The ABC Transporter BcatrB Affects the Sensitivity of *Botrytis cinerea* to the Phytoalexin Resveratrol and the Fungicide Fenpiclonil

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During pathogenesis, fungal pathogens are exposed to a variety of fungitoxic compounds. This may be particularly relevant to *Botrytis cinerea*, a plant pathogen that has a broad host range and, consequently, is subjected to exposure to many plant defense compounds. In practice, the pathogen is controlled with fungicides belonging to different chemical groups. ATP-binding cassette (ABC) transporters might provide protection against plant defense compounds and fungicides by ATP-driven efflux mechanisms. To test this hypothesis, we cloned *BcatrB*, an ABC transporter-encoding gene from *B. cinerea*. This gene encodes a 1,439 amino acid protein with nucleotide binding fold (NBF) and transmembrane (TM) domains in a [NBF-TM]₆ topology. The amino acid sequence has 31 to 67% identity with ABC transporters from various fungi. The expression of *BcatrB* is up regulated by treatment of *B. cinerea* germlings with the grapevine phytoalexin resveratrol and the fungicide fenpiclonil. *BcatrB* replacement mutants are not affected in saprophytic growth on different media but are more sensitive to resveratrol and fenpiclonil than the parental isolate. Furthermore, virulence of Δ *BcatrB* mutants on grapevine leaves was slightly reduced. These results indicate that *BcatrB* is a determinant in sensitivity of *B. cinerea* to plant defense compounds and fungicides.

The widely occurring plant pathogenic fungus *Botrytis cinerea* Pers.:Fr., anamorph of *Botryotinia fuckeliana* (De Bary) Whetzel, infects the fruits, flowers, or green tissues of at least 235 plant species (Jarvis 1977). Several of these hosts are known to produce defense compounds belonging to various chemical classes that act as constitutive or inducible chemical barriers (Osbourne 1999). *B. cinerea* is able to withstand toxic effects of plant defense compounds with varying structures such as stilbenes, isoflavonoids, coumarins, and sesquiterpenes. Pathogens can overcome the defense of their hosts by specific mechanisms such as enzymatic conversion of these

compounds (Schafer et al. 1989). The specificity of such enzymes might delimit the host range of pathogens. More general mechanisms that provide protection against a broad range of toxicants such as compartmentalization and reduction of accumulation would allow pathogens to cope with various defense compounds that occur in many different host species. Reduction of accumulation can be achieved with active efflux by ATP-binding cassette (ABC) transporters. These transporters accept various classes of secondary plant metabolites as substrates (Kolaczowski et al. 1998).

The superfamily of ABC transporters consists of membrane-bound proteins with an ATP-binding cassette (Higgins 1992). They use ATP to transport a wide spectrum of compounds over various membranes (Senior et al. 1995). Several subfamilies can be distinguished on the basis of the topology of hydrophobic domains made up of transmembrane helices (TM) and hydrophilic domains, comprising the nucleotide binding fold (NBF). The latter is characterized by the common nucleotide binding motifs, described by Walker et al. (1982), and the typical ABC signature. Some subfamilies of ABC transporters are named after their first-known member with a function in multidrug resistance (MDR). For instance, they can confer MDR to mammalian cancer cells causing resistance to unrelated drugs used in chemotherapy. In mammals, MDR can be mediated by overexpression of two subfamilies of ABC transporters, the P-glycoprotein or MDR1-like ABC transporters with a [TM₆-NBF]₂ topology, and the MDR-related proteins MRP with a TM_n-[TM₆-NBF]₂ topology (Ishikawa et al. 1997). In *Saccharomyces cerevisiae*, a pleiotropic drug resistance (PDR) network is involved in MDR (Balzi and Goffeau 1995). The PDR network is composed of transcriptional regulators such as PDR1 and PDR3 that activate expression of many genes, including *PDR5* and *SNQ2*, PDR-like ABC transporters with a [NBF-TM]₆ topology. ABC transporters with additional topologies have been described in *S. cerevisiae* (Decottignies and Goffeau 1997). Transporters in the PDR network provide *S. cerevisiae* with tolerance to different toxic compounds. This manifests itself as increased sensitivity upon disruption of one or more members of the network. ABC transporters present in *S. cerevisiae* can export a multitude of compounds comprising plant secondary metabolites (Kolaczowski et al. 1998). For this reason, ABC transporters of plant pathogens could play a significant

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role in pathogenesis by protecting against plant defense compounds or preventing suicidal effects of fungal toxins (De Waard 1997). Recently, this hypothesis was confirmed by the finding that the ABC transporter *ABC1* from *Magnaporthe grisea* is involved in pathogenesis on rice because disruption of the *ABC1* gene results in strongly reduced virulence (Urban et al. 1999). No substrate has yet been identified for *Abc1*, although it was proposed that this protein is involved in protection against unidentified plant defense compounds.

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

This paper focuses on the function of ABC transporters from *B. cinerea* in plant pathogenesis and protection against fungicides. We cloned and characterized the ABC transporter encoding gene *BcatrB*. Replacement of the *BcatrB* gene resulted in increased sensitivity to the grapevine phytoalexin resveratrol and a slight reduction in virulence on grapevine leaves. Additionally, increased sensitivity to the phenylpyrrole fungicide fenpiclonil was observed. Hence, we have shown that *BcatrB* may be a virulence factor of *B. cinerea*, which also can play a role in sensitivity to commercial fungicides.

RESULTS

Cloning of genes encoding ABC transporters.

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

A 5-kb *Eco*RV fragment and an overlapping 5.5-kb *Sal*I fragment were subcloned in pBluescript, a restriction map was

constructed, and the sequence was determined (Fig. 1). Assembly of sequences revealed a 4.3-kb ORF in which the presence of one intron was suggested on the basis of a sequence comparison. The presence of a 56-bp intron at position 1,116 to 1,171 was confirmed by reverse transcription-polymerase chain reaction (RT-PCR). The 5'-flanking region (833 bp) of *BcatrB* contains a conserved Kozak sequence (CCAUCAUGG) around the deduced translation start (Kozak 1984) and a TATA box (Chen and Struhl 1988) at position –220. In addition to these general promoter features, a single putative Pdr1p/Pdr3p binding element (Katzmann et al. 1996) is present at position –555, and a Ste11 and a Mat1–Mc binding site at position –165 (Kjaerulff et al. 1997). In yeast, these elements are involved in regulation of expression of ABC transporters involved in MDR and mating. No polyadenylation signal consensus sequence (AATAAA) in the 3'-flanking region was found.

BcatrB encodes a 1,439 amino acid protein with homology to ABC transporters of other fungi (Del Sorbo et al. 2000) such as *PDR5* and *SNQ2* from *S. cerevisiae* and *AtrA* and *AtrB* from *A. nidulans* (Table 1). The degree of homology between *BcatrB* and *AtrB* from *A. nidulans* is particularly high (67.5% identity). Alignment of the conserved amino acid stretches in the N- and C-terminal NBFs of the proteins (Table 2) demonstrates that the NBFs in both halves are highly conserved in *BcatrB* and ABC transporters from other fungi and yeasts. The conserved sequence stretches in fungal ABC transporters are longer than the general consensus described for other organisms (Higgins 1992; Walker et al. 1982). Characteristics of fungal ABC transporters such as a degenerated Walker motif in the C-terminal NBF and the presence of a cysteine instead of a lysine residue in the N-terminal Walker A motif also are found in *BcatrB*. Prediction of transmembrane helices of *BcatrB* with the prediction of transmembrane regions and orientation (TmPred) routine of the EMBL server (Hofmann 1993) yields a pattern that strongly resembles that of *PDR5* and *AtrB*. The protein consists of four domains, two hydrophobic TM₆s and two hydrophilic NBFs organized in a [NBF–TM₆]₂ topology.

Expression of *BcatrB* in vitro.

Northern blot analysis showed that *BcatrB* has a low level of basal expression in germlings grown in liquid-shake culture. Transcription of *BcatrB* is induced specifically by the phytoalexins resveratrol (50 mg per liter) and pisatin (20 mg per liter) from grapevine and pea, respectively, and the phenylpyrrole fungicide fenpiclonil (10 mg per liter) (Fig. 2). The fungicide tebuconazole (10 mg per liter) (Fig. 2) and the antibiotic cycloheximide (50 mg per liter; data not shown) give only weak induction. Transcript levels of actin in lanes 4, 5, and 9 (Fig. 2) are relatively low. This may be because the treatment of germlings with fenpiclonil and pisatin has a strong fungitoxic effect. The basal expression level of *BcatrB* in the azole-resistant isolate G25 and the benzimidazole-resistant isolate SAS405 did not differ significantly from the sensitive strains B3 and B05.10 (results not shown).

Replacement of *BcatrB*.

Functional analysis of *BcatrB* was studied in gene replacement mutants obtained with the construct pBBOHT in which a 2.6-kb *Xba*I–*Sal*I fragment of the *BcatrB* se-

quence is replaced by a 2.7-kb hygromycin resistance cassette (OHT) (Fig. 1). Transformation of the haploid *B. cinerea* strain B05.10 with pBBOHT yielded approximately 60 hygromycin-resistant colonies. Homokaryotic transformants were purified by three rounds of monospore isolation and alternating growth on selective and nonselective media. To verify the replacement of the *BcatrB* gene, DNA from the transformants was subjected to digestion with *Hind*III, Southern blotted, and hybridized with the 3.5-kb *Sal*I–*Eco*RV probe (Fig. 1). Restriction of DNA from strains with a wild-type copy of *BcatrB* with *Hind*III results in three fragments containing *BcatrB* sequences: a 2.8-kb fragment from the 5' side with flanking region, a 0.7-kb internal fragment, and a 7-kb fragment from the 3' side with flanking region. Restriction with *Hind*III of DNA from mutants with *BcatrB* replaced by a double crossover would result in two fragments containing *BcatrB* sequences, each approximately 5.2 kb long, without an internal fragment. Genome analysis of transformants revealed three major types of integration events: ectopic integrations, heterokaryons in which only a fraction of all nuclei contained the mutant copy, and homokaryons, in which all nuclei contained the mutant copy (Fig. 3). From type 3, two independent transformants were selected: Δ BcatrB4 and Δ BcatrB5. Northern analysis of both mutants confirmed that wild-type mRNA of *BcatrB* was not present, not even after treatment with resveratrol or fenpiclonil at concentrations that induce elevated transcript levels

in wild-type strains (Fig. 4). The absence of *BcatrB* in the replacement mutant strains was confirmed in three independent Northern analysis experiments.

Sensitivity to toxicants.

Vegetative growth of the mutants Δ BcatrB4 and Δ BcatrB5 on solid media (PDA, MEA, 1× Gamborg's B5) is similar to that of the wild-type strain B05.10 (data not shown). No difference in growth rate or timing and production of conidia was observed. In radial growth tests, the mutants showed increased sensitivity to the stilbene phytoalexin resveratrol when compared with the parental strain B05.10 (Table 3). All strains showed the characteristic brown discoloration associated with resveratrol conversion by laccase activity. No difference in sensitivity to the isoflavonoid phytoalexin pisatin from *Pisum sativum* was observed. The Δ BcatrB mutants did not show increased sensitivity to the antibiotic cycloheximide, the dicarboximide fungicide vinclozolin, and the azole fungicide imazalil. The Δ BcatrB mutants exhibited a small but significant increase in sensitivity to the phenylpyrrole fungicide fenpiclonil (Table 3). The increase in sensitivity was found for spore germination, germ-tube elongation (Fig. 5A), and colony formation (Fig. 5B), yet was hardly detectable for mycelial growth (Fig. 6). The increase in sensitivity of Δ BcatrB mutant spores is most apparent at concentrations of fenpiclonil of 0.05 to 0.1 mg per liter.

Fig. 1. Physical map of the *BcatrB* locus from *Botrytis cinerea* in **A**, genomic DNA and **B**, a phage clone. The conserved regions encoding the Walker A motif, ATP-binding cassette signature, and Walker B motif in the 5' and 3' halves of the open reading frame are indicated by black boxes. **B**, The intron is indicated by a shaded box. B = *Bst*XI; EI = *Eco*RI; EV = *Eco*RV; H = *Hind*III; S = *Sal*I; P = *Pst*I; and X = *Xba*I. Sites used for construction of the disruption construct are in bold. Arrows indicate replacement of a 2.6-kb *Xba*I–*Sal*I fragment by the hygromycin resistance cassette (2.7-kb *Xba*I–*Hind*III fragment) in the Δ BcatrB mutants. Black bars indicate the probes used for hybridization. The 0.7-kb *Hind*III fragment was used as a gene-specific probe in Northern and Southern blotting. The 3.5-kb *Pst*I–*Eco*RV fragment was used as a probe in Southern blotting to determine genomic organization in the transformants.

Virulence assays.

Virulence of the Δ BcatrB mutants was tested in wet chambers on detached grapevine leaves at 4°C (Table 4). The size of lesions caused by the control transformant T132 was not significantly different from the parental strain (exp. 1 and 2). In two independent experiments (exp. 2 and 3), the spreading necrotic lesions caused by mutants Δ BcatrB4 and Δ BcatrB5 were significantly smaller ($P < 0.05$ in Student's t test) than those formed by the parental strain B05.10. In experiment 3, the determined growth rate of the mutants Δ BcatrB4 (1.1 ± 0.9 mm per day) and Δ BcatrB5 (1.1 ± 1.1 mm per day) also was significantly lower than that of B05.10 (2.2 ± 1.2 mm per day).

DISCUSSION

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

nous toxic compounds or in plant pathogenesis. Indeed, our study shows that BcatrB is involved in protection against the grapevine phytoalexin resveratrol and the phenylpyrrole fungicide fenpiclonil. The finding that BcatrB protects against resveratrol is significant because it is known that susceptibility of grapevine to *B. cinerea* is inversely correlated with the resveratrol contents of the grapevine cultivar (Jeandet et al. 1995). The hypothesis that BcatrB can contribute to the virulence of *B. cinerea* on plants producing resveratrol was confirmed on grapevine leaves on which Δ BcatrB mutants showed slightly reduced virulence. Because virulence is not fully lost, we propose that BcatrB is one of the many factors that contribute to virulence. Recently, it has been described that Abc1, an ABC transporter homolog from the rice blast fungus *M. grisea*, is important for virulence on rice (Urban et al. 1999). The interaction between rice and *M. grisea* is accompanied by production of phytoalexins in the host (Kodama et al. 1992). Increased sensitivity to these plant defense compounds may explain the loss of virulence of *ABC1* mutants. Mutants do not show increased sensitivity to a number of phytoalexins in vitro, however, despite the ability of these compounds to induce expression of *ABC1* in the wild type. Therefore, an explanation for the loss of virulence in the

Table 1. Homology of ATP-binding cassette transporters^a

Source	Accession no.	Protein	BcatrA	AtrB	AtrA	Pdr5p	Snq2p	Cdr1p	Abc1	PMR1
Bc	AJ006217	BcatrB	36.5	67.5	32.7	31.3	33.3	32.3	33.1	32.2
Bc	Z68906	BcatrA	***	36.0	30.8	31.3	31.0	29.9	30.4	30.8
An	Z68905	AtrB		***	31.6	32.6	32.5	31.5	31.6	32.0
An	Z68904	AtrA			***	41.4	37.8	41.5	42.0	42.4
Sc	L19922	Pdr5p				***	35.9	53.6	43.3	45.5
Sc	X66732	Snq2p					***	36.5	33.9	35.8
Ca	X77589	Cdr1p						***	44.4	48.2
Mg	AF032443	Abc1							***	57.1
Pd	AB010442	PMR1								***

^a *Botrytis cinerea* (Bc), *Aspergillus nidulans* (An), *Saccharomyces cerevisiae* (Sc), *Candida albicans* (Ca), *Magnaporthe grisea* (Mg), and *Penicillium digitatum* (Pd) expressed as percentage identical amino acids.

Table 2. Alignment of the ATP-binding domain of BcatrB with homologous sequences in ATP-binding cassette (ABC) transporters^a

Protein	Source	Walker A	ABC signature	Walker B
N terminal				
		GxSGxGKS ^b	SGGQ	LxxDEXxSALD
BcatrB	Bc ^c	LLVLGRPGAGCTTLLK	GVSGGERKRVSIIEMLASRGSVWCWDNSTRGLD	
BcatrA	Bc	LLVLGRPGSGCSTFLK	GVSGGERKRVSIATLPTKKTVVSWDNSTRGLD	
AtrB	An	LLVLGRPGSGCTTLLK	GVSGGERKRVSIIECLGTRASVFCWDNSTRGLD	
AtrA	An	LLVLGRPGTGCSTFLK	GVSGGERKRVSIEMALAMTPFAAWDNSSRGLD	
PDR5	Sc	LVVLGRPGSGCTTLLK	GVSGGERKRVSIAEVSIKGFQWCWDNSTRGLD	
SNQ2	Sc	ILVLGRPGAGCSSLFLK	GVSGGERKRVSIAEALAAKGSICWDNSTRGLD	
CDR1	Ca	TVVLGRPGAGCSTLLK	GVSGGERGRVDIAEASLSGANIQWDNSTRGLD	
Abc1	Mg	LVVLGPFGSGCSTFLK	GVSGGERKRVITAEALSGAPLQCWDNSTRGLD	
PMR1	Pd	LIVLGPFGSGCSTFLK	GVSGGERKRVSIATLCSPLQCWDNSTRGLD	
C terminal				
		GxSGxGKS ^b	SGGQ	LxxDEXxSALD
BcatrB	Bc	LGALMGSSGAGKTTLL	LSVEQRKRLTIGVELVSKPSILIFLDEPTSGLD	
BcatrA	Bc	MVALMGASGAGKTTLL	LSVEQRKRVITIGVELAAKPNLLFLDEATSGLD	
AtrB	An	LGALMGSSGAGKTTLL	LSVEQRKRVITIGVELVSKPSILIFLDEPTSGLD	
AtrA	An	LTALMGVSGAGKTTLL	LNVEQRKLLTIGVELPPSKLLFLDEPTSGLD	
PDR5	Sc	LTALMGASGAGKTTLL	LNVEQRKRLTIGVELTAKPKLLVFLDEPTSGLD	
SNQ2	Sc	MTALMGESGAGKTTLL	LNVEQRKLLSIGVELVAKPKLLFLDEPTSGLD	
CDR1	Ac	ITALMGASGAGKTTLL	LNVEQRKRLTIGVELVAKPKLLFLDEPTSGLD	
Abc1	Mg	LTALMGVSGAGKTTLL	LNVEQRKRLTIGVELAAKPKLLFLVDEPTSGLD	
Pmr1	Pd	CTALMGVSGAGKTTLL	LNVEQRKRLTIGVELAAKPKLLFLDEPTSGLD	

^a Identical sequences in fungal ABC-transporters in bold.

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

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

ABC1 mutants of *M. grisea* is not yet known. It may be that *Abc1* provides protection against a yet-unidentified phytoalexin. Similar mechanisms may be involved in the protection of other plant pathogens against phytoalexins. For instance, activity of ABC transporters could explain the nondegradative tolerance of pisatin, a phytoalexin from *P. sativum*, in *Nectria haematococca* on the basis of energy-dependent efflux (Denny et al. 1987).

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

For several ABC transporter genes, a phenotype of single gene replacements becomes manifest only in fungicide-resistant strains in which the ABC-transporter gene responsi-

ble for resistance is overexpressed. In *P. digitatum* disruption of the *PMR1* gene in the azole-resistant strain LC2, which overexpresses *PMR1*, sensitivity to azole fungicides is restored to wild-type levels but not beyond those levels (Nakaune et al. 1998). The observation that replacement of *BcatrB* in a wild-type strain results in increased sensitivity to resveratrol and fenpiclonil indicates that *BcatrB* has a relatively high activity toward these compounds. Because the fungicide fenpiclonil is derived from the antibiotic pyrrolnitrin, the activity of *BcatrB* toward fenpiclonil may relate to the fact that *B. cinerea* needs to protect itself against natural toxins during saprophytic growth (Atlas and Bartha 1993). Among these are antibiotics such as pyrrolnitrin produced by *Pseudomonas* spp. (Ligon et al. 2000). It is possible that *BcatrB* is involved in protection against pyrrolnitrin, as shown for the structurally related fenpiclonil. If ABC transporters provide protection against natural toxicants, they could be significant in determining the broad host range and the saprophytic ability of *B. cinerea*.

Table 3. Effect of plant defense compounds and fungicides on radial growth of *Botrytis cinerea* on PDA medium

Compounds	Strains		
	B05.10 ^a	Δ BcatrB4 ^b	Δ BcatrB5 ^b
Pisatin	2.1 $\pm$ 0.5 ^c	2.5 $\pm$ 0.8	2 $\pm$ 0.6
Resveratrol	911 $\pm$ 383 ^c	290 $\pm$ 69 ^d	248 $\pm$ 53 ^d
Cycloheximide	2.2 $\pm$ 0.3 ^c	1.9 $\pm$ 0.6	1.8 $\pm$ 0.1
Fenpiclonil	12 $\pm$ 5 ^c	8.8 $\pm$ 2 ^d	7.7 $\pm$ 2 ^d
Vinclozolin	80 $\pm$ 20 ^c	70 $\pm$ 20	80.0 $\pm$ 10

^a Wild-type parental isolate.

^b *BcatrB* gene-replacement mutants.

^c 50% effective concentration (milligrams per liter) $\pm$ experimental error.

^d Significantly different from B05.10 ($P < 0.05$).

^e 50% effective concentration (micrograms per liter) $\pm$ experimental error.

Fig. 2. Expression of *BcatrB* in Northern blot experiments. RNA was hybridized with the 0.7-kb *Hind*III fragment of *BcatrB* (Fig. 1). Transcript levels of *BcatrB* in germlings of strain B05.10 after 60 min of mock-treatment (lane 1) or treatment with 0.1% methanol (lane 2), fenpiclonil (1, 3, and 10 mg per liter; lanes 3, 4, and 5), resveratrol (20 and 50 mg per liter; lanes 6 and 7), tebuconazole (10 mg per liter; lane 8), and pisatin (25 mg per liter; lane 9). *BcactA* shows hybridization with the *Botrytis cinerea* actin gene. *rRNA* shows methylene blue staining of ribosomal RNA.

Fig. 3. Southern blot with DNA from *Botrytis cinerea* strains SAS56 and B05.10 (wild type) and five putative Δ *BcatrB* mutants obtained by transformation with the construct pBBOHT. DNA was digested with *Hind*III and hybridized with a 3.5-kb *Pst*I-*Eco*RV probe from *BcatrB* (Fig. 1). Heterokaryotic transformant (lane 1), λ *Pst*I marker (lane 2), homo-karyotic transformants Δ *BcatrB*4, 5, 15 and 45 (lanes 3, 4, 5 and 6, respectively.), λ *Pst*I marker (lane 7), parental isolate B05.10 (lane 8), wild-type isolate SAS56 (lane 9), and λ *Hind*III marker (lane 10). Molecular size markers (kb) are indicated.

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

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

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

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

Experiment	Strain ^y			
	B05.10	T132	Δ BcatrB4	Δ BcatrB5
1	13.2 $\pm$ 2.9 a	12.7 $\pm$ 3.3 a		
2	8.4 $\pm$ 2.2 a ^z	8.6 $\pm$ 3.8 a	5.9 $\pm$ 2.3 b	6.0 $\pm$ 1.5 b
3	5.7 $\pm$ 2.0 a		3.6 $\pm$ 1.6 b	4.0 $\pm$ 1.6 b

^y B05.10 (parental wild-type strain), T132 (B05.10 transformed with hygromycin resistance cassette), and Δ BcatrB4 and Δ BcatrB5 (BcatrB gene-replacement mutants).

^z Average lesion size (diameter in millimeters). Values within one row followed by the same letter do not differ significantly ($P < 0.05$; student's *t* test).

Fig. 4. Expression of *BcatrB* in Northern blot experiments. RNA was hybridized with the 0.7-kb *Hind*III fragment of *BcatrB*. Transcript levels of *BcatrB* in parental strain B05.10, gene replacement mutants Δ BcatrB4 and Δ BcatrB5, and control strain Δ BcatrA-M7 are indicated. Germlings were mock treated (lanes 1), treated with methanol (0.1%; lanes 2), fenpiclonil (10 mg per liter; lane 3), or resveratrol (50 mg per liter; lane 4). *BcatrA* shows hybridization with the *Botrytis cinerea* actin gene. rRNA shows methylene blue staining of ribosomal RNA.

wild type. These results indicate that a yet-unidentified transporter is involved in resistance to azole fungicides.

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

MATERIALS AND METHODS

Culturing of fungal strains.

Strain SAS56, a monoascospore isolate (Van der Vlugt-Bergmans et al. 1993) and the haploid strain B05.10 derived from SAS56 (a gift from P. Büttner and P. Tudzynski, Institut für Botanik, Westfälische Wilhelms-Universität, Münster, Germany) were used as reference strains. T132 is a hygromycin-resistant strain derived from B05.10 by transformation with the plasmid pOHT containing the hygromycin resistance

cassette OHT (Van Kan et al. 1997). Δ BcatrA-M7 is a gene-replacement mutant derived from B05.10 in which *BcatrA* is replaced by the OHT cassette (Del Sorbo, *unpublished*).

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

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

All strains were cultured at 20°C in the dark on tPDA (potato dextrose agar amended with 300 g of homogenized tomato leaves per liter). After 3 days, the cultures were exposed to near-UV light for 16 h and incubated for 1 additional week in the dark. Conidia were collected from sporulating cultures to inoculate liquid cultures in Gamborg's B5 medium (Duchefa Biochemie, Haarlem, The Netherlands) supple-

Fig. 5. Effect of resveratrol and fenpiclonil on growth of *Botrytis cinerea*. **A**, Spore germination and germ-tube growth of strains B05.10 (parental strain) and Δ BcatrB4 (BcatrB replacement mutant) in B5 medium after 22 h incubation at 20°C. **B**, Colony formation on B5 agar amended with 0.1 mg of fenpiclonil per liter after 1 week incubation at room temperature. Untransformed control strains (spots 1, 2, and 3), Δ BcatrA mutants (spots 4, 5, and 6), T132 control (B05.10 transformed with hygromycin resistance cassette) (spot 7), and Δ BcatrB mutants (spots 8 and 9) are indicated.

mented with 1% sucrose and 10 mM ammonium phosphate (pH 6.5). Germlings from overnight cultures were used for induction experiments and DNA and RNA isolation. Hygromycin-resistant transformants were maintained on malt extract agar (Oxoid, Basingstoke, Hampshire, England) plates amended with 100 mg of hygromycin per liter (Sigma, St. Louis, MO, U.S.A.) and transferred to tPDA to harvest spores for experiments.

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

Molecular techniques.

DNA manipulations were performed according to standard methods (Sambrook et al. 1989). *Escherichia coli* strain DH5 α was used for propagation of constructs. The λ -EMBL3 system (Promega, Madison, WI, U.S.A.) was used to construct a library of *B. cinerea* strain SAS56 genomic DNA partially digested with *Sau*III.

The library was screened with a probe derived from the *S. cerevisiae* gene PDR5, provided by A. Goffeau (Louvain-la-Neuve, Belgium). This 1.5-kb *Bgl*II-*Taq*I fragment comprises the entire N-terminal ATP-binding cassette domain of PDR5. Membranes (Hybond-N⁺, Amersham, Arlington Heights, IL, U.S.A.) were hybridized with random-primed (GIBCO, Gaithersburg, MD, U.S.A.), [α -³²P]dATP-labeled probes at 56°C in modified Church buffer (Church and Gilbert 1984), and washed at 56°C in 1 $\times$ SSC. Fragments from positive phages were subcloned in pGEM3Z(+) (Promega) or pBluescriptII-SK (Stratagene, La Jolla, CA, U.S.A.). Sequencing was performed with the Thermo Sequenase II cycle sequencing kit (Amersham Pharmacia Biotech, Uppsala, Sweden).

Genomic DNA of *B. cinerea* was isolated as described previously (Drenth et al. 1993), digested with restriction enzymes, fractionated on 1.0% agarose-TAE gels, and transferred to Hybond-N⁺ membranes by capillary blotting (Sambrook et al. 1989). Blots were hybridized with random-primed, [α -³²P]dATP-labeled probes at 65°C in modified Church buffer, washed at 65°C in 0.2 $\times$ SSC, and exposed to X-OMAT AR films (Kodak, Rochester, NY, U.S.A.).

Gene expression.

For RNA induction experiments, precultures were grown in 300-ml round-bottom flasks (100 ml of B5 medium and 10⁶ conidia per ml) at 20°C and 180 rpm for 13 to 15 h. If necessary, mycelium of different cultures was pooled. Following distribution over fresh flasks, inducing agents were added from a 1,000 $\times$ concentrated stock solution in methanol. After additional incubation at 20°C and 180 rpm for 1 h, mycelium was harvested on glass-fiber filters and washed with ice-cold 10 mM sodium phosphate (pH 6.5) and sterile water with a vacuum manifold (Millipore, Bedford, MA, U.S.A.).

Total RNA was extracted from mycelium frozen in liquid nitrogen with guanidine hydrochloride (Logemann et al. 1987). Samples of 10 μ g of RNA were denatured with glyoxal in dimethyl sulfoxide and subjected to electrophoresis on a 1.4% agarose gel in 10 mM sodium phosphate. RNA was transferred to Hybond-N membranes by capillary blotting in 10 $\times$ SSC (Sambrook et al. 1989). Northern blots were hybridized in modified Church buffer at 65°C with random

primer [α -³²P]dATP-labeled probes. The gene-specific probes used were a 1.1-kb *Bam*HI-*Eco*RI fragment of *BcatrA*, a 0.7-kb *Hind*III fragment of *BcatrB*, and a 1.2-kb *Hind*III fragment of the constitutively expressed actin gene of *B. cinerea*.

Construction of the gene-replacement vector.

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

Toxicity assay.

For all toxicants, 50% effective concentration values (EC₅₀) for inhibition of radial growth were determined on PDA and solidified B5 medium, as described previously (Stehmann and De Waard 1996). Test compounds were added in a range of concentrations from 1,000 $\times$ stock solutions in methanol and resveratrol from freshly prepared 500 $\times$ stock solutions. Experiments were carried out in triplicate and repeated two or three times. Student's *t* test was used for statistical analysis.

Toxic activity of compounds in liquid B5 medium was determined in 96-well polystyrene plates (Greiner, Alphen a/d Rijn, The Netherlands). Wells were inoculated with 5 $\times$ 10³ spores in 100 μ l of medium and incubated at 20°C for 60 h. Growth was measured as an increase of absorbance at 405 nm in an EL312 microplate reader (BIO-TEK instruments, Winooski, VT, U.S.A.).

Virulence assay.

Virulence assays were performed on detached grapevine leaves placed in prewetted florist foam (Smithers-Oasis, Grünstadt, Germany) in humid chambers. Five (experiments 1 and 2) or four (experiment 3) leaves were inoculated with 2 $\times$

Fig. 6. Effect of fenpiclonil on germling growth of *Botrytis cinerea* strains B05.10 (parental strain, white), and the replacement mutants Δ BatrB4 (dashed up) and Δ BatrB5 (dashed down) in liquid B5 medium in multiwell plates, 60 h incubation at 20°C, and measured as absorbance at 405 nm. Solvent concentrations never exceeded 0.1% methanol.

10⁶ *B. cinerea* conidia per ml and preincubated in 1× B5 medium amended with 1% sucrose and 10 mM ammonium phosphate (pH 6.5) at 20°C for 2 h to synchronize germination. Four sectors were designated on the upper side of each leaf. Each sector was inoculated with 15 droplets (1 µl) of strains B05.10 (parental line), T132 (control transformant), ΔBcatrB4, or ΔBcatrB5 (BcatrB mutants). Lesion diameters were measured after incubation at 4°C for 6 days in experiments 1 and 2 and for 10 days in experiment 3. Mean values of lesion sizes were determined on the basis of figures of spreading lesions (> 1 mm). Student's *t* test was used for statistical analysis. In experiment 3, the growth rate of lesions was based on measurements made on days 9, 10, and 11.

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