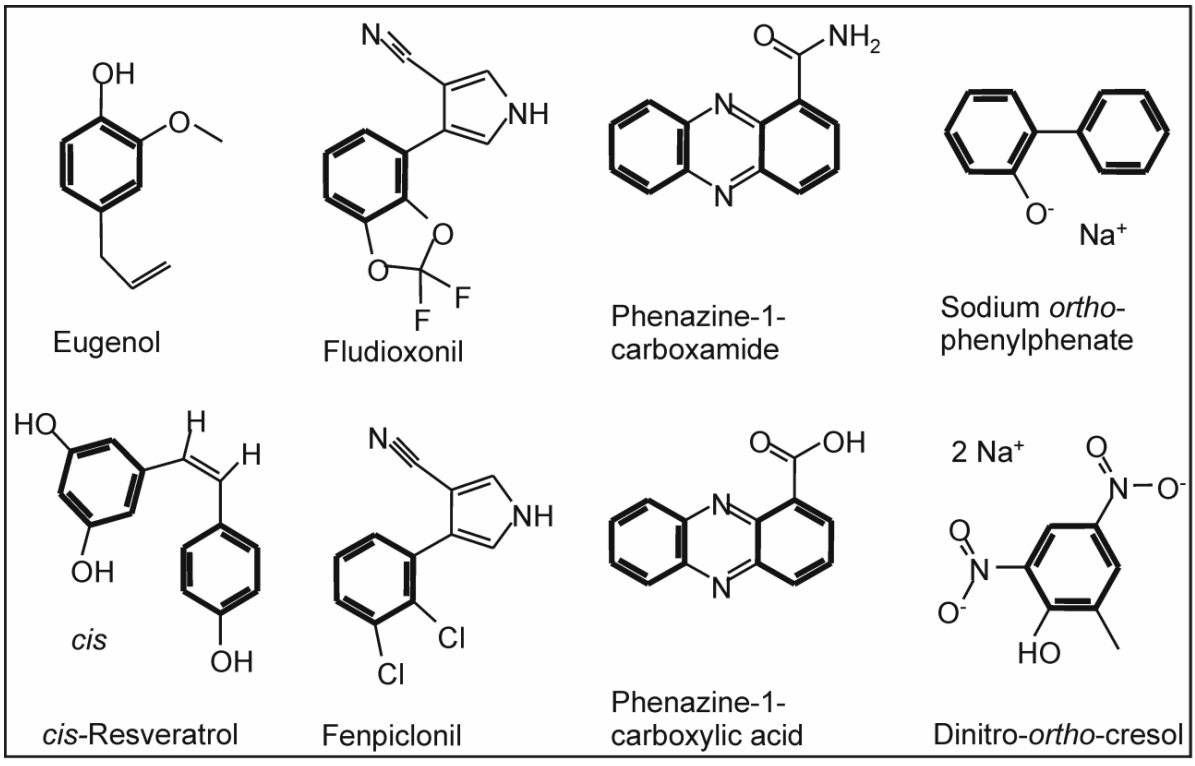

Figure 5. Structures of inducers and putative substrates of the ABC transporter BcatrB from *Botrytis cinerea*.

A natural function of BcatrB may be the protection of *B. cinerea* against aromatic fungitoxic compounds during pathogenesis and/or saprophytic growth. This may be a conserved trait in evolution of ABC transporters of filamentous fungi since the closest identified homolog of BcatrB, AtrB from *A. nidulans*, shows a similar function in multidrug resistance (Andrade *et al.* 2000). Two other homologues with similar substrate ranges are MgAtr5 from *M. graminicola* (Zwiers *et al.* 2003) and PMR5 from *P. digitatum* (Nakaune *et al.* 2002). This indicates that other homologues of BcatrB may function also in multidrug resistance and pathogenesis.

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Chapter 8

Virulence of *BcatrB* gene-replacement mutants of *Botrytis cinerea* on leguminous and solanaceous plant species

Henk-jan Schoonbeek and Maarten A. De Waard

ABSTRACT

Expression of the ABC transporter *BcatrB* from *Botrytis cinerea* is known to be induced by plant defence compounds produced by leguminous and solanaceous plant species. Therefore, we investigated whether BcatrB could act as a virulence factor of the pathogen on host plants of these families. To this purpose we determined the virulence of *BcatrB* replacement mutants on bean, pea, and peanut (*Leguminosae)*, and bell pepper fruits, potato tubers and tobacco and tomato leaves (*Solanaceae*). No differences in virulence were observed between *BcatrB* replacement mutants and control isolates. The BcatrB mutants were more virulent than the parental isolate on tomato cv. Vollendung but not on transgenic, resveratrol producing lines of this cultivar. These somewhat unexpected results will be discussed.

INTRODUCTION

The necrotrophic fungus *Botrytis cinerea* Pers.:Fr., anamorph of *Botryotinia fuckeliana* (De Bary) Whetzel, is the causal agent of grey mould and infects fruits, flowers or green tissues of at least 235 plant species (Jarvis 1977). It causes serious pre- and post-harvest diseases in a wide variety of plants including agronomically important crops such as grapevine, tomato, strawberry, cucumber, beans, bulb flowers and ornamentals. The broad host range implies that *B. cinerea* must possess multiple pathogenicity and/or virulence factors including toxins and enzymes, involved in breakdown of plant cell walls or protection to constitutive and inducible plant defences (Prins *et al.* 2000). Phytoanticipins and phytoalexins are antimicrobial compounds belonging to various chemical classes such as stilbenes, isoflavonoids, coumarines and sesquiterpenes (Osbourn 1999).

Mechanisms that provide pathogens protection against a broad range of antimicrobial compounds include metabolic inactivation, compartmentalisation, and reduced accumulation (Morrissey and Osbourn 1999). Reduced accumulation can be achieved with active efflux by ATP-binding cassette (ABC) transporters (Del Sorbo *et al.* 2000). ABC transporters are membrane-bound proteins with an ATP-binding cassette that couple ATP hydrolysis with transport of a broad spectrum of compounds over various membranes (Dassa and Bouige 2001; Senior *et al.* 1995). Since ABC transporters also accept secondary plant metabolites as substrates (Andrade *et al.* 2000; Kolaczkowski *et al.* 1998), they may function as virulence factors, as demonstrated for BcatrB from *B. cinerea* (Schoonbeek *et al.* 2001) and GpABC1 from *Gibberella pulicaris* (Fleissner *et al.* 2002). The ABC transporters ABC1 from *Magnaporthe grisea* and Mgatr4 from *Mycosphaerella graminicola* play a role in virulence on rice and wheat, respectively (Stergiopoulos *et al.* 2003; Urban *et al.* 1999), but the underlying mechanism has not yet been elucidated. Transporters could also play a role in pathogenesis by mediating the secretion of non host-specific and host-specific toxins. Such a function has been reported for members of the Major Facilitator Superfamily (MFS) of transporters, but not yet for ABC transporters. Examples are CFP from *Cercospora kikuchii*, which exports the phytotoxic polyketide cercosporin, (Callahan *et al.* 1999), TOXA from

Cochliobolus carbonum, involved in export of the host-selective cyclic tetrapeptide HC-toxin (Pitkin *et al.* 1996), and TRI12, a trichothecene efflux pump from *Fusarium sporotrichioides* (Alexander *et al.* 1999).

The ABC transporter BcatrB is described as a virulence factor of *B. cinerea* on grapevine leaves and plays a role in protection against the phytoalexin resveratrol (Schoonbeek *et al.* 2001). The expression of *BcatrB* is not only induced by resveratrol (Schoonbeek *et al.* 2001) but also by other plant defence compounds such as the phenylpropene eugenol, the isoflavanoid pisatin and the sesquiterpene rishitin (Schoonbeek *et al.* 2003). These data suggest that BcatrB is not only a virulence factor on grapevine leaves but also on other host plants that produce these phytoalexins. To test this hypothesis we compared the virulence of *BcatrB* gene replacement mutants with that of its parental strain B05.10 on a selection of host plants belonging to the *Leguminosae* and *Solanaceae*. *Leguminosae* produce isoflavonoid plant defence compounds (Dixon 2001). Plant defence compounds produced by *Solanaceae* include sesquiterpenes like capsidiol and rishitin (Mercier *et al.* 2001; Stoessl *et al.* 1972). We observed that the virulence of the *BcatrB* replacement mutants was not impaired on any of the plant species tested. We also investigated the virulence of *BcatrB* gene replacement mutants on transgenic tomatoes that express the vine stilbene synthase gene and produce resveratrol. These lines have a significantly reduced susceptibility to *Phytophthora infestans* but not to *B. cinerea* (Thomzik *et al.* 1997). We observed that the *BcatrB* replacement mutants have a similar virulence on the transgenic tomato lines as the wild type but possess an increased virulence on the parental line.

MATERIAL & METHODS

Culturing of strains

The monospore isolate SAS56 and the haploid strain B05.10 (Buttner *et al.* 1994) derived from SAS56 were gifts from Dr J.A.L. van Kan (Department of Phytopathology, Wageningen University, Wageningen, the Netherlands) and Prof. Dr P. Tudzynski (Institut für Botanik, Westfälische Wilhelms-Universität, Münster, Germany), respectively. Strain B05.10 is the parental isolate of the gene-replacement mutants ΔBcatrA-M7, ΔBcatrB4 and ΔBcatrB5 (Schoonbeek *et al.* 2001). Strain ΔBcatrA-M7 was kindly provided by G. Del Sorbo (ARBOVA, University of Naples, Naples, Italy). T132 is a hygromycin-resistant strain derived from B05.10 by transformation with the plasmid pOHT, containing the hygromycin-resistance cassette OHT (Van Kan *et al.* 1997). Isolate CH1.7 is a mono-ascospore isolate with decreased sensitivity to fludioxonil that overexpresses *BcatrB* (Vermeulen *et al.* 2001). The isolate was generated by Dr U. Hilber (Eidgenössische Forschungsanstalt, Wädenswil, Switzerland) and kindly provided by Dr K.M. Chin (Syngenta, Stein, Switzerland). Strains were maintained as described previously (Schoonbeek *et al.* 2001).

Virulence assay

Virulence of *B. cinerea* was tested in pathogenicity assays that allow comparison of different strains

as described by Benito and Ten Have (Benito *et al.* 1998; Ten Have *et al.* 1998). Conidia were harvested from 10-day-old cultures in 0.05% Tween 80 and resuspended in Gamborg's B5 medium (Duchefa Biochemie B.V., Haarlem, The Netherlands) supplemented with 1% sucrose and 10 mM ammonium phosphate (pH 6.5) to a final concentration of 10^6 conidia ml^{-1}. Conidia were preincubated at 20 °C for 2 h to synchronise germination. For each strain droplets (2 µl) were applied to the surface of the plants. Inoculated plant material was incubated in humid chambers in a climate room.

Growth and incubation conditions.

Common bean plants (*Phaseolus vulgaris*) were raised in a climate chamber at 20 °C for four weeks and placed in boxes. Sectors of the second and third set of leaves above the cotyledons were inoculated with *B. cinerea* strains B05.10, ΔBcatrA-M7, ΔBcatrB4 and ΔBcatrB5. Boxes were two incubated in the dark at 4 °C for two days and subsequently at 20 °C for 2 days.

Peanut plants (*Arachis hypogaea*) were raised in the greenhouse for six weeks and placed in boxes. Leaflets were inoculated pair wise with B05.10 and ΔBcatrB4 or B05.10 and CH1.7. Boxes were incubated in the dark at 4 °C for five days or in the light at 15 °C for three days.

Peapods (*Pisum sativum*) were purchased from the local market, inoculated on either the inside or the outside of the pod with strain B05.10, ΔBcatrA-M7, or ΔBcatrB4 and incubated at 15 °C in the light.

Potato tubers (*Solanum tuberosum* var. Bintje and Santé) were purchased from the local market and surface sterilised with 70% ethanol. Tuber slices were droplet-inoculated on the edge of the slice with B05.10, ΔBcatrA-M7, ΔBcatrB4, and ΔBcatrB5 and incubated in humid chambers in the dark at 15 °C for six days.

Sweet bell pepper (*Capsicum annuum* var. Mazurka) was grown in the greenhouse under commercial growing conditions. Green (unripe) and red (ripe) fruits were droplet inoculated and incubated at 15 and 20 °C in the dark. All fruits were inoculated with strains B05.10, ΔBcatrB4, ΔBcatrA-M7 and ΔBcatrB5 with four droplets of each strain.

Tobacco (*Nicotiana tabacum* var. Xanthi) was grown in the greenhouse for seven weeks and used in detached leaf assays. In a pilot experiment detached leaves were inoculated with droplets of B05.10 and ΔBcatrB4 with or without previous wounding with a sterile needle. Leaves were incubated in the light at 15 °C or in the dark at 4 and 15 °C for four days, or successively at 4 and 20 °C for two days. The least variation in lesion size for individual strains was obtained with unwounded leaves incubated in the dark at 15 °C. Therefore, these conditions were used for leaves, pair wise inoculated with B05.10 and ΔBcatrA-M7, ΔBcatrB4 or ΔBcatrB5.

Tomato plants (*Lycopersicon esculentum* cv. Moneymaker Cf4) were grown under greenhouse conditions for 6 weeks and used in detached leaf assays. The virulence of ΔBcatrA-M7, ΔBcatrB4, and ΔBcatrB5 was assessed in six independent experiments by pair wise inoculation with

B05.10. Leaves were incubated in the dark at 15 °C. Lesions were measured three and four days post inoculation (dpi).

Transgenic tomato plants (*L. esculentum* cv. Vollendung and the derived transgenic lines To25 and To42 (kindly provided by Dr. J. Thomzik, Bayer AG, Monnheim, Germany) were grown under PKII conditions in a greenhouse for six weeks and used in detached leaf assays. In experiment I leaves were inoculated with B05.10, ΔBcatrB4 or ΔBcatrB5, incubated in the light at 20 °C and measured three dpi. In experiment II leaves were pair wise inoculated with B05.10 and ΔBcatrA-M7 or B05.10 and ΔBcatrB4, incubated in the dark at 15 °C and measured four dpi.

Statistical analysis

For each strain, all measurements of lesions incited by a *B. cinerea* strain on a single plant part (leaf, tuber slice or fruit) were averaged and used as single "units" in statistical analysis. Differences between treatments were determined by ANOVA followed by Duncan's multiple range test (α = 0.05) (SAS Institute Inc., Cary, N.C., USA).

RESULTS

Virulence assays on leguminous plant species

Droplet inoculation of peanut leaves resulted in expanding lesions with an infection frequency of circa 50% for all strains tested. Lesions incited by strains B05.10, ΔBcatrB4 and CH1.7 were not significantly different in size (Fig. 1A).

Droplet inoculation of leaves from common bean resulted in an infection frequency of more than 95% for all strains tested. Lesions incited by strains B05.10, T132, ΔBcatrB4 and ΔBcatrB5 were not significantly different in size (Fig 1B).

Droplet inoculation of peapods from *P. sativum* resulted in an infection frequency of circa 20% for all strains tested. The size of lesions incited by the reference and the gene-replacement strains were also similar (data not shown).

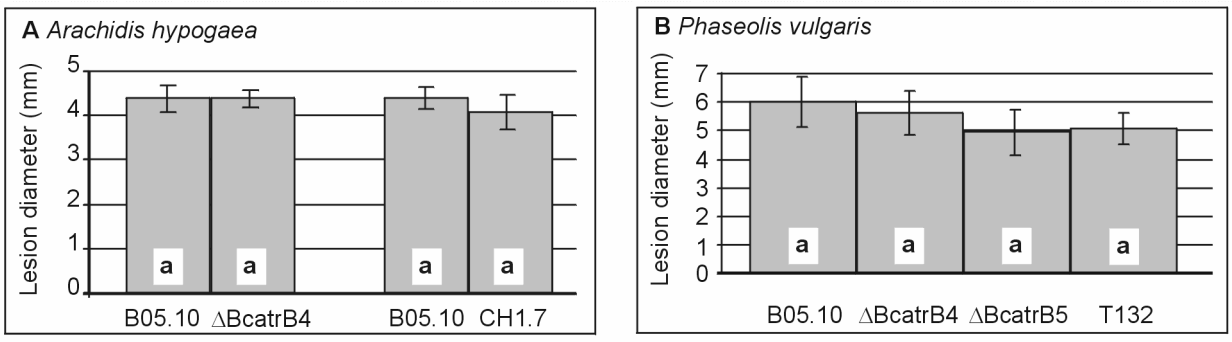

Figure 1. Virulence of *Botrytis cinerea* on leaves of the leguminous plants *Arachis hypogaea* (A) and *Phaseolus vulgaris* (B). The height of the bars indicates the lesion size (mm) incited by wild-type strain B05.10, *BcatrB* replacement mutants ΔBcatrB4 and ΔBcatrB5, transformation control strain T132 and *BcatrB* overexpressing strain CH1.7. Bars marked with different letters indicate a significant difference.

Virulence assays on solanaceous plant species

Droplet inoculation of potato slices resulted in browning of all inoculation sites at three dpi and an infection frequency with soft rot symptoms of *circa* 40% six dpi. No significant differences were observed in the size of lesions incited by B05.10 and the three mutant strains tested (Fig. 2A).

Droplet inoculation of pepper fruits resulted in an infection frequency of 100%. Growth of lesions incited by B05.10 at 20 °C was significantly slower on unripe peppers (4.0 ± 0.3 mm day^{-1}) than on ripe peppers (7.9 ± 0.6 mm day^{-1}). Similar results were obtained at 15 °C, although lesions were smaller. The virulence of B05.10 and all mutant strains proved to be similar (Fig. 2B).

Droplet inoculation of detached tobacco leaves resulted in an infection frequency between

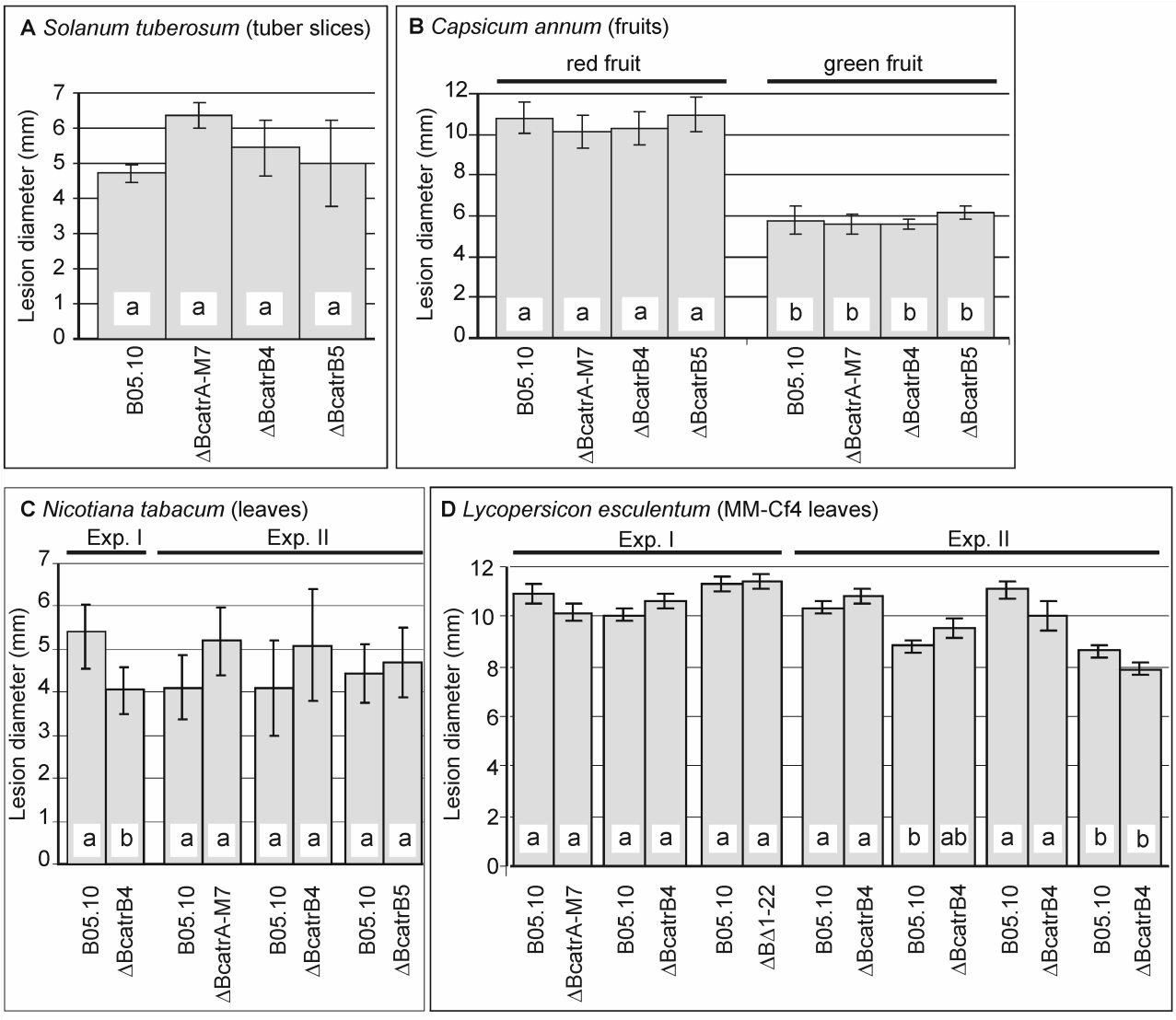

Figure 2. Virulence of *Botrytis cinerea* on solanaceous plant parts. Tuber slices of potato (*Solanum tuberosum*; A), red and green fruits of bell pepper (*Capsicum annum*; B), leaves of tobacco (*Nicotiana tabacum*; C), and leaves of tomato (*Lycopersicon esculentum* cv. Moneymaker Cf4; D). The height of the bars indicates the lesion size (mm) incited by wild-type strain B05.10, *BcatrA* gene-replacement mutant ΔBcatrA-M7, and *BcatrB* replacement mutants ΔBcatrB4 and ΔBcatrB5. Bars marked with different letters indicate a significant difference.

78 and 89%. There was no significant difference in frequency and size of expanding lesions between any of the strains tested (Fig. 2C).

The virulence of B05.10 was compared with that of ΔBcatrA-M7, ΔBcatrB4, ΔBcatrB5, and ΔBΔ1-22 on tomato variety Moneymaker. Droplet inoculation of detached tomato leaves resulted in an infection frequency of at least 88% in all experiments. In none of the experiments a significant difference in the size of lesions was observed (Fig. 2D).

Virulence assays on transgenic *L. esculentum*.

First, the virulence of *B. cinerea* strain B05.10 on detached leaves of cultivar Vollendung was compared with that on the transgenic lines To25 and To42, which produce resveratrol. Lesion sizes on Vollendung and the transgenic lines were not significantly different (Fig. 3). Surprisingly, the mutants ΔBcatrB4 and ΔBcatrB5 proved to be more virulent on Vollendung than B05.10 (Fig. 4A). In a second experiment it was shown that the increased virulence on Vollendung is specific for *BcatrB* replacement mutants since lesions incited by ΔBcatrB4 were larger than those from ΔBcatrA-M7 and B05.10 (Fig. 4B). The virulence on the lines To25 and To42 was similar for all strains tested except for ΔBcatrB5 on To25 (Fig. 4A).

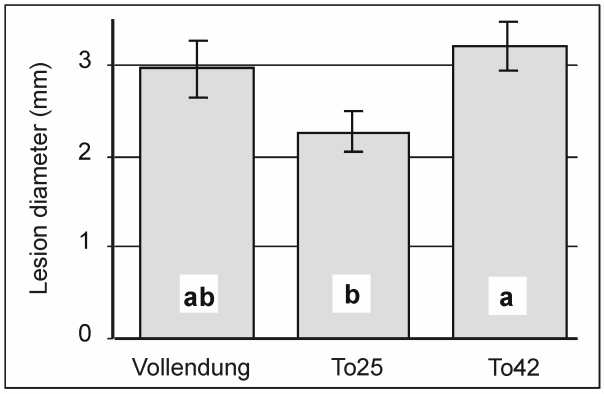

Figure 3. Virulence of *Botrytis cinerea* on leaves of tomato (*Lycopersicon esculentum* cv. Vollendung) and isogenic transgenic lines To25 and To42, which produce resveratrol. The height of the bars indicates the lesion size (mm) incited by wild-type strain B05.10. Bars marked with different letters indicate a significant difference.

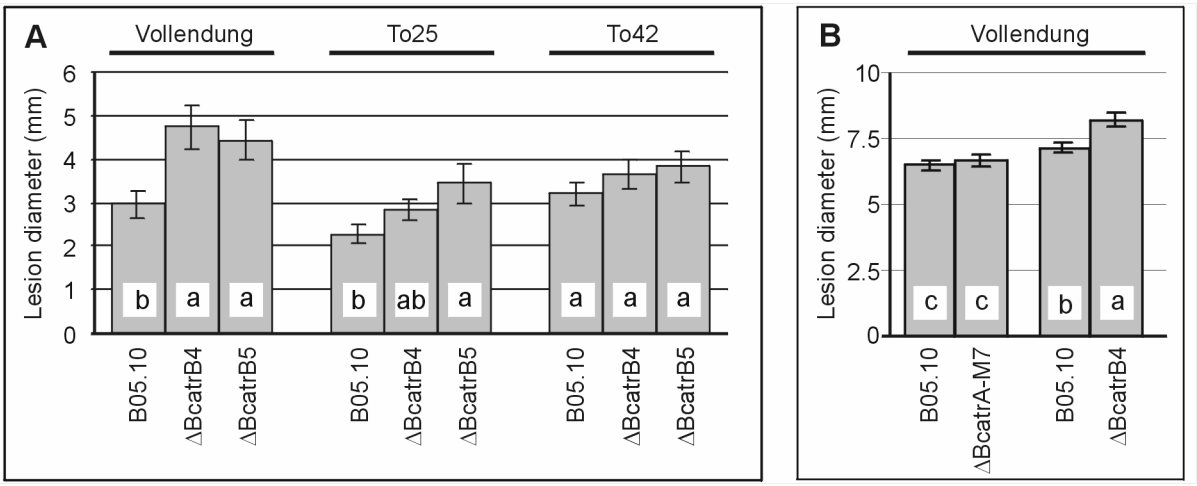

Figure 4. Virulence of *Botrytis cinerea* on leaves of tomato (*Lycopersicon esculentum* cv. Vollendung) and isogenic transgenic lines To25 and To42, which produce resveratrol at 20 °C (A) and 15 °C (B). The height of the bars indicates the lesion size (mm) incited by wild-type strain B05.10, *BcatrA* gene-replacement mutant ΔBcatrA-M7, and *BcatrB* replacement mutants ΔBcatrB4 and ΔBcatrB5. Bars marked with different letters indicate a significant difference.

DISCUSSION

Disease assays on a wide variety of plant species showed that the ABC transporter BcatrB is dispensable for full virulence of *B. cinerea* on plant species belonging to the *Leguminosae* and *Solanaceae*. These observations raise doubt on the hypothesis that a single ABC transporter provides sufficient protection to phytoalexins produced by multiple plant species (Del Sorbo *et al.* 2000; Morrissey and Osbourn 1999). The results suggest that the function of BcatrB as a virulence factor is limited to grapevine leaves (Schoonbeek *et al.* 2001).

Common plant defence compounds in the leguminous plant species tested are pisatin in pea (Fuchs *et al.* 1981), phaseolin and kievitone in bean (Fraile *et al.* 1982), and resveratrol in peanut (Cooksey *et al.* 1988). The main phytoalexins in the tested solanaceous plant species are sesquiterpenes such as rishitin, lubimin, and capsidiol (Adikaram *et al.* 1988; Kroon and Elgersma 1993; Moreau *et al.* 1992; Xie and Kuc 1986). *B. cinerea* can induce production of these compounds in the respective host plants (Glazener and Wouters 1981) and a high content of some of these compounds can affect the virulence of the pathogen (Stoessl *et al.* 1972). Previous studies have shown that plant defence compounds such as pisatin and rishitin induce expression of *BcatrB* (Schoonbeek *et al.* 2003), suggesting that they are substrates of the transporter and that BcatrB is a virulence factor of *B. cinerea* on various leguminous and solanaceous plants, as well. The conclusion that this hypothesis could not be validated may imply that *B. cinerea* has alternative mechanisms to cope with plant defence compounds that are more relevant in protection against phytoalexins than BcatrB. Such mechanisms may include the presence of insensitive target sites, compartmentalisation, and metabolic inactivation. An example of the latter mechanism is the P_{450}-dependent demethylation of pisatin, which is a major determinant in virulence of *Nectria haematococca* on pea (Wasmann and VanEtten 1996). All these mechanisms seem to be rather specific and do not explain why *B. cinerea* can cope with such a broad range of plant defence compounds.

It might also be that a role of BcatrB in virulence is still valid but remained unrevealed for the following reasons: a) the concentration of plant defence compounds in the plants tested may have been too low to hamper growth of any of the *B. cinerea* strains tested. This may especially have been the case for capsidiol in peppers, since its producion level depends on the inducing pathogen (Stoessl *et al.* 1972). The capacity of *B. cinerea* to induce production of capsidiol and pisatin is known to be variable but the induction by other pathogens is sufficient to reduce their virulence (Delserone *et al.* 1999; Dixon 2001; Xie and Kuc 1986). b) The conditions used to test the virulence of *B. cinerea* may have been so conducive for pathogenesis that phytoalexin production was too slow and localised to inhibit fungal growth. c) Inducers of *BcatrB* gene-expression are not necessarily substrates of BcatrB. d) Redundancy of ABC and/or MFS transporters with an overlap in substrate range may have compensated for the deficiency of BcatrB efflux activity. In the latter case reduced virulence on host plants and increased sensitivity to toxicants may only be observed in tests with multiple knock-out mutants. The validity of the latter explanation is supported by the

observation that certain phenotypes of ABC transporter mutants of *B. cinerea* (Hayashi *et al.* 2002) and *Candida albicans* (Sanglard *et al.* 1997) can only be observed in double knock-out mutants.

The virulence of *B. cinerea* strain B05.10 on the resveratrol-producing lines To25 and To42 was similar to that on the parental line Vollendung (Fig. 3). This observation corroborates a previous publication describing that the virulence of *B. cinerea* on tomato expressing stilbene synthase is not altered (Thomzik *et al.* 1997). Surprisingly, lesions caused by *BcatrB* replacement mutants on Vollendung are significantly larger than those caused by B05.10. This difference is less evident for lesions formed on To25, where it is only significant for ΔBcatrB5 and absent on To42 (Fig. 4A). There is no obvious explanation for this observation. The conclusion that BcatrB could not be identified as a virulence factor on the resveratrol-producing tomato lines To25 and To42, can be a consequence of the increased growth rate of the *BcatrB* replacement strains on tomato lines with a Vollendung background. This conclusion contrasts with the finding that BcatrB acts as a virulence factor on grapevine leaves by providing protection against resveratrol (Schoonbeek *et al.* 2001). Other factors that may affect the effectivity of resveratrol as a plant defence compound in this heterologous system are the resveratrol production level, which may be lower than in grapevine and thus insufficient to inhibit growth of *B. cinerea*. In addition, the inherent fungitoxicity of resveratrol is low and it requires activation by the laccase Bclcc2 (Schouten *et al.* 2002). The mechanism that activates the enzyme in *B. cinerea* may not function in plant species that do not naturally produce resveratrol.

The increased virulence of the *BcatrB* replacement mutants on tomato cv. Vollendung as compared to B05.10 is difficult to explain. First of all, the phenomenon is cultivar specific since no difference in virulence was observed on the tomato cv. Moneymaker *Cf4*. Secondly, the increased virulence is specific for *BcatrB* replacement mutants since it was not found for ΔBcatrA-M7, a gene-replacement mutant of the ABC transporter gene *BcatrA*, made with the same OHT-resistance cassette. One hypothetical explanation is that BcatrB secretes a compound that elicits a stronger defence response in Vollendung than in Moneymaker.

The observation that BcatrB does not act as a virulence factor on a range of leguminous and solanaceous plant species contrasts with reports in which ABC transporters, including homologues of BcatrB, are major contributors to virulence (Fleissner *et al.* 2002; Stergiopoulos *et al.* 2003; Urban *et al.* 1999; Zwiers 2002). In contrast to *B. cinerea*, all these pathogens have a narrow host specificity, which may imply that their ABC transporters have a relatively high substrate specificity for plant defence compounds and thus play an essential role in virulence of these organisms. Therefore, it may be that a putative function of BcatrB as a virulence factor is masked by the relatively low substrate specificity of multiple ABC transporters in *B. cinerea* and the presence of a wide array of other virulence mechanisms.

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Chapter 9

Fungal ABC transporters and microbial interactions in natural environments

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ABSTRACT

In natural environments, microorganisms are exposed to a wide variety of antibiotic compounds produced by competing organisms. Target organisms have evolved various mechanisms of natural resistance to these metabolites. In this study, the role of ATP-Binding Cassette (ABC) transporters in interactions between the plant pathogenic fungus *Botrytis cinerea* and antibiotic-producing *Pseudomonas* bacteria was investigated in detail. We discovered that 2,4-diacetylphloroglucinol, phenazine-1-carboxylic acid and phenazine-1-carboxamide (PCN), broad-spectrum antibiotics produced by *Pseudomonas* spp., induced expression of several ABC transporter genes in *B. cinerea*. Phenazines strongly induced expression of *BcatrB,* and ΔBcatrB mutants were significantly more sensitive to these antibiotics than their parental strain. Treatment of *B. cinerea* germlings with PCN strongly affected the accumulation of [^{14}C]fludioxonil, a phenylpyrrole fungicide known to be transported by BcatrB, indicating that also phenazines are transported by BcatrB. *Pseudomonas* strains producing phenazines displayed a stronger antagonistic activity *in vitro* towards ΔBcatrB mutants than to the parental *B. cinerea* strain. On tomato leaves, phenazine-producing *Pseudomonas* strains were significantly more effective in reducing grey mould symptoms incited by a ΔBcatrB mutant than by the parental strain. Collectively, these results indicate that fungal ABC transporters can play an important role in antibiotic-mediated interactions between bacteria and fungi in plant-associated environments. The implications of these findings for the implementation and sustainability of crop protection by antagonistic microorganisms are discussed.

INTRODUCTION

Soil and plant-associated environments harbour a wide variety of microorganisms that play an integral role in plant growth and in preservation of multiple ecosystem functions. To exist in natural environments, microorganisms must be able to endure all of the adverse abiotic and biotic conditions that are prevalent in a particular environment. Among the biotic factors, interactions within and among microbial communities are numerous and range from synergistic and mutualistic to antagonistic and parasitic. Many microorganisms isolated from soil and plant-associated environments produce secondary metabolites that adversely affect the growth or metabolic activity of other microorganisms (Fravel 1988). The significance of these antibiotics in microbial interactions *in situ*, however, has long been questioned because of the perceived constraints to antibiotic production in natural environments (Williams and Vickers 1986). Because of the biotic and abiotic complexity of soil and plant-associated environments, there were several inherent difficulties in demonstrating that antibiotics are produced by microorganisms *in situ*. Development of sensitive detection methods, including reporter gene systems and bioanalytical techniques, have now demonstrated unequivocally that antibiotic compounds are produced *in situ* by both introduced and indigenous microorganisms (Raaijmakers *et al.* 1999; Thomashow *et al.* 1997). In this context, considerable attention has been given to the antibiotics 2,4-diacetylphloroglucinol (DAPG),

phenazines and pyrrolnitrin (PRN), produced by plant growth-promoting fluorescent *Pseudomonas* species (Thomashow and Weller 1995). These antibiotics are produced in soil- and rhizosphere environments (Thomashow *et al.* 1997) and play a key role in interactions between *Pseudomonas* strains and soil-borne fungi (Cook *et al.* 1995; Whipps 2001).

Numerous *Pseudomonas* strains that produce antibiotics have been isolated from the rhizosphere of plants grown in soils from diverse geographical regions (Keel *et al.* 1996; McSpadden Gardener *et al.* 2000; Raaijmakers *et al.* 1997). The ability of plant-associated pseudomonades to produce antibiotics enables them to defend their habitats (Mazzola *et al.* 1992), whereas the existence of genotypic and physiological diversity among *Pseudomonas* spp. producing the same antibiotic (McSpadden Gardener *et al.* 2000; Picard *et al.* 2000; Raaijmakers and Weller 2001; Wang *et al.* 2001) provides a means by which populations of these microorganisms can adapt to diverse habitats. Although substantial progress has been made in the identification of genes involved in the biosynthesis and regulation of antibiotic production by *Pseudomonas* spp. (Bangera and Thomashow 1999; Delaney *et al.* 2001; Hammer *et al.* 1997; Mavrodi *et al.* 1998; Pierson *et al.* 1995) the responses of other microorganisms, and in particular defensive mechanisms, to these antibiotics have received little attention. Studies on the effect of therapeutic antibiotics, antimycotics and synthetic fungicides on human and plant pathogenic microorganisms have shown that target organisms can protect themselves by active efflux mechanisms that prevent the intracellular accumulation of these compounds to toxic concentrations (De Waard 1997; Del Sorbo *et al.* 2000; Sanglard *et al.* 1998; Van Bambeke *et al.* 2000). Active efflux mechanisms not only enable target organisms to tolerate exogenous antibiotics, but may also prevent self-intoxication in antibiotic-producing microorganisms (Andrade *et al.* 2000b; Mendez and Salas 2001). Among the active efflux mechanisms known, ATP-binding cassette (ABC) transporters are well studied (Dassa and Bouige 2001; Del Sorbo *et al.* 2000; Driessen *et al.* 2000; Rogers *et al.* 2001; Wolfger *et al.* 2001). ABC transporters are present from archae-bacteria to man and enable both influx and efflux of several compounds (Andrade *et al.* 2000a; Kolaczkowski *et al.* 1998; Lewis 2001). The natural functions of ABC transporters from plant pathogenic fungi comprise secretion of toxins that act as virulence factors (host-specific toxins or mycotoxins) and protection against plant defence compounds (phytoalexins) and synthetic fungicides (De Waard 1997). Over the last few years, these phenomena have been described for *Botrytis cinerea, Magnaporthe grisea* and *Mycosphaerella graminicola* (Schoonbeek *et al.* 2001; Urban *et al.* 1999; Zwiers and De Waard 2000).

It has frequently been postulated that ABC transporters may also play an important role in microbial interactions in natural environments (Andrade *et al.* 2000b; De Waard 1997; Del Sorbo *et al.* 2000). To date, however, their protective role in interactions between naturally occurring microorganisms has not been addressed. The objective of this study was to investigate whether fungal ABC transporters are involved in antibiotic-mediated interactions between bacteria and fungi in natural environments. More specifically, we studied interactions between *B. cinerea* and antibiotic-producing *Pseudomonas* spp. *in vitro* and *in situ*. *B. cinerea* is a fungus that infects a broad range of plant species and that resides in plant debris for its survival. During both the

parasitic and saprophytic stage of its life cycle, *B. cinerea* coexists and interacts with other microorganisms. In *B. cinerea*, at least 14 ABC transporters have been identified (Hayashi *et al.* 2001; Vermeulen *et al.* 2001), allowing the study of their role in interactions with antibiotic-producing *Pseudomonas* spp. We observed that broad-spectrum antibiotics produced by *Pseudomonas* spp. induced expression of several ABC transporter genes in *B. cinerea*. Phenazine antibiotics strongly induced expression of the ABC transporter gene *BcatrB* and ΔBcatrB mutants were significantly more sensitive to these antibiotics than their parental strain. On tomato leaves, phenazine-producing *Pseudomonas* strains were significantly more effective in reducing grey mould symptoms incited by a *BcatrB* mutant than by its parental strain. These results show, for the first time, that specific fungal ABC transporters play an important role in microbial interactions in plant-associated environments.

Table 1. Characteristics of bacterial and *Botrytis cinerea* strains

Strain	**Phenotype**
Botrytis cinerea	
B05.10	Haploid derivative of SAS56 with wild-type sensitivity (Buttner *et al.* 1994)
ΔBcatrA-M7	BcatrA gene-replacement mutant derived from B05.10 (Del Sorbo *et al.*)
ΔBcatrB4 and ΔBcatrB5	BcatrB gene-replacement mutants derived from B05.10 with increased sensitivity to fludioxonil (Schoonbeek *et al.* 2001)
Burkholderia cepacia	
B37w	(ATCC51671) Producer of PRN (Burkhead *et al.* 1994)
Pseudomonas chlororaphis	
PCL1391	Produces PCA and PCN (Chin-A-Woeng *et al.* 1998)
PCL1119	Mutant of PCL1391 with a Tn*5*-*luxAB* insertion in the *phz*B gene (Chin-A-Woeng *et al.* 1998)
Pseudomonas sp.	
JM13	No production of antibiotics known (Raaijmakers and De Souza; unpublished)
Phz24	Produces PRN, PCA, 2'OH-PCA (Raaijmakers and Lemanceau; unpublished)

RESULTS

Expression of ABC transporter genes.

The effect of the fungicide fludioxonil and the *Pseudomonas* antibiotics PRN, DAPG, PG, PCA, and PCN on expression of ABC transporter genes *BcatrA, B, C, D, E, F, G, I,* and *K* in *B. cinerea* germlings was studied by northern analysis. Treatment of germlings with fludioxonil or antibiotics for 20 min had no effect on the expression of *BcatrA* (Fig.1), *BcatrC, E, F, G, I,* and *K* (data not shown). Treatment with PRN did not induce expression of any of the ABC transporter genes tested, whereas fludioxonil, a fungicide derived from PRN (Ligon *et al.* 2000), resulted in highly elevated expression levels of *BcatrB* (Fig. 1). Treatment with the antibiotics DAPG, PG, PCA and PCN had a differential effect on expression of *BcatrB* and *BcatrD* (Fig. 1). DAPG and the phenazines PCA and PCN strongly induced *BcatrB* expression. Treatment with PG also resulted in elevated transcript levels of *BcatrB*, but expression levels were lower than those obtained with DAPG and PCA. DAPG and, to a lesser extent, PG also induced expression of *BcatrD*. The induction patterns obtained after 60 min of treatment were similar to the patterns obtained after 20 min (data not shown). Expression of the actin gene *BcactA,* an indicator of fungal growth and transcription, was constant for all treatments. These results clearly demonstrate that several antibiotics produced by *Pseudomonas* spp. induce expression of specific ABC transporter genes in *B. cinerea,* in particular *BcatrB*. For this reason, further studies on antibiotic-mediated interactions between *B. cinerea* and *Pseudomonas* spp. focussed on *BcatrB*.

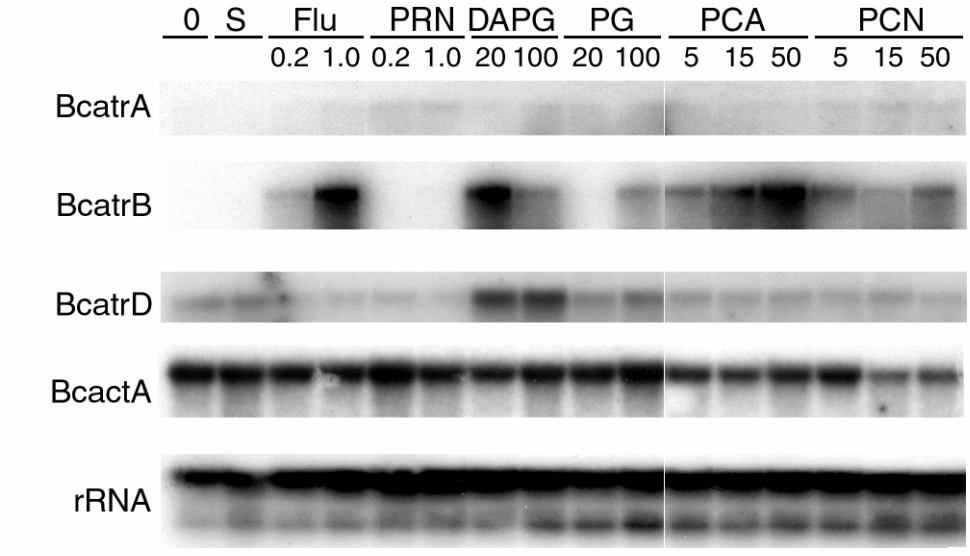

Figure 1. Northern analysis showing the effect of fludioxonil and antibiotics on expression of *BcatrA*, *BcatrB*, and *BcatrD* in *B. cinerea* strain B05.10.

Antifungal activity of antibiotics.

Compounds that induce expression of ABC transporter genes can also be substrates of the encoded proteins. In that case, replacement of the gene involved may cause an increased sensitivity to these compounds. The activity of fludioxonil, PRN, DAPG, PG, PCA and PCN was tested on radial mycelial growth of wild-type *B. cinerea* strain B05.10 and two independent ΔBcatrB mutants; a ΔBcatrA mutant was included as a control (Table 2). Both ΔBcatrB mutants were significantly more sensitive to PCA and PCN than their parental strain B05.10. Spore germination experiments confirmed this differential sensitivity to these phenazines (data not shown). The ΔBcatrB mutants were also more sensitive to fludioxonil than B05.10, as reported previously (Vermeulen *et al.* 2001), whereas no differences were observed in sensitivity to PRN and DAPG (Table 2). PG did not adversely affect mycelial growth of any of the *B. cinerea* strains at concentrations up to 500 μg ml^{-1}, suggesting that acetylation of PG significantly increases its fungicidal activity (data not shown). ΔBcatrA-M7 did not differ from its parental strain in sensitivity to any of the tested compounds, indicating that BcatrA is not involved in protection against any of these compounds. Collectively, these results strongly suggest that the ABC transporter BcatrB in *B. cinerea* not only provides protection to the fungicide fludioxonil but also to the antibiotics PCA and PCN.

Table 2. Effect of fludioxonil and antibiotics on radial mycelial growth of *B. cinerea* strain B05.10 and its derivatives.

Strain*	EC_{50} (mg $liter^{-1}$)				
	Fludioxonil	Pyrrolnitrin	DAPG	PCA	PCN
B05.10	0.0022 a†	0.019 a	17.4 a	39 a	40 a
ΔBcatrA-M7	0.0023 A	0.022 a	17.2 a	36 a	43 a
ΔBcatrB4	0.0013 B	0.019 a	16.9 a	13 b	19 b
ΔBcatrB5	0.0013 B	0.021 a	15.8 a	8.2 b	12 b

* Characteristics of the strains are described in Table 1.

† Different letters in the same column indicate a statistically significant difference (P=0.05).

Effect of phenazines on accumulation of [^{14}C]fludioxonil.

The effect of phenazines on the efflux activity of BcatrB was determined in [^{14}C]fludioxonil accumulation experiments with germlings of wild-type strain B05.10 (Fig. 2). Accumulation of [^{14}C]fludioxonil, a substrate of BcatrB, was transient: the maximum level of [^{14}C]fludioxonil accumulation was reached 20 min after initiation of the experiment and thereafter gradually declined and remained low from 60 min onwards (Fig. 2A). This transient accumulation is ascribed to rapid passive influx of [^{14}C]fludioxonil, resulting in induction of *BcatrB* expression and

subsequent active efflux. Addition of PCN 65 min after initiation of the experiment resulted in an instantaneous and transient increase in accumulation of [^{14}C]fludioxonil (Fig. 2A). This transient increase can be explained by competition of PCN with fludioxonil for export by BcatrB followed by additional induction of *BcatrB* and other transporters. A 60-min pre-treatment of germlings with PCN prior to addition of [^{14}C]fludioxonil resulted in a low and constant accumulation level of [^{14}C]fludioxonil (Fig. 2B), which is ascribed to induction of expression of *BcatrB* by PCN.

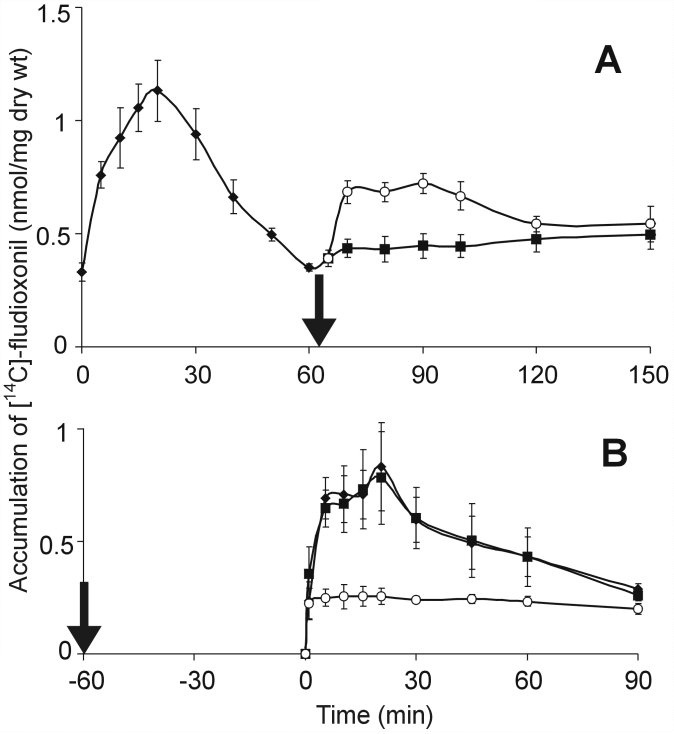

Figure 2. Effect of PCN on [^{14}C]fludioxonil accumulation by germlings of *B. cinerea* strain B05.10. Arrows indicate the addition of PCN 65 min after (A) or 60 min prior to (B) addition of [^{14}C]fludioxonil (1 mg liter^{-1}) at t=0. Treatments: control (♦), solvent control (0.1% DMSO; ■), and PCN (10 mg liter^{-1}; ○).

Role of BcatrB in *in vitro* interactions between *B. cinerea* and *Pseudomonas*.

Antagonism of the bacterial strains JM13, B37w, PCL1119, PCL1391, and Phz24 toward *B. cinerea* strains was determined in agar diffusion tests (Table 3). JM13 does not produce any known antibiotics and showed almost no activity against all *B. cinerea* strains tested. The antagonistic activity of B37w, known to produce PRN, was similar to all *B. cinerea* strains tested. Antagonistic activity of PCL1391 and Phz24, known to produce phenazines, was significantly stronger towards the mutants ΔBcatrB4 and B5 than to the parental strain B05.10 and ΔBcatrA-M7. In contrast, PCL1119, the phenazine-deficient mutant of PCL1391, had almost no activity towards the ΔBcatrB mutants and their inhibition was similar to that of the control strains. Microscopic observation of germling growth in the inhibition zones confirmed that PCL1391 had a relatively strong effect on spore germination and germ tube elongation of ΔBcatrB5 (Fig. 3). Similar observations were made in experiments with Phz24 and ΔBcatrB4. To ascertain that the inhibition zones incited by PCL1391 and Phz24 can be, at least in part, ascribed to activity of phenazines, the concentration of these antibiotics in the agar plates was determined by HPLC analysis. Inhibition zones around

inoculation sites of strain PCL1391 contained on average 30.1 µg ml^{-1} PCN and zones around Phz24 contained 37.9 µg ml^{-1} phenazines (32.5 µg ml^{-1} 2'-OH-PCA and 5.4 µg ml^{-1} PCA). These concentrations are approximately 2 times higher than the EC_{50} value of PCN for the ΔBcatrB mutants and 1.5 times lower than the EC_{50} value for the parental strain B05.10 (Table 2). In zones around the inoculation site of the other bacterial strains, including PCL1119, no phenazines were detected.

Table 3. Antagonistic activity of *Pseudomonas* and *Burkholderia* spp. against *B. cinerea* strain B05.10 and its derivatives in agar diffusion tests.

	Inhibition zone (mm)									
Strains*	JM13		PCL1119		PCL1391		Phz24		B37w	
B05.10	0.45	a†	1.25	a	1.46	a	14.17	a	7.68	a
ΔBcatrA-M7	0.32	a	0.43	b	1.12	a	14.46	a	7.01	a
ΔBcatrB4	0.37	a	0.74	ab	4.85	b	19.43	b	8.27	a
ΔBcatrB5	0.40	a	0.65	b	4.29	b	18.43	b	8.30	a

* Characteristics of the strains are described in Table 1.

† Different letters in the same column indicate a statistically significant difference (P=0.05).

Role of BcatrB in *in situ* interactions between *B. cinerea* and *Pseudomonas.*

The interactions between phenazine-producing *Pseudomonas* strains and *B. cinerea* strains B05.10 and ΔBcatrB4 was studied on tomato leaves. The virulence of B05.10 and ΔBcatrB4 on tomato leaves was similar in the control treatment (Fig. 4). Treatment of tomato leaves with cell suspensions of PCL1391 significantly reduced the percentage of expanding lesions caused by ΔBcatrB4, whereas no reduction was observed for parental strain B05.10 (Fig. 4A). PCL1119, the phenazine-deficient mutant of PCL1391, did not reduce the percentage of expanding lesions for both ΔBcatrB4 and B05.10. Furthermore, the effect of PCL1391 on the size and growth of the lesions caused by ΔBcatrB4 was significantly stronger than for B05.10 (Figs 4B, 4C). In contrast, the effect of PCL1119 on lesion size and growth was similar for B05.10 and ΔBcatrB4. In the repeat experiment, similar results were obtained: PCL1391 and also phenazine-producer Phz24 significantly reduced the size and growth of the lesions caused by ΔBcatrB4, whereas no reduction was observed for parental strain B05.10. HPLC analysis demonstrated that on leaves treated with PCL1391, PCN was produced at an average concentration of 5.6 µg g^{-1} leaf fresh weight. No phenazines were detected on untreated leaves or on leaves treated with PCL1119. These results indicate that the ABC transporter BcatrB provides protection to *B. cinerea* in phenazine-mediated interactions with *Pseudomonas* spp.

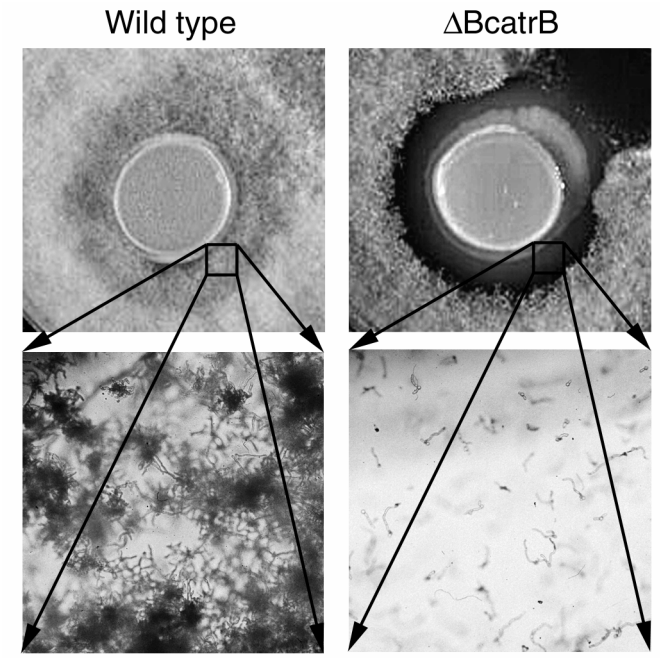

Figure 3. Antagonistic activity of *P. chlororaphis* strain PCL1391 against *B. cinerea* B05.10 and ΔBcatrB5, in agar diffusion tests. Inhibition of mycelial growth (top) and germination of conidia (400× magnification, bottom).

DISCUSSION

In natural environments, many microorganisms coexist in close physical association leading to numerous types of interactions. For their survival, microorganisms have developed both offensive and defensive strategies to effectively compete with other organisms. Various mechanisms that enable microorganisms to resist toxic compounds have been described in the area of medical microbiology and include enzymatic degradation or inactivation of antibiotic compounds (Davies 1994), alteration of their target sites (Spratt 1994), and active efflux (Nikaido 1994; Van Bambeke *et al.* 2000; Van Veen and Konings 1997). To date, however, the role of these protective mechanisms in microbial interactions in natural environments has received little attention. This study shows that the ABC transporter BcatrB from the plant pathogenic fungus *B. cinerea* provides protection against phenazine antibiotics produced by *Pseudomonas* spp. The phenazines PCA and PCN strongly induced *BcatrB* expression and ΔBcatrB mutants were more sensitive to phenazine antibiotics than their parental strain. In addition, accumulation of fludioxonil, a known BcatrB substrate (Vermeulen *et al.* 2001), was strongly affected by addition of PCN. These results indicate that phenazine antibiotics are substrates of the ABC transporter BcatrB in *B. cinerea*. In both *in vitro* and *in situ* experiments, the ΔBcatrB mutants of *B. cinerea* were significantly more sensitive to antagonism by the phenazine-producing *P. chlororaphis* strain PCL1391 than the parental strain B05.10. In contrast, a phenazine-deficient mutant of PCL1391 did not show this differential activity towards the wild type B05.10 and the ΔBcatrB mutant. Collectively, these results demonstrate, for the first time, that fungal ABC transporters can play an important role in microbial interactions in plant-associated environments and, more specifically, protect a fungal pathogen against antibiotic compounds produced by antagonistic bacteria.

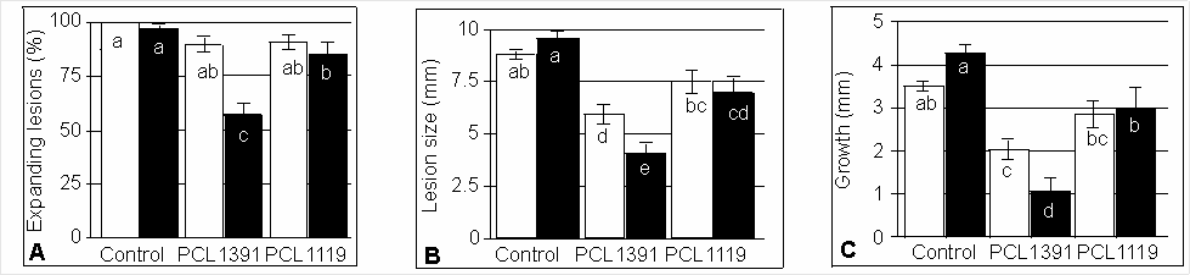

Figure 4. Antagonistic activity of *P. chlororaphis* PCL1391 and its phenazine-deficient mutant PCL1119 on disease development incited by *B. cinerea* strains B05.10 (white) and ΔBcatrB4 (black) on tomato leaves. Leaves were sprayed with sodium phosphate buffer (control), a suspension of PCL1391 or PCL1119 in the same phosphate buffer. Disease development is expressed as the percentage of inoculation sites developing into expanding lesions (A), the average lesion size at 4 days post inoculation (dpi; B), and the average growth of the lesions between 3 and 4 dpi (C). Different letters in columns indicate statistically significant differences between treatments (P=0.05).

Interestingly, BcatrB also provides protection against the grapevine phytoalexin resveratrol and phenylpyrrole fungicides (Schoonbeek *et al.* 2001; Vermeulen *et al.* 2001), indicating that one ABC transporter can transport multiple and structurally diverse compounds. ABC transporters with a wide substrate range have also been reported for yeasts and other filamentous fungi (Andrade *et al.* 2000a; Kolaczkowski *et al.* 1998; Moore *et al.* 2000; Rogers *et al.* 2001; Wolfger *et al.* 2001). At present, it is not known how multiple unrelated compounds can induce transcription of one particular gene and at the same time act as a substrate of the encoded protein. Our observation that fludioxonil, a fungicide derived from pyrrolnitrin (Ligon *et al.* 2000), differs from pyrrolnitrin in its induction of *BcatrB* and in activity towards ΔBcatrB mutants, is surprising and may provide an approach to further elucidate substrate specificity of BcatrB. In addition to phenazine antibiotics, also the antibiotics DAPG and PG induced expression of *BcatrB*. However, both Δ*BcatrB* mutants showed similar sensitivity to these compounds as their parental strain, indicating that induction of expression of a particular ABC gene does not necessarily imply that the encoded protein is a major transporter of that compound. This has also been observed for other inducers of *BcatrB* expression, including azole fungicides (Hayashi *et al.* 2001; Schoonbeek *et al.* 2001). Since DAPG also induces expression of *BcatrD*, this transporter may have operated in *BcatrB* mutants thereby compensating BcatrB efflux deficiency. The role of BcatrD and other transporters, including members of the Major Facilitator Superfamily, are currently being analysed to better understand their role in sensitivity of *B. cinerea* to DAPG, a key metabolite in interactions between several plant pathogens and fluorescent *Pseudomonas* strains (Walsh *et al.* 2001). The contribution of other protective mechanisms, including enzymes that reduce oxidative stress caused by antibiotics (Levy *et al.* 1992), will be included in these studies.

An interesting question arising from this study is how *B. cinerea* acquired resistance to phenazine antibiotics. We propose that this ability may have evolved from long-lasting selection pressure by phenazine-producing micro-organisms during the saprophytic and pathogenic stages of

the life cycle of *B. cinerea*. This may be a consequence of the fact that phenazine production is a widely distributed trait among different bacterial genera that are ubiquitous in soil and plant-associated environments (Turner and Messenger 1986). This hypothesis would imply that also *BcatrB* homologues are more prevalent among other saprophytic and pathogenic fungi. Studies on the distribution of *BcatrB* homologues in other fungi, including *A. nidulans, Fusarium oxysporum, Gaeumannomyces graminis* var. *tritici,* and *M. graminicola,* are ongoing in our laboratory and will further extend our insight in the role of ABC transporters in antibiotic-mediated interactions between pathogenic fungi and antagonistic microorganisms and, more generally, in ecological equilibria between microorganisms in natural environments.

Our observation that a plant pathogenic fungus harbours a resistance mechanism to defend itself against a natural antibiotic compound has several implications for the efficacy and implementation of antagonistic microorganisms for biocontrol purposes. It may suggest that if sufficient selection pressure is applied to target pathogens, either by repeated inundative applications of biocontrol agents or by introducing transgenic strains with enhanced antibiotic production, shifts in pathogen populations towards resistance may occur. Information on resistance development of pathogens to antibiotics produced by biocontrol agents is very limited. One of the few, but well-studied, examples is agrocin 84-resistance in *Agrobacterium tumefaciens* resulting from transfer of plasmid pAgK84 from the antagonistic strain *A. radiobacter* K84 to the pathogen (Stockwell *et al.* 1996). Repetitive treatment of *Astilbe* plants with the biocontrol agent *Bacillus subtilis* CL27 resulted in an increase in grey mould (Li and Leifert 1994). Recently, it was shown that introduction of a phenazine-producing *Pseudomonas* biocontrol strain exerted transient effects on the composition of the fungal rhizosphere microflora (Glandorf *et al.* 2001). Furthermore, Jones and Pettit (1987) observed variation in sensitivity among anastomosis groups of *Rhizoctonia solani* to the antibiotic gliotoxin produced by *Gliocladium virens*. Mazzola *et al*. (1995) showed that various isolates of the take-all fungus *G. graminis* var. *tritici* differ in their sensitivity toward DAPG and phenazines produced by *Pseudomonas* spp. In fact, the fungal isolates that were relatively insensitive to either DAPG or phenazines *in vitro* could no longer be suppressed *in situ* by *Pseudomonas* strains producing these antibiotics (Mazzola *et al.* 1995). These results suggest that at least certain pathogen populations have the potential to develop resistance to antibiotics produced by antagonistic micro-organisms. We postulate that the efficacy and sustainability of crop protection by antibiotic-producing biocontrol agents can be compromised by adaptation in target organisms and that overexpression of ABC transporters can act as one of the resistance mechanisms. One approach to counteract such a resistance development might be the selection of crop cultivars with traits that promote efficacy of biocontrol agents (Smith *et al.* 1999). This may apply to crop cultivars with enhanced production of secondary metabolites that act as modulators (*e.g.* flavonoids) of fungal ABC transporter activity (Conseil *et al.* 2000).

MATERIALS AND METHODS

Strains and cultural practices.

Characteristics of the bacterial and fungal strains used in this study are described in Table 1. *B. cinerea* strains were stored as conidial suspensions in 15% glycerol at -80 °C and cultured as described previously (Schoonbeek *et al.* 2001). Conidia for sensitivity assays, expression analysis, and accumulation experiments were harvested from 10-day-old cultures on malt extract agar plates (MEA; 50 g liter^{-1}; Oxoid, Basingstoke, Hampshire, England), amended with yeast extract (2 g liter^{-1}; Oxoid). Bacterial isolates were stored in 40% glycerol at -80 °C and cultured on King's medium B (King *et al.* 1954) (KMB) at 28 °C in the dark.

Chemicals.

Fludioxonil (technical grade) and [^{14}C]fludioxonil were kindly provided by Syngenta (Basel, Switzerland). The antibiotics used were phloroglucinol (1,3,5-trihydroxybenzene, PG; Eastman Kodak Company, Rochester NY), 2,4-diacetylphloroglucinol (DAPG, HPLC-purified), pyrrolnitrin (PRN; Syngenta, Basel, Switzerland), phenazine-1-carboxylic acid (PCA), and phenazine-1-carboxamide (PCN; Dr. T. F. C. Chin-A-Woeng, Leiden University, Leiden, The Netherlands). All other compounds were purchased from Sigma-Aldrich (Zwijndrecht, the Netherlands). Compounds were added to media from 1000-fold concentrated stock solutions in DMSO unless stated otherwise.

In vitro sensitivity assays.

The effective concentration of compounds resulting in inhibition of radial mycelial growth by 50% (EC_{50}) was determined on potato dextrose agar (PDA; Oxoid) as described previously (Stehmann and De Waard 1996). Experiments were repeated 3 times.

Gene expression in *B. cinerea*.

Expression analysis of ABC transporter genes in *B. cinerea* germlings was performed as described previously (Schoonbeek *et al.* 2001). Gene-specific fragments of *BcatrA, B, and D* and fragments corresponding to expressed sequence tags (ESTs) of *BcatrC*, *E, F, G, I, and K* were described previously (Hayashi *et al.* 2001; Vermeulen *et al.* 2001). Mycelium frozen in liquid nitrogen was used for RNA isolation with TRIzol (Life Technologies, Breda, The Netherlands). Hybridisations with *B. cinerea* 23S rRNA and *BcactA* (actin gene) were used as control (Prins *et al.* 2000).

Accumulation of [^{14}C]fludioxonil.

Accumulation experiments were performed as described previously (Vermeulen *et al.* 2001) with minor modifications. Standard germling suspensions (200 mg wet weight mycelium in 50 ml buffer (23.4 mM potassium phosphate (pH 6.0), 0.1 mM $CaCl_2$, 10 g liter^{-1} glucose) in 300 ml Erlenmeyer flasks) were shaken on a reciprocal shaker (120 rpm) at 20 °C. Experiments were initiated by

adding [^{14}C]fludioxonil to a final concentration of 0.4 µM (1 mg liter^{-1}; 250 Bq nmol^{-1}) from a 100x concentrated stock solution in methanol. The effect of phenazines (10 mg liter^{-1}) on [^{14}C]fludioxonil accumulation was determined by addition 60 minutes prior to or after the start of the incubation with the radiochemical. Experiments were performed in triplicate.

***In vitro* interactions between *Pseudomonas* and *B. cinerea*.**

All bacterial isolates were transferred from a single colony on KMB agar to 3 ml KMB broth and incubated (180 rpm) at 28 °C for 16 hr. Bacterial suspensions (0.5 ml), mixed with PDA (50 ml; 12 g PDA and 8 g agar liter^{-1}) at 42 °C, were added to Petri dishes (15 cm) and incubated in the dark at 25 °C for 10 days. Petri dishes (15 cm) containing half strength MEA (50 ml; 25 g MEA and 6 g agar liter^{-1}) mixed with fungal spores (5×10^4 spores ml^{-1}) at 42 °C were prepared on a horizontal surface. After solidification, agar plugs (diameter 18 mm) from plates with bacteria were placed on the plates containing fungal spores. After incubation at 19 °C in the dark for 3 days, the radius of the inhibition zone surrounding the bacterial plugs was measured. Tests were performed in quadruplicate and repeated 3 times.

***In situ* interactions between *Pseudomonas* and *B. cinerea*.**

The effect of phenazine-producing *Pseudomonas* strains on infection of tomato leaves by *B. cinerea* was tested using a standardised assay that allows comparison of two *B. cinerea* strains per leaf (Benito *et al.* 1998). Bacteria were spread on KMB agar, incubated at 25 °C for 2 days, washed and resuspended in sodium phosphate buffer (10 mM, pH 6.4) to an $OD_{600\ nm}$ of 1.0. Leaves were cut from 5-7-week old tomato plants (cv. Moneymaker-*Cf4*), placed in wet florist foam and incubated in plastic boxes. Leaves (four per treatment) were sprayed with suspensions of phenazine-producing strains PCL1391 or its phenazine-deficient mutant PCL1119 till run-off, using a DeVilbiss sprayer. The phenazine-producing strain Phz24 was included in a replicate experiment. Control leaves were sprayed with buffer. Leaves were incubated in boxes with closed lids for 26 hr at 15 °C, opened and dried prior to inoculation with *B. cinerea*. For each treatment, 24 leaflets were inoculated on the upper side with droplets (2 µl) of a conidial suspension (10^6 ml^{-1}) in Gamborg's B5 medium (Duchefa, Haarlem, The Netherlands) supplemented with 1% glucose and 10 mM ammonium phosphate (pH 6.5). The left half of each leaflet was inoculated with 5 droplets of B05.10 and the right half with 5 droplets of ΔBcatrB4. Boxes were closed and incubated at 15 °C in the dark and the diameter and growth of the lesions were determined after 3 and 4 days.

Quantification of phenazine antibiotics by HPLC.

In vitro and *in situ* phenazine production levels were determined by HPLC (C_{18} reverse phase 5 µm, 3.9×150 mm column, 616 pump, 600S controller, 996 photodiode array detector, Waters, Milford, MA) (Bonsall *et al.* 1997). Production levels in the *in vitro* assays were determined by extraction of the inhibition zone surrounding the bacterial plugs. *In situ* production levels of PCN were determined 5 days after application of bacterial strains to tomato leaves. Approximately five grams

of leaf fresh weight was extracted with 80% acetone, filtered, extracted twice with ethylacetate plus 0.1% TFA, air-dried and resuspended in 35% acetonitrile plus 0.1% TFA (Bonsall *et al.* 1997). For each treatment, two replicates were used. For quantification of phenazine production, a five-point standard curve generated by spiking known concentrations of PCN was used. A highly significant linear relationship was found for the standard curve (PCN=0.000125xA (r^2=0.99, P<0.0001), in which PCN represents the total amount of PCN (ng), and A represents the area of the PCN peak at 248 nm).

Statistical analysis.

Differences between treatments were determined by ANOVA followed by Duncan's multiple range test ($\alpha = 0.05$) (SAS Institute Inc., Cary, N.C.).

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Chapter 10

General discussion

ABUNDANCE OF ABC TRANSPORTER GENES IN *BOTRYTIS CINEREA*

This thesis describes the cloning, sequencing and functional analysis of the ABC transporter genes *BcatrB* and *BcatrD* from *Botrytis cinerea* with emphasis on their role in resistance to fungitoxic compounds and pathogenesis. Other ABC transporter genes identified in this pathogen are *BcatrA* (Del Sorbo *et al.*; Schoonbeek *et al.* 2001), *BcatrC*, *BcatrE-BcatrN* (Vermeulen *et al.* 2001), *BcatrO* (Accession number AF259075, G. Del Sorbo, unpublished data), *BMR1, BMR3*, and *BMR5* (Nakajima *et al.* 2001). *BcatrK* appears to be identical to *BMR1* and *BcatrC* to *BMR5*. Hence, the number of ABC transporter genes identified in *B. cinerea* amounts to 16. The actual number of ABC transporter genes is most likely higher since sequenced genomes of fungi revealed up to 58 ABC transporter genes (Table 1). In a preliminary analysis of a non-public genome sequencing project (Catlett *et al.* 2003), the number of ABC transporter genes in *B. cinerea* has been estimated to be 46 (Yoder and Turgeon 2001). This indicates that *B. cinerea* also possesses a large family of ABC transporter genes.

Table 1. Estimated numbers of ABC transporters in databases of fungal genomes.

Fungus	Number	Source: literature or website
Ashbya gossypii	17	Yoder and Turgeon, 2001
Aspergillus nidulans	29	Yoder and Turgeon, 2001
Aspergillus nidulans	49	http://www.broad.mit.edu/annotation/fungi/aspergillus/
Botrytis cinerea	46	Yoder and Turgeon, 2001
Cochliobolus sp.	51	Yoder and Turgeon, 2001
Fusarium sp.	54	Yoder and Turgeon, 2001
Fusarium graminearum	58	http://www.broad.mit.edu/annotation/fungi/fusarium
Magnaporthe grisae	35	http://www.broad.mit.edu/annotation/fungi/magnaporthe
Neurospora crassa	39	Yoder and Turgeon, 2001
Neurospora crassa	34	http://www.broad.mit.edu/annotation/fungi/neurospora
Saccharomyces cerevisiae	29	Decottignies and Goffeau, 1994

RELATION BETWEEN SEQUENCE HOMOLOGY AND PHYSIOLOGICAL FUNCTION

The 29 ABC transporters derived from the sequence of the genome of *Saccharomyces cerevisiae* can be classified in six clusters, including ten subclusters of distinct predicted topology and presumed function (Decottignies and Goffeau 1997). ABC transporters from *B. cinerea* and other filamentous fungi can be classified in a similar way (Del Sorbo *et al.* 2000; Stergiopoulos *et al.* 2002b). BcatrB and BcatrD from *B. cinerea* belong to the pleiotropic drug resistance (PDR)

transporters, characterised by a $(NBF\text{-}TMD_6)_2$ topology. The majority of ABC transporters from filamentous fungi described have this topology, or the reverse $(TMD_6\text{-}NBF)_2$ one. Transporters that belong to the latter group are described as MDR transporters, named after MDR1, the first transporter identified in human tumor cells (Ambudkar 1995). The phylogenetic relation between ABC transporters with the PDR topology is presented in a dendrogram (Fig. 1). The tree has 4-5 branches that may represent related clusters of fungal ABC transporters. ABC transporters from *B. cinerea* are present in different branches of the tree, indicating diversity of ABC proteins within one organism. From an evolutionary point of view, this might imply that the formation of different subclasses of ABC transporter occurred before speciation of fungi. Several ABC transporters involved in resistance to azole fungicides are clustered, such as AtrG, BcatrD and PdPMR1 from filamentous fungi and CaCDR1, CaCDR2, CgCDR1, and ScPDR5 from yeast species (Fig.1). It is, therefore, tempting to assume that ABC transporters with high primary sequence homology have similar physiological functions.

However, transporters with similar functions may also occur in different branches of the dendogram. This is the case for multidrug transporters, such as AtrB, BcatrB and PdPMR5 from filamentous fungi, and ScPDR5 and ScSNQ2 from *S. cerevisiae* which form two clusters on separate branches. Similarly, the ABC transporters BcatrB from *B. cinerea,* MagABC1 from *Magnaporthe grisea*, Mgatr4 from *Mycosphaerella graminicola,* and GpABC1 from *Gibberella pulicaris* are all involved in virulence on host plants (Fleissner *et al.* 2002; Schoonbeek *et al.* 2001; Stergiopoulos *et al.* 2003; Urban *et al.* 1999), but only MagABC1 and GpABC1 have a close phylogenetic relation (Fig.1).

PDR transporters from plant origin, AtPDR2, NpABC1 and NtPDR1, form a separate branch in the dendrogram (Fig. 1). This indicates that homologous ABC transporters from higher eukaryotes are more related to one another than to fungal ABC transporters. Plant ABC transporters can still have functions comparable to those from fungi, since they can be involved in transport of plant defense compounds or phytotoxins produced by pathogens. NpABC1 and NtPDR1 from *Nicotiana plumbaginifolia* and *Nicotiana tabacum*, respectively, appear to be involved in self-protection against secondary metabolites (Jasinski *et al.* 2001; Sasabe *et al.* 2002; Van Den Brule and Smart 2002). In conclusion, homology between ABC transporters could imply a similar function but one should realise that this statement is not generally valid.

REDUNDANCY OF TRANSPORTERS WITH SIMILAR FUNCTIONS

The presence of ABC transporters within an organism with a similar function may imply that loss of function of one particular transporter is compensated for by activity of a functional homologue. Consequently, single knock-out mutants of ABC transporter genes do not necessarily show a phenotype. Indeed, such a redundancy has been found for PDR5, YOR1 and SNQ2 from *S. cerevisiae*, (Balzi and Goffeau 1995; Kolaczkowski et al. 1998), and for CDR1 and CDR2 from *Candida albicans*, all involved in resistance to antimycotics (Krishnamurthy et al. 1998; Prasad et al. 1995; Sanglard et al. 1997).

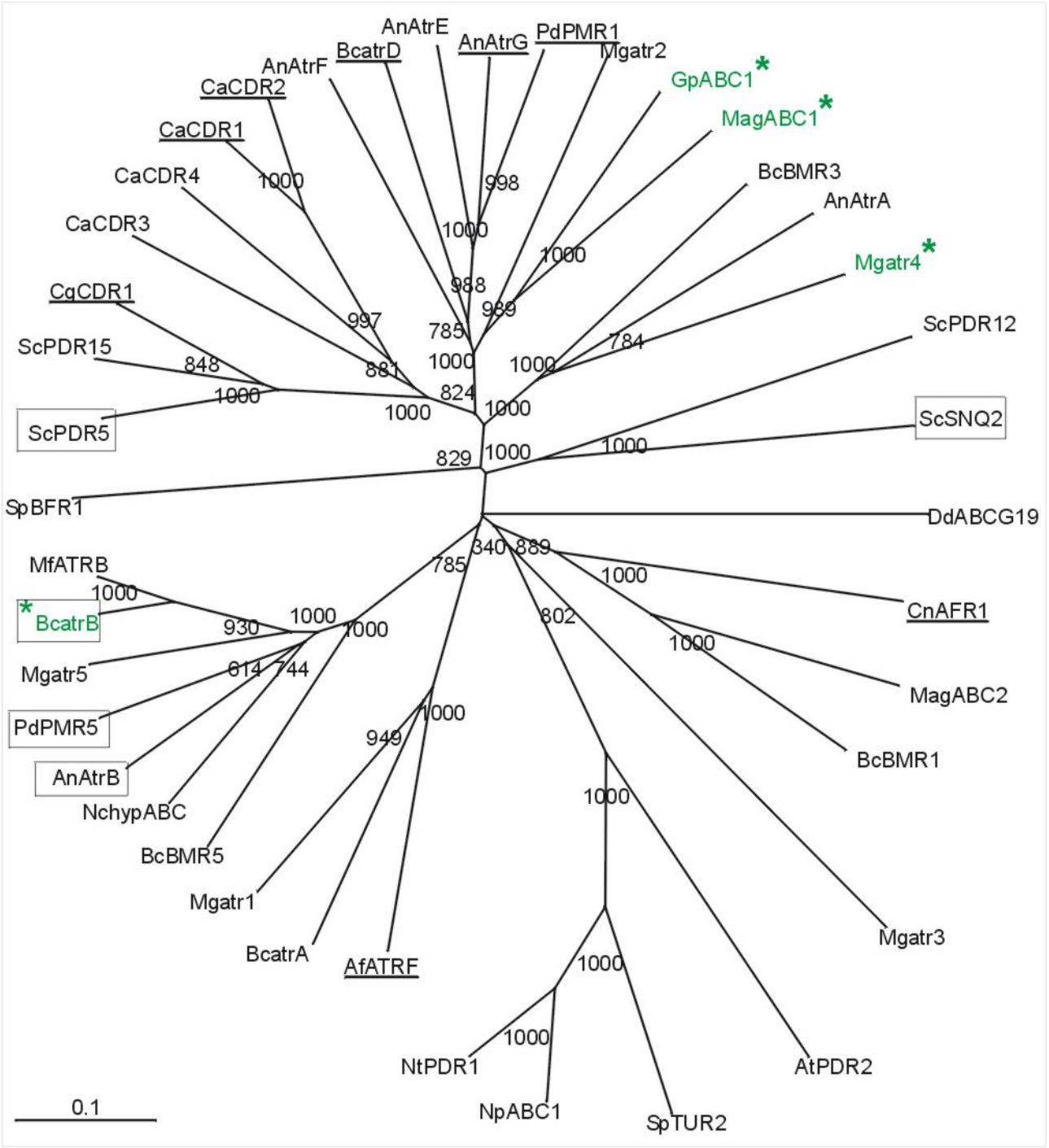

Figure 1. Unrooted phylogenetic tree, displaying the homology between ABC transporters with the PDR topology from filamentous fungi, and representative homologues from yeasts, plants, and a slime mould. Proteins with a designated function are underlined for a role in azole fungicide resistance, boxed for a role in multidrug resistance, and marked with an asterisk for a role in pathogenesis. Protein sequences were aligned using ClustalX v1.8 (Thompson *et al.* 1997) and TreeView (Win32 v1.6.6 by Roderic D. M. Page, 2001). Numbers at the nodes indicate the trustworthiness of the branches in the tree as determined by 1000-fold bootstrap analysis. Sequences are denoted with a short prefix indicating the species. Fungi: Cn, *Cryptococcus neoformans*; Af, *Aspergillus fumigatus*; An, *Aspergillus nidulans*; Bc, *Botrytis cinerea*; Gp, *Gibberella pulicaris*; Mag, *Magnaporthe grisea*; Mf, *Monilinia fructicola*; Mg *Mycosphaerella graminicola*; Nc, *Neurospora crassa*; Pd, *Penicillium digitatum*. Yeasts: Ca, *Candida albicans*; Cg, *Candida glabrata*; Sc, *Saccharomyces cerevisiae*; Sp, *Schizosaccharomyces pombe*. Plants: At, *Arabidopsis thaliana*; Np, *Nicotiana plumbaginifolia*; Nt, *Nicotiana tabacum*. Slime mould: Dd, *Dictyostelium discoideum*.

Redundancy in substrate specificity may also be true for transporters with a relatively low degree of primary homology. This may account for the different ABC transporters from *M. graminicola* involved in transport of azole fungicides (Zwiers et al. 2003). In *S. cerevisiae*, the redundancy in substrate specificity of ABC transporters even extends to MFS transporters, as demonstrated by the phenotype of multiple gene knock-out mutants of ABC and MFS transporter genes (Balzi and Goffeau 1995; Kolaczkowski et al. 1998; Mahé et al. 1996; Michalkova-Papajova et al. 2000; Rogers et al. 2001). We have demonstrated a similar redundancy in *B. cinerea* where both BcatrD and Bcmfs1 are capable of transporting azole fungicides and loss of Bcmfs1 can be compensated for by BcatrD activity (Hayashi et al. 2002a; Hayashi et al. 2002b). Redundancy of substrate specificity of transporters is probably essential for survival of fungi exposed to various toxic compounds. It might be possible that functionally related transporters are active under different physiological conditions. Similar observations have been made for members of the gene family of endopolygalacturonases of *B. cinerea*, which are induced under different environmental conditions (Manteau et al. 2003; Ten Have et al. 2001; Wubben et al. 2000).

EXPRESSION ANALYSIS OF ABC AND MFS TRANSPORTER GENES

Expression of fourteen ABC and three MFS transporter genes in *B. cinerea* was studied in Northern blot expression analysis experiments to select genes that are upregulated after treatment with toxic compounds. Short treatments of mycelial suspensions with compounds of different chemical classes showed distinct responses of these genes, depending on the incubation time, the concentration of the inducing compound and the genetic background of the *B. cinerea* strain used (Chapters 4, 5, 6, and 7).

In the wild-type *B. cinerea* strain B05.10, most genes were only induced by a limited number of compounds, often with similar structures. In contrast, *BcatrB* was induced by a broad variety of compounds. The nature of these inducers of gene expression can help to unravel the physiological function of BcatrB since inducers may also be a substrate. Strong inducers are the phenylpyrrole fungicides fludioxonil and fenpiclonil (Chapters 3 and 4), and phenazine antibiotics (Chapter 8), BcatrB is indeed involved in protection against these compounds. Similar results have been described for azole fungicides that induce expression of the genes coding for CDR1 and CDR2 in *C. albicans* (Krishnamurthy *et al.* 1998; Nakamura *et al.* 2001; Sanglard *et al.* 1997), for several fungitoxic compounds that induce *AtrB* in *A. nidulans* (Andrade *et al.* 2000), and for 4-nitroquinoline-N-oxide that induces *SNQ2* in *S. cerevisiae* (Mahé *et al.* 1996). PMR1 and PMR5 are ABC transporters from *P. digitatum* with high homology to BcatrD and BcatrB, respectively (Nakaune *et al.* 2002). *PMR1* expression is induced by treatment with fungitoxic compounds, such as azole fungicides, phloretin (the phytoanticipin of apples), camptothecin (an alkaloid) and oligomycin (an antibiotic). *PMR1* disruption mutants displayed increased sensitivity to several of these inducing compounds. *PMR5* is induced by several other compounds, such as thiabendazole and benomyl (benzimidazoles), dithianon (a quinone), resveratrol (the phytoalexin of grape) and camptothecin and PMR5 disruption mutants displayed increased sensitivity to these compounds.

Despite these numerous data, knock-out mutants of ABC transporter genes do not necessarily show a phenotype upon exposure to inducing compounds. This is, for instance, the case for azole fungicides that induce expression of *BcatrB* (Chapter 3, 4, and 5). This phenomenon can relate to aspecificity in activation of signalling pathways leading to transcription of transporter genes or to redundancy of transporters with an overlap in substrate specificity.

Most compounds induce expression of ABC or MFS transporter genes within 20 minutes of exposure. In the same timespan a rapid induction of efflux activity is observed (Chapters 4, 5 and 6). These observations suggest that transporters operate as a molecular first aid kit. The prompt response may provide the fungus time to escape the immediate effect of fungitoxic compounds and to develop additional detoxification mechanisms. These may include compartmentalisation, storage, enzymatic modification, or catabolic degradation.

BCATRD

The basal expression level of *BcatrD* in wild-type *B. cinerea* strains is low but can be weakly induced by eugenol and iprodione and strongly by 2,4-diacetylphloroglucinol (DAPG), cycloheximide and azole fungicides (Chapter 4, 5, 6, and 7). Basal expression levels in azole-resistant mutants, G25 and G66, are higher than in the azole-sensitive parent strain B3 (Chapter 5) and can be induced by the same compounds at higher concentrations, indicating that regulation of *BcatrD* expression is altered in these mutants. The expression level of *BcatrB* and other ABC transporter genes in these mutants is not elevated. The timing of maximal *BcatrD* expression in wild-type strains coincides with a decrease in accumulation of the azole fungicide oxpoconazole. This suggests that increased expression levels are positively correlated with increased efflux of the fungicide leading to resistance. Expression levels in wild-type and azole-resistant isolates is negatively correlated with the accumulation levels of oxpoconazole, indicating that efflux activity is involved in sensitivity and resistance. Further evidence for a role of BcatrD in azole efflux was obtained from gene expression and oxpoconazole accumulation studies with B05.10 and *BcatrD* gene-replacement and overexpression mutants. The relations found between the expression level of *BcatrD* in these mutants, the accumulation levels of oxpoconazole and azole sensitivity clearly indicate that BcatrD can be regarded as the main transporter of azole fungicides in *B. cinerea* (Hayashi *et al.* 2001; Hayashi *et al.* 2002a; Hayashi *et al.* 2002b).

BcatrD seems to belong to a subcluster of ABC transporters with relatively high substrate specificity. Its closest homologues are AtrE, AtrF, and AtrG from *A. nidulans* and PMR1 from *P. digitatum* (Fig. 1), which also play a role in protection against azole fungicides in azole-resistant mutants (Andrade 2000; Hayashi *et al.* 2002a; Nakaune *et al.* 1998). Expression of *AtrG, BcatrD,* and *PMR1* is induced by azoles in wild-type strains but, to a higher extent in azole-resistant mutants of these fungi (Andrade 2000; Hamamoto *et al.* 2001; Hayashi *et al.* 2001). All three proteins contribute to azole resistance by providing active efflux and seem to possess a narrow substrate specificity. Examples of functional homologues of BcatrD from *C. albicans* are CDR1 and CDR2 (Sanglard *et al.* 1997), but these proteins show lower primary homology (Fig. 1).

BCATRB

Expression of *BcatrB* in wild-type strains of *B. cinerea* is induced by a wide variety of compounds, including fungicides, plant defence compounds and microbial fungitoxic metabolites (Table 2). Induction can occur within a time span of 20 min and expression levels often decrease after longer exposure of fungal cell to these toxicants. Fludioxonil already induces *BcatrB* expression within 5 min, indicating that BcatrB, like BcatrD, may act as a molecular emergency kit, but with a much wider substrate range. BcatrB also provides protection against the natural compounds eugenol, resveratrol and phenazine-1-carboxylic acid (Chapter 3, 6 and 7).

BcatrB gene-replacement mutants have an increased sensitivity to several of the inducers tested, especially phenylpyrroles, resveratrol, eugenol and phenazines. However, this was not the case for azole fungicides, cycloheximide, pisatin and rishitin, indicating that BcatrB is not the major transporter of these compounds, despite their inducing activity (Chapter 2, 3 and 4). *BcatrB* overexpressing mutants CH1.7 and CH1.8 are less sensitive to fludioxonil and display relatively low accumulation levels of fludioxonil (Chapter 4). An obvious correlation between expression level of *BcatrB*, accumulation level of fludioxonil and sensitivity to phenylpyrrole fungicides was observed. These observations indicate that BcatrB is a true multidrug transporter, providing protection to a range of chemically unrelated compounds. It would be interesting to know the substrate range of PDR transporters that show a partial overlap in expression pattern, like BcatrG, which is also induced by resveratrol and fludioxonil, and BcatrC (BMR5), which is the closest homologue of BcatrB.

BcatrB has high homology (Fig. 1) with AtrB from *A. nidulans* (Del Sorbo *et al.* 1997), MfATRB from *Monilinia fructicola* (Schnabel *et al.* 2003), Mgatr5 from *M. graminicola* (Stergiopoulos *et al.* 2002a), and PMR5 from *P. digitatum* (Nakaune *et al.* 2002). Some members of this group are also multidrug transporters. The genes show distinct, but overlapping expression profiles and inducing compounds include cycloheximide, eugenol, resveratrol and phenylpyrrole fungicides (Andrade 2000; Schoonbeek *et al.* 2002; Stergiopoulos *et al.* 2002a; Vermeulen *et al.* 2001; Zwiers 2002). Both BcatrB and AtrB provide protection to resveratrol, phenazine antibiotics and phenylpyrrole fungicides, indicating that the transporters have a high functional homology (Andrade 2000; Andrade *et al.* 2000). Both BcatrB and PMR5 are involved in protection to fungicides and the plant defence compound resveratrol (Nakaune *et al.* 2002). Mgatr5 is involved in protection against the plant defence compounds resorcinol and resveratrol (Zwiers *et al.* 2003), but a role in virulence on wheat could not be established (Stergiopoulos *et al.* 2003). In the cluster of BcatrB homologs, BcatrB itself is the only transporter involved in virulence (Schoonbeek *et al.* 2001).

We conclude that BcatrB and its homologues are true multidrug transporters that provide protection against a wide range of natural and xenobiotic fungitoxic compounds, a function that might be fine-tuned for specific requirements in a particular fungus.

Table 2. Inducers of the ABC transporter gene *BcatrB* from *B. cinerea*.

Type of compound	Induction time (min)	Substrate	Chapter
Fungicides			
Cyprodinil (anilinopyrimidines)	15	No	4
Fenpiclonil and fludioxonil (phenylpyrroles)	15	Yes	3 and 4
Iprodione (dicarboximides)	15	No	4
Tebuconazole and oxpoconazole (azoles)	15	No	3, 4 and 5
Trifloxystrobin (strobilurins)	60	No	4
Plant defence compounds			
Camptothecin (alkaloids)	20	No	6
Eugenol (phenylpropenes/monoterpenes)	20	Yes	6
Pisatin (isoflavonoids)	60	No	3 and 6
Psoralen (furanocoumarins)	20	No	6
Resveratrol (stilbenes)	20	Yes	3 and 6
Rishitin (sesquiterpenes)	60	No	6
Microbial antibiotics			
Cycloheximide (glutarimides)	60	No	3 and 6
DAPG[1)] (polyketides)	20-60	No	7
PCA and PCN[2)] (phenazines)	20	Yes	7

[1)] DAPG = 2,4-diacetylphloroglucinol

[2)] PCA = phenazine-1-carboxylic acid and PCN = phenazine-1-carboxamide

ABC TRANSPORTERS AND VIRULENCE

BcatrB contributes to virulence of *B. cinerea* on grapevine by providing protection against resveratrol (Chapter 3). The protection is based on active efflux, preventing accumulation in fungal cells. This observation may imply that variation in sensitivity of *B. cinerea* to resveratrol and its derivatives like pterostilbene and ε-viniferin (Langcake and Pryce 1976; Pont and Pezet 1990)

relates to differences in efflux efficiency of BcatrB for these compounds. In contrast, BcatrB is not required for virulence of *B. cinerea* on pea and basil, which contain the plant defence compounds pisatin and eugenol, respectively. Both compounds induce expression and may even be substrates of BcatrB (Chapter 6 and 8). This discrepancy might be due to the possibility that the host plants used in our tests did not contain these products at concentrations required for inhibition of the pathogen. Alternative explanations are redundancy among transporters and the presence of detoxification mechanisms, comparable to the enzymatic degradation of wyerone and α-tomatin (Hargreaves *et al.* 1976; Lyon 1976; Quidde *et al.* 1998). Laccase activity in *B. cinerea* conidia can inactivate resveratrol (Breuil *et al.* 1998). However, a laccase that is active in mycelium of *B. cinerea* strain B05.10 converts resveratrol to isomers with increased fungitoxicity (Schouten *et al.* 2002). It might be that BcatrB transports one of these compounds and not only resveratrol (Chapter 6).

A role of ABC transporters in virulence has also been described for the ABC transporters GpABC1, MagABC1, and Mgatr4, from *G. pulicaris*, *M. grisae* and *M. graminicola*, respectively. GpABC1 and MagABC1 are PDR-like proteins closely related to each other (Fig. 1), but only distantly related to BcatrB. Disruption of MagABC1 or GpABC1 has a dramatic effect on virulence of the pathogen on rice and potato, respectively (Fleissner *et al.* 2002; Urban *et al.* 1999). *MagABC1* expression in *M. grisea* is upregulated by exposure to rice phytoalexins but the mutants do not display increased sensitivity to these compounds *in vitro*. Hence, the mechanism by which MagABC1 acts as a virulence factor is not known. *GpABC1* from *G. pulicaris* is induced by the phytoalexin rishitin and disruption of this gene leads to increased sensitivity to rishitin *in vitro* (Fleissner *et al.* 2002). For this reason, it is assumed that the reduced virulence of disruption mutants is due to increased sensitivity of the mutants to rishitin present in potato tubers. Although GpABC1 and MagABC1 are closely related to Mgatr2 and more distantly to Mgatr4, only gene disruption mutants of Mgatr4 of *M. graminicola* have a reduced virulence on several wheat cultivars (Stergiopoulos *et al.* 2003). This is manifested as a reduced colonisation of substomatal cavities. A correlation between *Mgatr4* disruption and sensitivity to plant defense compounds or production of phytotoxins could not be demonstrated. These results imply that only for BcatrB and GpABC1 a clear function in virulence through protection against plant defense compounds has been demonstrated.

ABC TRANSPORTERS AND COMPETITION

ABC transporters could also provide protection against natural toxic products that are relevant during saprophytism. This is especially important for *B. cinerea* since the saprophytic growth phase is important in its life cycle. Saprophytic growth of *B. cinerea* occurs on crop residues and other organic material. These ecological niches are also colonized by microorganisms that could antagonise growth of *B. cinerea*. This situation might imply that during evolution *B. cinerea* has acquired membrane transporters that provide protection against antibiotics produced by antagonistic microorganisms. Similarly, ABC transporters may provide protection against antagonistic metabolites during pre-infectional growth on the leaf surface. The validity of the latter hypothesis is

supported by our findings that BcatrB is a strong determinant of the *in vitro* and *in planta* sensitivity of *B. cinerea* to phenazine antibiotics produced by *Pseudomonas* and *Burkholderia* species (chapter 7). These results suggest that BcatrB can also provide protection against antibiotics produced by competing micro-organisms that co-colonise the infection site, as described for NorM from *Erwinia amylovora* (Burse *et al.* 2004). Similar roles in antibiosis during saprophytism or pathogenesis might be attributed to AtrB and Mgatr2 from *A. nidulans* and *M. graminicola*, respectively (Andrade 2000; Levy *et al.* 1992; Zwiers *et al.* 2003). Therefore, we suggest that the relevance of ABC transporters in protection against natural toxic compounds has a severe impact on microbial ecology (Duffy *et al.* 2003).

The observations that ABC transporters can provide protection against antibiotic compounds may also have implications for biocontrol efficacy (Duffy *et al.* 2003; Whipps 2001). Low biocontrol reproducibility is often ascribed to variability in climate or weather conditions and to variability of the biocontrol agents. However, our data indicate that insufficient biocontrol efficacy may also be due to inducible mechanisms in target organisms that protect them against antimicrobial compounds produced by biocontrol agents (Glandorf *et al.* 2001; Jones and Pettit 1987; Li and Leifert 1994; Mazzola *et al.* 1995; Stockwell *et al.* 1996). For that reason, we recommend that research on the role of ABC transporters in defence of plant pathogens against biocontrol agents should be intensified.

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Summary

Botrytis cinerea is the causal agent of grey mould disease on a wide variety of crops. It is a serious pathogenic fungus that is difficult to control since resistant plant are hardly available and it readily develops resistance to fungicides of various chemical classes. The life cycle of the pathogen, mechanisms of pathogenicity, disease control and fungicide resistance are described in Chapter 1.

Chapter 2 describes how active efflux systems with low specificity can contribute to decreased sensitivity of *B. cinerea* to various fungitoxic compounds. The major representatives of these export systems are proteins belonging to the ATP-binding cassette (ABC) and major facilitator superfamily (MFS) transporters. These proteins can actively export fungitoxic compounds from the cytoplasm and lower the intracellular concentration to non-toxic levels. The energy required for this transport is either derived directly from hydrolysis of ATP by ABC transporters, or indirectly, from the proton-motive force, by MFS transporters. In *Saccharomyces cerevisiae,* several transporter proteins involved in pleiotropic drug resistance have been described. However, reports on ABC transporters from filamentous fungi are limited.

The aim of this study was to investigate how transporters from *B. cinerea* protect this fungus against natural and synthetic fungitoxic compounds. A fragment of the well described ABC transporter gene PDR5 from *S. cerevisiae* was used to screen a genomic library of *B. cinerea* for homologous sequences. This screening revealed two candidate ABC transporter genes, *BcatrA* and *BcatrB*. Chapter 3 describes the cloning and initial characterisation of *BcatrB*. This gene indeed encodes a PDR-like ABC transporter, which consists of 1439 amino acids. Expression of *BcatrB* is upregulated upon incubation with various fungitoxic compounds. Gene-replacement mutants display increased sensitivity to the phenylpyrrole fungicide fenpiclonil and the grapevine phytoalexin resveratrol, implying a role in both fungicide resistance and virulence.

Twelve ABC and three MFS genes were found in a library of expressed sequence tags (ESTs), containing 6000 clones of cDNA from mycelium of a *B. cinerea* strain grown under nitrogen-starvation. Chapter 4 describes the cloning of the corresponding genomic fragments from strain B05.10. The expression of *BcatrA*, *BcatrB*, and the transporter genes from the EST library was analysed in untreated mycelium and in mycelium exposed to fungicides from various chemical classes. Many compounds differentially induced expression of the ABC and MFS transporter genes. Most striking results were obtained for *BcatrB*, which is strongly induced by the phenylpyrrole fungicide fludioxonil and overexpressed in the phenylpyrrole resistant strain CH1.7. *BcatrB* expression strongly correlates with the sensitivity of the strains tested and their ability to secrete the compound.

Chapter 5 and 6 describe the expression of the ABC and MFS transporter genes after treatment with azole fungicides. BcatrD is the strongest induced gene in wild-type strains B05.10 and B3 upon treatment with the azole oxpoconazole and has high basal levels of expression in the azole-resistant strains G25 and G66. Studies with these mutants indicate that BcatrD is a determinant in the sensitivity of *B. cinerea* to azole fungicides. Strains with higher expression levels

of *BcatrD* displayed reduced sensitivity and lower accumulation levels of oxpoconazole. Overexpression mutants of *BcatrD* in strain B05.10 also accumulated less oxpoconazole and displayed relatively low azole sensitivity. *BcatrD* replacement mutants in B05.10 showed the opposite phenotype.

The intrinsic role of ABC transporters during saprophytic growth and pathogenesis was studied by expression analysis of ABC and MFS transporter genes after treatment with plant defence compounds and antibiotics (Chapter 7). *BcatrB* expression in particular was induced by plant defence compounds, such as camptothecin, eugenol, psoralen, resveratrol and rishitin. *BcatrB* gene-replacement mutants showed increased sensitivity to eugenol but virulence on basil, a *B. cinerea* host plant that can produce eugenol, was not different from the wild type.

Studies on the role of BcatrA and BcatrB in virulence are described in more detail in Chapter 8. Virulence of gene-replacement mutants was compared with that of the parental strain B05.10 on *Leguminous* and *Solanaceous* host plants. No significant reduction in virulence of the mutant strains was observed. These results indicate that the multidrug exporter BcatrB is not essential for virulence on the tested host plants. The BcatrB mutants were slightly more virulent than the parental isolate on tomato (*Lycopersicon esculentum*) cv. Vollendung but not on transgenic, resveratrol-producing lines of this cultivar nor on tomato cv. Moneymaker *Cf4*. An explanation for this remarkable cultivar-dependent difference is not readily available.

Chapter 9 describes a role for ABC transporters in the protection of *B. cinerea* against antibiotics from competing microorganisms. Phenazine-1-carboxylic acid and phenazine-1-carboxamide, broad-spectrum antibiotics produced by *Pseudomonas* spp., strongly induced expression of *BcatrB*, and *BcatrB* gene-replacement mutants were significantly more sensitive to these antibiotics than their parental strain. Phenazines also interfered with the accumulation of [^{14}C]fludioxonil, a phenylpyrrole fungicide known to be transported by BcatrB, indicating that BcatrB also transports these antibiotics. *Pseudomonas* strains producing phenazines displayed a stronger antagonistic activity towards BcatrB mutants than to the parental *B. cinerea* strain. Collectively, these results indicate that fungal ABC transporters can play an important role in antibiotic-mediated interactions between bacteria and fungi in plant-associated environments, with considerable consequences for biocontrol strategies in agriculture.

In conclusion, we found that *B. cinerea* possesses several ABC and MFS transporters, which can be differentially induced by natural fungitoxic compounds and fungicides. BcatrD proved to be involved in protection against azole fungicides and BcatrB against plant defence compounds, phenylpyrrole fungicides and phenazine antibiotics. We predict that further functional analysis of MFS and ABC transporters will unravel additional physiological functions in saprophytic growth, virulence and multidrug resistance to fungicides.

Samenvatting

Botrytis cinerea veroorzaakt de grauwe schimmelziekte op een groot aantal gewassen. Het is een pathogene schimmel met een hoog aanpassingsvermogen die moeilijk te bestrijden is, vooral omdat er weinig resistentie tegen deze schimmel in genetische bronnen aanwezig is en de schimmel makkelijk resistentie ontwikkelt tegen chemisch niet-verwante fungiciden. De levenscyclus van de schimmel, pathogenese, gewasbescherming en resistentie tegen fungiciden worden in hoofdstuk 1 beschreven.

Hoofdstuk 2 beschrijft de wijze waarop actieve efflux systemen met een lage specificiteit kunnen bijdragen aan verminderde gevoeligheid van *B. cinerea* voor fungitoxische verbindingen. De belangrijkste voorbeelden van deze exportsystemen zijn eiwitten die behoren tot de superfamilies van ATP-binding cassette (ABC) en "major facilitator superfamily" (MFS) transporters. Deze eiwitten kunnen actief fungitoxische verbindingen uit het cytoplasma verwijderen en verlagen daarmee de intracellulaire concentratie tot een sublethaal niveau. De energie die benodigd is voor transport wordt door ABC transporters direct gegenereerd door hydrolyse van ATP en door MFS transporters verkregen uit de protonen gradient. Transporteiwitten met een rol in pleiotrope resistentie tegen fungitoxische verbindingen zijn vooral beschreven in *Saccharomyces cerevisiae*. Informatie over ABC transporters in filamenteuze schimmels is daarentegen zeer beperkt.

Het doel van dit onderzoek betreft een functionele analyse van transporters in *B. cinerea* bij bescherming tegen fungitoxische verbindingen van natuurlijke en synthetische oorsprong. Een fragment van het gen voor de goed gedocumenteerde ABC transporter PDR5 uit *S. cerevisiae* is gebruikt voor het opsporen van homologe sequenties in een genomische bank van *B. cinerea*. Door middel van heterologe hybridisatie zijn twee potentiële ABC transporter genen gevonden, *BcatrA* en *BcatrB*. Hoofdstuk 3 beschijft de klonering en initiële karakterisering van *BcatrB*. Het eiwit waar dit gen voor codeert is inderdaad een ABC transporter en heeft een lengte van 1439 aminozuren. De expressie van *BcatrB* werd verhoogd na incubatie van mycelium met diverse fungitoxische verbindingen. Genvervangingsmutanten vertoonden verhoogde gevoeligheid voor het fenylpyrrool fungicide fenpiclonil en het fytoalexine resveratrol uit druif, hetgeen een rol van BcatrB in zowel resistentie tegen fungiciden als in virulentie doet veronderstellen.

In een bibliotheek van "expressed sequence tags" (ESTs) met 6000 cDNA klonen van *B. cinerea*, gekweekt onder stikstof-limiterende omstandigheden, werden twaalf ABC en drie MFS genen gevonden. Hoofdstuk 4 beschrijft de klonering van de corresponderende genomische fragmenten uit isolaat B05.10. De expressie van *BcatrA*, *BcatrB* en de transporter genen ontdekt in de EST bibliotheek werd bestudeerd in onbehandeld mycelium en in mycelium blootgesteld aan fungiciden uit verschillende chemische groepen. Veel stoffen induceerden op verschillende wijze de expressie van ABC en MFS transporter genen. De meest opvallende resultaten werden gevonden voor *BcatrB*, dat sterk wordt geïnduceerd door het fenylpyrrool fungicide fludioxonil en een verhoogd expressieniveau vertoont in het fenylpyrrool-resistente isolaat CH1.7. *BcatrB* expressie

vertoonde een hoge correlatie met de gevoeligheid van de geteste isolaten en hun vermogen om deze verbinding uit te scheiden.

Hoofdstuk 5 en 6 beschrijven de expressie van de geïdentificeerde ABC en MFS transporter genen na behandeling met azool fungiciden. *BcatrD* werd het sterkst geïnduceerd in de wild-type isolaten B05.10 and B3 na behandeling met het azool fungicide oxpoconazool en heeft een hoog basaal expressie niveau in de azool-resistente isolaten G25 en G66. Studies met deze mutanten geven aan dat BcatrD een belangrijke rol speelt in de gevoeligheid van *B. cinerea* voor azool fungiciden. Isolaten die *BcatrD* sterk tot expressie brengen vertoonden verminderde gevoeligheid en lagere accumulatieniveaus voor oxpoconazole. *BcatrD*-overexpressiemutanten van isolaat B05.10 accumuleerden ook minder oxpoconazool en bezaten eveneens een verminderde gevoeligheid voor azolen. *BcatrD*-genvervangingsmutanten van B05.10 vertoonden juist het tegenovergestelde phenotype.

De natuurlijke rol van ABC transporters tijdens saprofytische groei en pathogenese werd bestudeerd door expressie-analyse van ABC en MFS transporter genen na behandeling met plantenafweerstoffen en antibiotica (Hoofdstuk 7). Vooral de expressie van *BcatrB* werd sterk geïnduceerd door plantenafweerstoffen, zoals camptothecine, eugenol, psoralen, resveratrol en rishitine. *BcatrB* genvervangingsmutanten vertoonden verhoogde gevoeligheid voor eugenol, maar de virulentie op basilicum, een waardplant van *B. cinerea* die eugenol kan produceren, was vergelijkbaar met het wild type.

Studies naar de rol van BcatrA en BcatrB in virulentie zijn verder beschreven in Hoofdstuk 8. De virulentie van genvervangingsmutanten is vergeleken met die van het moederisolaat B05.10 op waardplanten uit de families der *Leguminosae* en *Solanaceae*. Er werd geen significante vermindering in virulentie van de mutanten geconstateerd. Deze resultaten geven aan dat de multidrug transporter BcatrB niet van essentieel belang is voor de virulentie op deze waardplanten. De BcatrB genvervangingsmutanten waren wel enigszins virulenter dan B05.10 op de tomatencultivar Vollendung maar niet op transgene, resveratrol-producerende lijnen van deze cultivar noch op tomatencultivar Moneymaker *Cf4*. Een verklaring voor dit opmerkelijke cultivar-afhankelijke verschil is vooralsnog niet beschikbaar.

Hoofdstuk 9 beschrijft de rol van ABC transporters in de bescherming van *B. cinerea* tegen antibiotica van concurrerende microorganismen. Fenazine-1-carboxylzuur en fenazine-1-carboxamide, breed-spectrum antibiotica geproduceerd door *Pseudomonas* spp., bleken de expressie van *BcatrB* sterk te induceren, en BcatrB genvervangingsmutanten waren significant gevoeliger voor deze antibiotica dan de ouderstam. Fenazinen beïnvloedden ook de accumulatie van [^{14}C]fludioxonil, een fenylpyrrool fungicide dat door BcatrB wordt getransporteerd. Deze resultaten tonen aan dat fenazine antibiotica ook door BcatrB getransporteerd kunnen worden. De antagonistische activiteit van fenazine producerende *Pseudomonas* stammen was sterker tegen BcatrB genvervangingsmutanten dan tegen het *B. cinerea* moederisolaat. Al met al, geven deze resultaten aan dat ABC transporters van schimmels een belangrijke rol kunnen spelen in antibiotica-

afhankelijke interacties tussen bacterieën en schimmels in plant gerelateerde ecosystemen, hetgeen aanzienlijke consequenties kan hebben voor biologische bestrijding in land- en tuinbouw.

Samengevat, hebben we gevonden *B. cinerea* ABC en MFS transporters bezit, die op verschillende wijzen geïnduceerd worden door fungiciden en natuurlijke fungitoxische verbindingen. BcatrD blijkt betrokken te zijn bij bescherming tegen azool fungiciden en BcatrB tegen plantenafweerstoffen, fenylpyrrool fungiciden en fenazine antibiotica. We verwachten dat verdere functionele analyse van ABC en MFS transporters meer fysiologische functies bij saprofytische groei, pathogenese en multidrug resistentie zal identificeren.

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Training and supervision program completed at the Graduate School Experimental Plant Sciences

1. Participation in postgraduate courses and workshops:
   a) NIBI workshop "Project management, with writing, evaluation and presentation of a research project" (1997).
   b) EPS Autumn school "Sensible applications of reporter genes in plants" (1998).
   c) EPS Summer school "Disease resistance in plants" (1998).
   d) PhD student exchange "EPS-Julius-von-Sachs-Institut für Biowissenschaften Universität Würzburg" (Retzbach, Germany, 1999).
   e) Guide to digital scientific artwork, Genetics, Wageningen University (2001).

2. Poster presentations at international conferences:
   a) 12th International Botrytis Symposium (Wageningen, The Netherlands, 1996).
   b) 1st FEBS Advanced Lecture Course on ATP-Binding Cassette (ABC) Proteins: from Multidrug Resistance to Genetic Disease (Gosau, Austria, 1997).
   c) 12th International Symposium on Modern Fungicides and Antifungal Compounds (Reinhardsbrunn, Germany, 1998).
   d) 20th Fungal Genetics Conference (Asilomar, U.S.A., 1999).
   e) 9th Molecular Plant-Microbe Interactions Congress (Amsterdam, The Netherlands, 1999).

3. Oral and poster presentations at annual meetings:
   a) Annual SON Moleculaire genetica meetings (1996-2000).
   b) Annual EPS PhD students day (1997-2000).
   c) Annual SLW/ALW meetings (1997-2001).
   e) Willie Commelin Scholten dag (2000).
   e) EPS theme meeting (2001).

4. Participation in organisation:
   a) Representative of the Laboratory of Phytopathology in the EPS-PhD council (June 1999-December 2000).

Curriculum vitae

Henk-jan Schoonbeek werd op 27 juni 1973 geboren te Zoeterwoude-Rijndijk. Zijn jeugd heeft hij doorgebracht in Breda, alwaar hij in 1991 het V.W.O. diploma behaalde aan het Mencia de Mendoza Lyceum. Van 1991 tot en met 1996 studeerde hij Scheikunde aan de Rijksuniversiteit Leiden met als afstudeerrichting moleculaire genetica. Voor zijn afstudeerstage deed hij onderzoek naar DNA reparatie in hamstercellen (Dr. M. Tijsterman en Prof. Dr. P. van de Putte). Vanaf augustus 1996 tot augustus 2000 was hij door de Nederlandse Organisatie voor Wetenschappelijk Onderzoek (NWO-SLW) aangesteld als onderzoeker in opleiding binnen de leerstoelgroep Fytopathologie van Wageningen Universiteit. Hij heeft daar onderzoek verricht naar de rol van membraanpompen in de plantenpathogene schimmel *Botrytis cinerea*. Van september 2000 tot mei 2002 schreef hij de meeste publicaties die uit het promotieonderzoek voortkwamen en was hij op vrijwillige basis betrokken bij contractonderzoek naar de rol van membraanpompen bij gevoeligheid en resistentie van *B. cinerea* tegen fungiciden. Verder was hij van september 2001 tot mei 2002 als onderwijsassistent verantwoordelijk voor projectonderwijs. Vanaf mei 2002 is hij als pre-postdoc verbonden aan de leerstoelgroep voor Plantenbiologie (Prof. Dr. J.-P. Métraux) van de Universiteit van Fribourg (Zwitserland), alwaar hij de potentiële bijdrage van oxaalzuur-afbrekende bacteriën bij de bescherming van waardplanten tegen *B. cinerea* onderzoekt.

List of publications

Schoonbeek, H., G. Del Sorbo, and M.A. De Waard, 1999. *The role of ABC transporters in pathogenesis of* Botrytis cinerea. *In*: Modern Fungicides and Antifungal Compounds II. Eds. H. Lyr, P.E. Russell and H. Sisler. Intercept Ltd, Andover, UK, pp. 143-149.

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Cover: Conidiophores of *Botrytis cinerea* strain B05.10.