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

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 VIAbcG1a and VIAbcG1b. Overexpression of VIAbcG1a in Saccharomyces cerevisiae increased tolerance to β-pinene, while deletion of the VIAbcG1a homologous gene in Verticillium dahliae resulted in mutants with increased sensitivity to certain monoterpenes. Furthermore, the VIAbcG1a 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
transporter gene families has been observed in the genome of myco-associated proteins (MRP), and pleiotropic drug resistance (PDR) from several independent hybridization events between \( V.\ dahliae \) for example cotton, tomato, sugar beet, and olive (Pegg & Brady, 2017). More than 200 plant species can be infected by \( V.\ dahliae \), \( V.\ \) wilt disease in a plethora of cultivated and wild plants.

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).

\( V.\ dahliae \) species are ascomycete fungi responsible for \( V.\ dahliae \) wilt disease in a plethora of cultivated and wild plants. More than 200 plant species can be infected by \( V.\ dahliae \) for example cotton, tomato, sugar beet, and olive (Pegg & Brady, 2002). \( V.\ longisporum \) 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 \( B.\ napus \) roots exuding higher amounts of certain 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).

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 \( V.\ dahliae \) 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.
during exposure to β-pinene. This monoterpane 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 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. Asterisks (*) indicate statistically significant differences between 0% of monoterpene and the other percentages (0.05%, 0.25%, and 0.50%) according to Student’s t test (p < 0.05).

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).

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...
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 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 log2 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.
To investigate the potential role of VIAbcG1a and VIAbcG1b in *Saccharomyces cerevisiae* enhanced tolerance to β-pinene, the deletion strains (ΔPDR5, ΔPDR10, and ΔPDR15) 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 ≤ 0.001), 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, ΔPDR10 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 VIAbcG1a gene in Saccharomyces cerevisiae. (a) Phylogenetic analysis of VIAbcG1a and VIAbcG1b 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 ΔPDR5, ΔPDR10, and ΔPDR15 homologs to VIAbcG1a and VIAbcG1b on exposure to 0.003% β-pinene solubilized in dimethyl sulphoxide (DMSO). (c) Growth of S. cerevisiae VIAbcG1a overexpression strains in the WT background [WT (VIAbcG1a)] on exposure to β-pinene. (d–f) Growth of S. cerevisiae ΔPDR5 (VIAbcG1a), ΔPDR10 (VIAbcG1a), and ΔPDR15 (VIAbcG1a) strains overexpressing VIAbcG1a 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 > 0.9993) (Figure 4b).

Furthermore, S. cerevisiae strains overexpressing V. longisporum VIAbcG1a in the WT background [WT (VIAbcG1a)] and in the ΔPDR5, ΔPDR10, and ΔPDR15 deletion strains background [ΔPDR5 (VIAbcG1a), ΔPDR10 (VIAbcG1a), and ΔPDR15 (VIAbcG1a)] 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 (VIAbcG1a)] 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 ΔPDR5 (VIAbcG1a) strain was significantly higher at 22 hpi in medium supplemented with β-pinene as compared to ΔPDR5 (EV) (p < 0.001) (Figure 4d). However, no significant differences in growth rate were observed between ΔPDR10 (VIAbcG1a) or ΔPDR15 (VIAbcG1a) strains and empty vector ΔPDR10 (EV) or ΔPDR15 (EV) controls, respectively (p > 0.072) (Figure 4e,f). These data further support that the ABC transporter VIAbcG1a is involved in the β-pinene detoxification process.

### 2.6 | Deletion of the VIAbcG1a 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 VIAbcG1. We identified the VDAG_01167 gene in V. dahliae as a homolog to the V. longisporum VIAbcG1a and VIAbcG1b. VDAG_01167 showed 98% amino acid similarity, and structural analysis of the amino acid sequence showed domains identical to VIAbcG1. 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 VIAbcG1a overexpression strain (VIAbcG1a+) 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 (ΔVDAG_01167) was significantly decreased as compared to the WT (Figure 5), while biomass of the overexpression strain (VIAbcG1a+) 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 VIAbcG1a 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 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 VIAbcG1a overexpression strain was significantly reduced compared to the WT and VDAG_01167 overexpression 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 VIAbcG1 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
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*, *Cochliobolus 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-cineole, 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 ΔPDR5, 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 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 (ΔVDAG_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)
Likewise, increased fungal virulence of the VIAbcG1a+ 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 (10⁷ 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 monoterpenes 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.
One week 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 6890 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 10⁴ 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 (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 hmmsearch 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 (Ct) values according to the 2−ΔΔCt 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 OD₆₀₀ 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 OD600 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 were used divided in three biological replicates. 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 10⁶ conidia/ml, derived from V. dahliae WT, deletion (ΔVDAG_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 Véllez 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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How to cite this article: Rafiei, V., Ruffino, A., Persson Hodén, K., Tornkvist, A., Mozuraitis, R., Dubey, M. et al. (2022) *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](https://doi.org/10.1111/mpp.13162)