Fungal ABC Transporters and Microbial Interactions in Natural Environments

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

Soil and plant-associated environments harbor a wide variety of microorganisms that play an integral role in plant growth and in preservation of multiple ecosystem functions. To exist in natural environments, microorganisms must be able to endure all of the adverse abiotic and biotic conditions that are prevalent in a particular environment. Among the biotic

factors, interactions within and among microbial communities are numerous and range from synergistic and mutualistic to antagonistic and parasitic. Many microorganisms isolated from soil and plant-associated environments produce secondary metabolites that adversely affect the growth or metabolic activity of other microorganisms (Fravel 1988). The significance of these antibiotics in microbial interactions in situ, however, has long been questioned because of the perceived constraints to antibiotic production in natural environments (Williams and Vickers 1986). Because of the biotic and abiotic complexity of soil and plant-associated environments, there were several inherent difficulties in demonstrating that antibiotics are produced by microorganisms in situ. Development of sensitive detection methods, including reporter gene systems and bio-analytical techniques, now have demonstrated unequivocally that antibiotic compounds are produced in situ by both introduced and indigenous microorganisms (Raaijmakers et al. 1999; Thomashow et al. 1997). In this context, considerable attention has been given to the antibiotics pyrrolnitrin (PRN), 2,4-diacetylphloroglucinol (DAPG), and phenazines produced by plant growth-promoting fluorescent *Pseudomonas* spp. (Thomashow and Weller 1995). These antibiotics are produced in soil and rhizosphere environments (Thomashow et al. 1997) and play a key role in interactions between *Pseudomonas* strains and soilborne fungi (Cook et al. 1995; Whipps 2001).

Numerous *Pseudomonas* strains that produce antibiotics have been isolated from the rhizosphere of plants grown in soils from diverse geographical regions (Keel et al. 1996; McSpadden Gardener et al. 2000; Raaijmakers et al. 1997). The ability of plant-associated pseudomonads to produce antibiotics enables them to defend their habitats (Mazzola et al. 1992), whereas the existence of genotypic and physiological diversity among *Pseudomonas* spp. producing the same antibiotic (McSpadden Gardener et al. 2000; Picard et al. 2000; Raaijmakers and Weller 2001; Wang et al. 2001) provides a means by which populations of these microorganisms can adapt to diverse habitats. Although substantial progress has been made in the identification of genes involved in the biosynthesis and regulation of antibiotic production by *Pseudomonas* spp. (Bangera and Thomashow 1999; Delaney et al. 2001; Hammer et al. 1997; Mavrodi et al. 1998; Pierson et al. 1995), the responses of other microorganisms, and in particular defensive mechanisms, to these antibiotics have received little attention. Studies on the effect of therapeutic antibiotics, antimycotics, and synthetic fungicides on human- and plant-pathogenic microorganisms have shown that target organisms can protect themselves by active efflux mechanisms that prevent the intracellular accumulation of these compounds to toxic concentrations (Del Sorbo et al. 2000; De Waard 1997; Sanglard et al. 1998; Van Bambeke et al. 2000). Active efflux

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mechanisms not only enable target organisms to tolerate exogenous antibiotics, but also may prevent self-intoxication in antibiotic-producing microorganisms (Andrade et al. 2000a; Mendez and Salas 2001). Among the active efflux mechanisms known, ATP-binding cassette (ABC) transporters are well studied (Dassa and Bouige 2001; Del Sorbo et al. 2000; Driessen et al. 2000; Rogers et al. 2001; Wolfger et al. 2001). ABC transporters are present from archae-bacteria to man and enable both influx and efflux of several compounds (Andrade et al. 2000b; Kolaczowski et al. 1998; Lewis 2001). The natural functions of ABC transporters from plant-pathogenic fungi comprise secretion of toxins that act as virulence factors (host-specific toxins or mycotoxins) and protection against plant defense compounds (phytoalexins) and synthetic fungicides (De Waard 1997). Over the last few years, these phenomena have been described for *Botrytis cinerea*, *Magnaporthe grisea*, and *Mycosphaerella graminicola* (Schoonbeek et al. 2001; Urban et al. 1999; Zwiers and De Waard 2000).

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

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

RESULTS

Expression of ABC transporter genes.

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

Antifungal activity of antibiotics.

Compounds that induce expression of ABC transporter genes also can be substrates of the encoded proteins. In that case, replacement of the gene involved may cause an increased sensitivity to these compounds. The activity of fludioxonil, PRN, DAPG, PG, PCA, and PCN was tested on radial mycelial growth of wild-type *B. cinerea* strain B05.10 and two independent $\Delta BcatrB$ mutants; a $\Delta BcatrA$ mutant was included as a

Fig. 1. Northern analysis showing the effect of fludioxonil and antibiotics on expression of *BcatrA*, *BcatrB*, and *BcatrD* in *Botrytis cinerea* strain B05.10. Germlings received a 20-min treatment: mock control (0), solvent control (0.1% dimethyl sulfoxide, S), fludioxonil (Flu; 0.2 and 1 mg l⁻¹), pyrrolnitrin (PRN; 0.2 and 1 mg l⁻¹), 2,4-diacetylphloroglucinol (DAPG; 20 and 100 mg l⁻¹), phloroglucinol (PG; 20 and 100 mg l⁻¹), phenazine-1-carboxylic acid (PCA; 5, 15, and 50 mg l⁻¹), and phenazine-1-carboxamide (PCN; 5, 15, and 50 mg l⁻¹). Loading control: ribosomal 23S RNA and *BactA*.

Table 1. Effect of fludioxonil and antibiotics on radial mycelial growth of *Botrytis cinerea* strain B05.10 and its derivatives

Strain	EC ₅₀ (mg liter ⁻¹) ^a				
	Fludioxonil	Pyrrolnitrin	DAPG	PCA	PCN
B05.10	0.0022 a	0.019 a	17.4 a	39 a	40 a
$\Delta BcatrA$ -M7	0.0023 a	0.022 a	17.2 a	36 a	43 a
$\Delta BcatrB4$	0.0013 b	0.019 a	16.9 a	13 b	19 b
$\Delta BcatrB5$	0.0013 b	0.021 a	15.8 a	8.2 b	12 b

^a Effective concentration of compounds resulting in inhibition of radial mycelial growth by 50% (EC₅₀); DAPG = 2,4-diacetylphloroglucinol; PCA = phenazine-1-carboxylic acid; PCN = phenazine-1-carboxamide. Different letters in the same column indicate a statistically significant difference (*P* = 0.05).

control (Table 1). Both Δ BcatrB mutants were significantly more sensitive to PCA and PCN than their parental strain B05.10. Spore germination experiments confirmed this differential sensitivity to these phenazines (data not shown). The Δ BcatrB mutants also were more sensitive to fludioxonil than B05.10, as reported previously (Vermeulen et al. 2001), whereas no differences were observed in sensitivity to PRN and DAPG (Table 1). PG did not adversely affect mycelial growth of any of the *B. cinerea* strains at concentrations up to 500 $\mu\text{g ml}^{-1}$, suggesting that acetylation of PG significantly increases its fungicidal activity (data not shown). Δ BcatrA-M7 did not differ from its parental strain in sensitivity to any of the tested compounds, indicating that BcatrA is not involved in protection against any of these compounds. Collectively, these results strongly suggest that the ABC transporter BcatrB in *B. cinerea* provides protection not only to the fungicide fludioxonil but also to the antibiotics PCA and PCN.

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

The effect of phenazines on the efflux activity of BcatrB was determined in [^{14}C]fludioxonil accumulation experiments with germlings of wild-type strain B05.10 (Fig. 2). Accumulation of [^{14}C]fludioxonil, a substrate of BcatrB, was transient: the maximum level of [^{14}C]fludioxonil accumulation was

Fig. 2. Effect of phenazine-1-carboxamide (PCN) on [^{14}C]fludioxonil accumulation by germings of *Botrytis cinerea* strain B05.10. Arrows indicate the addition of PCN **A**, 65 min after or **B**, 60 min prior to addition of [^{14}C]fludioxonil (1 mg l^{-1}) at $t = 0$. Treatments: control = $\blacklozenge$, solvent control (0.1% dimethyl sulfoxide) = $\blacksquare$, and PCN (10 mg l^{-1}) = $\circ$.

reached 20 min after initiation of the experiment and thereafter gradually declined and remained low from 60 min onward (Fig. 2A). This transient accumulation is ascribed to rapid passive influx of [^{14}C]fludioxonil, resulting in induction of BcatrB expression and subsequent active efflux. Addition of PCN 65 min after initiation of the experiment resulted in an instantaneous and transient increase in accumulation of [^{14}C]fludioxonil (Fig. 2A). This transient increase can be explained by competition of PCN with fludioxonil for export by BcatrB followed by additional induction of BcatrB and other transporters. A 60-min pretreatment of germings with PCN prior to addition of [^{14}C]fludioxonil resulted in a low and constant accumulation level of [^{14}C]fludioxonil (Fig. 2B), which is ascribed to induction of expression of BcatrB by PCN.

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

Antagonism of the bacterial strains JM13, B37w, PCL1119, PCL1391, and Phz24 toward *B. cinerea* strains was determined in agar diffusion tests (Table 2). JM13 does not produce any known antibiotics and showed almost no activity against all *B. cinerea* strains tested. The antagonistic activity of B37w, known to produce PRN, was similar to all *B. cinerea* strains tested. Antagonistic activity of PCL1391 and Phz24, known to produce phenazines, was significantly stronger toward the mutants Δ BcatrB4 and B5 than to the parental strain B05.10 and Δ BcatrA-M7. In contrast, PCL1119, the phenazine-deficient mutant of PCL1391, had almost no activity toward the Δ BcatrB mutants and their inhibition was similar to that of the control strains. Microscopic observation of germing growth in the inhibition zones confirmed that PCL1391 had a relatively strong effect on spore germination and germ tube elongation of Δ BcatrB5 (Fig. 3). Similar observations were made in experiments with Phz24 and Δ BcatrB4. To ascertain that the inhibition zones incited by PCL1391 and Phz24 can be, at least in part, ascribed to activity of phenazines, the concentration of these antibiotics in the agar plates was determined by high-performance liquid chromatography (HPLC) analysis. Inhibition zones around inoculation sites of strain PCL1391 contained, on average, PCN at 30.1 $\mu\text{g ml}^{-1}$ and zones around Phz24 contained phenazines at 37.9 $\mu\text{g ml}^{-1}$ (2'-OH-PCA at 32.5 $\mu\text{g ml}^{-1}$ and PCA at 5.4 $\mu\text{g ml}^{-1}$). These concentrations are approximately two times higher than the effective concentration of compounds resulting in inhibition of radial mycelial growth by 50% (EC_{50}) of PCN for the Δ BcatrB mutants and 1.5 times lower than the EC_{50} for the parental strain B05.10 (Table 1). In zones around the inoculation site of the other bacterial strains, including PCL1119, no phenazines were detected.

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

The interactions between phenazine-producing *Pseudomonas* strains and *B. cinerea* strains B05.10 and Δ BcatrB4 was studied on tomato leaves. The virulence of B05.10 and Δ BcatrB4 on tomato leaves was similar in the control treatment (Fig. 4). Treatment of tomato leaves with cell suspen-

Table 2. Antagonistic activity of *Pseudomonas* and *Burkholderia* spp. against *Botrytis cinerea* strain B05.10 and its derivatives in agar diffusion tests

Strains	Inhibition zone (mm) ^a				
	JM13	PCL1119	PCL1391	Phz24	B37w
B05.10	0.45 a	1.25 a	1.46 a	14.17 a	7.68 a
Δ BcatrA-M7	0.32 a	0.43 b	1.12 a	14.46 a	7.01 a
Δ BcatrB4	0.37 a	0.74 ab	4.85 b	19.43 b	8.27 a
Δ BcatrB5	0.40 a	0.65 b	4.29 b	18.43 b	8.30 a

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

sions of PCL1391 significantly reduced the percentage of expanding lesions caused by Δ BcatrB4, whereas no reduction was observed for parental strain B05.10 (Fig. 4A). PCL1119, the phenazine-deficient mutant of PCL1391, did not reduce the percentage of expanding lesions for both Δ BcatrB4 and B05.10. Furthermore, the effect of PCL1391 on the size and growth of the lesions caused by Δ BcatrB4 was significantly stronger than for B05.10 (Fig. 4B and C). In contrast, the effect of PCL1119 on lesion size and growth was similar for B05.10 and Δ BcatrB4. In the repeat experiment, similar results were obtained: PCL1391 and phenazine-producer Phz24 significantly reduced the size and growth of the lesions caused by Δ BcatrB4, whereas no reduction was observed for parental strain B05.10. HPLC analysis demonstrated that, on leaves treated with PCL1391, PCN was produced at an average concentration of $5.6 \mu\text{g g}^{-1}$ of leaf fresh weight. No phenazines were detected on untreated leaves or on leaves treated with PCL1119. These results indicate that the ABC transporter BcatrB provides protection to *B. cinerea* in phenazine-mediated interactions with *Pseudomonas* spp.

DISCUSSION

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

Interestingly, BcatrB also provides protection against the grapevine phytoalexin resveratrol and phenylpyrrole fungicides (Schoonbeek et al. 2001; Vermeulen et al. 2001), indicating that one ABC transporter can transport multiple and structurally diverse compounds. ABC transporters with a wide substrate range have also been reported for yeasts and other filamentous fungi (Andrade et al. 2000a; Kolaczowski et al. 1998; Moore et al. 2000; Rogers et al. 2001; Wolfger et al. 2001). At present, it is not known how multiple unrelated compounds can induce transcription of one particular gene and at the same time act as a substrate of the encoded protein. Our observation

that fludioxonil, a fungicide derived from pyrrolnitrin (Ligon et al. 2000), differs from pyrrolnitrin in its induction of *BcatrB* and in activity toward Δ BcatrB mutants, is surprising and may provide an approach to further elucidate substrate specificity of BcatrB. In addition to phenazine antibiotics, the antibiotics DAPG and PG also induced expression of *BcatrB*. However, both Δ BcatrB mutants showed sensitivity to these compounds similar to that of their parental strain, indicating that induction of expression of a particular ABC gene does not necessarily imply that the encoded protein is a major transporter of that compound. This also has been observed for other inducers of *BcatrB* expression, including azole fungicides (Hayashi et al. 2001; Schoonbeek et al. 2001). DAPG also induces expression of *BcatrD*; therefore, this transporter may have operated in *BcatrB* mutants, thereby compensating for BcatrB efflux deficiency. The role of BcatrD and other transporters, including members of the Major Facilitator Superfamily, are currently being analyzed to better understand their role in sensitivity of *B. cinerea* to DAPG, a key metabolite in interactions between several plant pathogens and fluorescent *Pseudomonas* strains (Walsh et al. 2001). The contribution of other protective mechanisms, including enzymes that reduce oxidative stress caused by antibiotics (Levy et al. 1992), will be included in these studies.

An interesting question arising from this study is how *B. cinerea* acquired resistance to phenazine antibiotics. We propose that this ability may have evolved from long-lasting selection pressure by phenazine-producing microorganisms during the saprophytic and pathogenic stages of the life cycle of *B. cinerea*. This may be a consequence of the fact that phenazine production is a widely distributed trait among different bacterial genera that are ubiquitous in soil and plant-associated environments (Turner and Messenger 1986). This hypothesis would imply that *BcatrB* homologues also are more prevalent among other saprophytic and pathogenic fungi. Studies on the distribution of *BcatrB* homologues in other fungi, including *Aspergillus nidulans*, *Fusarium oxysporum*, *Gaeumannomyces graminis* var. *tritici*, and *Mycosphaerella*

Fig. 3. Antagonistic activity of *Pseudomonas chlororaphis* strain PCL1391 against *Botrytis cinerea* B05.10 and Δ BcatrB5, in agar diffusion tests. Inhibition of mycelial growth (top) and germination of conidia ($\times 400$ magnification, bottom).

graminicola, are ongoing in our laboratory and will further extend our insight into the role of ABC transporters in antibiotic-mediated interactions between pathogenic fungi and antagonistic microorganisms and, more generally, in ecological equilibria between microorganisms in natural environments.

Our observation that a plant-pathogenic fungus harbors a resistance mechanism to defend itself against a natural antibiotic compound has several implications for the efficacy and implementation of antagonistic microorganisms for biocontrol purposes. It may suggest that, if sufficient selection pressure is applied to target pathogens, either by repeated inundative applications of biocontrol agents or by introducing transgenic strains with enhanced antibiotic production, shifts in pathogen populations toward resistance may occur. Information on resistance development of pathogens to antibiotics produced by biocontrol agents is very limited. One of the few, but well-studied, examples is agrocin 84 resistance in *Agrobacterium tumefaciens* resulting from transfer of plasmid pAgK84 from the antagonistic strain *Agrobacterium radiobacter* K84 to the pathogen (Stockwell et al. 1996). Repetitive treatment of *Aspilbe* plants with the biocontrol agent *Bacillus subtilis* CL27 resulted in an increase in gray mold (Li and Leifert 1994). Recently, it was shown that introduction of a phenazine-producing *Pseudomonas* biocontrol strain exerted transient effects on the composition of the fungal rhizosphere microflora (Glandorf et al. 2001). Furthermore, Jones and Pettit (1987) observed variation in sensitivity among anastomosis groups of *Rhizoctonia solani* to the antibiotic gliotoxin produced by *Gliocladium virens*. Mazzola and associates (1995) showed that various isolates of the take-all fungus *Gaeumannomyces graminis* var. *tritici* differ in their sensitivity toward DAPG and phenazines produced by *Pseudomonas* spp. In fact, the fungal isolates that were relatively insensitive to either DAPG or phenazines in vitro could no longer be suppressed in situ by *Pseudomonas* strains producing these antibiotics (Mazzola et al. 1995).

These results suggest that at least certain pathogen populations have the potential to develop resistance to antibiotics produced by antagonistic microorganisms. We postulate that the efficacy and sustainability of crop protection by antibiotic-producing biocontrol agents can be compromised by adaptation in target organisms and that overexpression of ABC transporters can act as one of the resistance mechanisms. One approach to counteract such a resistance development might be the selection of crop cultivars with traits that promote efficacy of biocontrol agents (Smith et al. 1999). This may apply to crop cultivars with enhanced production of secondary metabolites that act as modulators (e.g., flavonoids) of fungal ABC transporter activity (Conseil et al. 2000).

MATERIALS AND METHODS

Strains and cultural practices.

Characteristics of the bacterial and fungal strains used in this study are described in Table 3. *Botrytis cinerea* strains were stored as conidial suspensions in 15% glycerol at -80°C and cultured as described previously (Schoonbeek et al. 2001). Conidia for sensitivity assays, expression analysis, and accumulation experiments were harvested from 10-day-old cultures on malt extract agar plates (MEA; 50 g l^{-1}) (Oxoid, Basingstoke, Hampshire, U.K.), amended with yeast extract (2 g l^{-1} ; Oxoid). Bacterial isolates were stored in 40% glycerol at -80°C and cultured on King's medium B (KMB) (King et al. 1954) at 28°C in the dark.

Chemicals.

Fludioxonil (technical grade) and $[^{14}\text{C}]$ fludioxonil were kindly provided by Syngenta (Basel, Switzerland). The antibiotics used were PG (1,3,5-trihydroxybenzene; Eastman Kodak Company, Rochester, NY, U.S.A.), DAPG (HPLC-purified), PRN (Syngenta), PCA, and PCN (T. F. C. Chin-A-Woeng,

Fig. 4. Antagonistic activity of *Pseudomonas chlororaphis* PCL1391 and its phenazine-deficient mutant PCL1119 on disease development incited by *Botrytis cinerea* strains B05.10 (white) and $\Delta\text{BcatrB4}$ (black) on tomato leaves. Leaves were sprayed with sodium phosphate buffer (control), or a suspension of PCL1391 or PCL1119 in the same phosphate buffer. Disease development is expressed as **A**, the percentage of inoculation sites developing into expanding lesions, **B**, the average lesion size at 4 days postinoculation (dpi), and **C**, the average growth of the lesions between 3 and 4 dpi. Different letters in columns indicate statistically significant differences between treatments ($P = 0.05$).

Table 3. Characteristics of bacterial and *Botrytis cinerea* strains

Strain	Phenotype ^z
<i>Botrytis cinerea</i>	
B05.10	Haploid derivative of SAS56 with wild-type sensitivity (Buttner et al. 1994)
$\Delta\text{BcatrA-M7}$	BcatrA gene-replacement mutant derived from B05.10 (Del Sorbo, unpublished data)
$\Delta\text{BcatrB4}$ and $\Delta\text{BcatrB5}$	BcatrB gene-replacement mutants derived from B05.10 with increased sensitivity to fludioxonil (Schoonbeek et al. 2001)
<i>Burkholderia cepacia</i>	
B37w (ATCC51671)	Producer of PRN (Burkhead et al. 1994)
<i>Pseudomonas chlororaphis</i>	
PCL1391	Producer of PCA and PCN (Chin-A-Woeng et al. 1998)
PCL1119	Mutant of PCL1391 with a Tn5-luxAB insertion in the <i>phzB</i> gene (Chin-A-Woeng et al. 1998)
<i>Pseudomonas</i> sp.	
JM13	No production of known antibiotics (Raaijmakers and De Souza, unpublished data)
Phz24	Producer of PRN, PCA, 2'OH-PCA (Raaijmakers and Lemanceau, unpublished data)

^z PRN = pyrrolnitrin, PCA = phenazine-1-carboxylic acid, PCN = phenazine-1-carboxamide.

Leiden University, Leiden, The Netherlands). All other compounds were purchased from Sigma-Aldrich (Zwijndrecht, The Netherlands). Compounds were added to media from 1,000-fold concentrated stock solutions in dimethyl sulfoxide unless stated otherwise.

In vitro sensitivity assays.

The EC₅₀ was determined on potato dextrose agar (PDA; Oxoid) as described previously (Stehmann and De Waard 1996). Experiments were repeated three times.

Gene expression in *B. cinerea*.

Expression analysis of ABC transporter genes in *B. cinerea* germlings was performed as described previously (Schoonbeek et al. 2001). Gene-specific fragments of *BcatrA*, *BcatrB*, and *BcatrD* and fragments corresponding to expressed sequence tags of *BcatrC*, *BcatrE*, *BcatrF*, *BcatrG*, *BcatrI*, and *BcatrK* were described previously (Hayashi et al. 2001; Vermeulen et al. 2001). Mycelium frozen in liquid nitrogen was used for RNA isolation with TRIzol (Life Technologies, Breda, The Netherlands). Hybridizations with *B. cinerea* 23S rRNA and *BactaA* (actin gene) were used as control (Prins et al. 2000).

Accumulation of [¹⁴C]fludioxonil.

Accumulation experiments were performed as described previously (Vermeulen et al. 2001) with minor modifications. Standard germling suspensions, consisting of 200 mg (wet weight) of mycelium in 50 ml of buffer (23.4 mM potassium phosphate [pH 6.0], 0.1 mM CaCl₂, glucose at 10 g l⁻¹) in 300-ml Erlenmeyer flasks, were shaken on a reciprocal shaker (120 rpm) at 20°C. Experiments were initiated by adding [¹⁴C]fludioxonil to a final concentration of 0.4 μM (1 mg l⁻¹; 250 Bq nmol⁻¹) from a 100× concentrated stock solution in methanol. The effect of phenazines (10 mg l⁻¹) on [¹⁴C]fludioxonil accumulation was determined by addition 60 min prior to or after the start of the incubation with the radiochemical. Experiments were performed in triplicate.

In vitro interactions

between *Pseudomonas* spp. and *B. cinerea*.

All bacterial isolates were transferred from a single colony on KMB agar to 3 ml of KMB broth and incubated (180 rpm) at 28°C for 16 h. Bacterial suspensions (0.5 ml), mixed with PDA (50 ml; 12 g of PDA and agar at 8 g l⁻¹) at 42°C, were added to petri dishes (15 cm) and incubated in the dark at 25°C for 10 days. Petri dishes (15-cm) containing half-strength MEA (50 ml; 25 g of MEA and agar at 6 g l⁻¹) mixed with fungal spores (5 × 10⁴ spores ml⁻¹) at 42°C were prepared on a horizontal surface. After solidification, agar plugs (diameter, 18 mm) from plates with bacteria were placed on the plates containing fungal spores. After incubation at 19°C in the dark for 3 days, the radius of the inhibition zone surrounding the bacterial plugs was measured. Tests were performed in quadruplicate and repeated three times.

In situ interactions

between *Pseudomonas* spp. and *B. cinerea*.

The effect of phenazine-producing *Pseudomonas* strains on infection of tomato leaves by *B. cinerea* was tested using a standardized assay that allows comparison of two *B. cinerea* strains per leaf (Benito et al. 1998). Bacteria were spread on KMB agar, incubated at 25°C for 2 days, washed, and resuspended in sodium phosphate buffer (10 mM, pH 6.4) to an optical density at 600 nm of 1.0. Leaves were cut from 5- to 7-week-old tomato plants (cv. Moneymaker-Cf4), placed in wet florist foam, and incubated in plastic boxes. Leaves (four per treatment) were sprayed with suspensions of phenazine-pro-

ducing strains PCL1391 or its phenazine-deficient mutant PCL1119 until run-off, using a DeVilbiss sprayer. The phenazine-producing strain Phz24 was included in a replicate experiment. Control leaves were sprayed with buffer. Leaves were incubated in boxes with closed lids for 26 h at 15°C, opened, and dried prior to inoculation with *B. cinerea*. For each treatment, 24 leaflets were inoculated on the upper side with droplets (2 μl) of a conidial suspension (10⁶ ml⁻¹) in Gamborg's B5 medium (Duchefa, Haarlem, The Netherlands) supplemented with 1% glucose and 10 mM ammonium phosphate (pH 6.5). The left half of each leaflet was inoculated with five droplets of B05.10 and the right half with five droplets of Δ*BcatrB4*. Boxes were closed and incubated at 15°C in the dark and the diameter and growth of the lesions were determined after 3 and 4 days.

Quantification of phenazine antibiotics by HPLC.

In vitro and in situ phenazine production levels were determined by HPLC (C₁₈ reverse phase 5 μm, 3.9-by-150 mm column, 616 pump, 600S controller, 996 photodiode array detector; Waters, Milford, MA, U.S.A.) (Bonsall et al. 1997). Production levels in the in vitro assays were determined by extraction of the inhibition zone surrounding the bacterial plugs. In situ production levels of PCN were determined 5 days after application of bacterial strains to tomato leaves. Approximately 5 g of leaf fresh weight was extracted with 80% acetone, filtered, extracted twice with ethylacetate plus 0.1% trifluoroacetic acid (TFA), air dried, and resuspended in 35% acetonitrile plus 0.1% TFA (Bonsall et al. 1997). For each treatment, two replicates were used. For quantification of phenazine production, a five-point standard curve generated by spiking known concentrations of PCN was used. A highly significant linear relationship was found for the standard curve (PCN = 0.000125 × A (*r*² = 0.99, *P* < 0.0001), in which PCN represents the total amount of PCN (ng), and A represents the area of the PCN peak at 248 nm.

Statistical analysis.

Differences between treatments were determined by analysis of variance followed by Duncan's multiple range test (*α* = 0.05) (SAS Institute Inc., Cary, NC, U.S.A.).

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