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BACTERIAL, FUNGAL AND VIRUS MOLECULAR BIOLOGY - RESEARCH PAPER

The AbcCl1 transporter of *Colletotrichum lindemuthianum* acts as a virulence factor involved in fungal detoxification during common bean (*Phaseolus vulgaris*) infection

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Abstract

Anthracnose, caused by *Colletotrichum lindemuthianum*, is a disease affecting the common bean plant, *Phaseolus vulgaris*. To establish infection, the phytopathogen must survive the toxic compounds (phytoanticipins and phytoalexins) that are produced by the plant as a defense mechanism. To study the detoxification and efflux mechanisms in *C. lindemuthianum*, the *abcCl1* gene, which encodes an ABC transporter, was analyzed. The *abcCl1* gene (4558 pb) was predicted to encode a 1450-amino acid protein. Structural analysis of 11 genome sequences from *Colletotrichum* spp. showed that the number of ABC transporters varied from 34 to 64. AbcCl1 was classified in the ABC-G family of transporters, and it appears to be orthologs to ABC1 from *Magnaporthe grisea* and FcABC1 from *Fusarium culmorum*, which are involved in pleiotropic drug resistance. A abcT3 (Δ*abcCl1*) strain showed reduction on aggressivity when inoculated on bean leaves that presented diminishing anthracnose symptoms, which suggests the important role of AbcCl1 as a virulence factor and in fungal resistance to host compounds. The expression of *abcCl1* increased in response to different toxic compounds, such as eugenol, hygromycin, and pisatin phytoalexin. Together, these results suggest that AbcCl1 is involved in fungal resistance to the toxic compounds produced by plants or antagonistic microorganisms.

Keywords Phytopathogen · Plant · Phytoalexin · Defense mechanism · Transporter

Introduction

The fungus *Colletotrichum lindemuthianum* (Sacc. & Magnus) Briosi & Cavara is the etiological agent of anthracnose in the common bean plant (*Phaseolus vulgaris* L.). Anthracnose is an important disease that contributes to reductions in bean yield in Brazil. Anthracnose is mainly combatted by using disease-resistant bean cultivars [1, 2]. However, this strategy is limited due to the large genetic variability in *C. lindemuthianum*, which results in frequent loss of resistance in commercial cultivars [3, 4]. Therefore, it is necessary to develop more efficient and long-lasting methods for anthracnose control, which likely requires a greater understanding of the pathogenic determinants of *C. lindemuthianum*.

A typical characteristic of phytopathogens is their ability to tolerate plant defense responses during the early stages of pathogenesis, which allows them to establish an infection. Multidrug resistance (MDR) is often observed in pathogenic fungi [5–7]. Among the non-degradative mechanisms of resistance, transmembrane efflux pump systems stand out, especially certain ABC transporter superfamily members, which have received considerable attention in recent years [7–10]. These transporters are capable of transporting an enormous quantity of cytotoxins through the plasma membrane against concentration gradients [11, 12]. This mechanism can provide protection against toxic compounds present in their natural habitat or prevent the cytoplasmic accumulation of toxic secondary metabolites produced by the fungi via MDR or pleiotropic drug resistance (PDR) [13]. In phytopathogenic fungi, these transporters can transport virulence factors, such as certain host-specific toxins,

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or promote protection against defensive plant compounds during infection [5]. The toxic agents are eliminated at membrane level before accumulated in the cytoplasm, being the drugs removed by the ABC transporters directly from the membranes into the extracellular space [14]. Another model suggests that these ABC transporters have a flippase activity, and can eliminate the toxic compounds by translocating them from the inner leaflet of the plasma membrane bilayer to the outer one [15].

ABC transporter genes that their products are involved in pathogenicity or resistance to antifungal compounds have been cloned and characterized in diverse phytopathogenic and model fungi, including *Gibberella pulicaris* (Fr.) Sacc. [16], *Mycosphaerella graminicola* (Fuckel) J. Schröt. [17, 18], *Monilinia fructicola* (G. Winter) Honey [19], *Magnaporthe grisea* (T.T. Hebert) M.E. Barr [20], *Botrytis cinerea* Pers. [21], *Blumeria graminis* f. sp. *tritici* (DC.) Speer [22], *Fusarium graminearum* [23], and *Colletotrichum gloeosporioides* (Penz) Penz. & Sacc. [10]. Additionally, genomic information and functional analyses have provided the tools to identify and classify *abc* genes into eight major subfamilies [8, 9, 24].

In this study, the *C. lindemuthianum abcCl1* gene, which encodes an ABC transporter, was characterized. As we have already sequenced the *C. lindemuthianum* genome, the genes in the genome of this important phytopathogen that encode ABC transporters were analyzed. In addition, a Δ*abcCl1* strain was isolated and its behavior during infection of bean leaves was analyzed in relation to changes in aggressiveness. The differential expression of the *abcCl1* gene during fungal growth in the presence of different toxic compounds also was evaluated by real-time PCR.

Materials and methods

Microorganisms

The A_2 2–3 isolate of race 89 of *C. lindemuthianum* (provided by Prof. Everaldo Gonçalves de Barros, Plant Molecular Genetics Laboratory, BIOAGRO, Universidade Federal de Viçosa, Brazil) was used as the wild-type strain. The isolate 83.501 of race 83 was included on the genome comparative analyses, and the isolate LV49 of race 81 was used to access the *abcCl1* gene sequence in the *C. lindemuthianum* species. The fungus was grown in potato dextrose agar (PDA) medium at 22 °C. To produce conidia, the mycelium was inoculated on a piece of a sterilized pod and incubated at 22 °C for 7 days [25]. A conidial suspension was prepared in sterile distilled water and filtered through sterile gauze to remove fragments of mycelium. Chemically competent *Escherichia coli* K12 DH5α cells were utilized for cloning. The plasmid pAN7.1 [26] was used as the template for amplify the *hph* (hygromycin B phosphotransferase) gene by PCR for use in gene inactivation.

Cloning and sequencing of the *abcCl1* gene from C. lindemuthianum

In an experiment previously conducted to characterize RAPD (random amplification of polymorphic DNA) sequences, a partial genomic library of *C. lindemuthianum* (data not shown) was created. One of the DNA sequences analyzed (437 bp, access number EI232168.1) showed identity to the *M. oryzae abc1* gene sequence and was used as a probe for cloning the complete *C. lindemuthianum abcCl1* gene from a phage lambda EMBL3 library [27].

For the cloning of the *abcCl1* gene from the genomic library was used the DNA hybridization to single plate methodology [28]. Phage DNA extraction was done from *E. coli* cultures lysed using the PEG/NaCl method [29]. The DNA preparations of the recombinant phages were digested with *SalI* (to release the phage insert) and subsequently hybridized with the 437 bp gene sequence. For probe labeling and detection of hybridization signals, the Gene Images Kits Random Primer II and Gene Images CDP-Star Detection Kits (Amersham Biosciences) were used following the manufacturer's guidelines. In this screening, five different clones were isolated (F1–F5), with homology to the *abcCl1* gene (data not shown).

The F3 DNA insert was subcloned into pBluescript II SK (Stratagene), to generate pABC-S, pABC-X2, pABC-X3, pABC-K, and pK9 plasmids (Fig. 1). These clones were sequenced and an 8403-bp fungal DNA sequence was obtained, containing the complete *abcCl1* gene, which encoded a putative ABC transporter of *C. lindemuthianum*. Specific oligonucleotides (Table S1) for DNA sequencing using primer walking were designed using the Primer3 program [30]. Plasmid DNA was extracted with the GeneJET™ Plasmid Miniprep Kit (Fermentas). The Big Dye Terminator (Amersham Life Science) system was used for sequencing in an ABI377 sequencer (Perkin-Elmer). The DNA sequences were analyzed and assembled using the DNAMAN (Lynnon Corporation) and Chromas (Technelysium Pty Ltd) programs and BLAST [31]. Putative transmembrane domains were analyzed using TM Finder [32]. Amino acid sequence alignments were performed using the CLUSTALW2 program [33]. The complete nucleotide sequence of the *abcCl1* (deposited name abcl1) gene was deposited in GenBank (http://www.ncbi.nlm.nih.gov/genbank) under accession number FJ753577.

The genomic library was made using genomic DNA from the LV49 isolate of race 81. However, after numerous years of laboratory manipulation, the infective capacity of the LV49 isolate in bean plants was reduced. Therefore, the A_2 2–3 isolate of race 89 was used in all tests performed in this

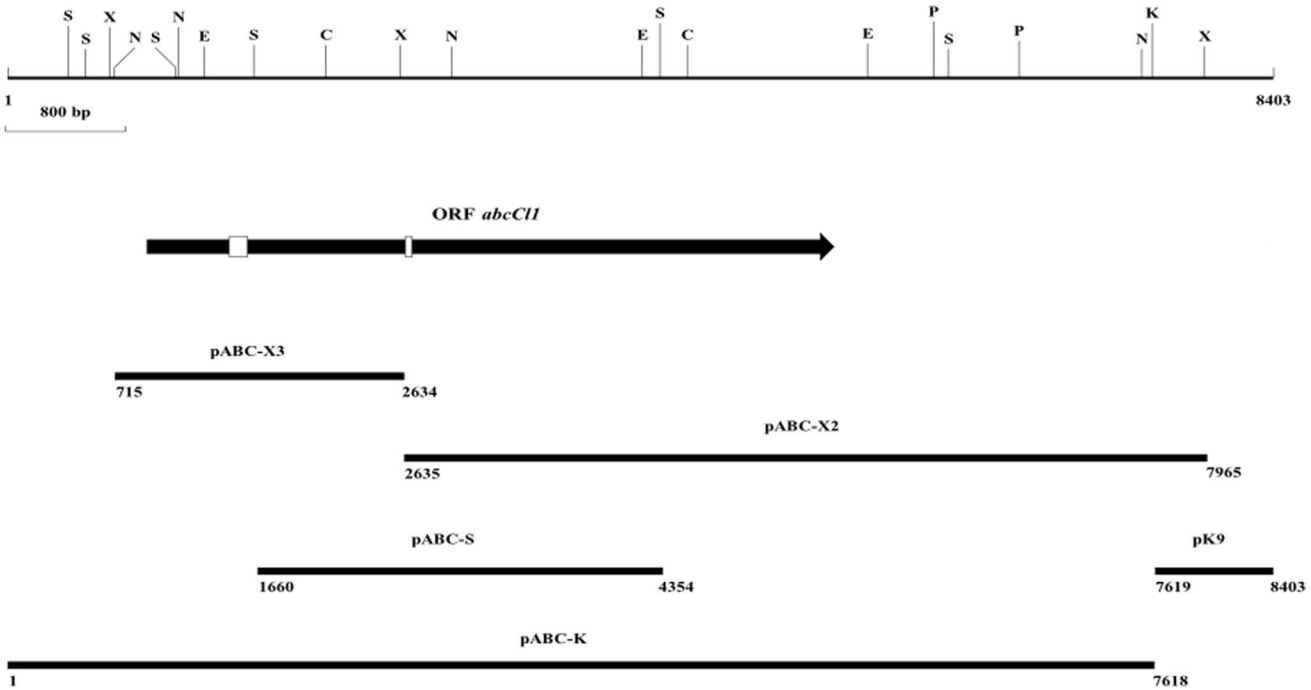

Fig. 1 Schematic representation of the genomic region where the *abcCl1* gene is present. At the top, there is a restriction map, and the position of *abcCl1* is shown by the black arrow. Boxes interrupting the ORF indicate introns. Restriction enzyme sites are indicated as follows: S, *Sal*I; X, *Xho*I; N, *Nco*I; E, *Eco*RV; P, *Sph*I; K, *Kpn*I; and C, *Sca*I. The DNA fragments are shown below, which were subcloned to generate pABC-S, pABC-X2, pABC-X3, pABC-K, and pK9

study since its genome sequence is available [34] and it has high virulence in the common bean plant (*P. vulgaris*). To improve our comparative analyses, we used a third isolate of race 83, 83.501, since we have also its genome sequence [34].

Genomic identification and phylogenetic analysis

Genes encoding ABC transporters were identified in the genomes of *C. lindemuthianum* (ASM169302v2), *Colletotrichum fioriniae* (JARH00000000.1), *Colletotrichum gloeosporioides* (GCA_000319635.1), *Colletotrichum graminicola* (NW_007361658.1), and *Colletotrichum higginsianum* (CACQ0200000000). Initially, a BLASTp (cutoffs: *E*-value, 10^{-5}; identity, 30%) was performed using the predicted proteome of this fungus as a query against a database of known ABC transporters (Table S2) created using sequences from UniProt (http://www.uniprot.org). The typical domains in ABC transporters were identified by using the Conserved Domains Database tool (https://www.ncbi.nlm.nih.gov/cdd/), and only proteins containing at least one copy of each domain (NBD/TMD) were considered complete ABC transporters. To classify the ABC transporters in the *C. lindemuthianum* genome and compare them to *abcCl1* gene orthologs, we performed a phylogenetic analysis using Geneious® 9 [35]. The AbcCl1 transporter was first identified in the *C. lindemuthianum* LV49 (belonging to the race 81) genome. Given the availability of *C. lindemuthianum* 83.501 and A_2-2–3 genomes (belonging to the races 83 and 89, respectively), we used them to identify the complete repertoire of ABC transporter-encoding genes in this species. In the first step, we identified the ABC transporter genes in these genomes and confirmed that the proteins have at least one NBD domain and one TMD domain. Afterwards, a phylogenetic analysis of *C. lindemuthianum* ABC transporters was performed with others ABC protein sequences from different fungi species. The alignment was performed using the NDB domain sequences of the major subfamilies of ABC transporters.

Inactivation of the *abcCl1* gene in *C. lindemuthianum*

Mutant strains were obtained by the Split-Marker strategy [36]. The oligonucleotides used to obtain the deletion of the *abcCl1* gene are shown in Table S1. Oligonucleotides F and R were designed using Primer3, and a 24-bp termination sequence was added to the 5′ region by using R1 and F2, which are complementary to the M13R and M13F primers, respectively. Two rounds of PCR were carried out using four oligonucleotides to amplify the *hph* gene (primers: M13R/NCL37 and M13F/NCL38), and four for the *abcCl1* gene (F1/R1 and F2/R2). To confirm the mutants,

the oligonucleotides FC and RC were used (Table S1). The PCR was performed with Platinum® Taq DNA polymerase High Fidelity (Invitrogen™) according to the manufacturer's instructions. *C. lindemuthianum* was transformed with the Split-Marker fragments according to the procedure described previously [27].

Southern blot analysis

DNA was extracted from *C. lindemuthianum* by adapting a protocol established by Specht et al. (1982), with modifications [37]. To obtaining mycelia, conidia of *C. lindemuthianum* were inoculated in Petri dishes containing GPYECH medium, and the dishes were incubated at 22 °C for 7 days. After incubation, the mycelium was collected, frozen in liquid nitrogen, and stored at − 80 °C until DNA extraction.

For Southern blot analysis, the total DNA was digested with the restriction enzyme *Eco*RI (Promega), which cuts once in the *hph* gene, but does not cut the *abcCl1* gene or the probe DNA. A 1090-bp DNA fragment of the 5′ region of the *abcCl1* gene not deleted from *C. lindemuthianum* was used as probe. The DNA digested were transferred to nylon membranes (Duralon-UV Membranes; Stratagene) by capillary action in 10 × SSC [29]. The probe was labeled with the Amersham Gene Images Alkphos Direct Labelling and Detection System Kit (GE Healthcare), and the membranes were hybridized at 65 °C. The labeling reactions as well as the hybridization and detection were performed according to the manufacturer's specifications.

Pathogenicity assay of the Δ*abcCl1* strain

A pathogenicity assay was carried out by adding 10^6 spores/ml to the abaxial region of susceptible detached leaves from a Rosinha bean plant 10 days old after emerging. The leaves were put in plate contained wet filter paper and incubated at 22 °C under a 16/8 h light (166 µE/s/m^2)/dark cycle, for 11 days. The negative control was sterile water. Symptoms were observed at 1, 3, 5, 6, 7, and 11 days after infection.

Analysis of the relative gene expression of *abcCl1*

To verify the effect of toxic compounds on *abcCl1* expression, conidia of the wild-type strain of *C. lindemuthianum* were inoculated in Erlenmeyer flasks containing 100 mL of GPYECH medium [38] and incubated at 22 °C with agitation. After growth, the mycelium was aseptically transferred to fresh GPYECH medium (50 mL) in the presence or absence of a toxic substance, and incubated for 8 h [39]. The following toxic compounds were tested: eugenol, 0.01% (Sigma-Aldrich); hygromycin, 10 µg/mL (Sigma-Aldrich); psoralen, 10–20 µg/mL (Sigma-Aldrich); camptothecin, 10–20 µg/mL (Sigma-Aldrich), and pisatin 10 µg/mL (kindly donated by Professor Hans VanEtten, Department of Plant Science, University of Arizona). Pure eugenol was added to the culture medium, while the other compounds were added from stock solutions with concentrations of 10 mg/mL prepared in water (hygromycin) or DMSO (psoralen and camptothecin), or 2 mg/mL in ethanol (pisatin). To evaluate the effect of the solvents on gene expression, the fungus was also cultivated for 8 h in the presence of DMSO (100 µL) and DMSO plus ethanol (50 µL and 250 µL, respectively). After incubation, the mycelia were filtered, washed in distilled water, frozen in liquid nitrogen, and stored at − 80 °C. Mycelia were obtained from independent duplicates for each evaluated condition.

To extract total RNA, mycelia, obtained as described above, were macerated in liquid nitrogen. Then, the RNA was isolated using TRI Reagent® (Sigma-Aldrich), according to the manufacturer's recommendations, treated with RNase-free DNase (Promega), and quantified by determining the 260 nm/280 nm ratio using spectrophotometer. RNA quality was evaluated by assessing the integrity of the RNA bands in a 1.0% agarose gel. A quantitative gene expression analysis was carried out by real-time PCR, using 1 µg of total RNA to prepare the first strand cDNA with the ImProm-II Reverse Transcription System Kit (Promega). Specific primers (CLABCq1 and CLABCq2) were used to amplify a 113 bp fragment of the *abcCl1* gene. All primers (Table S1) were designed using Primer Express (Applied Biosystems). Ribosomal RNA (rRNA; accession number: EU400134.1) was amplified using primers CLrRNA1 and CLrRNA2, and used as an endogenous control to normalize *abcCl1* gene expression levels. We performed the standard curve method to quantify gene expression, where cDNA obtained from mycelia grown in the absence of toxic compounds (calibrator) or in the presence of solvent was used. A standard curve was generated separately for each gene of interest and each reference gene. The relative quantification curve resulted for the gene of interest was normalized to endogenous gene in the same sample, and then the normalized numbers were compared between samples to get a fold change in expression (see FAQ in www.qiagen.com).

Reactions were performed in a 25 µL volume containing 1 × SYBR® Green PCR Master Mix (Applied Biosystems), 0.2 µM each primer, and 1 µL of cDNA. All samples were amplified in triplicate under the following cycle conditions: 50 °C for 2 min and 95 °C for 10 min, followed by 40 cycles of 95 °C for 15 s and 60 °C for 1 min. To confirm the presence of a single specific amplification product in each reaction, a melting curve was generated immediately after amplification. The amplification was conducted using an ABI 7500 Real-Time PCR system (Applied Biosystems). Relative expression data were analyzed in accordance with the standard-curve method (Applied Biosystems User Bulletin

#2; P/N 4,303,859). Standard curves were prepared for each primer group using 1:10 serial dilutions of the cDNA. Relative expression of the target gene (*abcCl1*) was calculated by dividing the normalized quantity of mRNA in each test condition by the normalized quantity of calibrator mRNA.

Results

Isolation and characterization of the *abcCl1* gene

The *abcCl1* gene (4558 pb) presents two putative introns of 129 bp (542–670) and 49 bp (1711–1759) and was predicted to encode a 1450-amino acid protein. The 5′ GT and 3′ AG splicing sites, predicted for the introns, are in accordance with typical intron sequences observed in fungi [40].

In the 5′ UTR region of *abcCl1* (929 bp), a CAAT motif was identified at − 370 bp (Kinghorn and Turner 1992). A 5′-GCCA$^{A}/_{G}$G-3′ PacC consensus binding sequence [41] was also identified at − 662 bp (Fig. S1). The PacC protein controls transcription of genes regulated by pH.

Analysis of the deduced protein sequence of *abcCl1* showed an ABC transporter that contains two nucleotide-binding domains (NBD) and two predominantly hydrophobic regions, each containing six transmembrane domains (TMD_6). These domains are organized in a $(NBD\text{-}TMD_6)_2$ topology. The transmembrane domains, identified with TM Finder [32], are located at the following positions: 482–496, 508–533, 568–580, 586–607, 621–641, and 732–750 in the N-terminus, and 1154–1170, 1182–1201, 1223–1246, 1273–1295, 1305–1316, and 1424–1442 in the C-terminus. Alignment with ABC transporters from other fungi (Fig. S2) showed that the AbcCl1 protein possesses, in both the N- and C-terminal NBDs, the sequence motifs (Walker A, Walker B, and ABC signature motifs) that participates in ATP binding and hydrolysis [42, 43]. Although there is asymmetry in the conservation of these motifs between the AbcCl1 NBDs (i.e. presence of one or more degenerate motifs in the N- or C-terminal NBD), it is in accordance with the found for others fungal ABC transporters involved in pleiotropic drug resistance [44].

Genomic identification and classification of the ABC transporters in *C. lindemuthianum*

The number of ABC transporters identified in *Colletotrichum* spp. genomes varied from 34 to 64 (Table 1); 34 ABC transporter-coding genes were identified in *C. lindemuthianum*. Among these, 23 were predicted to encode complete transporters, while the other 11 were one-half transporters. We then classified these ABC transporters into the eight major subfamilies (A to H) by using phylogenetic analysis (Fig. 2). Most of the ABC transporters in *C. lindemuthianum* isolate A_2 2–3 belong to the ABC-B and ABC-G subfamilies with 13 and 12 members, respectively, while five were ABC-C subfamily members, the two were ABC-D subfamily members, and one was an ABC-F subfamily member. The *abcCl1* NBDs of isolate 81 and its orthologs in isolate 89 (i89_g11125) were grouped together in two clades, with 99 and 100% bootstrap support. These clades belong to the ABC-G subfamily, which was previously reported to be involved in PDR. In support of this classification, these orthologs show the typical organization of this subfamily, with the NBD preceding the TMD [45]. The *abcCl1* gene identified in isolate A_2 2–3 is a protein containing 1519 residues, with two domains, NBD/TMD, which is a complete ABC transporter, and nucleotide analysis showed 96% identity with the AbcCl1 sequences from isolates 81 and 89. The *abcCl1* gene is present in all *Colletotrichum* species evaluated in this study, with lengths varying from 1507 to 1524 amino acids (Table 2). There is consensus in the domain organization among these proteins, which means that regardless of the variations in length, all orthologs are complete ABC transporters with 12 transmembrane helices, and 6 per TMD. Furthermore, a phylogenetic tree was generated using AbcCl1 orthologs and other ABC transporters previously reported to be involved in fungal virulence. The AbcCl1 protein sequences grouped in the same clade of proteins involved in PDR, exhibiting high proximity with ABC1 from *M. grisea* and FcABC1 from *F. culmorum* (Fig. 3).

Table 1 The number of ABC transporters in each *Colletotrichum* genome

	Complete	"half-transporters"	Total
C. fioriniae	32	19	51
C. gloesporioides	46	18	64
C. graminicola	35	13	48
C. higginsianum	26	34	60
C. lindemuthianum	23	11	34

Inactivation of the *abcCl1* gene

The *abcCl1* gene was deleted by using the Split-Marker strategy [36]. In the first round of PCR, pAN7.1 was used as a template, and 3101 and 1584 bp fragments corresponding to the *hph* gene, which was used as a selection marker, were obtained (Fig. S3). In addition, genomic DNA from *C. lindemuthianum* A_2 2–3 isolate (race 89) was used as a template to amplify the 3′ (1001 bp) and 5′ (1114 bp) portions of the *abcCl1* gene. In the second round of PCR, we obtained two fragments, 4215 and 2585 bp, which were used to transform protoplasts of *C. lindemuthianum* (Fig. S3).

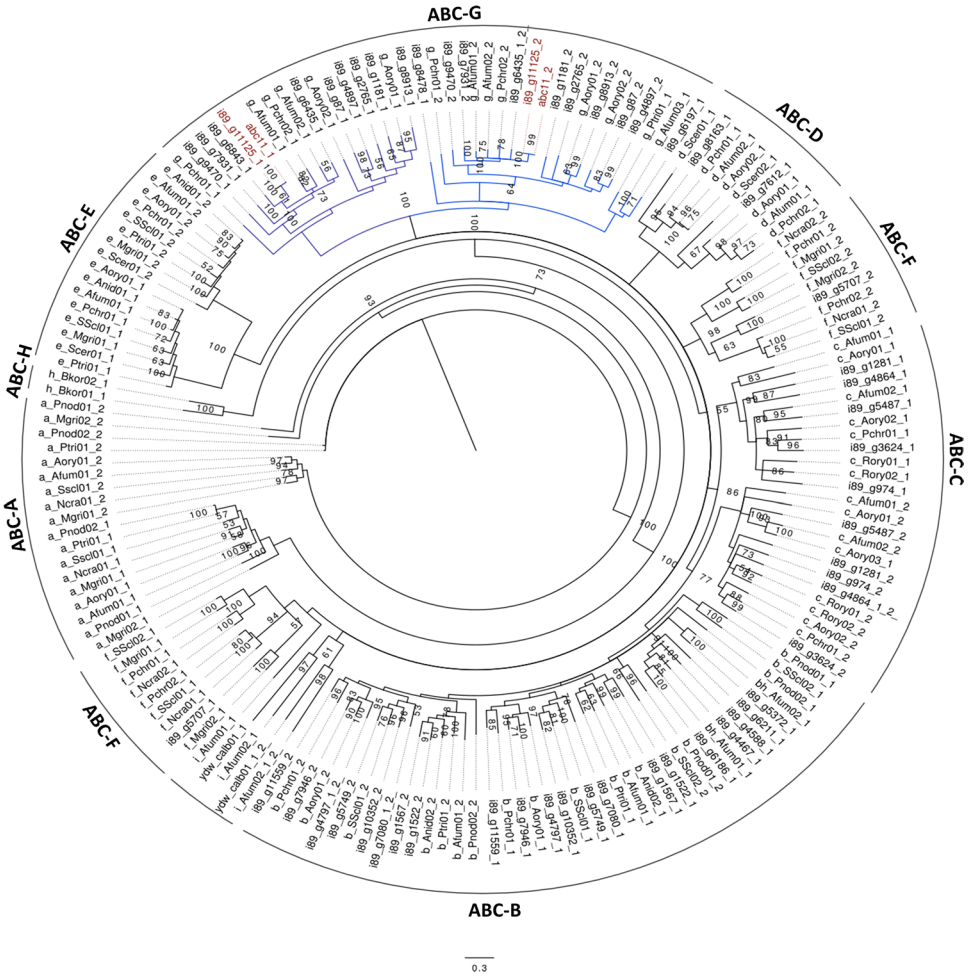

Fig. 2 Screening of all full-size ABC transporter proteins in *C. lindemuthianum* isolate A_2 2–3 (race 89). Phylogenetic analysis and classification of 23 genes encoding ABC transporters into the major subfamilies A–H. A neighbor-joining tree was generated using Geneious software with 4000 bootstrap replicates. Blue, ABC-G transporters associated with multidrug resistance; Red, *abcCl1* orthologs (races 81 and 89)

Table 2 The length and number of transmembrane segments in each AbcCl1 orthologous

Species	Length (number of residues)	TMs
C. lindemuthianum race 81	1519	12
C. lindemuthianum race 83	1519	12
C. lindemuthianum race 89	1519	12
C. sublineola	1523	12
C. nymphaea	1524	12
C. fioriniae	1524	12
C. simmondsii	1524	12
C. salicis	1523	12
C. higginsianum	1522	12
C. orbiculare	1523	12
C. gloesporioides	1507	12

Fourteen transformants were analyzed by PCR and Southern blot. Southern blot analyses showed ectopic integrations in transformants abcT1 (eight integrations) and abcT2 (three integrations), and only one mutant, transformant abcT3, showed deletion of the *abcCl1* gene without ectopic integration (Fig. 4a, b and c). *AbcCl1* gene deletion was also confirmed by PCR (Fig. 4d).

Pathogenicity assay of the Δ*abcCl1* strain

To identify the relationship between the *abcCl1* gene and the pathogenicity of *C. lindemuthianum* A_2 2–3 (race 89) in susceptible bean plants, we performed a pathogenicity assay by infecting bean leaves with the wild-type and abcT3 (Δ*abcCl1*) strains. Like the wild-type strain, leaves infected with the abcT3 (Δ*abcCl1*) strain showed the first symptoms after the fifth day of infection; according to previous studies, the fifth day is when the necrotrophic stage begins. However, on subsequent days, the abcT3 (Δ*abcCl1*) strain showed reduced aggressiveness, which was not observed for the wild-type infection (Fig. 5).

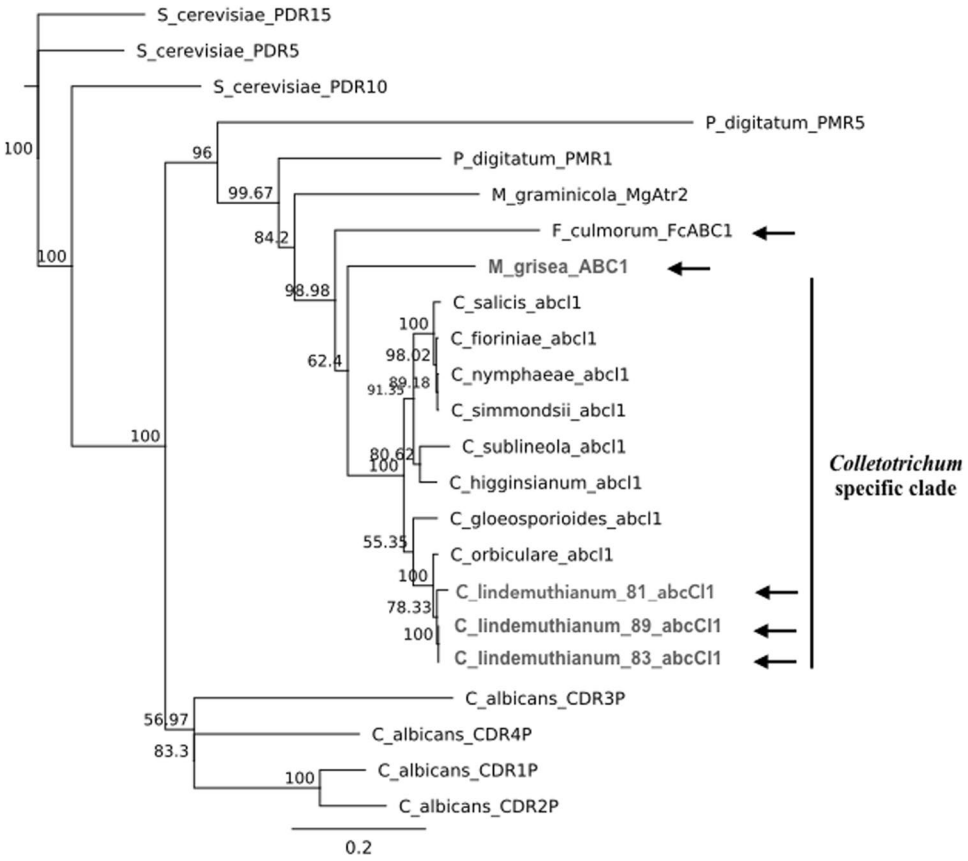

Fig. 3 Phylogenetic analysis of *abcCl1* orthologs related to pleiotropic drug resistance. Neighbor-joining trees were generated from ABC1 protein sequences using NCBI BLASTp and aligned with MAFFT. Confidence levels were calculated based on 4000 bootstrap replicates. A specific clade composed of *Colletotrichum* species is designated by the black vertical line. The arrows point to the AbcCl1 proteins of the three isolates of *C. lindemuthianum* (races 81, 83, and 89) used in this analysis as well as FcABC1 from *Fusarium culmorum* and ABC1 from *Magnaporthe grisea*

AbcCl1 gene expression in the presence of different toxic compounds

The wild-type A_2 2–3 isolate (race 89) of *C. lindemuthianum* was grown in the absence of toxic compounds and then transferred to fresh medium containing either solvent or a subinhibitory concentration of hygromycin, eugenol, pisatin, psoralen, or camptothecin, and incubated for 8 h. Total RNA was isolated, and relative *abcCl1* transcript levels were analyzed. Absence of DNA contaminants in the cDNA samples was confirmed with the CLABCq1 and CLABCq2 oligonucleotides, which were designed to bind near the intron–exon junctions of the predicted 49-bp intron of the *abcCl1* gene. Amplification of genomic DNA and cDNA samples with these primers showed a difference the bands sizes compatible with the 49 bp estimated for the intron, and verified the efficiency of the cDNA treatment with DNase (data not shown). The *abcCl1* transcript levels were induced by 12.6, 6.9, 3.8, and 2.2 times when the fungus was grown in the presence of eugenol (0.01%), hygromycin (10 µg/mL), pisatin (10 µg/mL), and camptothecin (20 µg/mL), respectively. Smaller increases in *abcCl1* expression were also observed after treatment with psoralen (20 and 10 µg/mL; 1.6 and 1.5 times, respectively) or camptothecin (10 µg/mL; 1.2 times, Table 3).

Discussion

Comparison of the protein sequence deduced from the *abcCl1* gene showed orthologs in all *Colletotrichum* species evaluated in this study as well as phylogenetic relatedness to ABC1 from *M. grisea* and FcABC1 from *F. culmorum* (Fig. 3), which were previously reported to be involved in virulence [47–50]. It was shown that ABC1 plays an important role in *M. grisea* virulence, and it is likely to be required at the initial stage of infection to export fungal toxins or as an efflux pump for external toxic substances of plant origin. Similarly, it was hypothesized that the protein encoded by *FcABC1* has a role in disease development by protecting fungi against the antifungal compounds produced by wheat. *M. grisea* and *C. lindemuthianum* are hemibiotrophic organisms that show extensive similarity in the initial infection of their host plants. Such similarity suggests that the products of these analogous genes are required for these microorganisms to establish disease. Indeed, an intergenic complementation experiment between *M. grisea* and *C. lindemuthianum* was successful [51]. Although *abcCl1* shows high similarity to *Abc1* and *FcABC1*, there is significant phylogenetic distance between them. The *abcCl1* gene product in *C. lindemuthianum* may have evolved to recognize bean-specific

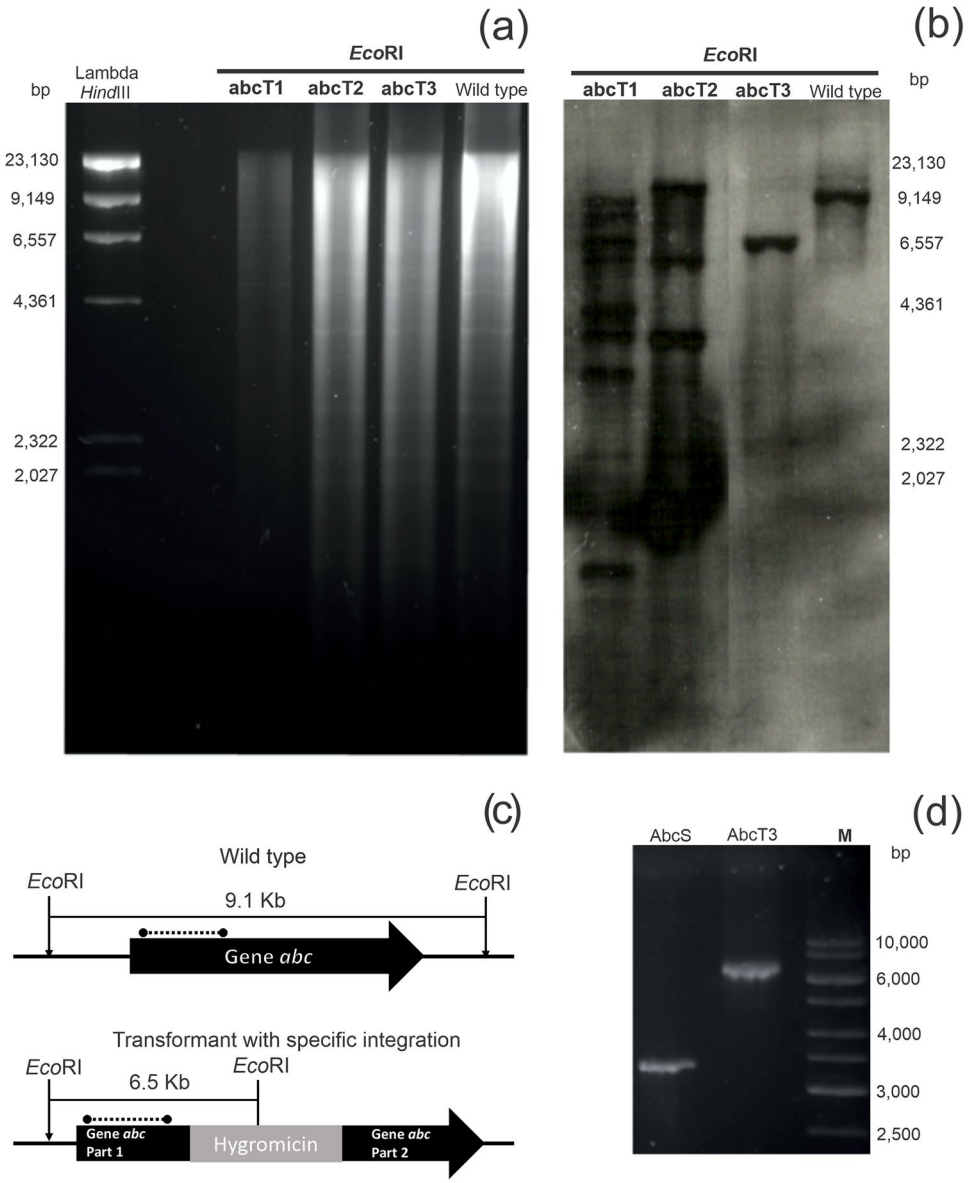

Fig. 4 Disruption of the *abcCl1* gene in *C. lindemuthianum*. (**a**) Agarose gel electrophoresis (0.8%) of total DNA digested with *Eco*RI, which does not cut the *abcCl1* gene. (**b**) Southern blot autoradiograph of *Eco*RI-digested genomic DNA from wild-type and *abcCl1* gene disruption construct transformants (*abc*T1–3). The blot was probed with in fragment in the 5′ region of *abcCl1*. (**c**) Restriction map of the *abcCl1* locus. The bottom line shows the gene disruption construct, Δ*abcCl1*. The *Eco*RI restriction site is indicated, as well as the position of the probe in the blot. (**d**) PCR amplification to confirm replacement of *abcCl1* with a hygromycin cassette using primers outside the *abcCl1* gene. AbcS, PCR product from the wild-type strain; AbcT3, PCR product from a hygromycin-resistant transformant. M, Marker: *GeneRuler*.™ 1 Kb DNA Ladder

antifungal compounds. Moreover, *abcCl1* is clade-specific for the *Colletotrichum* species complex, which provides more evidence for the possible function of AbcC1 against host-specific antifungal compounds. Taking into consideration the great variation in the number of ABC transporter-coding genes per *Colletotrichum* genome, it is reasonable to think that this repertoire may be involved in host range modeling. For example, *C. gloeosporioides*, *C. higginsianum*, and *C. fioriniae*, which are generalist pathogens, have more ABC transporter-coding genes than more specialist species, such as *C. lindemuthianum.* Although there is a smaller number of ABC transporter-encoding genes in the *C. lindemuthianum* genome, a major fraction of them is composed of genes encoding ABC-B and ABC-G transporters, which were previously reported to be involved in MDR and PDR, which is also called efflux-mediated resistance [45, 46].

The PacC protein acts as a transcriptional regulator of genes, repressing groups of genes in acid pH conditions and activating groups of genes in alkaline conditions [47]. In *C. gloeosporioides*, through comparative analysis of the transcriptome of wild-type and mutant (Δ*pacC*) strains it was demonstrated that PacC regulates (positively or negatively) up to 5% of the fungal genes, mainly genes that encode transporters, antioxidants, and enzymes involved in cell wall degradation [48]. In *C. lindemuthianum*, a Δ*pacCl* (*pacC* gene from *C. lindemuthianum*) mutants showed reduced growth in alkaline pH compared to the wild-type. Furthermore, despite the mutant being able to penetrate the host, it was unable to macerate plant tissue

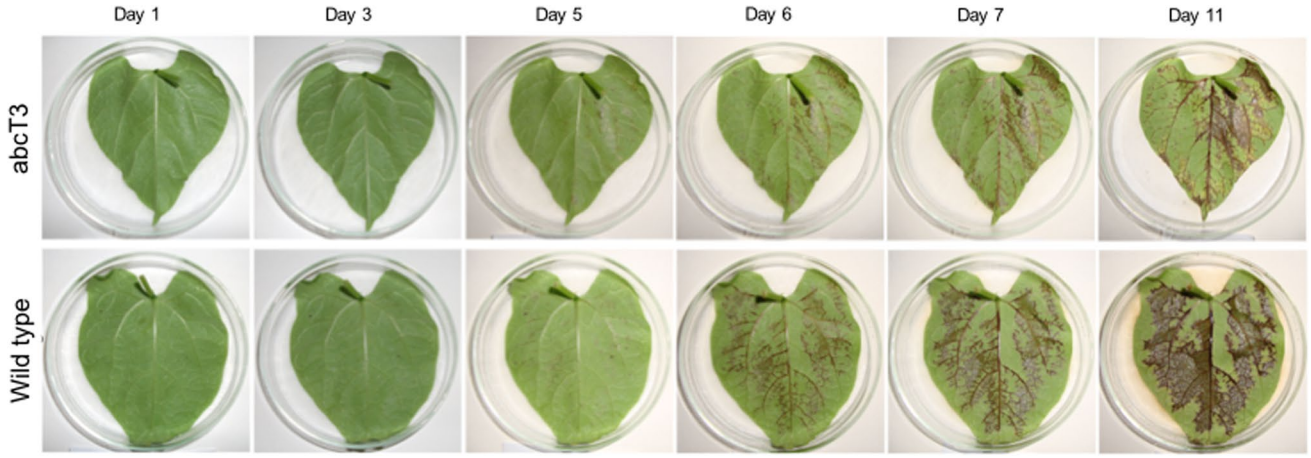

Fig. 5 Pathogenicity assay of *C. lindemuthianum* wild-type and abcT3 (Δ*abcCl1*) strains. Symptoms in common bean leaves during 11 days of infection by the two strains. For both infections, visible anthracnose lesions were detected at 5 days after inoculation; however, compared to the wild-type strain, the abcT3 (Δ*abcCl1*) strain was less aggressive, showing a reduced rate of disease progression

Table 3 Relative expression of *abcCl1* in the presence of different toxic compounds

Compound	*abcCl1* expression[a]		
	Mean C_T (*abcCl1*)	Normalized mean quantity (*abcCl1*)	Relative quantification[b]
None (calibrator)	22.366	0.006	1.0 ± 0.2
Hygromycin 10 µg/mL	18.923	0.038	6.9 ± 0.5*
Eugenol 0.01%	18.402	0.070	12.6 ± 1.2*
DMSO 50 µL Ethanol 250 µL (calibrator)	21.904	0.008	1.0 ± 0.1
Pisatin 10 µg/mL	19.203	0.029	3.8 ± 0.5*
Psoralen 10 µg/mL	19.977	0.012	1.5 ± 0.1
Camptothecin 10 µg/mL	20.862	0.010	1.3 ± 0.1
DMSO 100 µL (calibrator)	20.760	0.011	1.0 ± 0.05
Psoralen 20 µg/mL	19.333	0.018	1.6 ± 0.2*
Camptothecin 20 µg/mL	19.621	0.024	2.2 ± 0.2*

[a]Relative levels of *abcCl1* mRNA in each sample were normalized to ribosomal RNA (rRNA) as an endogenous control

[b]Amplification of the target and endogenous reference genes was performed in the same plate. The levels of *abcCl1* mRNA and rRNA were determined based on standard curves prepared for both the target (*abcCl1*) and endogenous control (rRNA). To obtain a normalized value, the target was divided by the endogenous reference. Normalized values of *abcCl1* for each experimental sample (fungus cultured in the presence of a toxic compound) were obtained by dividing the value by normalized value of *abcCl1* in the control (fungus cultured without toxic compound or in the presence of solvent), to generate relative expression. Relative quantification represents the number of times the gene is expressed compared to the non-treated sample (control). Results are the mean values of three repetitions

*Significant difference by the Tukey test at 5% probability in relation to the adopted control

and cause disease symptoms [27]. Thus, genes from *C. lindemuthianum* that encode enzymes and transporters necessary for pathogenesis may also be regulated by PacC, as observed in *C. gloeosporioides*. As an example, the Δ*pacCl* mutant of *C. lindemuthianum* was unable to produce or secrete extracellular lipases, which have already been reported as virulence factors in other pathogens such as *Fusarium graminearum* [49].

To defend themselves against toxic compounds of plant origin, phytopathogenic fungi can employ various mechanisms, including non-degradative detoxification [50–52]. This strategy appears to be used by *C. lindemuthianum*, and is mediated by at least one ABC transporter encoded by the *abcCl1* gene. Quantification of *abcCl1* expression by using real-time PCR showed increased gene expression in the presence of eugenol (a phenolic compound and

product of plant secondary metabolism) and hygromycin (Table 3). Expression of *abcCl1* also positively responded to psoralen (a phytoalexin), camptothecin (an alkaloid), and pisatin (a phytoalexin). These results suggest that *abcCl1* encodes an ABC transporter capable of making *C. lindemuthianum* resistant to different toxic compounds, and may be important for overcoming the defense mechanisms of the plant and establishing disease. Due to the greater *abcCl1* expression in the presence of diverse toxic substances, it is likely that the AbcCl1 transporter constitutes a primary line of defense for *C. lindemuthianum* against toxic compounds.

Kievitone and phaseollidin are isoflavonoid phytoalexins produced by the legume *P. vulgaris*, which need to be neutralized by the pathogens that infect the plant [50]. These and other phytoalexins produced by *P. vulgaris* constitute the most probable natural substrates for the AbcCl1 protein. Some of these compounds are also strong inhibitors of pisatin demethylase (PDA) activity, which is often encountered by pea phytopathogens [53]. During the initial stages of infection in *P. vulgaris*, *C. lindemuthianum* must be capable of tolerating the toxic effects of these phytoalexins likely by means of an efflux pump encoded by *abcCl1*, as demonstrated by the reduction in the virulence of the abcT3 (Δ*abcCl1*) strain on bean plant leaves (Fig. 5). Despite the absence of phytoalexins produced by the bean plant in the tests performed in this study, genetic expression analyses showed that the *abcCl1* gene is upregulated in response to pisatin, a phytoalexin also produced by leguminous *P. sativum* (Table 3). Induction of *abcCl1* expression by eugenol and pisatin, among other toxic compounds, indicates that these genes may have important functions during plant-microorganism interaction, providing resistance to the plant defense compounds.

Deletion of the *abcCl1* gene in *C. lindemuthianum* led to a decrease in the lesions produced in leaves during the pathogenicity assay, which indicates that this gene is necessary for penetration and the progression of infection [20]. Furthermore, inactivation of transporter genes can affect other physiological aspects, such as metabolism and nutrition [54]. Therefore, the AbcCl1 protein is a potential target for inhibitors, which, when used in conjunction with fungicides, could constitute an efficient anthracnose control method for the common bean plant against *C. lindemuthianum*. It was demonstrated that chlorpromazine and tacrolimus, two modulators of ABC transporter activity, displayed synergistic activity with the fungicide oxpoconazole, thus enhancing the fungitoxic activity against *B. cinerea* [55]. The authors showed that this synergism was positively correlated with enhanced accumulation of oxpoconazole in the mycelium due to inhibition of the ABC transporter BcatrD. Inhibitors of ABC efflux transporters have also been proposed as a novel disease management strategy for controlling the plant-pathogenic fungi *Pyrenophora tritici-repentis* [56] and *M. graminicola* [57].

Taken together, these findings indicate that the ABC transporter repertoire in *C. lindemuthianum* as well as other species may have evolved to contribute to the virulence of these pathogens. This highlights the importance of the products of *abcCl1* genes for cell detoxification in this fungus against plant molecules used in defense response, thus acting as a virulence factor. Although a deeper analysis is required to correlate the number of ABC transporters in the proteomes with host range.

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Code availability Not applicable.

Declarations

Ethics approval Not applicable.

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Consent for publication All authors gave consent to submit for publication.

Conflict of interest The authors declare no competing interests.

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