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ORIGINAL ARTICLE

A lavender ABC transporter confers resistance to monoterpene toxicity in yeast

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Abstract

Main conclusion **Functional expression of a multidrug resistance-type ABC transporter from *Lavandula angustifolia* improved yeast resistance to geraniol, a monoterpene constituent of lavender essential oil.**

Plant ATP-binding cassette (ABC) transporters are a large family of membrane proteins involved in active and selective transport of structurally diverse compounds. In this study, we functionally evaluated LaABCB1, a multidrug resistance (MDR)-type ABC transporter strongly expressed in the secretory cells of lavender glandular trichomes, where monoterpene essential oil constituents are synthesized and secreted. We used LaABCB1 to complement a yeast knockout mutant in which 16 ABC transporters were deleted. Expression of LaABCB1 enhanced tolerance of yeast mutants to geraniol, a key constituent of essential oils in lavenders and numerous other plants. Our findings suggest a role for the MDR-type ABC transporters in the toxicity tolerance of at least certain essential oil constituents in lavender oil glands.

Keywords ATP-binding cassette · *Lavandula* · Multidrug resistance · Monoterpene trafficking · Transmembrane protein

Introduction

Lavenders (*Lavandula*) are members of the *Lamiaceae* family widely grown for their essential oils (EOs), which is mainly constituted of a few monoterpenes, the C_{10} class of the isoprenoids or terpenoids. For example, the EOs of *L. angustifolia* cultivated in Poland were dominated by linalool (30.6%), linalyl acetate (14.2%), geraniol (5.3%), β-caryophyllene (4.7%), and lavandulyl acetate (4.4%) (Smigielski et al. 2009). Monoterpenes are particularly toxic to cells when accumulated at higher concentrations, and hence, their biosynthesis as well as accumulation is limited to specialized tissues called glandular trichomes or oil glands (McConkey et al. 2000). In these glands, a group of six-to-eight secretory cells actively produce and traffic monoterpenes into an epicuticular storage cavity that is demarcated by a plasma membrane. Indirect evidences suggest that these tissues are endowed with an active and selective monoterpene transport mechanism (Turner and Croteau 2004), although the exact mechanism and/or transporter proteins involved in the process are yet to be identified.

ATP-binding cassette (ABC) transporters are a large family of membrane proteins involved in active and selective transport of structurally diverse compounds (Rea 2007). The ABCB or permeability glycoprotein or multidrug resistance (MDR) sub-family of ABC transporters has been experimentally proven to transport structurally unrelated hydrophobic compounds including digoxin, vinblastine, doxorubicin, taxol (Ushigome et al. 2003), auxins, lipids (Buda et al. 2013), etc. In some instances, the in vitro transport of these substrates by MDR was selectively inhibited by monoterpenes. A case in point was the inhibition of [^{3}H] digoxin efflux by the human and mouse MDR1 by *Zanthoxyli* fructus major monoterpene constituents (*R*)-(+)-citronellal, (*S*)-(−)-ß-citronellol, ∂-terpinene, terpinolene,

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and (−)-ß-pinene (Yoshida et al. 2005, 2006). Although this selective inhibition of digoxin efflux by monoterpenes does not necessarily imply their role in monoterpene transport, it raises an important question about the role of MDR homologs expressed in tissues exclusively specialized for monoterpene synthesis and accumulation. Therefore, we decided to isolate MDR homologs, along with other ABC transporters such as the pleiotropic drug resistance transporter (PDR) homologs, expressed in lavender glandular trichomes and test if their heterologous expression in yeast confers terpene toxicity tolerance. Terpene toxicity tolerance by heterologous hosts expressing ABC transporters has been used previously to infer their biological role in terpene biosynthesis. Heterologous expression of NtPDR1, a candidate ABC transporter for terpene transport, in *Arabidopsis thaliana* conferred transgenic lines resistance to the diterpenes sclareol (Crouzet et al. 2013). On the other hand, deletion of the pleiotropic drug resistance transporter in the mountain pine beetle-fungal symbiont *G. clavigera* rendered them susceptibility to monoterpenes (Wang et al. 2013).

Selective enrichment of putative terpenoid biosynthetic cDNAs in glandular trichome-derived EST library has been previously used to identify and to functionally characterize several terpene synthases and prenyltransferases from *Lavandula* (Demissie et al. 2012, 2013). In an attempt to isolate cDNAs involved in *Lavandula* monoterpene trafficking and/or toxicity tolerance, we studied the above databases to identify ABCB (MDR) homologs expressed in the glandular trichomes of *Lavandula*. Here, we report that functional expression of the *L. angustifolia* ABCB1 cDNA, termed *LaABCB1,* in ABC transporter mutant yeast strains confers tolerance to geraniol toxicity. The function of LaABCB1 in yeast and its detection in secretory cells of *Lavandula* warrants further investigation of LaABCB1 and related transporter proteins for their in *planta* monoterpene trafficking and/ or toxicity mitigation role.

Materials and methods

LaABCB1 cloning and transcriptional activity study

Our previously reported *L. angustifolia* EST database (Lane et al. 2010) contained a full-length (4027 bp long) MDR homolog [*LaABCB1* (KJ135790)], which is conserved in *L. angustifolia, L. latifolia,* and *L.* × *intermedia* (a commercially important natural hybrid of *L. angustifolia* and *L. latifolia*). Transcript abundance in leaf, flower, and glandular trichome secretory cells of *L.* × *intermedia* was assessed by standard PCR based on the intensity of *LaABCB1* fragment amplified with set I primers (Table S1) and Taq DNA polymerase (New England Biolabs, USA). Briefly, total RNA was isolated from 100 mg fresh developing leaf, flower (stage 30% bloom), and glands isolated from floral tissues at 30% flowering stage as described in Demissie et al. (2013) using the RNeasy Plant Mini Kit (Qiagen; Valencia, CA, USA), and treated with DNase using the Qiagen on-column DNase digestion kit to remove genomic DNA. Total RNA was reverse transcribed with oligo d (T) primer and M-MuLV Reverse Transcriptase (New England Biolabs, Beverly, MA, USA) following the manufacturer's directions. The reverse transcribed cDNA was used as a template for transcript abundance study by standard PCR. The PCR program used was 95 °C for 1 min, followed by 30 cycles of 95 °C for 30 s, 60 °C for 30 s, and 72 °C for 90 s, and a 3 min final extension at 72 °C.

LaABCB1 expression in yeast

LaABCB1 was cloned into *Kpn*I and *Eco*RI restriction sites of pBEVY-L vector (Addgen, USA) and transformed into 16-ABC transporter knockout mutant strains RY0512 (Suzuki et al. 2011) using the Lithium acetate method (Gietz and Schiestl 2007). Briefly, *LaABCB1* ORF was amplified by PCR using primer set II (Table S1) to introduce *Kpn*I and *Eco*RI restriction sites at the corresponding 5′ and 3′ ends, respectively. Amplified genes were subsequently cloned into the pBEVY-L vector for expression under the yeast native ADH1 promoter. Competent yeast cells were prepared, transformed, and selected on yeast synthetic media lacking leucine as described by Gietz (2014). Growth inhibition effect of various monoterpenes on yeast mutants harboring the empty vector, or vectors expressing transporter or wild-type cells were assessed using plate assays as follows: yeast cells grown to OD600 of ~ 1.0 were streaked on YPD plate followed by placing three filter disks (BD Diagnostic Systems, USA) per plate at equal distance. The filter disks were impregnated with 20 µl of DMSO or 20 µl of DMSO containing 2.5, 5 and 7.5% geraniol (w/v) and incubated at 30 °C for 72 h. Inhibition zone was calculated by measuring the diameter of the yeast clear area around the filter disk. Yeast strains used in the study: RY0512 is MATa (or MAT[Δ]) adp1[Δ] snq2[Δ] ycf1[Δ] pdr15[Δ] yor1[Δ] vmr1[Δ] pdr11[Δ] nft1[Δ] bpt1[Δ] ybt1[Δ] ynr070w[Δ] yol075c[Δ] aus1[Δ] pdr5[Δ] pdr10[Δ] pdr12[Δ] can1[Δ]:GMToolkit-a (or -[Δ]) his3[Δ]1 leu2[Δ]0 ura3[Δ]0 met15[Δ]0 and RYO566 is MAT[Δ] lyp1[Δ] his3[Δ]1 leu2[Δ]0 ura3[Δ]0 met15[Δ]0.

Data analysis

One-way ANOVA and multiple mean separation modules of the GraphPad prism (version 7.0, Graphpad Software, San Diego, CA) were used to calculate *p* values and compare the means, respectively.

Results

LaABCB1 transcriptionally active in secretory cells

Transcripts corresponding to *LaABCB1* were detected in leaves, flowers, and glands, but were most prominent in flower and gland tissues (Fig. S1).

LaABCB1 expression in yeast confers resistance to geraniol

Preliminary differential toxicity screening of eight lavender major monoterpenes—linalool, linalool acetate, geraniol, borneol, camphor, limonene, and 1,8-cineole—revealed that only geraniol was significantly toxic to RYO512 (the 16 ABC transporter knockout mutant yeast strain) compared to its wild-type counterpart RYO566, making it the only testable monoterpene under this heterologous system. After 72 h of incubation with DMSO (the vehicle/solvent), RYO566 cells (WT) as well as RYO512 cells transformed with either the empty vector or with the vector harboring *LaABCB1* fully covered the whole plate (Fig. 1a, b). Similarly, 2.5% geraniol did not affect the growth of these cells either (Fig. 1b). However, treatment with 5% or 7.5% geraniol resulted in a significant radial growth inhibition zone (yeast clearance distance from monoterpene impregnated disk) in cells transformed with the empty vector compared to those transformed with *LaABCB1* and WT (Fig. 1a, b). The radial growth inhibition zone for RYO512 cells transformed with the empty vector was more than 50% higher than WT cells or those expressing LaABCB1 (Fig. 1b). There was no growth difference between WT (RYO566 strains) and RYO512-expressing *LaABCB1*. These results suggest that the observed growth inhibition effect of geraniol in mutant yeast strains is associated with the lack of endogenous ABC transporters, and that *LaABCB1* complemented the mutant cells most likely through its transporter activity.

Discussion

Currently, the cellular mechanisms mediating the secretion and/or toxicity tolerance of EO constituents are poorly understood. We hypothesized that ABC transporter homologs expressed in EO producing plant tissues could play a role in this process based on two lines of evidences. First, the trafficking of monoterpenes from their site of synthesis to their storage area in peppermint, a close relative of lavender, displays the peculiar features of ABC transporter-mediated transport; in that, it is unidirectional and shows selective retention (McCaskill et al. 1992; Turner and Croteau 2004). Second, the transcriptional activity of certain ABC transporters has been found to correlate well with isoprenoid synthesis/release and their heterologous expression improves the hosts' terpene tolerance. In this regard, NtPDR1 was isolated from tobacco tissues known to accumulate and/or release monoterpenes in response to fungal attack (Bienert et al. 2012) and *A. thaliana* transgenic lines expressing NtPDR1 were found to be resistant to some diterpenes like sclareol. Our search for ABC transporter homologs expressed in the glandular trichomes of *Lavandula* identified lavender PDR and MDR homologs. Upon expressing them in yeast cells, the MDR-type ABC transporter homolog, *LaABCB1*, improved the yeast cells' tolerance level against geraniol, while the PDR candidate failed to provide similar protection for unknown reasons (data not shown). Similarly, overexpression of NtPDR1 in *N. tabacum* BY2 cells failed to confer resistance against tobacco isoprenoids like geranylgeraniol and 1,8-cineole (Crouzet et al. 2013).

Although transcripts corresponding to *LaABCB1* were highly abundant in glandular trichomes of *L.* × *intermedia*, the gene was also expressed in other leaf and floral cells. This ubiquitous expression of *LaABCB1* could be due to the ability of this sub-family of ABC transporters to transport diverse metabolites. In this context, plant MDRs have shown transport substrate promiscuity towards structurally unrelated endogenous/exogenous secondary metabolites (Lefevre and Boutry 2018). For example, Kaneda et al. (2011) reported that AtABCB14, along with AtABCB15, is expressed in guard cells and possibly involved in polar transport of auxins. However, heterologous expression of AtABCB14 in *E. coli* and HeLA cells demonstrated its ability to transport malate (Lee et al. 2008). Another example in this instance is CjMDR1 (CjABCB1) which was confirmed to mediate the influx of berberine when expressed in *Xenopus laevis* oocytes. However, yeast cells expressing CjMDR1 were found to be sensitive to both berberine and 4-nitroquinoline N-oxide (Shitan et al., 2003).

Lavandula × *intermedia* is a natural hybrid of *L. angustifolia* and *L. latifolia* which themselves are genetically closely related (Lis-Balchin 2002). Therefore, it was not a surprise that transcripts corresponding to *LaABCB1* were detected in all three species (Fig. S1). Since the EOs of *L. angustifolia, L.* × *intermedia,* and *L. latifolia* are mainly constituted of the monoterpenes linalool, linalool acetate, geraniol, 1,8-cineole, camphor, and limonene (Lis-Balchin 2002; Smigielski et al. 2009), we screened the toxicity of these monoterpenes against the ABC transporter knockout mutant yeast strain (RYO512). RYO512 cells were found to be innately tolerant to all monoterpenes tested except geraniol (data not shown), making it the only testable monoterpene under this heterologous system. Upon exposure to geraniol, RYO512 cells expressing *LaABCB1* and its wild-type counterpart RYO566

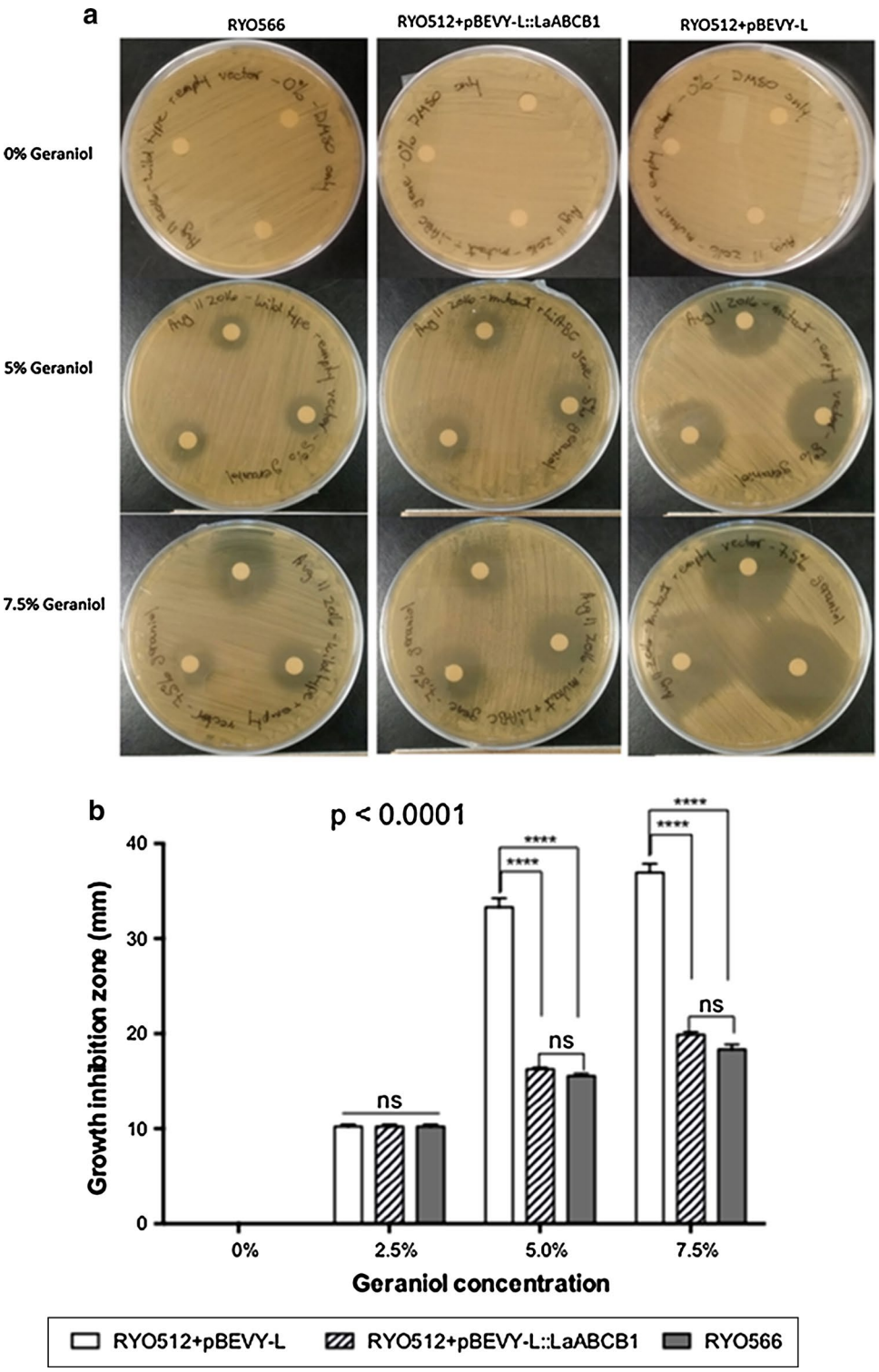

Fig. 1 Growth inhibition effect of geraniol, a *Lavandula* essential oil constituent monoterpene, on yeast strains expressing *LaABCB1*. **a** Effect of different geraniol concentration on yeast growth performance on plate and **b** growth inhibition zone (mm) established by different geraniol concentrations. RYO566: wild-type yeast strain; "RYO512 + pBEVY-L::*LaABCB1*": ABC transporter mutant yeast strain transformed with pBEVY-L vector containing *LaABCB1* ORF and "RYO512 + pBEVY-L": ABC transporter mutant yeast strain transformed with empty pBEVY-L vector. 0%—filter disk impregnated with DMSO alone, 2.5%—filter disk impregnated with DMSO containing 2.5% geraniol, 5%—filter disk impregnated with DMSO containing 5% geraniol, and 7.5%—filter disk impregnated with DMSO containing 7.5% geraniol. Error bars indicate standard means of error ($n = 9$) and asterisks indicate a significant level of growth inhibition by geraniol on empty pBEVY-L vector expressing yeast cells compared to pBEVY-L::*LaABCB1* transformed cells and wild-type strain ($p < 0.0001$)

showed significantly higher tolerance compared to RYO512 cells harboring the empty vector (Fig. 1a, b). This suggests that the observed growth inhibition effect of geraniol was, indeed, associated with the lack of endogenous ABC transporters in RYO512 which was complemented by *LaABCB1* expression. Supporting this outcome, geraniol acted similar to the classical MDR blocker verapamil; in that, it inhibited LaABCB1-mediated [^{3}H]-vinblastine efflux when expressed in *Xenopus laevis* oocytes (Demissie 2014). Taken together, these results indicate that ABC transporters (as exemplified by LaABCB1) may be involved in trafficking geraniol (and, perhaps, other monoterpenes) in lavenders.

In conclusion, we have identified a ubiquitously expressed lavender ABC transporter that can complement yeast ABC transporter mutants against geraniol toxicity. Although monoterpene toxicity reversal by ABC transporters in heterologous hosts or lack thereof has been used as an indicator of their transport activity (Wang et al. 2013), the properties of LaABCB1 expression in yeast together with its strong expression in the secretory cells of *L.* × *intermedia* glandular trichomes alone cannot conclusively establish its role in monoterpene trafficking and/or toxicity tolerance. This need to be confirmed by developing methods to directly measure LaABCB1-mediated in vitro monoterpene transport and by overexpressing or silencing *LaABCB1* through metabolic engineering in lavenders or related plants.

Author contribution statement ZAD and SSM were responsible for the experimental design, analysis, and manuscript preparation. ZAD, MT, and AMA performed all experiments and LSS was involved at cloning stage.

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