European Journal of Plant Pathology **109:** 1003–1011, 2003.

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

Henk-jan Schoonbeek[1,*], Johannes G.M. van Nistelrooy[1] and Maarten A. de Waard[1,**]
[1]*Laboratory of Phytopathology, Wageningen University, P.O. Box 8025, 6700 EE Wageningen, The Netherlands; *Current address: Department of Plant Biology, University of Fribourg, Rue Albert Gockel 3, CH-1700 Fribourg, Switzerland; **Author for correspondence (Phone: 31 317 483123; Fax: 31 317 483412; E-mail: maarten.dewaard@wur.nl)*

Accepted 19 August 2003

Key words: active efflux, expression analysis, ATP-binding cassette transporters, major facilitator superfamily, membrane transporters, fungicides and virulence

Abstract

The role of multiple ATP-binding cassette (ABC) and major facilitator superfamily (MFS) transporter genes from the plant pathogenic fungus *Botrytis cinerea* in protection against natural fungitoxic compounds was studied by expression analysis and phenotyping of gene-replacement mutants. The expression of 11 ABC (*BcatrA–BcatrK*) and three MFS genes (*Bcmfs1*, *Bcmfs2* and *Bcmfs4*) was studied. All genes showed a low basal level of expression, but were differentially induced by treatment with cycloheximide and the plant defence compounds camptothecin, eugenol, psoralen, resveratrol and rishitin. The latter compounds induced expression of *BcatrB* at a high level. Eugenol was more toxic to *BcatrB* gene-replacement mutants than to the control isolates. Eugenol also caused an instantaneous increase in mycelial accumulation of the fungicide fludioxonil, a known substrate of BcatrB. However, there was no difference in virulence between the wild-type and *BcatrB* gene-replacement mutants on *Ocimum basilicum*, a plant known to contain eugenol. The results indicate that BcatrB is a transporter of lipophilic compounds, such as eugenol, but its role in virulence remains uncertain.

Abbreviations: ABC transporter – ATP-binding cassette transporter; MFS – major facilitator superfamily; EST – expressed sequence tag; DNOC – dinitro-*o*-cresol (2-methyl-4,6-dinitrophenol); SOPP – sodium *o*-phenylphenate.

Introduction

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

The substrate range of ABC transporters can vary from a single compound, as for Mam1, which exports the M-factor mating pheromone from *Schizosaccharomyces pombe* (Christensen et al., 1997), to a wide spectrum of compounds with no identified common feature, as for PDR5 from *Saccharomyces*

cerevisiae (Kolaczkowski et al., 1998) and MDR1 from *Homo sapiens* (Chen and Simon, 2000). Fungal ABC transporters with a role in protection against toxicants belong to two subfamilies of full-size transporters, the PDR subfamily with the nucleotide binding domain (NBF) and transmembrane domains (TMD) organised in a $(NBF–TMD_6)_2$ topology and the MDR subfamily with a $(TMD_6–NBF)_2$ topology (Stergiopoulos et al., 2002). Information on the substrate range of ABC transporters from filamentous fungi is rather limited. AtrB and AtrD from *Aspergillus nidulans* are transporters with a broad range of substrates, including antibiotics, fungicides and plant defence compounds (Andrade et al., 2000). BcatrB from *B. cinerea* has a wide substrate range, comprising mainly aromatic compounds (Schoonbeek et al., 2001; 2002; Vermeulen et al., 2001), whereas sterol biosynthesis inhibiting (SBI) fungicides are the only substrates identified for BcatrD (Hayashi et al., 2001; 2002). Substrates of BMR1 from *B. cinerea* (also known as BcatrK (Vermeulen et al., 2001)) are polyoxin and iprobenfos (Nakajima et al., 2001). Multidrug resistance in *Penicillium digitatum* is provided by the ABC transporters PMR1 and PMR5 with a preference for SBI fungicides and resveratrol, respectively (Nakaune et al., 1998; 2002). Complementation studies with ABC transporter genes from *Mycosphaerella graminicola* in *S. cerevisiae* also suggest a redundancy in transporters of various natural and synthetic fungitoxic compounds (Stergiopoulos et al., 2002).

MFS proteins from filamentous fungi involved in transport of toxic products are drug-proton (H^+)-antiporters with 12 or 14 membrane spanning domains (Paulsen et al., 1996; Stergiopoulos et al., 2002). Their substrate specificity may be limited to endogenous toxins, thus providing self-protection to the producing organisms (Stergiopoulos et al., 2002). This has been suggested for transport of aflatoxin, cercosporin, HC-toxin and trichothecene, by AflT from *Aspergillus flavus*, CFP1 from *Cercospora kikuchii*, TOXA from *Cochliobolus carbonum* and Tri12 from *Fusarium sporotrichioides*, respectively. However, some MFS transporters from yeasts, such as BenR and FLU1 from *Candida albicans* and FLR1 from *S. cerevisiae* are involved in resistance to exogenous antifungal compounds (Stergiopoulos et al., 2002).

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

Materials and methods

B. cinerea *strains*

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

Compounds

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

Growth media and conditions

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

Expression analysis

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

Accumulation of [^{14}C]fludioxonil

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

Virulence assay

Virulence assays were performed on leaves of basil (*O. basilicum* cv. Genovese Gigante), grown under greenhouse conditions for 8 weeks. Seven (Exp. 1) or five (Exp. 2) intact plants with three pairs of expanded leaves (15 cm high) were placed in humid chambers. The upperside of the leaves was inoculated with droplets (2 μl) of spore suspensions of *B. cinerea* (5×10^5 ml^{-1}), preincubated in 1 × B5 medium amended with 1% glucose and 10 mM ammonium phosphate pH 6.5 at 20 °C for 2 h to synchronise germination. Each leaf was pairwise inoculated with three droplets of strain B05.10 (parental line) and three droplets of strain B05.10, ΔBcatrA-M7, ΔBcatrB4 or ΔBcatrB5. Lesion diameters were measured after incubation at 15 °C for 3 days in the dark (Exp. 1) or in the light (Exp. 2). Statistical analysis with Duncan's *t*-test was based on average values of spreading lesions (with a diameter over 1 mm) of each pair of leaves.

Results

Expression analysis

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

The effects of compounds that affected the expression of any of the genes tested (Table 1) were studied in more detail. Germlings were treated with the test compounds at two concentrations for 20 and 60 min (Figure 1). The results indicate that induction of

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

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

[a]Compounds added from 1000× concentrated stock solutions in methanol. Concentrations of compounds (mg l^{-1}) between brackets.
[b]Arbitrary quantification of expression: no expression detectable (−), very weak expression (±), weak expression (+), intermediate expression (++) and strong expression (+++).

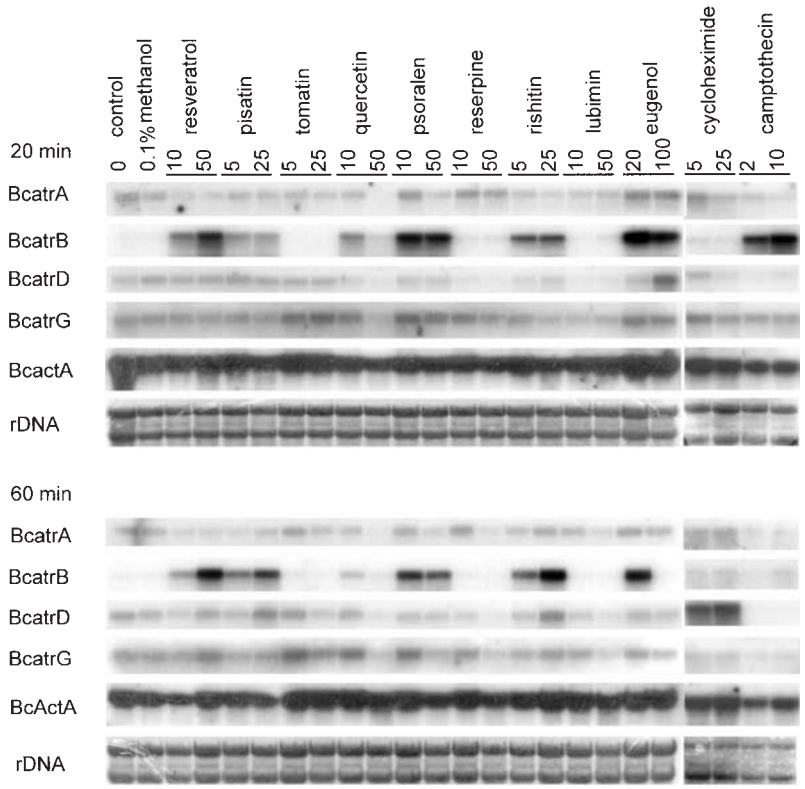

Figure 1. Expression analysis of the ABC transporter genes *BcatrA*, *BcatrB*, *BcatrD* and *BcatrG* after treatment of germlings from *B. cinerea* strain B05.10 with natural fungitoxic compounds for 20 and 60 min. Figures indicate concentration of compounds in mg l^{-1}. Blots were hybridised with *BcactA*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

expression can be time- and concentration-dependent. Generally, induction of *BcatrB* expression was relatively high after 20 min, except after exposure to pisatin. *BcatrD* expression was relatively high after 60 min, especially after treatment with cycloheximide. Transcript levels correlated positively with the concentrations of pisatin, resveratrol and rishitin tested. However, negative correlations were also observed, noticeably in the case of eugenol and *BcatrB*.

Toxicity assays

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

Accumulation of [^{14}C]fludioxonil

The interference of eugenol with efflux activity of BcatrB was studied by measuring its effect on accumulation of [^{14}C]fludioxonil, a well characterised substrate of BcatrB (Vermeulen et al., 2001). Resveratrol, another substrate of BcatrB (Schoonbeek et al., 2001), was used as a reference compound (Figures 2 and 3). The accumulation of [^{14}C]fludioxonil in control treatments proved to be as described previously (Vermeulen et al., 2001). In summary, accumulation by B05.10 was transient with a rapid initial increase followed by a low equilibrium level of circa 0.35 nmol mg^{-1} dry weight. Accumulation by ΔBcatrB4 rapidly increased and remained constant at circa 0.8 nmol mg^{-1} dry weight (data not shown). Addition of eugenol (100 mg l^{-1}) and resveratrol (100 mg l^{-1}) 65 min after addition of [^{14}C]fludioxonil resulted in a transient increase

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

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

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

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

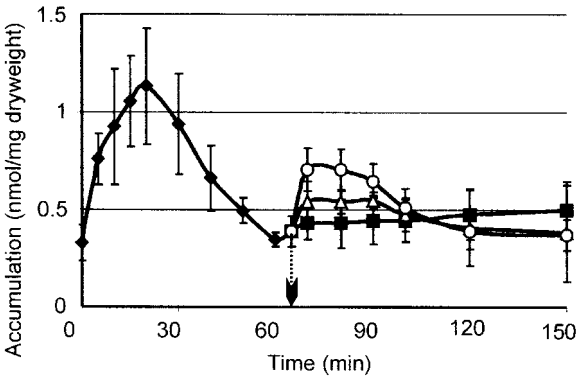

Figure 2. Effect of addition of eugenol and resveratrol on accumulation of [^{14}C]fludioxonil (1 mg l^{-1}) by germlings of *B. cinerea* strain B05.10. Compounds were added 65 min after addition of [^{14}C]fludioxonil (arrow). No treatment (♦), solvent control (■; 0.1% DMSO), eugenol (◯; 100 mg l^{-1})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

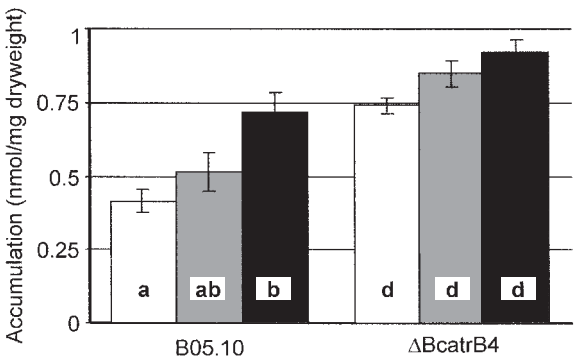

Figure 3. Effect of eugenol and resveratrol on accumulation of [^{14}C]fludioxonil (1 mg l^{-1}) by germlings of *B. cinerea* strains B05.10 and ΔBcatrB4. Compounds were added 65 min after addition of [^{14}C]fludioxonil. Control (0.1% DMSO; white), resveratrol (100 mg l^{-1}; grey) and eugenol (100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

in accumulation of [^{14}C]fludioxonil by B05.10 (Figure 2). The maximal increase was observed after 15 min. Therefore the effect of eugenol and resveratrol on [^{14}C]fludioxonil accumulation by B05.10 and ΔBcatrB4 was measured 15 min after addition of the compounds (Figure 3). Addition of eugenol, but not of resveratrol, resulted in a significant increase of the accumulation of [^{14}C]fludioxonil by B05.10. In contrast, neither compound had a significant effect on accumulation of [^{14}C]fludioxonil by ΔBcatrB4. Pre-treatment of germlings of B05.10 with eugenol or resveratrol induced [^{14}C]fludioxonil efflux capacity (data not shown).

Virulence assays

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

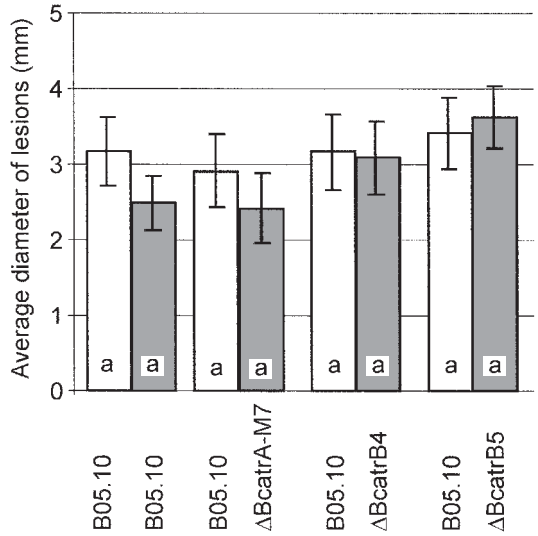

Figure 4. Virulence of *B. cinerea* strains on leaves of basil (*Ocimum basilicum* cv. Genovese Gigante) at 15 °C in the dark. Bars indicate the lesion size (mm) incited by pairwise inoculation with control strain B05.10 (white) and the test strains (grey) B05.10, *BcatrA* gene-replacement mutant ΔBcatrA-M7,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

Discussion

The presence of multiple ABC and MFS genes in *B. cinerea* (Vermeulen et al., 2001) suggests that a complex network of transporters might protect the pathogen against natural fungitoxic compounds (Stergiopoulos et al., 2002) and provide multidrug resistance to fungicides (De Waard, 1997), similar to the PDR-network in *S. cerevisiae* (Bauer et al., 1999). Expression analysis experiments described in this study demonstrate that multiple plant defence compounds induce several transporter genes from *B. cinerea*, especially *BcatrB*, indicating that these transporters indeed may contribute to protection of the pathogen against these toxic products.

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

The remarkable difference in expression pattern of transporter genes from *B. cinerea* after treatment with a range of compounds (Table 1) suggest that the encoded transporters differ in substrate specificity. The increased sensitivity of BcatrB replacement mutants to eugenol indicates that the high capacity to induce *BcatrB* expression indeed correlates with the potency of BcatrB to accept this compound as

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

The instantaneous increase of [^{14}C]fludioxonil accumulation after addition of eugenol and resveratrol (Figure 2) suggests that these compounds inhibit [^{14}C]fludioxonil efflux activity by competing for the fludioxonil binding site of the transporter protein. However, non-competitive inhibition via another binding site cannot be excluded. The transient nature of the elevated [^{14}C]fludioxonil accumulation suggests that eugenol and resveratrol induce additional efflux capacity, probably due to enhanced *de novo* synthesis of BcatrB. Eugenol and resveratrol not only induce expression of *BcatrB* (Table 1), but also of *BcatrD*, *BcatrG* and *BcatrK*. However, addition of eugenol or resveratrol to ΔBcatrB4 germlings did hardly affect the accumulation of [^{14}C]fludioxonil (Figure 3), suggesting that BcatrD, BcatrG and BcatrK do not significantly contribute to fludioxonil efflux activity. This is in agreement with the identification of BcatrB as the major efflux pump of fludioxonil in *B. cinerea* (Vermeulen et al., 2001).

Plant defence compounds identified as substrates for BcatrB are presented in Figure 5. Both eugenol and resveratrol are phenols with an aliphatic side chain (Langcake and Pryce, 1976; Miele et al., 2001). However, this structural moiety is not crucial in determining the substrate specificity of BcatrB since structurally related synthetic phenols such as SOPP and DNOC were not apparent substrates of the transporter. Other

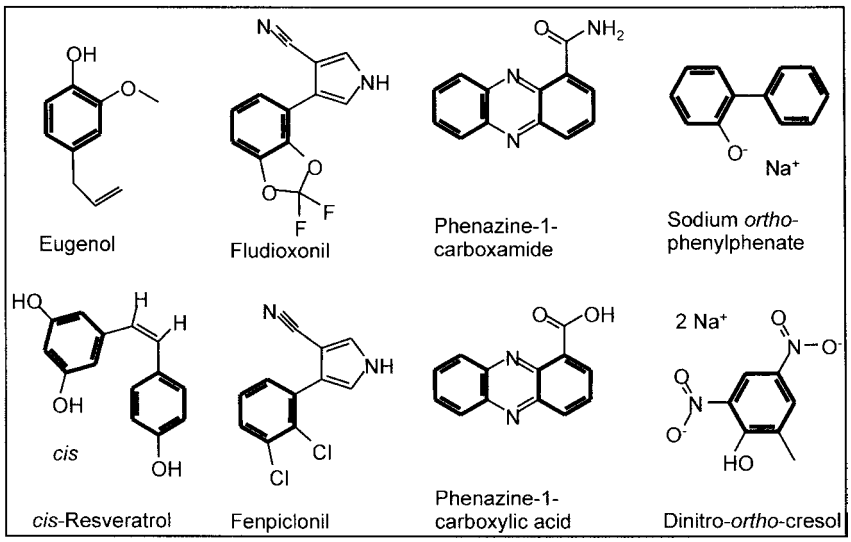

Figure 5. Structures of putative substrates of the ABC transporter BcatrB from *B. cinerea*.

substrates of BcatrB are the phenylpyrrole fungicides fenpiclonil and fludioxonil, and the phenazine antibiotics phenazine-1-carboxylic acid (PCA) and phenazine-1-carboxamide (PCN) (Schoonbeek et al., 2002). These are also aromatic compounds but lack a hydroxylated benzene ring (Figure 5). Hence, the only characteristic that known BcatrB substrates have in common is their aromatic character. This is in line with the hypothesis that substrates of multidrug ABC transporters only share a lipophilic character (Kolaczkowski et al., 1998).

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

Acknowledgements

H.S. was supported by the Council for Earth and Life Sciences (ALW), which is subsidised by The Netherlands Organisation for Scientific Research, project 805-22-462. We would like to thank Dr. P.J.G.M. De Wit for critically reading the manuscript.

References

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

Bauer BE, Wolfger H and Kuchler K (1999) Inventory and function of yeast ABC proteins: About sex, stress, pleiotropic drug and heavy metal resistance. Biochimica et Biophysica Acta 1461: 217–236

Buttner P, Koch F, Voigt K, Quidde T, Risch S, Blaich R, Bruckner B and Tudzynski P (1994) Variations in ploidy among isolates of *Botrytis cinerea*: Implications for genetic and molecular analyses. Current Genetics 25: 445–450

Chapeland F, Fritz R, Lanen C, Gredt M and Leroux P (1999) Inheritance and mechanisms of resistance to anilinopyrimidine fungicides in *Botrytis cinerea* (*Botryotinia fuckeliana*). Pesticide Biochemistry and Physiology 64: 85–100

Chen Y and Simon SM (2000) *In situ* biochemical demonstration that P-glycoprotein is a drug efflux pump with broad specificity. Journal of Cell Biology 148: 863–870

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

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

Dixon RA (2001) Natural products and plant disease resistance. Nature 411: 843–847

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

Fuchs HWM, Van der Lubbe JLM and Fuchs A (1981) The effect of cold storage, plant age and pod size on the ability of pea pods to accumulate pisatin. Acta Botanica Neerlandica 30: 250

Hayashi K, Schoonbeek H and De Waard MA (2002) The ABC transporter BcatrD from *Botrytis cinerea* determines sensitivity to sterol demethylation inhibitor fungicides. Pesticide Biochemistry and Physiology 73: 110–121

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

Jarvis WR (1977) *Botryotinia* and *Botrytis* species: Taxonomy, Physiology, and Pathogenicity: A Guide to the Literature, Canada Department of Agriculture, Harrow

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

Langcake P and Pryce RJ (1976) The production of resveratrol by *Vitis vinifera* [grapes] and other members of the *Vitaceae* as a response to infection or injury. Physiological Plant Pathology 9: 77–86

Miele M, Dondero R, Ciarallo G and Mazzei M (2001) Methyleugenol in *Ocimum basilicum* L. cv. *Genovese Gigante*. Journal of Agricultural and Food Chemistry 49: 517–521

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

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

Nakaune R, Hamamoto H, Imada J, Akutsu K and Hibi T (2002) A novel ABC transporter gene, *PMR5*, is involved in multidrug resistance in the phytopathogenic fungus *Penicillium digitatum*. Molecular Genetics and Genomics 267: 179–185

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

Prins TW, Tudzynski P, Von Tiedemann A, Tudzynski B, Ten Have A, Hansen ME, Tenberge K and Van Kan JAL (2000) Infection strategies of *Botrytis cinerea* and related necrotrophic pathogens. In: Kronstad JW (ed) Fungal Pathology (pp 33–64) Kluwer Academic Publishers, Dordrecht

Rosslenbroich HJ and Stuebler D (2000) *Botrytis cinerea* – history of chemical control and novel fungicides for its management. Crop Protection 19: 557–561

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

Schoonbeek H, Raaijmakers JM and De Waard MA (2002) Fungal ABC transporters and microbial interactions in natural environments. Molecular Plant–Microbe Interactions 15: 1165–1172

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

Stehmann C and De Waard MA (1996) Sensitivity of populations of *Botrytis cinerea* to triazoles, benomyl and vinclozolin. European Journal of Plant Pathology 102: 171–180

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

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

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

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

Zwiers LH, Stergiopoulos I, Gielkens MM, Goodall SD and De Waard MA (2003) ABC transporters of the wheat pathogen *Mycosphaerella graminicola* function as protectants against biotic and xenobiotic toxic compounds. Molecular Genetics and Genomics 269: 499–507