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A specialized ABC efflux transporter GcABC-G1 confers monoterpene resistance to *Grosmannia clavigera*, a bark beetle-associated fungal pathogen of pine trees

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Summary

- *Grosmannia clavigera* is a bark beetle-vectored pine pathogen in the mountain pine beetle epidemic in western North America. *Grosmannia clavigera* colonizes pines despite the trees' massive oleoresin terpenoid defences. We are using a functional genomics approach to identify *G. clavigera*'s mechanisms of adaptation to pine defences.
- We annotated the ABC transporters in the *G. clavigera* genome and generated RNA-seq transcriptomes from *G. clavigera* grown with a range of terpenes. We functionally characterized GcABC-G1, a pleiotropic drug resistance (PDR) transporter that was highly induced by terpenes, using qRT-PCR, gene knock-out and heterologous expression in yeast.
- Deleting *GcABC-G1* increased *G. clavigera*'s sensitivity to monoterpenes and delayed development of symptoms in inoculated young lodgepole pine trees. Heterologous expression of *GcABC-G1* in yeast increased tolerance to monoterpenes. *G. clavigera* but not the deletion mutant, can use (+)-limonene as a carbon source. Phylogenetic analysis placed GcABC-G1 outside the ascomycete PDR transporter clades.
- *G. clavigera* appears to have evolved two mechanisms to survive and grow when exposed to monoterpenes: GcABC-G1 controls monoterpene levels within the fungal cells and *G. clavigera* uses monoterpenes as a carbon source. This work has implications for understanding adaptation to host defences in an important forest insect–fungal system, and potentially for metabolic engineering of terpenoid production in yeast.

Introduction

The ascomycete *Grosmannia clavigera* (*Gc*) is a fungal pathogen of pine trees (*Pinus* spp.) and is vectored by the mountain pine beetle (*Dendroctonus ponderosae*, MPB). MPB, *Gc* and other vectored microorganisms form an interactive biological complex that has caused a rapid, large-scale decline of lodgepole pine (*Pinus contorta*) in western North America (Lee *et al.*, 2005). *Grosmannia clavigera* can kill trees and stain the sapwood blue or black when it is inoculated manually into trees at a certain density; such discoloration reduces the commercial value of lumber. In British Columbia alone, the MPB epidemic has already killed over 16 million hectares of lodgepole pine forests. With the recent spread of the MPB epidemic into forests east of the Rocky Mountains and an expansion of its host range into Jack pine (*Pinus banksiana*) (Cullingham *et al.*, 2011), the MPB–microbial complex now threatens the Canadian boreal forest. This large-scale disturbance has caused massive economic losses to forest-based industries, and has important implications for forest ecosystem stability and global atmospheric carbon balance (Kurz *et al.*, 2008).

While the MPB–*Gc* complex can successfully colonize > 20 different pine species, its preferred host is *P. contorta* (Safranyik *et al.*, 2010). Like all conifers, the pine hosts of the MPB epidemic have complex oleoresin-based chemical defences that protect these trees against most potential pests and pathogens (Keeling & Bohlmann, 2006a,b; Boone *et al.*, 2011; Bohlmann, 2012). The oleoresin of most conifers consists predominantly of monoterpenes and diterpene resin acids, with smaller amounts of sesquiterpenes. These terpenes can be fungistatic or fungicidal. Small lipophilic monoterpenes diffuse easily into and through eukaryotic cell membranes, interact with membranes and membrane-bound enzymes, and can change membrane fluidity and ultrastructure (Parveen *et al.*, 2004; Bakkali *et al.*, 2008; Witzke *et al.*, 2010). They can also cause fungal cells to swell, shrink and vacuolize (Soylu *et al.*, 2006). While antimicrobial properties of monoterpenes are documented, little is known about the mechanisms used by some microorganisms, particularly fungi that colonize conifers, to survive and grow in the presence of monoterpenes. The highly specialized MPB–*Gc* complex, which colonizes the monoterpene-rich environment of pine phloem and sapwood, requires mechanisms to overcome host defence

chemicals. To discover mechanisms involved in the ability of *Gc* to cope with host terpenes, we analysed the *Gc* genome and transcriptome to identify genes that are differentially expressed in response to terpene treatments (DiGuistini *et al.*, 2011). We noted that a gene annotated as an ATP-binding cassette (ABC) transporter, *GcABC-G1* (previously reported as GLEAN_8030), was highly up-regulated in the terpene-induced *Gc* transcriptome responses (DiGuistini *et al.*, 2011).

Fungal plant pathogens evolve a combination of strategies to colonize and survive in the unfavourable conditions occurring in their living environment, the host. These strategies include transporting toxic chemicals out of the cell or sequestrating them in cellular organelles, detoxifying host defence compounds by converting or modifying them and interfering with host signalling (Morrissey & Osbourn, 1999). Mechanisms can involve enzymes such as cytochrome P450s and membrane proteins such as ABC or MFS transporters (Han *et al.*, 2001; Coleman & Mylonakis, 2009). Fungal ABC transporters are well known for their roles in the secretion of harmful chemicals (Sipos & Kuchler, 2006; Coleman & Mylonakis, 2009; Coleman *et al.*, 2011). Typical ABC transporters consist of two transmembrane domains (TMDs) and two nucleotide-binding folds (NBFs); 'half-transporters' contain only one TMD and one NBF. ABC transporters are classified into subfamilies according to sequence homology and domain topology (Sipos & Kuchler, 2006; Lamping *et al.*, 2010). In eukaryotes, eight major subfamilies have been defined: ABC-A to ABC-H (Dean & Allikmets, 2001; Verrier *et al.*, 2008). Among these, full-size ABC-B, ABC-C and ABC-G are, respectively, referred to as multidrug resistance (MDR), multidrug resistance-associated protein (MRP) and pleiotropic drug resistance (PDR) (Paumi *et al.*, 2009; Kovalchuk & Driessen, 2010). Fungal PDR transporters are located in the cytoplasmic membrane and function as efflux pumps, contributing to drug resistance, chemical sensitivity and cellular detoxification (Coleman & Mylonakis, 2009; Lamping *et al.*, 2010).

Here, we characterized the responses of *Gc* to a range of individual terpenes and terpene mixtures. We identified and classified all putative ABC transporter genes in the *Gc* genome. We then profiled their expression and characterized the function of *GcABC-G1*, which was the gene most strongly induced by terpenes. Deleting this gene in *Gc*, and expressing it heterologously in *Saccharomyces cerevisiae* demonstrated that *GcABC-G1* confers tolerance to monoterpenes. GcABC-G1 appears to be specific to monoterpenes and to function as an efflux ABC transporter. Transcripts of *GcABC-G1* were detected in stem tissues of young lodgepole pine trees inoculated with *Gc*. The development of the symptoms in young trees that were inoculated with the deletion mutant was delayed relative to trees inoculated with the wild-type *Gc*, suggesting that *GcABC-G1* contributes to the fungus' ability to overcome host defence chemicals and survive in an environment that is highly unfavourable to most organisms. Plant terpenes, beyond being important in conifer defence (Keeling & Bohlmann, 2006a,b) and in the interactions of plants with other organisms (Gershenzon & Dudareva, 2007), are also being actively explored for metabolic engineering of biofuels and bioproducts in microbial hosts (Bohlmann & Keeling, 2008; Peralta-Yahya *et al.*, 2011). The discovery of a role of *GcABC-G1* in tolerance to monoterpenes may lead to applications for improved microbial production systems for terpenoids (Dunlop *et al.*, 2011; Ignea *et al.*, 2011).

Materials and Methods

Strains and plasmids

Grosmannia clavigera strain kw1407 (NCBI Taxonomy ID: 655863) was deposited at the University of Alberta Mycological Herbarium (UAMH1150). It is important to note that *G. clavigera* consists of two cryptic species, *Gc* and *Gs* (Alamouti *et al.*, 2011). The name *Gc* should be reserved for the holotype described in 1968. While the species described here belongs to the *Gs* group, which is not yet fully described, for continuity with our recent genomic work (DiGuistini *et al.*, 2011) we have used the name *Gc* in this manuscript. *Saccharomyces cerevisiae* wild type BY4741(MATa *his3Δ1 leu2Δ0 met15Δ0 ura3Δ0*) was provided by Dr Christopher Loewen, UBC. *Agrobacterium* GV3101, a laboratory stock, was used to transform *Gc*. Expression vector pESC-URA (Stratagene, Accession no. AF063585) was used for yeast expression.

Fungal media and growth condition

RNA-seq and quantitative reverse-transcription PCR analysis of *Gc* grown on complete medium (see the Supporting Information, Methods S1) treated with blends of monoterpenes and diterpenes were described by DiGuistini *et al.* (2011). For experiments to characterize monoterpene utilization, fungal spores were incubated for 3 d on 1% malt extract agar (MEA) overlaid with cellophane (cat#80611781; Amersham Biosciences), then the young mycelia were transferred to yeast nitrogen base (YNB) minimal medium (Methods S1) in glass plates, two (2 × 4 cm) strips of filter paper were placed inside the lid of the plate and 200 µl of individual monoterpenes (saturated, 1.4 g l^{-1} limonene) or a mixture of monoterpenes (MT) were added onto the filter paper. The plates were sealed with DuraSeal film (cat# 89031-573, Laboratory Sealing Film; VWR, Mississauga, Ontario, Canada) and incubated at 22°C in a sealed glass container, if necessary, monoterpenes were resupplied biweekly until the mycelia covered at least half of the media surface (3–4 wk). Fungal growth rate assays were carried out on MEA. For monoterpene treatments, we transferred plugs of actively growing fungal cultures into the centre of glass plates containing MEA medium and applied monoterpenes using the method described above; for other chemical assays, the chemicals were added after autoclaving the media. The optimal concentration of each chemical was determined by gradient tests. The final concentrations of the chemicals were: 3.3 µM for diterpenes (abietic acid, dehydroabietic acid and isopimaric acid), 2 µM for azoles (propiconazole, tebuconazole), 50 µM for flavonoids (quercetin and fisetin), 500 µM for antibiotics (cycloheximide, erythromycin), 7.5 µM for phenolic compounds (benzoic acid, salicylic acid, vanillic acid, gentisic acid) and 50 µM for phytoalexins (catechin, resveratrol and taxifolin).

Annotation of GcABC genes

HMMER3.0 (www.hmmer.janelia.org) was used to search the *Gc* genome and expressed sequence tags (ESTs) and retrieve gene models encoding proteins containing the conserved ABC motif sequences 'ABC-transporter (PF00005)', 'ABC-2 transporters (PF01061)' and 'ABC transporter transmembrane region (PF00664)'. The resulting gene model annotations were curated manually, considering alternatively spliced isoforms, exon-intron boundaries and coding starts/stops. Potential pseudo-genes were excluded. The *Magnaporthe grisea* ABC transporter collection (Kovalchuk & Driessen, 2010) was used as a query for TBLASN local searches and to confirm gene prediction and classification in *Gc*.

RNA-seq analysis

For each RNA-seq library, we collected samples from three biological replicates, extracted RNA separately and pooled the samples for paired-end sequencing on an Illumina GAIIx (Canada's Michael Smith Genome Science Center, Vancouver, BC, Canada). Sequence filtering, trimming, mapping to the reference genome and RNA-seq analyses were conducted on CLC Genomic Workbench v4 (http://www.clcbio.com) software. Differential expressed genes were identified by comparing the number of reads per kilobase of exon model per million mapped reads (RPKM) between treatment and control data sets; statistical analysis was carried out by Kal *et al.*'s *Z*-test (1999). Five RNA-seq data sets were analysed. Two were generated in previous work (DiGuistini *et al.*, 2011) and the third was generated for *Gc* growing on YNB with a mixture of monoterpenes as the sole carbon source. Results were normalized to the sample grown on mannose as a carbon source. To compare the response of *Gc* and the deletion mutant to a mixture of monoterpenes, both strains were grown on MEA and treated with a mixture of monoterpenes for 12 h before sampling, and gene expression was normalized to the untreated controls.

Quantitative reverse transcription PCR (qRT-PCR)

Differential gene expression was validated by qRT-PCR. Fungal mycelium samples were removed at 0, 6, 12, 36, 48 and 72 h from complete medium with mono/diterpenes (CM + T) and 7, 10, 14, 18 d from YNB minimal medium with a mixture of monoterpenes as sole carbon (YNB + MT); *GcABC-G1* expression was also measured in phloem of young pine trees inoculated with *Gc* for 4, 7, 14 d (see inoculation of pine, later). Phloem tissues next to the inoculation points were removed and immediately frozen in liquid nitrogen. Extraction of total RNA, cDNA synthesis and qPCR were performed as described by Hesse-Orce *et al.* (2010). Three biological and technical replicates were used for each time-point. Data collection and statistical analysis were carried out on the Bio-Rad CFX96 real-time PCR detection system.

Producing gene deletion mutants

ATP-binding cassette transporter mutants were generated using an *Agrobacterium*-mediated gene deletion procedure (Wang *et al.*, 2010). The whole gene open reading frame was replaced with the selective gene marker hygromycin B (*hph*). Gene replacements were verified by PCR amplification of adjacent regions, targeted region and selective marker gene; copy numbers were determined by Southern blot.

Heterologous expression of *GcABC-G1* in *S. cerevisiae*

The full-length cDNA of *GcABC-G1* was amplified and cloned into the yeast expression vector pESC-URA under the control of the *GAL1* promoter, using conventional digestion/ligation methods (H*in*DIII and B*am*HI). Yeasts were also transformed with the empty vector pESC-URA that was used as a control. For spot tests, yeast cells were induced in synthetic galactose (SG) broth, diluted to an OD_{600} of 0.1 and spotted on SG glass plates. Four filter paper discs (0.5 cm each) were placed in the centre of the plate and were loaded with 60 µl mixture or individual monoterpenes to provide a saturated environment. The plates were sealed with DuraSeal film and incubated face-up at 28°C until colonies showed. For survival tests, induced yeast cells were diluted to an appropriate density and spread on SG glass plate. The yeast cells were treated for 1 h with a specific monoterpene (5 µl) diluted in 245 µl ethanol; the solution was applied on five small filter papers placed inside the lid and the glass plates were sealed with DuraSeal film. After a 1-h incubation, the chemical was removed and plates were further incubated for 4 d at 28°C. The numbers of surviving cells with and without monoterpene treatments were counted.

Inoculation of young lodgepole pine trees with *Gc* or its *GcABC-G1* mutant

Five-year-old lodgepole pine trees were grown in the UBC greenhouse and maintained as described previously for other conifer saplings (Miller *et al.*, 2005). Trees were inoculated at six points along the stem with plugs of actively growing fungal mycelium on MEA medium. Circular bark plugs were removed from the outer stem tissue using a 5 mm diameter metal cork borer. Inoculations were done on opposite sides of the stem at locations that were 5, 10 and 15 cm above the base of the stem. Fungal inoculums consisting of 5 mm diameter circular MEA/mycelium plugs were inserted into each circular bark hole and a bark plug placed on top to close the hole. The inoculated stem section was sealed with Parafilm and an outer layer of duct tape (Wang *et al.*, 2010). For control treatments, we used MEA plugs without fungal mycelium. Symptoms, that is, wilting and discoloration of needles and growing shoot tips, were recorded weekly for 4 wk. After 4 wk after inoculation the stems were harvested, and needles, branches and outer bark tissue were removed. Discoloration of the inner stem tissue was recorded, and fungi (i.e. *Gc* or its mutant) were reisolated from the phloem and the inner stem. Replicate experiments were carried out in April 2011 and May and June 2012. For each replicate, seven trees were used for the control and 11 trees were inoculated with either *Gc* or the deletion mutant.

Results

ABC transporters in the *G. clavigera* genome

We identified 39 putative *ABC* protein genes in the *Gc* genome (DiGuistini *et al.*, 2011). All of the predicted proteins contained at least one NBF domain. These gene models were located in 16 contigs; 23 (59%) of those genes were also found in Sanger ESTs (Hesse-Orce *et al.*, 2010) and 37 (95%) in the RNA-seq transcriptome data (DiGuistini *et al.*, 2011). The transporter gene lengths varied from 999 to 5241 bp and intron numbers varied from zero to 14 per gene. Using functional domain predictions and TBLASTN searches of other fungal genomes, we classified these ABC proteins into subfamilies following the nomenclature used by the Human Genome Organization (HUGO), and named these subfamilies GcABC-A to GcABC-G. These subfamilies were further divided into groups based on sequence similarity and phylogenetic analyses (Kovalchuk & Driessen, 2010). Table 1 gives Genbank IDs, genome locations, intron numbers and predicted topology of all GcABCs. Among the 39 GcABC proteins, 24 were full transporters with more than one TMD or NBF, while eight were half transporters with either a TMD-NBF

Table 1 Inventory and key features of the ABC transporters from the genome of *Grosmannia clavigera*

GcABCs	Group	Genebank ID	Length (aa)	Contig no.	Gene introns	Predicted topology	EST evidence	RNA-seq evidence
GcABC-A1	A	EFX05787.1	1661	113	0	(TMD-NBF)2	/	Yes
GcABC-B1[a]	B-I	EFX02238.1	1417	156	2	(TMD-NBF)2	/	Yes
GcABC-B2[a]	B-III	EFW98992.1	1235	97	5	TMD-(TMD-NBF)2	/	/
GcABC-B3	B-IV	EFW99076.1	1360	89	1	(TMD-NBF)2	Yes	Yes
GcABC-B4[a]	B-I	EFX05555.1	721	113	1	TMD-NBF	Yes	Yes
GcABC-B5[a]	B-II	EFX00542.1	1014	173	2	TMD-NBF	Yes	Yes
GcABC-B6[a]	B-III	EFW99428.1	1039	82	5	TMD-NBF	/	Yes
GcABC-B7[a]	B-III	EFX01489.1	811	167	0	TMD-NBF	Yes	Yes
GcABC-C1	C-I	EFX03767.1	1747	140	1	(TMD-NBF)2	Yes	Yes
GcABC-C2	C-II	EFX02908.1	1718	144	2	(TMD-NBF)2	Yes	Yes
GcABC-C3	C-II	EFW99141.1	1513	89	5	TMD-TMD-NBF	Yes	Yes
GcABC-C4	C-III	EFX00086.1	1602	173	3	(TMD-NBF)2	Yes	Yes
GcABC-C5	C-IV	EFX06644.1	1595	108	0	(TMD-NBD)2	/	Yes
GcABC-C6	C-IV	EFX02441.1	1462	156	0	(TMD-NBF)2	Yes	Yes
GcABC-C7[a]	C-V	EFX06313.1/ EFX06672.1[b]	1460	108	14	(TMD-NBF)2	/	/
GcABC-C8[a]	C-V	EFX06639.1	1430	108	9	(TMD-NBF)2	/	Yes
GcABC-C9[a]	C-V	EFX04947.1	1416	132	2	(TMD-NBF)2	/	Yes
GcABC-C10[a]	C-V	EFX03081.1/ EFX02994.1[b]	1242	144	11	(TMD-NBF)2	/	Yes
GcABC-C11[a]	C-V	EFX02174.1/ EFX2266.1[b]	1501	156	1	(TMD-NBF)2	Yes	Yes
GcABC-C12	C-VI	EFW99233.1	1552	89	5	(TMD-NBF)2	Yes	Yes
GcABC-C13[a]	C-VII	EFX02817.1	1488	144	2	TMD-(TMD-NBF)2	Yes	Yes
GcABC-D1	D-1	EFW99459.1	735	82	1	TMD-NBF	/	Yes
GcABC-D2	D-2	EFX00928.1	817	168	2	TMD-NBF	Yes	Yes
GcABC-E1	E-1	EFX01682.1	609	161	5	NBF-NBF	Yes	Yes
GcABC-F1	F-I	EFX02105.1	619	156	2	NBF-NBF	Yes	
GcABC-F2	F-II	EFX02105.1	770	167	1	NBF-NBF	Yes	Yes
GcABC-F3	F-IV	EFX01944.1	1122	160	1	NBF-NBF	Yes	Yes
GcABC-F4	F-V	EFX04290.1	1055	140	1	NBF-NBF	Yes	Yes
GcABC-G1	G-I	EFX06115.1	1460	113	5	(NBF-TMD)2	/	Yes
GcABC-G2	G-I	EFX00255.1	1540	173	1	(NBF-TMD)2	Yes	Yes
GcABC-G3[a]	G-I	EFX03218.1	1494	144	4	(NBF-TMD)2	Yes	Yes
GcABC-G4	G-V	EFX01574.1	1507	161	1	(NBF-TMD)2	/	Yes
GcABC-G5[a]	G-V	EFW98765.1	1218	97	8	(NBF-TMD)2	/	Yes
GcABC-G6	G-V	EFX03933.1	1374	140	0	(NBF-TMD)2	/	Yes
GcABC-G7	G-V	EFW99599.1	1390	82	1	(NBF-TMD)2	/	Yes
GcABC-G8	G-VI	EFX00337.1	606	173	2	NBF-TMD	Yes	Yes
GcABC-G9	G-VII	EX05969.1	1118	113	3	NBF-TMD	Yes	Yes
GcABC-NC1	N.C-1	EFX01444.1	333	167	0	NBF	Yes	Yes
GcABC-NC2	N.C-2	EFW99237.1	646	89	0	NBF	Yes	Yes

[a]Annotation was corrected from the published genome.
[b]Two adjacent half transporters were annotated as one full transporter.

or an NBF-TMD domain arrangement. The remaining seven had one or two NBFs but lacked TMDs, and were considered not to be membrane proteins or not to have a transport function. We compared the numbers of proteins in each subfamily for *Gc*, the yeasts *S. cerevisiae* and *Yarrowia lipolytica*, the ascomycete rice pathogen *M. grisea*, and the ascomycete saprophyte *N. crassa* (Table S1). The total number of ABC transporters in these species was similar, ranging from 30 to 48. *Gc* has five group V members in both ABC-C and in ABC-G subfamilies, while *M. grisea* has only two ABC-C-group V members and one ABC-G-group V member. In contrast to mitochondrial and peroxisome transporters (GcABC-B, D), which were highly conserved across these species (70–80% identity), seven out of the 10 GcABC-C members shared < 50% amino acid identity with closely related fungal species.

Expression of GcABC transporter genes in response to terpenoids

To identify the ABC transporters involved in *Gc*'s response to terpenes, we compared RNA-seq expression profiles of all *GcABCs* for: (1) *Gc* grown on a complete medium with a blend of monoterpenes and diterpenes (CM + T) for 12 h and 36 h (DiGuistini *et al.*, 2011); and (2) *Gc* grown on a YNB medium with a mixture of monoterpenes (MT) as sole carbon source (YNB + MT). Fig. 1(a) shows GcABC transporter genes whose transcripts were significantly upregulated in at least one of these conditions (*Z* test *P*-value < 0.05). For CM + T, six GcABC transporter genes were upregulated at 12 h, while 11 were upregulated at 36 h. For YNB + MT, 10 *GcABCs* were upregulated. Of five *GcABCs* that were upregulated in all three conditions, two (*GcABC-G1, G2*) belonged to the ABC-G-group I transporters, and three (*GcABC-F1*, *F2*, *F3*) belonged to the ABC-F subfamily, whose members are not considered to be true transporters because of the absence of TMD (Kovalchuk & Driessen, 2010). Three *GcABCs* were upregulated only on the more restrictive YNB + MT medium, in which monoterpenes are the only available carbon source. These included a PDR (*GcABC-G3*), a putative vacuolar transporter (*GcABC-B5*) and a putative peroxisome transporter (*GcABC-D1*) that may be involved in fatty acid metabolism.

GcABC-G1 was the most strongly upregulated of the 39 *GcABCs*. Its abundance of transcript relative to controls increased at least 100-fold in all three conditions, and transcripts were almost 1500-fold more abundant in YNB + MT than in YNB + mannose, which we used as a control (Fig. 1a). We validated these results by qRT-PCR analysis at times up to 72 h for *Gc* growing on CM + T. The change of *GcABC-G1* transcript abundance was up to 115-fold after 6 h, reached a peak of 648-fold at 12 h, and was still above 50-fold at 72 h (Fig. 1b). *GcABC-G1* also showed increased transcript levels (> 100-fold) throughout fungal growth on YNB + MT compared with YNB + mannose (Fig. 1c). We noted that *GcABC-G1* was not induced by other stress treatments (e.g. oxidative, osmotic, nitrogen starvation, high temperature and lodgepole pine phloem extract).

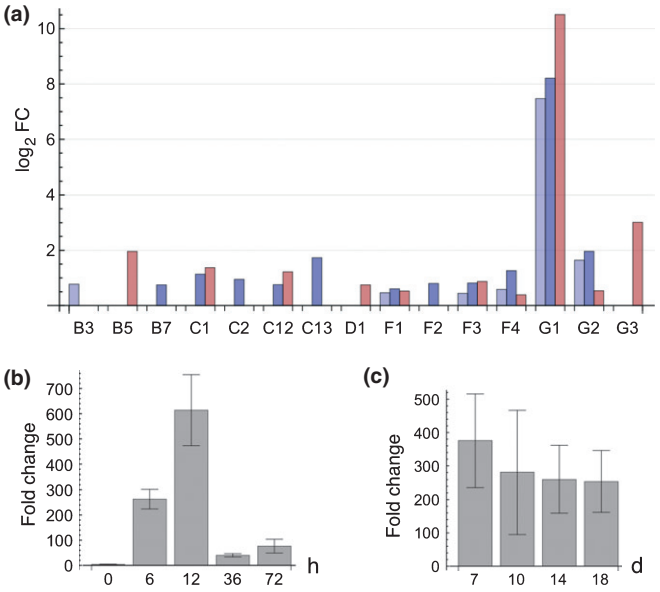

Fig. 1 Transcript abundance of selected GcABC transporter genes in response to terpene treatments. (a) RNA-seq result for the *GcABCs* that were upregulated for at least one type of terpene treatment. 12 h CM + T and 36 h CM + T: *Grosmannia clavigera* (*Gc*) mycelia grown on complete media (CM) and treated with mono/diterpene blend for 12 h (light blue bars) and 36 h (dark blue bars); upregulation indicates a *Z* test *P*-value < 0.05 (Kal *et al.*, 1999) for differential abundance and a fold change of at least 1.5× relative to the untreated control. YNB+MT (red bars): *Gc* mycelia grown for 10 d on YNB minimal media with a mixture of monoterpenes (MT) as sole carbon source; upregulation indicates a *Z* test *P*-value < 0.05 (Kal *et al.*, 1999) for differential abundance and a fold change of at least 1.5 × relative to the control grown on mannose. MT: (+)-limonene, (+)-3-carene, racemic α-pinene and (−)-β-pinene at a ratio of 5 : 3 : 1 : 1. (b, c) RT-qPCR validates the mRNA abundance of *GcABC-G1* on CM + T (b) and YNB + MT (c). Growth and treatment conditions were the same as for (a). mRNA abundance was normalized using β-tubulin, a housekeeping gene. Graphs show averages of three biological replicates; error bars show standard deviations. Except for 0 h CM + T, all time-points in (b) and (c) were significantly different from the controls ($P < 0.01$, Student's *t* test).

GcABC-G1 occupies a unique position in the phylogeny of PDR transporters

GcABC-G1 belongs to the ABC-G-group I transporters; in this group, three members were identified in *Gc* (Table 1). Maximum likelihood (ML) phylogenetic analyses of the predicted amino acid sequences for these three proteins (GcABC-G1, GcABC-G2 and GcABC-G3) with sequences for 80 ABC-G-group I transporters from 23 ascomycete species (Table S2), resolved four distinct clades. These included two clades that appear to be yeast-specific, one of which contains only the yeast *Yarrowia*, and two *Eurotiomycete–Sordariomycete* clades (Fig. 2). GcABC-G3 was placed in a *Eurotiomycete–Sordariomycete* clade showing an orthologous relationship with *Giberella zeae* (FGSG_03882). GcABC-G2 was placed in a *Sordariomycete*-specific subclade, which includes several transporters that have been reported as pathogenicity factors (e.g. *M. grisea* MGG_13624) or exporters of plant defence chemicals (*Nectria*

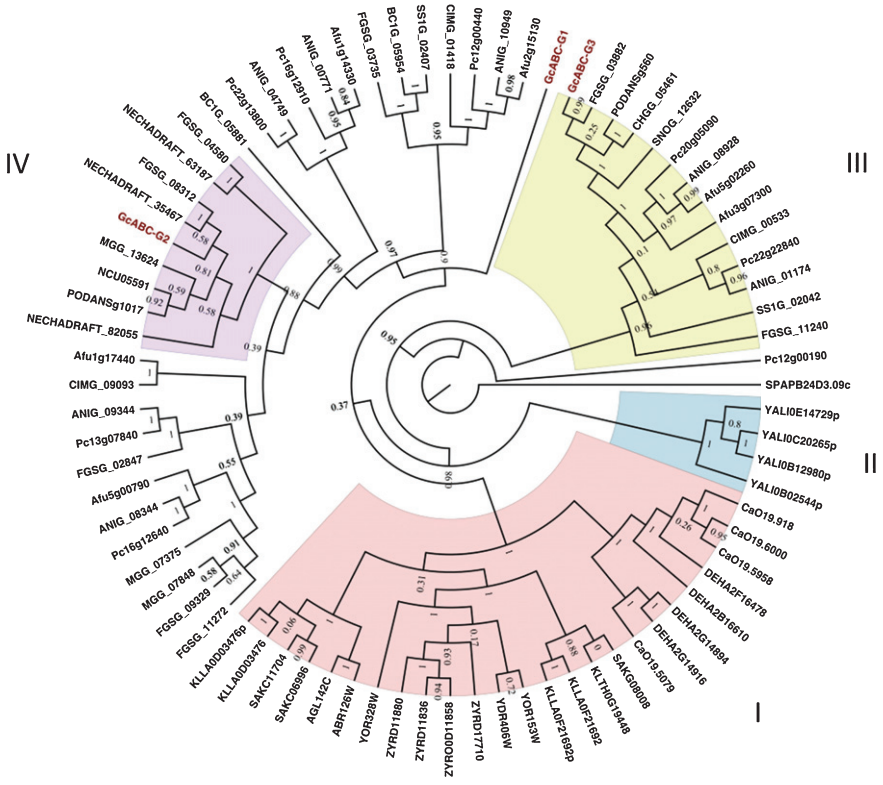

Fig. 2 Maximum likelihood phylogenetic tree of ABC-G group I transporters from 23 *Ascomycota* species. The species are listed in the Supporting Information, Table S3. Some specific clades are highlighted. I and II, yeast-specific clades, with II showing only *Yarrowia lipolytica*; III, *Eurotiomycetes–Sordariomycetes* clade; IV, *Sordariomycetes*-specific subclade.

haematococca NECHADRAFT_63187) in plant pathogens (Urban *et al.*, 1999; Coleman *et al.*, 2011). Whether GcABC-G2 has comparable functionality for *Gc* remains to be confirmed. By contrast, GcABC-G1 was placed outside of these four clades, and was separated from other sequences included in our ML analysis.

Deleting *GcABC-G1* increased fungal sensitivity to monoterpenes

To further functionally characterize *GcABC-G1*, we generated deletion mutants (Δ*gcabc-g1*) using the method previously developed for *Gc* (Wang *et al.*, 2010), and confirmed the deletion of the targeted gene by PCR and Southern blot (Fig. S1). On MEA, colony morphologies and growth rates were similar for *Gc* and Δ*gcabc-g1* (Fig. 3a). However, the mutant was more sensitive to monoterpenes than *Gc*. In the presence of individual monoterpenes the growth of *Gc* was not delayed by a racemic α-pinene, but was delayed by 1 d by (+)-3-carene, (+)-limonene, (−)-β-pinene and a mixture of monoterpenes (MT) (Fig. S2). In contrast, the mutant showed longer growth delays for all monoterpenes tested: 1 d for racemic α-pinene; 2 d for (+)-3-carene, (+)-limonene and MT, and up to 3 d for (−)-β-pinene (Fig. S2). We calculated fungal growth rates in the linear phase that followed such delays. Fig. 3(b) shows that the growth rates of Δ*gcabc-g1* and *Gc* were similar in the presence of racemic α-pinene (7 mm d^{-1}) and only slightly different with (+)-3-carene (5–6 mm d^{-1}). However, the growth rates of the mutant were 52% and 60% lower than *Gc* on (+)-limonene and (−)-β-pinene, respectively. The growth difference between *Gc* and Δ*gcabc*-g1 was significant at all monoterpene levels tested (Fig. S3). These results indicate that *GcABC-G1* supports the growth of *Gc* in the presence of certain monoterpenes.

To further assess GcABC-G1's role in the *Gc* response to monoterpenes, we compared the effects of monoterpenes on asexual spore germination in *Gc* and Δ*gcabc-g1* (Fig. 3c). For *Gc*, germination was not inhibited by racemic α-pinene or (+)-3-carene, and only partially reduced by (+)-limonene and (−)-β-pinene (70%). For the mutant, racemic α-pinene reduced spore germination by only 30%. However, (+)-3-carene, (+)-limonene, and (−)-β-pinene completely prevented spore germination; further, when we removed these monoterpenes after 6 d of incubation and continued the incubation, we found that 90% of Δ*gcabc-g1* spores had been killed.

In contrast to the monoterpenes, individual diterpenes (abietic acid, dehydroabietic acid, isopimaric acid) only slightly inhibited growth of *Gc* and Δ*gcabc-g1*, and growth rates were similar for *Gc* and Δ*gcabc-g1* (Fig. S4).

We had previously reported that *Gc* was able to grow on YNB with a mixture of monoterpenes as a sole carbon source (DiGuistini *et al.*, 2011). Here, we assessed which monoterpenes could support *Gc* growth and whether the utilization of specific monoterpenes was affected in Δ*gcabc-g1*. *Gc* grew on YNB with 200 μl of (+)-limonene as a sole carbon source (Fig. S5) but not with the same amount of racemic α-pinene or (−)-β-pinene. To observe if the deletion of *GcABC-G1* affects limonene utilization, we grew the *GcABC-G1* mutant in the same condition as *Gc* and found that Δ*gcabc-g1* was killed by 200 μl of (+)-limonene on YNB

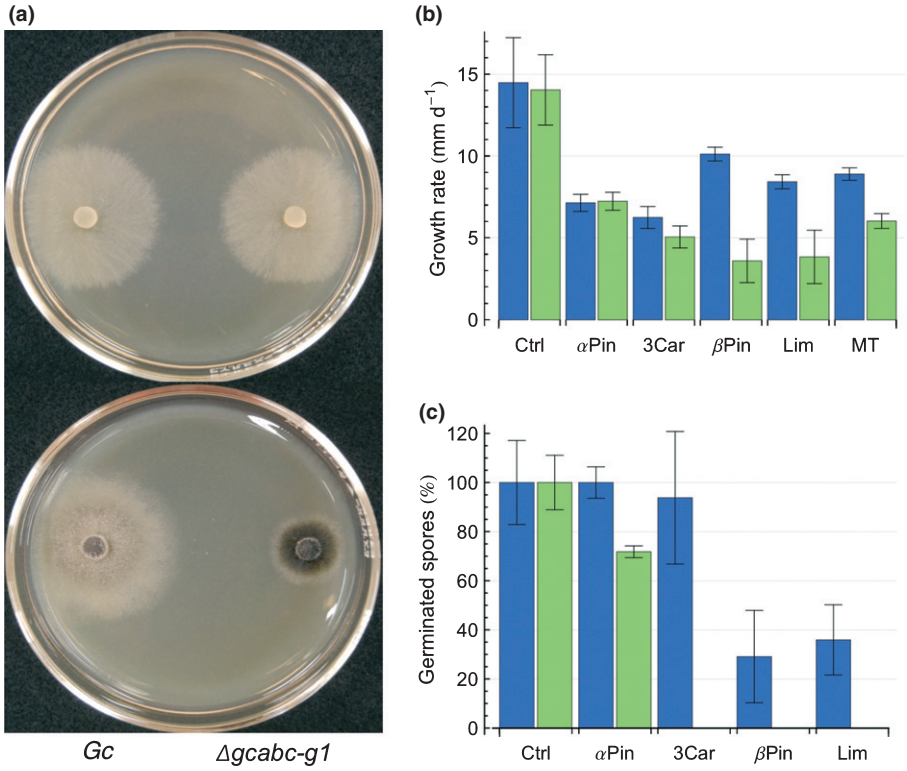

Fig. 3 Effects of monoterpenes on the growth of *Grosmannia clavigera* (*Gc*) and Δ*gcabc-g1* on malt extract agar (MEA). (a) Growth after 4 d without treatment (top) or after 7 d with a 200 μl mixture of monoterpenes (MT) treatment (bottom). (b) Mycelium growth rates with 200 μl of individual or a mixture of monoterpenes. Error bars are 95% confidence intervals on means. Student *t*-test indicated significant difference between *Gc* (blue bars) and Δ*gcabc-g1* (green bars) on Lim, 3Car, βPin and MT ($P < 0.01$), but not on Ctrl and αPin. (c) Asexual spore germination on MEA treated with 200 μl of individual monoterpenes. Germinated spores were counted after 3 d (control) and 6 d (treatment); percentages are relative to the non-treated control. Results are average of five replicates; error bars are standard deviations. Student *t*-test indicated significant difference between *Gc* and Δ*gcabc-g1* on all the monoterpenes ($P < 0.01$), but not on Ctrl. Ctrl, Control; αPin, racemic α-pinene; βPin, (−)-β-pinene; 3Car, (+)-3-carene; Lim, (+)-limonene; MT, (+)-limonene, (+)-3-carene, racemic α-pinene and (−)-β-pinene at a ratio of 5 : 3 : 1 : 1.

medium (Fig. S5), while at 10 μl the mutant survived, but did not grow.

Deleting *GcABC-G1* did not affect sensitivity to other potential PDR substrates

Based on sequence similarity and domain topology (Table 1), GcABC-G1 belongs to the PDR group of transporters, members of which are able to excrete a wide range of chemicals (Rogers *et al.*, 2001; de Waard *et al.*, 2006). To establish whether GcABC-G1 affects the tolerance of *Gc* to compounds other than monoterpenes, we assessed the growth rate of *Gc* and Δ*gcabc-g1* in the presence of other potential PDR substrates: azoles (propiconazole and tebuconazole), antibiotics (cycloheximide, erythromycin), flavonoids (fisetin, quercetin), simple phenolics (benzoic acid, salicylic acid, vanillic acid, gentisic acid) and phytoalexins (catechin, resveratrol, taxifolin). We selected these compounds based on the literature and determined experimentally which concentrations were affecting *Gc* growth rates. On MEA, none of the antibiotics, flavonoids or phytoalexins tested affected the growth of either *Gc* or the mutant. While the azoles and phenolics inhibited fungal growth, there were no inhibition differences between *Gc* and the mutant.

Heterologous *GcABC-G1* expression enhanced survival of *S. cerevisiae* in the presence of monoterpenes

We used heterologous expression of *GcABC-G1* in *S. cerevisiae* (*Sc*) to further assess the role of GcABC-G1 in monoterpene tolerance. *GcABC-G1* was expressed under the control of the *GAL1* promoter, which was induced in a synthetic galactose (SG) medium. *Sc* transformed with the vector only (*Sc-V*), or with the vector containing *GcABC-G1* (*Sc-ABC*), grew at similar rates on yeast extract-peptone-dextrose (YPD) and SG media. However, when *Sc-V* and *Sc-ABC* were spotted onto SG plates and treated with 60 μl of a mixture of monoterpenes, only *Sc-ABC* had grown after 7 d of incubation (Fig. 4a). Monoterpene treatments with > 60 μl per plate killed all yeast cells. These initial tests established that both *Sc-V* and *Sc-ABC* were more sensitive to monoterpenes than the pine pathogen *Gc*. We further tested the survival of *Sc-V* and *Sc-ABC* with individual monoterpenes using a dilution plate assay. When 10^5 yeast cells were spread on SG plates and incubated with 5 μl of individual monoterpene for 3 d, neither *Sc-V* nor *Sc-ABC* survived. When the duration of monoterpene treatments was reduced to 1 h, a sufficient number of *Sc-V* and *Sc-ABC* cells survived to allow for comparative analyses (Fig. 4b). Under conditions of 1-h treatment with

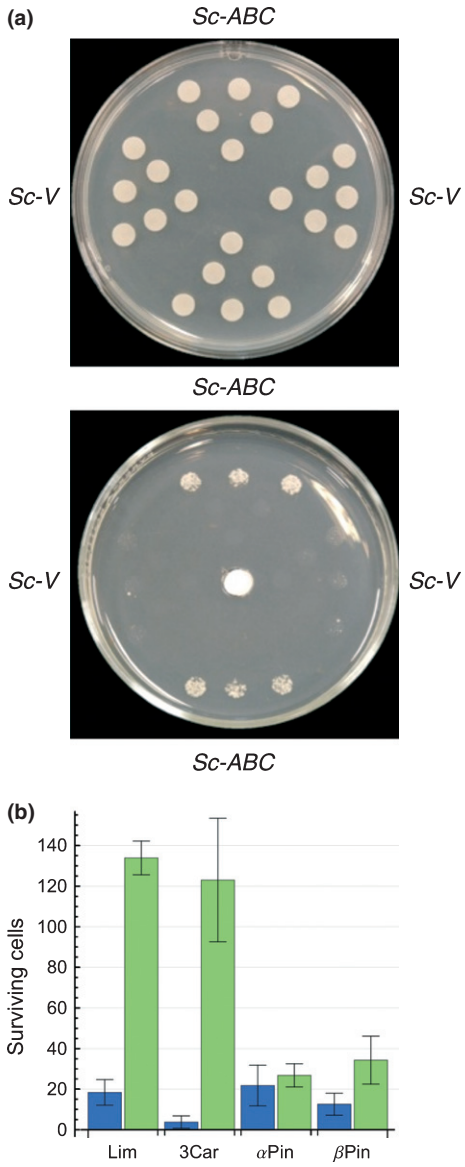

Fig. 4 *GcABC-G1* conferred monoterpene tolerance to *Saccharomyces cerevisiae* (*Sc*). (a) Spot test. Top plate: *Sc* cells transformed with the vector (*SC-V*) or with vector containing *GcABC-G1* (*Sc-ABC*) were spotted (10^5 per spot) on synthetic galactose (SG) media and incubated for 3 d. Bottom plate: *Sc-V* and *Sc-ABC* on SG medium with 60 μl limonene, after a 1-wk incubation. (b) Cell survival after 4 d: 10^5 *Sc-V* (blue bars) and *Sc-ABC* (green bars) cells were spread on SG and treated with 5 μl of individual monoterpenes for 1 h. Results are average of 10 replicates; error bars represent standard deviations. Student *t*-test indicated significant differences between *Sc* and Sc-ABC for all the monoterpenes ($P < 0.01$) except for αPin. Lim, (+)-limonene; 3Car, (+)-3-carene; αPin, racemic α-pinene; βPin, (−)-β-pinene.

(+)-3-carene, 30-times more *Sc-ABC* cells survived than *Sc-V*; for (+)-limonene and (−)-β-pinene, seven- and three-times more *Sc-ABC* cells survived than *Sc-V*. In contrast, with racemic α-pinene, the numbers of surviving cells were low and not significantly different for *Sc-V* and *Sc-ABC* (Fig. 4b). These results showed that the heterologous expression of *GcABC-G1* in *Sc* improved the survival of yeast cells in the presence of some but not all monoterpenes.

Table 2 Summary of *GcABC-G1*-dependent differences in effects of specific monoterpenes on *Grosmannia clavigera* (*Gc*) asexual spores and transformed *Saccharomyces cerevisiae* (*Sc*) cells

Monoterpenes	Observation (A), *Gc* spore survival	Observation (B), transformed *Sc* survival	Comparison of observation (A) and (B)
(+)-Limonene	+	+++	Consistent
(+)-3-carene	+++	+++	Consistent
Racemic α-pinene	+	Δ	–
(−)-β-pinene	+	+++	Consistent

+++, Difference between with and without *GcABC-G1* is > 50%, $P < 0.01$.
+, Difference between with and without *GcABC-G1* is < 50%, $P < 0.01$.
Δ, No significant difference between with and without *GcABC-G1*.

Together, the results obtained in independent experiments with *Gc* and *Sc* support a role for GcABC-G1 in tolerating (+)-limonene, (+)-3-carene and (−)-β-pinene, while effects varied for racemic α-pinene depending on the experimental system (Table 2).

Pathogenicity and detection of *GcABC-G1* transcripts in lodgepole pine inoculated with *Gc* or its *GcABC-G1* mutant (Δ*gcabc-g1*)

To test the effect of the deletion of the *GcABC-G1* gene on the development of symptoms in lodgepole pine, we carried out greenhouse inoculations on stems of young lodgepole pines with *Gc*, mutant Δ*gcabc-g1* or controls without fungus (Fig. 5). Two weeks after inoculation, several trees inoculated with *Gc* showed early symptoms of infection, that is, wilting of growing shoots and browning of needles (Fig. 5a-2); similar symptoms were observed 2–3 d later in the pines inoculated with Δ*gcabc-g1*. During the third and fourth week severe symptoms developed on branches and growing shoots for both *Gc* and Δ*gcabc-g1* (Fig. 5a-3). During the fourth week, all of the needles of several trees had become completely brown, and growing shoots had severely wilted (Fig. 5a-4). Although the numbers of trees that appeared healthy (Fig. 5a-1) was low for both treatments after 4 wk, trees treated with Δ*gcabc-g1* showed a 10–20% higher survival rate in all three experiments compared with trees inoculated with *Gc* (Fig. 5b). For both fungi, we also measured the typical blue/black discoloration in the stem cross-section of the young pine trees that is associated with fungal growth and melanin production. After 4 wk, we observed higher numbers of stem cross-sections with dark stains for *Gc* (81%) than for Δ*gcabc-g1* (21%) (Fig. S6). We were able to reisolate *Gc* and Δ*gcabc-g1* from stem cross-sections and from inner bark, but not from the controls inoculated with MEA agar plugs, confirming that the symptoms were caused by fungal growth and not by wounding.

To assess whether the *GcABC-G1* gene was expressed in *Gc* upon inoculation of trees we measured its transcript levels in stem tissues inoculated with *Gc* or Δ*gcabc-g1* as well as in the controls treated with MEA plugs only. No *GcABC-G1* gene transcripts were detected in the pine tissue treated with the Δ*gcabc-g1* or in

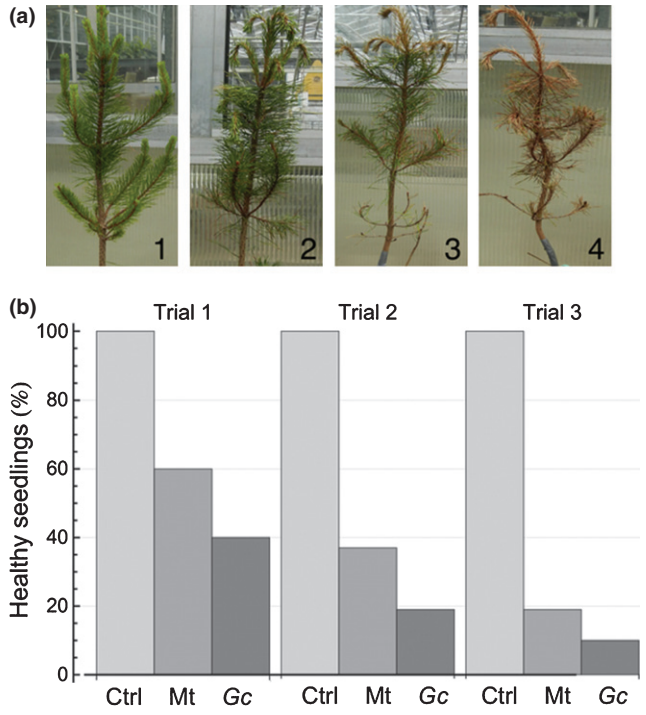

Fig. 5 Pathogenicity tests with young lodgepole pine (*Pinus contorta*) inoculated with *Gc* and Δ*gcabc-g1*. (a) Representative symptoms at different times after fungal inoculation. (1) Healthy tree. The tree in this image was inoculated with a malt extract agar (MEA) agar plug with no fungus. (2) Tree with wilting shoots and browning needles. (3) Tree with brown needles on branches and leader shoots. (4) Tree with all needles and shoots dead. (b) Percentage of healthy trees at 4 wk after inoculation with *Gc* or Δ*gcabc-g1*. Ctrl, agar plug inoculation without fungus; Mt, mutant Δ*gcabc-g1*. Because the three independent experiments were carried out at different times within 2 yr (April 2011, May 2012 and June, 2012), the development of the leader shoots of the healthy trees differed somewhat between the experiments, therefore we show the result of each experiment separately.

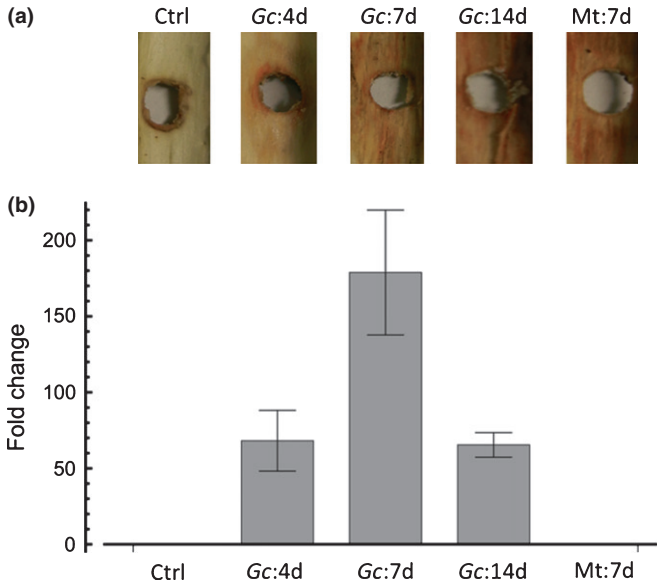

Fig. 6 Relative abundance of the fungal *GcABC-G1* transcript in the phloem of young lodgepole pine (*Pinus contorta*) trees inoculated with *Grosmannia clavigera* (*Gc*) or the *GcABC-G1* mutant (Δ*gcabc-g1*) for 4, 7 and 14 d. (a) Lesion next to the point of inoculation without fungus (control), *Gc* at 4, 7 and 14 d, or its *GcABC-G1* mutant at 7 d (Mt: 7d). (b) *GcABC-G1* transcript abundance at different times after inoculation. Error bars represent standard deviations from three technical replicates. The experiment was repeated three times; repeats showed similar trend to those shown (data not shown).

the controls. However, in trees inoculated with *Gc*, *GcABC-G1* transcripts were clearly detectable at 4, 7 and 14 d after inoculation. As transcript abundance was normalized to fungal β-tubulin transcripts, we were able to detect a temporal profile of increased relative abundance of the *GcABC-G1* transcript with a maximum 178-fold change at 7 d relative to *Gc* grown on MEA (Fig. 6). The observed induction of *GcABC-G1* gene transcripts when *Gc* grows in pine host tissue further supports a role of this gene in the pine–*Gc* interaction *in vivo*.

Comparing the monoterpene response transcriptomes of *Gc* and Δ*gcabc-g1*

GcABC-G1 appears to play a critical role in *Gc*'s tolerance to certain monoterpenes. To assess broader effects of the deletion of *GcABC-G1* in the *Gc*'s response to monoterpenes, we sequenced mRNA libraries of *Gc* and Δ*gcabc-g1* grown for 12 h on MEA, with and without a mixture of monoterpenes. In response to monoterpenes, RNA-seq analyses in Δ*gcabc-g1* identified transcripts for 1312 genes as significantly upregulated and 3459 as downregulated, while in *Gc* 961 genes were upregulated and 2501 genes were downregulated (P-value < 0.05) (Fig. S7). *Gc* and Δ*gcabc-g1* shared 846 upregulated genes (88% of the total upregulated in *Gc*; 64% of the total upregulated in Δ*gcabc-g1*) and 2218 downregulated genes (88% of the total downregulated in *Gc*; 64% of the total downregulated in Δ*gcabc-g1*). Using KEGG (http://www.genome.jp/kegg/) and InterProScan (http://www.ebi.ac.uk/Tools/pfa/iprscan/), we determined that the 466 genes that were upregulated only in the transcriptome of the mutant were mainly involved in metabolism (e.g. carbohydrate/lipid metabolism, macromolecular biosynthesis), genetic information processing (transcription, protein/RNA folding, sorting and degradation, DNA replication and repair), environmental information processing (transportation and signal transduction) and stress responses (Fig. S8). Further, in Δ*gcabc-g1*, 10 ABC transporters showed expression changes; nine of them were upregulated more highly in the mutant than in *Gc* (P-value < 0.05) (Table 3). Genes that were upregulated in both *Gc* and Δ*gcabc-g1* included an acetyl-CoA-acyltransferase, an alcohol dehydrogenase and genes involved in fatty acid metabolism that may be involved in degrading hydrophobic compounds. These genes were also upregulated when *Gc* was grown on YNB with monoterpenes as the sole carbon source.

Discussion

To survive and become established in a pine tree, the MPB symbiont *Gc* has to overcome preformed or induced host defence

Table 3 Comparison of GcABC transporter genes showing transcript abundance differences in *Grosmannia clavigera* (*Gc*) and Δ*gcabc-g1*

	Transcript abundance fold change[a]	
Gene ID	*Gc*	Δ*gcabc-g1*
GcABC-G1	594*	0
GcABC-G2	1.05	1.56*
GcABC-G9	−1.1	2.24*
GcABC-C8	34.96*	3.95
GcABC-C12	1.17	2*
GcABC-C11	2.64	7.39*
GcABC-C3	1.73	5.7*
GcABC-B5	4.43*	10.39*
GcABC-B3	2.37	6.19*
GcABC-D2	1.40	2.05*
GcABC-D1	2.07	4.44*

[a]The abundance was normalized to each strain's nontreatment control.
*Indicate significant values with $P < 0.05$.

chemicals (Bohlmann, 2012). Terpenoids, and specifically monoterpenes, are among the most abundant antimicrobial pine defence chemicals. In previous work, we reported that terpenes induce a stress response and activate a cluster of *Gc* genes that may be involved in detoxification or tolerance of host terpenes, and that monoterpenes can serve as a sole carbon source for *Gc* (DiGuistini *et al.*, 2011). In the present work, we demonstrated a role for GcABC-G1 in tolerance to certain monoterpenes, using a combination of growth experiments with a genetic deletion in *Gc* and heterologous expression in *S. cerevisiae*. We also inoculated young pine trees with *Gc* and the Δ*gcabc-g1* mutant to compare their pathogenicity and their expression of *GcABC-G1* in the host. We propose that *Gc* employs a combination of mechanisms to cope with host defence monoterpenes. The pathogen may control intracellular levels of monoterpenes by the induced expression of an efflux ABC transporter GcABC-G1, and it can metabolize monoterpenes and use them as a carbon source.

The *Gc* genome contains all ABC transporter subfamilies found in closely related species. As indicators of *Gc* having mechanisms for processing xenobiotics, the *Gc* ABC transporter subfamilies include the ABC-B, C and G subfamilies, whose members confer drug resistance in other fungi. RNA-seq expression analysis showed that transcript levels of members of the GcABC-F and GcABC-G subfamilies were upregulated when *Gc* was exposed to terpenes or grown in the presence of monoterpenes as the sole carbon source, but not under other stress conditions tested. The ABC-F subfamily has been reported as essential for cell viability and involved in ribosome biogenesis and translation, which would be activated while the fungus responded to sudden exposure to toxic compounds (Kovalchuk & Driessen, 2010). There are three ABC-G group I transporters in *Gc* (GcABC-G1, GcABC-G2 and GcABC-G3) and they are all upregulated in *Gc* in response to terpenes; GcABC-G1 was the most strongly induced in response to terpenes (i.e. > 100-fold change) while G2 (fourfold changes) and G3 (eightfold change) were expressed at a much lower level. GcABC-G2 has orthologues that are described as pathogenicity factors in *M. grisea* (MGG13624), *Gibberella pulicaris* (GpABC1), and *N. haematococca* (NECHADRAFT_63178) (Urban *et al.*, 1999; Fleissner *et al.*, 2002; Coleman *et al.*, 2011), but its function remains to be tested in *Gc* in the future. The GcABC-G1 protein sequence had the full $(NBF\text{-}TMD)_2$ domain organization that is common in PDR efflux transporters, which are localized in the cytoplasmic membrane in other fungal species; for example, the camalexin exporter BcatrB in *B. cinierea* and the pisatin exporter NhABC1 in *N. haematococca* (Stefanato *et al.*, 2009; Coleman *et al.*, 2011). The literature suggests that fungal PDRs evolved broad substrate specificities to export diverse antifungal compounds, including compounds derived from plants (Sipos & Kuchler, 2006; de Waard *et al.*, 2006; Cannon *et al.*, 2009). However, GcABC-G1 conferred resistance to monoterpenes but not to typical PDR substrates such as azoles, antibiotics, flavonoids, phytoalexins or simple phenolics. GcABC-G1 also had no close orthologues in the large set of ascomycete sequences analysed in this work, suggesting a specialized function as opposed to general xenobiotic transport. When *GcABC-G1* was deleted, the mutant's transcriptome showed an elevated stress response to monoterpenes that included upregulation of other ABC transporters. However, none of these were upregulated to the same extent as *GcABC-G1* in *Gc*, and none appeared to substitute functionally for *GcABC-G1* in the mutant.

GcABC-G1 appears to have evolved as a specialized monoterpene transporter that may allow *Gc* to better colonize a unique ecological niche: the monoterpene-rich tissues of living pine hosts. As Witzke *et al.* (2010) has shown that limonene can freely diffuse into biological membranes, it is plausible that monoterpenes can enter fungal cells by diffusion. While measuring both this volatile monoterpene and the fungal biomass were challenging, we were able to detect (+)-limonene in the young germinating spores of *Gc* or its mutant when exposed to this compound for 1 h, which is too short an exposure time for the fungus to have produced ABC transporter proteins. However, after 18 h, while the level of (+)-limonene remained high in the cells of the mutant, we detected only trace amounts of this monoterpene in *Gc* (Table S3). The data at 1 h are inconsistent with the ABC transporter importing monoterpenes, while the data at 18 h are consistent with induced *GcABC-G1* playing a role in reducing the concentration of monoterpene in the cells. We suggest that a similar process for controlling cellular monoterpene levels would occur in the pine phloem when the MPB vector disperses and so exposes fungal spores and young colonizing mycelia to monoterpenes, where strong inducible expression of *GcABC-G1* should provide an adaptive advantage in survival and growth.

ATP-binding cassette transporters, and particularly the ABC-G group I (PDR) transporters, play a role in plant infection by fungal pathogens by protecting pathogens from exogenous toxic compounds produced by hosts (Urban *et al.*, 1999). Given this, we compared the pathogenicity of *Gc* and its mutant on young pine trees, and measured the transcript abundance of *GcABC-G1* in pine stem tissue inoculated with *Gc* or the mutant. While both *Gc* and the mutant affected the health of young pine trees, symptoms were delayed by 3 d for the mutant, and the survival rate

was higher for the mutant than for *Gc*. These results suggest that *Gc* survives in and colonizes pine trees more efficiently than the mutant, which lacks the ABC transporter. It should not be surprising that the difference in effects caused by *Gc* or its mutant was not more pronounced, given that pine trees combat fungi with a range of chemical defences (Kolosova & Bohlmann, 2012). The results are consistent with our *in vitro* data for monoterpene treatments on MEA, in which the *GcABC-G1* mutant survived the treatment but required 2–3 d more than *Gc* to adapt to the chemical, and then grew more slowly than *Gc*. A role for the GcABC-G1 transporter in pine colonization is further supported by the induction profile of the fungal GcABC-G1 gene in young pine trees inoculated with *Gc*. The absence of transporter expression in young pine inoculated with the deletion mutant, and the early and peak expression at 7 d of the *GcABC-G1* gene, indicate that this transporter plays a role in the early phase of fungal colonization.

The unique ecological pine host niche colonized by *Gc* has high levels of monoterpenes and so would be unsuitable to most microorganisms. For example, in the broad range of niches in which *Sc* strains are found in nature, including grapevine berries, concentrations of terpenes are typically low, and, to this point, no mechanisms for coping with high concentrations of monoterpenes have been reported for *Sc*. The heterologous expression of *GcABC-G1* in *Sc* conferred increased resistance to monoterpenes, consistent with this transporter potentially being an efflux pump that may remove toxic monoterpenes from cells. In the current work, the four monoterpenes assessed were far more toxic to *Sc* than to *Gc* mycelia or germinating *Gc* spores. When exposed for shorter periods of time to certain monoterpenes, more cells survived for *Sc* transformed with *GcABC-G1* than for *Sc* transformed with only the vector. Metabolic engineering of *Sc* and other microorganisms is being actively pursued for the production of monoterpenes and other terpenoids of plant origin as high-value bioproducts and advanced biofuels (Kirby & Keasling, 2009; Fischer *et al.*, 2011; Zerbe *et al.*, 2012). While many plant species, and in particular the resin-producing conifers, develop specialized anatomical structures for sequestering large amounts of low molecular weight terpenoids (Bohlmann & Keeling, 2008), in engineered single-cell production systems, the toxicity of monoterpenes and biofuels may limit yield and performance (Dunlop *et al.*, 2011). As GcABC-G1 may be the first reported eukaryotic ABC transporter with a role in enhanced tolerance against monoterpenes, this gene may be of interest for protein and metabolic engineering geared towards improved terpenoid production in *Sc* and other systems.

Finally, we also demonstrated that *Gc* could process monoterpenes as a carbon source for growth. While conversion of monoterpenoids and diterpenoids into nontoxic compounds has been shown for *Penicillium caseifulvum*, *Aspergillus niger* and *Botryococcus braunii* (de Carvalho & da Fonseca, 2006), to our knowledge, fungal utilization of monoterpenes as carbon sources has not been reported in filamentous fungi. It is important to note that the *GcABC-G1* mutant did not grow at very low dosages of monoterpenes but was able to survive. Except for *GcABC-G1*, when *Gc* and the mutant were grown on MEA with monoterpenes, RNA-seq data showed that similar genes were upregulated (for example, acetyl-CoA-acyltransferase, alcohol dehydrogenase and genes involved in fatty acid metabolism). This suggests that the same enzyme-mediated metabolism pathways were induced in both *Gc* and its mutant. In ongoing work we are creating additional mutants in order to characterize the functions of these genes and their roles in monoterpene utilization.

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Supporting Information

Additional supporting information may be found in the online version of this article.

Fig. S1 PCR and Southern blot to confirm the deletion of the *GcABC-G1*.

Fig. S2 Growth curves of *Gc* and Δ*gcabc-g1* on MEA with and without monoterpenes.

Fig. S3 Growth rate of *Gc* and Δ*gcabc-g1* on MEA with different concentrations of (+)- limonene treatment.

Fig. S4 Individual diterpenes in MEA inhibited the growth of both *Gc* and Δ*gcabc-g1* at similar rates.

Fig. S5 Growth of *Gc* and Δ*gcabc-g1* with different carbon sources.

Fig. S6 Lodgepole pine stem sections stained by *Gc* and Δ*gcabc-g1* at 4 wk after inoculation.

Fig. S7 RNA-seq analysis of differentially expressed genes in *Gc* and the Δ*gcabc-g1* mutant in response to a mixture of monoterpenes.

Fig. S8 Functional grouping of genes exclusively upregulated in the Δ*gcabc-g1* library using InterproScan and KEGG annotation.

Table S1 Subfamily distribution of ABC transporter proteins in various fungal species

Table S2 List of species and proteins used for the phylogenetic analysis of the ascomycota ABC-G group I transporters

Table S3 The fungal intracellular limonene concentration after 1 h and 18 h incubation in *Gc* and its *GcABC-G1* mutant (Δ*gcabc-g1*)

Methods S1 Media composition for fungal growth; measurement of intracellular limonene.

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