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The ABC transporter BcatrB from *Botrytis cinerea* exports camalexin and is a virulence factor on *Arabidopsis thaliana*

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SUMMARY

Arabidopsis thaliana **is known to produce the phytoalexin camalexin in response to abiotic and biotic stress. Here we studied the mechanisms of tolerance to camalexin in the fungus *Botrytis cinerea*, a necrotrophic pathogen of *A. thaliana*. Exposure of *B. cinerea* to camalexin induces expression of *BcatrB*, an ABC transporter that functions in the efflux of fungitoxic compounds. *B. cinerea* inoculated on wild-type *A. thaliana* plants yields smaller lesions than on camalexin-deficient *A. thaliana* mutants. A *B. cinerea* strain lacking functional BcatrB is more sensitive to camalexin *in vitro* and less virulent on wild-type plants, but is still fully virulent on camalexin-deficient mutants. Pre-treatment of *A. thaliana* with UV-C leads to increased camalexin accumulation and substantial resistance to *B. cinerea.* UV-C-induced resistance was not seen in the camalexin-deficient mutants *cyp79B2/B3*, *cyp71A13*, *pad3* or *pad2*, and was strongly reduced in *ups1*. Here we demonstrate that an ABC transporter is a virulence factor that increases tolerance of the pathogen towards a phytoalexin, and the complete restoration of virulence on host plants lacking this phytoalexin.**

Keywords: grey mould, phytoalexin resistance, plant pathology, plant defence, pathogenicity, fungal infection.

INTRODUCTION

Plants attacked by pathogens produce phytoalexins, many of which contribute to the innate immune response of the plant (Dixon, 2001; Hammerschmidt and Kagan, 2001). The extensive variation in phytoalexins produced by different plants is considered to limit the host range of pathogens (Osbourn, 1999). The ability to detoxify phytoalexins contributes to the pathogenicity of bacteria and fungi. While specific degradation of one anti-microbial compound can enable for virulence on a particular host, and may actually be required for virulence, general detoxification mechanisms, such as efflux, are especially useful for pathogens with a broad host range (VanEtten *et al.*, 2001). One such pathogen is *Botrytis cinerea*, the causal agent of grey mould diseases on a wide variety of crop plants. This necrotrophic pathogen uses numerous pathogenicity factors to broaden its host range (Williamson *et al.*, 2007).

Under various conditions, *Arabidopsis thaliana* produces various levels of its major phytoalexin camalexin (Mert-Turk *et al.*, 2003; Glawischnig, 2007). Camalexin is mostly absent in healthy plants, but is synthesized in response to abiotic stresses such as treatment with silver nitrate (Glawischnig, 2007), UV-B irradiation (Nawrath and Métraux, 1999; Mert-Turk *et al.*, 2003; Glawischnig, 2007) and UV-C irradiation (unpublished results; see Table 1) or upon inoculation with pathogens (Tsuji *et al.*, 1992; Thomma *et al.*, 1999; Mert-Turk *et al.*, 2003). Several mutants have been found in which the production of camalexin is attenuated, and some of the genes corresponding to these phytoalexin-deficient (*pad*) mutants (Glazebrook and Ausubel, 1994) have been characterized. They include biosynthesis mutants, such as those in the cytochrome P450 enzymes CYP79B2 and CYP79B3 (double mutant *cyp79B2/B3*), CYP71A13 (*cyp71A13*) and CYP71B15 (*pad3*) (Zhao *et al.*, 2002; Schuhegger *et al.*, 2006; Nafisi *et al.*, 2007), and other mutants, including *pad1*, *pad4* (Glazebrook *et al.*, 1997) and *ups1* (Denby *et al.*, 2005). *pad2* (Glazebrook and Ausubel, 1994) harbours a mutation in the

Table 1 Camalexin content (ng g^{-1} fresh weight ± SEM) of *Arabidopsis thaliana* lines 24 h after UV treatment or *Botrytis cinerea* (B05.10) inoculation

Plant	Control	UV	B05.10
Col-0	2.6 ± 0.7^a	1558 ± 171^a	1262 ± 90^a
pad3	1.1 ± 0.5^a	5.3 ± 1.0^b	15.5 ± 7.3^b
cyp71A13	23 ± 10^a	43.3 ± 9.6^b	191.7 ± 3.1^b
cyp79B2B3	ND	ND	6.7 ± 3.0^b

Different letters within one column correspond to statistically significant differences (determined by Tukey's HSD test; $P < 0.05$).
ND, not detectable.

gene for γ-glutamylcysteine synthetase, and has reduced glutathione levels (Parisy *et al.*, 2007). It has been considered to be a regulatory mutant of plant defences, including camalexin production, but it might be that glutathione serves as a sulphur donor in camalexin synthesis. Both *pad2* and *ups1* can, under certain circumstances, produce some camalexin, but generally have reduced levels compared to Col-0 (Glazebrook and Ausubel, 1994; Denby *et al.*, 2005; Parisy *et al.*, 2007). We also utilized the *eds5* mutant, which is impaired in salicylic acid (SA) production and signalling, and is attenuated in responses to UV stress (Nawrath *et al.*, 2002). Despite reduced UV-induced responses, it can still accumulate camalexin. The availability of these mutants has made it possible to study the contribution of camalexin to defence in defined genetic backgrounds and compare them with the WT ecotype Col-0 (Glazebrook and Ausubel, 1994).

Although camalexin is produced in response to *Hyaloperonospora parasitica* and *Pseudomonas syringae*, this does not alter the resistance of Col-0 to these pathogens as *pad3* mutants do not display increased susceptibility (Zhou *et al.*, 1999; Mert-Turk *et al.*, 2003). In contrast, the virulence of *Alternaria brassicicola* is increased on *pad3* (Thomma *et al.*, 1999).

There are conflicting reports on the role of camalexin in resistance to *B. cinerea*. One report showed no increased sensitivity of *pad3* plants (Thomma *et al.*, 1999), but several other studies found that *pad* mutants are more readily infected by *Botrytis* (Ferrari *et al.*, 2003; van Baarlen *et al.*, 2007; Chassot *et al.*, 2008). A relationship between *in vitro* susceptibility and virulence on Col-0 has been reported (Kliebenstein *et al.*, 2005), making it likely that the effect of camalexin on *Botrytis* is isolate-dependent. This might be explained by variability in resistance mechanisms among the isolates, such as alterations of the target site of camalexin (as yet unknown), as observed with mutations leading to fungicide resistance (Rosslenbroich and Stuebler, 2000), or differential expression of detoxification mechanisms. Detoxification may be achieved by enzymatic conversion, as observed for several pathogens including the related *Sclerotinia sclerotiorum* (Pedras and Ahiahonu, 2002, 2005), or active efflux, as demonstrated for the phytoalexins resveratrol and eugenol (Schoonbeek *et al.*, 2001, 2003).

Energy-dependent export of toxic compounds is a protection mechanism that is found in all organisms (Higgins, 1992). In fungi, active efflux by ATP-binding cassette (ABC) and major facilitator (MFS) transporters provides resistance to endogenous and exogenous toxic compounds such as antibiotics, plant defence compounds and fungicides (Del Sorbo *et al.*, 2000; Rogers *et al.*, 2001). A role in pathogenesis has been found for a limited number of ABC transporters. Examples in plant pathogenic fungi include ABC1, ABC3 and ABC4 from *Magnaporthe grisea* on rice (Urban *et al.*, 1999; Sun *et al.*, 2006; Gupta and Chattoo, 2008), BcatrB from *B. cinerea* on grapevine (Schoonbeek *et al.*, 2001), GpABC1 from *Gibberella pulicaris* on potato (Fleissner *et al.*, 2002) and Mgatr4 from *Mycosphaerella graminicola* on wheat (Stergiopoulos *et al.*, 2003). Among fungi causing infections in humans, such as *Candida* and *Aspergillus* spp., ABC and MFS transporters are notorious for their contribution to azole fungicide resistance, rendering clinical therapy less efficient (Gulshan and Moye-Rowley, 2007). In *Cryptococcus neoformans*, the ABC transporter AFR1 provides protection against fluconazole and acts as a virulence factor, allegedly by protecting against anti-microbial substances produced by the host phagocytes (Sanguinetti *et al.*, 2006). Resistance to anti-fungal compounds is generally regulated at the transcriptional level: exposure to toxicants induces expression of those transporters that can export them (DeRisi *et al.*, 2000; Rogers *et al.*, 2001; Gulshan and Moye-Rowley, 2007).

In *B. cinerea*, ABC transporters contribute to virulence, but also to successful competition with other micro-organisms and multi-drug resistance. For example, BcatrB transports phenazine antibiotics (Schoonbeek *et al.*, 2002) and the fungicide fludioxonil (Vermeulen *et al.*, 2001), and BcatrD and Bcmfs1 transport azole fungicides (Hayashi *et al.*, 2002a,b).

The variation in camalexin sensitivity between *B. cinerea* isolates used in various studies might depend on the ability of each isolate to export camalexin. We tested whether ABC transporters of *B. cinerea* can transport camalexin, and verified the effect of mutations in such transporters on pathogenicity in *A. thaliana*. The use of both fungal ABC transporter mutants and *pad* mutants creates the unique possibility of evaluating a phytoalexin detoxification mechanism *in planta*, not only *in vitro*.

RESULTS

Camalexin induction of *BcatrB* expression

Treatment of *B. cinerea* germlings with 15 mg L^{-1} camalexin induced the expression of several ABC and MFS transporters present on a transporter macroarray after 30 or 120 mins (Figure S1; Table S2). A limited number of transporter genes

were induced at both time points. Other genes were induced at only one of the two time points. Several camalexin-induced genes were also induced by fludioxonil, a known substrate of BcatrB (Vermeulen *et al.*, 2001). Quantification by quantitative RT-PCR showed that induction of *BcatrB* occurred within 30 mins and was specific, as expression of *BcatrA*, *BcatrD* and *Bcmfs1* remained low (Figure 1a).

The expression of *BcatrB* in leaves of *A. thaliana* Col-0 infected with *B. cinerea* was determined by RT-PCR and reached the same level as the reference gene *BcEF1b* at 24 h post-inoculation (hpi), while *BcatrA* and *BcatrD* could not be detected at any time point. Strain BcatrBp803GUS-7, a *B. cinerea* transformant carrying a BcatrB promoter–GUS construct, showed little GUS activity on *pad3* but high GUS activity on Col-0 plants, demonstrating that the gene is actually expressed *in planta*, especially when camalexin is produced (Figure 1b).

Sensitivity of *B. cinerea* ABC transporter mutants to camalexin

The sensitivity of *B. cinerea* replacement mutants for *BcatrA*, *BcatrB* and *BcatrD* was determined using a radial growth test on potato dextrose agar. The wild-type (WT) isolate B05.10 had an EC_{50} of 6.2 mg L^{-1} and an EC_{95} of 26 mg L^{-1} (Figure 2). This sensitivity corresponds to that of *B. cinerea* isolates described as normally sensitive (Ferrari *et al.*, 2003; Kliebenstein *et al.*, 2005). B05.10-derived *BcatrB* mutants such as ΔBcatrB4 and the double mutant ΔBcatrB4D5 were more sensitive to camalexin (Figure 2). Mutants in which *BcatrA* or *BcatrD* were replaced by the same resistance cassette used for the BcatrB mutants did not show altered sensitivity.

Camalexin effect on BcatrB-mediated [^{14}C]-fludioxonil efflux

[^{14}C]-fludioxonil can enter the cell by passive influx, and accumulates transiently in germlings of the WT *B. cinerea* isolate B05.10. The initial accumulation peak is rapidly followed by induction of transport activity leading to active efflux and reduced [^{14}C]-fludioxonil levels in the germlings. After 1 h, a stable state is reached, with low internal levels of [^{14}C]-fludioxonil, during which passive influx is matched by active efflux (Figure 3). This active efflux mainly depends on BcatrB, as *BcatrB* mutants show little decrease in accumulation after the initial influx of [^{14}C]-fludioxonil (Figure 3) (Vermeulen *et al.*, 2001). Addition of a second substrate of BcatrB competing with the efflux capacity for [^{14}C]-fludioxonil, such as eugenol, resulted in increased accumulation, most likely because the additional substrate occupies the transporter that was exporting [^{14}C]-fludioxonil (Schoonbeek *et al.*, 2002, 2003). Treating a B05.10 culture equilibrated with [^{14}C]-fludioxonil with camalexin (20 or 40 mg L^{-1}) or eugenol (100 mg L^{-1}) caused increases in

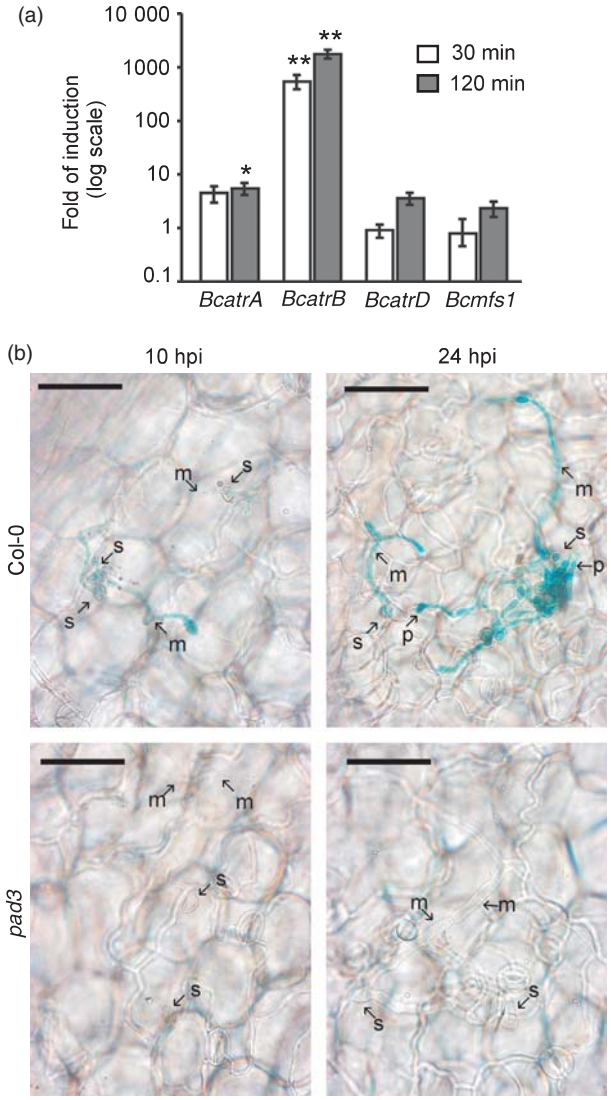

Figure 1. Induction of transporter genes in *Botrytis cinerea*.
(a) Increase in expression of *BcatrA*, *BcatrB*, *BcatrD* and *Bcmfs1* 30 and 120 mins after treatment of *B. cinerea* B05.10 germlings with 15 mg L^{-1} camalexin, compared to treatment with the solvent control (0.1% DMSO) at the same time point. Expression of each transporter gene was determined by quantitative RT-PCR and normalized against that of *BcEF1b*. Mean values from three replicates (±SEM) are presented on a logarithmic scale. An induction fold of one indicates no change in expression compared to the control treatment; asterisks indicate a significant difference between the treatment and control (*$P < 0.05$; **$P < 0.01$).
(b) Expression of BcatrB *in planta*: leaves of *A. thaliana* Col-0 and the camalexin-deficient mutant *pad3* were inoculated with spores of BcatrBp803-GUS-7, a *B. cinerea* transformant harbouring a BcatrB promoter–GUS fusion. Microscopic observations after staining with X-gluc at 10 and 24 hpi show that BcatrB is expressed on Col-0 but not on *pad3* (scale bar = 50 μm). Fungal structures are indicated by arrows and letters indicating spores (s), mycelium (m) and penetration structures (p). When not stained by X-gluc, these structures have a transparent appearance.

accumulation compared to the DMSO control after 15 mins of 106 ± 33%, 123 ± 30% and 94 ± 18%, respectively (Figure 3), with a significant difference between treatments

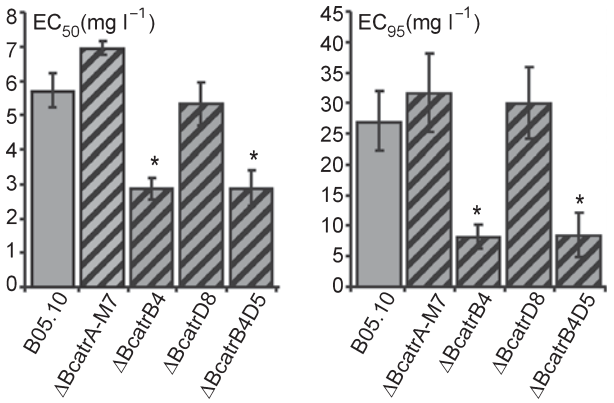

Figure 2. Sensitivity of *Botrytis cinerea* to camalexin.
EC_{50} and EC_{95} of B05.10 and the derived mutants ΔBcatrAM7, ΔBcatrB4, ΔBcatrD8 and the double mutant ΔBcatrB4D5 calculated from the dose–response curves of radial growth on potato dextrose agar plates to which various concentrations of camalexin had been added. Values are means ± SEM for at least four replicate experiments. Asterisks indicate a significant difference between the mutant and B05.10 ($P < 0.05$).

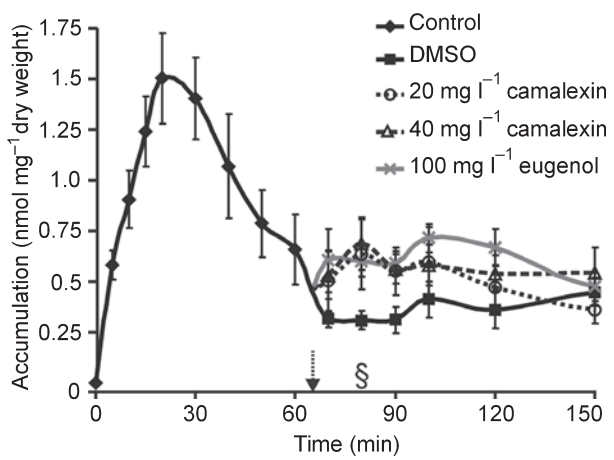

Figure 3. Effect of camalexin and eugenol on accumulation of [^{14}C]-fludioxonil.
The accumulation of [^{14}C]-fludioxonil by germlings of *Botrytis cinerea* strain B05.10 increases after addition of camalexin or eugenol 65 mins after [^{14}C]-fludioxonil (arrow), the increase in accumulation noted in the text was calculated after 15 mins (§). Values are means ± SEM for three replicate experiments.

and solvent control at $P < 0.11$. Thus both eugenol and camalexin compete for efflux by the same transporter as [^{14}C]-fludioxonil, BcatrB, indicating that BcatrB can transport these compounds.

Virulence of ABC transporter mutants on Col-0 and camalexin mutants

As BcatrB can export camalexin *in vitro*, it may contribute to the virulence of *B. cinerea* on *A. thaliana* lines that produce camalexin. We examined the role of BcatrB and other ABC transporters in virulence on Col-0, which has WT camalexin levels, and on the camalexin-deficient *pad3* mutant. B05.10 caused lesions of 5 mm in diameter on Col-0 compared to lesions of 6.5 mm on *pad3*, measured 3 days post-inoculation (dpi) (Figure 4). This indicates that B05.10 is an isolate of moderate to high virulence compared to those used in other studies (Kliebenstein *et al.*, 2005; van Baarlen *et al.*, 2007). The amount of camalexin produced by Col-0 after *B. cinerea* infection (Table 1) was enough to partially restrict the growth of B05.10 compared to *pad3* (Figures 4 and 5). The BcatrB mutants, however, could only produce lesions that were half the size of those produced by B05.10 on Col-0, but made lesions of equal size on *pad3* (Figure 4a,c). Mutants in *BcatrA* or *BcatrD* showed no reduction of lesion size. Only the double mutant ΔBcatrB4D5 displayed reduced virulence on Col-0, at a similar level as the single mutant ΔBcatrB4 (Figure 4c). Thus, BcatrB alone is required for full virulence on plants producing camalexin but not on those lacking the phytoalexin. At early time points of the infection process, such as 10–24 hpi, both B05.10 and ΔBcatrB4 behaved similarly on Col-0. Germination and first attempts to penetrate occurred with similar efficiency (Figure 4b). However, at 48 hpi, penetration and *in planta* growth were more vigorous for B05.10 than for ΔBcatrB4 (Figure 4b). On *pad3*, both isolates grew to the same extent within the plant tissue at all time points examined (Figure 4b). ΔBcatrB4 can initiate infection as well as B05.10 can, but is hindered when it comes in contact with camalexin. The virulence of BcatrB mutants was as high as that of B05.10 on *cyp79B2/B3* and *cyp71A13*, other *A. thaliana* mutant lines that are impaired in camalexin biosynthesis (Figure 5). In *cyp79B2/B3*, production of both camalexin and indole glucosinolates is impaired, which leads to strongly increased sensitivity to all *B. cinerea* isolates tested.

The virulence of B05.10 and the BcatrB mutant did not differ on the *A. thaliana* mutants *pad2* and *ups1*, which are impaired in camalexin production (Figure 6). This contrasts results obtained using Col-0 and *eds5*, *A. thaliana* lines that can make camalexin, on which the BcatrB mutants are less virulent than B05.10 (Figure 6).

UV-C induced resistance against *B. cinerea* depends on camalexin

We tested whether the size of *B. cinerea* lesions could be restricted by subjecting *A. thaliana* to a pre-treatment that elevates the camalexin levels. Col-0 plants can be induced to produce camalexin by exposure to UV-C light. We quantified the amount of camalexin produced by various *A. thaliana* lines after UV-C treatment or *B. cinerea* infection. All *A. thaliana* genotypes showed similar UV-induced symptoms after irradiation. On the first day after irradiation (which is the day of *B. cinerea* inoculation), no macroscopic

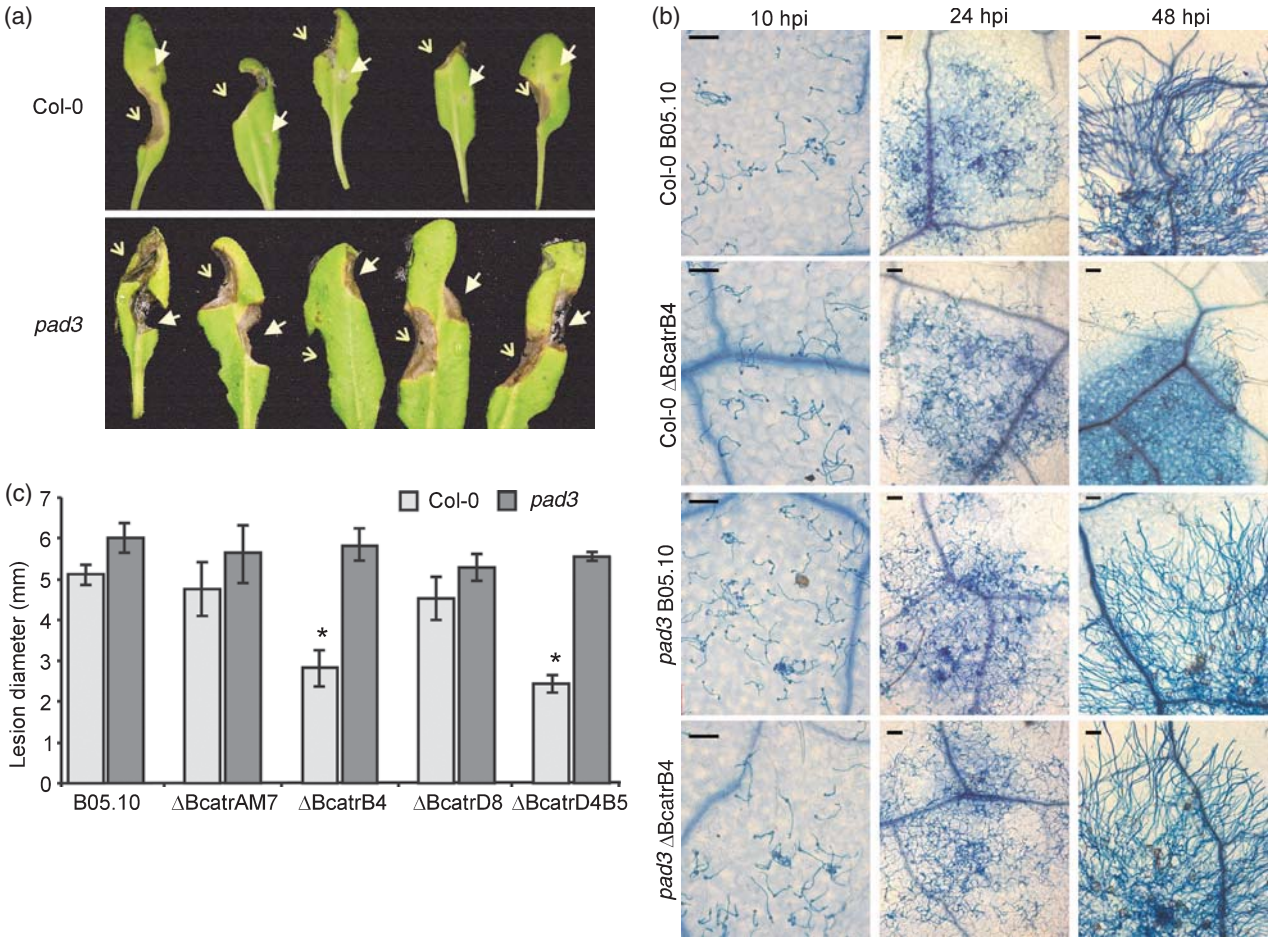

Figure 4. Disease symptoms of *Botrytis cinerea* on Col-0 and *pad3*.
Leaves of *Arabidopsis thaliana* Col-0 and the camalexin-deficient mutant *pad3* were pairwise-inoculated with spores of B05.10 and derived ABC transporter mutants.
(a) Macroscopic symptoms of the infection at 3 dpi caused by B05.10 (open arrow) and ΔBcatrB4 (closed arrow).
(b) Microscopic observations after staining with trypan blue at 10, 24 and 48 hpi; scale bar = 100 μm.
(c) Mean lesion diameter for all inoculation sites after inoculation with spores of B05.10 and various derived ABC transporter mutants, measured at 3 dpi. Values are means ± SEM for at least three replicate experiments. Asterisks indicate a significant difference between isolates on one host plant (determined by Tukey's HSD test; $P < 0.05$).

symptoms were observed, but symptoms became increasingly apparent between days 2 and 4, with a metallic sheen and yellowing of the leaves, and spontaneous, localized cell death, as observed by trypan blue staining (Figure 7 and Figure S2). In Col-0, both UV-C treatment and *B. cinerea* infection induced the expression of *CYP79B2*, *CYP71A13* and *PAD3*, genes involved in camalexin production, to a similar extent (Figure 8). Germlings of BcatrBp803GUS-7 were stained earlier and more intensely when growing on UV-C-treated Col-0 plants than on mock-treated Col-0 plants, and the staining on *pad3* plants was always low, even after UV-C treatment (Figure S3). This indicates that spores on UV-C-treated Col-0 plants are directly in contact with camalexin when they arrive on the surface of the leaf and start expressing *BcatrB*.

In Col-0, the basal level of camalexin was low, and its production was induced 24 h after UV-C treatment, to a level of 1.6 μg g^{-1} fresh weight. A similar level was reached after *B. cinerea* infection, reaching 1.3 μg g^{-1} fresh weight at 24 hpi (Table 1), and 4 μg g^{-1} fresh weight at 48 hpi (Table 2). No difference in camalexin accumulation was measured between plants infected with B05.10 and plants infected with any of the mutants tested (Table 2). As expected, very little camalexin was produced by the biosynthesis mutants *pad*3 and *cyp79B2/B3*, and only a small but significant amount was produced by *cyp71A13* (Table 1). UV-C treatment increased the resistance of Col-0 plants to *B. cinerea* B05.10 and even more to ΔBcatrB4, indicating that elevated camalexin levels limit infection by *B. cinerea* (Figure 5). On UV-C-treated *pad3*, *cyp79B2/B3* and *cyp71A13* plants, the lesion sizes caused by B05.10 and ΔBcatrB4 were comparable, and did not differ significantly from those on non-treated plants (Figure 5).

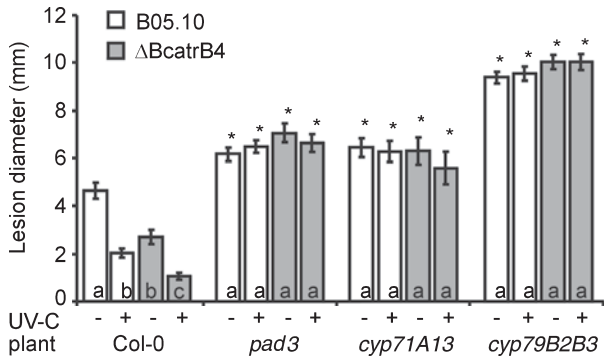

Figure 5. UV-C-induced resistance in *Arabidopsis thaliana* against *Botrytis cinerea.*
Lesion diameter at 3 dpi with *B. cinerea* isolates B05.10 and ΔBcatrB4 on *A. thaliana* Col-0, and on the mutants *pad3* and *cyp71A13*, which are deficient in camalexin biosynthesis, and *cyp79B2/B3*, which is deficient in camalexin and indole glucosinolate production, with or without pre-treatment with UV-C. Different letters indicate significant differences between treatments within one genotype (determined by Tukey's HSD test); asterisks indicate significant differences between Col-0 and the tested genotype for a given combination of UV treatment and isolate ($P < 0.05$).

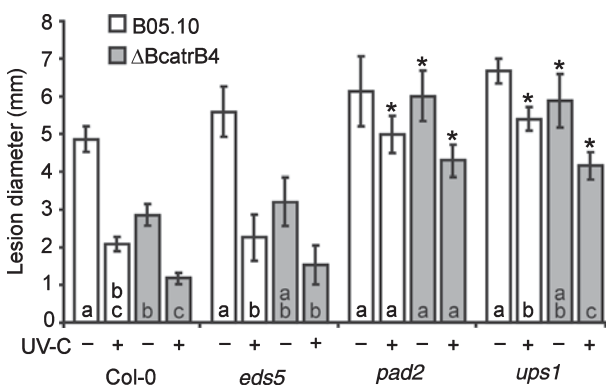

Figure 6. Effect of UV-C on susceptibility to *Botrytis cinerea* in Col-0 and regulatory mutants.
UV-C induced resistance against *B. cinerea* in Col-0 and the regulatory mutants *eds5*, *pad2* and *ups1*. Lesion diameters were measured at 3 dpi with *B. cinerea* isolates B05.10 and ΔBcatrB4, with or without pre-treatment with UV-C. Different letters indicate significant differences between treatments within one genotype (determined by Tukey's HSD test); asterisks indicate significant differences between Col-0 and the tested genotype for a given combination of UV treatment and isolate ($P < 0.05$).

UV-C-treated *eds5* plants were as resistant as UV-C-treated Col-0, but *ups1* plants showed only a small, but significant, decrease in lesion size after UV-C treatment, which was more pronounced for ΔBcatrB4 than for B05.10, while the decrease in lesion size after UV-C was not significant on *pad2* (Figure 6). These results appear to indicate that, despite other changes induced by UV-C, the resistance observed in Col-0 and *eds5* largely depends on camalexin production. Induction of resistance after UV-C treatment in *pad2* and *ups1* appears to be strongly or partially impaired, respectively, correlating with reduced induction of camalexin production.

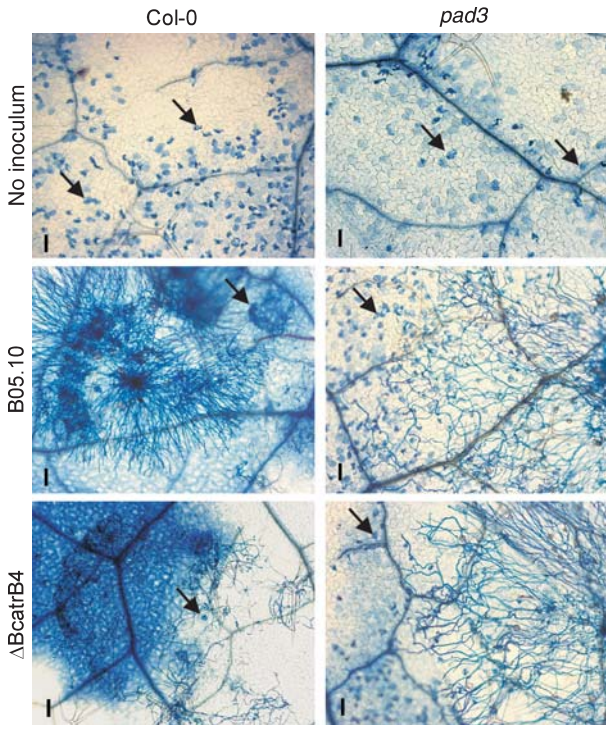

Figure 7. Disease symptoms caused by *Botrytis cinerea* are suppressed by UV-C on Col-0 but not on *pad3*.
Leaves of *Arabidopsis thaliana* Col-0 and the camalexin-deficient mutant *pad3* were treated with UV-C 24 h before pairwise inoculation with spores of B05.10 and the camalexin-sensitive mutant ΔBcatrB4. Microscopic observations after staining with trypan blue at 48 hpi show that hyphal growth in the leaf is reduced for B05.10 and mostly absent for ΔBcatrB4 on Col-0, whereas both isolates showed profuse hyphal growth in the leaf tissue on *pad3*. On both plant lines, the combination of UV-C and *B. cinerea* infection resulted in spontaneous cell death (arrow). Scale bar = 100 μm.

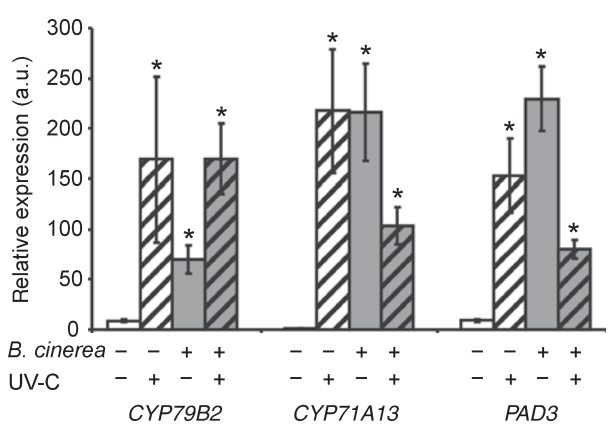

Figure 8. *Botrytis cinerea* and UV-C induce expression of camalexin biosynthesis genes.
Expression levels of genes involved in camalexin biosynthesis 16 h after treatment of Col-0 with UV-C and/or *B. cinerea*. Expression of *CYP79B2*, *CYP71A13* and *PAD3* was determined by quantitative RT-PCR and normalized against that of At4g26410. Values are mean relative expression levels (±SEM) in arbitrary units (a.u.) for three replicate cDNA samples; asterisks indicate significant differences between the control treatment and any given combination of UV-C and *B. cinerea* inoculation ($P < 0.05$).

Table 2 Camalexin content (ng g^{-1} fresh weight $\pm$ SEM) of *Arabidopsis thaliana* lines after inoculation with various *Botrytis cinerea* isolates or the inoculation medium alone (quarter-strength potato dextrose broth, 1/4 PDB)

Treatment	20 hpi	48 hpi
1/4 PDB	61 ± 32^a	210 ± 93^a
B05.10	748 ± 153^b	3814 ± 705^b
ΔBcatrB4	757 ± 167^b	2814 ± 535^b
MDR5_16	858 ± 270^b	3843 ± 835^b
MDR-ΔBcatrB1	700 ± 123^b	3099 ± 807^b
MDR-ΔBcatrB4	855 ± 131^b	3860 ± 811^b

Different letters within one column correspond to statistically significant differences (determined by Tukey's HSD test; $P < 0.05$).

Functionality of BcatrB in other *B. cinerea* isolates

To verify whether BcatrB is also functional in other *B. cinerea* isolates, we analysed isolate MDR5_16, which was isolated from grapevine, displays only low virulence on *A. thaliana*, and shows constitutively increased expression of BcatrB (M. Kretschmer, M. Leroch, A.-S. Walker, S. Fillinger, A. Mosbach, D. Mernke, J.M. Pradier, H.-J. Schoonbeek, P. Leroux, M.A. de Waard and M. Hahn, unpublished results). The isolate was more resistant to high concentrations of camalexin than B05.10 was (Figure S4). Nevertheless, lesions caused by MDR5_16 were small on all *A. thaliana* lines, independent of camalexin production, reaching an average lesion diameter of 2.1 ± 0.8 mm on Col-0. Remarkably, the lesion size of MDR5_16 on Col-0 after UV-C treatment was 1.9 ± 0.8 mm, indicating that this strain is not affected by the extra camalexin produced. BcatrB mutants in this background were more susceptible to camalexin than their parent, and were as susceptible as ΔBcatrB4 (Figure S4).

DISCUSSION

The outcome of interactions between plants and their pathogens is a delicate balance, tilting in favour of the plant if phytoalexins can keep the pathogens in check, or in favour of pathogens that possess efficient tools to withstand antimicrobial compounds produced by the plant (VanEtten *et al.*, 2001). The results presented here demonstrate that the concentration of camalexin produced during interaction between *A. thaliana* Col-0 and *B. cinerea* is within the discriminatory range for the outcome of infection. Alterations in the levels of camalexin have a profound effect on the outcome of the interaction. This is shown in mutants impaired in camalexin biosynthesis, in plants overexpressing camalexin after UV treatment in plants, or when camalexin efflux is perturbed in the fungus.

Pathogens with a broad host range, such as *B. cinerea*, are particularly advantaged by the ability to detoxify a wide spectrum of phytoalexins with various modes of action. Exposure to camalexin induces a variety of degradation mechanisms in fungal pathogens (Pedras and Ahiahonu, 2005). *Alternaria brassicicola* reacts to camalexin by upregulation of various genes. Some of the functions associated with these genes include maintenance of membrane integrity or transport processes (Sellam *et al.*, 2007). Here we show that *B. cinerea* exposed to camalexin induces several transporters linked with detoxification of a number of toxic compounds (De Waard *et al.*, 2006), in particular *BcatrB* (Figure 1). Induction of expression of a given ABC transporter gene by a compound does not necessarily mean that the protein transports it (Del Sorbo *et al.*, 2000; Rogers *et al.*, 2001; Schoonbeek *et al.*, 2003), or that it is the only transporter for this compound (Hayashi *et al.*, 2002b). Our results show that replacement of *BcatrB* increased the sensitivity of *B. cinerea* to camalexin (Figure 2) in two different backgrounds (Figure S4). In B05.10, the basal level of *BcatrB* expression is very low, but can be induced rapidly (within 5 mins) and to high levels after exposure to fungitoxic compounds (Schoonbeek *et al.*, 2001, 2003; Vermeulen *et al.*, 2001). Apparently, induction of BcatrB activity in B05.10 is fast and strong enough (Figures 1 and 3) to provide protection against the gradual increment in camalexin levels produced in Col-0 after infection. As fludioxonil efflux is dependent on BcatrB (Vermeulen *et al.*, 2001), conclusive evidence for involvement of BcatrB in the transport of camalexin is provided by the interference of camalexin with the active efflux of [^{14}C]-fludioxonil (Figure 3), similarly to the effect of the known BcatrB substrates phenazine-1-carboxamide, resveratrol and eugenol (Schoonbeek *et al.*, 2002, 2003). The transient accumulation in the mycelium of [^{14}C]-fludioxonil both before and after camalexin addition is in accordance with the rapid induction of *BcatrB* expression by fludioxonil (Vermeulen *et al.*, 2001) and camalexin (Figure 1), respectively.

The WT *B. cinerea* isolate B05.10 can infect Col-0, but gives slightly larger lesions on *pad3* (Figures 4 and 5), indicating that the amount of camalexin present in WT plants limits spreading. Lesions on *cyp79B2/B3* are even larger than on *pad3* (Figure 5), indicating that indole glucosinolates and camalexin work in concert in plant defence against *B. cinerea*, which corroborates results with other *B. cinerea* isolates (Kliebenstein *et al.*, 2005). The difference between Col-0 and *pad3* has been observed to various degrees in several studies (Thomma *et al.*, 1999; Ferrari *et al.*, 2003; van Baarlen *et al.*, 2007; Chassot *et al.*, 2008). Our data also agree with a study in which the *in vitro* resistance of several *B. cinerea* isolates to camalexin was shown to correlate with their ability to infect Col-0 (Kliebenstein *et al.*, 2005). The isolate most resistant to camalexin showed similar lesion sizes on Col-0 and *pad3*, while the isolates sensitive to camalexin produced much smaller lesions on Col-0 than on *pad3*. The sensitivity and virulence of B05.10 are moderate in comparison with other isolates.

The drastic effect of replacement of *BcatrB* on the lesion size on Col-0 but not on *pad3*, and the lack of effect of replacement of *BcatrA* and *BcatrD*, led us to the conclusion that BcatrB contributes to pathogenicity by protecting *B. cinerea* against camalexin, similar to its role in protection against resveratrol on grapevine (Schoonbeek *et al.*, 2001). Other ABC transporters have been implicated in fungal pathogenicity on plants, for example GpABC1 can protect *G. pulicaris* against rishitin on potato (Fleissner *et al.*, 2002). However, for most of these ABC transporters, the substrates are not known. Three ABC transporters are required for virulence of *M. grisea*, but the actual substrate transported during pathogenesis is not known for any of them. ABC1 is induced by rice phytoalexins, but ABC1 mutants are not more sensitive to these compounds (Urban *et al.*, 1999), ABC3 is required for host penetration, especially under conditions of oxidative stress (Sun *et al.*, 2006), and a mutant in ABC4 was demonstrated to be more sensitive to resveratrol but this phytoalexin is not found in *M. grisea* host plants (Gupta and Chattoo, 2008). In *Fusarium culmorum*, FcABC1 is required for virulence on wheat but not on barley, supposedly because the pathogen is more exposed to the plant defence compound DIMBOA in wheat (Skov *et al.*, 2004). None of these ABC transporter mutants was shown to perform better on plants lacking phytoalexins. The observation that virulence of BcatrB mutants was fully restored on camalexin-deficient plants offered the unique possibility of demonstrating unambiguously that BcatrB protects against phytoalexins *in planta*.

Several treatments that induce priming of resistance against *B. cinerea*, including treatment with oligogalacturonides (Ferrari *et al.*, 2007) or wounding (Chassot *et al.*, 2008), rely partially on camalexin accumulation. Treatment with UV light efficiently increased the camalexin levels in Col-0 plants (Table 1) and restricted the spread of B05.10 (Figure 5), possibly in combination with other defences. However, UV-C-induced resistance must depend on camalexin as it was not observed in mutants that are deficient in camalexin production (Figure 5). UV-C treatment alone increased camalexin levels (Table 1), building up a defensive barrier before inoculation with *B. cinerea*. This is in contrast with priming, where treatment alone does not induce defence but primes the plant to react faster upon pathogen infection (Conrath *et al.*, 2006).

UV treatment of crop plants has been reported to provide protection against *B. cinerea* and other pathogens (Mercier *et al.*, 1993; Nigro *et al.*, 1998; Bonomelli *et al.*, 2004; Charles *et al.*, 2008). In some cases, the treatment induced morphological changes and/or accumulation of phytoalexins, while in others it increased the responsiveness of tissues to the pathogen, but it has been difficult to distinguish the relative role of each effect. For example, in tomato, rishitin accumulation is both primed and initiated directly after UV treatment, but it is not the sole factor in *B. cinerea* resistance (Charles *et al.*, 2008). We observed that, after UV-C treatment, the morphological changes that occur in Col-0 also occurred in *pad3* (Figure 7 and Figure S2) and other camalexin-deficient plants that were fully susceptible to *B. cinerea*, but only Col-0 was resistant. We can conclude that only the increased level of camalexin in UV-C-treated Col-0 is responsible for the increased resistance, while other UV-C-induced changes, such as those that are impaired in *eds5* (Nawrath *et al.*, 2002), are not required (Figure 6).

The elevated *in vitro* camalexin tolerance of MDR5_16 corroborates the observation that this isolate is not affected by the higher levels of camalexin in UV-C-treated Col-0. This may imply that the constitutive over-expression of *BcatrB* in MDR5_16 is advantageous in situations of sudden exposure to high levels of its substrate, while B05.10, and possibly other WT isolates, which have a low basal expression level, can induce BcatrB fast enough to cope with moderate camalexin concentrations. *BcatrB* is expressed on *A. thaliana*, especially in the presence of camalexin, on Col-0 after 24 hpi and to a lesser extent on *pad3* (Figure 1 and Figure S3). Variation in *BcatrB* expression levels between isolates might be partially responsible for the differences observed in virulence on plants containing camalexin. As *BcatrB* is induced by many other plant-derived compounds (Schoonbeek *et al.*, 2001, 2003), it may also contribute to virulence on other hosts. The broad substrate specificity of BcatrB may help the fungus to cause disease on other host plants than Arabidopsis, and extend its host range to comprise crop plants such as grapevine. It might thus be a possible target for fungicides as suggested for other transporters (Reimann and Deising, 2005; Roohparvar *et al.*, 2007); modulators of transporter function can be used to increase the susceptibility of plant pathogenic fungi to fungitoxic compounds (Hayashi *et al.*, 2003).

Active export of camalexin by BcatrB is not necessarily the only protective mechanism in *B. cinerea*. While it temporarily removes the toxic compound from the fungal cell, further detoxification might be achieved by enzymatic conversion, such as the glucosylation found for *S. sclerotiorum* (Pedras and Ahiahonu, 2002) or the biotransformation observed in several other pathogens (Pedras and Ahiahonu, 2005). Active efflux might be a first line of defence, acting rapidly and helping the cell to survive until the phytoalexin is further detoxified by other enzymes. In conclusion, we consider BcatrB to be a virulence factor, the expression level of which determines the virulence.

EXPERIMENTAL PROCEDURES

Chemicals were purchased from Sigma-Aldrich (http://www.sigmaaldrich.com) and media components were purchased from Difco (http://www.bd.com/ds), unless stated otherwise. Camalexin was synthesized as described previously (Ayer *et al.*, 1992), and

verified by UV/VIS, UV fluorescence and ^{1}H-NMR spectroscopy against a standard provided by Ray Hammerschmidt (Department of Plant Pathology, Michigan State University, East Lansing, MI).

Cultivation of plants

Plants were grown under a 12 h light per 12 h dark cycle, with a night temperature of 16–18°C and a day temperature of 20–22°C (60–70% humidity) as described previously (Schoonbeek *et al.*, 2007), but in groups of four in 9 × 9 cm pots. WT plants are the *A. thaliana* accession Col-0 obtained from the Arabidopsis Biological Research Centre (Columbus, OH). The mutant *eds5* was *eds5-3* (Nawrath *et al.*, 2002), *pad2* was *pad2-1* (Parisy *et al.*, 2007), and *pad3* was *pad3-1* (Zhou *et al.*, 1999). The mutant *cyp79A13* was *cyp79A13-2* (Nafisi *et al.*, 2007) and was provided by Jane Glazebrook (Department of Plant Biology, University of Minnesota, MN); *cyp79B2/B3* (Zhao *et al.*, 2002) and *ups1* (Denby *et al.*, 2005) were provided by Daniel Kliebenstein (Plant Sciences, University of California, Davis, CA).

B. cinerea culture, sensitivity testing and [^{14}C]-fludioxonil accumulation

B. cinerea was cultured as described previously (Schoonbeek *et al.*, 2007). The haploid WT B05.10 was provided by Jan van Kan (Laboratory of Phytopathology, Wageningen University, Wageningen, The Netherlands). The B05.10 derived gene-replacement mutants ΔBcatrAM7 in BcatrA (Del Sorbo *et al.*, 2008), ΔBcatrB4 and ΔBcatrB5 in BcatrB (Schoonbeek *et al.*, 2001), and ΔBcatrD8 in BcatrD (Hayashi *et al.*, 2002a) have been described previously. The double mutant ΔBcatrB4D5 was produced by replacement of *BcatrD* with a nourseothricin resistance cassette in ΔBcatrB4, as described by Hayashi *et al.* (2002b). The fludioxonil-resistant field isolate MDR5_16 was isolated from vineyards in Western Germany. The MDR5_16-derived mutants MDR_ΔBcatrB1 and MDR_ΔBcatrB4 were generated by replacement of *BcatrB* with the same hygromycin resistance cassette as used in B05.10 (M. Kretschmer *et al.*, unpublished results). The BcatrB promoter–GUS fusion strain BcatrBp803GUS-7 was obtained by transformation of strain B05.HYG-3 with pAtrB803, which is derived from pBS.HYG-5 and contains the 803 bp upstream of the BcatrB start codon fused in-frame to the *uidA* gene from *Escherichia coli* (M. Kretschmer *et al.*, unpublished results). The recipient strain B05.HYG-3 and the plasmid pBS.HYG-5 (Noda *et al.*, 2007) were kind gifts from Celedonio Gonzalez (Biochemistry and Molecular Biology, University of La Laguna, La Laguna, Spain).

The camalexin sensitivity of *B. cinerea* was determined by measuring the hyphal growth on potato dextrose agar to which increasing concentrations of camalexin in DMSO (0.1% final concentration) had been added (Schoonbeek *et al.*, 2001). The effective concentrations for inhibition of growth by 50% (EC_{50}) or 95% (EC_{95}) were calculated using probit analysis in Microsoft Excel (http://www.microsoft.com). The EC values presented are the means of at least three independent experiments.

Induction studies in B05.10 were performed as described previously (Schoonbeek *et al.*, 2003). Germlings that had been grown for 16 h in liquid medium were treated with 15 mg L^{-1} camalexin (or 0.1% DMSO in the solvent control), harvested on Miracloth (Calbiochem; http://www.merckbiosciences.co.uk) after 30 or 120 mins, frozen in liquid nitrogen, and stored at −20°C until RNA extraction.

For GUS activity determination, inoculated leaves were detached and vacuum-infiltrated for 15 mins in X-Gluc staining buffer [50 mM sodium phosphate buffer pH 7.0, 10 mM EDTA, 0.5 mM $K_3Fe(CN)_6$, 0.5 mM $K_4Fe(CN)_6$, 0.5% w/v Triton X-100 and 0.5 mg ml^{-1} X-Gluc cyclohexylammonium salt (X-gluc Direct, http://www.x-gluc.com)]. Leaves were incubated for 24 h at 37°C, destained in three changes of chloral hydrate, and examined under a Leica DMR microscope (Leica-Microsystems, http://www.leica-microsystems.com) using a Zeiss axiocam (Zeiss, http://www.zeiss.de).

Accumulation of [^{14}C]-fludioxonil was determined as described previously (Schoonbeek *et al.*, 2002). To test the effect of various compounds on BcatrB-mediated efflux of [^{14}C]-fludioxonil, they were added to the germlings 65 mins after initiation of the experiment. Camalexin and eugenol were added from 1000 × concentrated stocks in DMSO; the control treatment was 0.1% DMSO.

Inoculation and disease evaluation

Inoculation of *A. thaliana* with *B. cinerea* was performed as described previously (Schoonbeek *et al.*, 2007), with minor modifications. For disease assays, 5–6-week-old plants were pair-wise-inoculated with a suspension of 2.5×10^5 spores ml^{-1} in quarter-strength potato dextrose broth (PDB, 6 g L^{-1}). Droplets (2 μl) of spore suspensions of the two different isolates to be compared were deposited on six leaves per plant, with at least eight plants per experiment. Lesion diameters were measured at 3 dpi. For camalexin measurements or gene expression experiments, the spore suspension was sprayed on whole plants; control plants were sprayed with quarter-strength potato dextrose broth.

To test the effect of UV-C light on camalexin production and *B. cinerea* resistance, plants were exposed to UV-C light (peak emission 254 nm) for 20 mins, at a distance of 20 cm using a tl-900 8 W lamp (CAMAG, http://www.camag.com), 24 h prior to inoculation with *B. cinerea*.

For determination of spore germination and fungal growth *in planta*, leaves were detached from inoculated plants, directly stained with trypan blue (Zimmerli *et al.*, 2001) and observed microscopically.

Camalexin and SA determination

Leaf material (approximately 150 mg) was collected in 2 ml tubes and assayed for camalexin as previously described (Nawrath and Métraux, 1999). HPLC was performed on a reverse-phase HPLC column (ABZ+, 25 cm × 4.6 mm; Supelco, http://www.sigmaaldrich.com/supelco.html). The amount of camalexin was calculated in ng g^{-1} fresh weight, with reference to the amount of internal standard. All extractions were performed at least in duplicate within one experiment, values from all samples taken in the same experiment were averaged. The data presented are the means of three independent experiments.

RNA extraction and real-time RT-PCR

RNA was prepared from plant and fungal material using TRIzol® reagent (Molecular Research Center Inc.; http://www.invitrogen.com). RNA (1 μg) was reverse-transcribed to cDNA (Omniscript® RT kit, Qiagen; http://www.qiagen.com). Fungal cDNA was hybridized to macroarrays using standard protocols (Kretschmer *et al.* in preparation).

Quantitative RT-PCR was performed using Absolute QPCR SYBR Green Mix (Abgene Ltd, http://www.abgene.com) on a Rotor-Gene 2000 (Corbett Life Science, http://www.corbettlifescience.com). Gene expression values were normalized to expression of the

A. thaliana expressed gene AT4G26410 (Czechowski *et al.*, 2005) or the *B. cinerea* gene for the elongation factor *BcEF1b* (Gioti *et al.*, 2006), using the primers listed in Table S1.

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SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article:

Figure S1. Expression levels of *Botrytis cinerea* ABC and MFS transporters present on the macroarray after treatment of B05.10 germlings with camalexin.

Figure S2. Microscopic observations of disease symptoms of *Botrytis cinerea* on Col-0 and *pad3* after UV-C treatment.

Figure S3. Effect of UV-C treatment on expression of BcatrB *in planta*.

Figure S4. Sensitivity of additional *Botrytis cinerea* isolates to camalexin.

Table S1. Primers and accession numbers used for quantitative RT-PCR.

Table S2. Genes present on the macroarray.

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