An ATP-Binding Cassette Pleiotropic Drug Transporter Protein Is Required for Xenobiotic Tolerance and Antagonism in the Fungal Biocontrol Agent *Clonostachys rosea*

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ATP-binding cassette (ABC) transporters mediate active efflux of natural and synthetic toxicants and are considered to be important for drug tolerance in microorganisms. In biological control agents (BCA), ABC transporters can play important roles in antagonism by providing protection against toxins derived from the fungal prey and by mediating the secretion of endogenous toxins. In the present study, we generated deletion and complementation strains of the ABC transporter *abcG5* in the fungal BCA *Clonostachys rosea* to study its role in xenobiotic tolerance and antagonism. Gene expression analysis shows induced expression of *abcG5* in the presence of the *Fusarium* mycotoxin zearalenone (ZEA), secreted metabolites of *F. graminearum*, and different classes of fungicides. Phenotypic analysis of *abcG5* deletion and complementation strains showed that the deletion strains were more sensitive towards *F. graminearum* culture filtrates, ZEA, and iprodione- and mefenoxam-based fungicides, thus suggesting the involvement of *abcG5* in cell protection. The $\Delta abcG5$ strains displayed reduced antagonism towards *F. graminearum* in a plate confrontation assay. Furthermore, the $\Delta abcG5$ strains failed to protect barley seedlings from *F. graminearum* foot rot disease. These data show that the *abcG5* ABC transporter is important for xenobiotic tolerance and biocontrol traits in *C. rosea*.

Filamentous fungi have evolved a diverse arsenal of mechanisms to cope with endogenous or exogenous toxic compounds, and one such mechanism is the removal of these compounds by active efflux mediated by ATP-binding cassette (ABC) transporter proteins (Del Sorbo et al. 2000; de Waard et al. 2006). ABC transporters are present in both prokaryotes and eukaryotes and utilize energy derived from ATP hydrolysis to shuttle metabolites and toxicants across cellular membranes (Del Sorbo et al. 2000; Sharom 2008). The function of ABC transporters depend on four conserved core domains: two cytoplasmic nucleotide binding domains (NBD) and two membrane-associated

transmembrane domains (TMD). The exception to this rule is the half-size transporters that possess only one NBD and one TMD domain (Biemans-Oldehinkel et al. 2006; Coleman and Mylonakis 2009; Lamping et al. 2010). A single NBD generally contains seven conserved functional motifs that are referred to as the Walker A motif or P-loop, Walker B motif, ABC signature motif or C-loop, Q-loop, pro-loop, H-loop, and D-loop. The TMD (TMD1 and TMD2) consists of 12 transmembrane segments (TMS1 to TMS12) in total, with six transmembrane segments in each TMD (Lamping et al. 2010). On the basis of domain topology and phylogeny, the fungal ABC transporter superfamily is divided into 10 families: ABC-A, ABC-B, ABC-C, ABC-D, ABC-E, ABC-F, ABC-G, ABC-H, Caf16p-like, and YDR061W-like (Kovalchuk and Driessen 2010). Three families (ABC-B, ABC-C, and ABC-G) are shown to be involved in efflux of toxic compounds and are referred to as multidrug resistance protein (MDR), multidrug resistance-associated protein (MRP), and the pleiotropic drug resistance protein (PDR) families, respectively (Coleman and Mylonakis 2009; Kovalchuk and Driessen 2010).

Functional characterizations of ABC transporters in filamentous fungi have shown involvement in cellular detoxification, xenobiotics and heavy metal tolerance, and in pathogenesis (Del Sorbo et al. 2000; de Waard et al. 2006; Coleman and Mylonakis 2009; Lamping et al. 2010; Paul et al. 2013). Similarly, in plant-pathogenic fungi, ABC transporters mediate resistance to fungicides and antimicrobial compounds produced by plants and are involved in virulence (de Waard et al. 2006; Coleman and Mylonakis 2009). Functional analysis of ABC transporters in the rice blast fungus *Magnaporthe oryzae* demonstrate the role of the ABC1, ABC3, and ABC4 proteins in tolerance to xenobiotics, cytotoxicity, and oxidative stress and during pathogenesis (Gupta and Chattoo 2008; Sun et al. 2006; Urban et al. 1999). Furthermore, mutants deficient in the ABC transporter gene *GpABC1* in the potato tuber pathogen *Fusarium sambucinum*, *Mgatr4* in the wheat leaf blotch pathogen *Mycosphaerella graminicola*, and *Bcatrb*, *BcatrD*, and *BcatrA* in the necrotrophic fungus *Botrytis cinerea* displayed reduced tolerance to phytoalexins and fungicides and reduced virulence (de Waard et al. 2006; Coleman and Mylonakis 2009; Del Sorbo et al. 2000; Schouten et al. 2008).

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The fungus *Clonostachys rosea* is a ubiquitous soilborne ascomycete known for its mycoparasitic, entomopathogenic, and nematophagous abilities (Li et al. 2004; Luongo et al. 2005; Rodriguez et al. 2011; Toledo et al. 2006; Zhang et al. 2008). The *C. rosea* IK726 strain, isolated from barley roots infected with *F. culmorum* in Denmark, has proven to be highly efficient as a biocontrol agent (BCA) against fungal pathogens of agricultural and horticultural crops (Jensen et al. 2004, 2007; Knudsen et al. 1995). In order to perform efficiently, BCA fungi must tolerate the cytotoxic and antifungal compounds produced not only by the fungal prey during direct antagonistic interactions but, also, during plant root colonization. However, limited information is available concerning the role of ABC transporters in BCA fungi during their interac-

tions with the fungal prey, other competing microorganisms, and host plants. Identification and functional characterization of the PDR transporter Taabc2 in the mycoparasitic fungus *Trichoderma atroviride* shows that this PDR transporter is involved in tolerance to fungicides, pathogen toxins, and in antagonism (Ruocco et al. 2009). Recently, deletion of genes encoding ABC transporters in the entomopathogenic fungus *Beauveria bassiana* reveals the role of ABC transporters in oxidative stress tolerance and virulence (Song et al. 2013).

In a recent study, we showed that several ABC transporter genes that belong to the ABC-B, ABC-C, and ABC-G families are induced in *C. rosea* during exposure to the mycotoxins deoxynivalenol and zearalenone (ZEA) or the fungicide boscalid (Kosawang 2013; Kosawang et al. 2014). In this study, we characterize the biological function of the PDR transporter *abcG5* in *C. rosea*. By analyzing the expression patterns and generating deletion and complementation strains of *abcG5*, we demonstrate the role of *abcG5* in tolerance to secreted metabolites of *F. graminearum*, the mycotoxin ZEA, and the chemical fungicides Chipco Green and Apron. In addition, our in vitro and in vivo results show that *abcG5* is necessary for the antagonistic and biocontrol activity of *C. rosea* against the fungal pathogen *F. graminearum*.

RESULTS

Sequence analysis of *abcG5*.

The full-length sequence of *abcG5* was identified previously (Kosawang et al. 2014). The *abcG5* open reading frame was predicted to encode a polypeptide composed of 1,436 amino acid (aa) residues with a predicted molecular mass of 161 kDa and an estimated isoelectric point at pH 6.89. The conserved domain analysis of the translated aa sequence using the simple modular architecture research tool (SMART) predicted that the *abcG5* protein was composed of two homologous halves, each with an NBD (aa positions 128 to 338 and 823 to 1,014) that precedes a TMD (aa positions 464 to 738 and 1,015 to 1,436) with six TMS each. This structure was further verified by conserved domain search. No N-terminal secretion signal peptide was found from analyses using SignalP. The *abcG5* protein showed highest similarity (74%) with the functionally characterized ABC-G family transporter nhABC1 (GenBank accession number XP_003048421.1) from *F. solani* and Gpabc1 (CAC40023.1) of *F. sambucinum*, followed by 64% similarity with ifT1 (AET72446) from the entomopathogenic fungus *Isaria fumosorosea* and ABC1 (AAB86640) from *Magnaporthe oryzae*. A Clustal W alignment of the *C. rosea* *abcG5* sequence with characterized ABC transporters from filamentous fungi (*Botrytis cinerea*, CAB52402.1; *Aspergillus nidulans*, CAA 93141.1; *Mycosphaerella graminicola*, XP_003852491.1; *Candida albicans*, CAA54692.1; *F. sambucinum*, CAC40023.1) identified the presence of conserved ABC signature (RGVSG GERKRV) and Walker B1 (CWDNSTRGLD) motif but a degenerate Walker A1 (GP/RPGA/SGCT/S) motif in the N-terminal NBD (aa position 128 to 338). The C-terminal NBD consisted of conserved ABC signature (GLNVEQRKRLTIG VEL), Walker B2 (DEPTSGLD), and Walker A2 (GVSGAGK TTLL) motifs (aa position 823 to 1,014).

Expression analysis of *abcG5*.

Real time reverse transcription quantitative polymerase chain reaction (RT-qPCR) on reverse transcribed mRNA showed no difference in *abcG5* expression between *C. rosea* self-interaction (Cr-Cr) and during *C. rosea* interaction with the fungal prey *Botrytis cinerea* (Cr-Bc) or *F. graminearum* (Cr-Fg) (Fig. 1A). In order to monitor the response to endogenous and exogenous secreted metabolites, *C. rosea* was inocu-

Fig. 1. Expression analyses of *abcG5* in *Clonostachys rosea*. **A**, During antagonistic interaction and **B**, in self or *F. graminearum* culture filtrates, mycelia grown in liquid Czapek-Dox (CZ) was used as control. **C**, In liquid CZ medium amended with zearalenone (ZEA), CZ medium amended with methanol was used as control. **D**, In liquid CZ medium amended with fungicides, CZ medium amended with water was used as control. For gene expression analysis during antagonistic interaction, mycelia were harvested at contact stage. For analysis in submerged shake flask cultures, total RNA was extracted from mycelia 2 h posttreatment in culture filtrates, ZEA, or fungicides. Relative expression levels for *abcG5* in relation to β -tubulin were calculated from the cycle threshold values and the primer amplification efficiencies, using the formula described by Pfaffl (Pfaffl 2001). Error bars represent standard deviation based on five biological replicates. Different letters indicate statistically significant differences ($P \leq 0.05$) within experiments based on the Tukey-Kramer test.

lated in culture filtrates from itself (*C. rosea*), or from *F. graminearum*, or in fresh media. Expression of *abcG5* was significantly ($P \leq 0.011$) upregulated in *C. rosea* mycelium grown both in self-culture filtrates and *F. graminearum*-culture filtrates compared with the control treatment. Interestingly, a significant ($P < 0.001$) induction of *abcG5* expression was detected in mycelium grown in self culture filtrates in relation to *F. graminearum* culture filtrates (Fig. 1B). Furthermore, expressions of *abcG5* were strongly induced ($P < 0.001$) when *C. rosea* was exposed to ZEA or the fungicides Amistar, Apron, Cantus, and Chipco Green at the tested concentrations (Fig. 1C and D).

Generation of *abcG5* deletion and complementation strains.

C. rosea abcG5 deletion mutants were generated by replacing *abcG5* with the hygromycin (*hygB*) selection cassette by *Agrobacterium tumefaciens*-mediated transformation (ATMT). Successful gene replacement in mitotically stable mutants was confirmed by PCR, using primers located within the *hygB* cassette together with primers located upstream and downstream of the construct (Supplementary Fig. S1A), as described in our previous work (Dubey et al. 2012, 2013a and b). PCR fragments of the expected size were amplified in all selected $\Delta abcG5$ strains, while no amplification was observed in wild type (WT). Furthermore, RT-PCR experiments using primers specific to the *abcG5* sequence demonstrated the complete loss of *abcG5* transcript in each mutant.

A $\Delta abcG5$ strain was complemented with the WT *abcG5* gene through ATMT. Successful integration of the nourseothricin (*nat1*) selection cassette in mitotically stable mutants was confirmed by PCR amplification of *nat1* (data not shown). RT-PCR from randomly selected *nat1*-positive *abcG5* strains, using *abcG5*-specific primer pairs, demonstrated restored *abcG5* transcription, while no transcripts were detected in the parental deletion strains. Three independent *C. rosea abcG5* mutant strains $\Delta abcG5A$, $\Delta abcG5B$, and $\Delta abcG5C$, along with the WT and the complemented $\Delta abcG5+$ strain were used in all phenotype analysis.

Deletion of *abcG5* results in decreased tolerance to xenobiotics.

No differences in growth rate were detected between deletion and WT strains on Czapek-Dox (CZ) medium. However, all deletion strains showed a significant ($P < 0.001$) reduction in growth rate on CZ medium amended with ZEA, Apron, or Chipco Green but not on CZ medium amended with Amistar or Cantus. The growth of deletion strains was completely abolished

by Chipco Green, whereas 45 and 33% reductions in growth rate were measured on Apron and ZEA medium, respectively (Fig. 2). In addition, we observed that colonies of the deletion strains had fluffier morphology on CZ medium, although the complemented strain $\Delta abcG5+$ did not show restoration in altered colony morphology (Supplementary Fig. S2).

Deletion of *abcG5* reduces antagonism towards *F. graminearum*.

In order to investigate the role of *abcG5* during antagonism, an in vitro plate confrontation assay was used in which mutants or WT strains and *F. graminearum* were confronted with each other. After 4 days postinoculation, *F. graminearum* exhibited a significant ($P < 0.001$) 28% increase in growth rate during confrontation with $\Delta abcG5$ strains in comparison with *C. rosea* WT strain (Fig. 3A and B). However, no differences in growth rate between *C. rosea* WT and deletion strains were measured during the same condition. On the basis of this result, we hypothesized that reduced antagonistic ability of the $\Delta abcG5$ strains towards *F. graminearum* was related to reduced efflux levels of toxic metabolites by the *C. rosea* deletion strains during the plate confrontation test. The hypothesis was tested by measuring the mycelial biomass production of *F. graminearum* in heat-treated (to inactivate secreted enzymes) culture filtrates of *C. rosea* WT, deletion, and complementation strains. Similarly to the plate confrontation assay, *F. graminearum* had significantly ($P \leq 0.016$) increased (15 to 22.5%) biomass in culture filtrates from $\Delta abcG5$ strains in comparison with culture filtrate from the WT strain (Fig. 3C). Furthermore, *C. rosea* strains and *F. graminearum* were allowed to interact with each other for 10 days in dual culture plate confrontation tests. The interaction between WT and *F. graminearum* resulted in an apparent inhibition zone, whereas no such zone appeared during interaction between deletion strains and *F. graminearum* (Fig. 3D). In another set of experiments, mycelial biomass production of *C. rosea* strains was measured in the culture filtrate of *F. graminearum* in order to test the tolerance of $\Delta abcG5$ and WT strains to the secreted metabolites from *F. graminearum*. In comparison with the WT, deletion strains had significantly ($P < 0.001$) less (42%) biomass in *F. graminearum* culture filtrates (Fig. 3E).

Deletion of *abcG5* reduces biocontrol efficiency against *F. graminearum*.

The biocontrol effect of WT and mutants strains was measured for *F. graminearum* foot rot disease on barley plants using an in vivo sand seedling test. Our results showed a significant ($P \leq 0.036$) 20% increase of disease severity in barley

Fig. 2. Growth rate of *Clonostachys rosea* wild type (WT), deletion, and complemented strains on Czapek-Dox (CZ) medium and CZ medium amended with toxin and fungicides. Strains were inoculated on solid agar medium and were incubated at 25°C, and the growth rate was recorded 3 days postinoculation. The experiments were carried out in three biological replicates. Different letters indicate statistically significant differences ($P \leq 0.05$) within experiments based on the Tukey-Kramer test.

seedlings previously seed coated with $\Delta abcG5$ strains as compared with seedlings from seeds coated with WT *C. rosea*. Disease symptoms on seedlings from seeds coated with $\Delta abcG5$ strains were, in turn, significantly milder compared with the symptoms on seedlings from control seeds (*F. graminearum* inoculation without any *C. rosea* strain) (Fig. 4).

DISCUSSION

Recently, *abcG5* was identified in *C. rosea*, through a suppression subtractive hybridization based transcriptome approach (Kosawang et al. 2014), as being induced by the *Fusarium* spp. mycotoxin ZEA. In this study, we characterize the physiological functions of *abcG5* by generating deletion mutants in *C. rosea*. The (NBD-TMS₆)₂ domain topology of *abcG5* is typical for the PDR ABC-G family, which is in line with results from a phylogenetic analysis that placed *abcG5* in the ABC-G family (Kosawang et al. 2014). Furthermore, *abcG5* show high aa sequence similarity with characterized ABC-G family transporters, including *Gpabc1* from *F. sambucinum*, *nhABC1* from *F. solani*, *ift1* from *Isaria fumosorosea*, and *ABC1* from *Magnaporthe oryzae*. Based on this, it is likely that *abcG5* encodes a G family PDR ABC transporter in *C. rosea*.

The mentioned ABC transporters with high similarity to *abcG5* are all involved in tolerance toward various toxic substances. *Gpabc1* is required for *F. sambucinum* tolerance to the potato phytoalexin rishitin and, consequently, for virulence on potato tuber (Fleissner et al. 2002). *nhABC1*, the ortholog to *Gpabc1* in *F. solani*, is involved in tolerance to rishitin and the pea phytoalexin pisatin and acts as a virulence factor on garden pea (Coleman et al. 2011). Furthermore, overexpression of *ift1* in *Isaria fumosorosea* results in increased tolerance to different types of chemical fungicides, including carbendazim, iprodione, and azoxystrobin (Song et al. 2012). Deletion of *ABC1* results in reduced pathogenicity of *Magnaporthe oryzae* on rice, although unlike the *ift1* deletion mutant in *Isaria fumosorosea*, the *Magnaporthe oryzae* $\Delta ABC1$ strain does not display reduced drug tolerance (Urban et al. 1999). Based on the high sequence similarity of *abcG5* with the mentioned ABC transporters, we hypothesize that *abcG5* may also be involved in drug tolerance in *C. rosea*.

Mycoparasitic *Clonostachys* spp. and *Trichoderma* spp. must be able to withstand toxic metabolites produced both by the mycoparasite itself but also by the fungal prey (Druzhinina

Fig. 3. Antagonism test against *Fusarium graminearum*. **A** and **D**, Plate confrontation assay against *F. graminearum*. Agar plugs of *Clonostachys rosea* strains (left side in the plate) and *F. graminearum* (right side in the plate) were inoculated on opposite sides in 9-cm-diameter agar plates and were incubated at 25°C. The experiment was performed in three replicates and photographs of representative plates were taken 4 and 10 days postinoculation, respectively. An arrowhead indicates the inhibition zone. **B**, Growth rate of *F. graminearum* in plate confrontation test against *C. rosea* strains. The experiments were carried out in three biological replicates and the growth rate was recorded 4 days postinoculation. **C**, Secretion assay of *C. rosea* strains. Fungal strains were grown in Czapek-Dox (CZ) broth for 10 days at 25°C. *C. rosea* culture filtrates were collected after removing mycelia mass, were heated at 80°C for 60 min to inactivate proteins, and were inoculated with a *F. graminearum* agar plug. Biomass production in culture filtrates was analyzed by determining mycelial dry weight 3 days postinoculation. **E**, Tolerance of *C. rosea* strains to secreted metabolites of *F. graminearum*. *F. graminearum* was grown in potato dextrose broth for 10 days at 25°C. Culture filtrates were collected after removing mycelial mass and were inoculated with *C. rosea* strains. Biomass production in culture filtrates was analyzed in triplicates by determining mycelial dry weight 3 days postinoculation. Experiments were performed in three biological replicates. Different letters indicate statistically significant differences ($P \leq 0.05$) within experiments based on the Tukey-Kramer test.

et al. 2011). For example, *C. rosea* produces the ZEA lactonohydrolase zhd101 that specifically detoxifies the antifungal *Fusarium* spp. mycotoxin ZEA (Takashi-Ando et al. 2002). Furthermore, both *C. rosea* and many *Trichoderma* spp. have the ability to live in soils as saprophytes and as endophytes in plants (Chatterton et al. 2008; Druzhinina et al. 2011; Lübeck et al. 2002). This may also select for mechanisms to withstand toxic metabolites produced by plants and competing soil microbes. Active efflux, driven by ATP hydrolysis, of toxic metabolites by ABC transporters with broad substrate specificity is considered to be an important aspect of the initial response of microorganisms during exposure to xenobiotics (Kretschmer et al. 2009). Consequently, broad-spectrum ABC transporters evolved to transport naturally occurring xenobiotics may also provide a certain level of tolerance towards man-made chemicals, such as agricultural pesticides.

C. rosea is able to tolerate relatively high concentrations of various chemical fungicides and pesticides in comparison with recommended commercial doses for controlling plant-pathogenic fungi (Jensen et al. 2011; Macedo et al. 2012; Roberti et al. 2006; Tzelepis and Lagopodi 2011). We hypothesize that ABC transporters may be one important factor that contributes to this ability, as the draft genome of *C. rosea* IK726 contains 85 different ABC transporter genes (Kosawang 2013). This number is substantially higher than in other filamentous fungi sequenced to date, including mycoparasitic *Trichoderma* spp. (48 to 62 genes). The numbers of MDR ABC-B, MRP ABC-C, and PDR ABC-G genes are particularly high (21, 19, and 28 genes, respectively), which suggests that ABC transporters mediate tolerance of *C. rosea* to endogenous and exogenous toxic metabolites.

Induced expression of *abcG5* by ZEA and a diverse group of fungicides, including Amistar, Apron, Cantus, and Chipco Green, indicate a role for *abcG5* in efflux of xenobiotics in *C. rosea*. This is verified by the reduced growth rate of $\Delta abcG5$ strains on media that contain ZEA, Apron, or Chipco Green. Furthermore, reduction in biomass of $\Delta abcG5$ strains in *F. graminearum* culture filtrates suggests that *abcG5* is not only involved in efflux of fungicides and ZEA but also in efflux of other secondary metabolites secreted by *F. graminearum*. However, reduction in biomass of $\Delta abcG5$ strains in culture filtrates might also be associated with presence of ZEA in secreted metabolites by *F. graminearum*. These data suggest that *abcG5* has low substrate specificity and is involved in protecting *C. rosea* from xenobiotics. Similar results are reported previously in the mycoparasitic fungus *T. atroviride* and the entomopathogenic fungus *Beauveria bassiana* (Ruocco et al. 2009; Song et al. 2013). In *T. atroviride*, the PDR ABC-G transporter *Taabc2* is found to be upregulated by mycotoxins, fungicides, and pathogen-related metabolites, and tolerance of the *Taabc2* deletion mutants to the fungicide Diclorum and metabolites produced by different phytopathogenic fungi is severely compromised (Ruocco et al. 2009).

The contradiction between induced expression of *abcG5* by Amistar and Cantus and the lack of observable growth reduction between $\Delta abcG5$ and WT strains on Amistar- and Cantus-containing media may be explained by an overlap in substrate specificity with other *C. rosea* ABC transporters that may transport azoxystrobin (active ingredient of Amistar) and boscalid (active ingredient of Cantus). Alternatively, *C. rosea* may compensate for the *abcG5* deletion by other mechanisms, such as increasing the number of mitochondria, as both azoxystrobin and boscalid target proteins involved in respiration while iprodione (active ingredient of Chipco Green) and mefenoxam (active ingredient of Apron) target lipid biosynthesis and RNA synthesis, respectively. This result is in line with data from *Beauveria bassiana* in which induced expression of

Pdr1 and *Pdr3* by fungicides azoxystrobin, carbendazim, and phosphinothricin was found, while deletion mutants of corresponding genes had significantly reduced growth rates on media amended with carbendazim but not with azoxystrobin (Song et al. 2013).

Our data also suggest that *abcG5* may function in secretion of endogenous metabolites that inhibit the growth of *F. graminearum* but not *C. rosea* itself. This is evident as *F. graminearum* grows faster during plate confrontations with the $\Delta abcG5$ strains compared to confrontations with the WT. At the same time, the *C. rosea* WT and *abcG5* deletion strains have the same growth rate on both solid CZ medium and during plate confrontations. The increased biomass production of *F. graminearum* in heat-treated culture filtrates from the $\Delta abcG5$ strains compared with the WT emphasize that secreted metabolites rather than secreted enzymes is responsible for this effect. Some peptaibol compounds with antifungal activity are identified in *C. rosea* (Rodriguez et al. 2011), although it is not known if they are heat stable. However, other peptaibols of fungal origin have been shown to be heat stable (Andersson et al. 2009). Induced expression of *T. atroviride Taabc2* under carbon starvation conditions that trigger secondary metabolite production suggest that this ABC transporter can also function in secretion of endogenous metabolites. This is validated by the complete loss of ability of the *Taabc2* deletion mutant to secrete the antifungal volatile compound 6-pentyl- α -pyrone produced by *T. atroviride* during mycoparasitic interactions (Ruocco et al. 2009). Similar results are also available for the rice blast fungus *Magnaporthe oryzae* in which inactivation of the MDR ABC-B family gene *ABC3* causes accumulation of endogenous toxic compounds in appressoria that results in a reduction in barley leaf penetration but in no effect on viability of the fungal mycelia (Sun et al. 2006).

We show that deletion of *abcG5* results in impaired ability to antagonize *F. graminearum*, both in dual plate confrontations and during in vivo interactions on barley seeds and roots. The reduced biocontrol ability may be due to a reduced ability to efflux exogenous toxins produced by *F. graminearum*, a reduced ability to secrete endogenous metabolites, or a combination of both. Reduction in virulence and, consequently, in biocontrol effect as a result from deletion of ABC transporter genes are reported in biocontrol fungi *T. atroviride* and *Beauveria bassiana* (Ruocco et al. 2009; Song et al. 2013). In *T.*

Fig. 4. In vivo bioassay to test the biocontrol ability of *Clonostachys rosea* strains against *Fusarium graminearum* foot rot disease on barley, using a sand seedling test. Barley seeds were coated with *C. rosea* conidia and were planted in moist sand together with a *F. graminearum* agar plug. Seedlings were harvested 2 weeks postinoculation, and disease symptoms were scored on a 0 to 4 scale. The experiment was performed in four biological replicates with 10 to 12 plants in each replicate. Different letters indicate statistically significant differences ($P \leq 0.05$) within experiments based on the Tukey-Kramer test.

atroviride, deletion of *Taabc2* results in reduced antagonistic ability towards *Botrytis cinerea*, *Rhizoctonia solani*, and *Pythium ultimum* (Ruocco et al. 2009).

It should be noted that the *C. rosea* $\Delta abcG5$ strains retain a certain ability to protect barley plants against *F. graminearum* foot rot disease in the current setup, which indicate that additional mechanisms are important for the biocontrol ability of *C. rosea*. This may include secretion of cell wall-degrading enzymes (Mamarabadi et al. 2008) or toxic peptaibols (Rodriguez et al. 2011). In summary, the data presented in this work demonstrate that *abcG5* is a PDR ABC-G transporter that is necessary for xenobiotic tolerance and successful antagonism in *C. rosea*. It is the first time that an ABC transporter is shown to be involved in biocontrol in *C. rosea*.

MATERIALS AND METHODS

Fungal strains and culture conditions.

C. rosea IK726 (WT) and mutants derived from it and *F. graminearum* 1104-14 were maintained on potato dextrose agar (PDA) medium (Oxoid, Cambridge, U.K.) at 25°C. CZ medium (Sigma-Aldrich, St. Louis) was used to grow fungi for gene expression and phenotypic analysis unless otherwise specified.

Sequence analysis.

Analyses for conserved domains were performed using the SMART protein analysis tool (Letunic et al. 2009) and conserved domain search (Marchler-Bauer et al. 2011). Signal P version 4.0 was used to search for signal peptide cleavage sites (Petersen et al. 2011). Amino acid sequence alignment was performed using ClustalW2 (Larkin et al. 2007). Analysis for conserved motifs was done manually.

Gene expression analysis.

Gene expression analysis of *abcG5* was carried out in *C. rosea* under four different conditions: i) during dual culture interaction with *F. graminearum*, ii) in culture filtrates of *C. rosea* and *F. graminearum*, iii) in liquid CZ medium amended with *Fusarium* mycotoxin ZEA, and iv) in liquid CZ medium amended with fungicides Cantus WDG (active ingredient boscalid, anilid group of fungicides; BASF Canada Inc., Mississauga, Canada), Amistar (active ingredient azoxystrobin, methoxyacrylates [strobilurin] group of fungicides; Syngenta, Basel, Switzerland), Chipco Green 75WG (active ingredient iprodione, dicarboximide group of fungicides; Bayer, Leverkusen, Germany), and Apron XL (active ingredient mefenoxam, acylalanins groups of fungicides; Syngenta). ZEA was dissolved in methanol, while fungicides were dissolved in sterile distilled water immediately before use.

For gene-expression analysis during dual culture interactions, the growing front (7 to 10 mm) of *C. rosea* mycelium was harvested at contact. *C. rosea* confronted with itself was used as control. For gene expression analysis in culture filtrates, an agar plug of *C. rosea* was precultivated for 5 days in 100-ml flasks containing 20 ml of liquid CZ medium on a rotary shaker (200 rpm [Infors HT Labotron, Rittergasse, Switzerland]) at 25°C, followed by harvesting by filtration through Miracloth, washing with sterile distilled water, and transfer to new flasks containing 20 ml of culture filtrates of *C. rosea* or *F. graminearum*. Mycelia transferred in fresh CZ medium were used as control treatment. Culture filtrates were obtained by growing *C. rosea* or *F. graminearum* in 200 ml of liquid CZ medium in 500-ml flasks as described above. At 10 days postinoculation, biomass was removed by filtering the culture through four layers of Miracloth followed by filter sterilization. For gene-expression analysis in medium containing ZEA

or fungicides, culture of *C. rosea* grown for 5 days in 20 ml of liquid CZ medium was transferred to CZ media amended with ZEA (10 µg/ml), Apron (mefenoxam, 2 µg/ml), Amistar (azoxystrobin, 7.5 µg/ml), Chipco Green (iprodione, 250 µg/ml), or Cantus (boscalid, 2,000 µg/ml). In control treatment, ZEA was replaced with an equal volume of methanol and fungicide was replaced by sterile distilled water. After 2 h, fungal mycelia were collected by vacuum filtration, were washed several times in distilled water to remove traces of toxin or fungicides, were frozen in liquid nitrogen, and were stored at -80°C.

RNA extraction was done using the Qiagen RNeasy kit following the manufacturer's protocol (Qiagen, Hilden, Germany). After DNaseI (Fermentas, St. Leon-Rot, Germany) treatment, 1 µg of total RNA was reverse transcribed in a total volume of 20 µl, using the Maxima first-stand cDNA synthesis kit (Fermentas). Transcript levels were quantified by RT-qPCR, using the SYBR green PCR master mix (Fermentas) and primer pair *abcG5* F and *abcG5* R (Supplementary Table S1) in an iQ5 qPCR system (Bio-Rad, Hercules, CA, U.S.A.), as described previously (Tzelepis et al. 2012). Melt curve analysis was performed after the qPCR reactions, to confirm that the signal was the result from a single product amplification. Relative expression levels for target genes in relation to β -tubulin, shown previously to be constitutively expressed (Mamarabadi et al. 2008), were calculated from the cycle threshold values and the primer amplification efficiencies by using the formula described by Pfaffl (2001). Gene expression analysis was carried out in five biological replicates, each based on two technical replicates.

Construction of disruption and complementation vectors.

Genomic DNA was isolated using a hexadecyltrimethylammonium bromide-based method (Nygren et al. 2008). Phusion DNA polymerase (Finnzymes, Vantaa, Finland) was used for PCR amplification of approximately 1-kb 5' flank and 3' flank regions of the *abcG5* gene from genomic DNA of *C. rosea*, using primer pairs ABC-ups F and ABC-ups R and ABC-ds F and ABC-ds R, respectively. Gateway entry clones of the purified 5' flank and 3' flank PCR fragments were generated as described by the manufacturer (Invitrogen, Carlsbad, CA, U.S.A.). The gateway entry clones for *hygB* (*hph*) and *nat1* resistance genes constructed during our previous studies were used (Dubey et al. 2012, 2013a and b). The Gateway LR recombination reaction was performed, using entry plasmid of respective fragments and destination vector pPm43GW (Karimi et al. 2005) to generate the disruption vectors, following the conditions described by the manufacturer (Invitrogen).

An *abcG5* complementation cassette was constructed by PCR amplification of the full-length sequence of *abcG5*, including 1-kb upstream and downstream regions from genomic DNA of *C. rosea* WT, using ABC comp F and ABC comp R. The amplified DNA fragments were purified and integrated into destination vector pPm43GW as described above, using Gateway cloning technology to generate complementation vectors.

ATMT and validation of transformants.

The disruption and complementation vectors were transformed into *Agrobacterium tumefaciens* AGL-1 as described before (Dubey et al. 2012). ATMT was performed based on a previous protocol for *C. rosea* (Utermark and Karlovsky 2008). Transformed strains were selected on plates containing *hygB* in case of deletion or *hygB* and *nat1* in the case of complementation. Putative transformants were repeatedly subcultured on PDA plates without the selectable agent five times, followed by re-exposure to selection antibiotics, in order to test for mitotic stability. Mitotically stable colonies were purified by two rounds of single-spore isolation.

A PCR screening approach of putative transformants was performed to validate the homologous integration of the disruption cassette. The primers used were specific to the *hph* gene (P3/P4), sequences flanking the deletion construct (ABC-ko F and ABC-ko R) and in combination (ABC-ko F and Hyg R, ABC-ko R and Hyg F). RT-PCR analysis was conducted on WT, deletion, and complemented strains using RevertAid premium reverse transcriptase (Fermentas) and primer pairs specific for *hph* (HygF_qPCR and HygR_qPCR), *nat1* (NatF_qPCR and NatR_qPCR), and *abcG5* (abcG5 F and abcG5 R).

Phenotypic analysis.

For growth-rate analysis, a 3-mm agar plug from the mycelial front was transferred to solid CZ medium or CZ medium containing ZEA (50 µg/ml, dissolved in ethanol) or fungicides with the same concentration of respective active ingredients used for gene expression analysis. Colony diameter was measured after 3 days of growth at 25°C. Antagonistic behavior against *F. graminearum* was tested using an in vitro plate confrontation assay. An agar plug of *C. rosea* strains were inoculated at the edge of a 9-cm CZ agar plate. After 3 days of incubation at 25°C, a plug of *F. graminearum* was placed at equal distance to the opposite edge of the plate. *F. graminearum* growth rate was measured after 4 days of interaction, and photographs of representative plates were taken 10 days postinoculation. For secretion assays, *C. rosea* or *F. graminearum* were grown for 10 days in liquid CZ or potato dextrose broth on a rotary shaker (200 rpm) at 25°C, and culture filtrates were obtained as described in the above. Heat inactivation of culture filtrates was performed for 60 min at 80°C (Bae et al. 2011). Heated culture filtrates were cooled to room temperature before inoculation of *F. graminearum*. Agar plugs of *F. graminearum* or *C. rosea* strains were inoculated in 50-ml conical flasks with 20-ml culture filtrates and were incubated at 25°C with constant shaking (100 rpm). Biomass production in culture filtrates was analyzed by determining mycelial dry weight 3 days postinoculation. The experiments were performed in three biological replicates unless otherwise specified. The complementation strain $\Delta abcG5+$ was included in all phenotype analyses to exclude the possibility that phenotypes derive from ectopic insertions.

Bioassay with *F. graminearum*.

An in vivo bioassay using a sand seedling test for Fusarium foot rot disease was performed in four biological replicates, and each replicate had 10 to 12 plants for each treatment, following the procedure described previously (Knudsen et al. 1995). In brief, surface-sterilized barley seeds were air dried in a laminar air-flow bench, followed by treatment with *C. rosea* conidia (1×10^6 conidia/ml) in sterile water, for 30 min on a rotary shaker (100 rpm). Water-soaked seeds were used as control. The seeds were sown in a 1- to 2-cm hole, 1 seed in each hole, in moistened sand in individual plastic pots ($5 \times 5 \times 5$ cm). Pathogen inoculation was carried out by placing a 5-mm PDA plug containing *F. graminearum* mycelia close to the seed (Roberti et al. 2008). PDA plugs without *F. graminearum* were used as control. After inoculations, the holes were filled with the moist sand. Pots were kept in a growth chamber with a photoperiod of 12 h of light, with $150 \mu\text{mol m}^{-2} \text{s}^{-1}$ light intensity, and 12 h of dark, $70\% \pm 5\%$ relative humidity, and $15 \pm 1^\circ\text{C}$ temperature. Seedlings were harvested 2 weeks post-inoculation, and disease symptoms were scored on a 0 to 4 scale described previously (Knudsen et al. 1995): 0 = healthy plants with no symptoms, 1 = slightly brown roots and coleoptiles, 2 = moderately brown roots and coleoptiles, 3 = severely brown roots and coleoptiles, and 4 = dead plants. The experiment was repeated two times.

Statistical analysis.

Analysis of variance was performed on gene-expression and phenotype data using a general linear model approach implemented in Statistica version 10 (StatSoft, Tulsa, OK, U.S.A.). Pairwise comparisons were made using the Tukey-Kramer method at the 95% significance level.

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