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Research Article

Over-expression of *ICE2* stabilizes cytochrome P450 reductase in *Saccharomyces cerevisiae* and *Pichia pastoris*

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Membrane-anchored cytochrome P450 enzymes (CYPs) are a versatile and interesting class of enzymes for industrial applications, as they are capable of regio- and stereoselectively hydroxylating hydrophobic molecules. However, CYP activity requires sufficient levels of suitable cytochrome P450 reductases (CPRs) for regeneration of catalytic capacity, which is a bottleneck in many industrial applications. Searching for positive effectors of membrane-anchored CYP/CPR function, we transformed and screened selected strains from a *Saccharomyces cerevisiae* knockout collection for *Hyoscyamus muticus* premnaspirodiene oxygenase (HPO; CYP) and *Arabidopsis thaliana* CPR (*At*CPR) expression levels, as well as for activity towards (+)-valencene. We found that in cells lacking the type III membrane protein Ice2p, *At*CPR was destabilized. Remarkably, over-expression of *ICE2* improved (+)-valencene hydroxylation to trans-nootkatol by 40–50%, both in resting cells and in vivo. Time-resolved immunoblot analysis and cytochrome c reductase activity assays revealed that Ice2 up-regulation stabilized *At*CPR levels and activity over extended periods of bioconversion. To underscore that we had identified a novel positive effector of recombinant CYP/CPR function, we confirmed the beneficial effect of *ICE2* over-expression for two further CYP/CPR combinations and the alternative host *Pichia pastoris*. Thus, we propose Ice2 up-regulation as a general tool for improving the applications of recombinant CYPs in yeasts.

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1 Introduction

Heterologous expression of membrane-anchored cytochrome P450 enzymes (CYPs) in microbial hosts for application in biocatalytic processes is challenging. However, particularly hydrophobic substrates are rather converted by membrane-anchored CYP/CPR systems than by their soluble counterparts [1, 2] rendering the membrane-attached activities highly interesting for industrial application. CYP activity is irrevocably linked to finely balanced NADPH cofactor recycling, to oxygen supply, to correct integration of heme iron into the active site of the CYP and to perfect interaction with the corresponding membrane-anchored cytochrome P450 reductase (CPR) that functions as electron donor [3–5]. The biotechnologi-

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Abbreviations: ***At*CPR,** *Arabidopsis thaliana* CPR; **CPR,** cytochrome P450 reductase; **CYP,** cytochrome P450 enzyme; **CYP2D6,** human cytochrome P450 2D6; **ER,** endoplasmic reticulum; **FPP,** farnesyl pyrophosphate; **hCPR,** human CPR; **HPO,** *Hyoscyamus muticus* premnaspirodiene oxygenase; **PM17,** *Mentha piperita* (–)-limonene-3-hydroxylase; **t*HMG1*,** truncated 3-hydroxy-3-methyl-glutaryl-coenzyme A reductase 1; **ValS,** (+)-valencene synthase

cal application of membrane-anchored CYP/CPR systems is furthermore hampered by limited operational stability of these activities. So far, solutions to this problem include fine-tuning of the relative expression levels of CYP/CPR [6] and covalent coupling of CYP and CPR [7, 8].

In this study, we have employed *Hyoscyamus muticus* premnaspirodiene oxygenase CYP71D55 (HPO), variant V482I A484I [9], and *Arabidopsis thaliana* CPR (*At*CPR) as a membrane-attached CYP/CPR model system. HPO/*At*CPR activity was monitored by trans-nootkatol formation from (+)-valencene [10, 11]. Searching for effectors of HPO/*At*CPR function, we have transformed and screened *S. cerevisiae* single-gene knockout strains from the EUROSCARF collection [12, 13] for HPO/*At*CPR activity and protein levels. Our most remarkable finding was that *At*CPR was prone to degradation in the Δ*ice2* knockout strain. Over-expressing *ICE2* increased HPO/*At*CPR-mediated bioconversion of (+)-valencene 1.4-fold in resting cells assays. Similarly, co-expression of *ICE2* in strains with intracellular (+)-valencene formation and conversion increased the trans-nootkatol yield by 40–50% to 30 mg L^{-1} of culture. Therefore, (+)-valencene synthase (ValS), a soluble enzyme originating from Alaska cedar heartwood (*Callitropsis nootkatensis*), had been introduced into baker's yeast [14]. Ice2p is a type III membrane protein with eight predicted transmembrane domains and an essential role in endoplasmic reticulum (ER) localization and inheritance in budding yeast [15, 16]. Furthermore, Ice2p has been suggested to play a role in intracellular zinc homeostasis [17] and, lately, also in neutral lipid transport [18]. A detailed study on the molecular function(s) of Ice2p beyond the description of phenotypes triggered by lack of Ice2 is missing. Strikingly, we have found that enhanced expression of Ice2 stabilized *At*CPR protein levels and activity over extended periods of (+)-valencene bioconversion. To test for general applicability of *ICE2* over-expression in stabilizing membrane-anchored CYP/CPR activities for biocatalytic application, we have extended our analysis to two alternative CYP/CPR combinations and *Pichia pastoris* as expression host and have detected CPR stabilization throughout.

2 Materials and methods

2.1 Chemicals

Unless stated otherwise, standard laboratory reagents were obtained from Sigma–Aldrich® (Steinheim, Germany) or Carl Roth GmbH & Co. KG (Karlsruhe, Germany) with the highest purity available. The pYES2 expression vector was purchased from Invitrogen (Carlsbad, USA). The pESC-URA expression vector was a kind gift of Howard Riezman (Geneva, Switzerland). Restriction enzymes were acquired from Thermo Scientific (St. Leon-Rot, Germany). Difco™ yeast nitrogen base w/o amino acids (YNB), Bacto™ tryptone and Bacto™ yeast extract were obtained from Becton Dickinson and Company (Schwechat, Austria). Geneticin sulfate (G418), Hygromycin B and Kanamycin monosulfate were ordered from Formedium™ (Norfolk, United Kingdom). Zeocin™ was purchased from InvivoGen (Eubio, Vienna, Austria). Sterile water was from Fresenius Kabi (Graz, Austria). Terpenoid standards were supplied by DSM Innovative Synthesis B.V. (Geleen, The Netherlands).

2.2 Microorganisms, plasmids, strain construction, and cultivation

For all cloning steps and plasmid replication, *E. coli* Top10 F' from Life Technologies (Vienna, Austria) was used. *S. cerevisiae* strains were constructed either in the BY4742 or in the W303 background (Supporting information, Table S1). Cloning and strain construction are described in the supplementary materials (Supporting information, File S1 and Tables S2 and S3). BY4742 and single gene knockout strains thereof were from the EUROSCARF collection (http://web.uni-frankfurt.de/fb15/mikro/euroscarf/) as a kind gift of Günther Daum (Graz, Austria). *S. cerevisiae* strains were cultivated in synthetic defined (SD) media (6.7 g yeast nitrogen base w/o amino acids; 1 g drop-out powder consisting of equal amounts of adenine, lysine, tyrosine, histidine, leucine, and tryptophane; 2% glucose). *P. pastoris* cultures were either grown in YPD (1% yeast extract, 2% peptone, and 2% glucose) or buffered complex glycerol medium, BMGY (1% yeast extract, 2% peptone, 100 mM potassium phosphate, pH 6.0, 1.34% YNB, 4×10^{-5} % biotin, 1% glycerol). BMMY (1% yeast extract, 2% peptone, 100 mM potassium phosphate, pH 6.0, 1.34%YNB, $4 \times 10^{-}$ % biotin, 1% methanol) was used for induction. Transformants were pinned onto plates containing up to 2 mg mL^{-1} Zeocin™ in screening for multi-copy gene integration events of CYP/CPR.

2.3 Quantitative RT-PCR

Three-hundred μL of galacatose-induced cell culture were harvested in a table top centrifuge and resuspended in RNAlater® as recommended by the supplier (Life Technologies, Vienna, Austria). Purified RNA extracts were prepared using ZR Fungal/Bacterial RNA MiniPrep™ kit (ZymoResearch, Freiburg, Germany). RNA quality and concentrations were determined via NanoDrop ND2000 (Thermo Scientific, St. Leon-Rot, Germany) and ethidium bromide gel electrophoresis. qRT-PCR was performed in a 2-step procedure. For reverse transcription, the Revert Aid Premium First Strand cDNA Synthesis Kit from Thermo Scientific (St. Leon-Rot, Germany) was used as described in the manual. The quantitative PCR step was carried out with the Maxima SYBR Green PCR MM (2×) from Thermo Scientific (St. Leon-Rot, Germany) applying

the respective forward and reverse primers (qRT-labeled primers; Supporting information, Table S2). Primers had been constructed with the help of the Primer3 program comprising the recommended guidelines (frodo.wi.mit.edu) [19, 20]. A no-template control and a reverse transcriptase minus (RT-) control were performed and tested negative for impurities or contaminant DNA. Results were evaluated based on the ΔC_t method via normalization of the observed values onto the house-keeping gene *CDC73* [21].

2.4 Resting cells assays for (+)-valencene, (–)-limonene and bufuralol conversion

To express protein for resting cells assays, 300-mL baffled shake flasks containing 50 mL of SD growth media were inoculated with *S. cerevisiae* strains to an OD_{600} of 0.1 and were shaken for 48 h at 130 rpm and 30°C. After centrifugation for 5 min at 1062×*g*, cell pellets were resuspended in 50 mL of SD induction media (containing 2% galactose and 0.7% raffinose instead of glucose) and were induced at 130 rpm and 30°C for 7 h. Randomly chosen *P. pastoris* transformants were cultivated in 96-well deep well plates (DWPs) as described [22]. In brief, cells were grown in 250 μL of BMGY medium for 24 h at 28°C, 320 rpm and 80% humidity. Induction was started by addition of 250 μL of BMMY (2% methanol). Methanol was added every 12 h to a final concentration of 1% until 48 h of induction.

For (+)-valencene and (–)-limonene bioconversions, 600 OD_{600} units of galactose-induced *S. cerervisiae* cells were harvested and resuspended in 2 mL of 50 mM potassium phosphate buffer, pH 7.4. Cell suspensions were split into two equal aliquots in PYREX® tubes and 20 μL of 100 mM (+)-valencene or 300 mM (–)-limonene in DMSO as well as 1% of Triton® X-100 (Amresco, Solon, Ohio) were added, respectively. PYREX® tubes were sealed loosely with screw caps to balance between air supply for cells and substrate volatilization during conversion. Biotransformations were carried out for 16 h at 170 rpm and 30°C. Terpenoids were extracted with 500 μL of ethyl acetate using a VXR basic Vibrax® (IKA®, Staufen, Germany) at room temperature and maximum speed for 30 min. After phase separation by centrifugation for 15 min at 2720×*g*, organic layers were verified via GC-MS and quantified via GC-FID (Supporting information, File S2). Pre-selected *P. pastoris* transformants were cultivated in shake flasks and assayed for conversion of 2 mM (+)-valencene or 6 mM (–)-limonene as described [11].

Bufuralol conversion assays employing *S. cerevisiae* were conducted exactly as reported [6]. *P. pastoris* transformants were cultivated in 96-DWPs as described. After 48 h of induction, OD_{600} values of cultures in each well were determined followed by centrifugation of the DWP at 3220×*g* for 5 min. The supernatants were discarded and the cell pellets were resuspended in 100 mM potassium phosphate buffer, pH 7.4. Prednisolone was added to the supernatants as an internal standard to a final concentration of 50 ng μL^{-1}. The reaction mix was analyzed by HPLC-MS according to the method described [6].

2.5 Biphasic (+)-valencene synthesis and bioconversion assay

Adapting the protocol of Cankar et al. [23], *S. cerevisiae* cells co-expressing HPO, CPR, ValS, and t*HMG1* were cultivated in 50 mL SD growth media in 100 mL shake flasks, starting at an OD_{600} of 0.1. After 24 h at 30°C and 170 rpm, 1 mL of induction solution (20% galactose and 7% raffinose) and 1 mL of supplement solution, that is 0.167 g yeast nitrogen base and 25 mg dropout powder dissolved in sterile ddH_2O, were added to the cell suspension. Five milliliters of n-dodecane were added directly to the flasks to form second, organic phases. Cells were cultivated for 24 h, followed by a second addition of 1 mL each of induction and supplement solutions. Cells were induced for up to 72 h, before the n-dodecane phases were subjected to GC analyses. Cultivation and biphasic bioconversion employing *P. pastoris* was performed according to published procedures [11].

2.6 SDS-PAGE/immunoblotting (Fiji quantification)

SDS-PAGE and immunoblotting were performed as described [11]. Immunoblot signal intensities were quantified with Fiji as biological image analysis platform [24]. Samples were loaded in triplicates and average signal intensities of HPO and CPR were normalized to the control strain set to 100%.

2.7 Cytochrome c reductase assay

CPR activity was estimated by its ability to reduce bovine heart cytochrome c [25]. *P. pastoris* and *S. cerevisiae* cell lysates were prepared by glass bead lysis according to the *Pichia* Expression Kit manual (Life Technologies, Vienna, Austria). Protein amounts in cell lysates were quantified using the Bio-Rad (Bradford) protein assay with bovine serum albumin as a standard. Cytochrome c reductase activity assays were conducted as described [6].

3 Results

3.1 Screening for effectors of CYP/CPR function in recombinant *S. cerevisiae* strains

An important feature of CYP function is their requirement for cytochrome P450 reductase (CPR) activities regenerating the catalytically active CYPs. There are reports suggesting mutual stabilization of membrane-associated CYPs and CPRs necessitating balanced stoichiometry upon heterologous expression of such CYP/CPR pairs

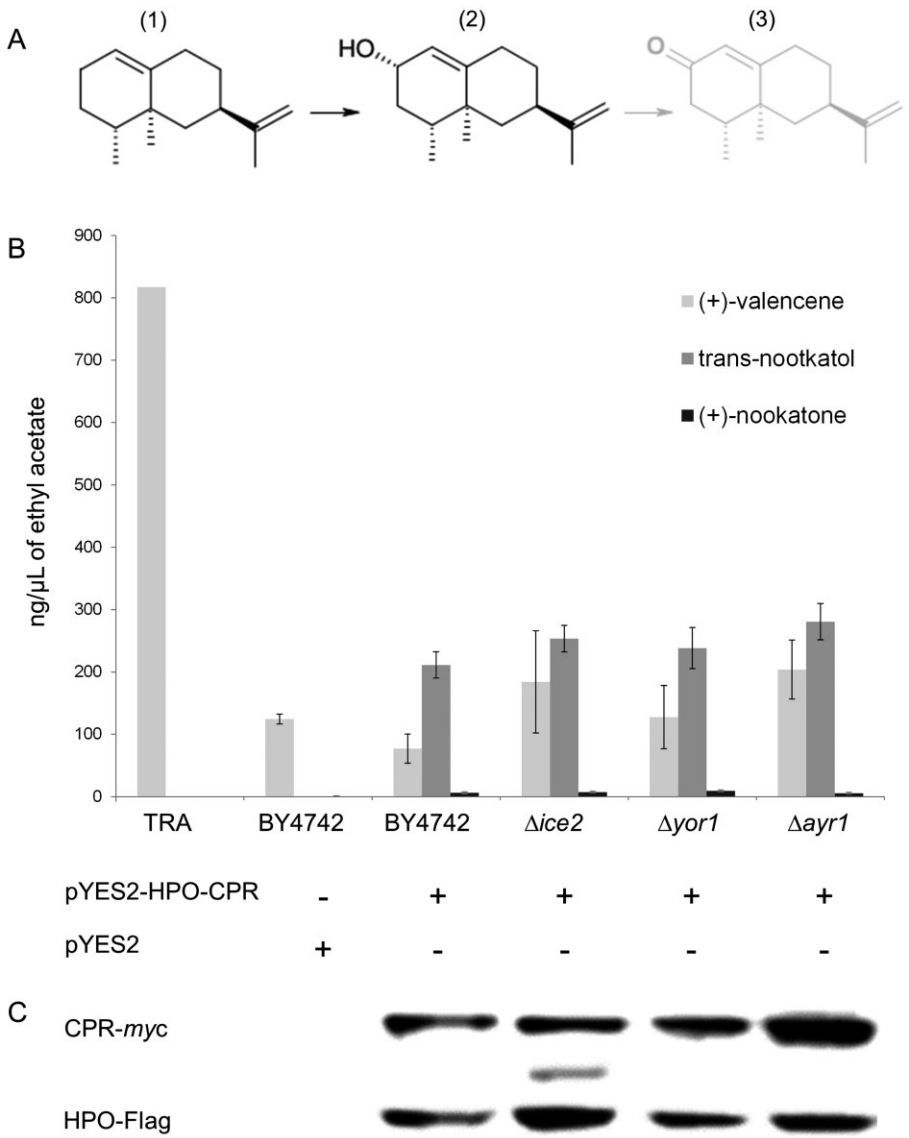

Figure 1. (+)-Valencene (1) biohydroxylation by HPO/*At*CPR activity recombinantly expressed in *S. cerevisiae*. trans-Nootkatol (2) formed by HPO/*At*CPR may be further oxidized to (+)-nootkatone (3) by an unidentified intrinsic activity of baker's yeast (**A**). Potential effectors of HPO/*At*CPR activity in *S. cerevisiae* were screened for by resting cells assays with a theoretically re-extractable amount (TRA) of 817 ng of (+)-valencene per µL of ethyl acetate (**B**). Assays on BY4742 (evc) indicated substrate loss within 20 h of assay. BY4742 and single knockout strains disrupted for *Δice2*, *Δyor1*, and *Δayr1*, respectively, in the same background were transformed with pYES-HPO-CPR to be assayed for (+)-valencene conversion. Results are given as mean values and standard deviations of resting cells assays performed in technical quadruplicates of two biological replicates. Immunoblot analyses were performed to reveal HPO/*At*CPR levels in total proteins of 1 OD_{600} unit after 6 h of induction (**C**).

[6, 26, 27]. We have functionally co-expressed HPO, variant V482I A484I [9], and *A. thaliana* cytochrome P450 reductase (*At*CPR) from a multicopy vector (pYES2-HPO-CPR) in *S. cerevisiae*. Recombinant yeast strains hydroxylated (+)-valencene to trans-nootkatol as expected (Figs. 1A and B). Terpenoids were verified by GC-MS (Supporting information, Fig. S1). Co-expression of HPO/*At*CPR from a single vector allowed to screen available yeast strain collections for effectors of HPO/*At*CPR protein levels and activities. Selected single-gene knockout strains of the EUROSCARF collection were transformed with the pYES2-HPO-CPR plasmid and were tested for altered (+)-valencene conversion in resting cells assays. Most transformants showed conversion rates indistinguishable from the reference strain BY4742 pYES2-HPO-CPR (Fig. 1B and data not shown). A severe drawback of external (+)-valencene addition in resting cells assays was the high volatility of (+)-valencene in aqueous environment. After 20 h of conversion, only 20% of the initially added (+)-valencene was re-extractable from control cells harboring the empty pYES2 vector (Fig. 1B). Because side-products of (+)-valencene hydroxylation were not detected in GC-MS, we ascribe the loss to evaporation. In equivalent experiments, only 30% of the added trans-nootkatol evaporated under the same conditions. Aliquots of galactose-induced cells were routinely checked for HPO-Flag (53 kDa) and CPR-*myc* (72 kDa) levels by immunoblotting. Thereby, we observed that CPR-*myc* appeared to be partially degraded in BY4742 *Δice2* (Fig. 1C), although trans-nootkatol formation by HPO/CPR activity was not significantly different in BY4742 *Δice2* as compared to the reference strain (Fig. 1B).

3.2 Over-expression of *ICE2* in *S. cerevisiae* W303

Among the various *S. cerevisiae* strains used for CYP-mediated bioconversions, it has particularly been the W303 strain that turned out to be the most applicable host [28–31]. Many of the standard lab strains of *S. cerevisiae*, but not the W303 strain, do carry a deletion in the *HAP1* gene. Hap1p is a transcription factor responsible for regulation of genes governing intracellular heme abundance in response to changes in available oxygen levels [32, 33]. A Δ*hap1* strain is supposedly less favorable for CYP expression and function as these are strongly dependent on optimal heme and oxygen supply. To analyze host strain effects, we performed HPO/*At*CPR-mediated resting cells conversion of (+)-valencene in parallel in the BY4742 and the W303 yeast strains harboring pYES-HPO-CPR. As expected, (+)-valencene conversion levels were two-fold higher in the W303 strain background yielding 400 ng trans-nootkatol per µL of ethyl acetate (Fig. 2A) than in the BY4742 strain background (Fig. 1B). In both strain backgrounds, the knockout of Δ*ice2* did not significantly influence the conversion of (+)-valencene to trans-nootkatol as compared to the corresponding reference strains. Since (+)-valencene conversion was substantially more efficient in the W303 strain background, we focused our further efforts on this host strain.

Triggered by the degradation products of CPR-*myc* observed in the BY4742 Δ*ice2* knock-out strain (Fig. 1C), we created strains expressing *ICE2* from a P_{GAL1} promoter inserted in front of the gene and quantified the effect

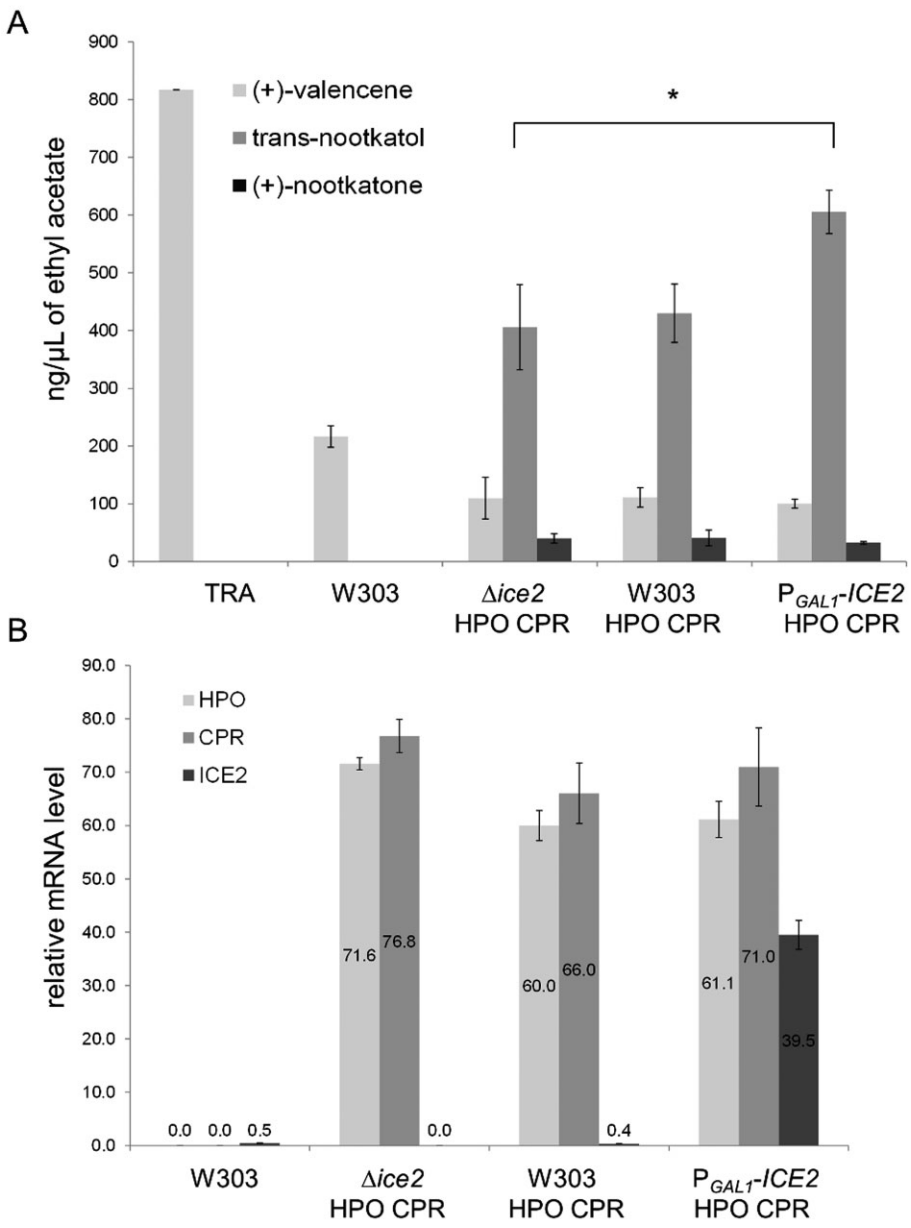

Figure 2. Assessing the effect of modulated *ICE2* expression levels on HPO/*At*CPR-mediated conversion of (+)-valencene. Control strain W303 and Δ*ice2* as well as P_{GAL1}-*ICE2* strains co-expressed HPO and *At*CPR from the pYES2 vector. Mean values and standard deviations are based on resting cells activity assays performed in three biological and four technical replicates each (**A**). The asterisk (*) indicates statistically significant difference in trans-nootkatol formation ($p < 0.01$, Student's *t* test). mRNA levels were quantified through real-time PCR analysis according to the $\Delta\Delta C_t$ method normalizing expression levels to the housekeeping gene *CDC73* (**B**).

on HPO/*At*CPR expression and (+)-valencene conversion, which were ideally controlled by the same promoter element. Interestingly, expressing *ICE2* from P_{GAL1} resulted in 1.4-fold improved (+)-valencene conversion independent of the host strain background ($p < 0.01$, Fig. 2A and data not shown). The manipulation of ICE2 expression levels did not significantly affect cellular growth characteristics, neither before nor after induction. To quantify expression of HPO, CPR, and *ICE2* on the mRNA level, quantitative real-time PCR was performed on all strains with modulated *ICE2* expression. RNA was extracted after 6 h of induction and results were normalized against the housekeeping gene *CDC73* in each sample. *ICE2* appeared to be expressed at low levels in wild type cells (Fig. 2B), which is consistent with studies calculating a total number of 606 molecules Ice2p per cell growing exponentially in YPD [34]. Strikingly, another ER-membrane spanning protein, Sec61p was suggested to be present in roughly 24 800 copies per cell. Expressing *ICE2* from the P_{GAL1} promoter resulted in 100-fold up-regulation of gene expression as compared to the strains with *ICE2* under the control of its own promoter (Fig. 2B). Interestingly, mRNA levels of HPO and *At*CPR were largely independent of Ice2 modulation. Thus, we hypothesized that the enhanced biohydroxylation of (+)-valencene observed for the W303 P_{GAL1}-*ICE2* pYES2-HPO-CPR strain was mediated by posttranscriptional effects of elevated ICE2 expression on HPO/*At*CPR protein levels and/or activity.

3.3 In vivo bioconversion of (+)-valencene in *S. cerevisiae*

Assessing HPO/*At*CPR-driven (+)-valencene hydroxylation in resting cells and quantifying the effect of P_{GAL1}-*ICE2* expression thereon is subject to one important uncertainty factor, which is the availability of the (+)-valencene substrate. It is not only the evaporation of the terpene in aqueous environment that needs to be taken into account (Figs. 1B and 2A), but also diverse transport processes into and out of *S. cerevisiae* cells. To a pure hydrocarbon compound like (+)-valencene, the plasma membrane of yeast should not pose a stringent barrier for entry into the yeast cell. However, there are plenty of export mechanisms described for *S. cerevisiae* efficiently expelling hydrophobic compounds from the cell interior, for example diverse ABC transporters [35, 36] and Pry proteins [37, 38]. Thus, the intracellular (+)-valencene concentration in resting cells assays is influenced by multiple factors that may be beyond the control of the experimenter. This situation obviously leads to the relatively high standard deviations in resting cells assays (Figs. 1B and 2A). Moreover, cell engineering, as for example over-expression of *ICE2*, might have an impact on the equilibrium of (+)-valencene transport across the yeast plasma membrane and, therefore, on intracellular availability of the compound. In order to avoid these uncertainties, we generated strains producing (+)-valencene intracellularly from FPP. Expression of sequence-optimized valencene synthase (ValS) from *C. nootkatensis* in *S. cerevisiae* W303 in our hands required co-expression of truncated *HMG1* in contrast to published work [14]. Strains expressing ValS without concomitant over-expression of t*HMG1* grew extremely slowly, probably due to massive withdrawal of FPP from ergosterol biosynthesis, which can impair cell growth [39–41]. In numerous related projects, over-expression of t*HMG1* has successfully enhanced production of terpenoids [42–45]. Cells co-expressing ValS, t*HMG1*, HPO, and *At*CPR were initially cultivated according to a described protocol [23], but cell growth was rather poor under these conditions. Conditions were adapted to pre-cultivating cells in 50 mL of glucose media for 24 h, followed by 24–72 h of induction. Overlaying the cell broth with 10% n-dodecane simultaneously with induction, entrapped all terpenoids formed during bioconversions [11, 46]. This elegant method prevents inhibitory effects of high concentrations of terpenoids on yeasts providing an on-line extraction step. High concentrations of terpenoids have recently been described to hamper (+)-valencene biohydroxylation in *S. cerevisiae* [10].

Thereby, the W303 *tHMG1* ValS pYES2-HPO-CPR strain produced roughly 16 mg of trans-nootkatol per liter of culture broth (Fig. 3). Remarkably, production of trans-nootkatol was increased by ~40% ($p < 0.01$) upon up-regulating *ICE2* resulting in a maximum productivity of 22 mg trans-nootkatol per liter of cell culture after 72 h (Fig. 3). Knocking out *ICE2*, on the other hand, did not influence the amount of terpenoids formed as compared to the reference strain. Consequently, modulation of Ice2 expression did have equivalent effects upon HPO/*At*CPR-mediated (+)-valencene hydroxylation in resting cells upon external addition of substrate (Fig. 2A) and in cells producing the substrate in vivo (Fig. 3). These results underscore that the beneficial effect of *ICE2* over-expression on HPO/*At*CPR function was not due to altering bioavailability of the (+)-valencene substrate, but was supposedly due to altering HPO/*At*CPR enzyme activity.

3.4 *ICE2* over-expression stabilizes *At*CPR levels and activity

To characterize the effects of *ICE2* over-expression in more detail, we created strains that expressed Ice2p-His_6 either from its endogenous promoter or from P_{GAL1}, and co-expressed HPO/*At*CPR, ValS, and t*HMG1*. These strains were subjected to time course bioconversion studies comparing HPO-Flag, CPR-*myc*, and Ice2p-His_6 levels by immunoblotting besides quantifying terpenoid formation by GC (Fig. 4). Samples taken from the n-dodecane phase after 24, 48, and 72 h of induction revealed that for the first 48 h of bioconversion there was only little difference in trans-nootkatol productivity (≤10%) between the strains that differed in the expression of Ice2p-His_6

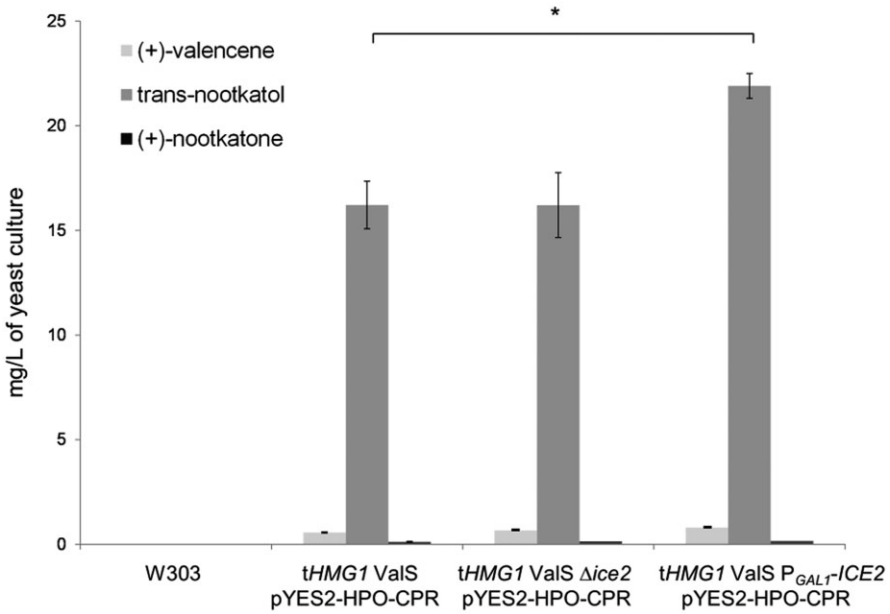

Figure 3. In vivo production and conversion of (+)-valencene in cells co-expressing ValS, t*HMG1*, HPO, and CPR and strains additionally harboring a deletion of *ICE2* or over-expressing *ICE2* from the P_{GAL1} promoter. Strains were compared to the control strain W303. Biotransformations were performed for 72 h in biological triplicates. Values are presented as means and standard deviations. The asterisk (*) indicates statistically significant difference in trans-nootkatol formation ($p < 0.01$, Student's *t* test).

(Fig. 4A). However, after 72 h of bioconversion, the strain expressing Ice2p-His_6 from the galactose-inducible promoter formed 300 ng trans-nootkatol μL^{-1} of n-dodecane (equivalent to 30 mg L^{-1} of yeast culture), which was about 50% more than produced by the strain expressing Ice2p-His_6 from its native promoter ($p < 0.01$, Fig. 4A). Immunoblot analysis showed that P_{GAL1}-driven *ICE2* overexpression was not only detectable on the mRNA level (Fig. 2B), but also translated into higher protein levels at each time point (Fig. 4C). The Ice2p-His_6 signals were hardly detectable when under the control of its native promoter in 24 h samples and further decreased at later time points. Ice2p-His_6 signals were much higher with a slight decrease in 72 h samples when the protein was expressed from P_{GAL1}. Most interestingly, HPO-Flag and, particularly, *At*CPR-*myc* protein levels were clearly more stable over 72 h of bioconversion in the strain expressing higher levels of Ice2p-His_6 (Fig. 4B). It is reasonable to assume that P_{GAL1}-driven Ice2p-His_6 expression enhanced (+)-valencene bioconversion by stabilizing *At*CPR levels, because HPO action requires CPR for regeneration. Thus, the strain harboring elevated levels of Ice2p-His_6 and detectable amounts of *At*CPR-*myc* will have further hydroxylated (+)-valencene until 72 h of conversion, in contrast to the strain that basically lacked *At*CPR-*myc* protein at the last time point. Yet, protein levels might not be representative of HPO/*At*CPR activity required for specific hydroxylation of (+)-valencene. Unfortunately, the active fraction of HPO could not be characterized by CO-difference spectroscopy [47] due to low signal intensities in whole cells, cell homogenates and microsomal fractions. Yet, we quantified the activities of cytochrome P450 reductase at different time points of bioconversion using the cytochrome c reductase assay. The evaluation of CPR activity is based on the reduction of oxidized cytochrome c and detecting reduced cytochrome at 550 nm [25]. Reference strains without co-expression of HPO and *At*CPR, that is strains P_{ICE2}-*ICE2*-His_6 (evc) and P_{GAL1}-*ICE2*-His_6 (evc) were found to have background reductase activities of 0.030 ± 0.004 and 0.042 ± 0.007 U per mg of total protein, respectively. This was in good agreement with values reported for the W303 strain [6]. Upon subtracting the background activity values, we realized that *At*CPR activity was virtually independent of *ICE2* expression at 24 h of terpenoid bioconversion experiments, but strongly decreased at low Ice2p levels after 48 h of galactose/raffinose induction (Fig. 4D). While over-expression of ICE2 from the inducible promoter stabilized roughly 50% of *At*CPR activity over 72 h of assay time, *At*CPR activity was not detectable in the strain expressing *ICE2* from its native promoter. These values corresponded very well to the *At*CPR-*myc* levels detected by immunoblotting (Fig. 4C). Thus, we propose that the positive effect of *ICE2* over-expression on HPO/*At*CPR-based (+)-valencene hydroxylation is mediated–at least partially–by stabilization of CPR activity during in vivo terpenoid formation.

Following up studies reporting an altered cellular ER membrane distribution in *S. cerevisiae* cells disrupted for *ICE2* [15, 16], electron microscopy images of engineered strains were generated (Supporting information, Fig. S2). Over-expression of membrane proteins usually results in formation of staggered ER-membranes, also called karmellae [48]. The same effect was observed for W303 wild type, Δ*ice2* and P_{GAL1}-*ICE2* strains overexpressing HPO and CPR (Supporting information, Fig. S2). As expected, knocking out *ICE2* did slightly reduce amounts of peripheral ER membranes (Supporting information, Fig. S2B), although the effect was not as explicit as shown

by Tavassoli et al. (2013). However, in the latter work electron microscopy images of an *ICE2-scs2* double knockout strain were presented. Notably, over-expression of *ICE2* did lead to a significant increase in ER membranes adjacent to the yeast plasma membrane (Supporting information, Fig. S2C).

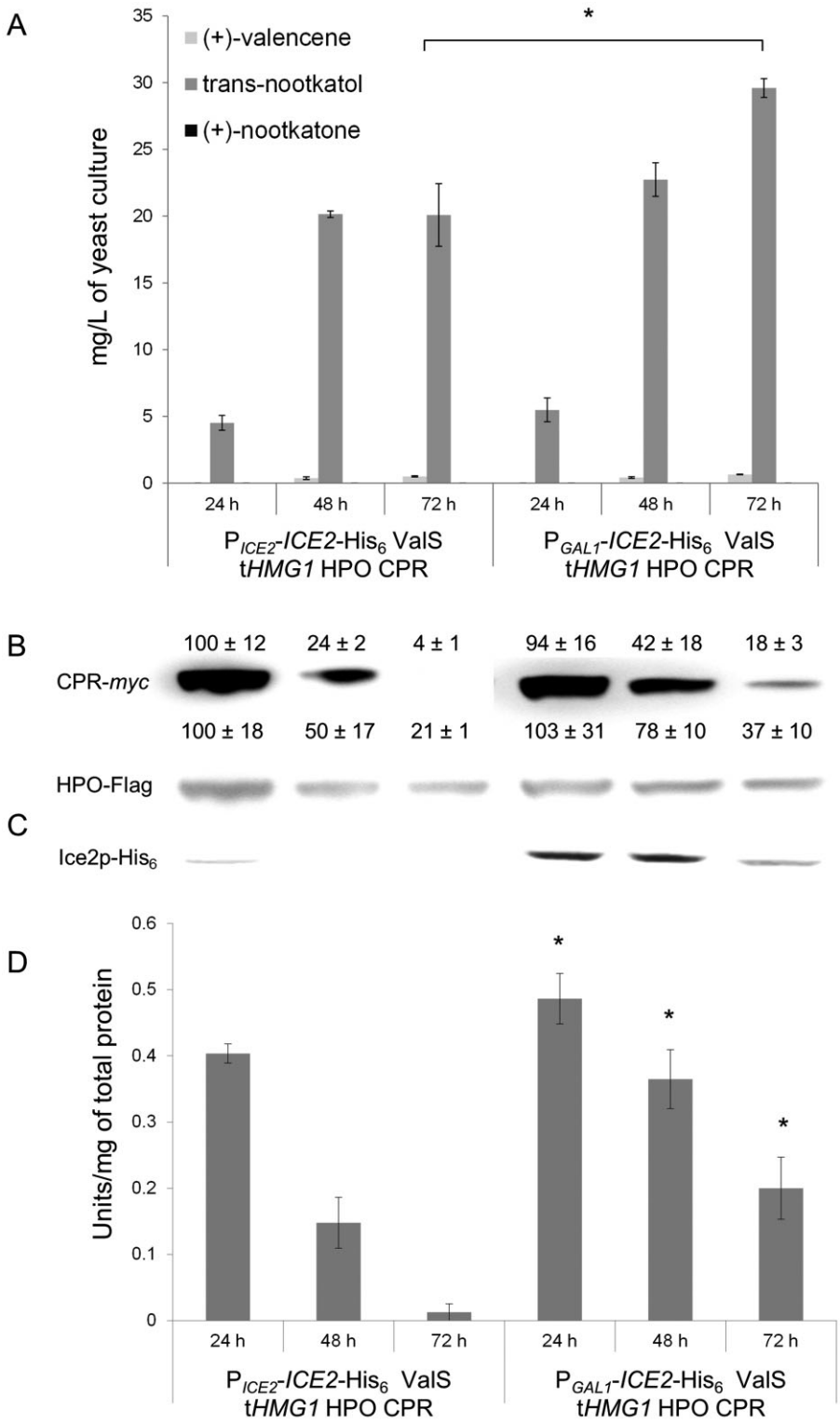

Figure 4. Comparing strains expressing Ice2 either from its endogeneous promoter or from P_{GAL1} for *in vivo* terpenoid production in the W303 t*HMG1* ValS HPO CPR strain background (**A**). Quantification of immunoblot signals was done for samples representing 1 OD_{600} unit of cells taken at time points 24, 48, and 72 h. Mean band intensities were calculated from quadruplicate immunoblots. A representative image is shown (**B**). Immunoreactive bands were normalized for the 24 h samples of the strains expressing Ice2 from its native promoter. At the same time points, expression of Ice2p-His_6 was tracked for strains expressing Ice2 from either its endogenous promoter or from the P_{GAL1}-promoter (2 and 1 OD_{600} units were loaded, respectively) (**C**). Cytochrome c reductase activity assay was done with the control strain W303 P_{ICE2}-*ICE2*-His_6 t*HMG1* ValS HPO CPR and the strain over-expressing *ICE2*-His_6. Background reductase activities of strains W303 *MATa* P_{ICE2}-*ICE2*-His_6 and W303 *MAT*α P_{GAL1}-*ICE2*-His_6 were subtracted. Data of technical quadruplicates with samples taken from two independent cultivations is shown as means and standard deviations. The asterisk (*) indicates statistically significant differences in trans-nootkatol formation (**A**) or *At*CPR activity (**D**) as compared to the corresponding strain with the endogenous *ICE2* promoter ($p < 0.01$, Student's *t* test).

3.5 General effect of *ICE2* over-expression on CPR stability

To test whether stabilization of recombinant CPR levels and/or activity is a general effect of *ICE2* over-expression we extended our analysis to alternative CYP/CPR combinations in *S. cerevisiae* and *P. pastoris*. Therefore, we expressed (–)-limonene-3-hydroxylase from *Mentha piperita* (PM17) together with *At*CPR as well as human cytochrome P450 2D6 (CYP2D6) combined with human reductase (hCPR). PM17 and CPR catalyze the conversion of (–)-limonene to (–)-trans-isopiperitenol [49, 50]. CYP2D6 and hCPR act as a main detoxifying system of drugs in the human liver [51], which is assessed by hydroxylation of the model substrate bufuralol to 1-hydroxybufuralol [6]. While (–)-limonene conversion was not enhanced in *S. cerevisiae* by over-expression of *ICE2* (Supporting information, Fig. S3A), hydroxylation of bufuralol, however, was improved 1.5-fold (Supporting information, Fig. S3B). Immunoblot analysis did not reveal any strain-dependent alterations of HPO and CPR levels (Supporting information, Fig. S3C). In contrast, analyses of CPR activities in *ICE2* over-expression strains showed a clear stabilization effect, particularly after 24 h of induction (Table 1). After 6 h of induction, the reductases showed consistently higher activities in the Ice2-upregulated strain. The Ice2 effect was even more striking if cells were induced for 24 h leading to three- to four-fold higher reductase activities in strains over-expressing *ICE2* compared to the wild-type situation (Table 1).

A *P. pastoris* strain producing (+)-valencene, trans-nootkatol and (+)-nootkatone was constructed by Wriessnegger et al. (2014) [11]. Co-expression of *PpICE2* did not improve terpenoid yields (Fig. 5A), but the *At*CPR level was slightly increased in the strain over-expressing *ICE2* (Fig. 5B). (–)-Limonene conversion was troublesome in *P. pastoris* (Fig. 5C) although (–)-limonene-3-hydroxylase and *At*CPR proteins were readily detected in immunoblots (Figs. 5C and E). Several studies are describing toxic effects of (–)-limonene on yeast cells [52, 53]. We speculate that *P. pastoris* might have more efficient mechanisms to get rid of potentially cell-toxic substances such as monoterpenoids than *S. cerevisiae* (own unpublished results). Indeed, conversion of (–)-limonene was only detected if *PpICE2* was co-expressed (Fig. 5C). *PpICE2* over-expression improved conversion of bufuralol 2.5-fold ($p < 0.01$, Fig. 5D). Interestingly, after 48 h of induction, we observed a higher CPR activity in all three *P. pastoris* co-expression systems (Table 1). Thus, in all CYP/CPR systems tested–in both yeasts–a significant stabilization of CPRs was observed in strains with up-regulated Ice2 suggesting that Ice2p is involved in stabilizing membrane protein levels and/or activities.

4 Discussion

S. cerevisiae has been shown to be a suitable host for either conversion of externally added (+)-valencene [10] or in situ production of the substrate combined with intracellular biotransformation to the desired products trans-

Table 1. Cytochrome reductase activities in U mg^{-1} of total protein in cell extracts of *S. cerevisiae* and *P. pastoris*

S. cerevisiae[a]						
Plasmid[b]	pYES2 HPO CPR		pESC-URA PM17 CPR		pESC-URA CYP2D6 hCPR	
P_{GAL1}-*ScICE2*[c]	–	+	–	+	–	+
6 h	1.10 ± 0.09	1.55 ± 0.02	2.38 ± 0.23	3.26 ± 0.22	7.13 ± 0.03	8.37 ± 0.49
Fold improvement[d]		**1.41**		**1.37**		**1.17**
24 h	0.36 ± 0.02	1.06 ± 0.02	0.22 ± 0.01	0.82 ± 0.07	0.28 ± 0.01	0.92 ± 0.14
Fold improvement		**2.94**		**3.73**		**3.29**
P. pastoris[e]						
Strain[f]	HCV[g]		PM17/CPR		CYP2D6/hCPR	
P_{AOX1}-*PpICE2*	–	+	–	+	–	+
48 h	0.26 ± 0.02	0.37 ± 0.01	0.26 ± 0.01	0.33 ± 0.01	0.52 ± 0.00	0.69 ± 0.01
Fold improvement		**1.42**		**1.31**		**1.32**

Note: Activity assays were performed with two biological and three technical replicates. Activity values are presented as mean and standard deviations.
a) *S. cerevisiae* was induced for 6 and 24 h, respectively, on 2% galactose and 0.7% raffinose.
b) *S. cerevisiae* strains expressed the following CYP/CPR combinations from single plasmids, pYES2 HPO CPR (*H. muticus* premnaspirodiene oxygenase, HPO; *A. thaliana* CPR); pESC-URA PM17 CPR (*M. piperita* (–)-limonene-3-hydroxylase, PM17; *At*CPR); pESC-URA CYP2D6 hCPR (human CYP2D6; human CPR).
c) *ScICE2* was over-expressed by inserting the galactose-inducible promoter in front of the genomic copy of *ICE2*.
d) Stabilizing effect of Ice2 over-expression on cytochrome P450 reductase activities.
e) *P. pastoris* strains were induced for 48 h with methanol and samples were drawn for cytochrome c reductase activity assays.
f) *ICE2* up-regulation was investigated in *P. pastoris* strains expressing the following CYP/CPR combinations and additional recombinant proteins from genomically integrated constructs, HCV (*H. muticus* premnaspirodiene oxygenase, HPO; *A. thaliana* CPR; ValS, *C. nootkatensis* (+)-valencene synthase); PM17/CPR (*M. piperita* (–)-limonene-3-hydroxylase, PM17; *At*CPR); CYP2D6/hCPR.
g) This experiment was performed in a biphasic system with 10% n-dodecane overlay.

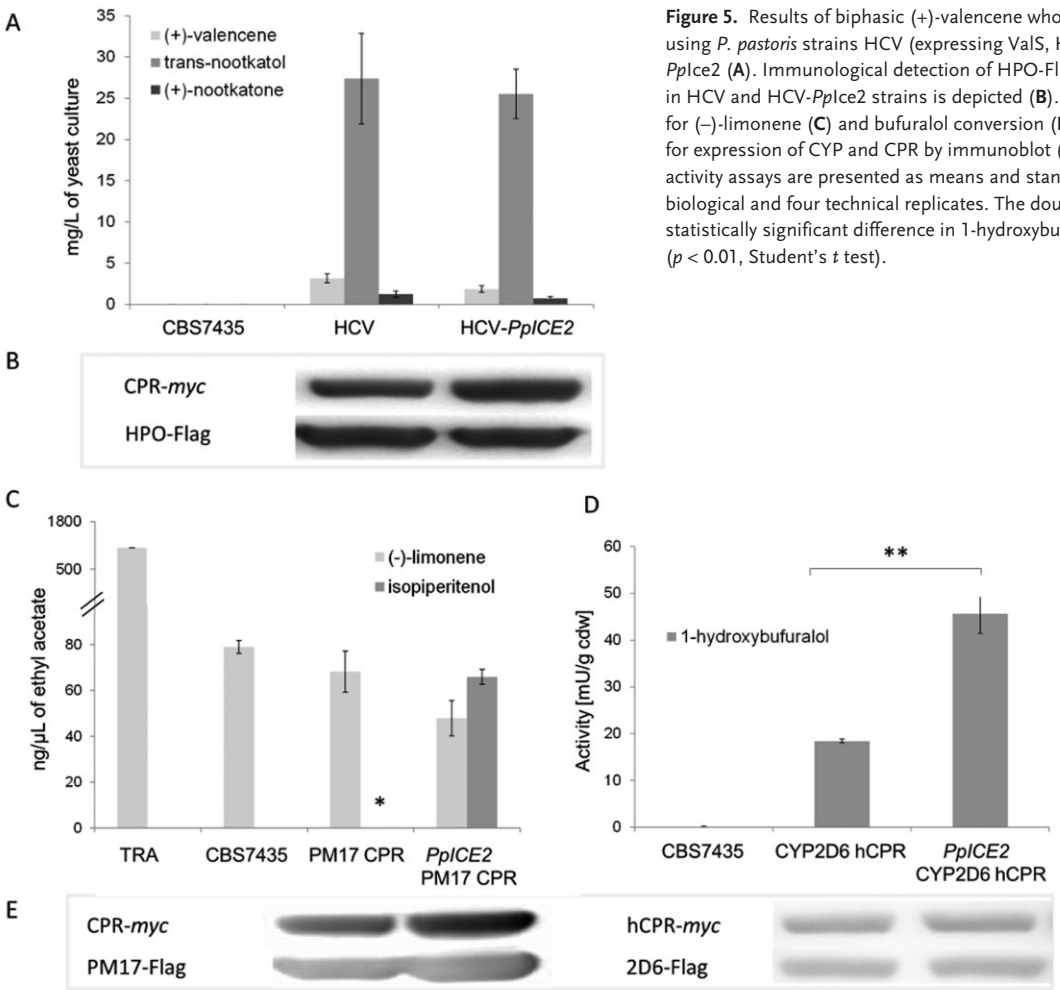

Figure 5. Results of biphasic (+)-valencene whole-cell hydroxylation assay using *P. pastoris* strains HCV (expressing ValS, HPO, and CPR) and HCV-*Pp*Ice2 (**A**). Immunological detection of HPO-Flag and CPR-*myc* proteins in HCV and HCV-*Pp*Ice2 strains is depicted (**B**). *P. pastoris* was also tested for (–)-limonene (**C**) and bufuralol conversion (**D**). Strains were analyzed for expression of CYP and CPR by immunoblot (**E**). Results of *P. pastoris* activity assays are presented as means and standard deviations of two biological and four technical replicates. The double asterisks (**) indicate statistically significant difference in 1-hydroxybufuralol formation ($p < 0.01$, Student's *t* test).

nootkatol and (+)-nootkatone [23, 54]. High amounts of terpenoids, in particular mono- and sesquiterpenoids, have been discussed to be detrimental for cell viability causing alterations of cell wall stability, intracellular accumulation of reactive oxygen species and wielding bad influence on membrane enzyme activities [10, 52, 53]. In our study, we obtained ~12 mg of trans-nootkatol per liter of yeast culture employing resting cells (Fig. 2A; 500 µL of 606 ng trans-nootkatol per µL ethyl acetate extract from 25 mL culture) closely matching the published value of 11 mg [10]. However, self-sufficiently (+)-valencene-producing and -converting cells yielded up to 30 mg L^{-1} trans-nootkatol (Fig. 3A) underscoring that phase transfer is a main issue in terpenoid formation and conversion. Additionally, intracellular production of terpenoid backbones is much easier to handle as it is less tedious and more efficient. Recently, the same (+)-valencene synthase we used in this work was published to yield 1.34 mg (+)-valencene per L of yeast culture [14]. Cankar et al. [23] produced 0.93 mg trans-nootkatol per L culture volume in vivo. Inspired by the cultivation protocol for *P. pastoris*, we optimized cultivation conditions by introducing a prolonged growth phase followed by milder induction conditions using less of the inducer. This protocol seemed to yield more robust cells, let them grow to higher cell densities before induction and, hence, efficiently redirected the metabolic flux to terpenoid production upon induction. Thus, we obtained 30 mg L^{-1} of trans-nootkatol, which is the highest value reported for *S. cerevisiae* so far.

Regio- and stereoselective functionalization of (hydrophobic) compounds, for example terpenoids, is of central interest in biocatalytic and metabolic engineering approaches. Highly specific hydroxylation reactions are performed by CYPs. Therefore, CYP activities have been in the focus of research for the last decades. On the one hand, the discovery and characterization of novel CYPs with superior properties is ongoing, and on the other hand, strategies to improve the applicability and yield of CYPs are desired. We set out to identify effectors of HPO/*At*CPR activity that would improve (+)-valencene hydroxylation. Screening a *S. cerevisiae* knock-out collec-

tion for strains that had altered HPO/*At*CPR levels and/or activity, we uncovered Ice2p as supposedly required for *At*CPR stability (Fig. 1C). Indeed, *ICE2* up-regulation led to improved cytochrome P450 activity in several CYP/CPR systems tested in both *S. cerevisiae* and *P. pastoris* (Figs. 2A, 3, 4A, 5, and Supporting information, Fig. S3). Throughout our bioconversion experiments, CPR activity was stabilized by *ICE2* over-expression (Table 1). There are a few publications that describe phenotypes of *ICE2* knockout cells and speculate on the cellular function of Ice2p [15–18]. However, altogether these studies present a very heterogeneous situation, which implies that the true molecular function(s) of Ice2p are still to be discovered. Here, we present evidence suggesting that stabilization of (recombinant) membrane proteins might be another facet of the role of Ice2p. A time-dependent analysis of samples revealed that the P_{GAL1}-*ICE2*-His_6 strain still produced trans-nootkatol after 48–72 h of conversion, which was not the case for the control strain (Fig. 4A). As indicated by activity assays and partially also by immunoblot analyses, the CPR proteins turned out to be more stable at later time-points when Ice2 was up-regulated in both, *S. cerevisiae* and *P. pastoris* strains (Table 1 and Fig. 4B). Evidently, stabilization of the reductase will not sustain hydroxylation activity on its own, because hydroxylation is performed by the CYP. Yet, it is assumed that the CPR moiety is responsible for higher mRNA stability of P450 enzymes [55] and better stabilization of P450 enzymes [56] and, therefore, is counteracting its cellular degradation.

4.1 Is Ice2p a stabilizer of (heterologous) membrane proteins ?

Previous studies have shown that *ICE2* knockout cells have changed patterns of cortical and peripheral ER membrane distribution compared to wild type cells [15]. We confirmed this effect by electron microscopy, but also showed that over-expression of *ICE2* did force ER membranes to proliferate and form staggered layers due to higher abundance of an ER membrane protein [48]. Over-expression of HPO and *At*CPR did lead to massively increased formation of karmellae layers in all of our strains, but no remarkable phenotypical differences were observed between the three strain backgrounds (Supporting information, Fig. S2). Moreover, the *ICE2* knockout mutant was shown to have a severe growth defect at low Zn^{2+} concentrations suggesting Ice2p to play a role in Zn^{2+} transport [17]. Lately, Ice2p was also implied in neutral lipid metabolism as well as transport from lipid droplets to the ER [18]. We do not find any obvious link between recombinant CPR stability/activity and either Zn^{2+} levels in the ER or neutral lipid metabolism in *S. cerevisiae*. However, we are tempted to speculate that Ice2p might be a more general stabilizer of membrane proteins as also indicated by enhanced HPO-Flag levels upon *ICE2* over-expression over time (Fig. 4C). Therefore, we wonder whether Ice2p might be involved in stabilizing (an) ER zinc transporter(s) or membrane proteins implicated in neutral lipid metabolism. Evidently, the question hovers, whether high abundance of Ice2p directly influences stability and/or activity of recombinant CPR, or maybe acts on another compound causing the stabilizing effect indirectly. The molecular role of Ice2p in stabilizing recombinant CPR remains to be clarified. Here, we present up-regulation of *ICE2* as a promising strategy to support CYP-based conversions in industrial/white biotechnology. We find that over-expression of *ICE2* stabilized CPR levels for three CYP/CPR pairs in both *S. cerevisiae* and *P. pastoris*, and we demonstrate enhanced substrate conversions in four of these six cases. Thus, we propose a stabilizing effect of Ice2p on recombinant CPRs, and speculate on a more general stabilizing effect towards membrane proteins.

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M M., I. K., D. M., and M. S. are employees of DSM, who has filed a patent application based on the results presented in this publication. A. E., M. W. -T., T.W., and H.P. are listed as co-inventors.

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Cover illustration

Special issue: Renewable Energy Crops, edited by Margit Laimer, Fatemeh Maghuly, Johann Vollmann and Nicolas Carels. Given the growing importance of environmental advantages of biofuel plants reducing greenhouse gas emissions as compared to fossil fuel consumption, second and third generation biofuels today are produced from both annual and perennial non-food biofuel crops, e.g. switchgrass, poplar and *Miscanthus*, or from new and non-food oil crops, like *Jatropha*, *Camelina* and oil palm. To achieve an economic production of biofuels for our future need of sustainable energy, genetic improvement of the plant material must be obtained by a range of different biotechnologies.

Biotechnology Journal – list of articles published in the April 2015 issue.

Research Article

Production of curcuminoids from tyrosine by a metabolically engineered *Escherichia coli* using caffeic acid as an intermediate

Joana L. Rodrigues, Rafael G. Araújo, Kristala L. J. Prather, Leon D. Kluskens, and Ligia R. Rodrigues

http://dx.doi.org/10.1002/biot.201400637

Research Article

Buffer-free therapeutic antibody preparations provide a viable alternative to conventionally buffered solutions: From protein buffer capacity prediction to bioprocess applications

Sven Bahrenburg, Anne R. Karow, Patrick Garidel

http://dx.doi.org/10.1002/biot.201400531

Research Article

Over-expression of *ICE2* stabilizes cytochrome P450 reductase in *Saccharomyces cerevisiae* and *Pichia pastoris*

Anita Emmerstorfer, Miriam Wimmer-Teubenbacher, Tamara Wriessnegger, Erich Leitner, Monika Müller, Iwona Kaluzna, Martin Schürmann, Daniel Mink, Günther Zellnig, Helmut Schwab and Harald Pichler

http://dx.doi.org/10.1002/biot.201400780

Research Article

Indole generates quiescent and metabolically active *Escherichia coli* cultures

Chih-Chin Chen, Rupali Walia, Krishna J. Mukherjee, Subhashree Mahalik and David K. Summers

http://dx.doi.org/10.1002/biot.201400381

Biotech Method

Optimized Sleeping Beauty transposons rapidly generate stable transgenic cell lines

Eric Kowarz, Denise Löscher, Rolf Marschalek

http://dx.doi.org/10.1002/biot.201400821