Received: 1 September 2021 | Revised: 19 October 2021 | Accepted: 1 November 2021

DOI: 10.1111/mpp.13162

Molecular Plant Pathology WILEY

ORIGINAL ARTICLE

A *Verticillium longisporum* pleiotropic drug transporter determines tolerance to the plant host β-pinene monoterpene

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Funding information
Carl Tryggers Foundation, Grant/Award Number: 19 and 374; Nilsson-Ehle Endowments; Helge Axelsson Johnson Foundation; Svenska Forskningsrådet Formas, Grant/Award Number: 942-2015-36

Abstract

Terpenes constitute a major part of secondary metabolites secreted by plants in the rhizosphere. However, their specific functions in fungal–plant interactions have not been investigated thoroughly. In this study we investigated the role of monoterpenes in interactions between oilseed rape (*Brassica napus*) and the soilborne pathogen *Verticillium longisporum*. We identified seven monoterpenes produced by *B. napus*, and production of α-pinene, β-pinene, 3-carene, and camphene was significantly increased upon fungal infection. Among them, β-pinene was chosen for further analysis. Transcriptome analysis of *V. longisporum* on exposure to β-pinene resulted in identification of two highly expressed pleotropic drug transporters paralog genes named *VlAbcG1a* and *VlAbcG1b*. Overexpression of *VlAbcG1a* in *Saccharomyces cerevisiae* increased tolerance to β-pinene, while deletion of the *VlAbcG1a* homologous gene in *Verticillium dahliae* resulted in mutants with increased sensitivity to certain monoterpenes. Furthermore, the VlAbcG1a overexpression strain displayed an increased tolerance to β-pinene and increased virulence in tomato plants. Data from this study give new insights into the roles of terpenes in plant–fungal pathogen interactions and the mechanisms fungi deploy to cope with the toxicity of these secondary metabolites.

KEYWORDS

ABC-transporters, *Brassica napus*, monoterpenes, soilborne pathogen, β-pinene

1 | INTRODUCTION

Plant roots exude a plethora of chemical compounds into the rhizosphere, the soil environment surrounding the root system. These compounds include ions, free oxygen, water, enzymes, and a diverse array of secondary metabolites that play important roles in microbe attraction to the roots (Bais et al., 2006; Hosseini et al., 2013). In the rhizosphere, plant roots interact with a variety of organisms such as insects, nematodes, and a vast range of microorganisms, such as fungi, bacteria, and protozoans. These interactions can be beneficial, pathogenic, or neutral for the plant (Campos-Soriano et al., 2012; Pineda et al., 2010; Zamioudis & Pieterse, 2012). Regarding the pathogenic interactions between plants and microbes, plant roots secrete antimicrobial compounds to defend themselves (Bais et al., 2006; Bednarek et al., 2005).

Terpenes, a class of volatile secondary metabolites, constitute a major portion of root exudates (Bais et al., 2006). They are

synthesized by terpene synthases from five-carbon isoprene units (C_5), leading to monoterpenes (C_{10}), sesquiterpenes (C_{15}), diterpenes (C_{20}), sesterterpenes (C_{25}), triterpenes (C_{30}), and carotenoids (C_{40}), while the modified ones contain oxygen in various functional groups and are called terpenoids. The role of these compounds in plant–plant and plant–herbivore signalling is widely proven, while their involvement in plant–fungal pathogen interactions is still obscure (Boncan et al., 2020). The involvement of certain terpenes in fungal behaviour such as growth and conidiation, either inhibitory or stimulatory, has previously been reported (Fiers et al., 2013; Kadoglidou et al., 2011; Lucini et al., 2006; Roos et al., 2015). The inhibitory effect of terpenes against microorganisms varies and the exact mechanisms are not fully understood. In bacteria, for example, the mode of action includes degradation of the cell wall, disruption of cytoplasmic membranes, increased permeability, and hydrolysis (Nazzaro et al., 2013).

Fungal pathogens have developed mechanisms to efflux endogenous and exogenous chemical compounds out of their cells using transmembrane transporter proteins, such as ATP-binding cassette (ABC) and major facilitator superfamily (MFS) ones (Coleman & Mylonakis, 2009; Dos Santos et al., 2014). The ABC transporters are well known for their role in drug resistance and cellular detoxification of fungal cells (Coleman & Mylonakis, 2009). They are divided into eight groups (A–H), and those belonging to groups B, C, and G are referred to as multidrug resistance (MDR), multidrug resistance-associated proteins (MRP), and pleiotropic drug resistance (PDR) (Kovalchuk & Driessen, 2010; Paumi et al., 2009). Expansion of transporter gene families has been observed in the genome of mycoparasitic fungi, followed by increased tolerance to xenobiotic chemical substances (Karlsson et al., 2015; Nygren et al., 2018).

Verticillium species are ascomycete fungi responsible for Verticillium wilt disease in a plethora of cultivated and wild plants. More than 200 plant species can be infected by *Verticillium dahliae*, for example cotton, tomato, sugar beet, and olive (Pegg & Brady, 2002). *Verticillium longisporum* is an amphidiploid hybrid derived from several independent hybridization events between *V. dahliae* or *V. dahliae*-like species (named D1 to D3) and an unknown species named A1 (Inderbitzin et al., 2011). This fungus was first reported in oilseed rape fields in southern Sweden and shows a preference for Brassicaceae plants (Eynck et al., 2007; Kroeker, 1970; Tzelepis et al., 2017). *V. longisporum*, like *V. dahliae*, can form long-lived, melanized resting structures called microsclerotia. The microsclerotia remain dormant until stimulated by root exudates from host roots and germinate through a still-unknown mechanism. The germinating microsclerotia can infect the host through the roots and colonize the vascular system. After entering the host, for example *Brassica napus*, *V. longisporum* incites stunting, chlorosis, and premature senescence and causes severe yield losses, especially in northern Europe (Depotter et al., 2016; Dunker et al., 2008; Johansson et al., 2006a).

The aim of this study was to investigate the role of plant terpenes in *B. napus*–*V. longisporum* interactions using a comprehensive approach of gas chromatography (GC), transcriptomics, heterologous gene expression, and generating fungal mutant strains. Our results showed that the production of certain monoterpenes was increased in *B. napus* upon infection with *V. longisporum*, triggering induction of a fungal ABC transporter gene. We also showed that this transporter is involved in tolerance against monoterpenes, giving new insights into the molecular mechanisms fungal phytopathogens deploy to cope with these plant defences.

2 | RESULTS

2.1 | *B. napus* roots exude higher amounts of certain monoterpenes upon infection with *V. longisporum*

To investigate the potential role of monoterpenes in *B. napus*–*V. longisporum* interactions, *B. napus* plants were infected with *V. longisporum* and symptoms were recorded 21 days postinoculation (dpi). The infected plants displayed clear stunting symptoms as compared to the noninfected ones (Figure 1a). To analyse the monoterpenes produced by plant roots, silastic probes were dipped to the soil next to the plant stems and analysed by gas chromatography-mass spectrometry (GC/MS). Our analysis revealed that among the monoterpenes identified, α- and β-pinene, 3-carene, and camphene showed a significant increased production in infected plants as compared to the mock-inoculated ones (Figure 1b, Figure S1).

2.2 | Certain monoterpenes showed strong fungistatic activity

Previous studies showed that certain monoterpenes can inhibit the conidial germination and mycelial growth in a variety of fungal species including *Verticillium* spp. (Kadoglidou et al., 2011; Lucini et al., 2006). Thus, we investigated the role of these monoterpenes on *V. longisporum* mycelial growth by measuring the mycelial biomass in growth media supplemented with different percentages of monoterpenes. A significant reduction in the mycelial dry weight was recorded in the media supplemented with all tested monoterpenes (Figure 1c). Because β-pinene showed significant fungistatic activity in all tested conditions, we further investigated whether it could also affect the conidial germination rate. To test this, *V. longisporum* conidia were grown in different β-pinene concentrations and a significantly reduced germination rate was observed in all tested concentrations (Figure 1d). These results suggest a clear fungistatic effect of these three monoterpenes against *V. longisporum* growth.

2.3 | Two fungal ABC-transporter-encoding genes were highly induced on early exposure to β-pinene

The specific mechanisms that fungi deploy to cope with plant terpenes are not fully understood and many aspects remain to be elucidated. To identify genetic factors associated with terpene tolerance, the *V. longisporum* transcriptomic response was analysed

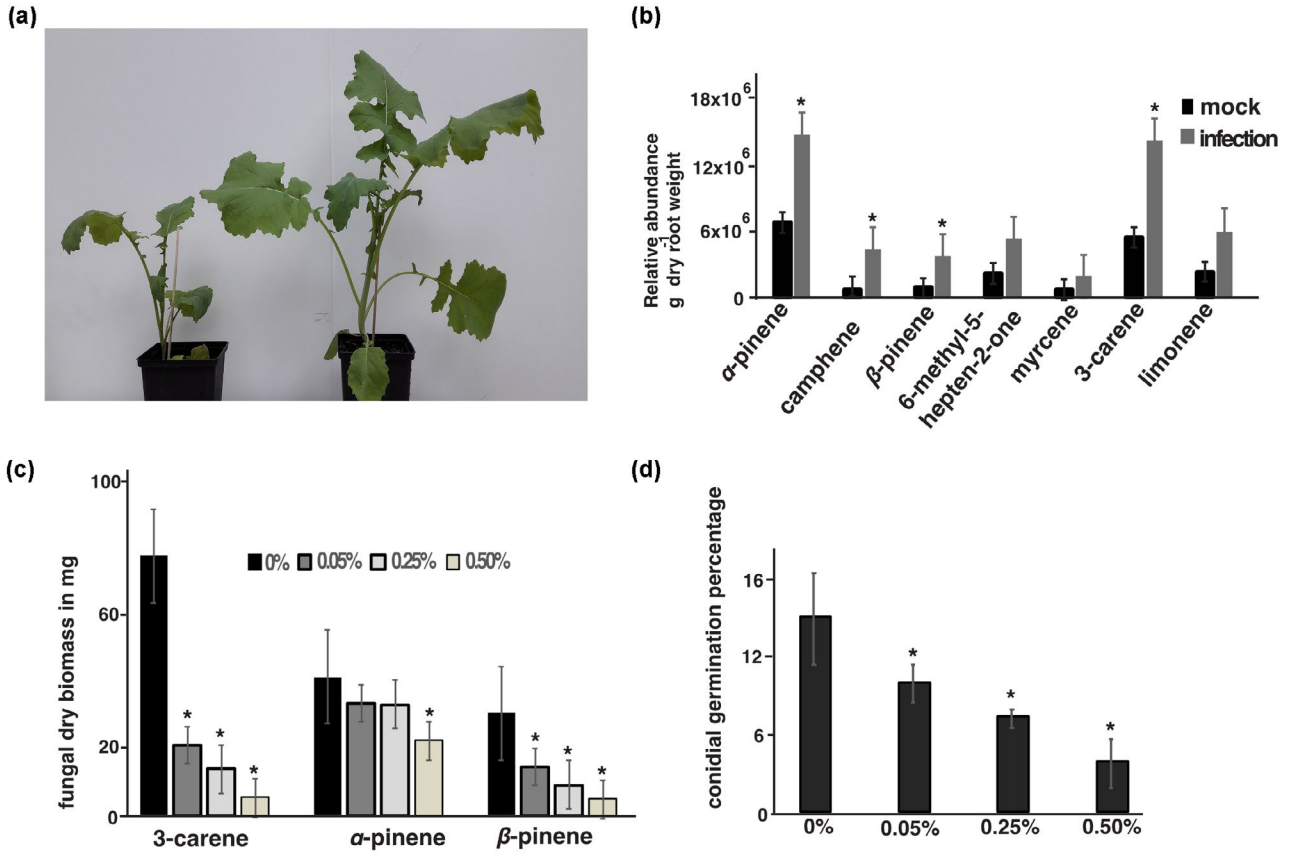

FIGURE 1 Assays in *Verticillium longisporum–Brassica napus* interactions. (a) Representative symptoms in infected (left) versus mock inoculated (right) *B. napus* plants, 21 days after infection with *V. longisporum*. (b) Relative abundance of the identified monoterpenes per gram of dry root in *V. longisporum*-infected and mock-inoculated plants. Error bars represent the standard error (*SE*) based on five biological replicates. Asterisks (*) indicate statistically significant differences between infected and mock-inoculated plants according to Student's *t* test ($p < 0.05$). (c) *V. longisporum* mycelial dry biomass on exposure to different concentration of monoterpenes previously identified in plant roots 5 days postinoculation. Mycelial growth on 0.5% dimethyl sulphoxide (DMSO) was used as a control. Error bars represent *SE* based on three biological replicates. Asterisks (*) indicate statistically significant differences between 0% of monoterpene and the rest of percentages (0.05%, 0.25%, and 0.50%) according to Student's *t* test ($p < 0.05$). (d) Percentage of *V. longisporum* conidial germination on exposure to different concentrations (0%, 0.05%, 0.25%, and 0.5%) of β-pinene 48 h postinoculation. Exposure to 0.5% DMSO was used as a control. Error bars represent *SE* based on three biological replicates. Asterisks (*) indicate statistically significant differences between 0% β-pinene and the other percentages (0.05%, 0.25%, and 0.50%) according to Student's *t* test ($p < 0.05$)

during exposure to β-pinene. This monoterpene was selected for transcriptome analysis as it showed relatively high inhibitory activity against *V. longisporum* mycelial growth. The RNA-sequencing (RNA-Seq) analysis revealed a cluster of 10 genes that were significantly induced in *V. longisporum* upon exposure to β-pinene in all tested time points (8, 24, 48 hours postinoculation [hpi]) as compared to 0 hpi control (Figure 2a, Table S1). This cluster includes genes putatively coding for an acetyltransferase (*DN17484*), an oxygenase (*DN5328*), and a glycolipid transfer protein (*DN15275*) (Figure 2a, Table S1). A putative glycosyl transferase family 4 (*DN14500*) gene and a putative transposase (*DN11913*) also showed induction 8 and 24 hpi (Figure 2a, Table S1). Finally, two genes coding for ABC transporters (*DN18642* and *DN18182*) were highly induced during early exposure to this monoterpene (Figure 2a, Table S1). Among the down-regulated genes, genes coding for putative glycoside hydrolases (GH) belonging to families 17 and 18 (*DN13345* and *DN17665*) were identified (Figure 2b, Table S2). The family GH17 includes enzymes with 1,3-β-glucosidase (EC 3.2.1.39), lichenase (EC 3.2.1.73), and exo-1,3-glucanase (EC 3.2.1.58) activities, while the GH18 family includes fungal enzymes with chitinolytic activity and endo-β-*N*-acetyl-glycosaminidases (ENGases) (Rafiei et al., 2021; Tzelepis & Karlsson, 2019). Furthermore, down-regulation of a gene coding for a putative hydrophobin (*DN10012*) was also observed (Figure 2b, Table S2). The RNA-Seq analysis was validated by reverse transcription quantitative PCR (RT-qPCR). Six genes were selected and the results followed the same induction patterns (Figure S2).

2.4 | Both induced ABC transporters belong to G-I subgroup

The role of drug resistance ABC transporters in the efflux of secondary metabolites has previously been demonstrated (Coleman

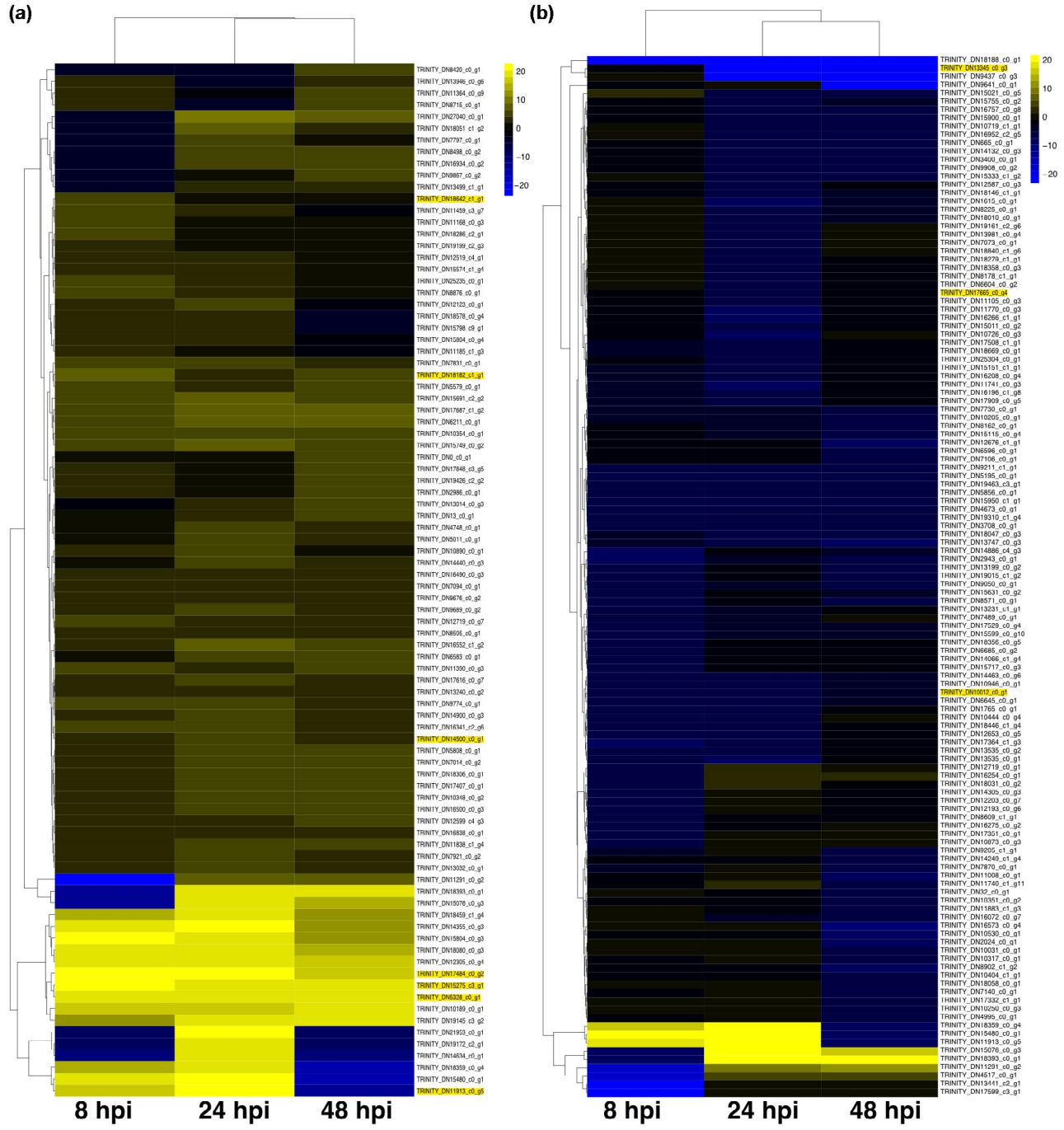

FIGURE 2 Transcription profiles of *Verticillium longisporum* genes on exposure to β-pinene in different time points 8, 24, and 48 h post-inoculation (hpi). (a) Up-regulated genes and (b) down-regulated genes. Data were normalized to the 0 hpi time point exposure (adjusted *p* value <0.05, absolute $\log_2$ fold change >2 for up-regulated genes and <−2 for the down-regulated ones). Yellow and blue represent up-regulated or down-regulated genes, respectively. The heatmaps show the 50 most up- or down-regulated genes. Genes mentioned in the text are highlighted with yellow

& Mylonakis, 2009). Thus, the two genes *BN1708_013935* (*DN18642*) and *BN1708_012316* (*DN18182*), encoding putative ABC-transporters, were selected for further analysis. Phylogenetic analysis showed that both ABC transporters belong to group G, referred to as pleiotropic drug resistance (PDR) (Figure 3a) (Kovalchuk & Driessen, 2010). Because group G is subdivided into seven subgroups (I–VII), a second phylogenetic analysis was conducted among homologs belonging to these subgroups. Our analysis showed that both the ABC transporters are paralogs of the same gene and clustered with ABC transporters of subgroup G-I. The gene models *BN1708_013935* and *BN1708_012316* were denominated as *VlAbcG1a* and *VlAbcG1b*, respectively (Figure 3b).

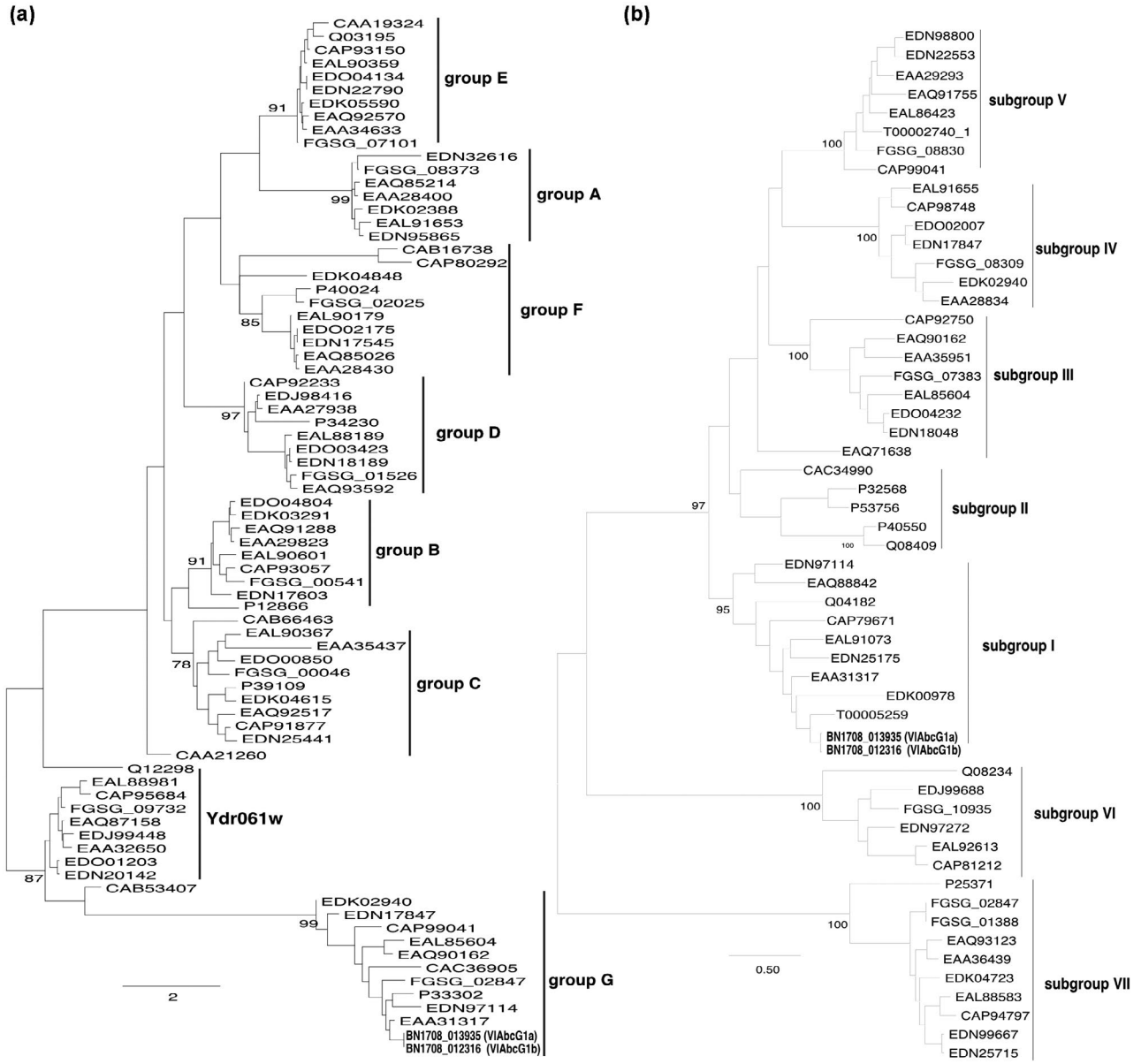

FIGURE 3 Phylogeny of the VlAbcG1a and VlAbcG1b ABC transporters. (a) Phylogenetic analysis of ABC transporters from different groups (A–G). (b) Phylogenetic analysis of ABC transporters from different G subgroups (I–VII). Analysis was conducted using the maximum likelihood with the LG+G amino acid substitution model based on amino acid sequences and 500 bootstraps. Number at nodes indicate the bootstrap values. Bar indicates the number of amino acid substitutions. Predicted amino acid sequences were aligned using the ClustalX algorithm and phylogeny was constructed in the MEGA X software. Bootstrap support values from 500 iterations are associated with the nodes

2.5 | Heterologous expression of *VlAbcG1a* in *Saccharomyces cerevisiae* enhanced tolerance to β-pinene

To investigate the potential role of VlAbcG1a and VlAbcG1b in monoterpene tolerance, homologs of VlAbcG1a and VlAbcG1b in *S. cerevisiae* were identified. The phylogenetic analysis showed that VlAbcG1a and VlAbcG1b are close to the three G-I ABC transporters Pdr5, Pdr10, and Pdr15 (Figure 4a). Then, the tolerance of *S. cerevisiae PDR5*, *PDR10*, and *PDR15* deletion strains to β-pinene was determined by measuring their growth rates in yeast extract peptone dextrose (YPD) medium supplemented with 0.003% β-pinene. The optimum concentration of β-pinene (0.003%) was selected based on successive screening of *S. cerevisiae* to this compound (Figure S3). The deletion strains (Δ*PDR5*, Δ*PDR10*, and Δ*PDR15*) showed significant reduction in growth rate in YPD medium supplemented with β-pinene between 10 and 16 hpi compared to *S. cerevisiae* wild type (WT) at the same time points ($p \leq 0.005$), indicating a role of these proteins in the efflux of β-pinene (Figure 4b). As expected from the phylogenetic analysis (Figure 4a), among the three deletion strains, Δ*PDR10* showed higher sensitivity to β-pinene even after 24 hpi ($p < 0.001$). However, the growth rate differences were no

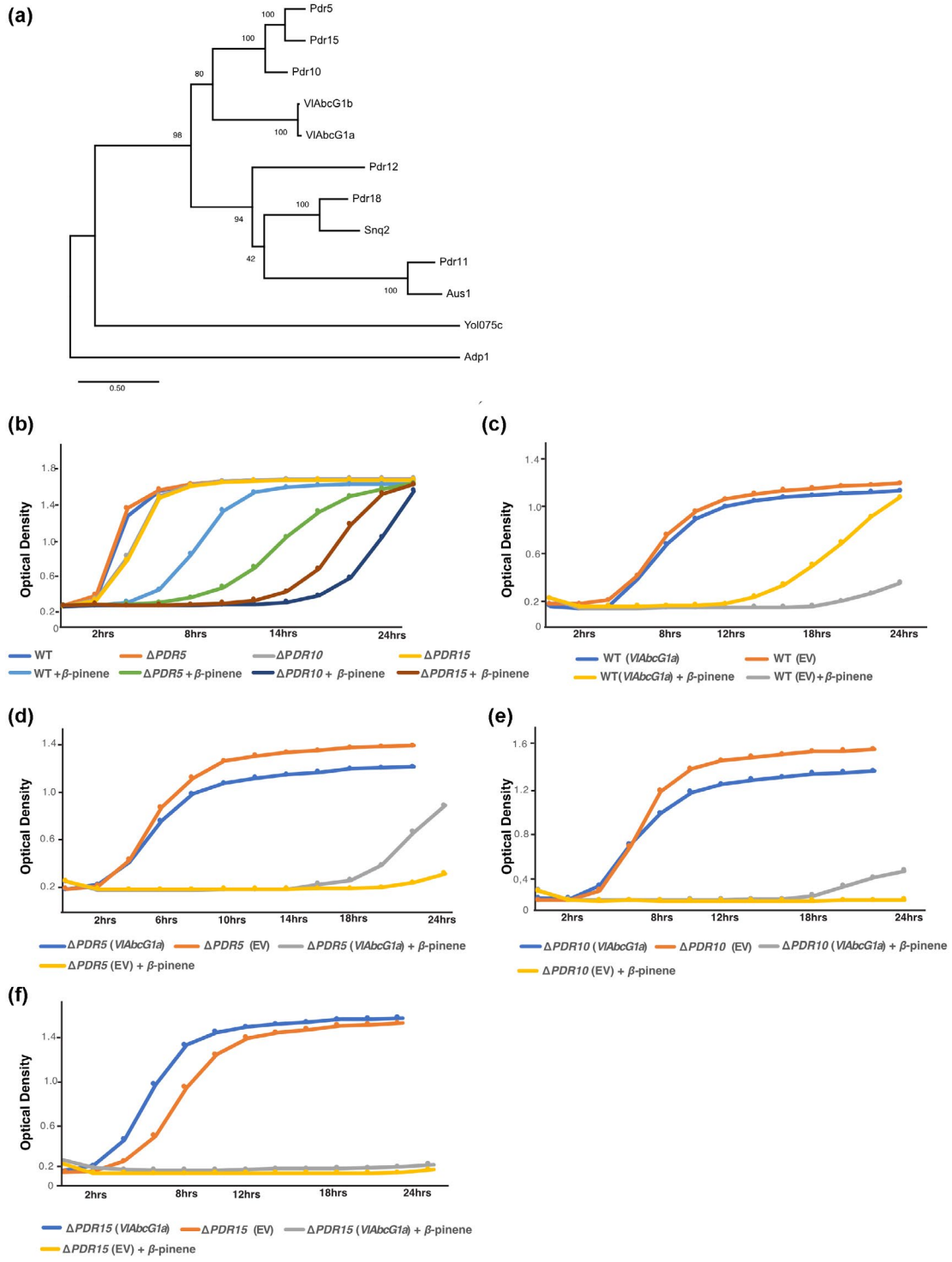

FIGURE 4 Analysis of the *VlAbcG1a* gene in *Saccharomyces cerevisiae*. (a) Phylogenetic analysis of *VlAbcG1a* and *VlAbcG1b* and ABC transporters from *S. cerevisiae*. Analysis was conducted using the maximum likelihood with the LG+G amino acid substitution model based on amino acid sequences and 500 bootstraps. Numbers at nodes indicate the bootstrap values. Bar indicates the number of amino acid substitutions. (b) Growth of *S. cerevisiae* wild-type (WT) and ABC-transporter deletion strains Δ*PDR5*, Δ*PDR10*, and Δ*PDR15* homologs to *VlAbcG1a* and *VlAbcG1b* on exposure to 0.003% β-pinene solubilized in dimethyl sulphoxide (DMSO). (c) Growth of *S. cerevisiae VlAbcG1a* overexpression strains in the WT background [WT (*VlAbcG1a*)] on exposure to β-pinene. (d–f) Growth of *S. cerevisiae* Δ*PDR5* (*VlAbcG1a*), Δ*PDR10* (*VlAbcG1a*), and Δ*PDR15* (*VlAbcG1a*) strains overexpressing *VlAbcG1a* upon exposure to β-pinene. In all assays, *S. cerevisiae* WT or PDR deletion strains transformed with the empty pYES-2 vector (EV) and grown in 0.003% DMSO were used as controls. Statistical analysis was conducted based on five biological replicates using the Fisher's test ($p < 0.05$)

longer detected after prolonged incubation for 24 h. No difference in growth rate between the WT and deletion strains was found in control YPD medium at the same time points ($p > 0.993$) (Figure 4b).

Furthermore, *S. cerevisiae* strains overexpressing *V. longisporum VlAbcG1a* in the WT background [WT (*VlAbcG1a*)] and in the Δ*PDR5*, Δ*PDR10*, and Δ*PDR15* deletion strains background [Δ*PDR5* (*VlAbcG1a*), Δ*PDR10* (*VlAbcG1a*), and Δ*PDR15* (*VlAbcG1a*)] were generated. The *S. cerevisiae* WT and PDR deletion strains transformed with the empty vector [WT (EV) and PDR (EV), respectively] were used as controls. A significant increase in growth of the *S. cerevisiae* [WT (*VlAbcG1a*)] strain was found in the presence of β-pinene compared to WT (EV) at 12 hpi ($p < 0.001$), while no differences were observed between the strains in the absence of the monoterpene ($p > 0.072$) (Figure 4c). Likewise, growth of the Δ*PDR5* (*VlAbcG1a*) strain was significantly higher at 22 hpi in medium supplemented with β-pinene as compared to Δ*PDR5* (EV) ($p < 0.001$) (Figure 4d). However, no significant differences in growth rate were observed between Δ*PDR10* (*VlAbcG1a*) or Δ*PDR15* (*VlAbcG1a*) strains and empty vector Δ*PDR10* (EV) or Δ*PDR15* (EV) controls, respectively ($p > 0.072$) (Figure 4e,f). These data further support that the ABC transporter VlAbcG1a is involved in the β-pinene detoxification process.

2.6 | Deletion of the *VlAbcG1a* homolog in *V. dahliae* increased susceptibility to monoterpenes

Because *V. longisporum* is a hybrid species and generation of gene deletion mutants is challenging, we selected *V. dahliae*, which is one of the its parent species, for functional analysis of VlAbcG1. We identified the *VDAG_01167* gene in *V. dahliae* as a homolog to the *V. longisporum VlAbcG1a* and *VlAbcG1b*. *VDAG_01167* showed 98% amino acid similarity, and structural analysis of the amino acid sequence showed domains identical to *VlAbcG1*. The biological role of *VDAG_01167* was characterized by generating gene deletion strains by homologous recombination. The *VDAG_01167* deletion strain with the correctly integrated deletion cassette was identified after screening more than 150 hygromycin-resistant fungal colonies using PCR as described before (Dubey et al., 2020) (Figure S4a). Furthermore, gene expression analysis showed no expression of the *VDAG_01167* gene in three independent single spore colonies, while expression was detected in the WT (Figure S4b). In addition, a *V. dahliae VlAbcG1a* overexpression strain (*VlAbcG1a*+) driven by the *gpdA* promoter was generated in the *V. dahliae* WT background. RT-qPCR analysis showed high expression levels of this gene in 10 selected single-spore hygromycin-resistant fungal colonies (Figure S4c). Among them the isolate number 6, which showed the highest expression levels, was chosen for the phenotypic and virulence assays.

First, we investigated whether deletion or overexpression affected colony morphology and fungal growth. In vitro assays on potato dextrose agar (PDA) plates showed no significant differences in growth rate and colony morphology between the WT and the mutant strains (data not shown). However, in the presence of α-pinene, β-pinene, or 3-carene biomass of the deletion strain (Δ*VDAG_01167*) was significantly decreased as compared to the WT (Figure 5), while biomass of the overexpression strain (*VlAbcG1a*+) was significantly increased only during exposure to β-pinene, further supporting the importance of this transporter in monoterpene efflux process.

2.7 | Overexpression of the *VlAbcG1a* in *V. dahliae* increased virulence in tomato plants

V. dahliae infection causes stunting in plants. This is used as a representative symptom to evaluate the virulence of *V. dahliae* strains in tomato plants (Fradin et al., 2009; Leonard et al., 2020). It is also known that tomato plants exude β-pinene (Falara et al., 2011). Thus, the role of Δ*VDAG_01167* in *Verticillium* virulence was investigated on tomato plants using pot experiments under glasshouse conditions infected with the *V. dahliae* strains. No significant differences in shoot length were recorded between plants infected with the WT and the *VDAG_01167* deletion strain 28 dpi (Figure 6a,b). However, the shoot length of tomato plants infected with the *VlAbcG1a*+ overexpression strain was significantly reduced compared to the WT and Δ*VDAG_01167* infected plants (Figure 6a,b), indicating a role of this gene in the fungal infection process. Reisolation of *V. dahliae* strains from infected plant stems, but not from the mock-inoculated ones, confirmed that the symptoms were caused by this pathogen. The virulence of these mutant strains was also evaluated on *Arabidopsis thaliana*. Our results did not show any significant difference in rosette growth between plants infected with WT and *V. dahliae* mutant strains 21 dpi (Figure S5a,b).

3 | DISCUSSION

Terpenes are secondary metabolites produced by a plethora of organisms, including plants. In plants the role of terpenes has mainly been reported in plant–insect interactions, where they are involved in defence against herbivores (Boncan et al., 2020; Herde et al., 2008; Hong et al., 2012). Fungistatic activity of certain monoterpenes such as carvacrol, carvone, and 1,8-cineole against many soilborne fungal species has previously been reported under in vitro conditions (Kadoglidou et al., 2011). However, studies about the exact role of plant terpenes in fungal pathogen–host interactions are scarce. In the current study we investigated the role of plant monoterpenes in *B. napus*–*V. longisporum* interactions and demonstrated a role of the PDR transporter VlAbcG1 in tolerance to these secondary metabolites. We propose that monoterpenes could play an important role in host–pathogen interactions, while *V. longisporum* deploys specific ABC transporters to cope with the emission of these compounds from *B. napus* roots to establish a successful infection.

First, we identified seven different monoterpenes emitted by *B. napus* roots, and four of them showed increased production on

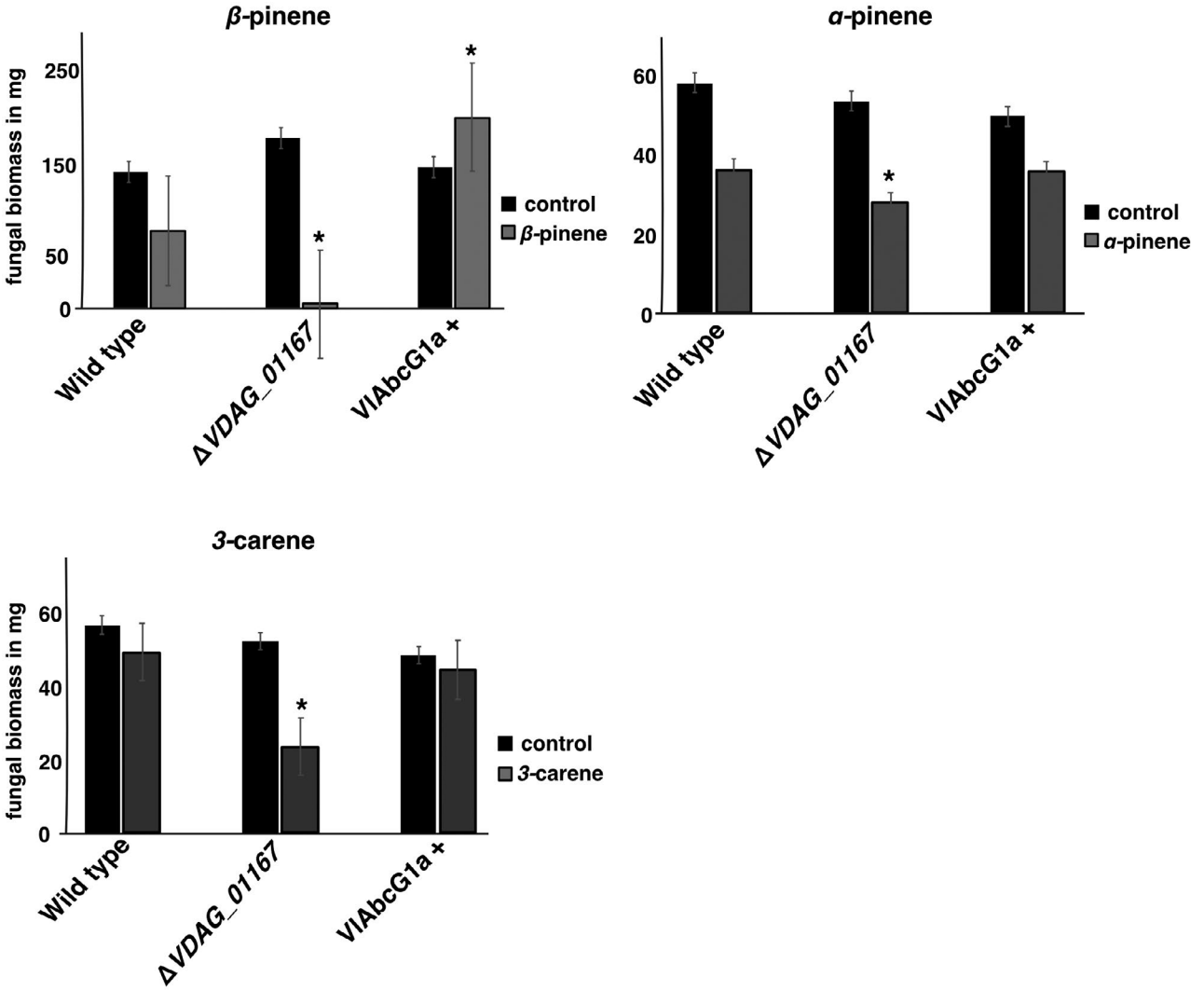

FIGURE 5 Functional analysis of the *VDAG_01167* gene in *Verticillium dahliae*, homolog to *VlAbcG1a*. *V. dahliae* mycelia from the wild type (WT), the *VDAG_01167* deletion (Δ*VDAG_01167*), and overexpression (VlAbcG1a+) strains, were exposed to 0.05% β-pinene, α-pinene, and 3-carene. Fungal biomass was measured 5 days after exposure. *V. dahliae* strains grown in 0.05% dimethyl sulphoxide (DMSO) were used as controls. Error bars represent *SE* based on five biological replicates. Asterisks (*) indicate statistically significant differences between columns of same colour according to Student's *t* test ($p < 0.05$)

infection with *V. longisporum*, indicating their involvement in plant resistance mechanisms against this pathogen. These results are in line with previous findings, where induced production of monoterpenes was reported in *A. thaliana* and barley roots during interactions with the plant pathogens *Alternaria alternata*, *Pseudomonas syringae*, *Cochliolobus sativus* and *Fusarium culmorum* (Fiers et al., 2013; Steeghs et al., 2004). Regarding the *A. thaliana*–*V. longisporum* interactions, Roos et al. (2015) showed that genes involved in terpene biosynthesis were induced on infection, indicating an involvement of these secondary metabolites in this pathosystem. Furthermore, induction of the *TPS23/27* gene, which is involved in synthesis of the monoterpene 1,8-cinole, was observed in *NDR1 A. thaliana* mutant lines, which show increased susceptibility to *V. longisporum* (Johansson et al., 2006b; Roos et al., 2015).

The strategies that plant-pathogenic fungi employ to overcome the toxic effects of terpenes are not well known. The fungistatic effect of 3-carene, α-pinene, and β-pinene on *V. longisporum* mycelial growth, which is similar to the previous reports in other fungal species, led us to investigate the mechanism associated with terpene tolerance in pathogenic fungi (Kadoglidou et al., 2011). We analysed the transcriptomic response of *V. longisporum* on exposure to β-pinene and identified two genes coding for a PDR transporter. The role of ABC transporters in the efflux of xenobiotic compounds in many fungal species has been previously shown (Nygren et al., 2018; Samaras et al., 2021). The increased β-pinene tolerance of *S. cerevisiae* strains overexpressing *VlAbcG1a* in the WT and Δ*PDR5*, background further support the crucial involvement of this transporter in the efflux of this secondary metabolite. In addition, the reduced ability of *V. dahliae VDAG_01167* (homolog to *VlAbcG1a*) deletion strain to tolerate monoterpenes, followed by the increased ability of the overexpression strains to tolerate β-pinene, corroborates the role of VlAbcG1a transporter in the detoxification process.

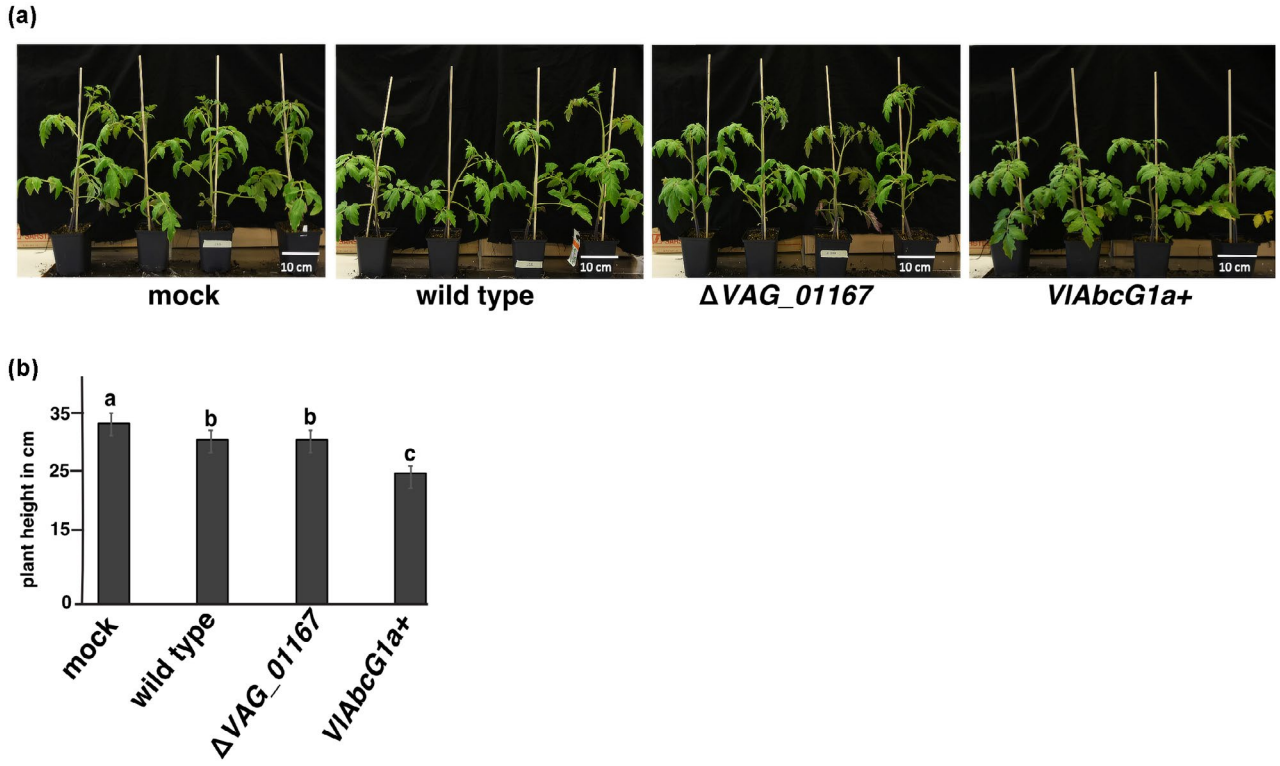

FIGURE 6 Virulence assay in tomato plants. (a) Representative plants infected with *Verticilium dahliae* wild type (WT), deletion (Δ*VDAG_01167*), and overexpression (*VlAbcG1a+*) strains or mock-inoculated 28 days post-inoculation (dpi). (b) Plant height in cm after infection with *V. dahliae* WT, deletion (Δ*VDAG_01167*), overexpression (*VlAbcG1a+*) strains, or mock-inoculated 28 dpi. Error bars represent *SE* based on three biological replicates each contains 10 plants. Letters (a, b, c) indicate statistically significant differences according to Student's *t* test ($p < 0.05$)

Likewise, increased fungal virulence of the *VlAbcG1a+* overexpression strain in tomato plants implies an active role of this transporter in fungal pathogenesis, possibly by protecting *Verticillium* from the toxic compounds produced by these plants, as has previously been demonstrated (Urban et al., 1999). Similarly, a role of group G-I ABC transporters in tolerance to monoterpenes and pathogenesis has previously been reported in *Grosmannia clavigera*, a bark beetle-associated fungal pathogen of pine trees (Wang et al., 2013).

In conclusion, in the current study we investigated the role of terpenes in *V. longisporum–B. napus* interactions. We showed that plant roots emitted higher amounts of certain monoterpenes during infection, and these compounds displayed fungistatic activity. A specific fungal ABC transporter was induced on exposure to a certain monoterpene and our functional analysis in *S. cerevisiae* and *V. dahliae* confirmed the involvement of this plasma membrane transporter in the detoxification process, although further studies are needed to elucidate the precise role of these secondary metabolites in plant defence mechanisms.

4 | EXPERIMENTAL PROCEDURES

4.1 | Fungal strains and growth conditions

The *V. longisporum* isolate VL1 (CBS110220) and *V. dahliae* strain JR2 were used in the current study (Fogelqvist et al., 2018; Steventon et al., 2002). Isolates were kept on potato dextrose agar (PDA; Difco) at 20°C in darkness and sporulation was induced on potato dextrose broth medium (PDB; Difco) at 20°C in 12 h light 7 dpi.

4.2 | Monoterpenes extraction and analysis

For monoterpenes extraction the solid-phase root zone extraction (SPRE) method was used with minor modifications (Kallenbach et al., 2014; Mohney et al., 2009; Weidenhamer et al., 2009). Briefly, *B. napus* 'Hannah' plants were grown in greenhouse conditions for 2 weeks at 20–25°C and 16 h light and 8 h darkness in commercial peat soil. Plants were carefully uprooted, washed with distilled water, and dipped in a *V. longisporum* conidial suspension (10^6 conidia/ml in distilled water) for 15 min. Mock-inoculated plants were treated in exactly the same way and dipped for 15 min only in distilled water. For monoterpene extraction from roots, silastic tubes (Thermo Fisher Scientific) were used. Probes were prepared by cutting the tubes into 5-cm lengths, soaked in hexane, and after swelling a wire was inserted to support the tube. Tubes were completely dipped in the soil 48 hpi with *V. longisporum* 5 cm from the plant stem. Ten probes were dipped per plant. The experiment was performed in five biological replicates with five plants per replicate. Pots containing only peat were used as controls to eliminate the presence of monoterpenes in soil.

Two weeks after inserting in the soil, probes were collected and washed with hexane, dichloromethane, acetonitrile, and distilled water by soaking them for 10 min in each, dried in the oven at 70°C and stored at −20°C until use. Before analysis, tubes were cut into small pieces and monoterpenes were extracted by sonication to an acetonitrile:water solution (65:35) for 10 min then analysed by GC-MS analysis as described previously by Roos et al. (2015), using a Hewlett Packard 6890N gas chromatograph coupled to a Hewlett Packard 5973 mass spectrometer (Agilent Technologies Inc). Monoterpenes were identified by comparing their mass spectral data and GC retention times with those from the National Institute of Standards and Technology (NIST) database and finally verified with those of synthetic standards. The relative amounts of these compounds were calculated as the ratio between the areas of the chromatograph peaks and the root dry biomass.

4.3 | Phenotypic analysis of *V. longisporum* on exposure to monoterpenes

To analyse the effect of monoterpenes in *V. longisporum* mycelial growth, a 5-mm agar plug, derived from 7-day-old PDA cultures, was inoculated in PDB containing 0.05%, 0.25%, and 0.5% 3-carene (90%), β-pinene (99%), or α-pinene (99%) (Sigma), on a rotary shaker at 25°C. Agar plugs inoculated in PDB containing 0.5% DMSO were used as a control treatment. Mycelial growth was determined by measuring mycelial dry weight 5 dpi. For conidial germination, a conidial suspension of *V. longisporum* at a final concentration of 10^4 conidia/ml was inoculated in PDB supplemented with 0.05%, 0.25%, and 0.5% of β-pinene, while exposure to 0.5% DMSO was used as a control. These concentrations were determined based on previous published data (Kadoglidou et al., 2011; Roos et al., 2015). The germination rate was calculated 2 dpi using a haemocytometer. Both assays were performed in three biological replicates.

4.4 | Transcriptomic analysis and validation of RNA-Seq data

For transcriptomic analysis, a *V. longisporum* 5-mm agar plug, derived from 1-week-old PDA culture, was precultured on PDB (Difco) for 5 days at 20°C and amended with 0.05% β-pinene. The mycelia were collected 0 (no exposure control), 8, 24, and 48 h after exposure. Total RNA was extracted from the collected mycelia using the TRIzol RNA extraction protocol (Thermo Fisher Scientific), according to the manufacturer's instructions. RNA strand-specific libraries were generated and sequenced using Illumina HiSeq 2500 at the SNP&SEQ Technology Platform, Science for Life Laboratory at Uppsala University, Sweden. The experiment was performed in three biological replicates. RNA-Seq analysis was performed according to the following procedure. FastQC v. 0.11.7 was used for quality control of the reads (Andrews, 2010). Transcripts were assembled by applying Trinity v. 2.5.1 with the following settings: --trimmomatic --quality_trimming_params TruSeq3-PE-2.fa:2:30:10 SLIDINGWINDOW:4:5 LEADING:5 TRAILING:5 MINLEN:25 --SS_lib_type RF (Grabherr et al., 2011). Transdecoder v. 5.0.1 was applied to identify candidate coding regions within the transcripts with peptide lengths longer than 80 amino acids (https://github.com/TransDecoder/TransDecoder). The function for each identified protein was predicted with blastp from the BLAST+ (v. 2.7.1) command line application and hmmscan from the HMMER application v. 3.1b2 (Camacho et al., 2009; Mistry et al., 2013). The abundance of each transcript was estimated with Kallisto v. 0.43.0 (Bray et al., 2016). Differential expression analysis was performed using DESeq2 v. 1.8.1 (Love et al., 2014). The number of reads is presented in Table S3. Variation among the samples is shown in Figure S6.

To validate RNA-Seq data samples collected from an independent trial, RNA extracted using the Spectrum plant total RNA Kit (Sigma) and 1 μg total RNA, treated with DNase I (Thermo Fisher Scientific), was reverse transcribed using an iScript cDNA synthesis kit (Bio-Rad). RT-qPCR analysis was conducted as described previously (Tzelepis et al., 2012). Primer efficiency was tested in a 10-fold dilution series of gDNA and were designed from predicted exons and listed in Table S4. Expression of genes was normalized using the expression levels of the *GAPDH* gene. Relative expression values were calculated from the threshold cycle (C_t) values according to the $2^{-\Delta\Delta Ct}$ method (Livak & Schmittgen, 2001).

4.5 | Phylogeny of ABC transporters

Amino acid sequences of VlAbcG1a, VlAbcG1b, and homologs from different fungal species were aligned with ClustalX (Thompson et al., 1997) and phylogenetic analysis was conducted using maximum likelihood implemented in MEGA X (Kumar et al., 2018), using the LG+G amino acid substitution model (Le & Gascuel, 2008). Statistical support for branches was supported by 500 bootstraps. The sequences used in this phylogenetic analysis are present in Table S5 and were retrieved from the NCBI database (https://www.ncbi.nlm.nih.gov/).

4.6 | Heterologous expression of *VlAbcG1a* in *S. cerevisiae*

To identify the homologs of VlAbcG1a and VlAbcG1b in *S. cerevisiae*, phylogenetic analysis was conducted as described above. For heterologous expression in WT and deletion strains, the *VlAbcG1a* gene was amplified using cDNA from *V. longisporum* and cloned into the pYES-2 vector driven by the *GAL1* promoter, while the empty pYES-2 vector was used as a negative control, followed by transformation using a polyethylene glycol-based protocol in *S. cerevisiae* BY4742 (Gietz & Schiestl, 2007). The positive transformants were selected on synthetic complete (SC) without uracil (−URA) medium. For gene induction, strains were precultured in SC−URA medium with 1% raffinose to reach the log phase. Then, the OD_{600} was adjusted to 0.3 and transferred to SC−URA medium supplemented with 2%

galactose. The growth of strains was investigated on exposure to 0.003% β-pinene and measuring the OD_{600} in SpectraMax Gemini XPS/EM microplate reader (Molecular Devices) at 30°C in a time-course assay. Five replicates per treatment were used.

4.7 | Construction of deletion and overexpression vectors, fungal transformation, and mutant validation

For the generation of gene deletion strains in *V. dahliae*, the homologous recombination approach was used. Approximately 1000 bp of the 5′ and 3′ flanking regions of the gene homologous to *VlAbcG1a* (*VDAG_01167*) were amplified using the high-fidelity Phusion polymerase (Thermo Fisher Scientific) and primers listed in Table S4. The entry clones were constructed using the MultiSite Gateway cloning technology according to manufacturer's instructions (Thermo Fisher Scientific) and ligated to the pPm43GW destination vector (Karimi et al., 2005) to generate a deletion cassette conferring resistance to hygromycin. Fungal transformation was conducted by an *Agrobacterium*-mediated protocol as previously described (Utermark & Karlovsky, 2008). Colonies grown on selective plates containing 50 μg/ml hygromycin were validated for homologous integration of the deletion cassette using the PCR approaches and primers listed in Table S4 (Dubey et al., 2020). For construction of the overexpression strains, the *VlAbcG1a* gene from *V. longisporum* cDNA was amplified using the primers listed in Table S4. The gene fragment was ligated to the pRFHUE-eGFP vector, driven by the *gpdA* constitutively expressed promoter (Crespo-Sempere et al., 2011), using GenArt Seamless cloning technology (Thermo Fisher Scientific) and transformed to *V. dahliae* using the *Agrobacterium*-mediated protocol (Utermark & Karlovsky, 2008). The expression levels of the *VlAbcG1a* gene in overexpression strains were investigated by RT-qPCR techniques as described above.

4.8 | Phenotypic analysis and virulence assays

The growth rate of *V. dahliae* mutant strains was measured on PDA plates and tolerance to β-pinene, α-pinene, and 3-carene (0.05%) was investigated on PDB cultures 5 dpi. For infection assays the *A. thaliana* Col-0 ecotype was used. In total, 18 plants per treatment were used divided in three biological replicates. Plants were grown on soil on short-day conditions (8 h light/16 h dark) at 22/17°C, and 2-week-old plants were carefully uprooted and dipped in a *V. dahliae* conidial suspension (10^6 conidia/ml in distilled water) for 15 min. Mock-inoculated plants were treated the same way and dipped for 15 min only in distilled water. Rosette growth was monitored 21 dpi. For the tomato infection assay, the cultivar Moneymaker was used. Plants were grown in greenhouse conditions in photoperiod of 16 h light and 8 h darkness and temperatures between 18 and 23°C on commercial peat soil. Ten-day-old plants were uprooted and dipped in a suspension containing 10^6 conidia/ml, derived from *V. dahliae* WT, deletion (Δ*VDAG_01167*), and overexpression (*VlAbcG1a*+) strains, while mock inoculation was conducted by dipping the roots only in distilled water for 15 min. Three biological replicates were used, each containing 10 plants. Plant shoot length was measured 28 dpi. To isolate *V. dahliae* strains from the tomato, stems were surface sterilized in 70% ethanol, followed by 10% bleach for 10 min and washed three times with autoclaved distilled water. Slices then were placed on PDA plates containing 50 μg/ml rifampicin and grown at 25°C until fungal growth was observed.

4.9 | Statistical analysis

One-way analysis of variance (ANOVA) was conducted on gene expression and phenotypic data using a general linear model implemented in SPSS v. 28 (IBM). Pairwise comparisons were made using the Fisher's or Student's *t* tests at the 95% significance level.

ACKNOWLEDGEMENTS

This work has been funded by the Swedish Research Council FORMAS (grant no. 942-2015-36), the Helge Axelsson Johnson Foundation, Nilsson-Ehle Endowments, and the Carl Tryggers Foundation (grant no. CTS 19:374). We would like to thank the Science for Life Laboratory, the National Genomics Infrastructure (NGI), Sweden, and UPPMAX for providing assistance in RNA sequencing and computational infrastructure. We are also thankful to Dr Heriberto Vélëz for his help in construction of the overexpression vector, and to Professor Hans Ronne and Dr Mattias Carlsson for providing us with the *S. cerevisiae* strains.

CONFLICT OF INTEREST

The authors declared no conflict of interest.

DATA AVAILABILITY STATEMENT

The RNA-Seq data used in this study has been deposited in the National Center for Biotechnology Information (NCBI) database under the accession number GSE158956. The data supporting the findings of this study are available from the corresponding author upon request.

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REFERENCES

Andrews, S. (2010) FastQC: a quality control tool for high throughput sequence data. Available at: http://www.bioinformatics.babraham.ac.uk/projects/fastqc/ [Accessed 23 November 2021].

Bais, H.P., Weir, T.L., Perry, L.G., Gilroy, S. & Vivanco, J.M. (2006) The role of root exudates in rhizosphere interactions with plants and other organisms. *Annual Review of Plant Biology*, 57, 233–266.

Bednarek, P., Schneider, B., Svatos, A., Oldham, N.J. & Hahlbrock, K. (2005) Structural complexity, differential response to infection, and tissue specificity of indolic and phenylpropanoid secondary metabolism in *Arabidopsis* roots. *Plant Physiology*, 138, 1058–1070.

Boncan, D.A.T., Tsang, S.S.K., Li, C., Lee, I.H.T., Lam, H.-M., Chan, T.-F. et al. (2020) Terpenes and terpenoids in plants: interactions with environment and insects. *International Journal of Molecular Sciences*, 21, 7382.

Bray, N.L., Pimentel, H., Melsted, P. & Pachter, L. (2016) Near-optimal probabilistic RNA-seq quantification. *Nature Biotechnology*, 34, 525–527.

Camacho, C., Coulouris, G., Avagyan, V., Ma, N., Papadopoulos, J., Bealer, K. et al. (2009) BLAST+: architecture and applications. *BMC Bioinformatics*, 10, 421.

Campos-Soriano, L., García-Martínez, J. & San Segundo, B. (2012) The arbuscular mycorrhizal symbiosis promotes the systemic induction of regulatory defence-related genes in rice leaves and confers resistance to pathogen infection. *Molecular Plant Pathology*, 13, 579–592.

Coleman, J.J. & Mylonakis, E. (2009) Efflux in fungi: la piece de resistance. *PLoS Pathogens*, 5, e1000486.

Crespo-Sempere, A., Lopez-Perez, M., Martinez-Culebras, P.V. & Gonzalez-Candelas, L. (2011) Development of a green fluorescent tagged strain of *Aspergillus carbonarius* to monitor fungal colonization in grapes. *International Journal of Food Microbiology*, 148, 135–140.

Depotter, J.R., Deketelaere, S., Inderbitzin, P., Tiedemann, A.V., Hofte, M., Subbarao, K.V. et al. (2016) *Verticillium longisporum*, the invisible threat to oilseed rape and other brassicaceous plant hosts. *Molecular Plant Pathology*, 17, 1004–1016.

Dos Santos, S.C., Teixeira, M.C., Dias, P.J. & Sa-Correia, I. (2014) MFS transporters required for multidrug/multixenobiotic (MD/MX) resistance in the model yeast: understanding their physiological function through post-genomic approaches. *Frontiers in Physiology*, 5, 180.

Dubey, M., Vélëz, H., Broberg, M., Jensen-Funck, D. & Karlsson, M. (2020) LysM proteins regulate fungal development and contribute to hyphal protection and biocontrol traits in *Clonostachys rosea*. *Frontiers in Microbiology*, 11, 679.

Dunker, S., Keunecke, H., Steinbach, P. & von Tiedemann, A. (2008) Impact of *Verticillium longisporum* on yield and morphology of winter oilseed rape (*Brassica napus*) in relation to systemic spread in the plant. *Journal of Phytopathology*, 156, 698–707.

Eynck, C., Koopmann, B., Grunewaldt-Stoecker, G., Karlovsky, P. & von Tiedemann, A. (2007) Differential interactions of *Verticillium longisporum* and *Verticillium dahliae* with *Brassica napus* detected with molecular and histological techniques. *European Journal of Plant Pathology*, 118, 259–274.

Falara, V., Akhtar, T.A., Nguyen, T.T.H., Spyropoulou, E.A., Bleeker, P.M., Schauvinhold, I. et al. (2011) The tomato terpene synthase gene family. *Plant Physiology*, 157, 770–789.

Fiers, M., Lognay, G., Fauconnier, M.L. & Jijakli, M.H. (2013) Volatile compound-mediated interactions between barley and pathogenic fungi in the soil. *PLoS One*, 8, e66805.

Fogelqvist, J., Tzelepis, G., Bejai, S., Ilback, J., Schwelm, A. & Dixelius, C. (2018) Analysis of the hybrid genomes of two field isolates of the soil-borne fungal species *Verticillium longisporum*. *BMC Genomics*, 19, 14.

Fradin, E.F., Zhang, Z., Juarez Ayala, J.C., Castroverde, C.D., Nazar, R.N., Robb, J. et al. (2009) Genetic dissection of *Verticillium* wilt resistance mediated by tomato Ve1. *Plant Physiology*, 150, 320–332.

Gietz, R.D. & Schiestl, R.H. (2007) Large-scale high-efficiency yeast transformation using the LiAc/SS carrier DNA/PEG method. *Nature Protocols*, 2, 38–41.

Grabherr, M.G., Haas, B.J., Yassour, M., Levin, J.Z., Thompson, D.A., Amit, I. et al. (2011) Full-length transcriptome assembly from RNA-Seq data without a reference genome. *Nature Biotechnology*, 29, 644–652.

Herde, M., Gartner, K., Kollner, T.G., Fode, B., Boland, W., Gershenzon, J. et al. (2008) Identification and regulation of TPS04/GES, an *Arabidopsis* geranyllinalool synthase catalyzing the first step in the formation of the insect-induced volatile C(16)-homoterpene TMTT. *The Plant Cell*, 20, 1152–1168.

Hong, G.J., Xue, X.Y., Mao, Y.B., Wang, L.J. & Chen, X.Y. (2012) *Arabidopsis* MYC2 interacts with DELLA proteins in regulating sesquiterpene synthase gene expression. *The Plant Cell*, 24, 2635–2648.

Hosseini, S., Heyman, F., Olsson, A., Broberg, D., Funck, J.D. & Karlsson, M. (2013) Zoospore chemotaxis of closely related legume-root infecting *Phytophthora* species towards host isoflavones. *Plant Pathology*, 63, 708–714.

Inderbitzin, P., Davis, R.M., Bostock, R.M. & Subbarao, K.V. (2011) The ascomycete *Verticillium longisporum* is a hybrid and a plant pathogen with an expanded host range. *PLoS One*, 6, e18260.

Johansson, A., Goud, J.K.C. & Dixelius, C. (2006a) Plant host range of *Verticillium longisporum* and microsclerotia density in Swedish soils. *European Journal of Plant Pathology*, 114, 139–149.

Johansson, A., Staal, J. & Dixelius, C. (2006b) Early responses in the *Arabidopsis–Verticillium longisporum* pathosystem are dependent on NDR1, JA- and ET-associated signals via cytosolic NPR1 and RFO1. *Molecular Plant-Microbe Interactions*, 19, 958–969.

Kadoglidou, K., Lagopodi, A., Karamanoli, K., Vokou, D., Bardas, G.A., Menexes, G. et al. (2011) Inhibitory and stimulatory effects of essential oils and individual monoterpenoids on growth and sporulation of four soil-borne fungal isolates of *Aspergillus terreus*, *Fusarium oxysporum*, *Penicillium expansum*, and *Verticillium dahliae*. *European Journal of Plant Pathology*, 130, 297–309.

Kallenbach, M., Oh, Y., Eilers, E.J., Veit, D., Baldwin, I.T. & Schuman, M.C. (2014) A robust, simple, high-throughput technique for time-resolved plant volatile analysis in field experiments. *The Plant Journal*, 78, 1060–1072.

Karimi, M., de Meyer, B. & Hilson, P. (2005) Modular cloning in plant cells. *Trends in Plant Science*, 10, 103–105.

Karlsson, M., Durling, M.B., Choi, J., Kosawang, C., Lackner, G., Tzelepis, G.D. et al. (2015) Insights on the evolution of mycoparasitism from the genome of *Clonostachys rosea*. *Genome Biology and Evolution*, 7, 465–480.

Kovalchuk, A. & Driessen, A.J.M. (2010) Phylogenetic analysis of fungal ABC transporters. *BMC Genomics*, 11, 177.

Kroeker, G. (1970) Vissnesjuka pa raps och rybs i Skåne orsakadav *Verticillium*. *Svensk Frötidning*, 19, 10–13.

Kumar, S., Stecher, G., Li, M., Knyaz, C. & Tamura, K. (2018) MEGA X: molecular evolutionary genetics analysis across computing platforms. *Molecular Biology and Evolution*, 35, 1547–1549.

Le, S.Q. & Gascuel, O. (2008) An improved general amino acid replacement matrix. *Molecular Biology and Evolution*, 25, 1307–1320.

Leonard, M., Kühn, A., Harting, R., Maurus, I., Nagel, A., Starke, J. et al. (2020) *Verticillium longisporum* elicits media-dependent secretome responses with capacity to distinguish between plant-related environments. *Frontiers in Microbiology*, 11, 1876.

Livak, K.J. & Schmittgen, T.D. (2001) Analysis of relative gene expression data using real-time quantitative PCR and the $2^{(-\Delta\Delta C(T))}$ method. *Methods*, 25, 402–408.

Love, M.I., Huber, W. & Anders, S. (2014) Moderated estimation of fold change and dispersion for RNA-seq data with DESeq2. *Genome Biology*, 15, 550.

Lucini, E.I., Zunino, M.P., Lopez, M.L. & Zygadlo, J.A. (2006) Effect of monoterpenes on lipid composition and sclerotial development of *Sclerotium cepivorum* Berk. *Journal of Phytopathology*, 154, 441–446.

Mistry, J., Finn, R.D., Eddy, S.R., Bateman, A. & Punta, M. (2013) Challenges in homology search: HMMER3 and convergent evolution of coiled-coil regions. *Nucleic Acids Research*, 41, e121.

Mohney, B.K., Matz, T., La Moreaux, J., Wilcox, D.S., Gimsing, A.L., Mayer, P. et al. (2009) *In situ* silicone tube microextraction: a new method for undisturbed sampling of root-exuded thiophenes from marigold (*Tagetes erecta* L.) in soil. *Journal of Chemical Ecology*, 35, 1279–1287.

Nazzaro, F., Fratianni, F. & Coppola, R. (2013) Quorum sensing and phytochemicals. *International Journal of Molecular Sciences*, 14, 12607–12619.

Nygren, K., Dubey, M., Zapparata, A., Iqbal, M., Tzelepis, G., Durling, M.B. et al. (2018) The mycoparasitic fungus *Clonostachys rosea* responds with both common and specific gene expression during interspecific interactions with fungal prey. *Evolutionary Applications*, 11, 931–949.

Paumi, C.M., Chuk, M., Snider, J., Stagljar, I. & Michaelis, S. (2009) ABC transporters in *Saccharomyces cerevisiae* and their interactors: new technology advances the biology of the ABCC (MRP) subfamily. *Microbiology and Molecular Biology Reviews*, 73, 577–593.

Pegg, G.F. & Brady, B.L. (2002) *Verticillium wilts*. Wallingford, UK: CABI Publishing.

Pineda, A., Zheng, S.J., van Loon, J.J., Pieterse, C.M. & Dicke, M. (2010) Helping plants to deal with insects: the role of beneficial soil-borne microbes. *Trends in Plant Sciences*, 15, 507–514.

Rafiei, V., Vélëz, H. & Tzelepis, G. (2021) The role of glycoside hydrolases in phytopathogenic fungi and oomycetes virulence. *International Journal of Molecular Sciences*, 22, 9359.

Roos, J., Bejai, S., Mozuraitis, R. & Dixelius, C. (2015) Susceptibility to *Verticillium longisporum* is linked to monoterpene production by TPS23/27 in *Arabidopsis*. *The Plant Journal*, 81, 572–585.

Samaras, A., Karaoglanidis, G.S. & Tzelepis, G. (2021) Insights into the multitrophic interactions between the biocontrol agent *Bacillus subtilis* MBI 600, the pathogen *Botrytis cinerea* and their plant host. *Microbiological Research*, 248, 126752.

Steeghs, M., Bais, H.P., de Gouw, J., Goldan, P., Kuster, W., Northway, M. et al. (2004) Proton-transfer-reaction mass spectrometry as a new tool for real time analysis of root-secreted volatile organic compounds in *Arabidopsis*. *Plant Physiology*, 135, 47–58.

Steventon, L.A., Fahleson, J., Hu, Q. & Dixelius, C. (2002) Identification of the causal agent of *Verticillium* wilt of winter oilseed rape in Sweden, *Verticillium longisporum*. *Mycological Research*, 106, 570–578.

Thompson, J.D., Gibson, T.J., Plewniak, F., Jeanmougin, F. & Higgins, D.G. (1997) The CLUSTAL_X windows interface: flexible strategies for multiple sequence alignment aided by quality analysis tools. *Nucleic Acids Research*, 25, 4876–4882.

Tzelepis, G., Bejai, S., Sattar, M.N., Schwelm, A., Ilback, J., Fogelqvist, J. et al. (2017) Detection of *Verticillium* species in Swedish soils using real-time PCR. *Archives of Microbiology*, 199, 1383–1389.

Tzelepis, G. & Karlsson, M. (2019) Killer toxin-like chitinases in filamentous fungi: structure, regulation and potential roles in fungal biology. *Fungal Biology Reviews*, 33, 123–131.

Tzelepis, G., Melin, P., Jensen Funck, J., Stenlid, J. & Karlsson, M. (2012) Functional analysis of glycoside hydrolase family 18 and 20 genes in *Neurospora crassa*. *Fungal Genetics and Biology*, 49, 717–730.

Urban, M., Bhargava, T. & Hamer, J.E. (1999) An ATP-driven efflux pump is a novel pathogenicity factor in rice blast disease. *EMBO Journal*, 18, 512–521.

Utermark, J. & Karlovsky, P. (2008) Genetic transformation of filamentous fungi by *Agrobacterium tumefaciens*. *Protocol Exchange*. Available at: http://www.nature.com/protocolexchange/protocols/427 [Accessed 23 November 2021].

Wang, Y., Lim, L., Di Guistini, S., Robertson, G., Bohlmann, J. & Breuil, C. (2013) A specialized ABC efflux transporter GcABC-G1 confers monoterpene resistance to *Grosmannia clavigera*, a bark beetle-associated fungal pathogen of pine trees. *New Phytologist*, 197, 886–898.

Weidenhamer, J.D., Boes, P.D. & Wilcox, D.S. (2009) Solid-phase root zone extraction (SPRE): a new methodology for measurement of allelochemical dynamics in soil. *Plant and Soil*, 322, 177–186.

Zamioudis, C. & Pieterse, C.M. (2012) Modulation of host immunity by beneficial microbes. *Molecular Plant-Microbe Interactions*, 25, 139–150.

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How to cite this article: Rafiei, V., Ruffino, A., Persson Hodén, K., Tornkvist, A., Mozuraitis, R., Dubey, M. et al. (2022) A *Verticillium longisporum* pleiotropic drug transporter determines tolerance to the plant host β-pinene monoterpene. *Molecular Plant Pathology*, 23, 291–303. https://doi.org/10.1111/mpp.13162