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ORIGINAL RESEARCH PAPER

Key cytomembrane ABC transporters of *Saccharomyces cerevisiae* fail to improve the tolerance to D-limonene

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Abstract ATP-binding cassette transporters (ABC) are important detoxification proteins and were proposed to play important roles in monoterpene resistance in *Saccharomyces cerevisiae*. In this work, the transcriptional levels of typical ABC transporters of *S. cerevisiae* under 85 mg D-limonene/l were evaluated using real-time quantitative PCR (q-PCR). Only the transcriptional level of *PDR5*, *YOR1* and *PDR15* were upregulated but overexpression of these genes in *S. cerevisiae* failed to improve D-limonene tolerance suggesting that other mechanisms are involved in tolerance of yeast to monoterpenes.

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Introduction

D-Limonene is an important monoterpene that is widely used in the food, pharmaceutical and cosmetic industries (Bakkali et al. 2008). *Saccharomyces cerevisiae* is widely used as a host microorganism for the production of ethanol and natural plant products (Herrero et al. 2008). Enhancing resistance to D-limonene is crucial to improve final yields or utilization of D-limonene containing citrus peel waste for biofuel production because of its toxicity to most of the microorganisms (Reddy et al. 2011). Different physical methods could be used to remove limonene prior to fermentation in laboratory scale but none of them, such as enzyme methods or flash evaporation, cannot eliminate limonene at an affordable cost (Pourbafrani et al. 2010). Moreover, these methods for removal of D-limonene could not be applied to the metabolic engineering of microorganisms for monoterpene production. Therefore, the enhancement of D-limonene tolerance by *S. cerevisiae* could facilitate the utilization of citrus peel waste in the production of bioethanol.

ABC transporters are membrane-intrinsic primary active pumps. Pleiotropic drug resistance (PDR) is a subfamily of the ABC transporters that can identify and transport different types of substrates, including

antibiotics, flavones, terpenoids and xenobiotic chemicals (Jungwirth and Kuchler 2006). Yor1p, Ste6p and members of the PDR subfamily are main ABC transporters in the *S. cerevisiae* plasma membrane, which are encoded by *YOR1*, *SET6*, *PDR12*, *PDR5*, *SNQ2*, *AUS1*, *PDR15*, *PDR11*, and *PDR10*. Artemisinic acid production can be enhanced through overexpression of *PDR15*, *YOR1*, and *SNQ2* (Ro et al. 2008) and expression 1 of *PDR5* and *YOR1* of *S. cerevisiae* could be induced by thymol, which is a monoterpene phenol that is similar to D-limonene (Bi et al. 2010). Therefore, the main cytomembrane ABC transporter genes and two main regulators (*PDR1* and *PDR3*) in *S. cerevisiae* were investigated to identify their roles in D-limonene resistance in this work.

Materials and methods

Microorganisms and media

Saccharomyces cerevisiae CEN.PK2 (MATa/MATα *ura3*-52/*ura3*-52; *trp1*-289/*trp1*-289; *leu2*-3,112/*leu2*-3,112; *his3*Δ1/*his3*Δ1; *MAL2*-8^C/*MAL2*-8^C; *SUC2*/*SUC2*) from EUROSARF (Frankfurt, Germany) was used in this study. *Escherichia coli* JM109 was used to construct the expression plasmids. The plasmids used in the study were the pMD19-T Simple vector and the bacterial expression vector pYX212. YPD media (10 g yeast extract/l, 20 g peptone/l, and 20 g glucose/l) and YNBM media (20 g glucose/l, 5 g $(NH_4)_2SO_4$/l, 1.7 g yeast nitrogen base without amino acids (YNB)/l, 20 mg L-tryptophan/l, 30 mg L-leucine/l, and 20 mg L-histidine/l) were used for *S. cerevisiae* growth.

D-Limonene resistance test

D-Limonene (85–510 mg/l) was added in DSMO (Dambolena et al. 2008) to cultures when *S. cerevisiae* was about 0.45 g dry cell wt (DCW)/l. The same amount of DMSO was added to culture medium as control.

Real-time quantitative PCR analysis

Two hours after adding 85 mg D-limonene/l to cultures of the yeast, total RNA was extracted using RNAiso plus total RNA extraction reagent (Takara), PrimeScript RT reagent kit (Takara), and SYBR Premix Ex Taq (Takara). The primers and their amplicon efficiencies are shown in Supplementary Table 1. The housekeeping gene *ACT1* was used as a reference gene. The experiment was performed using the Light Cycler 480 II (Roche). q-PCR analysis was performed with an initial denaturation step at 95 °C for 40 s, followed by 40 cycles of amplification at 95 °C for 5 s and annealing/extension at 57 °C for 30 s, and cooling at 50 °C for 30 s. The results were analyzed using Light Cycler software (Roche).

Plasmid construction and transformation of *S. cerevisiae*

ABC genes in *S. cerevisiae* were PCR-amplified using the primers listed in Supplementary Table 2. The fragments were digested with corresponding restriction enzymes and inserted into the multi-cloning site of pYX212 (Zhou et al. 2009). The resultant plasmids were named pY-PDR3, pY-PDR12, pY-PDR1, pY-PDR5, pY-SNQ2, pY-AUS1, pY-PDR15, pY-PDR11, pY-STE6, pY-YOR1, pY-PDR10, respectively. *S. cerevisiae* CEN-PK2 transformants with these plasmids were named 3D-PDR3, 3D-PDR12, 3D-PDR1, 3D-PDR5, 3D-SNQ2, 3D-AUS1, 3D-PDR15, 3D-PDR11, 3D-STE6, 3D-YOR, 3D-PDR10, respectively. The transformants with vector pYX212 was named 3D, as control. The purified plasmids (Zhou et al. 2010) extracted from yeast transformants were further identified by PCR using the primers shown in Supplementary Table 3. The transcriptional level of these ABC genes of the *S. cerevisiae* transformants compared with the control were evaluated using q-PCR when the cell was ~0.45 g DCW/l.

The role of the ABC transporters on D-limonene resistance

To identify the effects of the ABC transporter genes, the resistances of the *S. cerevisiae* transformants and the control were compared. 85 mg D-limonene/l was added in the media when the cell was ~0.59 g DCW/l. The biomass of the strains was analyzed after 4 and 8 h. When the *S. cerevisiae* transformants was were ~0.32 g DCW/l, the medium was serially diluted

tenfold, and 2.5 µl was spotted onto YNBM-agar plates with 0 and 85 mg D-limonene/l separately (Dambolena et al. 2008), and incubated at 30 °C for 36 h. Three independent experiments were carried out in every completed experiment.

Results and discussion

Tolerance of *S. cerevisiae* under different concentration of D-limonene stress

Figure 1 shows the growth of *S. cerevisiae* under different concentrations of D-limonene: 510 mg D-limonene/l was lethal to but even 85 mg D-limonene/l inhibited growth but was not lethal. Based on these observations, 85 mg D-limonene/l was used as a stress concentration for q-PCR analysis.

Limonene plays a key role in the antimicrobial effect of lemon extracts and other essential oils. *Pichia subpelliculosa* and *Candida lusitaniae* had higher minimal inhibitory concentrations than *S. cerevisiae* (Conte et al. 2007). *Schizosaccharomyces pombe* was more sensitive to limonene than *S. cerevisiae* while the *Geotrichum candidum* was relatively insensitive (Tserennadmid et al. 2011). Tolerance of these yeasts is very low according the requirement of industrial usage (>100–∼500 mg D-limonene/l).

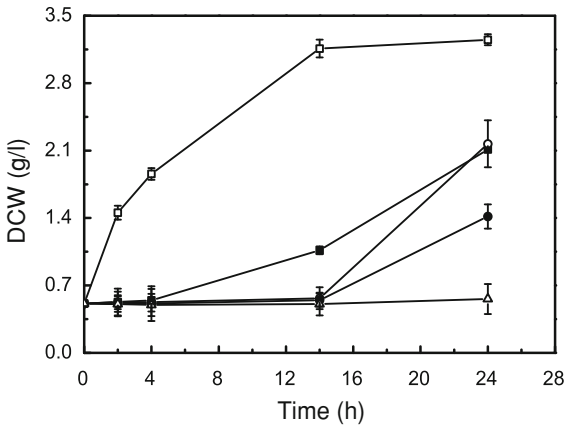

Fig. 1 Growth curves of *S. cerevisiae* under different D-limonene stress. The growth curve of *S. cerevisiae* CEN-PK2 in media with different D-limonene concentrations: zero D-limonene (*open square*), 85 mg D-limonene/l (*solid square*), 170 mg D-limonene/l (*open circle*), 340 mg D-limonene/l (*solid circle*), and 510 mg D-limonene/l (*open triangle*)

Transcriptional analysis of main ABC genes in *S. cerevisiae*

The transcriptional levels of the main ABC genes are shown in Fig. 2. The upregulation of these three genes may have been due to the relatively short-life span of these transporters, which are primarily active during the early and major growth phases, as their levels quickly decreased when cells exited the main growth phase (Jungwirth and Kuchler 2006). Therefore, inconspicuous differences in growth conditions may influence the expression levels. Most of these cytomembrane ABC transporter genes are regulated by *PDR1* and *PDR3* (Jungwirth and Kuchler 2006). However, *PDR1* and *PDR3* did not show the same tendency as *PDR5*, *PDR15*, and *YOR1*. These results indicated that the upregulation of these genes should not be attributed to their transport function.

Construction of *S. cerevisiae* transformants over-expressing ABC transporter genes

Saccharomyces cerevisiae transformants 3D-PDR3, 3D-PDR12, 3D-PDR1, 3D-PDR5, 3D-SNQ2, 3D-AUS1, 3D-PDR15, 3D-PDR11, 3D-STE6, 3D-YOR1 and 3D-PDR10, were constructed (Fig. 3b). The transcriptional levels of genes in these *S. cerevisiae* transformants are upregulated by varying degrees (Fig. 3c). The subcellular localization of these transporters and their primary substrates are shown in Fig. 3a.

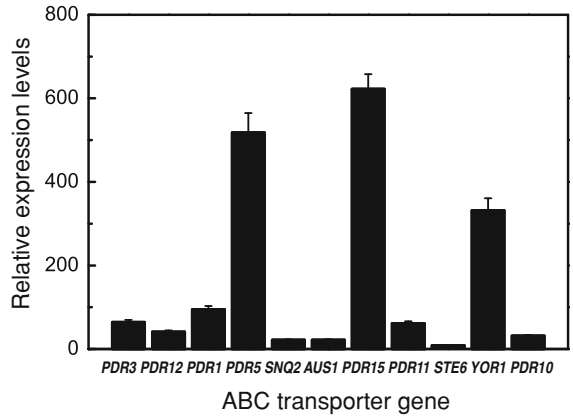

Fig. 2 Transcriptional levels of the key ABC transporter genes under D-limonene stress. *PDR5*, *PDR15* and *YOR1* were upregulated more than two-fold, while the other genes were down-regulated

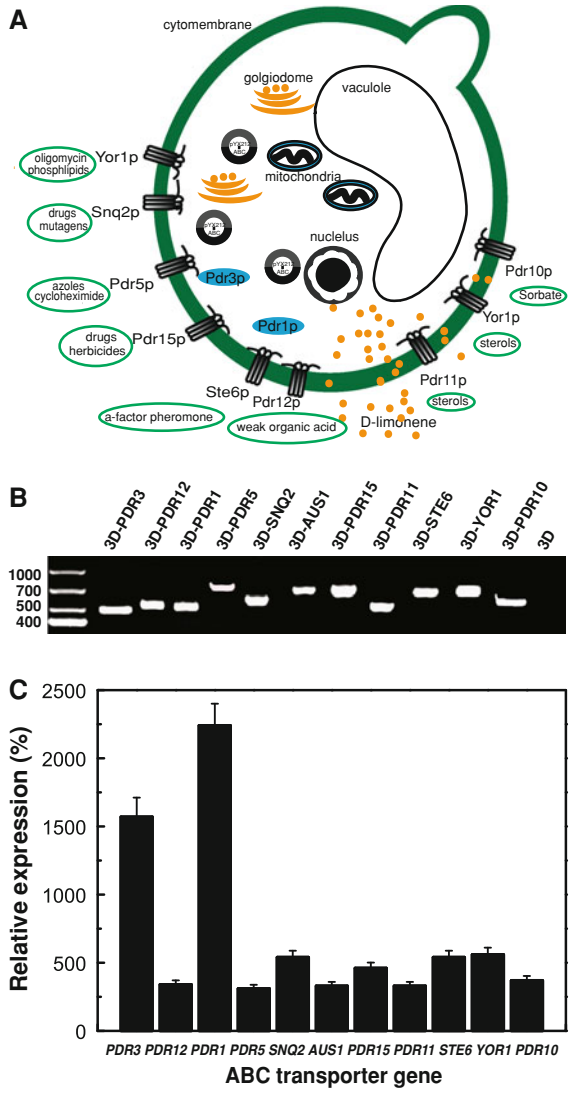

Fig. 3 Typical known targets of key ABC transporters and their relative transcriptional levels in corresponding overexpression transformants. **a** The subcellular localization of the cytomembrane ABC transporters and their typical target chemicals. **b** The result of the confirmation PCR, whose templates were the purified plasmids extracted from the transformants. The size of the fragments was shown in Table S3. **c** The relative expression levels of ABC transporter genes in *S. cerevisiae* after overexpression. All genes were upregulated, but to varying degrees compared to the control

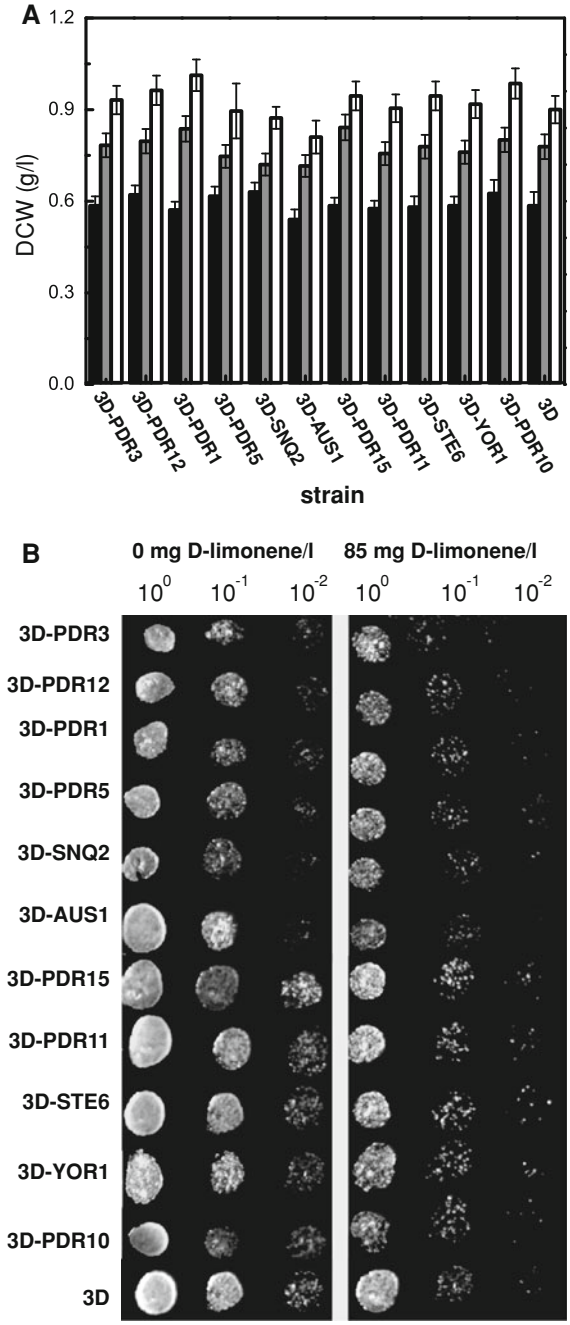

Fig. 4 Growth conditions of strains over-expressing different ABC transporter genes. **a** The *black* columns represent the DCW of the yeast before stress. The *white* columns represent the DCW after 4 h of stress, and the *grey* columns are that at 8 h. **b** YNBM plates with zero D-limonene (*left*) and 85 mg D-limonene/l (*right*)

Effects of overexpression of ABC transporter genes on D-limonene tolerance

Saccharomyces cerevisiae transformants had the same initial concentration before adding D-limonene and, 4 and 8 h later, the DCW of these strains were still similar (Fig. 4a). However, the transformants did not show greater tolerance than the control when growing on the YNBM-agar plates with D-limonene (Fig. 4b).

Although the most potentially active transporter Pdr5p can transport a large number of substrates, previous studies have shown that 4-nitroquinoline oxide, resazurin, and helminthosporal could not be recognized by the transporters due to the size of the molecules (Golin et al. 2002). However, all of these molecules are larger than D-limonene. Pdr15p and Yor1p are lipid transporters and homologues of Pdr5p. Snq2p and Pdr15p can actively transport several drugs. The substrates of Aus1p, Pdr10p and Pdr11p are mainly sterols (Gupta et al. 2011).

In conclusion, Although some of these substrates of these ABC transporters have similar characteristic with D-limonene, none of the ABC transporters can transport D-limonene. D-Limonene is a small hydrophobic molecule that can diffuse quickly across the bilayer (Fig. 3a), allowing little time for interaction with these transporters (Golin et al. 2002). The failure to improve monoterpene resistance by enhancing ABC transporters suggests that we should explore new routes to improve monoterpene tolerance, such as rational modification of cell membrane components, to expand the metabolic engineering strategies for both monoterpene accumulation and utilization of materials containing monoterpenes.

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