Identification of a New Biocontrol Gene in *Trichoderma atroviride*: The Role of an ABC Transporter Membrane Pump in the Interaction with Different Plant-Pathogenic Fungi

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Successful biocontrol interactions often require that the beneficial microbes involved are resistant or tolerant to a variety of toxicants, including antibiotics produced by themselves or phytopathogens, plant antimicrobial compounds, and synthetic chemicals or contaminants. The ability of *Trichoderma* spp., the most widely applied biocontrol fungi, to withstand different chemical stresses, including those associated with mycoparasitism, is well known. In this work, we identified an ATP-binding cassette transporter cell membrane pump as an important component of the above indicated resistance mechanisms that appears to be supported by an extensive and powerful cell detoxification system. The encoding gene, named *Taabc2*, was cloned from a strain of *Trichoderma atroviride* and characterized. Its expression was found to be upregulated in the presence of pathogen-secreted metabolites, specific mycotoxins and some fungicides, and in conditions that stimulate the production in *Trichoderma* spp. of antagonism-related factors (toxins and enzymes). The key role of this gene in antagonism and biocontrol was demonstrated by the characterization of the obtained deletion mutants. They suffered an increased susceptibility to inhibitory compounds either secreted by pathogenic fungi or possibly produced by the biocontrol microbe itself and lost, partially or entirely, the ability to protect tomato plants from *Pythium ultimum* and *Rhizoctonia solani* attack.

Trichoderma spp. are ubiquitous filamentous fungi known for their ability to colonize a variety of niches, as well as antagonize and control numerous plant-pathogenic fungi. They also have direct beneficial effects on plants, including enhancement of growth, nutrient assimilation, and systemic resistance (Woo et al. 2006; Yedidia et al. 1999, 2000). It has been widely recognized that *Trichoderma* spp. use multiple mechanisms and different modes of action when they interact with plant

and other microbes (Brunner et al. 2005; Harman et al. 2004a; Sivasithamparam and Ghisalberti 1998; Woo et al. 1999).

The antimicrobial and mycoparasitic activities of *Trichoderma* spp. biocontrol strains have been investigated in depth, also by using functional genomics techniques (Lu et al. 2004; Marra et al. 2006; Rey et al. 2004), and several “virulence” factors capable of killing the host fungus have been identified (Benítez et al. 2004; Daniel and Filho 2007; Liu and Yang 2007; Lorito et al. 1994; Sharon et al. 2001; Vinale et al. 2006; Wilhite et al. 2001). In addition, the ability of these fungi to grow well in a medium amended with plant-pathogen-derived (i.e., *Botrytis cinerea*, *Rhizoctonia solani*, or *Fusarium oxysporum*) secondary metabolites or mycotoxins is a common finding. However, the mechanisms by which *Trichoderma* spp. protect themselves from the host and their own toxins are still unknown. The only molecule for which a protective role has been proposed thus far is the cell-wall-bound protein encoded by the gene *Qid74*, which may help to isolate the fungal cell from the negative effects of degrading enzymes and other toxins (Rosado et al. 2007).

Resistance to exogenous and endogenous toxic compounds is one of the keys to the ecological success of *Trichoderma* spp. (Lorito et al. 1996b). In fact, various strains belonging to this genera are among the most resistant microbes to natural and synthetic chemicals and toxins, and are able to rapidly degrade some of them, including hydrocarbons, chlorophenolic compounds, polysaccharides, and pesticides (Harman et al. 2004a and b; Rigot and Matsumura 2002). These beneficial fungi are among the first microbes to recolonize the soil after sterilization by methyl bromide, being more tolerant to this chemical than most other fungi or bacteria. Several integrated pest management (IPM) applications have been suggested for the control of fungal plant diseases by combining biological and chemical methods, including treatments of *Trichoderma virens* combined with metalaxyl or thiram to control the attack of *Pythium ultimum* on cotton or *R. solani* on tobacco (Chet et al. 1997), and of *T. harzianum* combined with captan used against *Verticillium dahliae* infecting potato (Chet and Inbar 1994). Moreover, many commercially applied *Trichoderma* strains are indicated as compatible with several fungicides, including captan, chlorothalonil, iprodione, thiophanate methyl, metalaxyl, chlorpyrifos, vinclozolin, (for instance, see *T. harzianum* T22 online). Increased resistance to drugs also has been described for *Trichoderma* spp. that attack immunocom-

This paper is dedicated to the memory of Giovanni Del Sorbo.

Nucleotide sequence data for gene *Taabc2* is available in the GenBank database under accession number AY911669.

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promised patients. Kratzer and collaborators (2006) demonstrated reduced activity of fluconazole and amphotericin B against clinical infections caused by *Trichoderma* isolates. In contrast, a novel beneficial treatment has been proposed using a strain of *T. virens* combined with an appropriate antifungal drug against the nail fungus *Trichophyton rubrum* (Omero et al. 2004).

The resistance of *Trichoderma* spp. to antimicrobial compounds also aids the fungi to stably colonize the roots and interact with plant tissues. This may result in a type of “priming” effect, with the plant being stimulated to produce various antimicrobial compounds, including pathogenesis-related proteins and phytoalexins, to which the biocontrol fungus appear to be at least partially tolerant (Yedidia et al. 1999, 2000, 2003). Also in this case, little is known about the mechanisms that permit *Trichoderma* spp. to survive in plant tissues without being apparently affected by defense compounds.

ATP-dependent permeases, composed of at least one highly conserved ATP-binding cassette (ABC) and one less conserved transmembrane (TM) domain, mediate the transport of many different substrates and, thus, the secretion of a wide variety of toxic compounds across biological membranes against a concentration gradient (Driessen et al. 2000). The differences in substrate specificity are determined by the TM domains (Chang 2003) which recognize and mediate the passage of the transported compounds.

Table 1. Primers used in this study^a

Primer name	Sequence (5'–3')
Pdaspl	ATGGGKGYGAGCGGKGCWGGKAA
Pdaspl2	ATGGGKGYGTCCGGKGCWGGKAA
Pdsp1	GCAGARGGYTGRTGGATSGT
Pdsp2	GATGCTGGGCTGRTGGATGGT
TABC2for1	TGGCTCCATCCAGGTTGAC
TABC2rev1	CGGCAGCGAGCTTCGTAAG
SLABgII	<u>AGATCT</u> CACACGGAACCTTTGAATGTGGA
SLBStuI	<u>AGGCTT</u> TGTTAGTGGTTAAGAGACCAC
SLCXbaI	<u>TCTAGA</u> CTGTCCGTTTGTACGAAAG
SLDNarI	<u>GGCGCC</u> CAAGAGTTCCAATAACGACAG
HPHforw	CATGGATGCGATCGCTGC;
HPHrev	GTCTAGAAAGAAGGATTACCTC
TrAct1F	GGTATGGGTCAGAAGGACT
TrAct1R	CCTTTCGGACGTCGACATC

^a Inserted restriction sites are underlined.

Several ABC transporter genes have been detected and characterized in filamentous fungi, and they have been associated with the movement of different substrates, including plant defence (i.e., phytotoxins), mating factors, antibiotics, chemical pesticides, and heavy metals (Andrade et al. 2000a and b; Del Sorbo et al. 2000, 2008; De Waard et al. 2006; Fleissner et al. 2002; Stergiopoulos et al. 2003; Urban et al. 1999). In particular, the role of the ABC transporter genes *ABC1* of *Magnaporthe grisea* and *BcatrB* of *B. cinerea* in pathogenicity and fungus–bacterium interaction, respectively, has been demonstrated (Schoonbeek et al. 2002; Urban et al. 1999). Very little is known about the function of these important permeases in biocontrol interactions mediated by bacteria (Abbas et al. 2004, Lee and Cooksey 2000; Martins dos Santos et al. 2004) and, further, there are no extended reports about the isolation and characterization of ABC transporter genes in biocontrol fungi.

In the present article, we suggest that ABC transporters are key factors in the multiple interactions established by *Trichoderma* biocontrol strains with other microbes and a potentially toxic or antagonistic environment. We report the cloning and functional characterization of the first ABC transporter gene from a biocontrol fungus, which was named *Taabc2* and isolated from a *Trichoderma atroviride* strain (P1). We demonstrate by expression studies and targeted knock-out that this gene is required for a full antagonistic or biocontrol effect. It provides resistance or tolerance to some chemical fungicides as well as to pathogen toxins and, possibly, avoids autointoxication of the biocontrol agent by the inhibitory compounds produced during antagonistic interactions.

RESULTS

Isolation and sequence analysis of a *Trichoderma* ABC transporter gene.

We identified and cloned the full sequence of an ABC transporter gene named *Taabc2* from *T. atroviride* P1. We first isolated a 560-bp fragment by using combinations of degenerated primers designed (discussed below) on the conserved sequence of the ABC of fungi and yeast. The partial sequence showed very high homology or similarity to ABC transporter genes found in other fungi, with identity or positive scores ranging from 87 to 95% for genes from *Gibberella zeae*, *Neurospora*

Fig 1. Targeted disruption of the *Taabc2* gene from *Trichoderma atroviride* P1. **A**, Wild-type *Taabc2* locus. Asterisks indicate the 393-bp fragment used as a probe in Southern and Northern analyses. **B**, *Hph* cassette of the disruption vector containing homologous portions of *Taabc2*. **C**, *Taabc2* locus after knock-out.

crassa, and *Aspergillus nidulans* (data not shown). Subsequently, the primers TABC2for1 and TABC2rev1 (Table 1), designed on the 560-bp fragment, were used to make a 393-bp probe (Fig. 1) for screening a genomic DNA library of *T. atroviride* P1. The 20-kb insert of one selected library clone was digested with *EcoRI*, and the four fragments obtained were subcloned and sequenced. Two of these fragments combined provided a 6,535-bp sequence that contained the entire *Taabc2* gene. It included a coding region of 4,270 bp, interrupted by two introns of 61 bp (at +697) and 55 bp (at +1655), 1,624 bp of the promoter, and 641 bp of the terminator. No restriction sites were found for *Bam*HI, *Bgl*II, *Kpn*I, and *Not*I. Southern analysis indicated that the gene is present as a single copy in the genome of *T. atroviride* P1 (Fig. 2A). The sequence of the *Taabc2* 5'-upstream region contained two predicted TATA boxes (at -174 and -946) and several putative transcription-factor binding sites (HSF, NIT2, ADR1, STRE, MYC, MATA1, and StuAP), also found in the promoter of *ech42*, an endochitinase-encoding gene directly involved in mycoparasitism and biocontrol in the same *T. atroviride* strain (Lorito et al. 1996a; Mach et al. 1999; Woo et al. 1999). The BLASTP analysis of the predicted protein performed with the entire sequence (1,384 amino acids) confirmed the BLAST results obtained with the originally cloned 560-bp fragment. The proteins with the highest scores were ABC transporters from different phytopathogenic fungi, with the exception of *N. crassa*, and, in most cases, known to be involved in multidrug resistance (MDR) and transport of toxic compounds (Table 2). The best match to

a protein with a known function was ATRB of *A. nidulans*, a multidrug transporter of many agricultural fungicides and some natural toxicants (Andrade et al. 2000a and b). One protein structure analysis identified 12 TM domains corresponding to the standard architecture of an ABC transporter with the longest cytosolic portion (amino acids 678 to 1,089) containing the ATP binding site. The ABC signature and the Walker A and Walker B domains were completely conserved, as indicated by the clustalW alignment with eight other ABC transporters from filamentous fungi (data not shown). The topology of the *Taabc2*-encoded protein matched that of a typical pleiotropic drug transporter ([NBD-TMS₆]₂), with the protein divided in two homologous halves each containing a nucleotide-binding domain (NBD) that precedes six TM domains.

Expression analysis.

Reverse-transcription polymerase chain reaction (RT-PCR) indicated that the expression of the *T. atroviride* *Taabc2* gene was stimulated only by benomyl and dicloran but not prochloraz (Fig. 3A) at the concentrations tested. *Taabc2* transcription was strongly activated when *T. atroviride* was grown in the presence of culture filtrates of the fungal plant pathogens *B. cinerea*, *R. solani*, or *P. ultimum*, as well as sterilized mycelia of *B. cinerea* or *R. solani* as the sole carbon source added to the growth medium (Fig. 3B).

Northern analysis confirmed that *Taabc2* expression is induced by both the culture filtrates and biomass of *B. cinerea* or *R. solani* (Fig. 4, lanes 6 through 8) and, if *T. atroviride* is

Fig. 2. Molecular characterization of the *Taabc2* locus in *Trichoderma atroviride* P1 wild type (P1wt) and $\Delta Taabc2$ mutants. **A**, Southern analysis of *T. atroviride* P1wt *Taabc2*. Left lane, λ HindIII marker (kb); right lane, strain P1wt genomic DNA digested with *Bam*HI (no sites in the gene). **B**, Polymerase chain reaction products from amplification with primers specific for the *hph* cassette: M = 1 Kb plus DNA ladder, A1 to A6 and B2 to B10 = $\Delta Taabc2$ mutants, N = pAN7-1 plasmid, P1wt = wild type. **C**, Southern analysis of DNA digested with *Xba*I and probed with the *hph* gene: A1 to A4 and B2 to B10 = $\Delta Taabc2$ mutants, P1wt = wild type, M = λ HindIII marker (kb). **D**, Southern analysis of DNA digested with *Bgl*II and probed with the *Taabc2* 393-bp probe: M = marker (kb), A1, A4, and B2 to B10 = $\Delta Taabc2$ mutants, P1wt = wild type. Experiments were performed at least twice.

Table 2. BlastP analysis of the deduced amino acid sequence of the *Trichoderma atroviride* *Taabc2* gene.

Identities (%)	Positives (%)	Gaps (%)	Accession no.	Matching ATP-binding cassette transporter proteins, source
75	87	0	XP389006	Hypothetical protein, <i>Gibberella zeae</i>
73	84	0	XP380480	Hypothetical protein, <i>Neurospora crassa</i>
71	82	0	CAA93141	ATRB, <i>Aspergillus nidulans</i>
70	82	0	AA180009	MFABC1, <i>Monilinia fructicola</i>
71	82	1	CAB52402	BCATRB, <i>Botrytis cinerea</i>
68	82	1	BAB59028	PMR5, <i>Penicillium digitatum</i>
66	79	1	AAK62640	ATR5, <i>Mycosphaerella graminicola</i>

exposed even at a relatively low concentration (5 ppm), to beauvericin, a mycotoxin produced during habitat colonization by *Beauveria bassiana* and *Fusarium* spp. (Fig. 4, lanes 1 through 3). Interestingly, the addition of little amounts of glucose (Fig. 4, lanes 10 and 11), fructose (Fig. 4, lanes 12 and 13), and *N*-acetyl-D-glucosamine (Fig. 4, lanes 14 through 16) stimulated accumulation of *Taabc2* mRNA.

Experiments with green fluorescent protein (GFP) mutants of *T. atroviride* P1, which contained the reporter gene under the control of the cloned *Taabc2* promoter (914 bp), confirmed the activation of the ABC transporter gene by dicloran, relatively low amounts of glucose, and fructose but not sucrose, added to the salt medium (SM), or by *R. solani* culture filtrate alone (Fig. 5). Dual culture tests performed on agarized SM + 1% (wt/vol) sucrose (SMS) medium as described by Zeilinger and associates (1999) also demonstrated that *Taabc2* expression is directly stimulated by the presence of the living host (*R. solani*) already before the contact of the two confronting colonies (5 mm apart) (Fig. 5F), similarly to other biocontrol-related genes (Mach et al. 1999; Zeilinger et al. 1999).

Effect of targeted knock-out of *Taabc2*.

Southern and PCR analysis confirmed the presence of the *hph* cassette (Figs. 1 and 2B and C) and the absence of a complete *Taabc2* gene in the transformation progenies (Fig. 2D), the majority of which showed single and not multiple insertions (Fig. 2C). Five of the single-insert progenies were randomly selected, and the lack of *Taabc2* transcripts was confirmed by Northern and RT-PCR analysis (data not shown) and used for the characterization of the $\Delta Taabc2$ mutant phenotype. The growth rate on SMS, potato dextrose agar

Fig 5. Induced fluorescence of *Trichoderma atroviride* P1 green fluorescent protein mutants, obtained by using the *Taabc2* promoter and grown in the presence of: **A**, salt medium (SM) + 1% (wt/vol) sucrose; **B**, SM + 1% glucose; **C**, SM + 1% fructose; **D**, SM + dicloran at 5 ppm; **E**, *Rhizoctonia solani* culture filtrate; and **F**, *R. solani* mycelium (dual culture) before contact with the pathogen colony.

Fig. 3. Expression of the ATP-binding cassette transporter gene *Taabc2* from *Trichoderma atroviride* P1 in the presence of different fungicides or fungal hosts, culture filtrates (CF), or mycelia. **A**, Growth substrate, salt medium + 1% (wt/vol) sucrose (SMS), contained benomyl, 50 ppm (lane 1); procloraz, 50 ppm (lane 2); dicloran, 50 ppm (lane 3); and no fungicide control with SMS alone (lane 4). **B**, Growth substrate SMS contained *Botrytis cinerea* CF (lane 1), *Rhizoctonia solani* CF (lane 2), *Pythium ultimum* CF (lane 3), no amendment control with SMS alone (lane 4), *B. cinerea* mycelia at 1% (wt/vol) (lane 5), and *R. solani* mycelia at 1% (wt/vol) (lane 6).

Fig. 4. Northern analysis of *Taabc2* expression when *Trichoderma atroviride* P1 was grown in the presence of different fungal toxins, pathogen culture filtrates (CF) or mycelia, or with different carbon sources. Lane 1, salt medium + 1% (wt/vol) sucrose (SMS) + beauvericin at 5 ppm for 10 min before RNA extraction; lane 2, SMS + beauvericin at 5 ppm for 20 min before RNA extraction; lane 3, SMS + beauvericin at 5 ppm for 40 min before RNA extraction; lane 4, no treatment control on SMS (1% sucrose) only; lane 5, no treatment control on SMS (3% sucrose) only; lane 6, SMS + *Botrytis cinerea* mycelia; lane 7, SMS + *Rhizoctonia solani* CF; lane 8, SMS + *B. cinerea* CF; lane 9, no treatment control on SMS (1% sucrose) only; lane 10, salt medium (SM) + 0.2% glucose; lane 11, SM + 4% glucose; lane 12, SM + 0.5% fructose; lane 13, SM + 1% fructose; lane 14, SM + 0.1 M *N*-acetyl-D-glucosamine (NAG); lane 15, SM + 0.01 M NAG; lane 16, SM + 0.001 M NAG.

(PDA), or potato dextrose broth (PDB) of the selected knock-out mutants was substantially the same as the wild-type P1 (P1wt) (data not shown), with the exception of $\Delta Taabc2B6$ on PDB. In all cases, the conidia produced by the mutants were similar in color, form, and size to those of P1wt but had a germination rate reduced by approximately 30%. In contrast to the wild type, the mutants were completely inhibited by the chemical fungicide dicloran applied at a relatively low concentration (10 ppm) (Fig. 6A) and grew poorly in salt media containing only 1% fructose, 0.2% glucose, or 0.5% colloidal chitin as the sole carbon source (Fig. 6B). These results are in agreement with the fact that the above drug and sugars at the applied concentrations stimulate the expression of the native *Taabc2* gene.

In order to investigate the role of *Taabc2* during mycoparasitism and antagonism, *Botrytis cinerea*, *R. solani*, and *P. ul-*

mum were grown in PDB liquid media, the fungal biomass was removed, and the culture filtrates were inoculated with spores of the *T. atroviride* $\Delta Taabc2$ mutants, P1 containing the *hph* cassette, or P1wt (Fig. 7). Considering the amount of mycelia recovered after 1 week of incubation compared with the wild type and controls (fungal biomass obtained in P1wt culture filtrates) (Fig. 7), all the mutants tested grew less in the presence of metabolites and molecules that may stimulate mycoparasitism or antagonism (i.e., cell wall fragments) accumulated in the culture filtrates by any of the phytopathogenic fungi (Fig. 7, representative mutants). Similar results were obtained when *T. atroviride* P1 $\Delta Taabc2$ mutants and the wild type were exposed to the toxins beauvericin, fusaproliferin, and aflatoxin B1, B2, and G1 produced by *B. bassiana* or *Fusarium* spp. during both plant tissue colonization and antagonistic or mycoparasitic interactions with *Trichoderma* spp. (Fig. 8). $\Delta Taabc2$

Fig. 6. Effect of *Taabc2* knock-out on the growth of *Trichoderma atroviride* P1 on different agarized substrates. **A**, Potato dextrose agar (PDA) containing no fungicide (Control) or dicloran (1 and 10 ppm); **B**, salt medium (SM) containing the indicated sugar with compound as the sole carbon source. Controls include a representative mutant transformed with the empty vector (P1 hph). Fungi were observed for approximately 1 week, until the fastest growing isolate reached the petri plate edge.

Fig. 7. Comparative growth of *Trichoderma atroviride* strains P1 wild type (P1wt) and $\Delta Taabc2$ mutants in the culture filtrates of P1 and three plant pathogenic fungi. Fungal biomass was obtained in potato dextrose broth for all the fungi and removed after 10 days of incubation. The culture filtrate was filter sterilized, then inoculated with spores of the *T. atroviride* strains. After 7 days, the $\Delta Taabc2$ mutants, P1 hph, and P1wt biomass were collected, lyophilized, and weighed. Only two representative mutants are shown. Controls include biomass obtained in culture filtrates from P1wt instead of a pathogen, and a representative mutant transformed with the empty vector (P1 hph). Bars indicate standard deviation from three experiments with five replicates per treatment.

mutants were much less tolerant than the wild type to all tested mycotoxins and, in particular, to aflatoxins B1 and G1 and fusaproliferin (Fig. 8).

The effect of the *Taabc2* knock-out on the direct antagonistic ability of *T. atroviride* was also investigated by using a standard dual-culture assay, in which mutants or the wild-type strain were allowed to grow opposite to different fungal hosts on agarized substrate for at least 1 month. Also, in this case, the ability of the selected $\Delta Taabc2$ mutants to overgrow, inhibit, and degrade the mycelia of *B. cinerea*, *R. solani*, or *P. ultimum* was strongly reduced compared with the effective mycoparasitic action of the untransformed strain P1wt (Fig. 9). Coherently, the lack of *Taabc2* function significantly reduced the protective effect of *T. atroviride* P1 against *R. solani* or *P. ultimum* attack on tomato plants (Fig. 10). The biocontrol effect was reduced by approximately 25% against the Oomycete (Fig. 10A) and completely lost against the Basidiomycete (Fig. 10B). This is in agreement with the fact that *R. solani* culture filtrate produced both a high level of *Taabc2* expression (Figs. 3, lane 2 and 4, lane 7) and a significant inhibition of the $\Delta Taabc2$ mutant growth (Fig. 7).

DISCUSSION

Fungi belonging to the genus *Trichoderma* are known and extensively studied because they are capable of actively interacting with different substrates and living organisms, including plants and microbes (Benítez et al. 2004; Harman et al. 2004a). We considered that the innate ability of these fungi to tolerate relatively high concentrations of a variety of synthetic and natural toxic compounds, including its own antibiotics, and also to act as strong competitors of different microorganisms depends on efficient cell detoxification mechanisms supported by a complex system of membrane pumps. In a recent proteomic study, we found that, in different *Trichoderma* spp., there are a number of ABC transporter genes upregulated during the three-way interaction with various plants and fungal pathogens, which possibly supports both antagonistic activity and root colonization (Marra et al. 2006).

In this work, we fully sequenced and characterized the first ABC transporter gene from the *Trichoderma* genus and named it *Taabc2*. Sequence similarities indicate that this gene belongs to a group of pleiotropic drug resistance (PDR) factors with overlapping substrate specificity, including *AtrB*, *BcatrB*, *MfatrB*, and *Pmr5*. The highest homology scores with characterized ABC proteins (Table 2) were with ATRB from *A. nidulans*, MFABC1 (MFATR) from *Monilinia fructicola*, BCATR of *B. cinerea*, and PMR5 from *Penicillium digitatum*, suggesting that TAABC2 has a role in the resistance to and transport of various toxins and antibiotics. In fact, there seems to be a general correspondence, at least in fungi, among the sequences of ABC transporters and their physiological functions (i.e., in regards to the class of transported substrates) (De Waard et al. 2006). *AtrB* was found to encode for an MDR transporter capable of providing resistance to azole fungicides, cycloheximide, and pisatin (Andrade et al. 2000a and b; Angermayr et al. 1999; Del Sorbo et al. 1997). Similarly, *Mfatr1* and *Pmr5* are known to be involved in the resistance to demethylation inhibitor fungicides, whereas $\Delta Pmr5$ knock-out mutants also displayed increased sensitivity to thiabendazole, benomyl, dithianon, resveratrol, and camptothecin (Nakaume et al. 2002; Schnabel et al. 2003). In *T. atroviride*, *Taabc2* was upregulated by benomyl and dicloran (Fig. 3) but not by the azole fungicide procloraz. Accordingly, $\Delta Taabc2$ mutants were more sensitive than the wild type to dicloran, indicating a role of this gene in the mechanisms of cell detoxification from xenobiotic compounds. Another function of *Taabc2* could be to make *T. atroviride* more competitive toward other microbes by providing tolerance to bacterial or fungal toxins and by supporting the secretion of the biocontrol agent antibiotics (self-detoxification function). The expression of this gene was stimulated by beauvericin (Fig. 4), and the relative knock-out mutants were more sensitive to this and other mycotoxins, including aflatoxins B1, B2, and G1, as well as fusaproliferin (Fig. 8). Notably, the addition of beauvericin at up to 10 ppm did not interfere with the mycoparasitic and antagonistic activity of the wild-type strain against *B. cinerea* and different *Fusarium* spp. that are natural beauvericin producers (data not

Fig. 8. Effect of different mycotoxins on the mycelial growth of a representative *Trichoderma atroviride* $\Delta Taabc2$ mutant, indicated as percent inhibition relative to the growth of the wild-type strain P1 subjected to the same treatments. The tests were performed on agarized salt medium + 1% sucrose (SMS). Toxins were applied at a concentration of 5 ppm, which did not produce significant growth inhibition of the wild-type P1 (data not shown). Controls include treatment with the solvent used for dissolving the mycotoxins (MeOH) and a representative mutant transformed with the empty vector (P1 hph). Fungi were grown for approximately 1 week, until the fastest growing strain reached the petri plate edge. Bars indicate standard deviation from three experiments with five replicates per treatment.

shown). Also, in the case of *A. nidulans*, Andrade and colleagues (2000b) demonstrated that *AtrD* protects the cell from valinomycin, a potassium-selective cyclododecapeptide ionophore that is structurally related to beauvericin. *BcatrB* of *B. cinerea*, another ABC transporter gene with high similarity to *Taabc2*, was found to be involved in competition between microorganisms (Schoonbeek et al. 2002). In particular, this gene protected the pathogenic fungus from phenazine antibiotics produced by *Pseudomonas* spp. Similarly, the ability of *Erwinia chrysanthemi* to compete in planta against saprophytic bacteria was reduced by the inactivation of the ABC transporter gene *ybiT* (Llama-Palacios et al. 2002).

Taabc2 may also support the secretion of *T. atroviride*'s own mycotoxins, as demonstrated for CFP of *Cercospora kikuchii* (Callahan et al. 1999) and *PhlE* of *Pseudomonas fluorescens* (Abbas et al. 2004), even though the *T. atroviride* permease belongs to the ABC transporter and not to the major facilitator superfamily. We didn't specifically investigate this hypothesis because of the large variety of antibiotics produced by strain P1, but there are several indications in favor of it. i) *Taabc2* was co-induced with biocontrol-related "virulence factors" (enzymes and antibiotics) (described below) in conditions of carbohydrate starvation given by media containing low amounts of glucose, fructose, or chitin as the sole carbon source (Mach et al. 1999), as well as in the presence of mycotoxins and metabolites produced by other microbes (Figs. 3 and 4). ii) The 5' upstream region of *Taabc2* contains putative consensus sequences, such as stress-related elements (STRE) and mycoparasitism-related elements (MYC1-3) (Cortes et al. 1998; Delgado-Jarana et al. 2002), typically found on the promoter of mycoparasitism-related genes also in strain P1 (Lorito

et al. 1996a; Woo et al. 1999). iii) The $\Delta Taabc2$ mutants grew and competed poorly in conditions conducive to the production of antibiotics, such as starvation (Figs. 6 and 9) or in the presence of signals from fungal hosts (*R. solani*, *B. cinerea*, and *Pythium ultimum*) (Fig. 7). In fact, the lack of function of this particular ABC transporter associated with the stimulated synthesis of *T. atroviride*'s own toxins may have caused an auto-intoxication of the fungus (Figs. 6, 7, 9, and 10) similar to that reported by Abbas and associates (2004) about the role of the permease-encoding *PhlE* gene in *Pseudomonas fluorescens*. Accordingly, the growth on glucose-rich PDA or PDB medium was not impaired (Figs. 6 and 7), because the expression of many mycoparasitism-related genes is repressed in high-carbon conditions (Lorito et al. 1996a). iv) We have evidence that one of the selected $\Delta Taabc2$ knock-out mutants is unable to release 6-pentyl- α -pyrone, a volatile antibiotic normally secreted by the wild-type strain during mycoparasitism (F. Vinale and M. Lorito, unpublished data).

A variety of "offensive" compounds secreted by antagonistic fungi have been identified not only in *Trichoderma* spp. but also in other mycoparasitic species. These "virulence factors" include a large number of different enzymes (mainly chitinases, glucanases, proteases, and cellulases) (Benítez et al. 2004; Brunner et al. 2003; Howell 2003; Mendoza-Mendoza et al. 2003; Viterbo et al. 2002; Woo et al. 1999) and up to 200 different antibiotics and mycotoxins (Daniel and Filho 2007; Sivasithamparam et al. 1998; Vey et al. 2001). However, a successful interaction, antagonistic or mycoparasitic with other microbes or symbiotic with the plant, requires the activation of defence mechanisms for cell detoxification and protection of the fungus metabolism and structures. Unfortunately, very little

Fig. 9. Antagonistic activity in vitro of *Trichoderma atroviride* wild-type strain P1 (P1wt) and a representative $\Delta Taabc2$ mutant against various plant pathogens on salt medium + 1% sucrose (SMS). Confrontation assays were left to run for at least 1 month. Control: *T. atroviride* P1wt, $\Delta Taabc2A4$, and a representative empty vector-transformed mutant (P1 hph), grown alone without plant pathogen. Letters indicate the site of the inoculum of *T. atroviride* P1wt or the indicated mutant (T), *Rhizoctonia solani* (R), *Botrytis cinerea* (B), or *Pythium ultimum* (P).

information is available today on the “protective factors” used by biocontrol fungi, including *Trichoderma* spp. For instance, it has been suggested that a cell-wall-bound protein named QID74 (Rosado et al. 2007) or some specific proteases (Delgado-Jarana et al. 2000) avoid the degradation of *T. harzianum* cell walls by enzymes released during mycoparasitism. On the other hand, antagonistic fungi and, in particular, mycoparasitic species manage in many cases to be tolerant to a variety of mycotoxins and antibiotics, including their own, and also to some chemical fungicides. Therefore, it is likely that these microbes possess an extensive and effective membrane pump system that actively removes many different harmful compounds from the cell, which may explain at least in part the ecological success of *Trichoderma* spp. It has been demonstrated

that ABC transporter-encoding genes support both the growth and the pathogenic action of important plant pests such as *B. cinerea*, *Mycosphaerella graminicola*, and *Magnaporthe grisea* (Del Sorbo et al. 2008; Schoonbeek et al 2002; Stergiopoulos et al. 2003; Urban et al. 1999). Similarly, targeted knock-out of the *Taabc2* gene strongly reduced the antagonistic activity of a *T. atroviride* strain, both in vitro and in vivo, against fungi belonging to diverse fungal groups—an Ascomycete, a Basidiomycete, and an Oomycete.

The data presented in this article indicate that TAABC2 and, in general, ABC transporters help *T. atroviride* to successfully antagonize or colonize various pathogens or plants in many different environmental conditions (Marra et al. 2006) and, thus, play a key role in *Trichoderma*-mediated biocontrol.. Ob-

Fig. 10. Effect of *Taabc2* knock-out on the in vivo biocontrol activity of *Trichoderma atroviride* P1. The wild-type strain (P1wt) and two representative $\Delta Taabc2$ mutants were applied as a seed coating, and the plant protection effect is indicated as the percentage of tomato seed germination in soil infested with **A**, *Pythium ultimum* or **B**, *Rhizoctonia solani*. Control = no fungal treatment (no pathogen no antagonist); pathogen = pathogen alone without seed treatment with *T. atroviride*; P1 hph = seed treatment with a representative empty vector-transformed mutant instead of P1wt or a $\Delta Taabc2$ strain. The standard deviation bars were obtained from at least three different experiments, with five replicates and a total of 15 treated seeds per treatment.

viously, the genes encoding for these permeases may prove to be useful new tools for genetic improvement of microbial agents or disease resistance in plant.

MATERIAL AND METHODS

Fungal strains.

T. atroviride P1 (ATCC 74058), isolated from wood chips and selected for its tolerance to the fungicide iprodione (Tronsmo 1991), was used in this study. Pathogen *B. cinerea* 309 isolated from tobacco, *R. solani* 1556 from tomato, and *Pythium ultimum* 2312 from tomato were obtained from the culture collection of the Department ArBoPaVe of the University of Naples. Fungi were grown on PDA, colonies were allowed to sporulate, and conidia were harvested and used to inoculate liquid media (100 ml) in glass flasks (1×10^8 spores/flask), which were incubated at 25°C on a rotary shaker at 150 rpm. PDB was used for the pathogens, whereas the *T. atroviride* wild type and mutants were grown in SMS (composition per liter: 680 mg of KH_2PO_4 , 870 mg of K_2HPO_4 , 200 mg of KCl, 1 g of NH_4NO_3 , 200 mg of CaCl_2 , 200 mg of $\text{MgSO}_4 \times 7\text{H}_2\text{O}$, 2 mg of FeSO_4 , 2 mg of MnSO_4 , 2 mg of ZnSO_4 , and 10 g of sucrose) (Schirmbock et al. 1994) unless differently indicated. This media was eventually amended with glucose, fructose, or N-acetyl-D-glucosamine (NAG) instead of sucrose, or with toxins, fungicides, pathogen mycelia, and so on.

Cloning of ABC transporter genes.

Fungal DNA extraction was performed from mycelia collected by filtration with Miracloth (Calbiochem, La Jolla, CA, U.S.A.) and by using a standard phenol-chloroform procedure (Sambrook et al. 1989). Degenerated primers Pdsp1, Pdsp2, Pdsp1, and Pdsp2 (Table 1) were synthesized based on the conserved ABC sequences of fungal and yeast ABC transporter genes. PCR amplification was performed, unless differently indicated, by using: 100 ng of *T. atroviride* P1 DNA as template. Amplicons of approximately 500 bp were excised from the gel and cloned in the pGEMT Easy vector (Promega Corp., Madison, WI, U.S.A.). The resulting plasmids were sequenced by MWG Biotech AG (Ebersberg, Germany) and subjected to BLASTX analysis. Plasmid DNA was purified from *Escherichia coli* cultures using an alkaline lysis method (Sambrook et al. 1989) or a Qiaprep spin miniprep kit (Qiagen, Hilden, Germany) following the manufacturer's directions. Primers TABC2for1 and TABC2rev1 (Table 1) were designed on the sequence of one of the clones that matched ABC transporter genes and used to isolate the entire corresponding gene (named *Taabc2*). This probe was obtained by using the PCR digoxigenin (DIG) system kit (Roche, Basel, Switzerland). The 393-bp fragment obtained was used to screen a *T. atroviride* P1 Lambda genomic DNA library following the protocol described on the Lambda Library user manual (Clontech, Mountain View, CA, U.S.A.).

Southern analysis of the *Taabc2* locus.

DNA (5 µg) of *T. atroviride* P1wt or of the selected transformants was digested overnight with the appropriate restriction enzyme (Promega Corp.), separated by electrophoresis on agarose (1%) gel, and transferred onto Hybond N⁺ nylon membrane (Amersham Biosciences, Uppsala, Sweden). Hybridization and visualization were performed following the manufacturer's directions (Roche); 20 ng of the 393-bp DIG-labeled probe were used per milliliter of the hybridization buffer DIG easy Hyb and were left to react at 42°C for 18 h. Membranes were washed twice for 15 min in 2× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate) with 0.1% sodium dodecyl sulfate (SDS) at room temperature and twice for 30 min in 0.1×

SSC with 0.1% SDS at 42°C. Band detection was obtained according to the manufacturer's instructions (Roche) and using the antiDIG Ap-conjugate antibody and the substrate CDP-Star.

Gene expression analysis.

Induction of ABC transporter genes in *T. atroviride* P1 was determined by RT-PCR and Northern analysis. RNA extraction was performed on mycelia grown for 2 days in SMS augmented with benomyl (50 ppm) (DuPont Agricultural Products, Wilmington, DE, U.S.A.), procloraz (50 ppm) (Aventis Crop Science, Wolfenbuttel, Germany), dicloran (50 ppm) (Sariaf Gowan, Faenza, Italy), autoclaved *B. cinerea* mycelia (1% wt/vol), or autoclaved *R. solani* mycelia (1% wt/vol). For the other tested conditions, the fungus was grown in SMS for 48 h and then exposed for 10, 20, or 40 min to beauvericin (5 ppm) (Sigma-Aldrich, St. Louis) prior to RNA extraction; salt medium (SM) + 0.2% glucose; SM + 4% glucose; SM + 0.5% fructose; SM + 1% fructose; SM + 0.1, 0.01, or 0.001 M NAG; and culture filtrates of *B. cinerea*, *R. solani*, or *P. ultimum* obtained by filtering (0.22 µm) cultures of the phytopathogens grown in PDB at 25°C for 10 days. Total RNA was extracted by using the TRI Reagent buffer (Sigma-Aldrich), separated electrophoretically on a 0.8% formaldehyde-agarose gel, and transferred onto Hybond N⁺ nylon membrane (Amersham Bioscience). Northern analysis was performed by using the DIG-labeled 393-bp *Taabc2* probe and membrane prehybridization, hybridization, and development protocols recommended by the DIG-labeling kit (Roche). mRNA and cDNA for the RT-PCR analysis were prepared from total RNA by using the Genelute mRNA Miniprep kit (Sigma-Aldrich) and the AccuScript High Fidelity First Standard cDNA Synthesis Kit (Stratagene, La Jolla, CA, U.S.A.), respectively. Amplification was obtained by using the TABC2for1 and TABC2rev1 (Table 1) specific primers designed on the partial sequence identified as a portion of an ABC transporter gene. Actin was used as a housekeeping gene and its cDNA was amplified with the TrAct1F and TrAct1R primers (Table 1). All gene expression data were confirmed by performing each experiment at least twice.

Construction of the *T. atroviride* mutants.

Targeted disruption of *Taabc2* was obtained by using the construct *pΔTabc2* (Fig. 1), which is based on pAN7-1 (Punt et al. 1987) and contains the *hph* cassette positioned between two portions of the *T. atroviride* gene. Four primers (SLABgIII, SLBStuI, SLCXbaI, and SLDNarI) (Table 1) were used to amplify the two *Taabc2* fragments (Fig. 1): a 962-bp fragment (SLA-SLB) of the promoter region in which *Bgl*II and *Stu*I sites were introduced, and a 921-bp fragment (SLC-SLD) near the end of the gene in which *Xba*I and *Nar*I sites were introduced (Fig. 1). The two fragments were ligated into pAN7-1 following *Bgl*II-*Stu*I (SLA-SLB) and *Xba*I-*Nar*I (SLC-SLD) digestion (Fig. 1). The resulting plasmid was transferred into *T. atroviride* P1 by a standard, polyethylene glycol-mediated, protoplast transformation procedure (Punt et al. 1987). The transformants were selected on PDA + hygromycin at 100 ppm and subjected to three rounds of monospore selection as well as subculturing in the absence and again in the presence of the antibiotic, in order to ensure the homokaryotic stage. Stable transformation and homologous insertion, with the absence of intact copies of *Taabc2*, was confirmed by PCR by using the primer pair HPHrev/HPHforw (Table 1), and by Southern analysis with both the 393-bp *Taabc2* fragment and the *hph* probe.

T. atroviride P1 GFP mutants were obtained by transforming the fungus with the construct p*Taabc2*::GFP obtained by ligating 914 bp of the *Taabc2* promoter region to the GFP

gene:*tchb2* terminator fragment that was released from the plasmid pZEGA3 (Zeilinger et al. 1999) with an *XbaI*-*HindIII* cut. Mutants were characterized as described by Zeilinger and associates (1999) and used to test GFP expression in the presence of different carbon sources, toxins, and pathogen mycelia. In particular, the activation of *Taabc2* during the interaction with a living *R. solani* colony was tested in dual-culture assays following the method described by Zeilinger and associates (1999). The expression of the reporter gene was monitored both before and after the contact of the two confronting fungi (a *Taabc2::gfp* mutant and *R. solani*).

Characterization of knock-out mutants.

Antagonistic activity was tested on both PDA and SMS by a standard plate confrontation assay, in which the *T. atroviride* wild type and mutants were grown in the presence of *R. solani*, *B. cinerea*, or *P. ultimum* for at least 1 month. Resistance to pathogen toxins was determined by exposing P1wt and its mutants to culture filtrates of P1wt itself, *B. cinerea*, *R. solani*, or *P. ultimum*. Culture filtrates containing metabolite mixtures were obtained by growing each fungus in PDB at 25°C for 10 days in a rotary shaker (150 rpm); then, the biomass was removed and the culture filtrate was filter sterilized and inoculated with 30 µl of a *T. atroviride* (wild type or transformants) 1×10^8 /ml conidia suspension. After 7 days of incubation, the *T. atroviride* biomass of each sample was collected, lyophilized, and weighed.

The wild-type and mutant strains were tested to determine the susceptibility to fungicides and various mycotoxins by growing the fungi in petri dishes containing agarized substrate: SMS (control), SM + 1% fructose, SM + 0.2% glucose, or SM + 0.5% chitin as the sole carbon sources; and SMS amended with the fungicides dicloran (1 to 10 ppm) or of various mycotoxins (aflatoxin B1, aflatoxin B2, aflatoxin G1, fusaproliferin, and beauvericin) at 5 ppm provided by A. Ritieni (Department of Food Science, University of Naples "Federico II"). The mycotoxins were tested at a concentration (5 ppm) that had no inhibitory effect on the growth of *T. atroviride* P1wt on SMS.

Mutant and wild-type biocontrol activity against the pathogens *R. solani* and *P. ultimum* were tested in vivo as described by Brunner and associates (2005) on tomato. Data presented are from at least three different experiments, with five replicates (15 seeds) for each treatment.

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