ABC and MFS transporters from *Botrytis cinerea* involved in sensitivity to fungicides and natural toxic compounds

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LIST OF ABBREVIATIONS

ABC	ATP-binding cassette
ALDP	Adrenoleukodystrophy protein
ATP	Adenosine 5'-triphosphate
BLAST	Basic local alignment search tool
CCCP	Carbonyl-cyanide *m*-chlorophenylhydrazone
CO	Carbon monooxide
CYP	Cytochrome P450
DHA	Drug-H^+ antiporter
DMI	Demethylation inhibitor
DNA	Deoxyribonucleic acid
EC_{50}	Effective concentration inhibiting growth by 50%
EMBL	European molecular biology laboratory
EST	Expressed sequence tag
GC	Gas chromatography
HC toxin	*Helminthosporium carbonum* toxin
HMG-CoA	Hydroxymethylglutaryl-Coenzyme A
LD_{50}	Lethal dose eliminating population by 50%
LSD	Least significant difference
MAO	Mono-amine oxidase
MDR	Multidrug resistance
MEA	Malt extract agar
MeOH	Methanol
MFS	Major facilitator superfamily
MIC	Minimum inhibitory concentration
MRP	Multidrug resistance-related protein
MS	Mass spectrometry
NBF	Nucleotide-binding fold
4-NQO	4-Nitroquinoline-*N*-oxide
OHT	OliC promoter-hygromycin resistance cassette-tubulin terminator
ONT	OliC promoter-nourseothricin resistance cassette–tubulin terminator
ORF	Open reading frame

PCR	Polymerase chain reaction
PDA	Potato dextrose agar
$P450_{14DM}$	Cytochrome P450 sterol 14α-demethylase
PDR	Pleiotropic drug resistance
PGR	Plant growth regulator
PR	Pathogenesis-related
RNA	Ribonucleic acid
rRNA	Ribosomal RNA
RT-PCR	Reverse transcription polymerase chain reaction
SBI	Sterol biosynthesis inhibitor
SDS	Sodium dodecyl sulphate
SHAM	Salicylhydroxamic acid
SSC	Sodium chloride-sodium citrate
TLC	Thin layer chromatography
TMD	Trans-membrane domain

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OUTLINE OF THE THESIS

ATP-binding cassette (ABC) and major facilitator superfamily (MFS) transporters are membrane proteins present in all living organisms. One of their physiological functions in fungi involves active efflux of endogenous toxins from the cytoplasm and cell membranes into the outer environment. In this way transporter proteins reduce the intracellular concentration of the toxins and may provide protection against their fungitoxic activity. Transporters can also provide protection against exogenous toxic compounds by preventing the accumulation of toxic compounds in cell membranes and at the intracellular target sites of the compounds. ABC and MFS transporters may not only provide protection against natural toxic compounds but also against synthetic toxic chemicals, such as fungicides that inhibit fungal sterol demethylation (DMIs).

The aim of this thesis is to identify ABC or MFS transporters involved in the efflux of DMI fungicides, and to discover compounds, which show synergism with DMI fungicides by modulating the activity of ABC or MFS transporters.

In Chapter 2, the new imidazole fungicide oxpoconazole, which is effective against *Botrytis cinerea* under field conditions, is introduced. We demonstrate that oxpoconazole is effective against plant pathogenic fungi belonging to *Ascomycetes* and *Deuteromycetes,* and that benzimidazole- and dicarboximide-resistant isolates do not show cross-resistance to oxpoconazole. The resistance level to oxpoconazole in DMI-resistant isolates of *Sphaerotheca fuliginea* is low as compared to other DMIs tested. We show that the mode of action of oxpoconazole is based on inhibition of P450-dependent sterol C14-demethylase activity, which is the target enzyme of DMIs.

Chapter 3 describes a screen for ABC and MFS transporter genes in an EST library of *B. cinerea* for a putative function in DMI efflux by analyzing their basal and DMI-induced expression using wild-type and two laboratory-generated DMI-resistant isolates. The ABC transporter gene *BcatrD* was selected for further studies since basal transcript levels of *BcatrD* showed a positive correlation with sensitivity to DMI fungicides in these isolates. Transcription of *BcatrD* can be induced by DMI fungicides and the induced transcription level also correlates with sensitivity of the mutants to DMIs. The MFS transporter gene *Bcmfs1* is an additional gene of interest since it can be induced by treatment with DMI fungicides.

Chapter 4 describes the validation of the function of the ABC transporter BcatrD in efflux

of DMI fungicides. To this end, phenotypes of gene replacement and overexpression mutants were studied. *BcatrD* replacement mutants have an increased and overexpression mutants a decreased sensitivity to DMI fungicides as compared to the parental isolate. We also demonstrate that the initial accumulation level of oxpoconazole correlates with the sensitivity to DMIs and with basal and oxpoconazole-induced expression levels of *BcatrD* in wild-type and mutants. The substrate range of BcatrD is limited since only DMI fungicides were shown to be substrates of the transporter out of many compounds tested. We propose that BcatrD is a determinant of sensitivity of *B. cinerea* to DMI fungicides.

In Chapter 5, the construction of replacement and overexpression mutants of the MFS transporter gene *Bcmfs1* is described. The phenotypic characterization of these mutants demonstrates that Bcmfs1 functions as a multidrug transporter. Substrates of Bcmfs1 comprise natural toxic compounds (camptothecin and cercosporin) and various systemic fungicides including DMIs. Loss of oxpoconazole efflux activity could not be demonstrated in single replacement mutants of *Bcmfs1*. However, this could be demonstrated in *BcatrD* and *Bcmfs1* double replacement mutants. Bcmfs1 may be involved in protection of *B. cinerea* against plant defence compounds during pathogenesis and against fungitoxic antimicrobial metabolites during saprophytic growth.

In Chapter 6, the effects of modulators on ABC or MFS transporter activity are described. We found that chlorpromazine and tacrolimus display synergism with oxpoconazole. Their synergistic activity is low in replacement mutants and relatively high in overexpression mutants of *BcatrD*. The synergism in overexpression mutants is ascribed to an enhancing effect of chlorpromazine and tacrolimus on accumulation of oxpoconazole in the fungus. We assume that the compounds inhibit oxpoconazole efflux by modulating BcatrD activity.

Finally, all results presented in this thesis are discussed in Chapter 7. We conclude that compounds which modulate the activity of ABC or MFS transporters show synergism with DMI fungicides in filamentous fungi. We speculate that such compounds can be useful in countermeasures to avoid or delay resistance development to DMI fungicides. ABC and MFS transporters can also be regarded as new target sites for disease control agents with an indirect action, as they may be involved in secretion of host-specific toxins or in protection against plant defence products.

Chapter 1

General introduction

The pathogen *Botrytis cinerea*

Botrytis cinerea Pers. ex Fr. is the causal agent of grey mould. The name of the asexual stage or anamorph is derived from the Greek name 'βοτρυς', which means a bunch of grapes. The name of the sexual stage or teleomorph is *Botryotinia fuckeliana* (de Bary) Whetzel (83). *B. cinerea* can produce apothecia under specific laboratory conditions (70), but the ascocarps are rarely observed under field conditions (135). Therefore, the anamorphic name *B. cinerea* is commonly used.

B. cinerea can infect at least 235 plants species, including ornamentals, vegetables, fruits, and in particular grapevine. In contrast, the host range of other *Botrytis* species is rather restricted (106). *B. cinerea* can infect host plants easily via wounds (118). Infection through natural openings (187) or directly via the cuticle has also been reported (138). To establish successful infection of healthy tissue, supplementation with nutrients is required. Successful infection requires 93 to 100% relative humidity (22). The optimal temperature for germination of *Botrytis* conidia ranges from 12 to 22°C, but germination can be observed as low as at 0 to 5°C (84). Conidia of *B. cinerea* germinate and grow under dark conditions. Light affects various stages of growth of *B. cinerea.* The production of conidia is particularly induced by near UV light (55).

B. cinerea can be observed wherever host plants are grown from subtropical areas to temperate zones. Conidia of *B. cinerea* are spread by wind, air currents in greenhouses, or insects (84). The fungus overwinters as mycelium or sclerotia in dead crop tissue and other organic debris. In spring, conidia are formed from overwintering mycelium or sclerotia and start epidemics. In case of grapevine, they may infect blossoms, colonize dead flower parts, and penetrate young grapevine berries. In the berries, the fungus may remain dormant until the fruit sugar content increases to a level that supports fungal growth. Symptoms develop easily under cool and moist conditions. Initial symptoms often look like brown water-soaked lesions. As the disease progresses, webs or masses of grey mycelium and conidia are frequently observed on dead tissue (Figure 1).

Grey mould caused by *B. cinerea* is one of the economically most important diseases on grapes since it reduces the quantity and quality of grapes (163). Rotten grapes also reduce the quality of wine due to a decrease in monosaccharides content (*e.g.* glucose, fructose), an increase of other metabolites (*e.g.* glycerol, gluconic acid), and enhanced oxidation of phenolic compounds. Under specific conditions, *B. cinerea*-infected grapes produce high quality sweet

wine such as Sauternes (France), Tokaji (Hungary), and Trockenbeerenauslese (Germany and Austria).

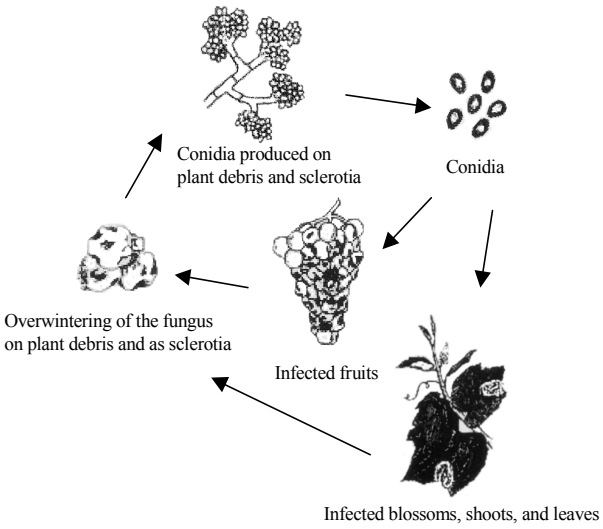

Figure 1. Infection cycle of *Botrytis cinerea* on grape vine.

Management practices to control grey mould diseases involve growing resistant varieties, cultural practices, and biological and chemical control.

Resistant varieties. Several attempts to breed plant cultivars tolerant to *B. cinerea* have been reported. Plant lines with a thick cuticle or rapidly ripening fruit might be useful in breeding programs. The role of plant genes encoding pathogenesis-related (PR) proteins such as chitinases and β-1,3 glucanases has also been investigated (184, 185). Also the involvement of cell wall degrading enzymes such as pectin methylesterase (143), pectin lyase (116), exo- and endopolygalacturonases (88), and cellulase (19) have been studied (176).

Cultural practices. The main strategies to reduce grey mould by cultural methods under green house and field conditions are, i) reduction of the humidity by ventilation, lowering of the water supply, and temperature control, ii) decrease of inoculum by removing dead, decayed, or infected materials, iii) reduction of wounding by birds, insects, fungal infection, hail, and frost, iv) reduction of crop density in order to create an unfavorable microclimate for *B. cinerea*, v) limited nutrients conditions, and vi) the use of UV films in greenhouses to prevent induction of conidia formation.

Biological control. *Aureobasidium*, *Bacillus* (56), *Gliocladium*, *Pichia* (147), *Pseudomonas* (5), *Trichoderma,* and *Ulocladium* (93) species have been investigated for biocontrol of *B. cinerea* (49). Although there are many successful reports, biological control is

not yet a reliable method for *B. cinerea* control in practice. A general drawback of biocontrol agents is that they can not maintain their control activity in crops under field conditions that favor *B. cinerea*.

Chemical control. Fungicides are the main strategy for chemical control of grey mould. In addition to fungicides, $NaHCO_3$ and $KHCO_3$ have been used in practice as plant protective agents. Although many botryticides have been introduced, their efficacy is not durable because of resistance development. For this reason, the perfect botryticide is still to be discovered.

In summary, none of the disease control strategies by themselves are sufficient for optimal grey mould control. In addition, chemical control is re-evaluated by pressure from society to reduce or eliminate pesticide input into environment (124). For theses reasons, the ultimate solution for grey mould control is probably integrated pest management (35).

Botryticides and resistance development

Several chemical classes of botryticides have been developed since the 1950s when conventional fungicides such as aromatic hydrocarbons and dithiocarbamates were introduced. These fungicides have a specific mode of action and have no systemic activity in plants. Since the 1970s, systemic fungicides with a specific mode of action such as the benzimidazole and dicarboximide fungicides were introduced. During the last decade anilinopyrimidines, fluazinam, *N*-phenylcarbamates, phenylpyrroles, and strobilurin-related compounds were introduced as botryticides. In general, fungicides that inhibit sterol biosynthesis (SBIs) have a limited efficacy against *B. cinerea* under field conditions (166, 167). Recently, fenhexamid was reported as a SBI botryticide with a new target site in sterol biosynthesis (33). *B. cinerea* developed resistance against most botryticides with a specific mode of action, especially the benzimidazoles and dicarboximides. For these reasons, fungicides from these groups are hardly used in disease control. Fungicides that represent the different classes of botryticides will be discussed and representatives of the fungicides are presented in Figure 2.

Anilinopyrimidines. This class of fungicides, introduced in the middle of the 1990s, comprises cyprodinil, mepanipyrim, and pyrimethanil. First reports of the mode of action of mepanipyrim (114) and pyrimethanil (113) indicate that the compounds inhibit the secretion of hydrolyzing enzymes such as cutinase, pectinase, and cellulase from fungal hyphae. These enzymes are required for the fungus to penetrate plant cells. Inhibition of methionine biosynthesis was also proposed as a mode of action of anilinopyrimidines (108). More

specifically, pyrimethanil seems to decrease the accumulation of methionine and to induce the accumulation of cystathionine. This observation indicates that pyrimethanil effects methionine biosynthesis by inhibition of cystathionine β–lyase (63).

Isolates with a high degree of resistance have been detected in French and Swiss vineyards (77, 101). In Switzerland, a failure of disease control was reported after a long term trial of anilinopyrimidine fungicides (61). At least three different phenotypes of anilinopyrimidine-resistant mutants were observed according to their sensitivity to other chemically unrelated fungicides, indicating the possible involvement of ABC transporters in resistance (28).

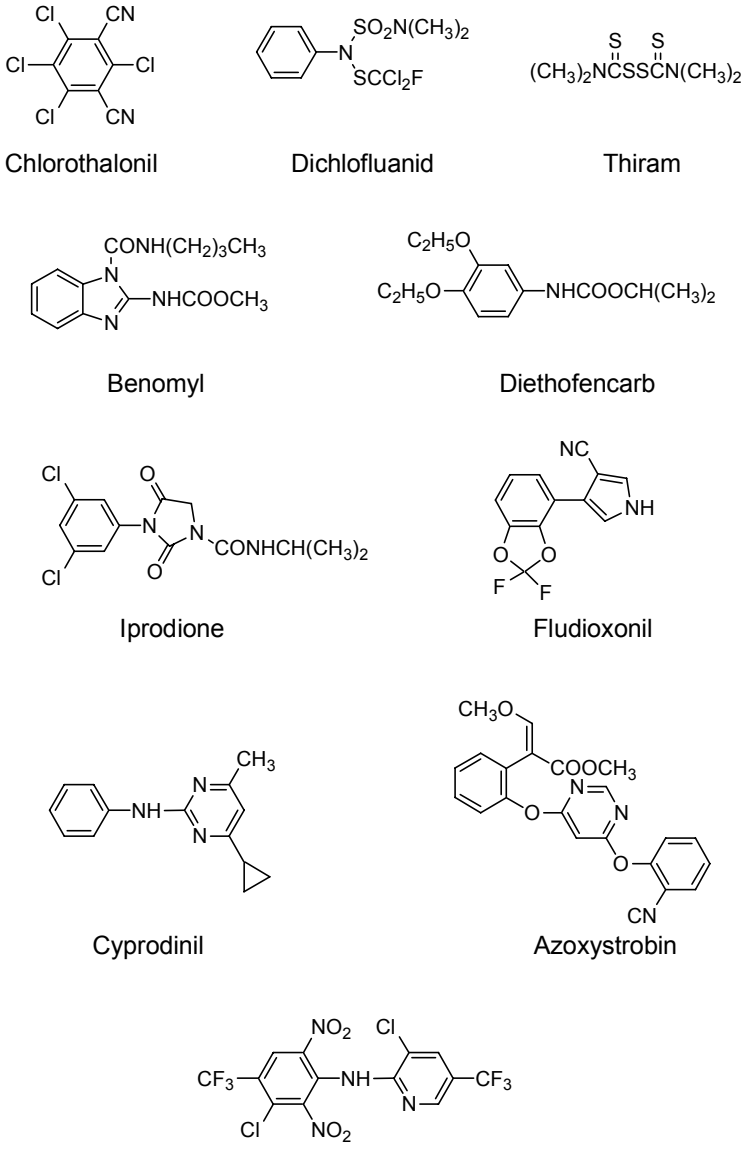

Figure 2. Chemical structures of botryticides.

Benzimidazoles. Benzimidazole fungicides were introduced in the 1960s. Representatives of this class of fungicides are benomyl, carbendazim, and thiophanate-methyl. Thiophanate-methyl is not a benzimidazole derivative by itself but it can be readily converted to carbendazim, which is the active principle of benomyl (155, 189).

The mode of action of carbendazim is based on inhibition of mitosis (73). In *Aspergillus nidulans,* benzimidazoles block mitosis in a similar way as described for colchicine in mammals and plant cells (32). The inhibition of mitosis is ascribed to complex formation between benzimidazoles and β-tubulin, which is required for microtubule formation (32).

The mechanism of resistance to benzimidazoles in *B. cinerea* can be based on a single amino acid change at position 198 of the β-tubulin protein from glutamic acid to alanine, valine or lysine (57, 81). The resistance level in isolates with such a mutation is extremely high and results in failure of disease control. Other amino acid changes at positions 200 can be responsible for relatively low resistance levels (103).

***N*-Phenylcarbamates.** *N*-Phenylcarbamates such as barban were initially known as herbicides. Barban revealed an exclusive fungitoxicity to benzimidazole-resistant strains of various fungi (99). Screening of derivatives of *N*-phenylcarbamates resulted in the discovery of compounds active only against benzimidazole-resistant strains of *B. cinerea*. Diethofencarb was developed as a commercial fungicide (172). The compound inhibits fungal mitosis as reported for benzimidazole fungicides.

Mutants of *B. cinerea* resistant to benzimidazole fungicides can become resistant to diethofencarb as well. Several phenotypes of such mutants with double resistant to diethofencarb and benzimidazoles have been reported (81). The mechanism of resistance to diethofencarb is similar as for benzimidazoles and relates to amino acid substitutions at position 198 of the β-tubulin protein.

Dicarboximides. Fungicides of this class comprise iprodione, procymidone, and vinclozolin. These fungicides have a common chemical structure, *N*-(3,5-dichlorophenyl)-dicarboximide, and a similar antifungal spectrum including a strong activity against *B. cinerea*.

The mode of action of dicarboximide fungicides is not yet fully understood. Morphological disorders of hyphae and germ tubes upon treatment with the fungicides are often observed. Antifungal activity is reversed by free radical scavengers such as α-tocopherol suggesting that the mode of action of dicarboximides may relate to lipid peroxidation (127). Glutathione

synthetase as a target enzyme of dicarboximides has been also proposed (54). Laboratory-generated dicarboximide-resistant strains show cross-resistance to aromatic hydrocarbons, tolclofos-methyl, and phenylpyrroles (100).

Resistance levels in field isolates of *B. cinerea* correlated with catalase activity suggesting that resistance to dicarboximides might relate to enhanced activity of oxidative enzymes (164). In addition molecular characterization of a dicarboximide-resistant mutant of *Ustilago maydis* revealed a gene with high homology to a gene encoding serine (threonine) protein kinase. The results suggest that protein kinase and signal transduction can play a role in resistance to dicarboximides (128). Dicarboximide-resistant field isolates of *B. cinerea* showed a point mutation in codon 365 of a histidine kinase gene, which resulted in a single amino acid change from isoleucine to asparagine, arginine, or serine (103). The encoded protein is probably involved in fungal osmoregulation (130).

Fluazinam. This fungicide has a broad antifungal spectrum against *Oomycetes* and fungi belonging to *Ascomycetes* and *Deuteromycetes* and is used to control various plant diseases such as downy mildews, late blight, scabs, and grey mould.

Its mode of action is based on uncoupling of the mitochondrial oxidative phosphorylation (2). Reactivity with sulfhydryl groups may also be relevant (1). The evolution of mutants of *B. cinerea* with a high degree of resistance to fluazinam has been reported in Japan (173).

Multi-site inhibitors. Multi-site inhibitors with activity against *B. cinerea* are aromatic hydrocarbons (*e.g.* quintozene and dicloran), dithiocarbamates (*e.g.* mancozeb and thiram), captan, chlorothalonil, and dichlofluanid. These fungicides are also described as conventional fungicides, since they are non-selective and non-systemic. The term “multi-site inhibitors” indicates that the compounds inhibit simultaneously multiple sites in fungal cells by reacting with sulfhydryl groups or other reactive substituents. The sites may include respiration, biosynthesis of proteins, lipids, and nucleic acids, formation and maintenance of cellular and mitochondrial structures.

Because of their multi-site action, resistance to multi-site inhibitors has not become a practical problem. In rare cases, resistance to dichlofluanid in *B. cinerea* has been observed. It can be mediated by at least two genes, *Dic1* and *Dic2*. The gene products are possibly involved in detoxification of dichlofluanid (136).

Phenylpyrroles. Fenpiclonil and fludioxonil are the only compounds, which belong to phenylpyrroles. They are derived from the antifungal antibiotic pyrrolnitrin. Fludioxonil is used

as a foliar fungicide and in seed treatments to control *Fusarium*, *Tilletia* and other seed-borne pathogens.

Pyrrolnitrin may inhibit energy production by uncoupling of the oxidative phosphorylation in fungal respiration (97). The uncoupling was also observed with fenpiclonil in *B. cinerea* but concentrations required for this action were much higher than those for its fungitoxicity (100). Treatment of fenpiclonil induces accumulation of the membrane potential probe tetraphenylphosphonium bromide in *F. sulphureum* suggesting that it effects membrane potential and transport processes (85). Fenpiclonil inhibited incorporation of glucose and mannose into *F. sulphureum* suggesting interference with transport-associated phosphorylation of glucose (86).

In laboratory-generated mutants resistant to phenylpyrroles, the ABC transporter gene *BcatrB* is constitutively expressed, while sensitive isolates have no basal level of expression. Accumulation of fludioxonil by the mutant was relatively low as compared to the sensitive isolates (188). Disruption of *BcatrB* increased the sensitivity to fenpiclonil (153). For these reasons the ABC transporter BcatrB is of potential importance for resistance development in practice. Mutants with a high degree of resistance to phenylpyrroles, dicarboximides, and aromatic hydrocarbons are null mutants of the *os-1* gene which encodes putative osmo-sensing histidine kinase in *Neurospora crassa* (126).

Strobilurins. Strobilurins are synthetic analogs of strobilurin A, an antifungal antibiotic produced by *Strobilurus tenacellus* (12). Azoxystrobin, kresoxim-methyl, metominostrobin, and trifloxystrobin belong to this class of fungicides.

Strobilurins inhibit the electron transfer in the cytochrome bc_1 complex (complex III) of the mitochondrial respiratory chain (6, 194). They inhibit the Qo site of the cytochrome bc_1 complex (66). Inhibitors of this respiratory pathway induce the alternative respiratory pathway also known as the cyanide-insensitive pathway. Natural antioxidants present in plants such as flavonoids and other phenolic compounds might inhibit the alternative pathway and contribute to their high efficacy under field conditions (115).

Sequence analysis of the *cyt b* gene from sensitive and resistant isolates of *Mycosphaerella fijiensis* revealed a change in amino acid 143 from glycine to alanine in resistant isolates (159). Several other mutations in the *cyt b* gene from bacteria, algae, yeasts, protozoa, and animals with single nucleotide polymorphisms are described as well (67). However, such changes were often not found in strobilurin-resistant isolates of *Venturia inaequalis*. A short-cut mechanism in

the cytochrome bc_1 complex or an upregulation of an undefined energy-producing metabolic pathway located upstream of the bc_1 complex may constitute other resistance mechanisms to strobilurins (168).

Sterol biosynthesis inhibitors (SBIs)

SBI fungicides are effective in control of plant diseases caused by *Ascomycetes*, *Basidiomycetes*, and *Fungi Imperfecti*. All SBIs lack activity against *Oomycetes* since they do not synthesize sterols and hence, lack the target site required for activity (52). Initially, SBIs became especially known for their high effectiveness against powdery mildew of cereals, fruits, and vegetables. At present, SBIs with differential selective activity against a wide range of pathogens are available. Most of the commercial SBI fungicides do not show sufficient control of diseases caused by *B. cinerea*, while they do have a high antifungal activity *in vitro*. An explanation for this discrepancy is still not available (165). SBIs with field efficacy against *B. cinerea* are fenhexamid, oxpoconazole, prochloraz, and tebuconazole.

SBIs were initially applied as inhibitors of cholesterol biosynthesis in treatments of arteriosclerosis in mammals. Cholesterol biosynthesis inhibitors were also reported to inhibit the growth of yeast and filamentous fungi (53, 110). The first SBI fungicide developed for disease control in agriculture was triarimol (141). Since that time more than 30 SBI fungicides have been developed. SBIs have also been introduced as antimycotics for control of mammalian pathogens (*e.g.* ketoconazole, miconazole).

Sterols function as important components of cell membranes and are synthesized by a conserved pathway starting from acetate (Figure 3) (112). The main sterols in mammals, plants, and fungi are cholesterol, β-sitosterol and stigmasterol, and ergosterol, respectively (Figure 4). Sterols are not only important for structural strength of cell membranes but also for maintenance of appropriate membrane fluidity, regulation of membrane permeability, and activity of membrane-bound enzymes (31, 71, 123, 183).

SBI fungicides can be categorized as inhibitors of squalene epoxidase, 14α-demethylase, Δ^{14}-reductase, and $\Delta^{8}\rightarrow\Delta^{7}$-isomerase. Recently, the hydroxyanilide fungicide fenhexamid was described as a 3-keto reductase inhibitor (33). Chemical structures of the various classes of SBIs will be discussed and are shown in Figure 5.

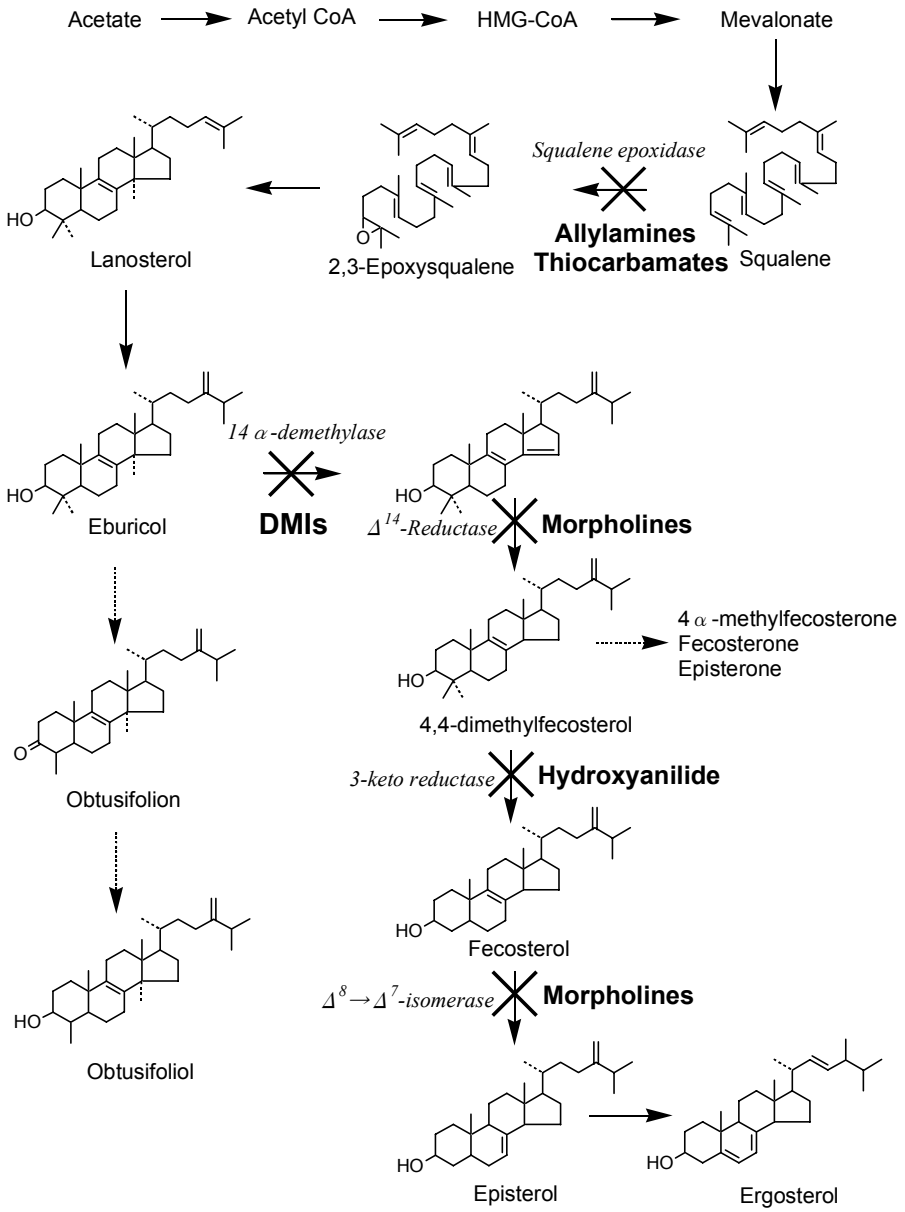

Figure 3. Ergosterol biosynthesis pathway and inhibition sites by compounds.

: normal biosynthesis pathway
: abnormal biosynthesis pathway induced by sterol biosynthesis inhibitors
: inhibition sites of sterol biosynthesis inhibitors

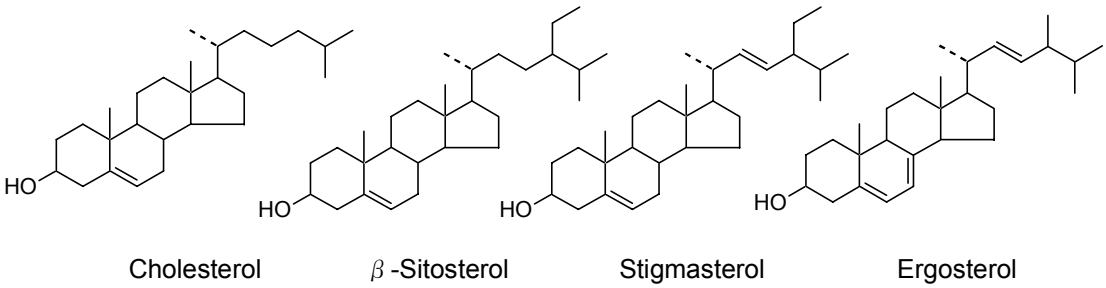

Figure 4. Common sterols in mammalians, plants, and fungi.

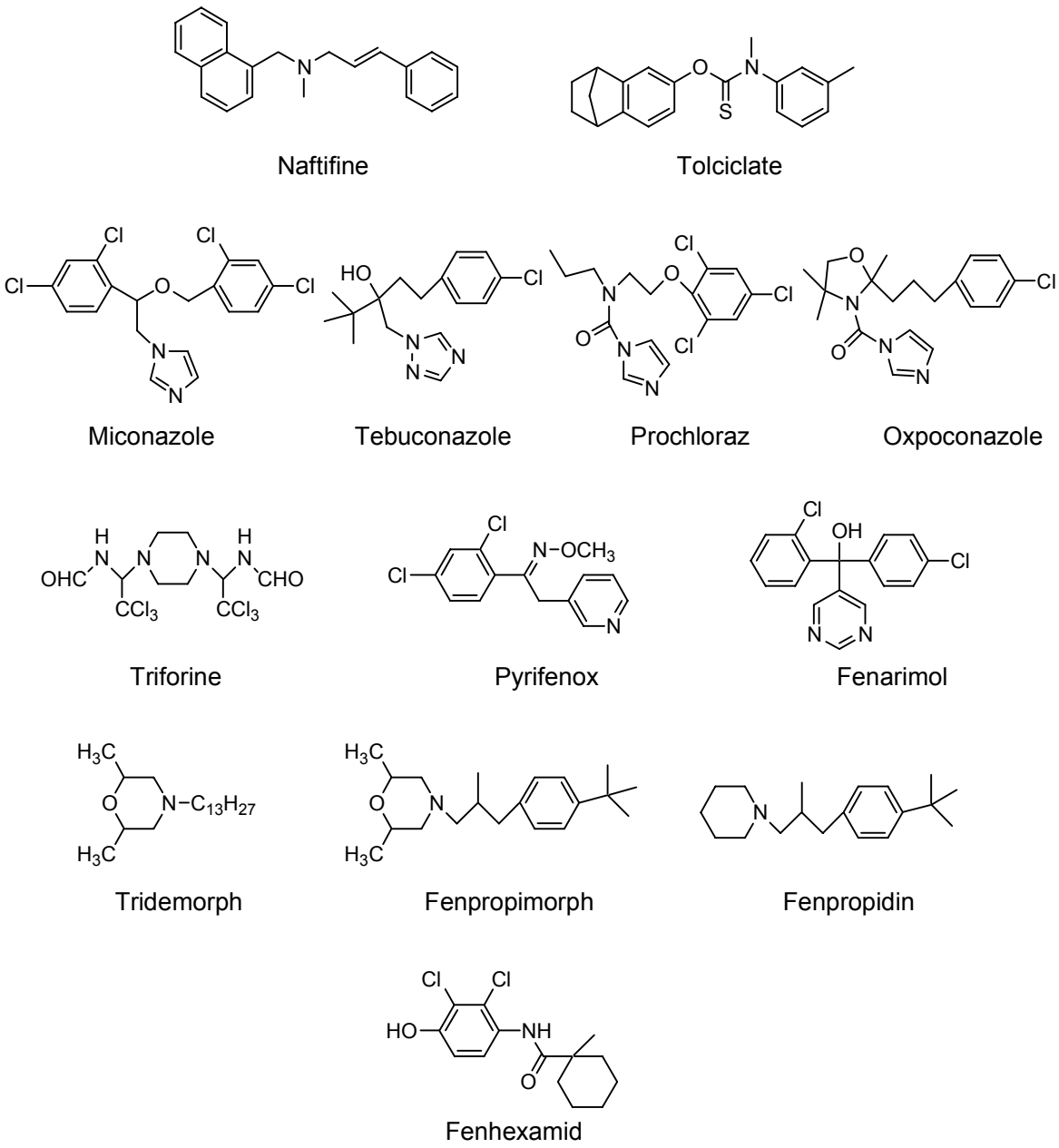

Figure 5. Chemical structures of sterol biosynthesis inhibitors.

Squalene epoxidase inhibitors. Allylamines (*e.g.* naftifine, terbinafine) and thiocarbamates (*e.g.* tolciclate, tolnaftate) inhibit the conversion of squalene into 2,3-epoxysqualene catalyzed by squalene epoxidase (Figure 3) (145). *ERG1* is the gene coding this enzyme in *Saccharomyces cerevisiae*. Deletion of this gene leads to cell death (82). Squalene epoxidase inhibitors are exclusively used as antimycotics. The selective action between mammalian and fungal squalene epoxidase is based on a relatively high affinity of the compounds to the target enzyme of mammals.

14α-Demethylase inhibitors (DMIs). This class of fungicides includes derivatives of triazoles (*e.g.* tebuconazole) and imidazoles (*e.g.* oxpoconazole and prochloraz). For this reason, these fungicides are also described as azole fungicides. DMIs also include fungicides with other heterocyclic rings such as piperazine (*e.g.* triforine), pyridine (*e.g.* pyrifenox), and pyrimidine (*e.g.* fenarimol).

The mode of action of DMI fungicides is the inhibition of cytochrome P450-mediated 14α-demethylase ($P450_{14DM}$) in the sterol biosynthetic pathway (Figure 3). P450 is a heme iron-containing protein which shows a maximal absorption at 450 nm in the presence of carbon monoxide (CO) after reduction with dithionite (21). Many P450-dependent enzymes are involved in metabolism of xenobiotics and the synthesis of endogenous lipophilic substrates such as steroids and fatty acids (122, 183). P450s involved in the metabolism of xenobiotics have low substrate specificity and inhibition of their activity may cause synergism or antagonism of fungicide action. $P450_{14DM}$ is an enzyme catalyzing C14 demethylation of lanosterol (yeast) or eburicol (filamentous fungi) with high substrate specificity.

The activity of DMIs often depends on the stereo-isomeric configuration around the second (β) atom of the side chain attached to the nitrogen atom in the azole ring. The configurations around the first (α) and third (γ) atoms may also affect the activity suggesting that their fungitoxic action is due to stereo-specific binding of the azole ring to the heme iron of the $P450_{14DM}$ (180). The *N*1-substituent is also important for inhibitory activity. It is believed that the *N*1-substituent interacts with the apoprotein of the enzyme. The structural flexibility of DMIs may indicate that the apoprotein pocket can be occupied by a variety of structures.

DMIs may also inhibit $P450_{14DM}$ activity in plants and hence, inhibit gibberellin biosynthesis. Inhibition of *ent*-kaurene oxidase may result in growth retardation and phytotoxicity (160). A limited number of DMIs (*e.g.* uniconazole) have been developed as plant growth regulator (PGR) (20, 95). This PGR effect is reversed by exogenous supply of gibberellic acid (140). DMI fungicides may also display phytotoxicity to particular plant species by inhibition of the production of gibberellin (24).

Resistance to DMIs in fungi has been reported frequently (44). The main mechanisms involved in DMI resistance are increased efflux activity of DMIs mediated by ATP-binding cassette (ABC) and major facilitator superfamily (MFS) transporters (120, Chapter 4 and 5) and overproduction of the target enzyme $P450_{14DM}$ by tandem repeat mutations in the promoter region of the gene (72). Other resistance mechanisms of DMIs are listed in Table 1.

Table 1. Mechanisms of insensitivity and resistance to DMI fungicides. Modified from De Waard (44)

Mechanism	Reference
Detoxification	64
Protonation	158
Increased efflux from mycelium	39
Lack of activation	65
Defect in sterol 14α-demethylation	191
Circumvention of toxic sterol formation	174
Overproduction of $P450_{14DM}$	89
Deposition in cellular compartments	78
Tolerance of toxic sterols	192
Induced resistance response	162
Decreased affinity of $P450_{14DM}$	96, 182

Δ^{14}-Reductase and $\Delta^{8} \rightarrow \Delta^{7}$-isomerase inhibitors (morpholines). 4-Substituted 2,6-dimethylmorpholines are fungicides with a high efficacy against powdery mildew of cereals. Compounds with a high efficacy are tridemorph and fenpropimorph.

The mode of action of tridemorph in *B. cinerea* is based on inhibition of $\Delta^{8}\rightarrow\Delta^{7}$-isomerase activity (90). In *U. maydis* and *S. cerevisiae* the mode of action of morpholines can also be due to inhibition of Δ^{14}-reductase activity (15, 92). The primary mode of action depends on the fungal species and the growth conditions. The activity of morpholines to both target sites is ascribed to structural similarity of ionized morpholines with high-energy intermediates in sterol biosynthesis (16). Some additional inhibition sites of morpholines such as $\Delta^{24(28)}$ reduction and squalene cyclisation were summarized by Hollomon (79).

In general, morpholine fungicides are considered to have a low risk for development of resistance. They have been used for more than 30 years without performance difficulties although shifts in sensitivity against *Erysiphe graminis* to fenpropimorph in France (10), the Netherlands (43), and Germany and Switzerland (104) have been reported. Morpholine fungicides are often mixed with DMI fungicides to combat resistance development to DMIs (75).

3-Keto reductase inhibitor. In *B. cinerea,* fenhexamid reduced the content of ergosterol and induced accumulation of 3-keto compounds such as 4α-methylfecosterone, fecosterone, and episterone. This observation suggests that fenhexamid inhibits the activity of 3-keto reductase involved in C4 demethylation (33).

At least three different phenotypes of fenhexamid resistant isolates have been reported. In Hyd R1 resistant phenotypes, a cytochrome P450 monooxygenase, similar to the one that activates phosphorothiolate fungicides (170), is involved in fenhexamid detoxification. The

resistance mechanisms of Hyd R2 and R3 phenotypes remain to be solved, but may involve a P450 different from the one identified in phenotype Hyd R1 (103).

ATP-binding cassette (ABC) and major facilitator superfamily (MFS) transporters

To survive adverse natural conditions, microorganisms may increase their competitive ability by the production of toxins and may possess mechanisms that provide protection against natural toxic compounds. To this end, organisms evolved active transporter mechanisms that mediate the transport of endogenous and exogenous toxic compounds and prevent intracellular accumulation. In phytopathogenic fungi, transporters can be specifically involved in transport of endogenous fungal toxins, plant defence compounds, and fungicides.

Two major classes of transporters involved in secretion of these compounds are ATP-binding cassette (ABC) transporters and major facilitator superfamily (MFS) transporters (38). ABC transporters are regarded as primary transporters since they use the energy of ATP hydrolysis to drive transport of compounds (76). MFS transporters use the proton or sodium motive force to transport compounds (131). For this reason MFS transporters are cited as secondary transporters (48). Full-size ABC transporter proteins harbor two similar halves each containing one nucleotide binding fold (NBF) and six transmembrane domains (TMD) (Figure 6). The topology of important fungal ABC transporters is either $[TMD_6\text{-}NBF]_2$ or $[NBF\text{-}TMD_6]_2$. These types of transporters consist of 1300-1600 amino acid residues giving 140-170 kDa proteins. Also half-sized transporters have been reported.

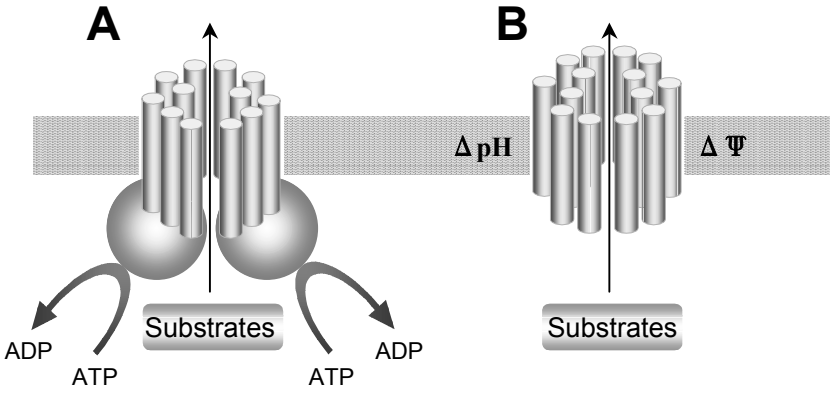

Figure 6. Representation of ABC transporter with a $[NBF\text{-}TMD_6]_2$ topology (A) and MFS transporter with 14 TMDs (B).

The sequence of the NBF domain is highly conserved during evolution and comprises the Walker A and B motif and the ABC signature (190, 80). At least 48 human ABC transporters can be classified into seven subfamilies (http://nutrigene.4t.com/humanabc.htm) (50). Yeast

ABC proteins were classified in six clusters (34), and constitute a pleiotropic drug resistance (PDR) network of genes involved in many physiological processes (18). An overview of ABC transporters in fungi and yeasts is given in Table 2.

MFS transporters have 12 or 14 TMDs (Figure 6). Theses types of transporters are composed of 400-800 amino acid residues with a molecular mass of 45-90 kDa. Saier *et al*. (146) divided MFS transporter proteins into at least 29 subfamilies. Transporters of three subfamilies belong to drug transporters. Analysis of structures and functions of these subfamilies revealed that each subfamily transports a specific class of substrates and possesses subfamily-specific signature sequences and conserved motifs. MFS transporters in fungi and yeasts are given in Table 3.

ABC and MFS transporters are known for their ability to transport chemically unrelated compounds. In fungi, ABC transporters involved in multidrug resistance (MDR) are AtrB (7) and AtrD (8) from *A. nidulans*, BcatrB (153, 154), BMR1 (105, 119), and BMR3 (105) from *B. cinerea*, ABC1 from *Leptosphaeria maculans* (175), MgAtr1 and MgAtr2 from *M. graminicola* (196), and PMR1 (120) and PMR5 (121) from *Penicillium digitatum*. ABC transporters that provide protection against plant defence compounds are BcatrB from *B. cinerea* (153), and abc1 from *Gibberella pulicaris* (58). MFS transporters in fungi are known for their ability to provide self-protection against their own toxins. These are aflT from *A. flavus* (27), CFP from *Cercospora kikuchii* (26), dotC from *Dothistroma pini* (23), TOXA from *Cochliobolus carbonum* (134), and Tri12 from *G. zeae* (4).

DMI fungicides are well known substrates of fungal ABC transporters. ABC transporters with the ability to transport DMI fungicides are AtrB from *A. nidulans* (7), BcatrD from *B. cinerea* (Chapter 4), CDR1 and CDR2 from *C. albicans* (139, 150), PMR1 from *P. digitatum* (120), and PDR5 (17) and SNQ2 from *S. cerevisiae* (94). Bcmfs1 from *B. cinerea* (Chapter 5), CaMDR1 (149) and FLU1 (25) from *C. albicans,* and FLR1 from *S. cerevisiae* (3) are MFS transporters involved in DMI transport.

Inhibitors of ABC or MFS transporter activity may show synergism with fungicides, which are substrates of these transporters (107). Similarly, inhibitors of ABC or MFS transporters which transport pathogenicity factors (*e.g.* fungal or plant toxins, peptides) can be lead compounds in the discovery of disease control agents with an indirect mode of action (46, Chapter 6). Validation of some of the statements provided above is one of the major goals of the research on ABC and MFS transporters described in this thesis.

Table 2. ABC transporter genes from fungi and yeasts involved in efflux of drugs and natural toxic compounds. Modified from Del Sorbo *et al.* (38)

Microbe	Gene	Accession No.[a]	Subfamily[b]	Protein topology[c]	Function / effect	Reference
Aspergillus fumigatus	*mdr1*	U62934	MDR	$(TMD_6\text{-}NBF)_2$	Resistance to cilofungin	178
	mdr2	U62936	MDR	$TMD_6\text{-}NBF$	Not involved in MDR	178
	ADR1	-	-	-	Upregulated by itraconazole	161
Aspergillus nidulans	*AtrA*	Z68904	PDR	$(NBF\text{-}TMD_6)_2$		37
	AtrB	Z68905	PDR	$(NBF\text{-}TMD_6)_2$	MDR	7
	AtrC	AF071410	MDR	$(TMD_6\text{-}NBF)_2$	Up-regulated by various toxicants	8
	AtrC2	AF082072	MDR	$(TMD_6\text{-}NBF)_2$	Up-regulated by cycloheximide	11
	AtrD	AF071411	MDR	$(TMD_6\text{-}NBF)_2$	MDR	8
	AtrE	AJ309280	PDR	$(NBF\text{-}TMD_6)_2$		9
	AtrF	AJ309281	PDR	$(NBF\text{-}TMD_6)_2$		9
	AtrG	AJ309282	PDR	$(NBF\text{-}TMD_6)_2$		9
Botrytis cinerea	*BcatrA*	Z68906	PDR	$(NBF\text{-}TMD_6)_2$	Up-regulated by cycloheximide	36
	BcatrB	AJ006217	PDR	$(NBF\text{-}TMD_6)_2$	Sensitivity to phenylpyrroles, resveratrol, and compounds secreted by *Pseudomonas* species	153,154 188
	BcatrD	AJ272521	PDR	$(NBF\text{-}TMD_6)_2$	DMI sensitivity	Chapter 4
	BMR1	AB028872	PDR	$(NBF\text{-}TMD_6)_2$	MDR	105,119
	BMR3	AB091178	PDR	$(NBF\text{-}TMD_6)_2$	MDR	105
	BMR5	AB091179	PDR	$(NBF\text{-}TMD_6)_2$		105
Candida albicans	*CDR1*	X77589	PDR	$(NBF\text{-}TMD_6)_2$	MDR	139
	CDR2	U63812	PDR	$(NBF\text{-}TMD_6)_2$	MDR	150
	CDR3	U89714	PDR	$(NBF\text{-}TMD_6)_2$	Expressed in opaque-phase of growth	14
	CDR4	AF044921	PDR	$(NBF\text{-}TMD_6)_2$	Not involved in DMI resistance	62
Gibberella pulicaris	*abc1*	AJ306607	PDR	$(NBF\text{-}TMD_6)_2$	Virulence on potato	58
Leptosphaeria maculans	*ABC1*	-	-	-	MDR	175
	ABC2	-	-	-	Not involved in MDR	175
Magnaporthe grisea	*ABC1*	AF032443	PDR	$(NBF\text{-}TMD_6)_2$	Essential for pathogenicity	181
Mycosphaerella graminicola	*MgAtr1*	AJ243112	PDR	$(NBF\text{-}TMD_6)_2$	Up-regulated by cycloheximide and eugenol	196
	MgAtr2	AJ243113	PDR	$(NBF\text{-}TMD_6)_2$	Up-regulated by eugenol and imazalil	196
	MgAtr3	AF364105	PDR	$(NBF\text{-}TMD_6)_2$		169
	MgAtr4	AF329852	PDR	$(NBF\text{-}TMD_6)_2$		169
	MgAtr5	AF364104	PDR	$(NBF\text{-}TMD_6)_2$		169

Penicillium digitatum	*PMR1*	AB010442	PDR	$(NBF\text{-}TMD_6)_2$	DMI Resistance	120
	PMR5	AB060639	PDR	$(NBF\text{-}TMD_6)_2$	MDR	121
Saccharomyces cerevisiae	*PDR5*	L19922	PDR	$(NBF\text{-}TMD_6)_2$	MDR	17
	PDR12	U39205	PDR	$(NBF\text{-}TMD_6)_2$	C1-C7 organic acids resistance	133
	PDR15	U32274	PDR	$(NBF\text{-}TMD_6)_2$	Inducible upon stress	195
	SNQ2	X66732	PDR	$(NBF\text{-}TMD_6)_2$	MDR	156
	PXA1	Z73503	ALDP	$TMD_6\text{-}NBF$	Required for β-oxidation of fatty acids	157
	PXA2	Z28188	ALDP	$TMD_6\text{-}NBF$	Required for β-oxidation of fatty acids	157
	BAT1	Z73153	MRP	$(TMD_6\text{-}NBF)_2$	Bile acid transporter	129
	YCF1	Z48179	MRP	$(TMD_6\text{-}NBF)_2$	Multidrug and heavy metals resistance	171
	YOR1	Z73066	MRP	$(TMD_6\text{-}NBF)_2$	MDR	91
	ATM1	Z49212	MDR	$TMD_6\text{-}NBF$	Mitochondrial DNA maintenance	98
	STE6	Z28209	MDR	$(TMD_6\text{-}NBF)_2$	Secretion of the **a** mating factor	111
	YEF3	U20865	YEF3	$(NBF)_2$	Interaction with aminoacyl-tRNA	148
	GCN20	D50617	YEF3	$(NBF)_2$	Interaction with tRNA and Gcn2p	186
Schizosaccharomyces pombe	*Abc1*	Y09354	PDR	$(TMD_6\text{-}NBF)_2$		29
	Mam1	U66305	MDR	$(TMD_6\text{-}NBF)_2$	Secretion of mating factor	29
	bfr1	S76267	PDR	$(NBF\text{-}TMD_6)_2$	MDR	117
	pmd1	D10695	MDR	$(TMD_6\text{-}NBF)_2$	MDR	125
Venturia inaequalis	*ViABC1*	AF227914	PDR	$(NBF\text{-}TMD_6)_2$		152
	ViABC2	AF227915	PDR	$(NBF\text{-}TMD_6)_2$		152
	ViABC3	AF375878	MDR	$(TMD_6\text{-}NBF)_2$		152
	ViABC4	AF375879	MDR	$(TMD_6\text{-}NBF)_2$		152

a: GenBank accession numbers.

b: Names of subfamilies based on sequence similarity with human (ALDP, MDR, MRP) or yeast (PDR, YEF3) ABC transporters. ALDP: adrenoleukodystrophy protein, MDR: multidrug resistance, MRP: multidrug resistance-associated protein, PDR: pleiotropic drug resistance, YEF3: yeast elongation factor 3.

c: NBF, nucleotide binding fold; TMD_6, transmembrane domain with six transmembrane spans.

Table 3. MFS transporter genes from fungi and yeasts involved in efflux of drugs and natural toxic compounds. Modified from Del Sorbo *et al.* (38)

Microbe	Gene	Accession No.[a]	TMD[b]	Function / effect	Reference
Aspergillus flavus	*AflT*	AF268071	-	Secretion of aflatoxin	27
Botryotinia fuckeliana	*Bcmfs1*	AF238225	14	MDR	Chapter 5
Candida albicans	*BenR/CaMDR1*	X53823/ Y14703	12	MDR	60
	FLU1	AF188621	12	Fluconazole resistance	25
Candida maltosa	*CYHR*	M64932	12	MDR	151
Cercospora kikuchii	*CFP*	AF091042	14	Secretion of cercosporin	26
Cochliobolus carbonum	*TOXA*	L48797	14	Secretion of HC toxin	134
Gibberella pulicaris	*Rin6*	AJ132188	-	Up-regulated by rishitin	193
Gibberella zeae	*Tri12*	AY102605	14	Secretion of trichotecenes	4
Mycosphaerella pini	*dotC*	AF448056	-	Secretion of dothistromin	23
Saccharomyces cerevisiae	*FLR1*	Z35877	12	MDR	3
	ATR1	M20319	14	MDR	68
	SGE1	U02077	14	MDR	51
Schizosaccharomyces pombe	*Car1*	Z14035	12	MDR	87

a: GenBank accession numbers.

b: TMD, number of transmembrane domains.

Fungicide mixtures

Since the 1970s, fungicides with systemic and site-specific activity, sometimes called modern fungicides, have been often used. Modern fungicides have a relatively low environmental toxicity as compared to conventional fungicides and can be applied at lower rates of active ingredients. Hence, they contribute to environmental safe agriculture. On the other hand, modern fungicides have caused resistance problems (102). Countermeasures to avoid or delay the risk of resistance development are largely based on the use of fungicide mixtures. The main purpose of mixtures is extending the antifungal spectrum of commercial products. Mixtures can also extend the spray interval time. This type of mixtures is especially useful in control of rice blast caused by *Magnaporthe grisea*. The use of mixtures which show synergistic interaction may result in a reduction in amount of active ingredients and can also act as a countermeasure against resistance.

Synergistic interactions between components in a mixture can relate to one of the following mechanisms (41): (a) non- mediated diffusion across the plasma membrane, (b) carrier-mediated transport across the plasma membrane, (c) energy-dependent efflux from the fungal cell, (d) transport to the target site, (e) activation, (f) detoxification, (g) affinity for the target-site, (h) circumvention of the target site, and i) compensation of the target-site. Examples of synergisms in fungicide mixtures are listed in Table 4.

Table 4. Examples of synergism in fungicide mixtures. Modified from De Waard (42)

Components in mixture	Pathogen	Reference
Anilazine / zinc or copper	*Botrytis cinerea*	69
	Colletotrichum coccodes	
Carboxin / mancozeb	Various	13
Chloroneb / thiram	*Pythium ultimum*	144
Copper / zineb	*Plasmopara viticola*	177
Dimethyldithiocarbamates / complex-forming agents	*Botrytis cinerea*	109
Dodine / captan	*Venturia inaequalis*	137
	Xanthomonas prunei	47
Elemental sulfur / surfactants	*Botrytis cinerea*	132
Ethazole / pentachloronitrobenzene	*Pythium aphanidermatum*	142
Fenarimol / CCCP[a]	*Aspergillus nidulans*	40
Fluconazole / cyclosporine	*Candida albicans*	107
Oxpoconazole / chlorpromazine, tacrolimus	*Botrytis cinerea*	Chapter 6
Phosphorothiolate / phosphoramidate	*Pyricularia oryzae*	179
Strobilurins, Antimycin A / SHAM[b]	*Botrytis cinerea*	74
Zineb / polyram	*Plasmopara viticola*	59

a: Carbonyl cyanide 3-chlorophenylhydrazone.

b: Salicylhydroxamic acid.

Synergism may be classified as pseudo- or true synergism. Pseudo synergism occurs with compounds that affect the distribution of active ingredient on the plant surface, prevent run-off from leaves, or stimulate the uptake into plant tissue. True synergism is the case if components of a mixture directly react with each other, or if one of the compounds influences the physiology of the pathogen in such a way that it enhances the toxicity of the other compound (45). Until now, the global use of fungicide mixtures with a synergistic action in practice is rather limited (42). Deployment of mixtures of a multi-site inhibitor with a site-specific fungicide are probably the best strategy to lower the risk of resistance development and widen the fungicidal spectrum. This has been the main policy for the development of fungicide mixtures in practice (30).

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Chapter 2

Oxpoconazole fumarate: a new imidazole fungicide with a broad antifungal spectrum

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This chapter is a compilation of original research and data from the following manuscripts:

Anonymous, 2000. New imidazole SBI fungicide Al Shine WP, pp. 9.
Nishimura, T. and Ikemi, N. 2000. Oxpoconazole fumarate (AL-SHINE). Nouyaku-zihou 524: 6-9 (in Japanese).
Morita, T. and Nishimura, T. 2001. Development and fungicidal activity of new fungicide “oxpoconazole fumarate”. Syokubutuboueki 55: 384-388 (in Japanese).

ABSTRACT

Oxpoconazole fumarate (AL-SHINE®) is a new imidazole fungicide developed by Ube Industries, Ltd. and Otsuka Chemical Co., Ltd., and registered in Japan in 2000. The fungicide has a broad antifungal spectrum against fungi belonging to *Ascomycetes* and *Deuteromycetes*. In particular, it has an outstanding efficacy against *Botrytis cinerea* under field conditions. Fungal mutants of *B. cinerea* resistant to benzimidazole and dicarboximide fungicides did not display cross-resistance to the fungicide. Isolates of *Sphaerotheca fuliginea* resistant to sterol demethylation inhibiting (DMI) fungicides, showed cross-resistance to oxpoconazole fumarate. However, the resistance level to oxpoconazole fumarate was lower as compared to other DMIs tested. Evidence is presented that the mode of action of oxpoconazole fumarate is based on inhibition of P450 dependent sterol C14-demethylase ($P450_{14DM}$) activity, an enzyme involved in fungal ergosterol biosynthesis.

INTRODUCTION

Botrytis cinerea belongs to one of the most ubiquitous plant pathogens. The host-range of this pathogen comprises at least 235 plant species (5). Many crops (*e.g.* citrus, cucumber, eggplant, grapevine, pea, peach, and strawberry) suffer from significant losses caused by grey mould diseases, incited by *B. cinerea* during cultivation, transport, and storage. One of the most effective methods to control these diseases is fungicide treatment.

Although conventional fungicides such as chlorothalonil, dichlofluanid, or thiram are still used to control *B. cinerea*, most of these compounds are weak botryticides. Systemic fungicides such as benzimidazoles and dicarboximides have been used for grey mould control since the 1970s. During the last decade anilinopyrimidines, fenhexamid, fluazinam, phenylpyrroles, and strobilurins were introduced as new botryticides. However, their efficacy against *B. cinerea* is hampered by rapid emergence of resistance (9, 10). DMI fungicides have hardly been developed for control of *B. cinerea.* The only compound known is the imidazole prochloraz, which is used for *Botrytis* control in ornamentals.

Recently, another imidazole fungicide oxpoconazole fumarate (AL-SHINE®) (Figure 1) was developed by Ube Industries, Ltd. and Otsuka Chemical Co., Ltd. in Japan. The fungicide has high activity against grey mould in a wide variety of crops. An overview of chemical and physical properties of oxpoconazole fumarate is summarized in Table 1. Data on the mammalian toxicology of oxpoconazole fumarate summarized in Table 2, indicate that the fungicide has low

mammalian toxicity (11). The toxicity to birds, aquatic organisms, and other beneficial organisms is also low (results not shown).

The results presented in this study indicate that oxpoconazole fumarate has a wide *in vitro* antifungal spectrum. Under field conditions, it showed excellent properties for control of grey mould and several other diseases. The study also demonstrates that the mode of action of the fungicide is based on inhibition of P450 dependent sterol C14-demethylase ($P450_{14DM}$) activity, an enzyme involved in the fungal sterol biosynthesis pathway. Hence, the fungicide can be classified as a sterol demethylation inhibiting (DMI) fungicide.

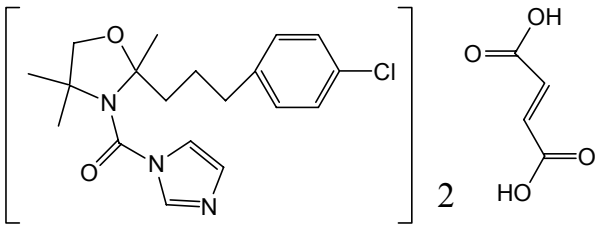

Figure 1. Chemical structure of oxpoconazole fumarate.

Table 1. Chemical and physical properties of oxpoconazole fumarate

Molecular formula		$C_{42}H_{52}Cl_2N_6O_8$
Molecular weight		839.82
Appearance		Colorless crystalline solid
Melting point		Melting point: 123.6-124.5°C
Log Pow[a]		3.69 (25°C, pH 7.5)
Vapor pressure		$5.42x10^{-6}$ Pa (25°C)
Stability		Stable for 14 days at 55°C
		Slightly unstable in acid
		Stable in base
		Slightly unstable in light
Solubility in	water	0.0895 g L^{-1} (25°C)
	methanol	109 g L^{-1} (25°C)
	ethanol	57.6 g L^{-1} (25°C)
	acetone	109 g L^{-1} (25°C)
	ethylacetate	39.0 g L^{-1} (25°C)

a: Partition coefficient between *n*-octanol and phosphate buffer pH7.4

Table 2. Mammalian toxicology of oxpoconazole fumarate

Acute oral LD_{50}	Mouse	1073 mg kg^{-1} (♂), 702 mg kg^{-1} (♀)
	Rat	1424 mg kg^{-1} (♂),1035 mg kg^{-1} (♀)
	Dog	>3000 mg kg^{-1} (♂)
Acute dermal LD_{50}	Rat	>2000 mg kg^{-1} (♂, ♀)
Acute inhalation LD_{50}	Rat	>4398 mg m^{-2} (♂, ♀)
Eye irritation	Rabbit	Slight irritation
Skin irritation	Rabbit	Non-irritation
Dermal sensitization	Guinea pig	Non-sensitization

MATERIALS AND METHODS

Chemicals

Oxpoconazole, triadimefon, and triflumizole (technical grade) were synthesized by Ube Industries, Ltd. A 20% WP formulation of oxpoconazole was also prepared by Ube Industries, Ltd. Formulated products of iprodione (ROVRAL®; 50% WP) and triflumizole (TRIFMINE®; 30% WP) were commercially obtained.

Fungi and fungitoxicity tests

Fungi tested were maintained as fungal stock in Ube Industries, Ltd. Various resistant phenotypes of *B. cinerea* isolates were kindly provided by Akira Kiso (Institute of Japan Plant Protection Association). The *B. cinerea* isolates were maintained on potato dextrose agar (PDA) plates at 20°C.

Test microorganisms were incubated on PDA plates at appropriate temperatures for 2 to 10 days. Mycelial segments (3 X 3 mm) were removed and transferred to PDA plates amended with oxpoconazole fumarate at various concentrations. Plates were incubated at appropriate temperatures for the test organisms and the diameter of fungal colonies was measured after incubation. The values were used to compose dosage-response curves from which fungicide concentrations that inhibit growth by 50% (EC_{50}) and the minimal inhibitory concentration (MIC) were calculated.

Leaf disc test on cucumber powdery mildew

Leaf disc assays were performed as described previously (12). Spore suspensions from wild-type and triadimefon-resistant isolates of *Sphaerotheca fuliginea* were sprayed on cucumber leaf discs (diameter 1 cm). The inoculated leaf discs (n = 5) were transferred to solutions amended with fungicides at different concentrations at 20°C for 8 days. The fungicide solutions were prepared from 1000X concentrated solutions of technical grade compounds in methanol. The efficacy of the fungicides was assessed microscopically (X50) by assessing disease incidence according to the following scale: 0 (no lesion), 1 (less than 5% of leaf area infected), 2 (6-25% of leaf area infected), 3 (26-50% of leaf area infected), 4 (51-75% of leaf area infected), and 5 (more than 75% of leaf area infected). The disease incidence was calculated with the formula: Σ index (0-5) / 25 X 100. EC_{50} values were calculated from dosage-response curves. Resistance factors were calculated as the ratio between the EC_{50} of

fungicides for the triadimefon-resistant isolate and the wild-type isolate.

Activity of oxpoconazole under field conditions

Field trials were performed according to governmental guidelines for official field trials in Japan. Field trials on grey mould control in grapevine were performed at Hiroshima Agricultural Technology Center in 1995. The efficacy of fungicides against grey mould of citrus was studied at Yamaguchi Citrus Experiment Station in 1998. Activity against powdery mildew of grapevine was determined at Nagano Tyuusin Agricultural Experiment Station in 1998.

Incorporation of [2-^{14}C] acetic acid and thin-layer chromatography (TLC) analysis

The tests were performed according to methods described previously (7). Spore suspensions of *B. cinerea* (10^6 spores ml^{-1}) were incubated in potato dextrose broth at 25°C for 24 h. Methanol solutions of oxpoconazole fumarate (10 µl) and [2-^{14}C] acetic acid (18.5 kBq) were added to cultures of *B. cinerea* (1.5 ml) and incubated at 25°C for 2 h. Total lipids were extracted according to methods described previously (1, 6, 16). The extracts were spotted on TLC plates (Silica Gel $60F_{254}$, Merck, Whitehouse Station, NJ, USA) and developed with *n*-heptane : isopropyl ether : acetic acid (60 : 40 : 4, v/v/v) as a solvent. Autoradiograms of TLC plates, made by exposure to BAS5000 films overnight, were analyzed with a densitometer (BAS5000, Fiji Film, Tokyo, Japan).

Gas chromatographic (GC) analysis of sterols

GC analysis was performed according to methods described by Kato (1981). Spore suspensions of *B. cinerea* (10^6 spores ml^{-1}) were incubated in potato dextrose broth at 25°C for 24 h. Oxpoconazole fumarate was added and incubated at 25°C for 5 h. The mycelium was centrifuged at 10,000x g for 10 min. Wet mycelium (0.1 g) was suspended in 60% ethanol including 10% KOH and heated at 70°C for 1 h. Non-saponifiable lipids were extracted three times with hexane from cooled saponification mixtures. Cholesterol (100 mg L^{-1}) was added as internal standard. The extract was analyzed by GC (GC-17A, Shimadzu, Kyoto, Japan) and the lipids were identified with a GC-mass spectrometer (GCMS-QP5000, Shimadzu).

RESULTS

Antifungal activities

The fungitoxic activity of oxpoconazole fumarate against a range of fungi, yeast, and bacteria are listed in Table 3. The results demonstrate that oxpoconazole fumarate has a broad *in vitro* spectrum of antifungal activity and affects most of the *Ascomycetes* and *Deuteromycetes* tested. EC_{50} values of the compound for inhibition of growth of *Alternaria citri, Botryosphaeria berengeriana, Botryosphaeria dothidea, B. cinerea, Cercospora beticola, Cochliobolus miyabeanus, Diaporthe citri, Monilinia fructicola, Physalospora piricola, Pyrenophora graminea, P. teres, Rhizoctonia solani, Sclerotinia sclerotiorum, Venturia inaequalis,* and *V. nashicola* were less than 0.1 mg L^{-1}, indicating that these plant pathogenic fungi are particularly sensitive to oxpoconazole fumarate. Oxpoconazole fumarate showed weak activity against *Basidiomycetes* while *Oomycetes* tested were also relatively insensitive. The compound is also not active against bacteria. Activity of oxpoconazole fumarate against wild-type and various benzimidazole- and dicarboximide-resistant strains of *B. cinerea* was similar.

Leaf disc assay

Cross-resistance of DMI-resistant isolates of *S. fuliginea* to oxpoconazole fumarate was tested in leaf disc assays. DMIs tested were oxpoconazole fumarate, triadimefon, and triflumizole. The resistance factors, calculated as the ratio between the EC_{50} values of DMIs for control of the triadimefon-resistant isolates and the wild-type isolate, indicated cross-resistance to the fungicides tested. The cross-resistance level to oxpoconazole fumarate was relatively low as compared to other DMIs tested (Table 4).

Table 4. Resistance factor of *Sphaerotheca fuliginea* to DMI fungicides in cucumber leaf disc assays

Fungicide	Resistance factor[a]
Oxpoconazole fumarate	17
Triflumizole	78
Triadimefon	270

a: Ratio of EC_{50} values between triadimefon resistant and sensitive isolates in cucumber leaf disc assay.

Table 3. Antifungal spectrum of oxpoconazole fumarate in radial growth experiment

Microorganism		EC_{50} (mg L^{-1})	MIC (mg L^{-1})
Protoctista	***Oomycetes***		
	Phytophthora infestans	12.0	>100
	Pythium sp.	18.1	>100
Fungi	***Ascomycetes***		
	Botryosphaeria berengeriana	0.041	11.1
	Botryosphaeria dothidea	0.008	11.1
	Cochliobolus miyabeanus	0.027	3.7
	Gibberella fujikuroi	0.11	3.7
	Glomerella cingulata	0.53	11.1
	Monilinia fructicola	0.002	0.14
	Physalospora piricola	0.066	11.1
	Pyrenophora graminea	0.02	3.7
	Pyrenophora teres	0.079	11.1
	Saccharomyces cerevisiae	-	1.23
	Sclerotinia sclerotiorum	0.096	1.23
	Valsa ceratosperma	0.13	3.7
	Venturia inaequalis	0.004	33.3
	Venturia nashicola	0.019	0.4
	Basidiomycetes		
	Corticium rolfsii	5.411	>100
	Helicobasidium mompa	2.180	33.3
	Deuteromycetes		
	Alternaria citri	0.063	33.3
	Alternaria mali	0.151	11.1
	Botrytis cinerea (S, S, HR)[a]	0.085	3.7
	Botrytis cinerea (HR, MR, S)[a]	0.058	3.7
	Botrytis cinerea (HR, S, MR)[a]	0.096	3.7
	Botrytis cinerea (HR, MR, S)[a]	0.087	3.7
	Botrytis cinerea (HR, MR, WR)[a]	0.261	3.7
	Cercospora beticola	0.04	11.1
	Colletotrichum lagenarium	0.714	11.1
	Diaporthe citri	0.042	1.23
	Elsinoe ampelina	0.234	11.1
	Penicillium digitatum	0.114	3.7
	Penicillium italicum	1.79	100
	Pseudocercospora herpotrichoides (W, S)[b]	0.134	3.7
	Pseudocercospora herpotrichoides (W, R)[b]	0.210	3.7
	Pseudocercospora herpotrichoides (R, S)[b]	2.117	33.3
	Pseudocercospora herpotrichoides (R, R)[b]	1.230	33.3
	Pyricularia oryzae	0.17	3.7
	Rhizoctonia solani	0.008	>100
	Septoria tritici	0.197	33.3
Bacteria	*Xanthomonas campestris* pv. *citri*	-	>100
	Pseudomonas sp.	-	>100

a: *Botrytis cinerea* phenotype to benzimidazoles (left letter), dicarboximides (middle letter), and *N*-phenylcarbamates (right letter). S: sensitive, WR: weakly resistant, MR: moderately resistant, and HR: highly resistant.

b: *Pseudocercospora herpotrichoides*. First letter: wheat-type (W) or rye-type (R); second letter: sensitivity to benzimidazoles. S: sensitive and R: resistant.

Field performance

The activity of oxpoconazole fumarate in control of grey mould of grapevine under field conditions was tested at Hiroshima Agricultural Technology Center in 1995. The results indicate that the efficacy of the compound is at least comparable with the reference fungicide iprodione (Table 5-1). Its activity against grey mould of citrus performed at Yamaguchi Citrus Experiment Station in 1998 was also similar as compared to iprodione (Table 5-2). A field experiment at Nagano Tyuusin Agricultural Experiment Station in 1998 demonstrated that oxpoconazole fumarate is also active against powdery mildew of grapevine incited by *Uncinula necator* (Table 5-3).

Table 5-1. Activity of oxpoconazole fumarate against grey mould of grapevine (cv. Campbell early) at Hiroshima Agricultural Technology Center

Fungicide[a]	Infected branches (%)[b]
Control	21.3
Oxpoconazole fumarate (100 mg L^{-1} [c])	3.0
Iprodione (333 mg L^{-1} [c])	4.7

a: Application date: 16 May 1995

b: Date of assessment: 4 June 1995

c: Concentration of formulated fungicide

Table 5-2. Activity of oxpoconazole fumarate against grey mould of citrus (cv. Koutuwase) at Yamaguchi Citrus Experiment Station

Fungicide[a]	Infected flowers (%)[b]	Infected fruits (%)[b]
Control	20.3	10.8
Oxpoconazole fumarate (100 mg L^{-1} [c])	5.5	4.9
Iprodione (333 mg L^{-1} [c])	3.0	3.6

a: Application date: 16 May 1998

b: Date of assessment: 23 May 1998 (flowers), 10 June1998 (fruits)

c: Concentration of formulated fungicide

Table 5-3. Activity of oxpoconazole fumarate against powdery mildew of grapevine (cv. Kyohou) at Nagano Tyuusin Agricultural Experiment Station

Fungicide[a]	Infected branches (%)[b]
Control	21.0
Oxpoconazole fumarate (66 mg L^{-1} [c])	0.0
Triflumizole (150 mg L^{-1} [c])	0.0

a: Application dates: 29 May 1998, 5 June 1998, 17 June 1998

b: Date of assessment: 13 July 1998

c: Concentration of formulated fungicide

Mode of action

The effect of oxpoconazole fumarate on sterol biosynthesis was studied by investigating the incorporation of [2-^{14}C] acetic acid in mycelium of *B. cinerea.* Separation of extracted total lipids by TLC revealed several distinct bands (Figure 2). The respective bands were identified by comparison of their R_f values with reference compounds (7). The densitometer measurements of the spots on the TLC plates indicate that the incorporation ratio between C4,4-dimethyl sterols (band 5) and C4-desmethyl sterols (band 3) in the water control and the oxpoconazole fumarate treatment at 0.1 and 1 mg L^{-1} amounts 0.6, 21.6, and 63.2, respectively (Table 6). These results indicate that treatment of mycelium with oxpoconazole fumarate decreased the incorporation of radioactivity into C4-desmethyl sterols and increased the incorporation into C4,4-dimethyl sterols.

The non-saponifiable lipids were also analyzed by GC. In control samples, two major peaks were observed with retention times of 21.8 (peak 1) and 25.6 min (peak 2). In oxpoconazole-treated samples two additional peaks with retention times of 30.8 (peak 3) and 38.3 min (peak 4) were detected (Figure 3). These four peaks were identified by MS as cholesterol (peak 1, internal standard), ergosterol (peak 2), obtusifoliol (peak 3), and eburicol (peak 4). In samples from oxpoconazole fumarate treated mycelium, the ergosterol peak was relatively low and the obtusifoliol and eburicol peaks were relatively high as compared to the control samples. The peak area of the internal standard was similar in the control and oxpoconazole treatment (Figure 3, Table 7). The results indicate that oxpoconazole fumarate inhibits synthesis of ergosterol by inhibition of P450 dependent C14-sterol demethylation.

DISCUSSION

The studies on the effect of oxpoconazole fumarate on sterol synthesis in *B. cinerea* indicate that the compound inhibits the biosynthesis of C4-desmethyl sterols by inhibition of P450 dependent C14-demethylation of eburicol. Data that support this hypothesis are the depletion of ergosterol synthesis upon treatment with oxpoconazole fumarate and the accumulation of eburicol and obtusifoliol (Figure 3). Similar effects on sterol biosynthesis of *B. cinerea* were observed by the DMI fungicide imazalil (3, 14). The results indicate that oxpoconazole fumarate can be regarded as a new member of the large group of modern fungicides named sterol demethylation inhibitors (DMIs).

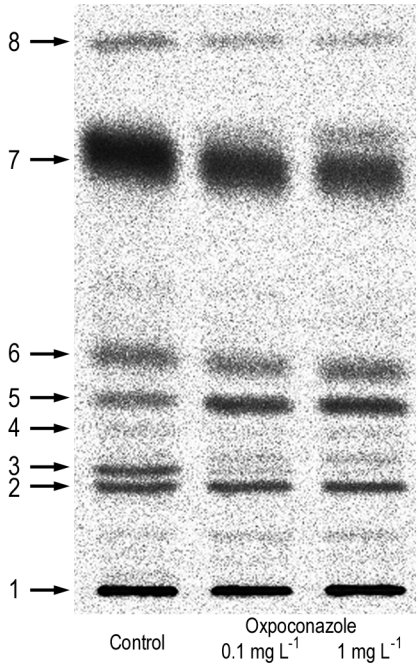

Figure 2. Autoradiogram of a thin-layer chromatographic separation of lipids extracted from oxpoconazole-treated mycelium of *Botrytis cinerea* incubated with [2-^{14}C] acetic acid for 2 h.
1: origin (complex lipids), 2: diglycerides, 3: C4-desmethyl sterols, 4: C4-methyl sterols, 5: C4,4-dimethyl sterols, 6: free fatty acids, 7: triglycerides, 8: sterol esters

Table 6. Effect of oxpoconazole fumarate on the incorporation of [2-^{14}C] acetic acid into lipids of *Botrytis cinerea*

Treatment	Lipid class	Radioactivity unit	Ratio between di- and desmethyl sterols
Control	C4,4-dimethyl sterols	1529	0.6
	C4-methyl sterols	254	
	C4-desmethyl sterols	2424	
	Total lipids	92530	
Oxpoconazole fumarate (0.1 mg L^{-1})	C4,4-dimethyl sterols	4271	21.6
	C4-methyl sterols	88	
	C4-desmethyl sterols	197	
	Total lipids	68910	
Oxpoconazole fumarate (1 mg L^{-1})	C4,4-dimethyl sterols	5292	63.2
	C4-methyl sterols	168	
	C4-desmethyl sterols	84	
	Total lipids	66390	

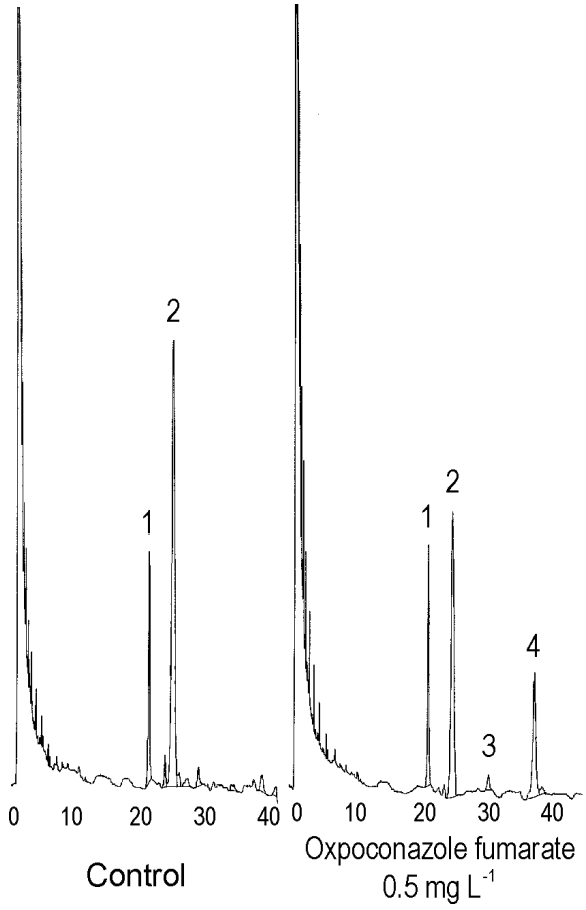

Figure 3. GC analysis of non-saponifiable lipids extracted from *Botrytis cinerea* treated with oxpoconazole fumarate (0.5 mg L^{-1}). 1: cholesterol (internal standard), 2: ergosterol, 3: obtusifoliol, 4: eburicol

Table 7. Effect of oxpoconazole fumarate on the peak area of lipids extracted from culture of *Botrytis cinerea* detected by GC

Compound	Peak area			
	Cholesterol	Ergosterol	Obtusifoliol	Eburicol
Control	9875	34230	ND	ND
Oxpoconazole fumarate (0.5 mg L^{-1})	10198	21117	915	10280

ND: not detected.

The antifungal spectrum of oxpoconazole fumarate seems to include more representatives of *Ascomycetes* and *Deuteromycetes* (Table 3) as compared to other DMI fungicides. The wide spectrum of the fungicide also includes *B. cinerea* and *Pseudocercospora herpotrichoides.* Only a limited number of DMI fungicides are effective against these pathogens and the diseases they incite. Another DMI effective against these pathogens is prochloraz. Oxpoconazole fumarate and prochloraz share an imidazole moiety in their molecular structure. It might be that this

imidazole moiety is relevant for field activity against *B. cinerea* and *P. herpotrichoides*. Oxpoconazole fumarate has almost no effect against *Oomycetes* and bacteria. This is a typical character of DMI fungicides that can be explained by the fact that these organisms lack a sterol biosynthetic pathway.

Many DMIs have a high intrinsic activity against *B. cinerea in vitro*. However, their efficacy in control of grey mould under field conditions is not satisfactory. The reason for this discrepancy is still unknown (15). The present results demonstrate that oxpoconazole fumarate is both active *in vitro* and under field conditions against *B. cinerea*. For these reasons, oxpoconazole fumarate was developed as a commercial fungicide for grey mould control, since chemical control of grey mould under field conditions is a serious problem. The main reason for this situation is resistance development in *B. cinerea* to fungicides from different chemical classes (9).

Resistance development in *B. cinerea* to benzimidazole and dicarboximide fungicides is most notorious (8). The pathogen did not show cross-resistance to oxpoconazole fumarate, benzimidazole, and dicarboximide fungicides since the activity of oxpoconazole against benzimidazole- and dicarboximide-resistant strains of *B. cinerea* was similar as compared to the wild-type (Table 3). In view of this character, oxpoconazole fumarate is expected to show significant field performance against grey mould caused by benzimidazole- and dicarboximide-resistant *B. cinerea* strains.

The risk for resistance development in plant pathogens to DMI fungicides is relatively low. Still, long-term treatment of pathogens may result in a step-wise buildup of DMI resistant populations. This phenomenon has been described for various powdery mildews (2, 4, 13). Our experiments demonstrate that isolates of *S. fuliginea*, resistant to the DMIs triadimefon and triflumizole are also resistant to oxpoconazole fumarate. However, the resistance factor to the latter fungicide was relatively low as compared to other DMI fungicides tested (Table 4). This may imply that oxpoconazole fumarate may have a field activity against powdery mildew populations that are resistant to other DMI fungicides.

Oxpoconazole also proved to be effective against powdery mildew of grapevine incited by *U. necator* (Table 5-3). Excellent control of powdery mildew diseases is a common property of many DMIs. However, the combined activity of oxpoconazole fumarate against both powdery mildew (Table 5-3) and grey mould (Table 5-1, Table 5-2) makes this fungicide attractive for disease control in crops in which both diseases occur simultaneously. This is the case for

grapevine. Oxpoconazole is also officially registered for control of other common diseases such as rusts, scabs and diseases caused by *Alternaria, Monilinia*, *Penicillium,* and *Phomopsis*. Hence, its broad field efficacy may make it possible that a single application of the fungicide provides control of multiple diseases in the same crop. This implies that the use of oxpoconazole fumarate may significantly reduce the number of applications with other fungicides.

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Chapter 3

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

Hayashi, K., Schoonbeek, H., Sugiura, H., and De Waard, M. A. 2001. Pesticide Biochemistry and Physiology 70: 168-179.

ABSTRACT

Azole-resistant mutants of *Botrytis cinerea* have a multidrug resistance 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 transient accumulation pattern similar to that of the wild-type isolate. The putative role of 10 additional ABC and 3 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 also correlated with resistance levels. 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 defence compounds, and mating factors (9). Major groups of transporters involved in drug resistance can be divided into two superfamilies: the ATP-binding cassette (ABC) superfamily (21, 7) and the major facilitator superfamily (MFS) (28). ABC transporters directly utilize the energy generated by ATP hydrolysis to pump substrates across membranes against a concentration gradient (18). On the other hand, MFS transporters are secondary transporters, driven by the proton motive force over membranes (29). Inhibitors of ABC and

MFS transporter activity may act as synergists with fungicides that are substrates for these transporters (17). Inhibitors of transporter activity in plant pathogens may also result in decreased secretion of virulence factors or increased accumulation of plant defence 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 (22, 40). 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* (12, 13, 15, 36), *Botrytis cinerea* (6, 37), *Candida albicans* (32), and *Penicillium italicum* (14, 16). 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* (2, 3, 8), *C. albicans* (30, 33), *Mycosphaerella graminicola* (41), and *P. digitatum* (26). A role of ABC transporters in resistance of *Saccharomyces cerevisiae* to dicarboximides was also reported (25).

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 a 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 (37). This resistance mechanism was also found for tebuconazole in anilinopyrimidine-resistant field isolates of *B. cinerea* that show cross-resistance to azole fungicides (6). 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 in azole-resistant mutants of *B. cinerea*. Previously, we have reported on the ABC transporter genes of *BcatrA* and *BcatrB* from *B. cinerea* (35). Furthermore, we detected the presence of 10 additional ABC (*BcatrC-BcatrN*) and 3 MFS (*Bcmfs1-Bcmfs4*) genes by analysis of an EST library from *B.*

cinerea (39). This study describes the expression analysis of all these ABC and MFS genes in a wild-type isolate and in 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) (38). These strains were kindly provided by B. N. Ziogas (University of Athens, Greece). Strain B05.10 is a haploid wild-type strain (5) and ΔBcatrB4 is a *BcatrB* replacement mutant generated from strain B05.10 in our laboratory (35).

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, DE, USA), ISK Bioscience Co. (Mentor, OH, USA), Aventis (Lyon, France), Bayer AG (Leverkusen, Germany), and Syngenta (Stein, Switzerland), respectively. Cycloheximide was purchased from Sigma (St. Louis, MO, 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 to 3 days. Then, plates were irradiated with near-UV light for 24 h to induce formation of conidia and incubated at 20°C for another 3 to 7 days. 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 hematocytometer. Conidial suspensions (approximately 10^6 spores ml^{-1}) were spread on synthetic agar medium (23) 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. Fungicide 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

three agar plugs and incubated at 20°C for 2 to 3 days. EC_{50} values of fungicides were calculated from dose-response curves with Excel 97. Statistical analysis of the EC_{50} values was performed with the LSD (*t* test) 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 that of 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) (19) to a final density of 2×10^6 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 (11, 16). Accumulation experiments were initiated by the addition of [^{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 three times in 30 s with 5 ml of the same buffer. Radioactivity in mycelium was extracted with scintillation liquid (LUMASAFE PLUS; LUMAC*LSC B.V., Groningen, The Netherlands) for 1 day and counted in a liquid scintillation spectrometer (BECKMAN LS6000TA; Beckman Coulter Inc., CA, USA).

Effects of compounds on uptake of [^{14}C]oxpoconazole were determined by addition from a 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* (Bitton, F., Levis, C., Fortini, D., Pradier, J. M., and Brygoo, Y. Genoscope, Centre National de Sequençage, *Botrytis cinerea* strain T4cDNA library under conditions of nitrogen deprivation; EMBL Accession Nos. AL110624 to AL117185; unpublished, 1999), 10 ESTs with homology to ABC transporter genes and 3 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 by use of pGEM-T Vector Systems (Promega, Madison, WI, USA). The sequences of these inserts were determined with BigDye Terminator sequence kits (Perkin-Elmer Corp., CN, USA). DNA manipulations were performed according to standard methods (34). *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 a 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 to 16 h. Cultures were divided into 20 ml aliquots in 50 ml Erlenmeyer flasks containing approximately 100 mg wet weight (approximately 10 mg dry weight) of germlings. Fungicides were added to germlings from 1000X concentrated stock solutions 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 12,000x g at 4°C for 10 min to remove extracellular material and polysaccharides. Supernatants were transferred, mixed with chloroform (0.2 ml), and centrifuged at 12,000x g at 4°C for 15 min to separate the aqueous and organic phases. 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 12,000x g 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 to 200 µl of RNAse-free water. The concentration of RNA was determined by measurement of 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), dimethyl sulfoxide (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 with a SEA 2000 gel electrophoresis apparatus (Elchrom Scientific AG, Cham, Switzerland) for 2 h at 0.4 A (3–4 V cm^{-1}). RNA was blotted on to Hybond-N^+ membranes (Amersham Pharmacia Biotech, Uppsala, Sweden) by capillary transfer (34) in 10X standard saline citrate (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 No. Z68906; nucleotides 3411–4344) and *BcatrB* (Accession No. AJ006217; nucleotides 2246-2942) were also used. The DNA fragments were purified with the QIAquick gel extraction kit (Qiagen GmbH, Hilden, Germany). Purified DNA fragments (30 ng) were radioactively labeled with 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 then 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 to 7 days with Kodak Scientific Imaging Film, X-OMAT LS. Membranes were reused in a northern analysis experiments after being stripping in boiling 1% SDS. Hybridization with 28S rRNA from *B. cinerea* (31) was used as loading control. Ethidium bromide staining of agarose gels demonstrated that the quality of RNA was good (results 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 a radial growth test (Table 1). Both mutants displayed cross-resistance to all tested azole fungicides (oxpoconazole, prochloraz, and tebuconazole). The resistance ratio for all tested azoles was higher for strain G25 than for strain G66. A low

degree of cross-resistance to the dicarboximide iprodione and the antibiotic cycloheximide was also found. Sensitivity to the benzimidazole fungicide carbendazim was similar for all isolates tested. This was also found for the anilinopyrimidine fungicide pyrimethanil (results not shown).

The fungitoxic activity of the tested azole fungicides to ΔBcatrB4 was slightly, although not significantly, higher than that to the parent isolate B05.10. The activity of iprodione, carbendazim, and cycloheximide to strains B05.10 and ΔBcatrB4 was similar (Table 1).

Accumulation of oxpoconazole

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

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 (Figure 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 in 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 (results not shown).

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

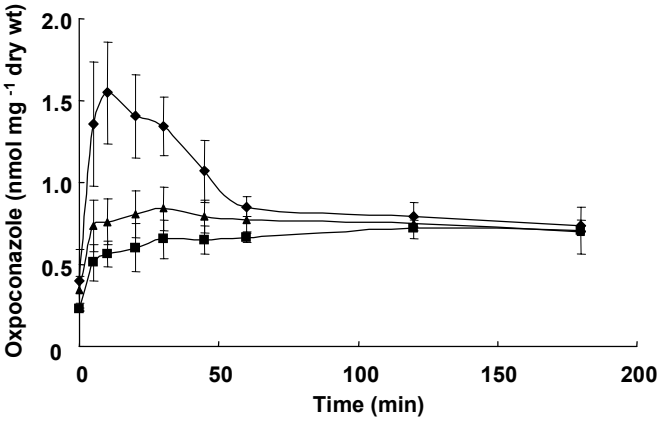

Figure 1. Accumulation of oxpoconazole (30 μM) by germlings of *Botrytis cinerea* wild-type strain B3 (◆) and azole-resistant mutants G25 (■) and G66 (▲).

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

Experiment	Strain [a]	Oxpoconazole		Prochloraz		Tebuconazole		Iprodione		Carbendazim		Cycloheximide	
		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 the azole-resistant mutants G25 and G66 and the 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 Experiment A or B indicate that values do not differ significantly ($P = 0.05$).

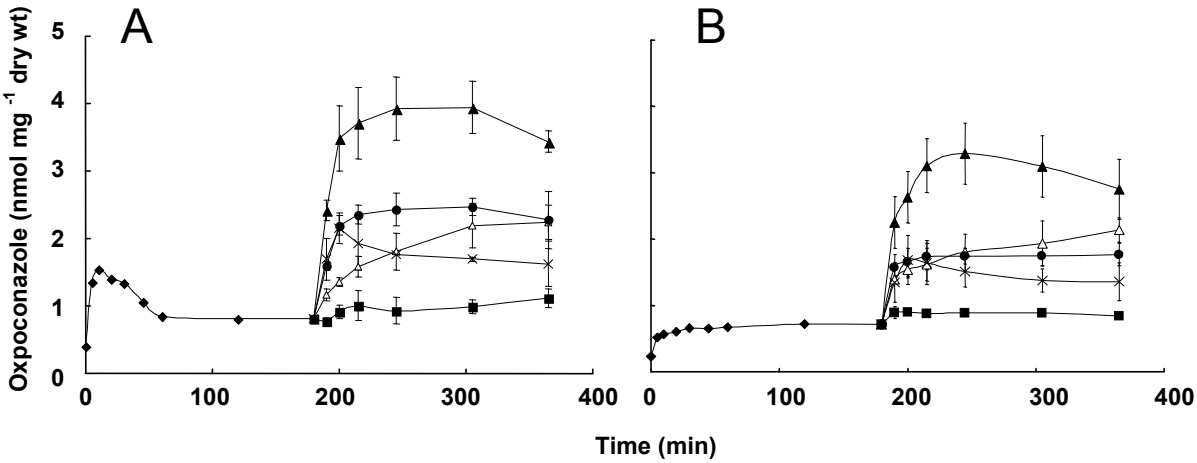

Figure 2. Effects of various compounds on the accumulation of oxpoconazole (30 µM) by germlings of *Botrytis cinerea* strains B3 (A) and G25 (B). No treatment: (◆). Treatments: methanol control (0.1%, ■), captan (100 µM, △), copper sulfate (10 µM, ●), fluazinam (10 µM, ▲), and trifloxystrobin (10 µM,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

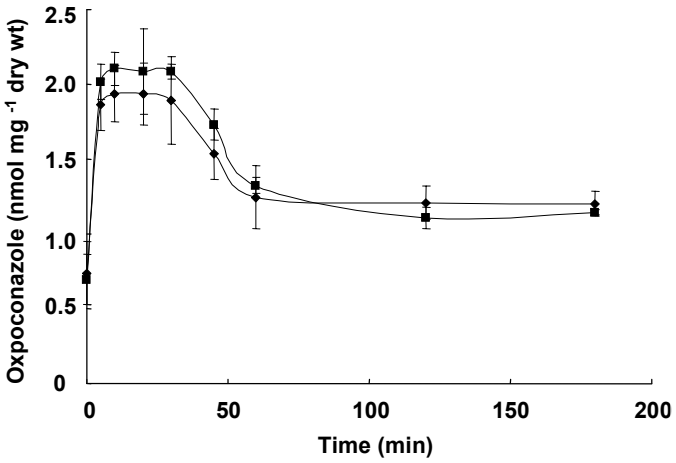

Figure 3. Accumulation of oxpoconazole (30 µM) by germlings of *Botrytis cinerea* strains B05.10 (◆) and ΔBcatrB4 (■).

Expression analysis

Expression of ABC and MFS transporter genes in the wild-type isolate B3 and the azole-resistant mutants G25 and G66 was studied in northern analysis experiments with gene-specific fragments (Table 2) as probe (39).

Basal transcript levels differed significantly for the various genes tested (Figure 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 for *BcatrB* and *BcatrK* (Figure 4). The other genes tested showed transcript signals (Figure 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 in mutants G25 and G66, except for *BcatrD* and *Bcmfs1*.

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 No.	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 [$(NBF\text{-}TMD_6)_2$ topology]; MDR: MultiDrug Resistance [$(TMD_6\text{-}NBF)_2$ topology]; 1/2 MDR: [$(TMD_6\text{-}NBF)$ topology]; MRP: Multidrug Resistance-related Protein [$TMD_n\text{-}(TMD_6\text{-}NBF)_2$ 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 No. AB28872) (24).

d: Similar to *BcatrK*.

e: Not cloned.

Additional experiments on induction of expression of *BcatrD* in the wild-type isolate B3 by oxpoconazole (30 mg L^{-1}) showed that an increase of transcripts could already be observed after 5 min of treatment. The highest transcript levels were found after 15–30 min of treatment (Figure 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 in azole-resistant mutants became clearly differential (Figure 4). For these reasons we studied the effects of all tested azole fungicides on transcription of ABC and MFS genes after 15 and 60 min of incubation at 3 mg L^{-1}.

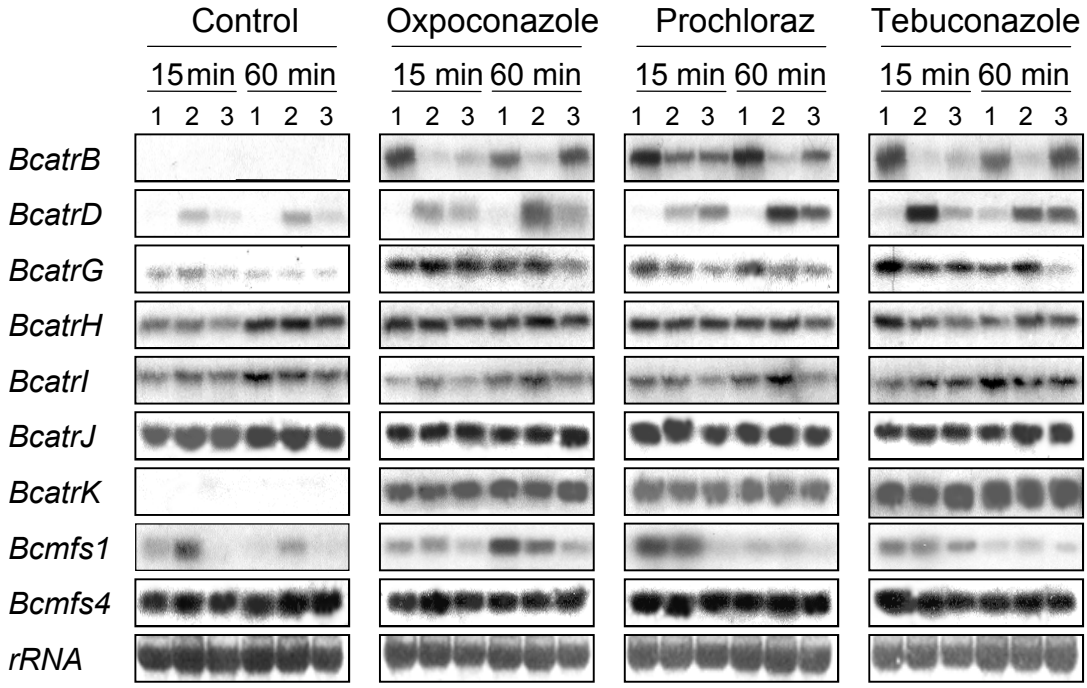

Figure 4. Effect of azole fungicides on transcript levels of ABC and MFS genes in northern blot analysis with RNA from germlings of *Botrytis cinerea*. Control (0.1% methanol): basal levels of expression. Treatments (3 mg L^{-1}): azole fungicides oxpoconazole, prochloraz, and tebuconazole. Northern 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 rRNA.

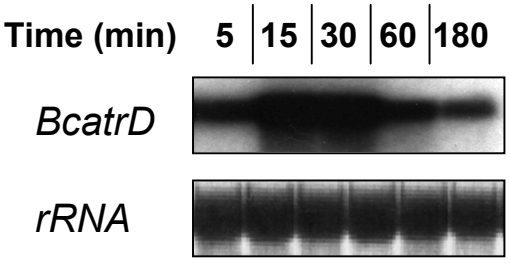

Figure 5. Time-course of *BcatrD* transcription in wild-type strain B3 of *Botrytis cinerea* after treatment of germlings with oxpoconazole (30 mg L^{-1}). Treatment for 5, 15, 30, 60, and 180 min. Equal loading of lanes with RNA was checked by subsequent probing of the same blot with 28S rRNA.

None of the tested azole fungicides induced transcript levels of *BcatrA, BcatrC, BcatrE, BcatrF, BcatrL, BcatrM, BcatrN,* and *Bcmfs2* to detectable levels in any of the tested strains (results not shown). The treatments did not markedly influence the transcript signals of *BcatrH, BcatrI, BcatrJ,* and *Bcmfs4* (Figure 4). In contrast, the treatments induced high transcript levels of *BcatrB, BcatrD, BcatrG, BcatrK,* and *Bcmfs1* in wild-type strain B3 (Figure 4). Differential effects of azoles on transcript levels among the tested strains 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 (Figure 4).

The effect of compounds from other classes of fungicides was tested on expression of *BcatrB*, *BcatrD,* and *Bcmfs1* only (Figure 6). Carbendazim enhanced transcript levels of *BcatrD* in mutants G25 and G66. The effect was strongest upon treatment for 60 min. Cycloheximide had an effect on transcript levels of *BcatrD* in both mutants similar to that of carbendazim. In addition, the antibiotic also induced *BcatrB,* but relatively stronger in the wild-type than in the mutants. This effect was observed only 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 the signals were relatively strong in strain B3.

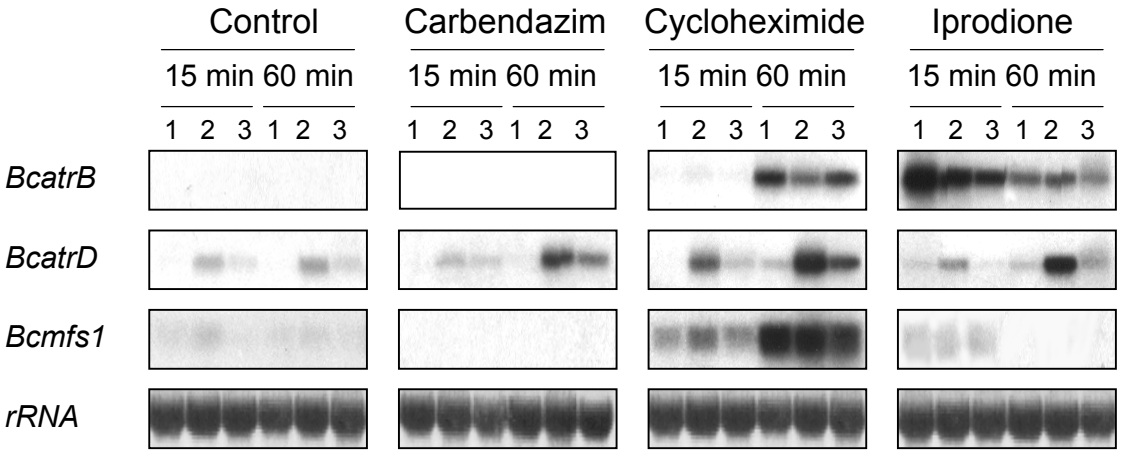

Figure 6. Effect of different fungicides on transcript levels of *BcatrB, BcatrD,* and *Bcmfs1* in northern blot analysis with RNA from germlings of *Botrytis cinerea*. Control (water): basal levels of expression. Treatments: carbendazim (30 mg L^{-1}), cycloheximide (50 mg L^{-1}), and iprodione (30 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 rRNA.

DISCUSSION

Mutants of *B. cinerea* selected for resistance to azoles have a low degree of cross-resistance to unrelated 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 (17). 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 that 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 (12, 14). The fact that various respiratory inhibitors (captan, copper sulfate, fluazinam, and trifloxystrobin) enhance the accumulation of oxpoconazole in both wild-type and azole-resistant strains 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* (37) and in azole-resistant field isolates of *B. cinerea* (6).

Field isolates of *B. cinerea* with a reduced sensitivity to azoles are strains SD29 and D12 (37) and strains with an AniR3 phenotype (6, 23). 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 that of the wild-type isolate (37). Remarkably, accumulation of the anilinopyrimidine fungicide pyrimethanil by wild-type and AniR3 strains was similar, whereas initial accumulation of the azole fungicide tebuconazole (100 μM) by AniR3 strains was significantly lower than that by wild-type strains (6). However, the transporter involved in efflux of azoles in AniR3 mutants is probably different from that 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 cannot be due to overexpression of *BcatrB* because the gene replacement mutant ΔBcatrB4 shows an accumulation of

oxpoconazole similar to that of the wild-type isolate B05.10 (Figure 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 tested azole fungicides upregulate expression stronger in mutants 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* (26). BcatrD could also be induced with the nonazole 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. This hypothesis is tested by characterization of the phenotype of gene replacement and overexpression mutants of *BcatrD* in Chapter 4.

Some fungicides induce expression of particular ABC genes, whereas 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 cannot act as a substrate of the encoded transporter protein. This phenomenon has been described before (2, 35).

Transcription of *BcatrG* and *BcatrK* was induced by azole fungicides, but induced transcript levels were similar in the wild-type isolates and in both mutants (Figure 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 than in 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 (1) 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* also. 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 (4, 10, 27). 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 (Figure 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* (20).

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Chapter 4

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

Hayashi, K., Schoonbeek, H., and De Waard, M. A. 2002. Pesticide Biochemistry and Physiology 73: 110-121.

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. ex Fr., anamorph of *Botryotinia fuckeliana* (De Bary) is the causal agent of many grey mould diseases of worldwide importance. The pathogen has an extremely wide host range (5), 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 (21). 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) (30). 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 (10) of the target enzyme $P450_{14DM}$. These mechanism has been reported in *Candida albicans* (19, 20) and *Penicillium digitatum* (16). Resistance can also be due to decreased accumulation of DMIs in mycelium as a result of active efflux, as demonstrated in *Aspergillus nidulans* (8) and *P. italicum* (9). The efflux can be mediated by ATP-binding cassette

(ABC) and major facilitator superfamily (MFS) transporters (7). 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 (13). ABC transporters involved in energy-dependent efflux of DMIs have been described for *A. nidulans* (6), *C. albicans* (23), *Mycosphaerella graminicola* (36), and *P. digitatum* (22). 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* remains to be elucidated (4). An MFS transporter involved in resistance to DMIs was reported in *Saccharomyces cerevisiae* (1). 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 (11). MDR mediated by ABC transporters in fungi has been described for *A. nidulans* (2), *B. cinerea* (25) and *P. digitatum* (22).

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 (26), it is not clear what the reason for the poor field performance of DMIs is (27). 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* (17, 34) and demonstrated that *BcatrD* and *Bcmfs1* showed higher transcript levels in DMI-resistant laboratory mutants as compared to the parental strain (17). 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, USA), and fenarimol (Eli Lilly and Company, Indianapolis, IN, USA) 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, USA). Progesterone was purchased from BDG Chemical Ltd. (Poole, England).

Fungal strains

B. cinerea strain B05.10 provided by P. Tudzynski (Institut für Botanik, Westfälische Wilhelms-Universität, Münster, Germany), is a haploid strain derived from SAS56 isolated by F. 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 (25). These strains were maintained on MEA plates (malt extract agar; Oxoid, 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 to 7 days.

Library screening

A genomic library of strain SAS56 in λEMBL3 was kindly provided by A. ten Have (Laboratory of Phytopathology, Wageningen University, Wageningen, The Netherlands) and screened with an EST gene fragment from *BcatrD* (Figure 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 (24). *Escherichia coli* strain DH5α was used for propagation of the constructs. DNA was sequenced with the BigDye (Perkin-Elmer, CT, USA) and DyEnamic ET (AP-biotech, USA) Terminator

Cycle Sequencing kits. The sequence of fragments of *BcatrD* present in a purified phage was also determined by the primer-walk method. First primers' sequences were based on the EST.

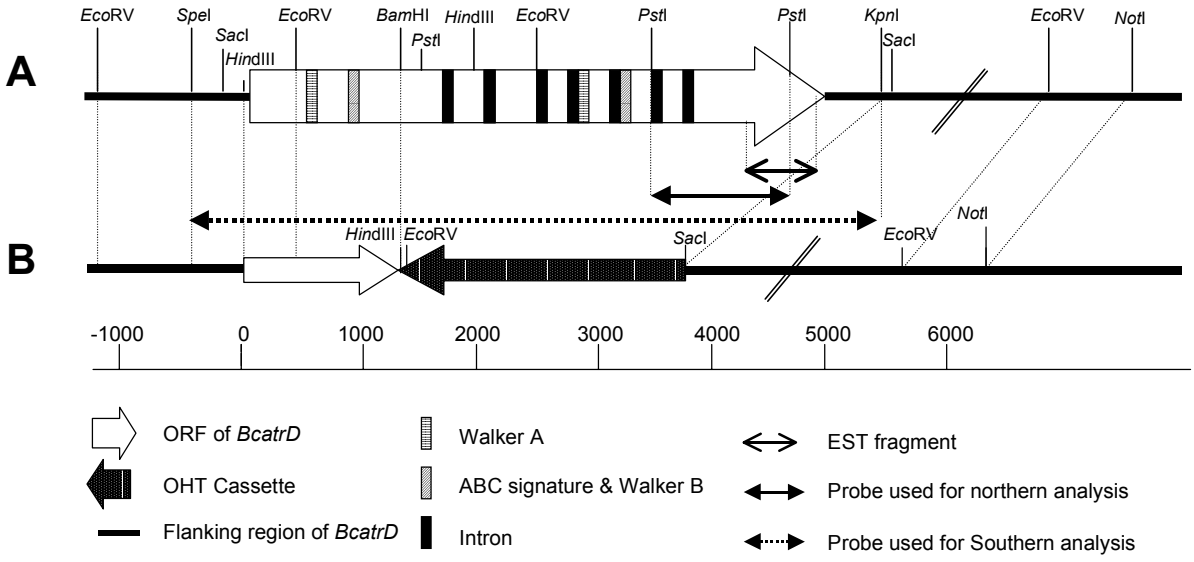

Figure 1. Physical map of *BcatrD* from *Botrytis cinerea* wild-type strain B05.10 (A) and a replacement mutant of *BcatrD* (B).

cDNA synthesis

cDNA was amplified by RT-PCR using the SUPERSCRIPT One-Step RT-PCR with PLATINUM *Taq* system (Life Technologies, 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, USA).

Multiple alignment of cDNA was performed by ClustalW analysis provided by the European Bioinformatics Institute (29). 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.

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 (Figure 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* tublin terminator, kindly provided by J. A. L. van Kan and 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 (Figure 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 fragment 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 (Figure 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 (33). 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.* (12). 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-N^+ membranes (Amersham Pharmacia Biotech, Uppsala, Sweden). The 6.1 kb *Spe*I-*Kpn*I fragment (Figure 1) was labeled as described previously (17) and hybridized with the Southern blot at 65°C overnight.

Northern blot analysis was performed almost same as described previously with slight modification (17). 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 (24). The 1.1 kb *Pst*I fragment was used as a gene specific probe (Figure 1).

Sensitivity assay

Sensitivity tests were performed as described previously (4). 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 (9). 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 PLUS, LUMAC*LSC B.V., Groningen, The Netherlands) for 1 day and counted in a liquid scintillation spectrometer BECKMAN LS6000TA (Beckman Coulter, CA, USA).

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 (Figure 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; Accession No. 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 (Figure 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 (31). 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 (14). 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* (Figure 2). Hydropathy analysis (18) indicated that BcatrD has two hydrophilic domains including the nucleotide-binding fold (NBF) and 12 hydrophobic domains including transmembrane domains (TMD), which are typical for ABC transporters with a $[NBF\text{-}TMD_6]_2$ topology (35). Walker A, B and ABC signatures were found in the hydrophilic regions of BcatrD (Table 1).

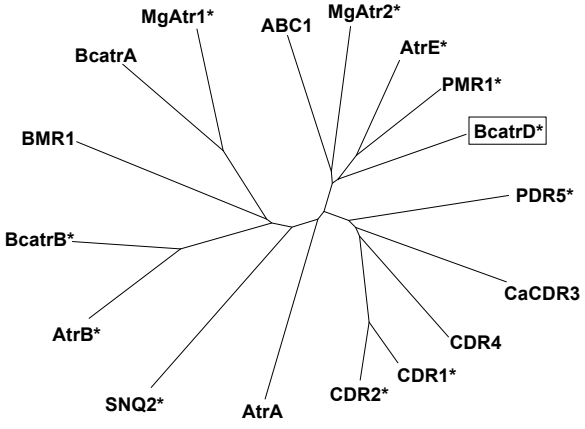

Figure 2. Dendrogram of 17 fungal and yeast ABC transporter proteins with the $[NBF\text{-}TMD_6]_2$ topology. AtrA (46.2%, Z68904), AtrB (33.0%, Z68905), and AtrE (60.5%, AJ276241) from *Aspergillus nidulans*, BcatrA (32.8%, Z68906), BcatrB (33.1%, AJ006217), BcatrD (100%, AJ272521), and BMR1 (33.2%, AB028872) from *Botrytis cinerea*, CDR1 (46.8%, X77589), CDR2 (45.4%, U63812), CDR3 (45.4%, U89714), and CDR4 (47.9%, AF044921) from *Candida albicans*, ABC1 (54.5%, AF032443) from *Magnaporthe grisea,* MgAtr1 (34.2%, AJ243112) and MgAtr2 (53.2%, AJ243113) from *Mycosphaerella graminicola*, PMR1 (56.4%, AB010442) from *Penicillium digitatum,* and PDR5 (46.5%, L19922) and SNQ2 (37.2%, X66732) from *Saccharomyces cerevisiae.* Between brackets: identity percentages with BcatrD and EMBL database accession numbers. Asterisks indicate proteins involved in efflux of DMI fungicides.

Table 1. Alignment of the ATP-binding domain of *BcatrD* and other ABC transporters from filamentous fungi[a]

N-terminus

Protein	Walker A	ABC-signature	Walker B
BcatrD	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	
ABC1	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	
ABC1	926-LTALMGVSGAGKTTLLD	1035-GLNVEQRKRLTIGVELAAKPPLLLFVDEPTSGLD	
MgAtr1	934-MVALMGASGAGKTTLLN	1039-SLGVEQRKRLTIGVELAAKPSLLLFLDEPTSGLD	
MgAtr2	870-LTALMGVSGAGKTTLLD	979-GLNVEQRKRLTVGVELAAKPQLLLFLDEPTSGLD	
PMR1	857-CTALMGVSGAGKTTLLD	966-GLNVEQRKRLTIGVELAAKPQLLLFLDEPTSGLD	
	**** *********	* ***** * **** * * * ** *****	

a: Identical sequences are marked with asterisks. Accession numbers are mentioned in Figure 2. AtrA, AtrB, and AtrE are from *Aspergillus nidulans*, BcatrA, BcatrB, BcatrD, and BMR1 from *Botrytis cinerea*, ABC1 from *Magnaporthe grisea*, MgAtr1 and MgAtr2 from *Mycosphaerella graminicola*, and PMR1 from *Penicillium digitatum*.

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 (Figure 1). Lanes with DNA from the parental strain B05.10 showed three bands (4.3, 2.0, and 1.8 kb) (Figure 3).

Homokaryotic transformants were expected to show two bands (1.8 and 0.7 kb) (Figure 1). This was observed in lanes with DNA from two transformants (ΔBcatrD-8 and ΔBcatrD-12) (Figure 3). The reference strain HR-9, obtained by transformation with pLob1, and strain ΔBcatrB4 revealed the same hybridization pattern as the parental strain B05.10 (Figure 3).

Transcript levels of *BcatrD* in the parental isolate B05.10 and the various mutants were studied with the *BcatrD* specific *Pst*I probe (Figure 1). Basal transcript levels were not detectable in any of the gene replacement mutants tested (Figure 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 reference strains HR-9 and ΔBcatrB4. No transcripts were observed in the replacement mutants ΔBcatrD-8 and ΔBcatrD-12 (Figure 4).

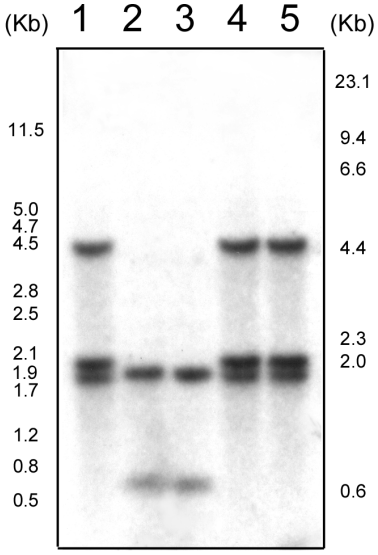

Figure 3. Southern blot analysis with DNA of *Botrytis cinerea* parental strain B05.10 (lane 1), two *BcatrD* replacement mutants ΔBcatrD-8 (lane 2) and ΔBcatrD-12 (lane 3), and two reference strains HR-9 (lane 4) and ΔBcatrB4 (lane 5). Genomic DNA (5 µg) was digested with *Eco*RV and hybridized with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

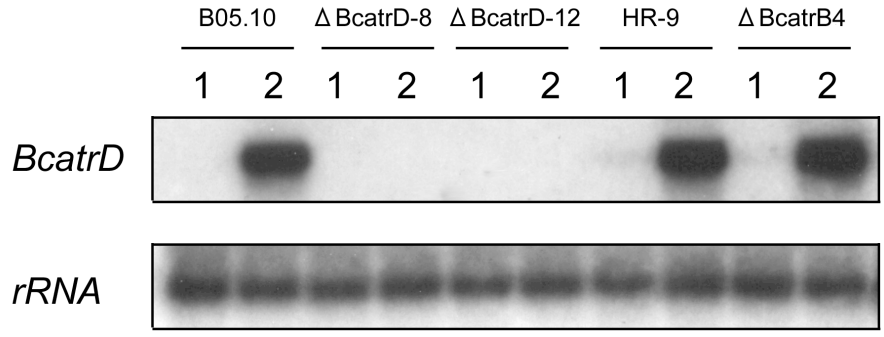

Figure 4. Northern analysis of replacement mutants of *BcatrD* with RNA from germlings of *Botrytis cinerea* parental strain B05.10, two *BcatrD* replacement mutants ΔBcatrD-8 and ΔBcatrD-12, and two reference strains HR-9 and ΔBcatrB4. Basal levels of expression (lanes1). Treatment with 30 mg L^{-1}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

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* (Figure 5). Codes of representative mutants are indicated between brackets. Transcript levels of *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).

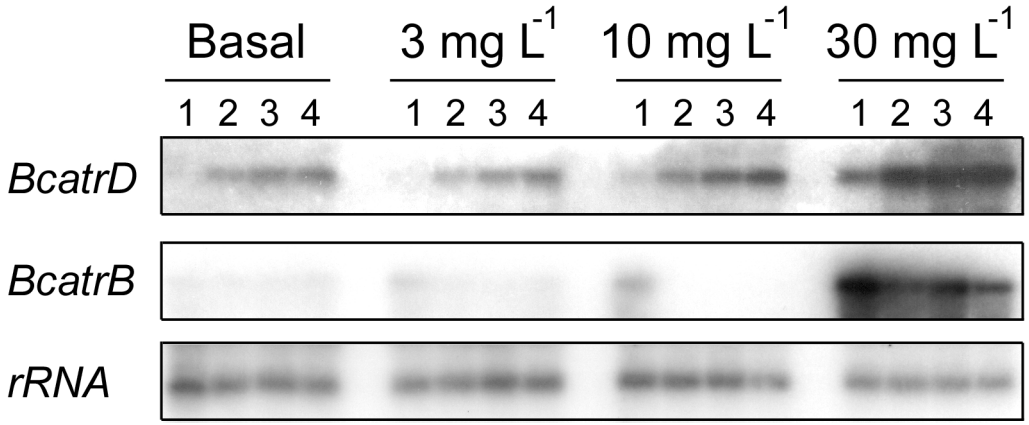

Figure 5. Northern analysis of overexpression mutants of *BcatrD* with RNA from germlings of *Botrytis cinerea* parental strain B05.10 (lanes 1), and the *BcatrD* overexpression mutants OVD-15 (lanes 2), OVD-21 (lanes 3), and OVD-2 (lanes 4). The figure shows basal levels of expression and induced expression after treatment with 3, 10, and 30 mg L^{-1} oxpoconazole. RNA was hybridized with a 1.1 kb *Pst*I probe from *BcatrD* (Figure 1) and 0.7 kb *Hind* III probe from *BcatrB* (25). 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, Figure 6). The overexpression

mutants showed a decreased sensitivity to all DMIs tested (Table 2, Figure 6). 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 (reference 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} value (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 ($P = 0.05$).

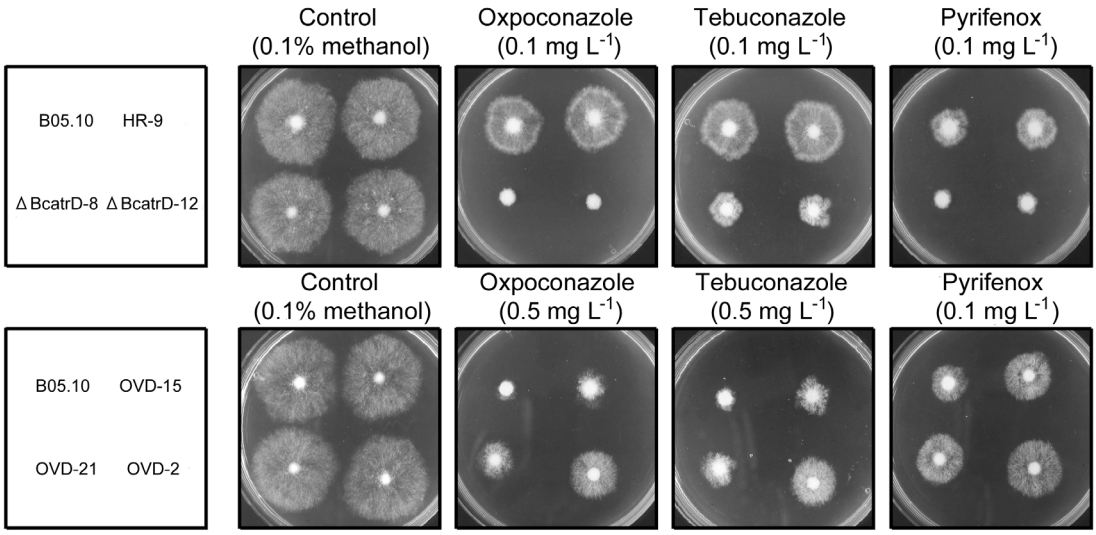

Figure 6. Activity of DMI fungicides from different chemical classes [oxpoconazole (an imidazole), tebuconazole (a triazole), and pyrifenox (a pyridine)] in radial growth experiments of wild-type strain B05.10, reference transformant HR-9, gene replacement mutants (ΔBcatrD-8 and ΔBcatrD-12), and gene 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. *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 (Figure 7).

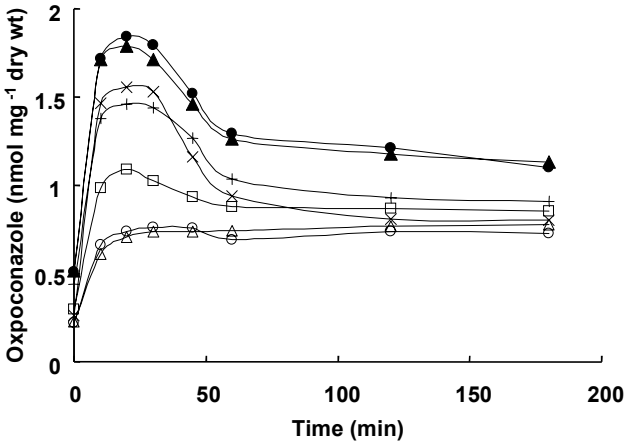

Figure 7. Accumulation of oxpoconazole (30 μM) by germlings of *Botrytis cinerea* wild-type strain B05.10 (+), reference transformant HR-9 (×), replacement mutants ΔBcatrD-8 (●) and ΔBcatrD-12 (▲), and overexpression mutants OVD-15 (□), OVD-21 (○), and OVD-2 (△).

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 that of 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 $[NBF\text{-}TMD_6]_2$ 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 $[NBF\text{-}TMD_6]_2$ topology (Figure 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. Example 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 (32), and substrate specificity of MgAtr2 from *M. graminicola* and PMR1 from *P. digitatum* also seems to be limited (L.-H. Zwiers, personal communication, 22).

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 (17). However, *BcatrD* replacement mutants still display a transient accumulation of oxpoconazole in time (Figure 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* (17).

Besides DMIs, the anilinopyrimidine fungicide cyprodinil, the dicarboximide fungicide iprodione, and the antibiotic cycloheximide also induce transcription of *BcatrD* (17, 34). 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 (25). 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 (17). Another possibility is that activity of other ABC or MFS transporters compensates for the decrease in activity of BcatrD (25).

The overexpression mutants with increased transcript levels of *BcatrD* as compared to B05.10 have relatively low transcript levels of *BcatrB* (Figure 5). This observation can probably be ascribed to the fact that the mutants accumulate less oxpoconazole. 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* (3) which probably carry a mutation in a gene regulating expression of ABC transporter genes.

A common function of transporters in pathogenic fungi is protection against natural toxic compounds (*e.g.* plant defence compounds or antibiotics) (7). 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 that of parental strain B05.10, indicating that plant defence products in tomato leaves do not seem to act as substrates of BcatrD. These results also support the hypothesis that BcatrD has narrow substrate specificity. Natural substrate(s) of BcatrD may be found by testing the sensitivity of *BcatrD* replacement 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 (15). 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 (28). The reason for this variation in sensitivity can probably be attributed to different factors, which have not been fully understood (27). 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 (4, 21) can be attributed to overexpression of *BcatrD* or other transporter genes.

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Chapter 5

Bcmfs1, a novel major facilitator superfamily transporter from *Botrytis cinerea*, provides tolerance towards the natural toxic compounds camptothecin and cercosporin and towards fungicides

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ABSTRACT

Bcmfs1, a novel major facilitator superfamily (MFS) gene from *Botrytis cinerea,* was cloned and replacement and overexpression mutants were constructed to study its function. Replacement mutants showed increased sensitivity to the natural toxic compounds camptothecin and cercosporin, produced by the plant *Camptotheca acuminata* and the plant pathogenic fungus *Cercospora kikuchii,* respectively. Overexpression mutants displayed decreased sensitivity to these compounds and to structurally unrelated fungicides such as sterol demethylation inhibitors (DMIs). A double replacement mutant of *Bcmfs1* and the ATP-binding cassette (ABC) transporter gene *BcatrD* was more sensitive to DMI fungicides than a single replacement mutant of *BcatrD*, known to encode an important ABC transporter of DMIs. The results indicate that Bcmfs1 is a MFS multidrug transporter involved in protection against natural toxins and fungicides and has a substrate specificity that overlaps with the ABC transporter BcatrD. Bcmfs1 may be involved in protection of *B. cinerea* against plant defence compounds during the pathogenic phase of growth on host plants and against fungitoxic antimicrobial metabolites during its saprophytic phase of growth.

INTRODUCTION

Microorganisms in their natural environment need to protect themselves from adverse effects caused by natural toxic compounds. This also accounts for *Botrytis cinerea* Pres. ex Fr. [anamorph of *Botryotinia fuckeliana* (De Bary)], a plant pathogenic fungus with a wide host range that can also grow as a saprophyte (8). Thus, the fungus has to cope with natural toxic compounds produced by host plants during pathogenesis and with antagonistic microorganisms during the saprophytic phase. ATP-binding cassette (ABC) and major facilitator superfamily (MFS) transporters can enable the fungus to survive exposure to toxic compounds. These membrane-bound proteins are known to provide protection against a wide range of natural toxic compounds and xenobiotics (12). ABC transporters use the energy of ATP hydrolysis to transport compounds over membranes. They may have a broad substrate range including unrelated chemicals such as sugars, inorganic ions, heavy metals, peptides, amino acids, oligopeptides, polysaccharides, proteins, and drugs (18). Transporters located in the plasma membranes can transport toxic compounds from the inner leaflet of these membranes to the outer environment of cells, thereby reducing accumulation of the compounds in the cells (14). ABC transporter activity in filamentous fungi involved in energy-dependent efflux of fungicides has been demonstrated for

Aspergillus nidulans (3) and *B. cinerea* (34). Overexpression of ABC transporters can result in resistance to sterol demethylation inhibitors (DMIs) as reported for *A. nidulans* (3, 11), *B. cinerea* (17), *Candida albicans* (24), *Penicillium digitatum* (20), and *Saccharomyces cerevisiae* (18). MFS transporters may also prevent accumulation of toxic compounds in cells, but their activity is driven by the proton-motive force over membranes (21). MFS transporters from *C. albicans* (5) and *S. cerevisiae* (1) are involved in protection against exogenous toxic compounds, such as DMIs. In filamentous fungi, a number of MFS transporters are known to mediate the secretion of endogenously produced toxins (22) such as aflatoxin, cercosporin, *Helminthosporium carbonum* toxin (HC toxin), and trichothecene by *A. flavus* (Chang, P. K., Yu, J., Bhatnagar, D., and Cleveland, T. E., Abstr. 99th Gen. Meet. Am. Soc. Microbiol., abstr. O-31, p. 501, 1999), *Cercospora kikuchii* (6), *Cochliobolus carbonum* (23), and *Fusarium sporotrichioides* (2), respectively. This may result in self-protection of the producing organisms against these compounds. So far, a role of MFS transporters of filamentous fungi in protection against synthetic drugs, such as fungicides, has not been reported.

Recently, we demonstrated that *B. cinerea* possesses multiple ABC and MFS transporter genes (16, 34) and showed that the ABC transporter BcatrB plays a role in protection against the plant defence compound resveratrol and phenylpyrrole fungicides (29). Similarly, the ABC transporter BcatrD provides protection against DMIs (17). Overexpression of these transporters in laboratory-generated mutants resulted in multidrug resistance (MDR) to fungicides and unrelated chemicals (16). This mechanism may also apply to fungicide resistance development under field conditions (7, 19).

In this paper, we describe the isolation of the MFS gene *Bcmfs1* from *B. cinerea.* We constructed *Bcmfs1* replacement and overexpression mutants and phenotyped these mutants for sensitivity to compounds from different chemical classes. The differential sensitivity of the mutants to the DMI fungicide oxpoconazole correlated with expression levels of *Bcmfs1* and with accumulation of the fungicide by germlings of the mutants. We propose that Bcmfs1 functions in protection against natural toxins, DMI fungicides, and other unrelated compounds. Hence, Bcmfs1 is the first MFS multidrug transporter of a filamentous fungus for which multiple substrates have been described.

MATERIALS AND METHODS

Fungal strains

B. cinerea strain B05.10 (4), provided by P. Tudzynski (Institut für Botanik, Westfälische Wilhelms-Universität, Münster, Germany), is a haploid strain derived from SAS56 isolated by F. Faretra (Università of Bari, Bari, Italy). B05.10 was used as the parental isolate in all experiments. B05.10 and mutants constructed (Table 1) were maintained on malt extract agar plates (Oxoid Ltd., Basingstoke, Hampshire, England) amended with 0.2% yeast extract (Oxoid) at 20°C. Formation of conidia was induced by irradiation with near-UV light for 24 h after 3 days of incubation and prolonged incubation for 3 to 7 days. Conidial suspensions were stored in 15% glycerol at -20°C.

Plasmids carrying a hygromycin resistance cassette (pLob1) and a nourseothricin resistance cassette (pNR2) were gifts from J. van Kan, S. Schouten, and I. Kars (Laboratory of Phytopathology, Wageningen University, Wageningen, The Netherlands). pNR2 is derived from pNR1 (kindly provided by P. and B. Tudzynski, Westfälische Wilhelms-Universität, Münster, Germany). pLob1 carries the *Escherichia coli* hygromycin phosphotransferase-encoding gene *hph*, and pNR2 carries the *Streptomyces noursei* nourseothricin acetyltransferase-encoding gene *nat*-1 (Werner-Bioagents, Jena, Germany) under control of the *A. nidulans* oliC promoter and a *B. cinerea* β–tubulin transcription terminator fragment.

Compounds

Oxpoconazole, iprodione, and prochloraz (technical grade) were obtained from Ube Industries, Ltd. (Ube, Yamaguchi, Japan), captan and tebuconazole were obtained from Bayer AG (Leverkusen, Germany), cyprodinil and pyrifenox were obtained from Syngenta (Stein, Switzerland), fenarimol was obtained from Eli Lilly and Company (Indianapolis, IN, USA), and fluazinam was obtained from ISK Bioscience Co. (Mentor, OH, USA). Barbaloin, camptothecin, cercosporin, colchicine, cycloheximide, 8-methoxypsoralen, reserpine, rhodamine 6G, rose bengal, toluidine blue, and vincamine were purchased from Sigma (St. Louis, MO, USA), globulol and patchoulol were purchased from Fluka Chemie AG (Buchs, Switzerland), and hypericin and pseudohypericin were purchased from Planta Naturstoffe (Wien, Austria).

Cloning of *Bcmfs1*

Cloning of *Bcmfs1* was performed following an approach similar to that described for *BcatrD* (17). An Expressed Sequence Tags (EST) fragment of *Bcmfs1* (Figure 1A) obtained by PCR amplification was used for screening of a genomic library of strain SAS56 in λEMBL3, provided

by A. ten Have (Laboratory of Phytopathology, Wageningen University, Wageningen, The Netherlands). Positive and purified phages were subcloned in pBluescript II SK and sequenced. DNA manipulations were performed according to standard methods (25). *E. coli* strain DH5α was used for propagation of the constructs.

Table 1. *Botrytis cinerea* strains used in this study

Strain	Character	Reference
B05.10	Wild-type strain	4
ΔBcatrB4	*BcatrB* replacement mutant derived from B05.10 carrying the hygromycin resistance cassette	29
ΔBcatrD-8	*BcatrD* replacement mutant derived from B05.10 carrying the hygromycin resistance cassette	17
ΔBcmfs1-16 and ΔBcmfs1-18	*Bcmfs1* replacement mutant derived from B05.10 carrying the hygromycin resistance cassette	This study
OV1-23, OV1-48, and OV1-13	*Bcmfs1* overexpression mutant derived from B05.10 carrying the hygromycin resistance cassette with a low, medium, and high level of resistance to oxpoconazole, respectively	This study
HR-9	Reference strain derived from B05.10 carrying an ectopic integration of the hygromycin resistance cassette	17
ΔBΔ1-22	*BcatrB* and *Bcmfs1* double replacement mutant derived from ΔBcatrB4 carrying both the hygromycin and nourseothricin resistance cassettes	This study
ΔDΔ1-45	*BcatrD* and *Bcmfs1* double replacement mutant derived from ΔBcatrD-8 carrying both the hygromycin and nourseothricin resistance cassettes	This study
HNR-4	Reference strain derived from B05.10 carrying an ectopic integration of both the hygromycin and nourseothricin resistance cassettes	This study

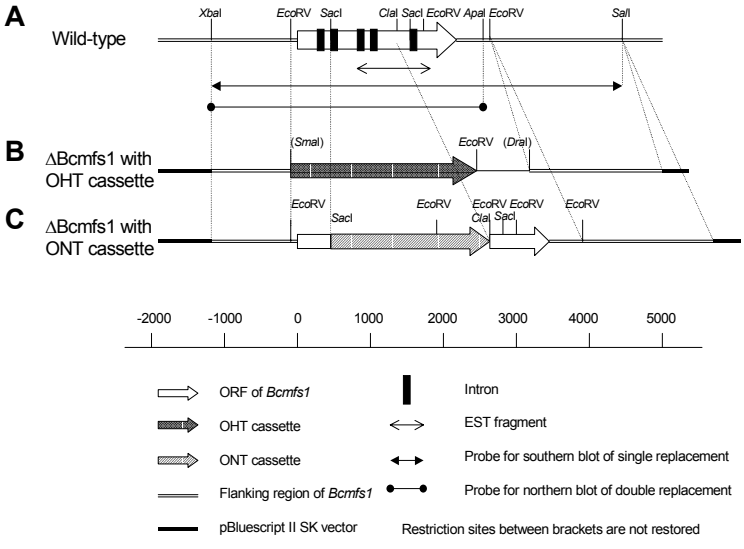

Figure 1. Physical map of *Bcmfs1* in genomic DNA from *Botrytis cinerea* wild-type strain B05.10 (A), and replacement mutant of *Bcmfs1* with the hygromycin resistance (OHT) cassette (B), or the nourseothricin resistance (ONT) cassette (C). Southern blots of DNA from single replacement mutants and double replacement mutants of *Bcmfs1* were hybridized with the 5.5 kb *Xba*I-*Sal*I fragment and the 3.6 kb *Xba*I-*Apa*I fragment, respectively. Northern blots were hybridized with the EST fragment of *Bcmfs1*.

cDNA was amplified by reverse transcription-PCR (RT-PCR) using the SUPERSCRIPT One-Step RT-PCR with PLATINUM *Taq* system (Life Technologies Inc., Breda, The Netherlands). Multiple alignment of protein sequences derived from cDNA was performed by ClustalW analysis (http://www2.ebi.ac.uk/clustalw/) provided by the European Bioinformatics Institute (31). Homology of the putative protein sequence derived from *Bcmfs1* cDNA with other MFS proteins was calculated using the Clustal method by the program Megalign (DNAstar, Madison, WI, USA).

Construction of *Bcmfs1* replacement mutants

In a 8.5 kb *Xba*I-*Sal*I subclone (5.5 kb *Xba*I-*Sal*I fragment and 3.0 kb pBluescript II SK vector) containing the full-length *Bcmfs1* gene and its flanking regions, the 2.4 kb *Eco*RV fragment containing *Bcmfs1* was replaced by the hygromycin resistance cassette (OHT cassette) from pLob1 to construct the replacement vector pΔ1-H (Figure 1B). Before transformation, the plasmid was linearised with *Xho*I. Transformation was performed as described for replacement of the *BcatrD* gene (17).

Construction of double replacement mutants

In a 8.5 kb *Xba*I-*Sal*I subclone (5.5 kb *Xba*I-*Sal*I fragment and 3.0 kb pBluescript II SK vector), the 1.1 kb *Sac*I-*Cla*I fragment was replaced by the nourseothricin-resistance cassette (ONT cassette) from pNR to construct the replacement vector pΔ1-N (Figure 1C). For the construction of double replacement mutants, protoplasts from ΔBcatrB4 (29) or ΔBcatrD-8 (17) were transformed with 1 µg of pΔ1-N. Before transformation, the plasmid was linearised with *Xho*I. The selection and purification of putative transformants were performed by three successive transfers on malt extract agar amended with 100 mg L^{-1} of nourseothricin (Werner bioagents, Jena-Cospeda, Germany) followed by single-spore isolation.

Construction of *Bcmfs1* overexpression mutants

A subclone containing the 5.5 kb *Xba*I-*Sal*I fragment (Figure 1A) in pBluescript II SK (3.0 kb) was used to generate overexpression mutants by co-transformation with pLob1 to protoplasts of B05.10 as described previously (17).

Southern and northern blot analysis

Southern and northern blot analyses were performed as described before (17). In Southern blot experiments, 5 µg genomic DNA was digested with *Eco*RV and hybridized with the 5.5 kb *Xba*I-*Sal*I probe to characterize *Bcmfs1* single replacement mutants or the 3.6 kb *Xba*I-*Apa*I probe to characterize double replacement mutants as presented in Figure 1A. In northern blot analysis experiments, 10 µg total RNA was loaded on agarose gel (1.6%), and the blots were hybridized with the EST fragment of *Bcmfs1* (Figure 1A).

Phenotype assay

The phenotype of replacement and overexpression mutants was studied by investigating their sensitivity to compounds in radial growth experiments as described previously (7). 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 in the dark for 3 days. The sensitivity to camptothecin, cercosporin, and other photosensitizers was investigated in the light as well as in the dark. Effective concentrations inhibiting radial growth by 50% (EC_{50}s) of chemicals were calculated from dose-response curves using Excel 97. Experiments were repeated three times, and statistical analysis of the EC_{50}s was performed by the LSD (*t* test).

Accumulation of oxpoconazole

Accumulation experiments were performed as described previously (13). Germling suspensions (4 mg wet weight per ml) 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 and 180 rpm 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 various 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 PLUS, LUMAC*LSC B.V., Groningen, The Netherlands) for 1 day and counted in a liquid scintillation spectrometer BECKMAN LS6000TA (Beckman Coulter Inc., CA, USA).

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 spotted onto the surface of the tomato leaves. The wild-type isolate B05.10 and the mutants were inoculated on two halves of the same leaf. 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 *Bcmfs1*

Screening of a phage library of *B. cinerea* with an EST fragment of *Bcmfs1* (Figure 1A) resulted in the selection of a phage containing the full-length *Bcmfs1* gene (Accession No. AF238225). Comparison of the sequence of genomic DNA and cDNA revealed that *Bcmfs1* contains a 1797 bp open reading frame (ORF) interrupted by 5 introns. The introns vary in size from 53 to 92 bp and are distributed over the whole ORF of *Bcmfs1* (Figure 1A). The 5' and 3' sequences of these introns match known intron sequences from filamentous fungi (32). The putative ORF of *Bcmfs1* has two ATG codons at the 5' end. Hence, the first codon may not belong to the ORF of *Bcmfs1*. BLAST database (http://www.ncbi.nlm.nih.gov/BLAST/) provided by the National Center for Biotechnology Information demonstrated that Bcmfs1 is homologous to other MFS transporters, such as aflT from *A. parasiticus*, CFP from *C. kikuchii*, TOXA from *C. carbonum*, and ORF10 from *A. terreus* (Figure 2). Hydropathy analysis (http://www.ch.embnet.org/software/TMPRED_form.html) provided by Swiss Institute of Bioinformatics predicts that Bcmfs1 has 14 trans membrane domains (TMDs) (results not shown).

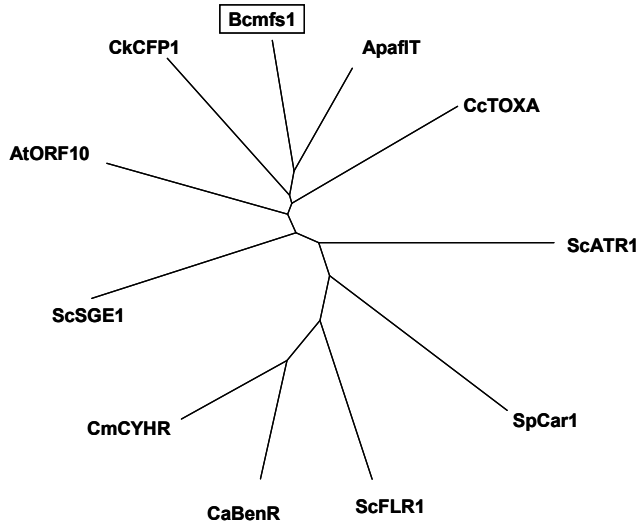

Figure 2. Dendrogram of 11 MFS transporter proteins. aflT (54.1%, AAK58582) from *Aspergillus parasiticus*, ATR1 (14.4%, M20319), FLR1 (13.5%, P38124), and SGE1 (19.9%, U02077) from *Saccharomyces cerevisiae*, Bcmfs1 (100%, AAF64435) from *Botrytis cinerea*, BenR (13.5%, X53823/Y14703) from *Candida albicans*, Car1 (14.3%, Z14035) from *Schizosaccharomyces pombe*. CFP (36.6%, AAC78076) from *Cercospora kikuchii*, CYHR (12.1%, M64932) from *Candida maltosa*, ORF10 (35.6%, AAD34558) from *Aspergillus terreus*, and TOXA (35.4%, AAB366607) from *Cochliobolus carbonum*. Between brackets: identity percentages with Bcmfs1 and EMBL database accession numbers.

***Bcmfs1* single replacement mutants**

Protoplasts of *B. cinerea* strain B05.10 were transformed with linearised pΔ1-H (Figure 1B). Thirty putative transformants were selected and purified by single spore isolation. Southern blot analysis of genomic DNA from these transformants revealed that several strains were homokaryotic transformants with a site-specific integration, including ΔBcmfs1-16 and ΔBcmfs1-18 (Figure 3A). The reference strain HR-9 carrying the OHT cassette and BcatrD replacement mutant ΔBcatrD-8 (17) showed the same bands as the parental strain B05.10 (Figure 3A). The expression of the *Bcmfs1* replacement mutants ΔBcmfs1-16 and ΔBcmfs1-18 was investigated by northern analysis with the EST fragment of *Bcmfs1* (Figure 1A) as probe. The basal level of expression of *Bcmfs1* in the parental strain B05.10, the reference strain HR-9, and ΔBcatrD-8 was low but strongly induced after treatment of germlings with oxpoconazole (30 mg L^{-1}) for 60 min. In ΔBcmfs1-16 and ΔBcmfs1-18, neither basal nor oxpoconazole-induced expression was observed (Figure 4A).

Radial growth tests demonstrated that ΔBcmfs1-16 and ΔBcmfs1-18 had an increased sensitivity to the alkaloid camptothecin (a fungitoxic compound from *Camptotheca acuminata*) and the perylenequinone cercosporin (a host-specific toxin produced by *C. kikuchii*), while the reference strain HR-9 displayed almost the same sensitivity to these compounds as the parental strain B05.10 (Figure 5A, Table 3). As expected, the fungitoxic activity of the photosensitizer cercosporin was higher in the light than in the dark (Figure 6). The fungitoxic activity of other photosensitizers, such as cercosporin analogues (hypericin and pseudohypericin), rose bengal, toluidine blue, 8-methoxypsoralen, and alkaloids (barbaloin, colchicine, reserpine, and vincamine) was similar to all strains tested and was not influenced by light (results not shown). The activity of fungicides (captan, cyprodinil, fluazinam, iprodione, oxpoconazole, prochloraz, and tebuconazole) and other fungitoxic compounds (cycloheximide and rhodamine 6G) tested was similar for the wild-type strain and all *Bcmfs1* single replacement mutants tested (results shown only for ΔBcmfs1-16 in Table 2). Similar results were obtained with the botrydial analogues patchoulol or globulol (33) (results not shown).

Double gene replacement mutants

Protoplasts of *BcatrB* replacement mutant ΔBcatrB4 (29) and the *BcatrD* replacement mutant ΔBcatrD-8 (17) were transformed with linearised pΔ1-N (Figure 1C). Southern analysis of genomic DNA from the putative transformants digested with *Eco*RV revealed that ΔBΔ1-22 and

ΔDΔ1-45 were homokaryotic transformants (Figure 3B). Northern analysis of the transformants demonstrated that basal and oxpoconazole-induced expression of *Bcmfs1* was not detectable in mutants ΔBΔ1-22 and ΔDΔ1-45 (Figure 4B).

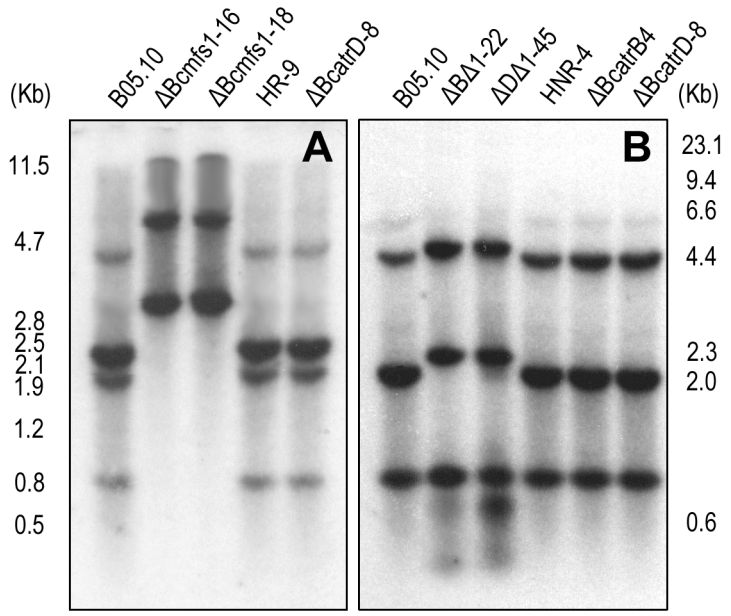

Figure 3. Southern blot analysis of DNA from *Botrytis cinerea.* Genomic DNA (5 μg) was digested with *Eco*RV and hybridized with a 5.5 kb *Xba*I-*Sal*I probe (A) and with a 3.6 kb *Xba*I-*Apa*I probe (B).

Table 2. Activity of compounds on radial growth of *Botrytis cinerea*

Chemical class	Compound	EC_{50} (mg L^{-1})[a] of compound for:			
		B05.10	ΔBcmfs1-16	OV1-13	HR-9
DMIs	Oxpoconazole	0.151 a	0.189 a	0.330 b	0.161 a
	Prochloraz	0.031 ab	0.025 a	0.088 c	0.032 ab
	Tebuconazole	0.161 a	0.151 a	0.337 b	0.136 a
Anilinopyrimidine	Cyprodinil	0.0029 a	0.0022 a	0.0060 b	0.0025 a
Dicarboximide	Iprodione	0.057 a	0.054 a	0.148 b	0.056 a
Phthalimide	Captan	6.29 a	6.30 a	12.6 b	5.58 a
Phenylpyridylamine	Fluazinam	0.0021 a	0.0021 a	0.0037 b	0.0019 a
Antibiotic	Cycloheximide	3.42 b	3.55 b	1.94 a	3.10 ab
Xenobiotic	Rhodamine 6G	1.53 ab	1.88 b	1.16 a	1.47 ab

a: Means followed by the same letters in the same rows indicate that figures do not differ significantly ($P = 0.05$).

The sensitivity of the double replacement mutant ΔDΔ1-45 to the DMI fungicide oxpoconazole was higher than that of the single replacement mutant ΔBcatrD-8 (Figure 5B, Table 3). This was not observed for the double replacement mutant ΔBΔ1-22 (Figure 5B). Differential activities against ΔDΔ1-45 and ΔBcatrD-8 were also observed with other DMI fungicides, such as fenarimol, prochloraz, pyrifenox, and tebuconazole (Table 3). In contrast, camptothecin did have activity similar to that of ΔBcmfs1-16, ΔBΔ1-22, and ΔDΔ1-45 (Figure 5B).

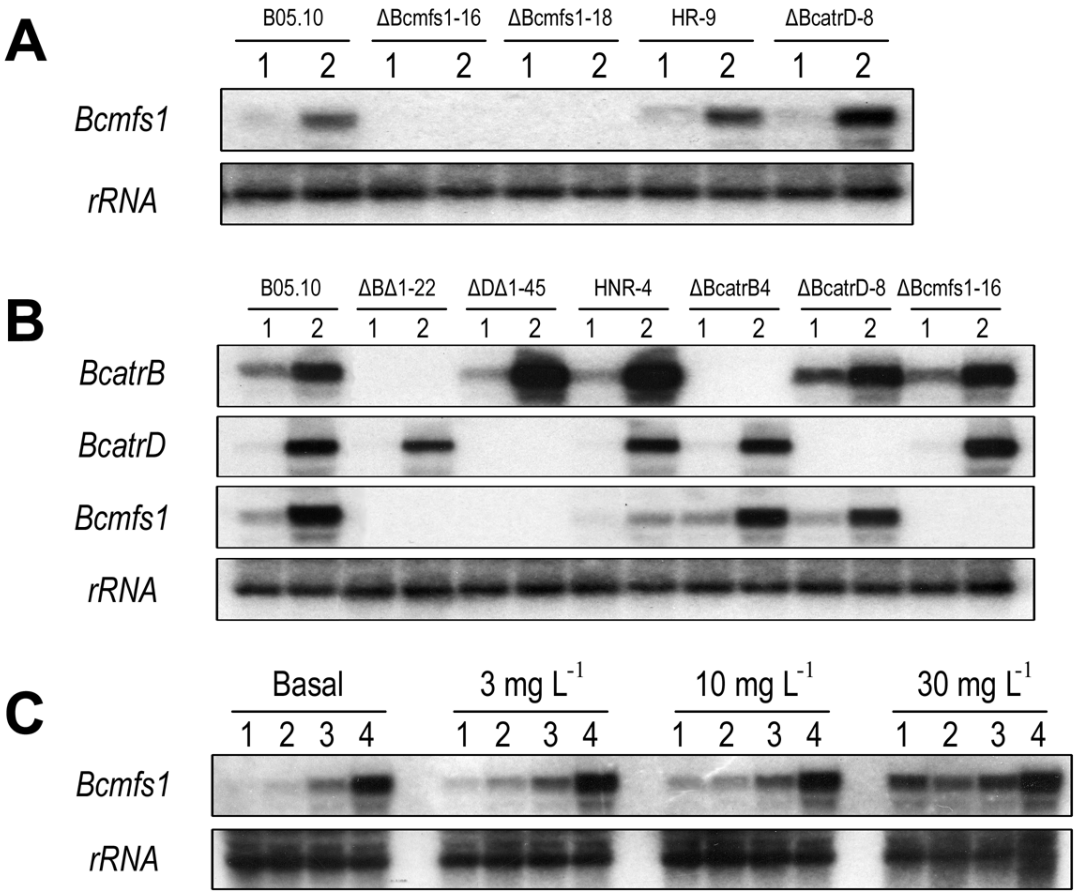

Figure 4. Northern blot analysis of total RNA (10 µg) from germlings of *Botrytis cinerea*. (A) Parental strain B05.10, *Bcmfs1* replacement mutants ΔBcmfs1-16 and ΔBcmfs1-18, reference mutant HR-9, and *BcatrD* replacement mutant ΔBcatrD-8. Basal levels of expression (lanes 1) and expression levels after treatment with 30 mg L^{-1} oxpoconazole (lanes 2) are shown. (B) Parental strain B05.10, *BcatrB* and *Bcmfs1* double replacement mutant ΔBΔ1-22 and *BcatrD* and *Bcmfs1* double replacement mutant ΔDΔ1-45, reference mutant HNR-4, *BcatrB* replacement mutant ΔBcatrB4, *BcatrD* replacement mutant ΔBcatrD-8, and *Bcmfs1* replacement mutant ΔBcmfs1-16. Basal levels of expression (lanes 1) and expression levels after treatment with 30 mg L^{-1} oxpoconazole (lanes 2) are shown. (C) Parental strain B05.10 (lanes 1), and the *Bcmfs1* overexpression mutants OV1-23 (lanes 2), OV1-48 (lanes 3), and OV1-13 (lanes 4). Basal and induced expression levels after treatment with 3, 10, and 30 mg L^{-1} oxpoconazole are shown. RNA was hybridized with the EST probe specific for *Bcmfs1* (Figure 1A). Equal loading of lanes with RNA was checked by subsequently probing the same blot with 28S rRNA.

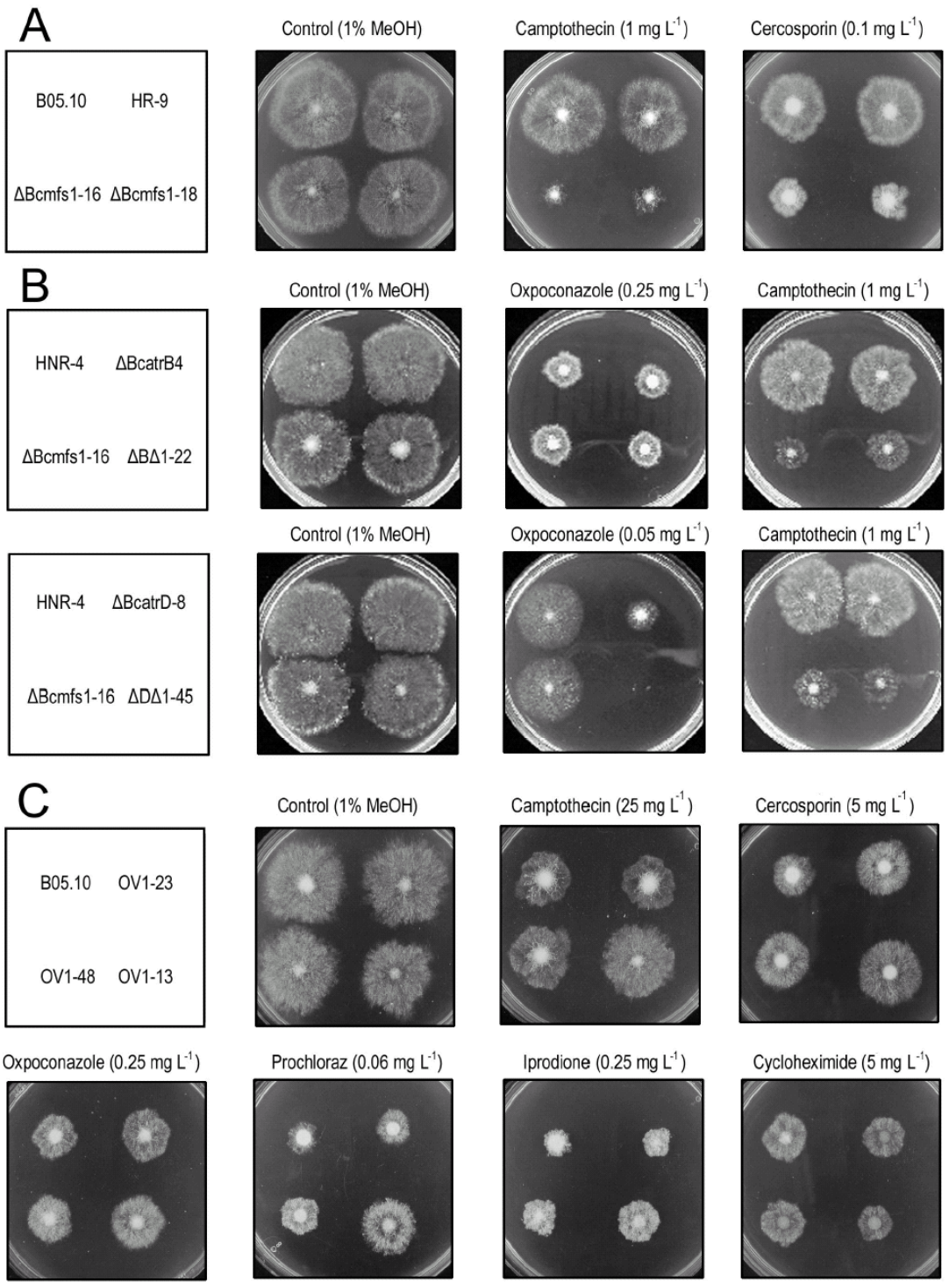

Figure 5. Activity of compounds in radial growth experiments. (A) Parental strain B05.10, reference mutant HR-9, and *Bcmfs1* replacement mutants ΔBcmfs1-16 and ΔBcmfs1-18. (B) Reference mutant HNR-4, single gene replacement mutants (ΔBcatrB4, ΔBcatrD-8, and ΔBcmfs1-16), and double gene replacement mutants (ΔBΔ1-22 and ΔDΔ1-45). (C) Wild-type strain B05.10 and *Bcmfs1* overexpression mutants (OV1-23, OV1-48, and OV1-13).

Table 3. Activity of compounds on radial growth of *Botrytis cinerea*

Chemical class	Compound	EC_{50} (mg L^{-1}) of compound for:			
		HNR-4[a]	ΔBcatrD-8	ΔBcmfs1-16	ΔDΔ1-45
DMIs	Oxpoconazole	0.114 c[b]	0.035 b	0.107 c	0.017 a
	Prochloraz	0.026 c	0.014 b	0.016 b	0.005 a
	Tebuconazole	0.110 c	0.087 b	0.105 c	0.035 a
	Pyrifenox	0.046 b	0.036 b	0.044 b	0.021 a
	Fenarimol	0.869 c	0.374 b	0.780 c	0.242 a
Alkaloid	Camptothecin	>10 b	>10 b	0.68 a	0.70 a

a: EC_{50} values of compounds for wild-type strain B05.10 and HNR-4 do not differ significantly (results not shown).

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

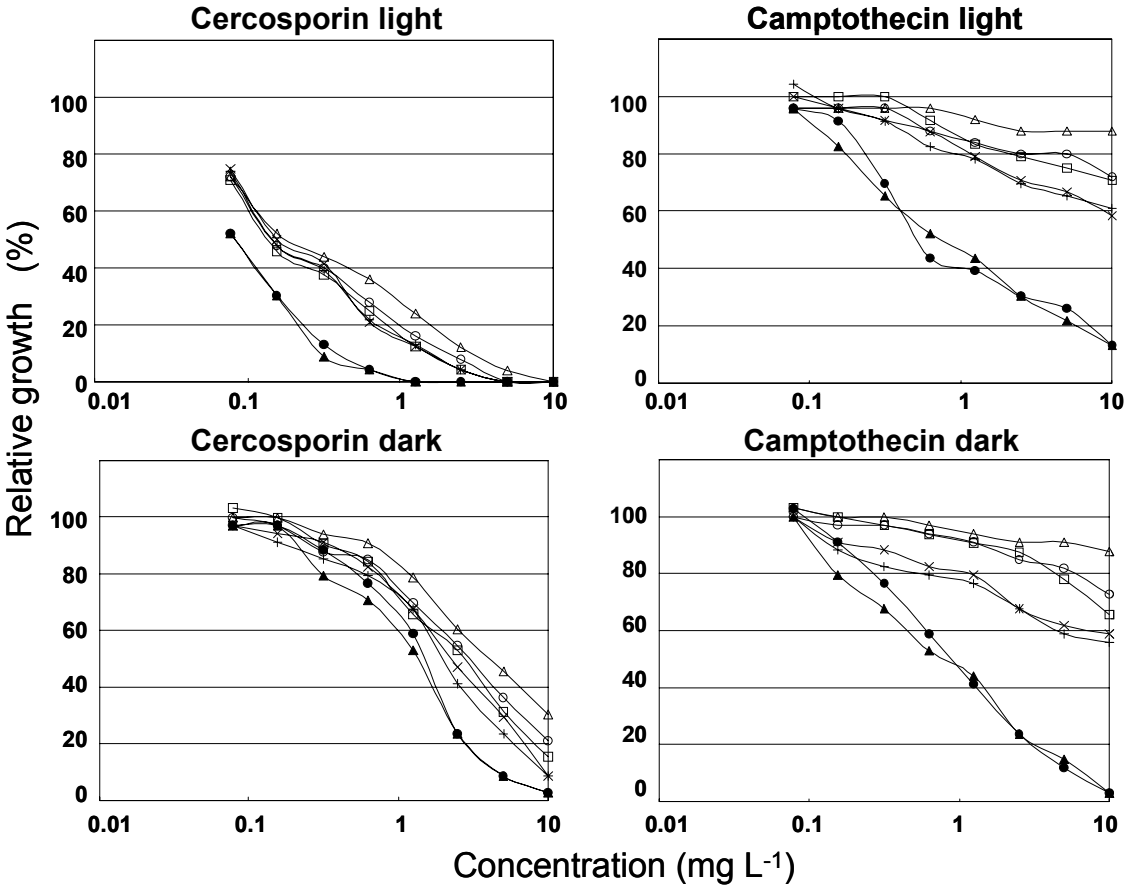

Figure 6. Activity of cercosporin and camptothecin on relative growth of *Botrytis cinerea* wild-type strain B05.10 (+), reference transformant HR-9 (×), two *Bcmfs1* replacement mutants, ΔBcmfs1-16 (●) and ΔBcmfs1-18 (▲), and *Bcmfs1* overexpression mutants OV1-23(□), OV1-48(○), and OV1-13 (△).

Bcmfs1 overexpression mutants

Protoplasts of *B. cinerea* strain B05.10 were transformed with the plasmid carrying the full-length *Bcmfs1* gene (Figure 1A). The expression of *Bcmfs1* in putative transformants was investigated

by northern blot analysis. Transformants with three different levels of expression were selected and arbitrarily classified as low (OV1-23), medium (OV1-48), and high (OV1-13) (Figure 4C). Induced expression levels of *Bcmfs1* in these transformants after treatment with oxpoconazole (3, 10, and 30 mg L^{-1}) for 60 min correlated with the basal levels of expressions (Figure 4C). Overexpression mutants possessed a decreased sensitivity to camptothecin, cercosporin, DMI fungicides, cyprodinil, iprodione, captan, and fluazinam compared to wild-type strain B05.10. Surprisingly, the same mutant showed an increased sensitivity to the antibiotic cycloheximide (Figure 5C, Table 2).

Accumulation of oxpoconazole

The accumulation of oxpoconazole by germlings of *B. cinerea* wild-type strain B05.10 was transient in time. The mutants tested also showed this phenomenon, though to a varying extend. The initial accumulation (up to 40 min of incubation) of oxpoconazole by all strains tested correlated with sensitivity to oxpoconazole in radial growth experiments (Figure 7, Tables 2 and 3). The correlation coefficient (R^2) between accumulation of oxpoconazole after 20 min of incubation and the EC_{50} of oxpoconazole for the different strains was calculated to be 0.813. Strikingly, the steady-state levels of accumulation of oxpoconazole (after 60 min of incubation) by ΔBcatrD-8 and ΔDΔ1-45 always remained higher than that by all other strains tested (Figure 7). Furthermore, the level of accumulation by ΔDΔ1-45 was significantly higher than that of ΔBcatrD-8.

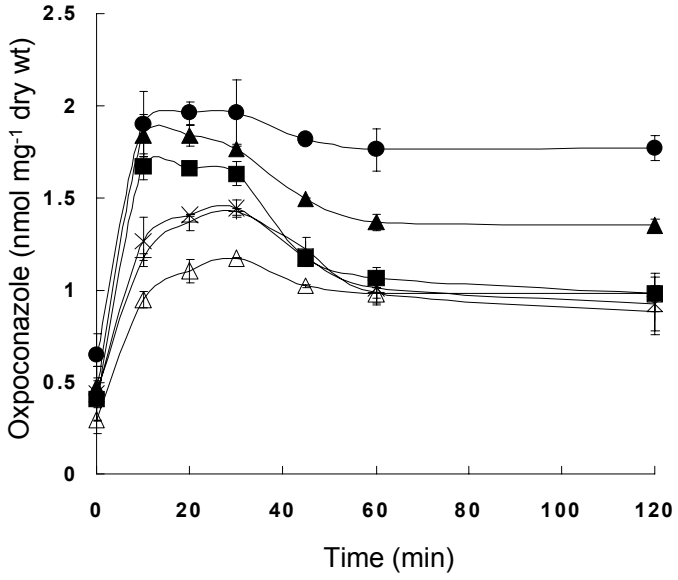

Figure 7. Accumulation of oxpoconazole (30 μM) by germlings of *Botrytis cinerea* wild-type strain B05.10 (+), reference transformant HNR-4 (×), *BcatrD* single replacement mutants ΔBcatrD-8 (▲), *Bcmfs1* single replacement mutants ΔBcmfs1-16 (■), *BcatrD* and *Bcmfs1* double replacement mutant ΔDΔ1-45 (●), and *Bcmfs1* overexpression mutants OV1-13 (△).

Virulence assay

Virulence of all mutants tested (ΔBcmfs1-16, ΔBcmfs1-18, ΔBΔ1-22, ΔDΔ1-45, OV1-23, OV1-48, OV1-13, HR-9, and HNR-4) on detached tomato leaves was similar to that of the parental strain, B05.10 (results not shown).

DISCUSSION

Bcmfs1 is a new member of the MFS genes family encoding transporters with 14 TMDs. Phenotypic characterization of replacement and overexpression mutants indicated that the transporter provides protection against the alkaloid camptothecin, the photosensitizer cercosporin (a perylenequinone toxin), and DMI fungicides. Accumulation of the DMI fungicide oxpoconazole by germlings of these mutants and the parental strain, B05.10, and their sensitivity to oxpoconazole correlated with the expression of *Bcmfs1*. These results demonstrate that Bcmfs1 is a multidrug transporter involved in protection against a wide range of chemicals.

Bcmfs1 has a high homology with aflT from *A. parasiticus*, CFP from *C. kikuchii,* and TOXA from *C. carbonum.* The homology with CFP may reflect why both Bcmfs1 and CFP are involved in protection against cercosporin. Like Bcmfs1, MFS proteins from yeasts, such as BenR (15) and FLU1 (5) from *C albicans* and FLR1 (1) from *S. cerevisiae*, transport DMI fungicides. However, these three yeast MFS proteins have a low level of homology to Bcmfs1, suggesting that there is no obvious relation between homology and substrate specificity.

The role of *Bcmfs1* in transport of DMI fungicides only became obvious after functional inactivation in a ΔBcatrD mutant (Figure 5B, Table 3). A similar phenomenon has been reported with *C. albicans* for the ABC transporter CDR2, which showed a phenotype only in mutants with a ΔCDR1 background (28). This can be ascribed to redundancy of transporters with an overlap in substrate specificity. Previously, we demonstrated that BcatrD is the major transporter of DMI fungicides in *B. cinerea* (17). Hence, we assume that the lack of phenotype of ΔBcmfs1 mutants with respect to sensitivity to DMIs is due to compensating activity of BcatrD. However, such compensating activity does not seem to be accompanied by increased transcription of *BcatrD,* since basal and induced transcript levels of the gene were similar in strain B05.10 and ΔBcmfs1 (Figure 4B). The conclusion that Bcmfs1 mediates transport of DMIs is supported by the observation that the *Bcmfs1*-overexpressing mutants showed a significant reduction in DMI sensitivity (Figure 5C, Table 2). BcatrB is not a DMI transporter (29, 34). Still, expression of *BcatrB* is induced by treatment with DMI fungicides (16), indicating that inducers of expression

of *BcatrB* are not necessarily a substrate of the encoded proteins. Similar phenomena have been described for other ABC genes (17, 29).

Bcmfs1-overexpressing mutants display reduced sensitivity to various unrelated fungicides (Figure 5C, Table 2). This suggests that the multidrug transporter Bcmfs1 has low substrate specificity for these products and that loss of the Bcmfs1 function in deletion mutants can be compensated for by other transporters. Mutant OV1-13 has a slightly increased sensitivity to cycloheximide (Figure 5C, Table 2). A similar phenomenon was observed in MDR mutants of *A. nidulans* with resistance to DMIs and increased sensitivity to dithiocarbamate fungicides and the antibiotic phleomycin (3). We hypothesize that the increased sensitivity displayed by the overexpression mutant could be due to the fact that MFS transporters function not only as efflux but also as influx transporters (14).

The accumulation of oxpoconazole by germlings of *B. cinerea* was transient in time. The initial accumulation (up to 40 min) by OV1-13 was lower than that by B05.10 (Figure 7) and the accumulation by all strains correlated with their sensitivity to the fungicide (Figure 7, Table 2). Steady-state level of oxpoconazole accumulation (after 60 min) by ΔDΔ1-45 was significantly higher than that by ΔBcatrD-8. These observations indicate that mutations in *Bcmfs1* indeed functionally affect efflux of oxpoconazole. Besides BcatrD and Bcmfs1, additional transporters in *B. cinerea* may exist that play a role in efflux of DMI fungicides. This assumption is based on the observation that the double replacement mutant ΔDΔ1-45 still displays a transient accumulation profile (Figure 7) which suggests that efflux activity still proceeds to a weak extent. The transporter gene involved might be *BcatrG* and/or *BcatrK* since expression of these genes was induced by DMI fungicides (16). Such a situation would indicate that multiple transporter proteins mediate the transport of a particular compound. A similar phenomenon has been described for *C. albicans*, which possesses at least four transporter genes involved in efflux of DMIs. These include the ABC transporter genes *CDR1* (24) and *CDR2* (27) and the MFS genes *CaBenR* (26), and *FLU1* (5).

It is probable that *B. cinerea* developed transporter systems during evolution to cope with natural toxic compounds. However, in this context it is difficult to understand why camptothecin and cercosporin are substrates of Bcmfs1. Camptothecin is an alkaloid compound with antitumour activity isolated from Chinese tree *C. acuminata.* This plant is not known as a host of *B. cinerea.* It might be that plant species within the wide host range of *B. cinerea* contain the same or related alkaloids. Cercosporin is a natural photoactivated toxin produced by *Cercospora*

species (9), and similar compounds are not known for *B. cinerea*. It is not likely that they are produced during pathogenesis, since necrotic symptoms incited by *B. cinerea* are light independent. Bcmfs1 is also not a general transporter of photosensitizers, as shown for Snq2 (35). Hence, the potency of Bcmfs1 for transport of cercosporin is hard to explain. A number of MFS transporters from filamentous fungi homologous to Bcmfs1 can function as virulence factors. This is reported for the cercosporin transporter from *C. kikuchii* (6) and the transporter of trichothecene from *F. sporotrichioides* (2). MFS genes involved in secretion of HC toxin and trichothecene are located in a gene cluster carrying genes encoding enzymes involved in biosynthesis of these toxins. A role of Bcmfs1 in secretion of endogenous toxins is not obvious, since DNA sequences flanking *Bcmfs1* did not reveal the presence of genes involved in toxin biosynthesis. Botrydial, produced by *B. cinerea,* is toxic to sweet pepper (10). This toxin might be a substrate of Bcmfs1, although disruption of *Bcmfs1* did not increase the sensitivity to botrydial analogues, such as patchoulol and globulol (33). The virulence of all Bcmfs1 mutants tested on detached tomato leaves was similar to that of the parental strain, B05.10. For these reasons, the intrinsic function of Bcmfs1 is still obscure. Such a function might become obvious upon testing the virulence of replacement mutants on a wide range of host plants. These studies are being performed in current research, but so far no phenotype with respect to host virulence has been found. It is possible that Bcmfs1 functions in protection against antibiotics produced by antagonistic microorganisms during its saprophytic phase of growth. Such a function has recently been reported for BcatrB of *B. cinerea* in protection against phenazine antibiotics produced by *Pseudomonas* species. (30). This hypothesis is currently being tested for Bcmfs1 and other ABC and MFS transporters of *B. cinerea.*

ACKNOWLEDGEMENT

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Chapter 6

Modulators of membrane drug transporters potentiate the activity of the DMI fungicide oxpoconazole against *Botrytis cinerea*

Hayashi, K., Schoonbeek, H., and De Waard, M. A. 2003. Pest Management Science 59: 294-302.

ABSTRACT

Modulators known to reduce multidrug resistance in tumor cells were tested for their potency to synergize the fungitoxic activity of the fungicide oxpoconazole, a sterol demethylation inhibitor (DMI), against *Botrytis cinerea*. Chlorpromazine, a phenothiazine compound known as a calmodulin antagonist, appeared the most potent compound. Tacrolimus, a macrolide compound with immunosuppressive activity, was also active. The synergism of chlorpromazine negatively correlated with the sensitivity of the parent strain and mutants of *B. cinerea*. The synergism was highest in a mutant that overexpressed the ATP-binding cassette transporter BcatrD, known to transport DMI fungicides such as oxpoconazole. The synergism of chlorpromazine positively correlated with its potency to enhance the accumulation of oxpoconazole in *BcatrD* mutants. These results indicate that chlorpromazine is a modulator of BcatrD activity in *B. cinerea* and suggest that mixtures of DMI fungicides with modulators may represent a perspective for the development of new resistance management strategies.

INTRODUCTION

Fungicide mixtures are widely used in commercial products. The main advantages of mixtures are that they can extend the antifungal spectrum of the single products and delay resistance development to the individual components. Fungicide mixtures may also display a synergistic interaction by which the amount of active ingredients can be reduced (14). If a synergist could annul the mechanism of resistance to a particular fungicide, the synergistic activity of a mixture would be limited to the fungicide-resistant subpopulation of a pathogen. Such synergists need not necessarily be fungitoxic by themselves and could be useful as an anti-resistance strategy.

Reports on synergism in mixtures of commercial fungicides are rare (14). The most obvious cases were observed for phenylamide fungicides in mixtures with mancozeb and cymoxanil against *Phytophthora infestans* and *Plasmopara viticola* (21). Experimental synergists that show activity in *in vitro* experiments have been described frequently. The classical example is the synergism in mixtures of phosphoramidate and phosphorothiolate fungicides to *Pyricularia oryzae*. The synergism has been ascribed to inhibition of phosphoramidate metabolism by a phosphorothiolate (36). The fungitoxic activity of mitochondrial bc_1 complex inhibitors such as metominostrobin (Qo site inhibitor), and antimycin A (Qi site inhibitor) can be synergized in *Botrytis cinerea, Cochliobolus miyabeanus, Monilinia fructicola*, and *P. oryzae* by salicylhydroxamic acid that inhibits the cyanide-insensitive respiration pathway (27). Mixtures of

stereoisomers of the sterol biosynthesis inhibitors cyproconazole or tebuconazole also display synergism (20). This has been ascribed to the binding of the most active isomer to the $P450_{14DM}$ target site, whereas the less active isomers may saturate other P450s (34).

Modulators of the activity of ATP-binding cassette (ABC) transporters have been reported as synergists of drugs and fungicides against multidrug resistant (MDR) tumor cells of mammals (39) and demethylation inhibitor (DMI)-resistant fungi (16), respecively. Resistance to drugs can be mediated by the overproduction of specific ABC transporters, resulting in reduced drug accumulation in cells. Modulators inhibit ABC transporter activity, which trap the drugs inside the cells and drug resistance is annulled. Modulators that potentiate the activity of drugs against MDR of tumor cells can be classified as anthracycline and *Vinca* alkaloid analogs, calcium channel blockers, calmodulin antagonists, cyclosporines, steroids and hormonal analogs, and miscellaneous hydrophobic, cationic compounds (19). These compounds share few structural similarities, but are all extremely lipophilic or amphipathic. This suggests that ABC transporters may have one or more specific receptor sites for these modulators (19). Respiratory inhibitors and uncouplers can also be regarded as modulators, although their activity is probably based on a reduction in the synthesis of ATP required to drive the activity of energy-dependent ABC transporters (12, 13). Combinations of DMI fungicides with compounds that display synergism are fenarimol and carbonyl cyanide 3-chlorophenylhydrazone (CCCP) in activity against *Aspergillus nidulans* (11), fenarimol and vanadate against *Penicillium italicum* (12), itraconazole and hydrocortisone against *A. fumigatus* (32), fluconazole and cyclosporine against *Candida albicans* (25), and fluconazole and tacrolimus against *Cryptococcus neoformans* (9).

A natural function of fungal ABC transporters is to provide protection against plant defence products during pathogenesis. This has been demonstrated for the ABC transporters BcatrB, abc1, and ABC1 from *B. cinerea* (33), *Gibberella pulicaris* (18), and *Magnaporthe grisea* (37), respectively. In these instances ABC transporters can be regarded as virulence factors, and modulators of their activity may also control diseases, since they could annul the protecting activity against plant defence compounds (17). Major facilitator superfamily (MFS) transporters can act as virulence factors in a similar way. Examples are aflT, a putative aflatoxin transporter from *A. parasiticus* (6), CFP, a cercosporin pump from *Cercospora kikuchii* (5), dotC, a dothistromin transporter from *Dothistroma pini* (3), TOXA, a putative HC-toxin transporter from *C. carbonum* (31), and Tri12, trichothecenes pump from *Fusarium sporotrichioides* (2). Hence, inhibitors of these transporters might also display disease control activity. However, the

modulators of ABC transporters mentioned above have not been reported as inhibitors of MFS transporter activity. It might be that compounds that dissipate the proton motive force, the driving force of MFS transporters, can act as modulators of these transporters.

We are interested in ABC and MFS transporters from the fungus *B. cinerea*. This phytopathogen is known for its wide range of host plants, suggesting that it has adopted multiple mechanisms to cope with diverse plant defence compounds. One of these mechanisms could be based on the activity of ABC or MFS transporters that prevent the accumulation of plants defence compounds in mycelium. *B. cinerea* is also notorious for its rapid development of fungicide resistance. Recent data suggest that laboratory generated DMI-resistant mutants (28) and field isolates (23) of *B. cinerea* display multidrug resistance related to overproduction of specific ABC transporters. In this case, modulators of ABC and MFS transporters might become relevant for anti-resistance strategies in the control of *B. cinerea*.

We have previously demonstrated by phenotypic characterization of replacement and overexpression mutants that the ABC transporter BcatrD (29) and the MFS transporter Bcmfs1 (30) are involved in sensitivity to DMIs and MDR. The goal of the present study is to describe the interaction between modulators of ABC transporters and oxpoconazole. The studies were performed with the replacement and overexpression mutants in crossed-paper strip experiments. The results demonstrate that combinations of oxpoconazole and chlorpromazine (a phenothiazine compound with antagonistic activity against calmodulin) and tacrolimus (a macrolide compound with immunosuppressive activity) display synergistic activity. To validate the mechanism of synergistic activity, the effect of chlorpromazine on the accumulation of oxpoconazole by germlings of *B. cinerea* was investigated. We found a correlation between sensitivity of the strains to oxpoconazole and the potency of chlorpromazine to enhance the accumulation of oxpoconazole in mycelium. These results indicate that synergism between oxpoconazole and chlorpromazine can be ascribed to inhibition of the ABC transporter BcatrD, which is primarily responsible for efflux of oxpoconazole from mycelial cells.

MATERIALS AND METHODS

Fungal strains

B05.10 is a haploid *B. cinerea* strain provided by P. Tudzynski (Institut für Botanik, Westfälische Wilhelms-Universität, Münster, Germany) (4). B05.10 was used as the reference strain in all experiments. ΔBcatrD-8 and ΔBcmfs1-16 are replacement mutants of *BcatrD* and *Bcmfs1*,

respectively. ΔDΔ1-45 is a double replacement mutant of *BcatrD* and *Bcmfs1*. OVD-2 and OV1-13 are overexpression mutants of *BcatrD* and *Bcmfs1*, respectively. All mutants were derived from B05.10 (Table 1) and maintained on MEA plates (malt extract agar, Oxoid Ltd., Basingstoke, Hampshire, England) amended with 0.2% yeast extract (Oxoid) at 20°C. Conidia formation was induced after 3 days of incubation by irradiation of cultures in Petri-dishes with near-UV light for 24 h and continued incubation for 3 to 7 days.

Table 1. *Botrytis cinerea* strains used in this study

Strain	Genotype	Reference
B05.10	Parental strain	4
OVD-2	*BcatrD* overexpression mutant derived from B05.10	29
OV1-13	*Bcmfs1* overexpression mutant derived from B05.10	30
ΔBcmfs1-16	*Bcmfs1* replacement mutant derived from B05.10	30
ΔBcatrD-8	*BcatrD* replacement mutant derived from B05.10	29
ΔDΔ1-45	*BcatrD* and *Bcmfs1* double replacement mutant derived from ΔBcatrD-8	30

Compounds

The DMI fungicide oxpoconazole (Figure 1) was synthesized by Ube Industries, Ltd. (Ube, Yamaguchi, Japan). Compounds known to modulate the activity of ABC transporters and tested for synergism with oxpoconazole were chlorpromazine, CCCP, colchicine, primaquine, progesterone, propranolol, quinacrine, quinidine, quinine, reserpine, thioridazine, and verapamil. These compounds were purchased from Sigma (St. Louis, MO, USA). Tacrolimus (FK506), known to increase the activity of DMI antifungal agents against DMI-resistant *C. albicans* (24), was a kind gift from Fujisawa Pharmaceutical Co., Ltd. (Osaka, Japan).

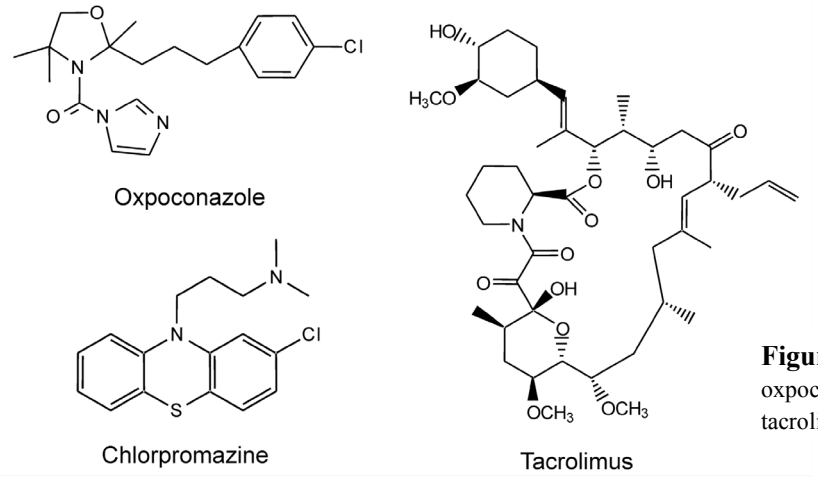

Figure 1. Chemical structures of oxpoconazole, chlorpromazine, and tacrolimus.

Crossed-paper strip experiments

Crossed-paper strip experiments were performed as described previously (11, 35). Conidial suspensions of *B. cinerea* were mixed with synthetic medium (7) at 50°C and immediately poured into Petri dishes to make agar plates (diameter 9 cm). Strips of filter paper (0.7 X 8 cm) were impregnated with acetone solutions of the test compound (Table 2), air-dried, and placed cross-wise on test agar plates. Test plates were incubated at 20°C for 3 days. The interaction between the fungitoxic action of compounds at the crossing of the paper strips was assessed visually (11).

Table 2. Compounds that alter multidrug resistance in tumors and tested in crossed-paper strips experiments with *Botrytis cinerea* for synergistic activity with oxpoconazole

Category	Compound	Concentration[a]	Physiological activity
Acridine	Quinacrine	1000	MAO inhibitor, antimalarial drug
Alkaloid	Colchicine	1000	Mitosis inhibitor
	Quinidine	1000	Potassium channel blocker,
	Quinine	1000	antimalarial drug
Indole alkaloid	Reserpine	1000	*Antihypertensive activity*
Macrolide	Tacrolimus	5000	Immunosuppressive activity
Phenothiazine	Chlorpromazine	1000	Calmodulin antagonist
	Thioridazine	1000	
Benzene acetonitrile	Verapamil	1000	Calcium channel blocker
Phenylhydrazone	CCCP[b]	1000	Uncoupler
Propanole	DL- Propranolol	1000	β-Adrenergic antagonist
Quinoline	Primaquine	1000	Antimalarial drug
Steroid	Progesterone	1000	Steroid hormone

a: Concentration (mg L^{-1}) in acetone solution used for impregnation of the paper strips.
b: Carbonyl cyanide 3-chlorophenylhydrazone.

Accumulation of oxpoconazole

Accumulation experiments were performed with suspensions of germlings of *B. cinerea* grown as described previously (15). 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}). Accumulation experiments were initiated by the addition of [^{14}C]oxpoconazole (30 μM initial external concentration, 750 Bq $nmol^{-1}$) to the germling suspension on a reciprocal shaker at 20°C. Accumulation of oxpoconazole was determined in germlings collected by filtration. Radioactivity in germlings was counted in a liquid scintillation spectrometer (BECKMAN LS6000TA; Beckman Coulter Inc., CA, USA). The effect of synergistic compounds on uptake of [^{14}C]oxpoconazole was determined by the addition of the compounds from 1000X concentrated solutions in methanol 185 min after addition of [^{14}C]oxpoconazole to the germling suspensions.

Accumulation values of oxpoconazole in mycelium (expressed as nmoles of oxpoconazole per mg dry weight of germlings) are means from three independent experiments.

Quantification of interaction

The activity of the compounds against *B. cinerea* was determined in radial growth experiments as described previously (29). EC_{50} values of chemicals were calculated from dosage-response curves using Excel 97. Interactions between compounds in mixtures were calculated according to Colby (8) and Wadley (38). Analysis according to Colby is made with the formula $E = X_A Y_B/100$ in which X_A and Y_B represents growth as a percentage of the control with compound A at concentration p and compound B at concentration q, respectively. E is the calculated growth as a percentage of the control for mixture A and B at concentration p and q. Analysis according to Wadley was made with the formula $EC_{50exp} = (a + b)/ (a/ EC_{50\ (A)} + b/ EC_{50\ (B)})$ that enabled the calculation of the expected effective concentration of the mixtures inhibiting radial growth by 50% (EC_{50exp}). A and B are the two compounds and a and b represent the concentration of the compounds in the mixture. In both analyses the synergism ratio R is calculated as the ratio between the theoretical values and observed values. Experiments were performed twice.

RESULTS

Crossed-paper strip experiments

Compounds known to interfere with the activity of ABC transporters (Table 2) were screened for synergistic activity with oxpoconazole (30 mg L^{-1}) in crossed-paper strip experiments. Chlorpromazine and tacrolimus (Figure 1) showed synergism with oxpoconazole (Figure 2). Additional experiments showed that thioridazine, another phenothiazine compound, gave results similar to chlorpromazine.

The width of the inhibition zone of oxpoconazole (vertical paper strips) for the wild-type strain B05.10 and five mutants generated from B05.10 (Figure 2) correlated with the EC_{50} values of oxpoconazole (Table 3). Combinations of oxpoconazole with chlorpromazine (horizontal paper strips) clearly resulted in synergistic interactions with strains OVD-2, OV1-13, B05.10, and ΔBcmfs1-16. The synergistic activity with strains ΔBcatrD-8 and ΔDΔ1-45 was minor or absent (Figure 2A). The synergistic activity of tacrolimus (horizontal strip paper) with oxpoconazole was only significant in strain OVD-2. Slight synergism was also observed with strains OV1-13, B05.10, and ΔBcmfs1-16 (Figure 2B). Strips impregnated with chlorpromazine (1000 mg L^{-1})

showed no inhibition zone by itself. In contrast, tacrolimus (5000 mg L^{-1}) caused a wide inhibition zone in which conidial germination but no mycelial growth was observed.

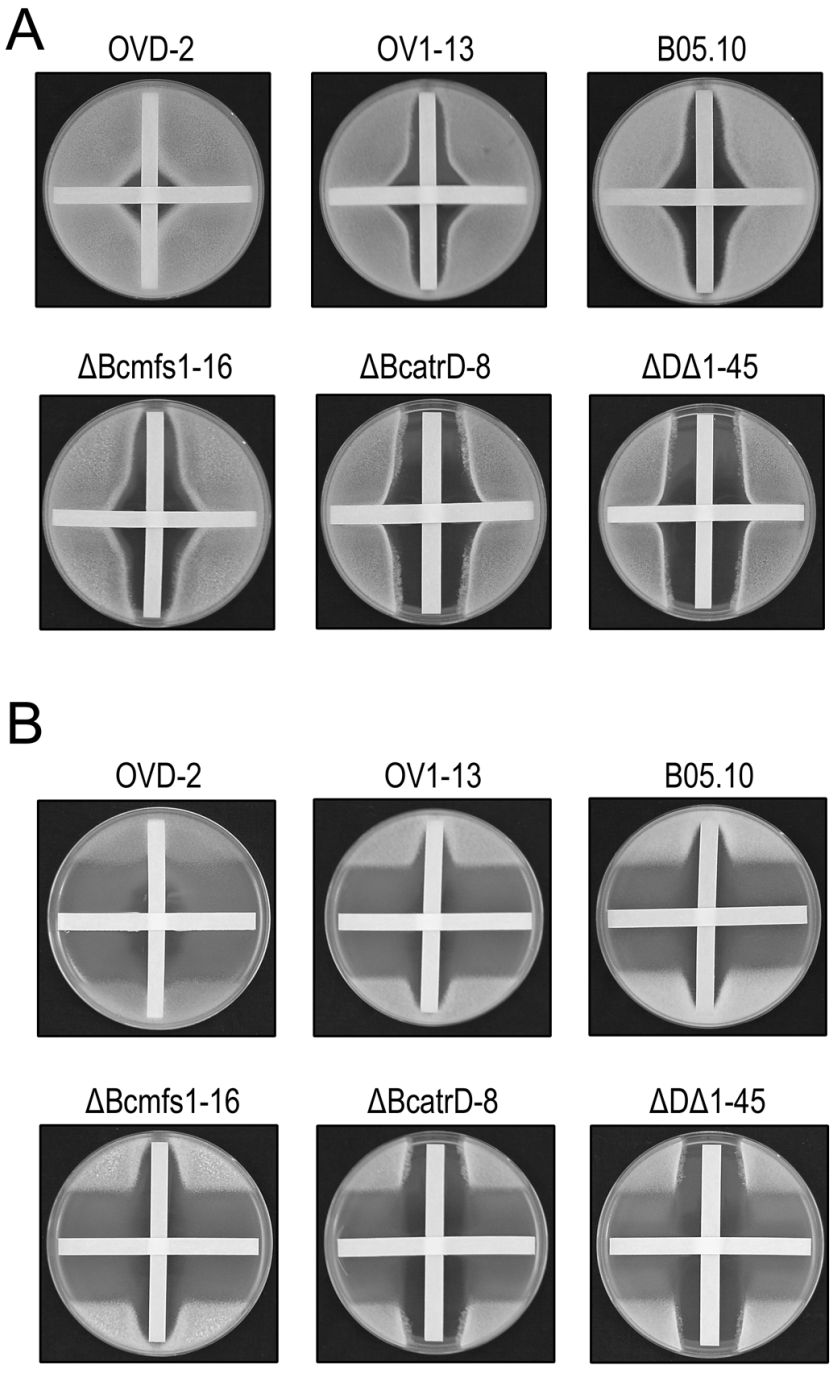

Figure 2. Synergistic activity between oxpoconazole and (A) chlorpromazine and (B) tacrolimus to *Botrytis cinerea* in crossed-paper strip experiments. Strains tested are listed in Table 1. The vertical strips were impregnated with acetone solutions of oxpoconazole (30 mg L^{-1}) and the horizontal strips with (A) chlorpromazine (1000 mg L^{-1}) or (B) tacrolimus (5000 mg L^{-1}).

Table 3. EC_{50} values of oxpoconazole and chlorpromazine alone and in mixture on radial growth of *Botrytis cinerea* and synergism ratios as analyzed according to Wadley (38)

Strain	EC_{50} (mg L^{-1})				Synergy ratio R[c]
	Observed			Theoretical[b]	
	Oxpoconazole	Chlorpromazine	Mixture[a]		
OVD-2	0.331	8.22	1.81	5.60	3.10
OV1-13	0.166	7.80	2.47	4.10	1.66
B05.10	0.156	7.88	2.30	4.00	1.74
ΔBcmfs1-16	0.135	6.30	2.15	3.33	1.55
ΔBcatrD-8	0.054	7.24	1.60	2.00	1.25
ΔDΔ1-45	0.026	6.83	1.04	1.13	1.08

a: Mixture of oxpoconazole and chlorpromazine (1:50).

b: Theoretical EC_{50} of the mixture according to Wadley analysis.

c: Synergy ratio R = Theoretical EC_{50} (mg L^{-1}) / observed EC_{50} (mg L^{-1}) of mixture.

Effect of modulators on accumulation of oxpoconazole

The accumulation levels of oxpoconazole by the wild-type strain B05.10, the overexpression strain OVD-2 and the double gene replacement mutants ΔDΔ1-45 differed significantly in time, as described previously (Figure 3) (29, 30). Chlorpromazine and tacrolimus were added under equilibrium conditions with respect to accumulation of oxpoconazole, 185 min after the start of the accumulation experiment. Chlorpromazine instantaneously enhanced the oxpoconazole accumulation. The effect on accumulation was concentration-dependent and always transient at the lowest concentration tested (0.1 mM). Tacrolimus also enhanced oxpoconazole accumulation but its effect was less pronounced than that of chlorpromazine. In the same experiment, addition of tacrolimus (0.3 mM) at 180 min after the addition of oxpoconazole enhanced accumulation of the fungicide to 1.8 ± 0.21, 2.2 ± 0.18, and 3.8 ± 0.28 nmol mg^{-1} dry weight of mycelium for strains OVD-2, B05.10, and ΔDΔ1-45 at t = 360 min, respectively (results not shown).

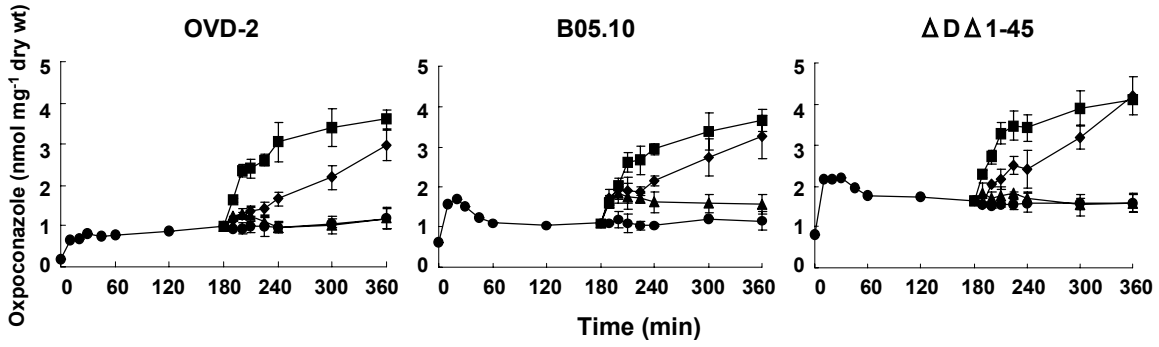

Figure 3. Effect of chlorpromazine on the accumulation of oxpoconazole by germlings of *Botrytis cinerea*. Strains tested are described in Table 1. Chlorpromazine was added 185 min after the addition of oxpoconazole at 0.1 mM (▲), 0.3 mM (◆), 1.0 mM (■). Control treatment (●).

Quantification of synergism

EC_{50} values of oxpoconazole and chlorpromazine in radial growth of B05.10 were 0.156 and 7.88, respectively, implicating that the ratio between the two values was approximately 50. This ratio was used in mixtures of oxpoconazole and chlorpromazine to determine the theoretical response of mixtures according to Wadley (Table 3) and Colby (Table 4). With both methods the highest synergism was observed for the overexpression mutant OVD-2, while mutants with a non-functional *BcatrD* gene did not display any synergistic interaction. Correlation coefficients (R^2) between the synergy ratio R (ratio between the theoretical and observed response) according to the Wadley and Colby analysis and the EC_{50} values of oxpoconazole for radial growth of all strains tested were calculated to be 0.96 and 0.93, respectively (Figure 4A).

Table 4. Effect of oxpoconazole and chlorpromazine alone and in mixture on growth rate of *Botrytis cinerea* in radial growth experiments and synergism ratios as analyzed according to Colby (8)

Strain	Growth rate as percentage of control				
	Observed			Theoretical[b]	Synergy ratio R[c]
	Oxpoconazole (0.1 mg L^{-1})	Chlorpromazine (5 mg L^{-1})	Mixture[a]		
OVD-2	77	66	22	50.8	2.31
OV1-13	69	53	24	36.5	1.52
B05.10	61	61	23	37.2	1.62
ΔBcmfs1-16	64	58	24	37.1	1.55
ΔBcatrD-8	31	63	19	19.5	1.03
ΔDΔ1-45	3	58	3	1.7	0.57

a: Mixture of oxpoconazole (0.1 mg L^{-1}) and chlorpromazine (5 mg L^{-1}).
b: Theoretical growth rate of the mixture according to Colby analysis.
c: Synergy ratio R = Theoretical growth rate (%) / observed growth rate (%) of mixture.

Correlation coefficients (R^2) between the Wadley and Colby synergy ratios and the ratio between oxpoconazole accumulation in the presence and absence of chlorpromazine were determined in an additional experiment in which all test strains described in Table 1 were included. Chlorpromazine was tested at a sublethal concentration of 0.1 mM and added 120 min after addition of oxpoconazole. The oxpoconazole accumulation ratio was calculated as the ratio between accumulation in the presence of chlorpromazine at t = 165 min and in the absence of chlorpromazine at t = 20 min. A high ratio, as found for the overexpression mutants OVD-2 and OV1-13, indicates that these strains have a low initial accumulation level due to high oxpoconazole efflux activity which is sensitive to chlorpromazine (Table 5). The correlation coefficients (R^2) for the Wadley and Colby synergy ratios and accumulation ratio were 0.90 and 0.70, respectively (Figure 4B).

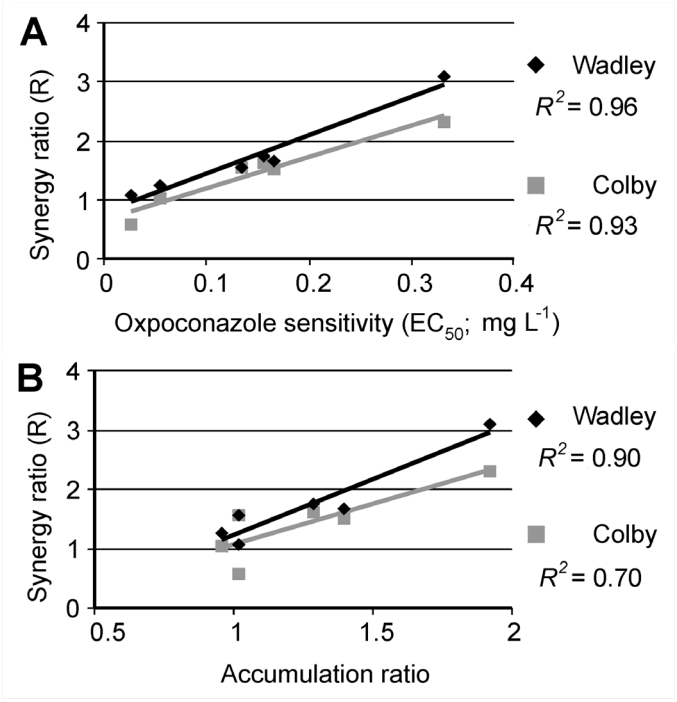

Figure 4. Correlation between (A) EC_{50} values of oxpoconazole to radial mycelial growth (Table 3) or (B) the accumulation ratio of oxpoconazole by germlings of *B. cinerea* (Table 5) and the synergy ratio R of wild-type and mutant strains (Table 1), as calculated according to analytical methods of Wadley (Table 3) and Colby (Table 4).

Table 5. Ratio between initial and induced accumulation of oxpoconazole in the presence of chlorpromazine (0.1 mM)

Strain	Initial accumulation without chlorpromazine[a]	Induced accumulation with chlorpromazine[b]	Accumulation ratio[c]
OVD-2	0.70 ± 0.14	1.35 ± 0.32	1.92
OV1-13	1.11 ± 0.22	1.55 ± 0.24	1.40
B05.10	1.37 ± 0.11	1.76 ± 0.39	1.29
ΔBcmfs1-16	1.66 ± 0.27	1.69 ± 0.23	1.02
ΔBcatrD-8	1.84 ± 0.30	1.75 ± 0.16	0.95
ΔDΔ1-45	1.88 ± 0.21	1.91 ± 0.17	1.02

a: Oxpoconazole accumulation measured 20 min after addition of the fungicide.
b: Chlorpromazine added 120 min after addition of oxpoconazole and oxpoconazole accumulation measured 165 min after addition of the fungicide.
c: Ratio between induced and initial accumulation of oxpoconazole.

DISCUSSION

Crossed-paper strip and radial growth experiments indicated that calmodulin antagonists such as chlorpromazine and thioridazine, and the immunosuppressive compound tacrolimus, synergize the fungitoxic activity of the DMI fungicide oxpoconazole against *B. cinerea*. We also showed that the synergism negatively correlated with sensitivity of the *B. cinerea* strains tested to oxpoconazole and positively correlated with enhanced accumulation of oxpoconazole in mycelium. These results indicate that the mechanism of synergism can be ascribed to inhibition of

the activity of the ABC transporter BcatrD. Hence, the results presented describe a clear case of potentiation of the activity of a DMI fungicide by enhancing its accumulation in fungal cells. Chlorpromazine is already known as a modulator of activity of ABC transporters in tumor cells of mammals (1) and in *C. albicans* (25). Our results indicate for the first time that such modulators also have a modulating effect on the activity of ABC transporters in filamentous fungi. Our test strains also included mutants with modified expression of the MFS transporter gene *Bcmfs1*. The synergism observed with these mutants (OV1-13 and ΔBcmfs1-16) is similar to that observed for the parent strain B05.10. Therefore, we conclude that the activity of this transporter is not inhibited by chlorpromazine, indicating that the activity of the compound is specific for ABC transporters.

Crossed-paper strip experiments offer an easy way to investigate the synergistic fungitoxic action of two compounds. This makes the method suitable for qualitative screening of the synergistic properties of many compounds and enabled us to find the synergistic properties for chlorpromazine and tacrolimus. However, the degree of synergism in crossed-paper strip experiments is highly dependent on the chemical properties of the compound such as water solubility and rate of diffusion into the agar (14). For these reasons, we quantitatively determined the synergism of chlorpromazine in radial growth experiments according to analytical methods as described by Wadley and Colby. Both analyses confirm that chlorpromazine synergizes the fungitoxic activity of oxpoconazole.

Synergism of chlorpromazine was most pronounced in mutants with a relatively high expression level of *BcatrD*. This observation is in line with previous results that describe BcatrD as the major transporter of DMI fungicides such as oxpoconazole (29). Since chlorpromazine instantaneously affects the accumulation of oxpoconazole, we propose that BcatrD has a specific binding site for chlorpromazine that may be identical to the oxpoconazole binding site (26). We assume that binding of chlorpromazine to BcatrD reduces the transport of oxpoconazole. Tacrolimus also potentiates the activity of oxpoconazole, though to a lesser extent than chlorpromazine. The modulating effect of tacrolimus on the activity of drugs has been described before and correlates with enhanced accumulation of itraconazole by DMI-resistant strains of *C. albicans* (24). Tacrolimus proved to be a stronger synergist of itraconazole in activity against *C. albicans* than chlorpromazine (25), which contrasts with the results of the present experiments. The differential effect of chlorpromazine and tacrolimus in different organisms has not been described before, and may relate to differences in the binding affinity of the modulators to the

ABC transporter involved in DMI transport.

Other known modulators of mammalian ABC transporters such as verapamil, quinine, and propranolol (19) did not exhibit synergism with oxpoconazole against *B. cinerea*. It may be that uptake of these compounds by *B. cinerea* is too low for activity. Alternatively, the binding affinity of the compounds to BcatrD may be too low. These modulators are known to potentiate the activity of antitumour drugs in multidrug resistant tumor cells. ABC transporters involved in multidrug resistant tumor cells such as MDR1 and MRP1 have a topology in which the transmembrane domains (TMD) and nucleotide binding fold (NBF) are arranged in a $[TMD_6\text{-}NBF]_2$ and $TMD\text{-}[TMD_6\text{-}NBF]_2$ configuration, respectively (22). DMI transporters from fungi such as BcatrD from *B. cinerea* have a $[NBF\text{-}TMD_6]_2$ configuration (10, 29). Hence, the modulating activity of verapamil, quinine, and propranolol may be limited to transporters with the $[TMD_6\text{-}NBF]_2$ configuration.

The synergism between oxpoconazole and MDR modulators such as chlorpromazine and tacrolimus as described in this report is only of academic interest. The use of these modulators as medical drugs precludes their use in agriculture. However, the observations may provide a "proof of concept" for modulators that could be useful in agriculture by enhancing the activity of fungicides against wild-type and MDR populations of plant pathogens. Compounds described in this research might also act as lead compounds in the chemical synthesis of modulators of interest in either clinical or agricultural situations.

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Chapter 7

General discussion

The aim of the research described in this thesis was to isolate ATP-binding cassette (ABC) and major facilitator superfamily (MFS) transporter genes from *Botrytis cinerea* involved in efflux of fungicides that inhibit sterol biosynthesis and to discover compounds, which can modulate efflux of DMIs by both transporters. This research could contribute to a better understanding of mechanisms involved in fungicide sensitivity and resistance and provide clues to counteract resistance development by the use of compounds that synergise the activity of DMI fungicides.

Expression of transporter genes from *B. cinerea*

Chapter 3 describes a screen for putative ABC and MFS genes from *B. cinerea* involved in transport of DMI fungicides. Basal expression of these genes in a wild-type isolate and DMI-resistant mutants, and induced expression after treatment with various DMI fungicides were investigated. We considered that basal expression levels would be more relevant for the phenotype of mutants than induced expression levels, because replacement or overexpression mutants of the same genes do not necessarily show a phenotype for compounds that induce expression of ABC transporter genes (2, 22, Chapter 4). Such genes might encode transporters with no or a low substrate specificity for the inducing compounds. Consequently, their function can be compensated by redundancy of transporters with a relatively high substrate specificity. ABC genes that are induced by DMI fungicides but do not obviously bear relevance for transport by the encoded proteins are *BcatrB*, *BcatrG*, and *BcatrM* (Chapter 3). The fact that one compound can induce expression of multiple ABC transporter genes indicates that they are co-regulated. This is in agreement with results described for ABC transporters from other organisms (13, 14, 20). We also found that the ABC transporter gene *BcatrD* and the MFS transporter gene *Bcmfs1* are simultaneously induced by the same DMI fungicides (Chapter 3) and that the encoded proteins both transport DMIs (Chapter 4 and 5). This observation indicates that co-regulation of ABC and MFS transporter genes is possible. This phenomenon has not been described before for other organisms.

***BcatrD* and ABC transporters**

Among the ABC transporters studied in this thesis, BcatrD seemed to be the most significant one for transport of DMIs since basal expression levels of *BcatrD* in three *B. cinerea* isolates correlated with sensitivity to DMI fungicides. This assumption was confirmed by phenotyping of gene-replacement and overexpression mutants with respect to fungicide sensitivity (Chapter

4). A DMI transporter reported before from *Penicillium digitatum* is PMR1 (17). The homology between BcatrD and PMR1 is rather high (56.4%). The substrate specificity of BcatrD and PMR1 is also similar and only differs with respect to camptothecin, a natural toxic product from *Camptotheca acuminata*. Replacement mutants of *BcatrD* have a similar sensitivity to camptothecin (Chapter 4), while *PMR1* disruption mutants exhibit increased sensitivity against this natural toxic compound (18). AtrE, AtrF, and AtrG from *Aspergillus nidulans* are also homologues of BcatrD (Figure 1). The above mentioned proteins cluster with transporters from taxonomically distinct filamentous fungi (15). DMI transporters are also found in yeasts. An example is CDR1 (46.8%) from *Candida albicans* located on a branch of the dendrogram different from BcatrD (Figure 1). It is also interesting to note that fungal DMI transporters do not need to be closely related to BcatrD. Examples are AtrB from *A. nidulans* and MgAtr2 from *Mycosphaerella graminicola.* These two ABC transporters are multidrug transporters with a wide substrate range. The results indicate that ABC genes may play a significant role in protection of *B. cinerea* and other micro-organisms against DMIs and other fungitoxic compounds.

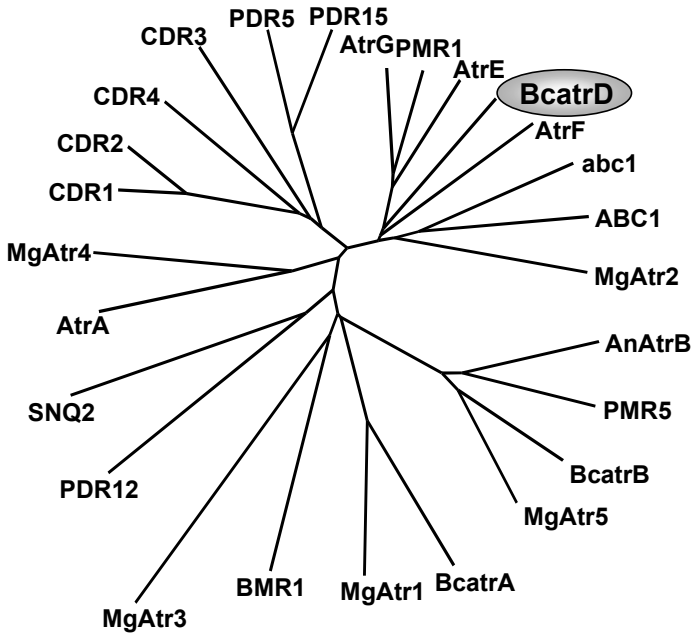

Figure 1. Dendrogram of 26 fungal and yeast ABC transporter proteins with the $[NBF\text{-}TMD_6]_2$ topology. Multiple alignment was performed by ClustalW analysis provided by the European Bioinformatics Institute. AtrA, AtrB, AtrE, AtrF, and AtrG are ABC transporters from *Aspergillus nidulans*, BcatrA, BcatrB, BcatrD, and BMR1 from *Botrytis cinerea*, CDR1, CDR2, CDR3, and CDR4 from *Candida albicans*, abc1 from *Gibberella pulicaris,* ABC1 from *Magnaporthe grisea*, MgAtr1, MgAtr2, MgAtr3, MgAtr4, and MgAtr5 from *Mycosphaerella graminicola*, PMR1 and PMR5 from *Penicillium digitatum*, and PDR5, PDR12, PDR15, and SNQ2 from *Saccharomyces cerevisiae*.

Bcmfs1 and MFS transporters

Bcmfs1 is highly homologous to aflT from *A. parasiticus*, CFP from *Cercospora kikuchii*, and TOXA from *Cochliobolus carbonum* (Figure 2), which function in secretion of aflatoxin, cercosporin, and HC-toxin, respectively (6, 5, 19). This observation suggests that MFS transporters are especially involved in secretion of endogenous toxins and that Bcmfs1 may play a role in secretion of toxins from *B. cinerea.* However, such a physiological role has not yet been found. *aflT* and *TOXA* cluster in the genome with genes involved in the biosynthetic pathways of aflatoxin and HC-toxin, respectively. This observation might suggest that *Bcmfs1* is also located in a gene cluster that encodes a toxin, but such a gene cluster could not be identified yet. Similar results were observed for *CFP* (5). CFP is regarded as a virulence factor since disruption of the encoding gene resulted in reduced virulence on soybean (5). However, virulence of replacement mutants of *Bcmfs1* from *B. cinerea* on tomato is similar to that of the parental strain. Therefore, the natural function of Bcmfs1 is still unclear (Chapter 5).

The substrate range of Bcmfs1 seems to be quite broad since chemically and biologically unrelated compounds such as natural toxic compounds (camptothecin and cercosporin) and fungicides (anilinopyrimidines, dicarboximides, and DMIs) can be substrates of Bcmfs1. Therefore, Bcmfs1 was proposed as the first multidrug transporter identified in a filamentous fungus (Chapter 5). Other MFS transporters involved in efflux of DMI fungicides are BenR (10) and FLU1 (4) from *C. albicans* and FLR1 from *Saccharomyces cerevisiae* (1). The sequence homology of Bcmfs1 with these DMI transporters is low (Figure 2).

The results suggest that *Bcmfs1* is a unique gene without known functional homologues in other fungi. The results also indicate that ABC and MFS transporters may have similar functions in protection against exogenous toxic compounds.

ABC and MFS transporters as new targets of crop protection agents

ABC or MFS transporters can be regarded as putative targets of crop protection agents. Inhibitors of transporter activity may synergize the activity of commercial fungicides, act as plant disease control agents by increasing the efficacy of plant defence products, or block the secretion of virulence factors of plant pathogens.

Inhibitors that synergize activity of commercial fungicides. Fungicides known to be secreted by ABC or MFS transporters are DMIs (*e.g.* tebuconazole, oxpoconazole, and prochloraz) (17,

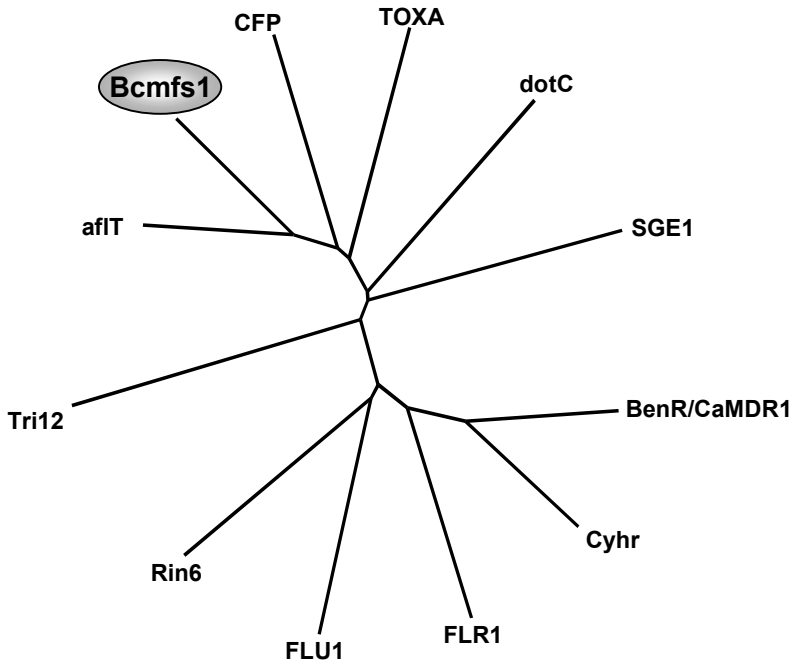

Figure 2. Dendrogram of 12 fungal and yeast MFS transporter proteins. Multiple alignment was performed by ClustalW analysis provided by the European Bioinformatics Institute. aflT is an MFS transporter from *Aspergillus flavus*, Bcmfs1 from *Botrytis cinerea*, BenR/CaMDR1 and FLU1 from *Candida albicans*, TOXA from *Cochliobolus carbonum*, CFP from *Cercospora kikuchii,* CYHR from *Candida maltosa*, dotC from *Dothistroma pini*, Rin6 from *Gibberella pulicaris,* Tri12 from *Gibberella zeae*, and FLR1 and SGE1 from *Saccharomyces cerevisiae*.

Chapter 4), phenylpyrroles (fenpiclonil and fludioxonil) (22, 26) and several fungicides from other classes (2, Chapter 5). In mammalian tumour cells, a wide range of compounds is described with an inhibitory activity against ABC transporters. Such inhibitors can be classified as anthracycline and *Vinca* alkaloid analogs, calcium channel blockers, calmodulin antagonists, cyclosporines, steroids and hormonal analogs, and miscellaneous hydrophobic, cationic compounds (11). The activity of plant antimicrobials (*e.g.* berberine, resveratrol, and rhein) can be potentiated more than 100 fold by MDR inhibitors (MC_{207110} and/or INF_{271}) against human pathogens, such as *Pseudomonas aeruginosa, Escherichia coli,* and *Salmonella enterica* (25). In the human pathogen *C. albicans*, cyclosporine and fluphenazine potentiate the activity of fluconazole (16). These inhibitors reduce the efflux of fungicides that are substrates of the transporters and ultimately enhance the accumulation of the compounds inside the microorganisms. Enhanced accumulation will result in a faster and more efficient interaction of fungicides with their target and, hence, act as synergists of fungicides against wild-type isolates. If resistance to fungicides is based on overproduction of transporters, the inhibitors may abolish

the resistance mechanism and, hence, be used as a countermeasure against resistance development to fungicides (Figure 3) (8).

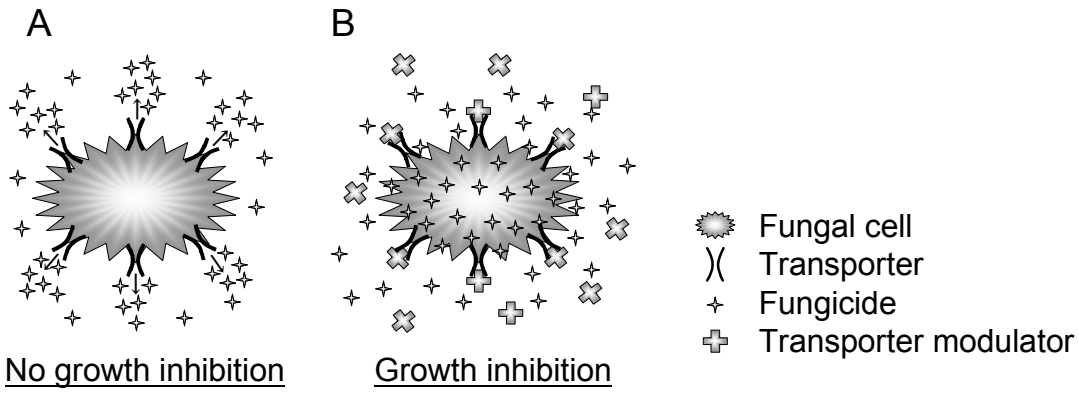

Figure 3. Cartoon of fungicide synergism by modulators of transporter activity. (A) Fungicide secretion from fungal cells in the absence of a modulator. Result: no growth inhibition by the fungicide. (B) Fungicide accumulation in the presence of a modulator. Result: growth of the fungus is arrested.

Our results indeed indicate that inhibitors of drug transporter activity can act as synergists of fungicides. We demonstrated that the ratio between the EC_{50} values of oxpoconazole for radial growth of an overexpression mutant of *BcatrD* and a double replacement mutant of *BcatrD* and *Bcmfs1* in *B. cinerea* amounted almost 20. Hence, synergistic activity by inhibitors of ABC transporter activity might also approach this ratio (Chapter 4 and 5). However, the value of synergists that modulate the activity of ABC transporter activity in fungicide resistance management is doubtful since a major resistance mechanism in plant pathogens to DMI fungicides is overproduction of the target enzyme $P450_{14DM}$. This can be caused by mutations in the promoter region of the encoding *CYP51* gene in *P. digitatum* (12). Overproduction of $P450_{14DM}$ is also found in DMI-resistant field isolates of *Venturia inaequalis* (21). Hence, inhibition of drug transporter activity alone may be not sufficient to prevent resistance development under field conditions.

In human, P450 oxidase CYP3A4, responsible for the metabolic degradation of the majority of pharmaceutical agents, and the multidrug transporter MDR1 are coordinately regulated by the same steroid and xenobiotic receptor (SXR), which plays a central role in regulating *CYP3A4* transcription (24). Synold *et al.* (24) also found that ecteinascidin-743, a marine-derived natural compound, antagonizes SXR activity by suppressing the induced expression of both *CYP3A4* and *MDR1*. It is possible that P450s and efflux transporter genes are

also co-regulated in phytopathogenic fungi. If so, compounds that reduce the expression of both genes in fungi might also act as strong synergists of fungicides.

Inhibitors that increase the efficacy of plant defence products. Phytoanticipins and phytoalexins are plant defence compounds. Transporters from plant pathogens can provide protection against plant defence compounds by efflux of the products into the outer environment. In this way the transporters can prevent accumulation of plant defence compounds in fungal cells and reduce their activity. The ABC transporter BcatrB from *B. cinerea* is a transporter of the phytoanticipin resveratrol produced by grape vine (23). The ABC transporter abc1 from *Gibberella pulicaris* can secrete rishitin, a phytoalexin of potato (9). Both transporters can thus be regarded as virulence factors since disruption of the genes reduced virulence of the pathogens on their respective hosts. These results suggest that transporters can secrete a range of natural toxic compounds. Compounds that inhibit the activity of ABC transporters of plant defence compounds will increase the accumulation of these products in plant pathogens. As a result, such inhibitors can act as plant disease control agents. In this way natural defence mechanisms of host plants are exploited (Figure 4). Hence, agrochemical companies should search for candidate fungicides with such a mode of action.

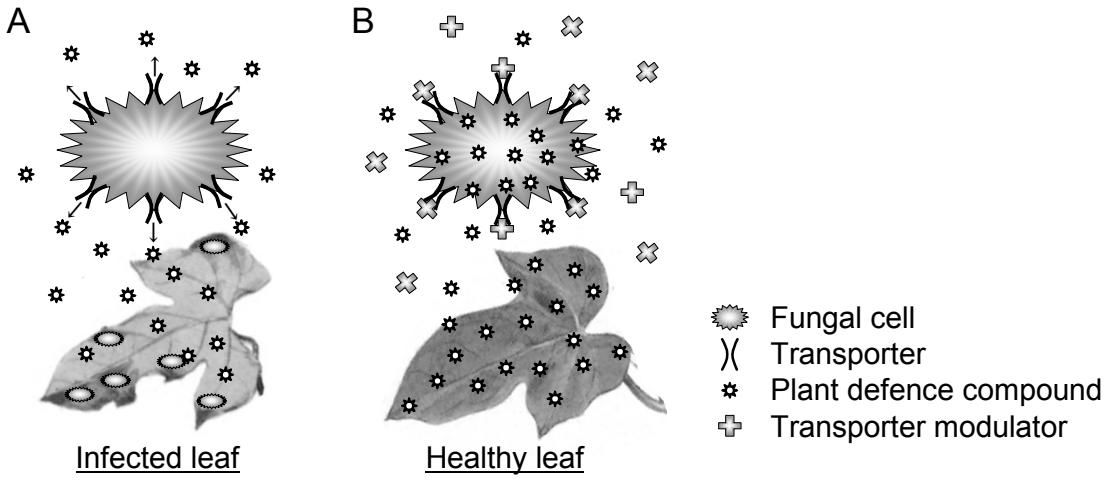

Figure 4. Cartoon of indirect disease control by modulators of transporter activity through interference with plant defence mechanisms. (A) Secretion of plant defence compounds in the absence of a modulator. Result: growth of the pathogen is not inhibited and the host is susceptible. (B) Accumulation of plant defence compounds in fungal cells in the presence of a modulator. Result: growth of the pathogen is inhibited and the host is resistant.

Inhibitors that block the secretion of virulence factors of plant pathogens. Several MFS transporters are involved in the efflux of secondary metabolites or toxins of fungi. aflT from *A. flavus* mediates the efflux of the mycotoxin aflatoxin (6), CFP from *C. kikuchii* the host specific toxin cercosporin (5), and TOXA from *C. carbonum* the host specific HC-toxin (19). Disruptants of *CFP* displayed a decreased virulence on soybean leaf. Recently, dotC from *Dothistroma pini* was reported to secrete dothistromin, a precursor of aflatoxin. Dothistromin is regarded as a virulence factor to infect needles of *Pinus radiata* (3). These data suggest that MFS transporters can function as a virulence factor in many plant pathogens. Compounds that inhibit such transporters may act as plant disease control agents as they interfere with essential virulence factors required for host plant colonization (Figure 5). Hence, agrochemical companies also should try to validate this hypothesis.

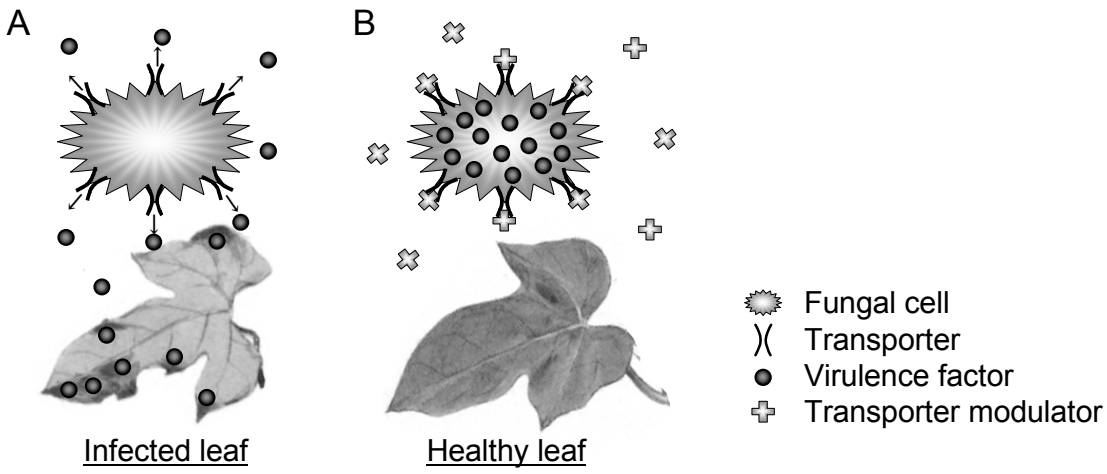

Figure 5. Cartoon of indirect disease control by modulators of transporter activity through inhibition of secretion of virulence factors (host specific toxins). (A) Secretion of virulence factors from fungal cells. Result: disease symptom development (chlorosis) and host plant colonization. (B) Inhibition of secretion of virulence factors in the presence of a modulator. Result: indirect disease control by inability of the fungus to attack the host.

Physiological functions of BcatrD and Bcmfs1

Experiments described in this thesis did not reveal the natural function of BcatrD and Bcmfs1. Their function can be identified by investigating the sensitivity of replacement and overexpression mutants to a broader range of natural toxic compounds than tested in this thesis, and by studying the virulence of these mutants on hosts belonging to different plant species. The identification of host plants on which BcatrD or Bcmfs1 mutants demonstrate a reduced virulence would provide interesting clues to the nature of the natural substrates of the proteins

during pathogenesis. Virulence test on such plants would also provide ways to test whether transporter modulators such as chlorpromazine or tacrolimus can act as plant disease control agents against wild-type isolates of *B. cinerea.* In this way, one could verify the hypothesis that ABC transporter modulators can act as new crop protection agents.

Discovery of transporter modulators

A direct and easy way to discover compounds that modulate the activity of drug transporters is the use of overexpression mutants in crossed-paper strip tests as described in Chapter 6. By comparison of the synergistic activity of compounds to a wild-type isolate and to overexpression and replacement mutants, the modulating activity for a particular transporter protein can be assessed. In this way, we identified chlorpromazine and tacrolimus as a modulator of BcatrD (Chapter 6).

Modulators may exhibit different modulating mechanisms. One mechanism might be based on reduction of the transcription of transporter genes. DNA microarray analysis with transporter genes could contribute to high-throughput detection of compounds with such functions. Another putative mechanism of modulators is competitive inhibition at the substrate-binding site of the transporter protein with physiological substrates. The mechanism could decrease the activity of transporters and result in a higher accumulation inside the fungal cells. This type of modulation can be identified by measuring the effect of modulating compounds on the accumulation of substrates.

The value of knockout mutant of transporter genes in drug discovery

Knockout mutants may exhibit increased sensitivity to fungitoxic products. Hence, such mutants can be useful in screening programs to find candidate fungicides in chemical libraries, since their growth will be inhibited stronger by toxic compounds than that of the corresponding wild-type isolates. Multiple knockout mutants of different transporter genes can be even more useful. One multiple knockout mutant of *S. cerevisiae* is already available (7). Similar mutants are under construction for *A. nidulans* (De Waard, personal communication).

Comparison of the sensitivity of replacement and overexpression mutants of transporter genes with that of the wild-type isolate can reveal whether the fungitoxic activity of candidate compounds affects the activity of transporters. This is important to know since fungicides, which can be substrates of transporters are prone to acquire resistance mediated by

overexpression of transporters. To avoid this, candidate fungicides should be screened for their ability to act as a substrate of transporters.

Conclusions

In this thesis, we isolated two transporter genes involved in efflux of DMI fungicides from *B. cinerea.* One is the ABC transporter gene *BcatrD* and the other the MFS transporter gene *Bcmfs1*. We propose that BcatrD is the major transporter involved in efflux of DMIs and that Bcmfs1 is only of minor significance. Compounds that can modulate the activity of DMI fungicides by interfering with ABC transporters were identified. We anticipate that the results may contribute to the development of new crop protection agents.

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SUMMARY

ATP-binding cassette (ABC) and major facilitator superfamily (MFS) transporters are two major classes of proteins involved in drug resistance. ABC transporter proteins are primary transporters that use the energy generated by ATP hydrolysis to transport drugs over membranes, while MFS transport proteins are secondary transporters that use the proton motive force as an energy source. The substrate range of both transporters is very broad and may include ions, amino acids, peptides, sugars, secondary metabolites, and drugs. The goal of this thesis was to identify ABC and MFS transporter genes from *Botrytis cinerea,* which are involved in transport of sterol demethylation inhibitor (DMI) fungicides, and to discover compounds, which can modulate the activity of such transporters. Such modulators may be useful in practice to counteract resistance development to DMIs.

An overview of characteristics of *B. cinerea*, commercial fungicides used in control of the pathogen, modes of action of botryticides, and resistance development to fungicides that inhibit sterol biosynthesis is described in Chapter 1. This chapter also describes the relevance of ABC and MFS transporters in fungicide resistance and the importance of fungicide mixtures to delay resistance development.

The DMI fungicide oxpoconazole, developed by Ube industries, Ltd., is introduced in Chapter 2. This fungicide is effective against diseases that are commonly controlled by DMIs, such as rusts and scabs. In addition, it is effective against grey mould caused by *B. cinerea* under field condition. *B. cinerea* is known for its ability to acquire resistance to fungicides easily. ABC and MFS transporters may play a role in such a resistance development. The physiological functions of these transporters may be to cope with natural plant toxins since the pathogen has an extraordinary wide host range.

Chapter 3 describes fourteen ABC and three MFS transporter genes from *B. cinerea*. Two of the ABC transporters were described previously and the others were identified in an EST library of the fungus. Their role in DMI resistance was investigated by studying basal and induced expression in wild-type and DMI-resistant strains of *B. cinerea.* From this screen, it appeared that *BcatrD* was the most probable ABC transporter gene encoding a putative DMI transporter since basal expression in three isolates correlated with sensitivity to DMIs. Induced expression after treatment with DMIs also correlated with sensitivity to DMIs. The MFS transporter gene *Bcmfs1* might encode another DMI transporter since its basal expression in DMI resistant strains was also higher than that in the wild-type strain.

Summary

The functional analysis of *BcatrD* and *Bcmfs1* is described in Chapters 4 and 5, respectively. This was achieved by phenotyping of gene replacement and overexpression mutants generated from the haploid wild-type strain B05.10. Replacement and overexpression mutants of *BcatrD* displayed an increased and decreased sensitivity to DMIs, respectively. Overexpression mutants of *Bcmfs1* also exhibited a decreased sensitivity to these fungicides while *Bcmfs1* replacement mutants showed similar sensitivity to DMIs as compared to the wild-type. To clarify the role of Bcmfs1 in DMI-sensitivity in more detail, we constructed a double replacement mutant of *BcatrD* and *Bcmfs1*. The double replacement mutant was more sensitive to DMIs than the single replacement mutant of *BcatrD*. These results suggest that BcatrD functions as a major DMI transporter and that Bcmfs1 is of minor importance.

Accumulation of oxpoconazole by germlings of the mutants is also described in Chapter 4 and 5. Accumulation of oxpoconazole was transient in time as observed before for other DMIs and other filamentous fungi. The transient accumulation pattern is the result of passive influx and inducible active efflux of fungicides by transporters. The initial accumulation (20 min after the addition of oxpoconazole) level of oxpoconazole in the strains tested correlated with sensitivity to oxpoconazole. The steady state levels (more than 60 min after the addition of oxpoconazole) of accumulation by *BcatrD* and *Bcmfs1* replacement mutants were higher than that by the wild-type isolate. These results indicate that BcatrD and Bcmfs1 mediate the sensitivity of *B. cinerea* to oxpoconazole by reducing the accumulation of the fungicide in mycelial cells.

Known modulators of human ABC transporters were tested for their potency to modulate the activity of DMIs (Chapter 6). This activity was investigated qualitatively in crossed-paper strips tests. Chlorpromazine and tacrolimus proved to be modulators of BcatrD since these compounds showed obvious synergism with oxpoconazole against *BcatrD* overexpression mutants. The synergistic activity negatively correlated with the sensitivity to oxpoconazole of the strains tested, and positively correlated with the accumulation level of oxpoconazole after treatment with the modulator chlorpromazine. These results indicate the existence of compounds that can modulate the activity of fungal ABC transporters and synergize the activity of DMI fungicides.

Replacement mutants of *Bcmfs1* also displayed an increased sensitivity to the natural toxic compounds camptothecin (a plant defence compound from *Camptotheca acuminata*) and cercosporin (a fungal toxin of *Cercospora kikuchii*). This observation may imply that Bcmfs1 is

involved in protection of *B. cinerea* against plant defence compounds or in secretion of virulence factors. However, the virulence of all gene replacement mutants of *B. cinerea* tested on detached leaves of tomato was similar. These results indicate that BcatrD and Bcmfs1 are not involved in virulence on tomato. Virulence tests on other host plants are required to elucidate whether any of the two transporters is involved in secretion of virulence factors during pathogenesis.

In conclusion, the results presented in this thesis demonstrate that fungicide transporters with an overlap in substrate specificity exist in *B. cinerea*. The intrinsic function of these transporters may involve protection of the pathogen against natural toxic compounds or secretion of endogenous toxic metabolites. Modulators of fungal transporters can affect of interfere with the transport of these products or fungicides. This implies that modulators can act as synergists of fungicides or may function as plant disease control agents with an indirect activity (Chapter 7).

SAMENVATTING

ATP-bindingscassette (ABC) en major facilitator superfamily (MFS) transporters zijn twee belangrijke klassen van eiwitten die betrokken zijn bij resistentie tegen bestrijdingsmiddelen en andere toxische verbindingen (drugs). ABC transporters zijn primaire transporteiwitten die de energie nodig voor transport van toxische verbindingen over membranen, genereren door hydrolyse van ATP. MFS transporters zijn secundaire transporteiwitten die de energie van de elektrochemische gradiënt over membranen benutten. De substraatspecificiteit van beide typen van transporters is vrij breed. Bekende substraten zijn ionen, aminozuren, peptiden, suikers, secundaire metabolieten, en drugs. Het doel van dit onderzoek was om ABC en MFS transporter genen van *Botrytis cinerea* te identificeren, die betrokken zijn bij transport van fungiciden die de sterol demethylering remmen (DMIs) en om stoffen te ontdekken die de activiteit van zulke transporters kunnen moduleren. Dergelijke modulatoren kunnen in de praktijk nuttig zijn om resistentieontwikkeling tegen DMIs tegen te gaan.

Hoofdstuk 1 geeft een overzicht van de karakteristieken van *B. cinerea*, commerciële fungiciden die voor de bestrijding van het pathogeen gebruikt worden, werkingsmechanismen van fungiciden en van resistentieontwikkeling tegen DMI fungiciden. In dit hoofdstuk worden ook het belang van ABC en MFS transporters in resistentie tegen fungiciden en het belang van mengsels om resistentieontwikkeling tegen te gaan, beschreven.

Het DMI fungicide oxpoconazool, ontwikkeld door Ube industries, Ltd., wordt geïntroduceerd in hoofdstuk 2. Het fungicide is effectief tegen ziekten die gewoonlijk ook door andere DMIs worden bestreden, zoals roesten en schurft. Oxpoconazool is onder veldcondities echter ook effectief tegen grauwe schimmelziekten die worden veroorzaakt door *B. cinerea*. De schimmel staat bekend om zijn vermogen om snel resistentie tegen fungiciden te ontwikkelen. ABC en MFS transporters kunnen bij resistentieontwikkeling een rol spelen. Waarschijnlijk is een fysiologische functie van deze transporters het bieden van bescherming tegen natuurlijke, fungitoxische stoffen die in de vele waardplanten van *B. cinerea* voorkomen.

Hoofdstuk 3 beschrijft veertien ABC en drie MFS transporter genen van *B. cinerea.* Twee van deze ABC genen zijn eerder beschreven. De overige werden geïdentificeerd in een EST bank van de schimmel. Hun rol in resistentie tegen DMIs werd onderzocht door bestudering van de basale en geïnduceerde expressie in wild-type en DMI-resistente stammen. De resultaten van deze screening wijzen erop dat *BcatrD* waarschijnlijk het belangrijkste ABC transporter gen is dat codeert voor een DMI transporter omdat de basale expressie in de drie stammen correleerde

met gevoeligheid voor DMIs. Het MFS transporter gen *Bcmfs1* zou kunnen coderen voor een andere DMI transporter omdat de basale expressie ervan in resistente stammen ook hoger was dan in het wild-type.

De functionele analyse van *BcatrD* en *Bcmfs1* wordt beschreven in de hoofdstukken 4 en 5. De analyse werd uitgevoerd door fenotypische karakterisering van deletie- en overexpressiemutanten van de haploide wild-type stam B05.10 van *B cinerea*. Deletie- en overexpressiemutanten van *Bcmfs1* vertoonden respectievelijk een toe- en afname in gevoeligheid voor DMIs in vergelijking tot het wild-type. Om de rol van *Bcmfs1* in gevoeligheid voor DMIs verder te onderzoeken, werd een dubbele deletiemutant van *BcatrD* en *Bcmfs1* geconstrueerd. De resultaten van de fenotypische karakterisering van deze mutant wijzen erop dat BcatrD functioneert als een belangrijke DMI transporter en dat Bcmfs1 in dit opzicht een ondergeschikte rol speelt.

Accumulatie van oxpoconazool door kiemlingen van mutanten van *B. cinerea* wordt ook beschreven in hoofdstuk 4 en 5. De accumulatie van oxpoconazool was transient in de tijd, zoals dat eerder voor andere DMIs en filamenteuze schimmels is waargenomen. Het transiente accumulatiepatroon is het resultaat van passieve influx en induceerbare, actieve efflux van het fungicide door transporters. Het initiële accumulatieniveau van oxpoconazool (20 min na toevoeging) in de stammen correleerde met de gevoeligheid voor het fungicide. Het accumulatieniveau in de steady state (meer dan 60 min na de toevoeging) van *BcatrD* en *Bcmfs1* deletiemutanten was hoger dan van het wild-type. Deze resultaten wijzen erop dat BcatrD en Bcmfs1 invloed hebben op de gevoeligheid van *B. cinerea* voor oxpoconazool doordat zij de accumulatie van het fungicide in het mycelium verlagen.

Bekende modulatoren voor menselijke ABC transporters werden getest op hun vermogen om de activiteit van DMI fungiciden te versterken. Dit werd onderzocht in agar-diffusieproeven met papierstroken, geïmpregneerd met oxpoconazool en teststoffen. Chlorpromazine en tacrolimus bleken modulatoren van BcatrD te zijn omdat deze stoffen duidelijk synergisme vertoonden tegen *BcatrD* overexpressiemutanten. De synergistische activiteit vertoonde een negatieve correlatie met de gevoeligheid van de geteste stammen voor oxpoconazool en een positieve correlatie met het accumulatieniveau van oxpoconazool na behandeling met de modulator chlorpromazine (hoofdstuk 6). Deze resultaten tonen aan dat er stoffen bestaan die de activiteit van ABC transporters in schimmels kunnen moduleren en de activiteit van DMI fungiciden kunnen versterken.

Deletiemutanten van *Bcmfs1* vertoonden ook een verhoogde toename in gevoeligheid voor de natuurlijke, toxische verbindingen camptothecine (een afweerstof van de plant *Camptotheca acuminata*) en cercosporine (een toxine van de schimmel *Cercospora kikuchii*). Deze waarneming kan betekenen dat Bcmfs1 betrokken is bij de bescherming van de schimmel tegen afweerstoffen van planten of bij de secretie van virulentiefactoren. De virulentie van alle geteste deletiemutanten op bladeren van tomaat was echter identiek aan die van het wild-type. Deze resultaten wijzen erop dat BcatrD en Bcmfs1 niet betrokken zijn bij virulentie op tomaat. Virulentietesten op andere gastheerplanten van *B. cinerea* zijn vereist om te bepalen of de transporters inderdaad als virulentiefactoren kunnen fungeren.

Samenvattend, tonen de resultaten van dit onderzoek aan dat *B. cinerea* transporters van fungiciden bezit met een overlap in substraatspecificiteit. De natuurlijke functie van deze transporters is waarschijnlijk het bieden van bescherming tegen natuurlijke, toxische verbindingen of secretie van endogene toxische metabolieten van de schimmel. Modulators van transporters in schimmels kunnen het transport van deze stoffen en fungiciden moduleren. Dit betekent dat modulatoren als synergist werkzaam kunnen zijn en kunnen fungeren als bestrijdingsmiddelen met een indirecte werking (hoofdstuk 7).

要　旨

薬剤耐性に関与しているトランスポーターは ATP-binding cassette（ＡＢＣ）トランスポーターと major facilitator superfamily (ＭＦＳ)トランスポーターの２つのスーパーファミリーに大別される。ＡＢＣトランスポーターは、ＡＴＰの加水分解の際に発生するエネルギーを薬剤排出のエネルギーとして用いるため、第１トランスポーター (primary transporter)と呼ばれ、ＭＦＳトランスポーターは、プロトンの濃度勾配を薬剤排出機構としており、第２トランスポーター(secondary transporter)と呼ばれている。これらのトランスポーターの基質は、イオン、アミノ酸、ペプチド、糖、二次代謝物、および薬物等であり、多種多様の化合物がトランスポーターの基質として認識されている。この論文の目的は、植物病原菌である灰色カビ病菌(*Botrytis cinerea*)より、菌類のステロールの１４位の脱メチル化反応を阻害するＤＭＩ剤（アゾール系殺菌剤）の排出に関与する、ＡＢＣおよびＭＦＳトランスポーター遺伝子を見つけ、また、それらトランスポーターの阻害剤を探すことである。そのようなトランスポーター阻害剤によって、ＤＭＩ剤の耐性菌問題の克服ができると期待された。

第１章には、灰色カビ病菌およびその防除法、灰色カビ病防除剤の作用機構および耐性機構、ステロール合成阻害剤（ＤＭＩ剤を含む）の作用機構および耐性機構、ＡＢＣおよびＭＦＳトランスポーターと殺菌剤耐性の関連、そして、耐性の進展を遅らすことに有意義な混合剤の重要性について紹介した。

第２章には、宇部興産（株）によって研究開発された、ＤＭＩ殺菌剤であるオキスポコナゾール（オーシャイン®）を紹介した。この殺菌剤は、他の市販ＤＭＩ剤と同様に各種サビ病、黒星病等に効果があるのみならず、灰色カビ病菌によって引き起こされる、灰色カビ病にも効果が認められる。灰色カビ病菌は、殺菌剤に対して容易に耐性を獲得し、また、２００以上の植物に感染する事例が知られていることから、殺菌剤、多様な自然界の毒物および灰色カビ病菌自身の毒素を排出するための効率よいトランスポーターシステムを構築していると考えられる。

第３章では、既知のＡＢＣトランスポーター遺伝子と新たに灰色カビ病菌のＥＳＴライブラリーより選抜した、計１４種のＡＢＣおよび３種のＭＦＳトランスポーター遺伝子について述べた。野生株および室内淘汰で得られたＤＭＩ剤耐性株を用い、それらの遺伝子の構成的な発現量と、ＤＭＩ剤で誘導された発現量を調べることでＤＭＩ剤耐性における役割について検討した。その結果、ＡＢＣトランスポーター遺伝子 *BcatrD* の構成的な発現量およびＤＭＩ剤で誘導された発現量が、菌株のＤＭＩ剤への感受性と相関しており、*BcatrD* 遺伝子が、ＤＭＩ剤トランスポーターとして最も有力であると

考えられた。また、ＭＦＳトランスポーター遺伝子 *Bcmfs1* の構成的な発現量も、ＤＭＩ剤耐性株で野生株より多いことが認められ、*Bcmfs1* 遺伝子も、ＤＭＩ剤トランスポーターとしての可能性が示唆された。

第４章および第５章では、野生株から *BcatrD* と *Bcmfs1* 遺伝子の破壊株および過剰発現株を作出し、表現型を調べる事により、*BcatrD* と *Bcmfs1* 遺伝子の機能を調べた。*BcatrD* 遺伝子の破壊株は、ＤＭＩ剤への感受性が増加し、過剰発現株では、感受性が低下していたことより、ＡＢＣトランスポーターBcatrD の、ＤＭＩ剤トランスポーターとしての機能が明らかとされた。一方、*Bcmfs1* 遺伝子の破壊株のＤＭＩ剤への感受性は変化しなかったが、過剰発現株のＤＭＩ剤への感受性は低下した。ＤＭＩ剤に対する *Bcmfs1* 遺伝子の機能を明らかとするために、*BcatrD*、*Bcmfs1* 両遺伝子の破壊株を作出した。*BcatrD*、*Bcmfs1* 両遺伝子の破壊株は、*BcatrD* 単独遺伝子の破壊株より、ＤＭＩ剤への感受性が増加した。これらの結果より、ＡＢＣトランスポータ BcatrD は、ＤＭＩ剤の排出に大きく関与しており、一方、ＭＦＳトランスポータ Bcmfs1 は、ＤＭＩ剤の排出に関与しているが、その働きは大きくないと考えられた。

第４章および第５章には、灰色カビ病遺伝子変異株菌体内へのオキスポコナゾールの取り込みについても述べた。オキスポコナゾールの菌体内への取り込みは、受動的な取り込みとトランスポーターによる能動的な排出の結果、トランジエントな経時変化を示した。オキスポコナゾールを添加、２０分後の初期の取り込み量は、菌株のオキスポコナゾールへの感受性と相関した。また、オキスポコナゾールを添加、６０分後以降は、取り込みと排出の均衡が取れている定常状態であると考えられるが、この時の *BcatrD* および *Bcmfs1* 遺伝子破壊株へのオキスポコナゾールの取り込み量は、野生株への取り込み量より多く、トランスポーターBcatrD および Bcmfs1 は、オキスポコナゾールの菌体内への取り込みを減少させる事で、灰色カビ病菌の感受性に影響を与えていると考えられた。

第６章では、ヒトにおいて知られているＡＢＣトランスポーター阻害剤を用い、灰色カビ病菌のトランスポーター阻害作用をろ紙交差法にて検討した。その結果、クロルプロマジンおよびタクロリムスがオキスポコナゾールと協力作用を示した。これらの協力作用は、*BcatrD* 遺伝子の過剰発現株で顕著に認められ、*BcatrD* 遺伝子の破壊株では、ほとんど認められなかった。また、平板培地での菌叢生育法より算出された協力係数は、オキスポコナゾールへの感受性およびクロルプロマジン処理前後のオキスポコナゾールの菌体内への取り込み量と相関関係を示した。これらの結果は、菌類のＡＢＣトランスポーターを阻害し、ＤＭＩ剤と協力作用を示す化合物が存在していることを示唆する

ものと考えられた。

Bcmfs1 遺伝子の破壊株では、野生株に比べカンプトテシン（*Camptotheca acuminata* 由来の植物防御化合物）およびサーコスポリン（*Cercospora kikuchii* 由来の菌毒素）への感受性が増加した。この結果は、ＭＦＳトランスポーターBcmfs1 が、植物防御化合物からの防御、または病原性物質の排出に関与している可能性を示唆している。しかしながら、*BcatrD* および *Bcmfs1* 遺伝子破壊株の、トマトの切り取り葉への病原性は、野生株と同様であり、両遺伝子はトマトへの病原性には関与していないと考えられた。これらのトランスポーターの病原性への関与を証明するには、他の植物を使用した遺伝子破壊株の病原性試験が必要であると考えられた。

この論文には、灰色カビ病菌には、多様な化合物を基質として共有している殺菌剤排出トランスポーターが存在していることを述べてある。これらトランスポーターの本来の機能は、この病原菌を自然界の毒性物質からの防御すること、または菌体内の毒性代謝物を排出することであると考えられる。これらトランスポーターの阻害剤は殺菌剤およびこれらの天然毒性物質の排出に影響を及ぼすことで、殺菌剤との協力剤もしくは間接的に病原菌を防除できる化合物の創製につながると考えられる（第７章)。

CURRICULUM VITAE

Keisuke Hayashi was born on March 16, 1967 in Shimonoseki, Yamaguchi, Japan. In 1990 he obtained his B.Sc. degree in agriculture at the University of Tsukuba (Tsukuba, Ibaraki, Japan). He worked at the Laboratory of Food Biochemistry under the supervision of Prof. Isao Kusakabe. The research topic was the enzyme system involved in complete hydrolysis of xylan. In the same year, he started his professional career in agrochemical research at Ube Industries, Ltd. (Ube, Yamaguchi, Japan). In 1998, the company decided to send him to the Netherlands to study a new product of the Company, the fungicide oxpoconazole. In April 1999, he started his Ph.D. study at the Laboratory of Phytopathology of Wageningen University. The title of the research topic was "ABC and MFS transporters from *Botrytis cinerea* involved in sensitivity to fungicides and natural toxic compounds". He finished the experimental part of the research in June 2001 and returned to his company where he completed his Ph.D. thesis and started research in pharmacology and pharmacokinetics in the pharmaceutical department of the company.

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