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Challenges and pitfalls of P450-dependent (+)-valencene bioconversion by *Saccharomyces cerevisiae*

Carole Gavira [a], René Höfer [a], Agnès Lesot [a], Fanny Lambert [b], Joseph Zucca [b], Danièle Werck-Reichhart [a,*]

[a] Institute of Plant Molecular Biology of CNRS UPR2357, University of Strasbourg, 28 rue Goethe, F-67083 Strasbourg Cedex, France
[b] Laboratoire de Recherche en Biotechnologies, V. MANE & Fils, Bar-Sur-Loup, France

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ABSTRACT

Natural nootkatone is a high value ingredient for the flavor and fragrance industry because of its grapefruit flavor/odor, low sensorial threshold and low availability. Valencene conversion into nootkatol and nootkatone is known to be catalyzed by cytochrome P450 enzymes from both prokaryotic and eukaryotic organisms, but so far development of a viable bioconversion process using either native microorganisms or recombinant enzymes was not successful. Using an *in silico* gene-mining approach, we selected 4 potential candidate P450 enzymes from higher plants and identified two of them that selectively converted (+)-valencene into β-nootkatol with high efficiency when tested using recombinant yeast microsomes *in vitro*. Recombinant yeast expressing CYP71D51v2 from tobacco and a P450 reductase from arabidopsis was used for optimization of a bioconversion process. Bioconversion assays led to production of β-nootkatol and nootkatone, but with low yields that decreased upon increase of the substrate concentration. The reasons for this low bioconversion efficiency were further investigated and several factors potentially hampering industry-compatible valencene bioconversion were identified. One is the toxicity of the products for yeast at concentrations exceeding 100 mg L^{-1}. The second is the accumulation of β-nootkatol in yeast endomembranes. The third is the inhibition of the CYP71D51v2 hydroxylation reaction by the products. Furthermore, we observed that the formation of nootkatone from β-nootkatol is not P450-dependent but catalyzed by a yeast component. Based on these data, we propose new strategies for implementation of a viable P450-based bioconversion process.

1. Introduction

(+)-Nootkatone has organoleptic properties highly valuable for the flavor and fragrance industry. It is extensively used as ingredient, mainly for its grapefruit flavor and its low perception threshold (Haring et al., 1972). Present in trace amounts in plants, it is one of the constituents of Alaska cedar heartwood (*Chamaecyparis nootkatensis*; Barton, 1976), vetiver (*Vetiveria zizanioides*; Andersen, 1970) and of some citrus (*Citrus*; MacLeod and Buigues, 1964) oils. Industrial needs have currently to be met by chemical synthesis, mainly from the corresponding sesquiterpene hydrocarbon (+)-valencene, readily available from orange industry, using unsafe oxidizing agents like *tert*-butyl peracetate (Wilson and Shaw, 1978) or *tert*-butyl hydroperoxide in combination with surface-functionalized silica-supported metal catalysts (Salvador and Clark, 2002). The resulting (+)-nootkatone thus cannot be marketed as a "natural" product and does not satisfy the increasing demand of natural aromatic compounds.

In order to match this demand, many efforts have been invested in biotechnological processes using bacteria, fungi or plants (reviewed in Fraatz et al. (2009)). Several of these involve enzymes from the superfamily of cytochrome P450 monoxygenases, many of which were documented for introducing an oxygen atom into allyl groups, and prime candidates for converting (+)-valencene into (+)-nootkatone. The identification of efficient and regioselective P450 enzymes matching industrial requirements for achieving this task nevertheless remains a challenge. A screening of 125 bacterial P450 enzymes led to the selection of one best candidate, CYP109B1 from *Bacillus subtilis* (Girhard et al., 2009), but the reaction suffered of the formation of multiple over-oxidized products. Those are likely to appear when the active site allows different binding orientations for the substrate or product, as suggested for $P450_{BM-3}$ from *Bacillus megaterium* engineered to metabolize (+)-valencene (Sowden et al., 2005). Conversely, $P450_{cam}$ mutants from *Pseudomonas putida* were more selective but less active. The use of P450 inhibitors also suggested P450-dependence of the (+)-valencene to α-nootkatol and (+)-nootkatone conversion by cultures of the

* Corresponding author. Fax: +33 368 851 921.
E-mail addresses: carole.gavira@ibmp-cnrs.unistra.fr (C. Gavira), rene.hofer@ibmp-cnrs.unistra.fr (R. Höfer), Fanny.LAMBERT@MANE.com (F. Lambert), Joseph.ZUCCA@MANE.com (J. Zucca), daniele.werck@ibmp-cnrs.unistra.fr (D. Werck-Reichhart).

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ascomycete *Chaetomium globosum*. In this case again, a large proportion of further oxygenated odorless compounds was obtained (Kaspera et al., 2005). Plant cytochromes P450, which are assumed to be involved in (+)-nootkatone biosynthesis *in planta* (del Río et al., 1992), seem to metabolize (+)-valencene with both regioselectivity and high efficiency. CYP71AV8 from chicory root (*Cichorium intybus*) co-expressed with a valencene synthase from *Citrus sinensis* in yeast resulted in the formation of β-nootkatol as the main product (Cankar et al., 2011), whereas (+)-nootkatone was the major product from a chicory roots microsome assay (De Kraker et al., 2003). Around 1 mg L^{-1} nootkatol and 0.04 mg L^{-1} (+)-nootkatone was obtained from a recombinant CYP71AV8-expressing yeast culture. CYP71D55, a premnaspirodiene oxygenase from *Hyoscyamus muticus*, was also found to catalyze the conversion of valencene to β-nootkatol more efficiently than premnaspirodiene oxidation. This efficiency has been further improved by targeted mutagenesis modifying amino acids located in regions of the active site involved in substrate recognition, but yields from recombinant yeast bioconversion were not reported (Takahashi et al., 2007).

Despite these encouraging results, no industrial process exploiting plant P450 enzymes has emerged so far. This work was initiated to select a plant P450 catalyzing the conversion of (+)-valencene into nootkatol and (+)-nootkatone with good efficiency and regioselectivity, and to optimize the production of (+)-nootkatone in engineered yeast using a bioconversion process. By a gene mining approach, we selected among candidates a novel plant P450 catalyzing efficient conversion of (+)-valencene into β-nootkatol *in vitro* and tested valencene bioconversion by the P450-transformed yeast. Poor performance of whole-cell bioconversion compared to *in vitro* activity led us to dissect the factors limiting P450-dependent (+)-valencene bioconversion in recombinant yeast. Based on these data, which most likely explain why yeast-based processes for nootkatone production were so far not successful, we can propose further strategies that may lead to economically viable P450-catalyzed bioconversion of (+)-valencene into (+)-nootkatone.

2. Materials and methods

2.1. Sesquiterpenoids

(+)-Valencene, β-nootkatol, (+)-nootkatone, (−)-nootkatene and isolongifolene were provided by V. Mane Fils (Le Bar-sur-Loup, France). (+)-Valencene and (−)-nootkatene were obtained by distillation of Valencia orange peel (*C. sinensis*) and Alaska cedar heartwood (*C. nootkatensis*) essence oil, respectively. (+)-Nootkatone was synthesized by (+)-valencene oxidation (Wilson and Shaw, 1978). β-Nootkatol was synthesized by reduction of (+)-nootkatone with lithium aluminim hydride as previously described (Shaffer et al., 1975).

Structures and stereochemistry of (+)-valencene, (−)-nootkatene, (+)-nootkatone and β-nootkatol were confirmed by NMR (Supplementary information, Figs. S1–S24 and Table S1–S4). The spectra of (+)-valencene, (+)-nootkatone and (−)-nootkatene were recorded on a Bruker Avance III 600 MHz spectrometer, at 14.09 T, at the resonating frequencies of 600.13 MHz for ^{1}H and 150.90 MHz for ^{13}C, using a BBI Bruker 5 mm gradient – probe. The temperature was regulated at 298 K and no spinning was applied to the NMR tube. The spectra of β-nootkatol were recorded on a Bruker Avance II 500 MHz spectrometer, at 11.75 T, at the resonating frequencies of 500.13 MHz for ^{1}H and 125.76 MHz for ^{13}C, using a DCH Bruker 5 mm gradient—cryoprobe. The temperature was regulated at 300 K. Every compound was analyzed using the following series of experiments in $CDCl_3$: 1D ^{1}H acquisition sequence, 1D ^{13}C decoupled from ^{1}H acquisition sequence, Double quantum 2D homonuclear shift correlation using gradient pulses for selection – COSY (Ancian et al., 1997), 2D homonuclear correlation via dipolar coupling – NOESY (Jeener et al., 1979), 2D ^{1}H–^{13}C correlation via double INEPT transfer with decoupling during acquisition and with multiplicity editing – HSQC (Parella et al., 1997), 2D ^{1}H–^{13}C correlation via heteronuclear zero and double quantum coherence with low-pass J filter – HMBC (Cicero et al., 2001).

2.2. Genes sequences, plasmid insertion and yeast strain

The complete coding sequences of *CYP71D51v2*, *CYP71D4*, *CYP71D1*, and *CYP71D326* are available from the NCBI or GenBank Nucleotide databases under the accession numbers DQ350345, AJ296346, EU541505 and XM_002522675, respectively. They were optimized for yeast expression (Supplementary information, Figs. S25–S28) and synthesized by GeneCust Europe (Dudelange, Luxembourg). Alignment of protein sequences with CYP71AV8 (GenBank ID: HQ166835) and CYP71D55 (GenBank ID: EF569601) was carried out using Clustal W with BioEdit version 7.0.9.0 (Hall, 1999). The USER™ cloning technique (Nour-Eldin et al., 2006) was used to insert *CYP71D51v2* into the USER compatible yeast expression vector pYeDP60u2 (Höfer et al., submitted). The *Saccharomyces cerevisiae* WAT11 strain, expressing the ATR1 cytochrome P450 reductase from *Arabidopsis thaliana* under the galactose-inducible promoter *GAL10-CYC1*, is a gift from Dr. D. Pompon (Urban et al., 1997).

2.3. Heterologous expression in yeast and isolation of microsomal membranes

The WAT11 yeast strain was transformed with pYeDP60u2 harboring *CYP71D51v2*, *CYP71D4*, *CYP71D1 or CYP71D326* sequences as described (Gietz et al., 1992). A transformed colony was grown at 28 °C for 17 h in 30 mL of minimum SGI medium (1 g L^{-1} bactocasamino acids, 7 g L^{-1} yeast nitrogen base, 20 g L^{-1} glucose and 20 mg L^{-1} tryptophan). The culture was diluted 10 times in YPGE (10 g L^{-1} bactopeptone, 10 g L^{-1} yeast extract, 15 g L^{-1} glucose and 3% ethanol by volume). After 30 h at 28 °C, the medium was replaced by the same volume of YPI medium (10 g L^{-1} bactopeptone, 10 g L^{-1} yeast extract, 20 g L^{-1} galactose) for galactose induction of gene expression at 28 °C for approximately 17 h.

Microsomes were prepared on ice. Yeast cells harvested from 200 mL culture by centrifugation at 7000 rpm for 10 min at 4 °C were washed with TEK buffer (50 mM Tris–HCl pH 7.5, EDTA 1 mM, KCl 100 mM) and resuspended in 2 mL of TES (50 mM Tris–HCl pH 7.5, EDTA 1 mM, sorbitol 600 mM) supplemented with 4.7 mM of 2-mercaptoethanol and 10 g L^{-1} bovine serum albumin, fraction V (buffer A). In a 50 mL Falcon tube, glass beads (0.4–0.6 mm diameter, Sartorius, Germany) were added until skimming the top of the cell suspension. Cells were broken by handshaking 5 times for 1 min. Beads were washed twice with 25 mL of the buffer A. Cell debris and cell walls were removed by 10 min centrifugation of the pooled extract at 7500 rpm and 4 °C. After filtration on Miracloth (22–25 μm pore size, Calbiochem, Maryland), the microsomal membranes were recovered by 45 min ultracentrifugation at 30,000 rpm and 4 °C. Pellets, corresponding to the microsomal fraction, were resuspended in TG buffer (50 mM Tris–HCl pH 7.5, 30% glycerol by volume) with a Potter-Elvehjem homogenizer. Aliquots of 200 μL were stored at −20 °C.

2.4. Differential spectroscopy

Measurements were carried out on a Cary 300 UV–Vis spectrophotometer (Varian, Les Ulis, France) at room temperature. Difference spectra were recorded from $Na_2S_2O_4$-reduced microsomes in TG buffer (50 mM Tris–HCl pH 7.5, 30% glycerol by volume). A baseline was measured with reduced microsomes in the two cuvettes before saturation of the sample cuvette with CO. Differential absorbance was then recorded from 400 nm to 500 nm and P450 quantified using the absorption coefficient at 450 nm $\varepsilon = 91\ mM^{-1}\ cm^{-1}$ determined by Omura and Sato (1964).

Ligand binding spectra were obtained from recombinant yeast microsomes diluted to a final CYP71D51v2 concentration of 600 nM. A baseline was recorded from native oxidized microsomes. β-Nootkatol diluted in ethanol was then added to the sample cuvette to a final concentration ranging from 20 μM to 400 μM, and the same volume of solvent (maximum of 2% by volume) was added to the reference. Differential absorbance was recorded between 370 nm and 500 nm. Dissociation constant (K_d) was calculated from the linear equation resulting of the double reciprocal plot of the differential absorbance between 420 nm and 390 nm *versus* β-nootkatol concentration.

2.5. Enzyme assays

Enzyme assays were incubated in closed 2 mL glass vials for 20 min at 28 °C using a thermo mixer (Eppendorf, Le Pecq, France) under a constant shaking at 700 rpm. In a final reaction volume of 300 μL, 30 μL of yeast microsomes were diluted in phosphate saline buffer (75 mM pH 6.8 (PBS)) in the presence of 200 μM of (+)-valencene or β-nootkatol (dissolved in ethanol not exceeding 0.8% of assay volume) and 1 mM of NADPH. Assays without NADPH or with microsomes prepared from yeast transformed with an empty vector were incubated as negative controls. The reaction was quenched on ice and 100 μM of isolongifolene in ethanol was added as internal standard. The mixture was extracted with 500 μL of pentane in a thermo mixer at 1400 rpm during 5 min at 20 °C. After 10 min centrifugation at 4400 rpm, the organic phase was recovered for GC-FID and GC–MS analysis.

For kinetic parameters determination, triplicate assays were incubated with (+)-valencene concentrations ranging from 50 μM to 200 μM. Formation of β-nootkatol was quantified after 5 min of incubation using an internal standard. $K_{m,\ app}$ and $V_{max,\ app}$ were deduced from Hanes–Woolf representation.

The inhibition of the reaction by (+)-nootkatone was determined for 20 min assays with 200 μM of (+)-valencene in the presence of 200 μM of (+)-nootkatone. NADPH oxidation resulting from (+)-valencene metabolism by CYP71D51v2 in presence or absence of β-nootkatol or (+)-nootkatone was evaluated by monitoring changes in NADPH absorption at 350 nm. Yeast microsomes were diluted in 1 mL TG buffer containing 50 μM of NADPH to 55 nM of CY71D51v2 and the mix was divided in two cuvettes of the spectrophotometer. After checking for constant differential absorbance, 100 μM of valencene was added in the assay cuvette and a corresponding volume of solvent in the reference. NADPH oxidation was calculated from the rate of absorbance decrease at room temperature (approx. 20 °C) using an $\varepsilon_{350\ nm} = 5600\ M^{-1}\ cm^{-1}$ (Urban et al., 1994). To evaluate β-nootkatol or nootkatone inhibition, these compounds were added to both cuvettes at a 100 μM concentration before initiation of the reaction. Using the same conditions, the turnover of (+)-valencene hydroxylation by CYP71D51v2 was also determined from β-nootkatol produced after addition of an internal standard, extraction with 500 μL of pentane and analysis on GC-FID.

2.6. Bioconversion

(+)-Valencene bioconversion by yeast expressing CYP71D51v2 was compared to that obtained with a control transformed with the pYeDP60u2 empty vector. β-Nootkatol bioconversion by the control yeast was evaluated after 24 h of incubation. Tests were performed using 100 mL yeast culture. After induction of CYP71D51v2 expression, cells were transferred to fresh YPI and appropriate concentrations of (+)-valencene or β-nootkatol were supplied in ethanol (2% by volume). The cultures were incubated for 24 h at 28 °C with shaking at 160 rpm. For quantification of the products accumulated in yeast cells and in the medium, 1 mL of acetone was added to 1 mL of the culture before vortexing and extraction. For quantification of products present in the medium, yeast cells were discarded by centrifugation at 7000 rpm for 10 min before extraction of 1 mL of the supernatant. In order to check if β-nootkatol was adsorbed to the yeast surface, the cell pellet was washed with alkaline buffer as previously described (Ro et al., 2006). 400 μM of isolongifolene was added as internal standard. Then, substrate and products were extracted with 2 mL of a mixture of pentane/ethyl acetate (85:15, v/v). The organic layer was recovered and concentrated under argon to a volume of 200 μL before GC–MS and GC-FID analysis.

2.7. Sesquiterpenoid partition

Partition of substrate and products between the culture medium and soluble and membrane fractions (cell wall, cytosol and internal membranes) of the yeast expressing CYP71D51v2 was measured after 24 h of bioconversion of 80 mg L^{-1} of valencene. The yeast and culture medium were separated by centrifugation at 7000 rpm for 10 min. Microsomes were prepared as described above, replacing TEK and TES with PBS. After cell disruption, yeast cell wall/debris and microsome pellets were washed and resuspended in PBS buffer before extraction. The cytosol fraction was recovered after ultracentrifugation. Isolongifolene (400 μM) was added to each fraction before extracting with a mixture of pentane/ethyl acetate (85:15, v/v). The organic layer was recovered and concentrated under argon to a volume of 200 μL before GC–MS and GC-FID analysis.

2.8. Viability assays

After 17 h of induction, control yeast cells (pYeDP60u2-transformed) were transferred to fresh YPI and aliquoted in 100 mL before addition of (+)-valencene (10 mg L^{-1}, 80 mg L^{-1}, 400 mg L^{-1} or 4000 mg L^{-1}), β-nootkatol or (+)-nootkatone (10 mg L^{-1}, 100 mg L^{-1} or 1000 mg L^{-1}) in ethanol (2% by volume) and incubated at 28 °C for 24 h. Control yeast culture was supplemented with the same volume of ethanol. Aliquots of 5 mL of culture were harvested, and cells were collected by centrifugation, washed in SGI before serial dilution of 1 mL in SGI and plating of two 100 μL samples on YPI. After 3 days at 28 °C, the number of colony forming units (cfu) was recorded. Toxicity was evaluated by comparison with cultures incubated with ethanol only.

2.9. Quantification and identification of products

For quantitative analysis, 1 μL of extract was injected in a 3900 GC-FID (Varian, Les Ulis, France) equipped with a DB-5 column (30 m, 0.25 mm, 0.25 μm; Agilent Technologies, Massy, France). The injector was used in the split mode with a ration of 50 and was set at 280 °C. Hydrogen gas flow was 1.2 mL min^{-1}. The oven temperature was maintained 0.5 min at 50 °C, increased 20 °C min^{-1} until 100 °C, then 4 °C min^{-1} until 300 °C and finally,

held at this temperature for 5 min. β-nootkatol formed during enzyme assays was determined using isolongifolene as internal standard. Mixes of all components present in enzyme assay (with 50 μM of isolongifolene) except NADPH and substrates, but supplemented with 25 μM, 50 μM, 75 μM, 100 μM, 200 μM, 300 μM or 400 μM of β-nootkatol were extracted in triplicate and analyzed in GC-FID. The ratios of peak areas for β-nootkatol to internal standard were plotted against ratios of β-nootkatol to isolongifolene concentrations to obtain a calibration curve. For quantification of bioconversion products, similar calibration curves were generated using extracts of either 1 mL of control yeast culture plus 1 mL of acetone or 1 mL of culture medium in bioconversion conditions, supplemented with isolongifolene and different concentrations of β-nootkatol or (+)-nootkatone. Calibration coefficients obtained for the acetone-free extraction of culture medium were also applied for quantification of substrate and products in yeast subcellular fractions (cell wall, cytosol and microsomes).

Products were identified on GC-FID and GC–MS by comparing retention times and mass spectra with those of authentic standards. Mass spectra were acquired on a Clarus 680 GC coupled to a Clarus 600T mass spectrometer (Perkin Elmer, Courtaboeuf, France). A scan range of 50–300 *m*/*z* acquiring in 0.25 s at 70 eV was used.

3. Results

3.1. Selection of a plant P450 enzyme candidate for (+)-valencene C2 oxidation

Two plant P450 enzymes were previously reported to regioselectively introduce an oxygen atom at the 2 position of (+)-valencene. Those are the premnaspirodiene oxygenase CYP71D55 from *H. muticus* (Takahashi et al., 2007) and CYP71AV8 from chicory (*C. intybus*) (Cankar et al., 2011). They share 49% amino acid identity and seem to result from convergent evolution in unrelated taxa. Assuming that they would bear the structural features required for supporting (+)-valencene regiospecific 2-oxygenation, a BLAST search using their amino acid sequences was used as a starting point to identify potential (+)-valencene 2-oxygenases from other plants. This led to a short list of four candidates from different plants: CYP71D4 from *Solanum tuberosum*, CYP71D51v2 from *Nicotiana tabacum*, CYP71D1 from *Catharanthus roseus* and CYP71D326 from *Ricinus communis*. Their protein sequence alignments and identities with CYP71D55 and CYP71AV8 are provided in Supplemental Fig. S29 and Table S5, respectively. All four coding sequences were synthesized, with codon optimization according to yeast codon usage, inserted into the pYeDP60u2 vector and expressed in the WAT11 yeast strain, which is transformed for stable expression of *A. thaliana* P450 reductase ATR1 (Urban et al., 1997). Microsomes were prepared from yeast cells induced for P450 and P450-reductase expression, and successful P450 expression assessed by differential spectrophotometry. Differential CO spectra confirmed the expression of 0.9 nmoles mL^{-1} of microsomes for CYP71D4 and 6 nmoles mL^{-1} of microsomes for CYP71D51v2 (Fig. 1), but no differential absorbance was detected upon expression of CYP71D1 and CYP71D326. Nevertheless, all four enzymes were found to generate oxidized compounds when tested for (+)-valencene metabolism, compared to control microsomes prepared from yeast transformed with an empty pYeDP60u2 vector. Apparently poorly expressed in yeast, CYP71D1 and CYP71D326 generated also low amounts of products and showed a low regiospecificity (Supplementary information, Fig. S30). Higher activity and regioselectivity was observed with CYP71D51v2 and CYP71D4 that both generated the same products (Fig. 2A). The main product was identified as β-nootkatol based on comparison of retention time and mass spectrum with a synthetic standard (Fig. 2C, Supplementary information, Figs. S19–S24 and Table S4). From the 3 minor products, one could be identified as (+)-nootkatone (Fig. 2C), and the two others with a *m*/*z* of 202 are valencene desaturation products, one of them with the mass spectrum and retention time of (−)-nootkatene (Supplementary information, Fig. S31). Formation of the latter compounds most likely results from P450-catalyzed hydrogen abstraction followed by electron transfer and formation of an allylic carbocation (Supplementary information, Fig. S32), a mechanism previously proposed for alkylbenzenes desaturation by a bacterial CYP102A1 (Whitehouse et al., 2008). The second unidentified desaturation product is thus likely to be 2,3-dihydro-valencene but could not be isolated in amounts large enough for confirmation via NMR. Two minor products with the same retention times and mass spectra are also formed after reduction of (+)-nootkatone with lithium aluminum hydride (17% of **4** and 15% of **5** as evaluated by GC). As a consequence, the same carbocations seem to be formed upon acidification.

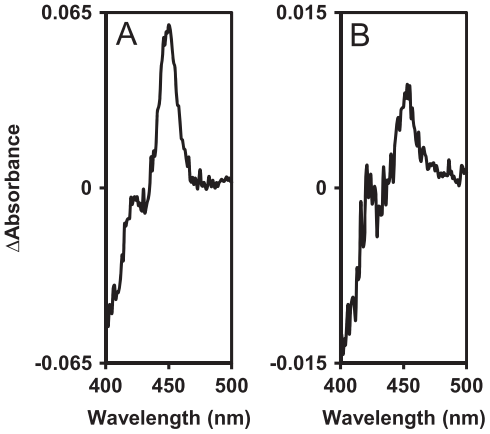

Fig. 1. Carbon monoxide difference spectra recorded with microsomes prepared from yeast expressing CYP71D51v2 (A) or CYP71D4 (B). Microsomes diluted in TG buffer were reduced with an excess of solid sodium dithionite before recording a baseline. The sample was saturated with carbon monoxide and the difference spectrum was recorded.

Since CYP71D51v2 was showing the highest expression in yeast, swift conversion of (+)-valencene to β-nootkatol and a low formation of side products, it was selected for further studies. To evaluate its potential to catalyze the conversion of β-nootkatol into (+)-nootkatone, microsomes of recombinant yeast were incubated with β-nootkatol as a substrate. Only minor amounts of (+)-nootkatone were obtained in an NADPH-dependent manner (Fig. 2B). Evaluation of the kinetic constants indicated that (+)-valencene to β-nootkatol conversion proceeded with a $K_{m,app}$ of 52 ± 2 μM, a k_{cat} of 5.8 ± 0.8 s^{-1} and a catalytic efficiency (k_{cat}/K_m) of 0.15 s^{-1} μM^{-1} (Fig. 3). The amounts of (+)-nootkatone formed from β-nootkatol were too low for determination of the kinetic parameters of the reaction.

3.2. Bioconversion of (+)-valencene in yeast by CYP71D51v2

Given its high turnover, CYP71D51v2 was tested for its performance in bioconversion. The CYP71D51v2 transformed yeast was grown to high cell density, induced for expression and first incubated with 80 mg L^{-1} (400 μM) of (+)-valencene supplied in ethanol (2%) for 24 h before extraction of the whole culture (medium plus yeast). β-nootkatol was the main product detected in the extract. It was formed only when yeast was expressing CYP71D51v2 (Fig. 4). The bioconversion yield in this initial assay was 5 mg L^{-1} of β-nootkatol.

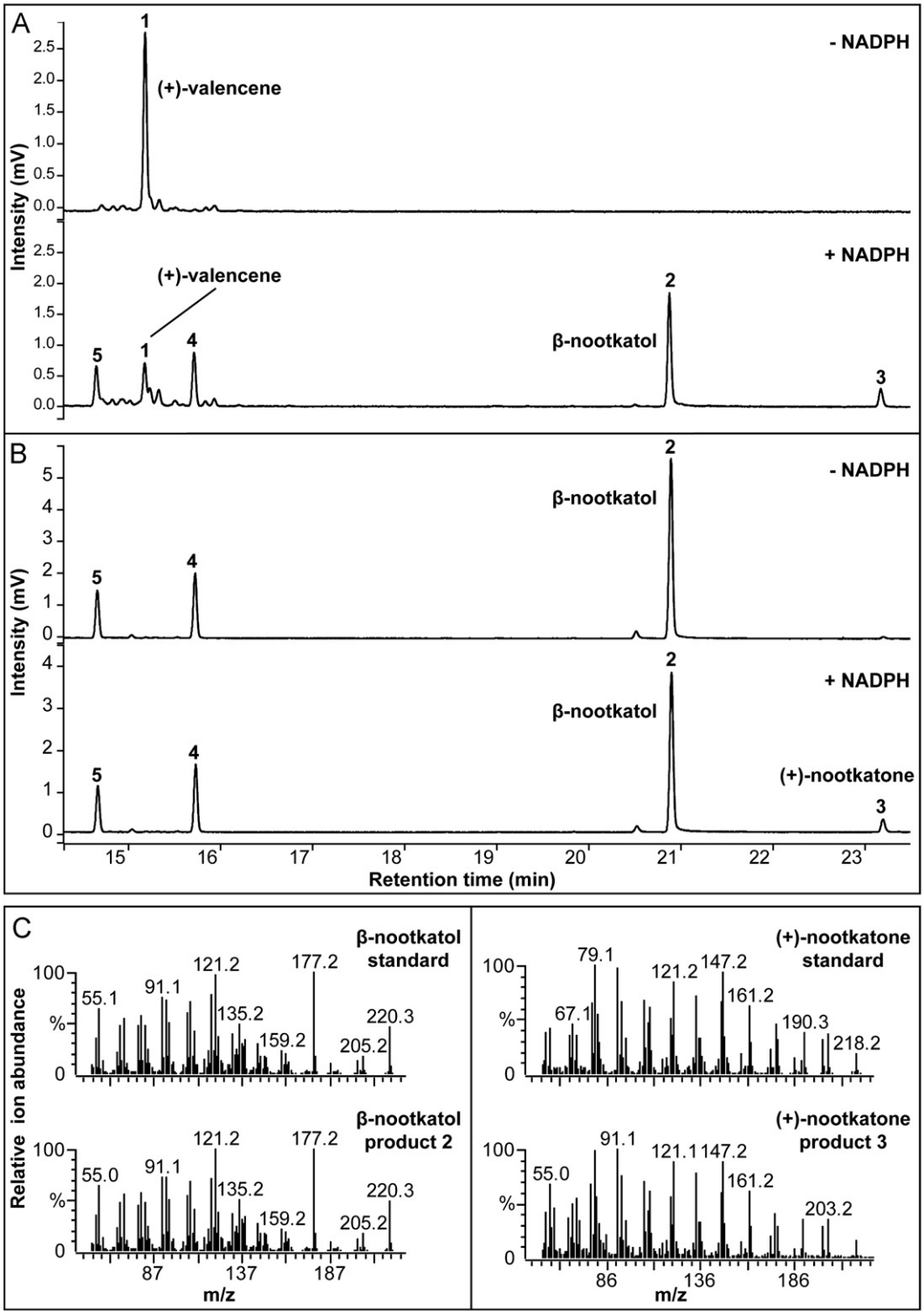

Fig. 2. Analysis and identification of the products of (+)-valencene and β-nootkatol conversion by microsomes prepared from yeast expressing CYP71D51v2. GC-FID analysis of the products obtained after 20 min incubation at 28 °C of 66 nM of CYP71D51v2 in yeast microsomes with 200 μM of (+)-valencene (A) or β-nootkatol (B) in the presence or absence of 1 mM NADPH. (C) Products identification by mass spectrometry. Mass spectra of the products are compared with mass spectra of authentic standards. 1: (+)-valencene, 2: β-nootkatol, 3: (+)-nootkatone, 4: (−)-nootkatene, 5: unknown compound. A similar profile was obtained with CYP71D4 and (+)-valencene. The mass spectra of compounds 4 and 5 are shown in supplemental file S31.

Surprisingly, whereas very minor amounts of (+)-nootkatone were obtained in the microsome assay, up to 3 mg L^{-1} (+)-nootkatone were obtained in the bioconversion extract, together with an unidentified product with a *m*/*z* of 220 (Fig. 3, Supplementary information, Fig. S33). To test whether the production of (+)-nootkatone resulted from the activity of CYP71D51v2 or from a dehydrogenase or oxidative activity present in yeast, a β-nootkatol bioconversion assay was carried-out with the control WAT11 yeast transformed with the pYeDP60u2 empty vector. Interestingly, this assay demonstrated that the formation of (+)-nootkatone and of the unknown product from β-nootkatol was catalyzed by the control yeast, independently from the

presence of CYP71D51v2 (Fig. 5). After 24 h of bioconversion by control yeast, 7 mg L^{-1} (+)-nootkatone were obtained from 80 mg L^{-1} β-nootkatol.

To test if the CYP71D51v2-transformed yeast might be suitable for a viable industrial process that would require the production of nootkatol/nootkatone in the g L^{-1} range, the induced culture was incubated in the presence of increasing concentrations of (+)-valencene. A maximum yield around 6 mg L^{-1} of β-nootkatol and (+)-nootkatone was obtained for a (+)-valencene concentration of 200 mg L^{-1}. The yield dropped to 4.5 mg L^{-1} for a (+)-valencene concentration of 400 mg L^{-1} and only trace amounts of products were obtained using a (+)-valencene concentration of 3 g L^{-1}. This suggested that substrate or products toxicity may limit the bioconversion process.

3.3. Toxicity of the substrate and products

Sesquiterpenoids have been reported to show antifungal activities (Engström et al., 1999; Manter et al., 2007; Yao et al., 2003; Yokose et al., 2004) and their biosynthesis is often induced upon fungal infection (Huffaker et al., 2011; Vögeli and Chappell, 1988). The first hypothesis we investigated to explain the low bioconversion efficiency was thus the possible toxicity of the substrate and products. Viability assays were carried out using yeast transformed with the empty pYeDP60u2 vector, cultivated for 24 h in the presence of increasing concentrations of (+)-valencene, β-nootkatol or (+)-nootkatone, and keeping a constant solvent concentration. For yeast grown in the presence of 10 mg L^{-1} of substrate or products, more than 80% of viable cells were recovered compared to the control (Fig. 6). (+)-Valencene was tolerated at concentrations up to 4 g L^{-1}, with 73 ± 4% of viable cells recovery after 24 h. On the other hand, β-nootkatol and (+)-nootkatone led to loss of cell viability at 1 g L^{-1}, with already 45 ± 3% viability loss at 100 mg L^{-1} in the case of β-nootkatol.

Based on these data, a bioconversion conducted with 80 mg L^{-1} of (+)-valencene should not result in more than 15% yeast viability loss after 24 h. At higher (+)-valencene concentrations, the sum of products does not exceed much 10 mg L^{-1}. The toxicity

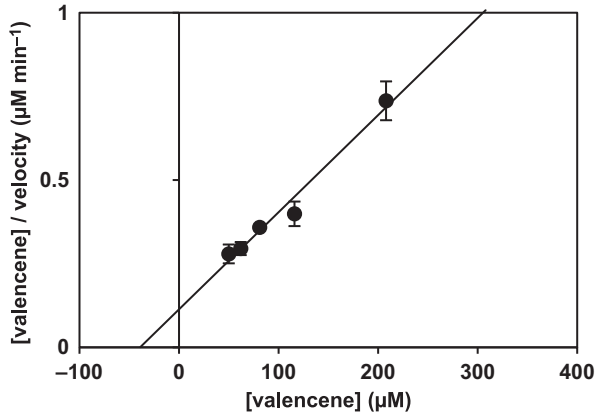

Fig. 3. Kinetic of (+)-valencene conversion by CYP71D51v2. Assays were incubated 5 min with 700 nM of CYP71D51v2 and in triplicate for each concentration of substrate.

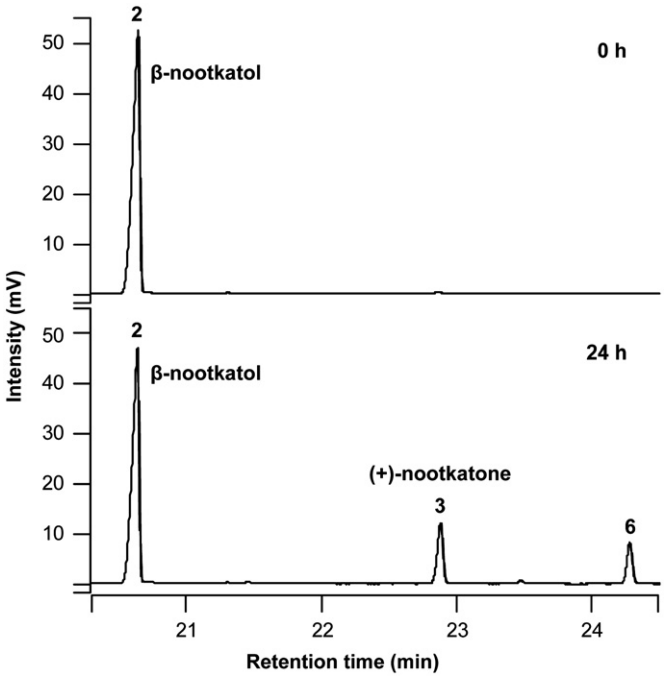

Fig.5. GC analysis of the bioconversion products of β-nootkatol by the control yeast. Yeast was grown for 3 days before addition of 80 mg L^{-1} of β-nootkatol and further incubation for 24 h. Yeast cells and culture medium were simultaneously extracted with a mix of acetone, pentane and ethyl acetate (33:57:10, v/v). Products were identified in GC–MS. 2: β-nootkatol, 3: (+)-nootkatone, 6: unknown compound. The mass spectrum of the compound 6 is shown in supplemental file S33.

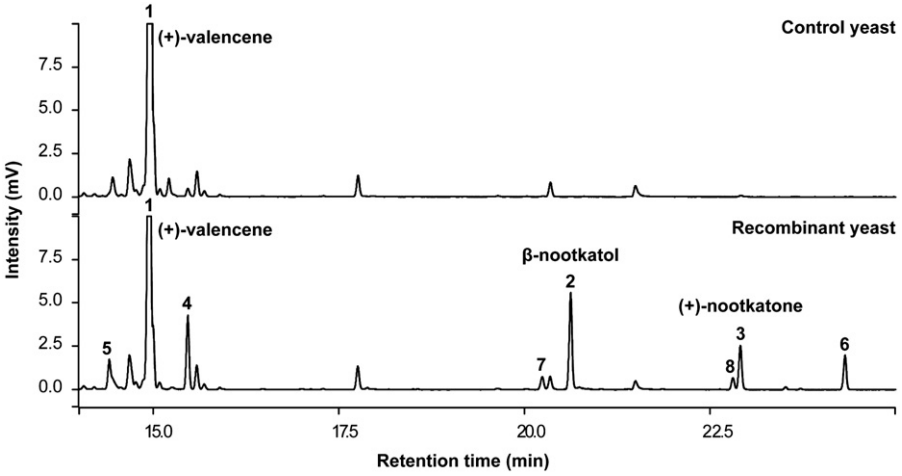

Fig. 4. GC analysis of the bioconversion products of (+)-valencene by yeast overexpressing CYP71D51v2. Yeast was grown for 2 days, induced for expression for 17 h, before addition of 80 mg L^{-1} of (+)-valencene and further incubation for 24 h. Yeast cells and culture medium were simultaneously extracted with a mix of acetone, pentane and ethyl acetate (33:57:10, v/v). Products were identified in GC–MS. 1: (+)-valencene, 2: β-nootkatol, 3: (+)-nootkatone, 4: (−)-nootkatene, 5 and 6: unknown compounds, 7 and 8: minors unknown compounds.

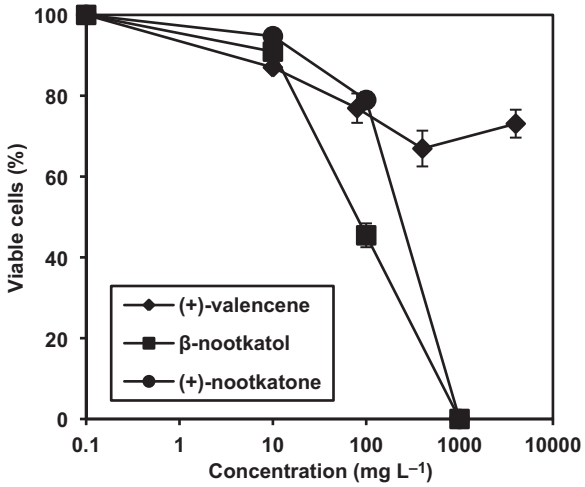

Fig. 6. Yeast viability after 24 h in the presence of (+)-valencene, β-nootkatol or (+)-nootkatone. The control *S. cerevisiae* WAT11 strain transformed with the empty vector pYeDP60u2 was grown and incubated for 24 h with the substrate or products (added as ethanolic solution) in the same conditions as the bioconversion assay. The number of viable cells was estimated from the number of colonies growing on complete solid medium by comparison to a control incubated for 24 h with the solvent.

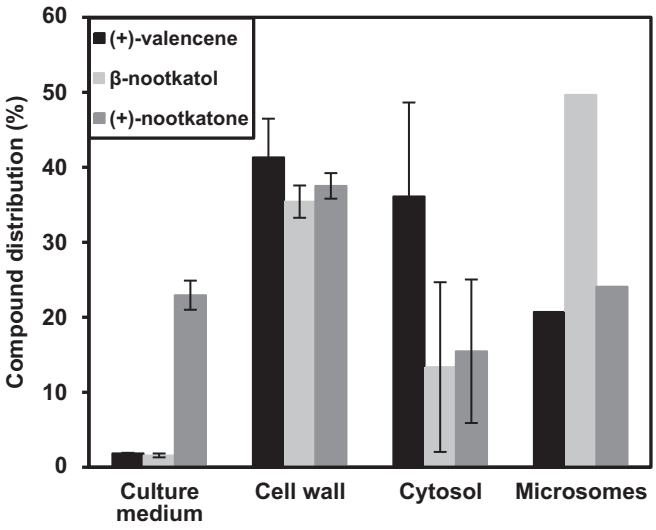

Fig. 7. Partition of (+)-valencene and products after 24 h of bioconversion by the CYP71D51v2-tranformed yeast. The initial concentration of (+)-valencene was 80 mg L^{-1}. Yeast cells were disrupted with glass beads and the fractions separated by differential centrifugation. Substrate and products were quantified after extraction with a mixture of pentane and ethyl acetate (85:15, v/v).

of valencenoids is thus not sufficient to explain the low efficiency of bioconversion observed in our experiments.

3.4. Subcellular localization of the substrate and products

Nootkatone and especially nootkatol were previously reported to accumulate in the fungal biomass of *C. globosum* and to be poorly released in the culture medium (Kaspera et al., 2005). In addition, the antibacterial activity of monoterpenols such as carvacrol and thymol has been suggested to rely on a membrane permeation/destabilizing effect based on 1-*N*-phenylnaphtylamine uptake, detergent sensitization and lipid and ATP release (Helander et al., 1998). This led us to investigate the substrate and product distribution in the yeast membranes and soluble fractions during bioconversion. (+)-Valencene was found accumulated in yeast, most of which was trapped in the cell wall (41%) and microsomal membrane (21%) fractions (Fig. 7). While a significant proportion (38%) of (+)-nootkatone accumulated in the cell wall fraction, it was the most efficiently released in the culture medium or was possibly formed by enzymes such as laccases adsorbed on the outer surface of the cell wall. Conversely, a very minor proportion (2%) of β-nootkatol was found in the culture medium, whereas 35% and 50%, respectively, were found associated with the cell wall and the microsomal fractions. It has been previously shown that artemisinic acid produced by *CYP71AV1*-transformed yeast is adsorbed onto the external surface of the cell wall and is readily removed from the cell pellet by washing with an alkaline pH 9 buffer (Ro et al., 2006). Similar washes performed on *CYP71D51v2*-transformed yeast did not release any product. β-Nootkatol thus appears trapped and concentrated in the endomembranes.

To reduce β-nootkatol/nootkatone accumulation in the endomembranes, two approaches were tested. The first was to add γ-cyclodextrins (5 mM to 500 mM) in the bioconversion medium in order to trap the products, without success. The second was to perform bioconversion in the presence of 4% of dimethyl sulfoxide (DMSO), previously reported to enhance product formation in bacteria (Girhard et al., 2009), which led to a 7 and 2.3 fold increase in β-nootkatol and (+)-nootkatone release in the incubation medium (Fig. S34), with only limited increase in overall products formation, to reach 11 mg L^{-1} of β-nootkatol and 4 mg L^{-1} of (+)-nootkatone.

3.5. Inhibition by the reaction products

(+)-Nootkatone was previously described as an inhibitor of the human drug metabolizing cytochrome P450 enzymes CYP2A6, CYP2C19 (Tassaneeyakul et al., 2000), CYP3A4 and CYP2C8 (Sime et al., 2000) at low concentrations, and drug formulation including valencenoids was patented for drug stabilizing effect via delayed metabolism and clearance. Product inhibition would also provide a reasonable explanation for the low yields observed in our experiments since significant amounts of nootkatone are obtained upon bioconversion. We consequently decided to investigate CYP71D51v2 inhibition by (+)-nootkatone and β-nootkatol. The latter was included in these experiments since alcohols are documented ligands of P450 enzymes (Beyeler et al., 1988; Ortiz de Montellano and Correia, 1995).

Both alcohols and ketones have been reported to induce a shift of the Soret to approximately 415 nm when they form a coordination with the heme iron in the active site of P450 enzymes, giving rise to so-called modified type II or reverse type I differential spectra (Jefcoate, 1978; Schenkman et al., 1967). The capacity of (+)-nootkatone and β-nootkatol to bind CYP71D51v2 was thus first investigated by recording the difference spectra the compounds induce when added to the yeast-expressed enzyme. Whereas no consistent modification in UV–visible absorbance was observed upon addition of (+)-nootkatone, a characteristic reverse type I spectral shift was obtained with β-nootkatol (Fig. 8A), with a maximum at 415 nm and a minimum at 383 nm. This shift is expected to reflect a high- to low-spin transition of the heme iron as a result of oxygen coordination (Ortiz de Montellano and Correia, 1995; Schenkman et al., 1967). This shift was saturable and proportional to the β-nootkatol concentration in the assay. The apparent dissociation constant of β-nootkatol deduced from the saturation curve was 66 ± 9 μM (Fig. 8B), which compares with a K_m for (+)-valencene to β-nootkatol conversion was estimated to be around 52 μM. Intracellular β-nootkatol concentration can be roughly estimated from the experiment in Fig. 7, considering the collected yeast biomass (around 1 g from 100 mL of culture). Cytoplasmic concentration is

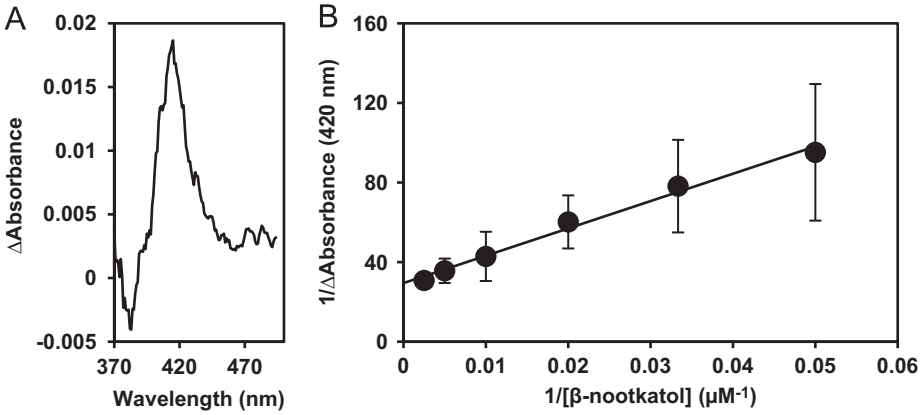

Fig. 8. β-nootkatol binding-induced difference spectrum obtained with microsomes of yeast expressing CYP71D51v2. The two cuvettes contained 600 nM of CYP71D51v2 in TG buffer. A baseline was recorded before adding 200 μM of β-nootkatol to the assay cuvette and an equivalent volume of the solvent to the reference (A). Different concentrations of β-nootkatol were added to plot the linear regression (B).

expected to be higher than 300 μM, while the total concentration in yeast cells, membranes included, would be around 1.1 mM. This implies a strong competition of substrate and product for the active site of CYP71D51v2.

In order to further check the capacity of (+)-nootkatone to inhibit CYP71D51v2, it was tested on enzyme activity. Added at the same concentration as (+)-valencene (200 μM) in the assay, it inhibited the 2-hydroxylation reaction by 37 ± 2%.

For a more direct comparison of the inhibition of the reaction by β-nootkatol and (+)-nootkatone using the same assay, the CYP71D51v2 activity was evaluated by monitoring NADPH consumption resulting from (+)-valencene metabolism (Table 1). In the case of a well coupled reaction, one molecule of NADPH is expected to be oxidized for the formation of one molecule of oxygenated product. As shown in Table 1, valencene 2-hydroxylation by CYP71D51v2 occurs with four molecules of NADPH oxidized for producing one molecule of β-nootkatol, implying significant uncoupling of the reaction. In the presence of β-nootkatol or (+)-nootkatone, the rate of the reaction estimated from NADPH consumption is decreased by 40% and 28%, respectively, implying stronger inhibition by β-nootkatol compared to (+)-nootkatone.

Accumulation of the products, in particular β-nootkatol, is thus likely to be an important factor limiting rates and yields of the CYP71D51v2-dependent (+)-valencene to β-nootkatol bioconversion.

Table 1
Comparison of the turnover of (+)-valencene hydroxylation by CYP71D51v2 evaluated from the rate of NAPDH oxidation in absence or presence of β-nootkatol or (+)-nootkatone (200 μM). NADPH oxidation was evaluated from the change in absorbance at 350 nm induced by the addition of 200 μM (+)-valencene in the assay and is compared to the rate of β-nootkatol formation in the same reaction by GC-FID.

Monitoring	Added compound	Turnover (min^{-1})
NADPH consumption	None	58 ± 2
NADPH consumption	β-nootkatol	35 ± 2
NADPH consumption	(+)-nootkatone	42 ± 2
β-nootkatol production	None	13 ± 2

4. Discussion

With the aim to identify an enzyme efficiently converting (+)-valencene into (+)-nootkatone, we selected four candidate genes from different plant species, which encoded proteins with 48 to 92% identity with previously reported valencene 2-oxygenases (Supplementary information, Table S5). While all four of them showed some valencene oxygenase activity, only two, both isolated from Solanaceae (tobacco and potato) with 88 and 92% identity to the previously described CYP71D55 from henbane, were found well expressed in yeast and showed high and regioselective 2-oxygenase activity. Both of them converted valencene into β-nootkatol, but did not allow significant formation of nootkatone from β-nootkatol, as previously observed for the *H. muticus* premnaspirodiene oxygenase (HPO) (Takahashi et al., 2007). Potato, as henbane, accumulates solavetivone, the natural product of HPO, as a major compound upon elicitation, while capsidiol is the dominant elicitor-induced sesquiterpene in tobacco (Vögeli and Chappell, 1988). Capsidiol is the product of 5-*epi*-aristolochene oxidation by tobacco CYP71D20 that has only 80% identity with CYP71D55 (Ralston et al., 2001). However, spirovetivone conjugated derivatives were recently isolated from the leaves of *N. tabacum* (Feng et al., 2009, 2010). CYP71D51v2 and CYP71D4 are thus likely to be orthologs of henbane CYP71D55 in tobacco and potato.

The best expressed of the two candidates, CYP71D51v2, was selected for full identification of products and determination of catalytic potential *in vitro* and in bioconversion assays. CYP71D51v2 produced β-nootkatol as a major product with only traces of the α epimer (Fig. 9). This production occurred with a quite high uncoupling of the reaction (i.e. unproductive catalytical cycles leading the oxidation of NADPH without production of β-nootkatol), suggesting that the distance of the (+)-valencene 2-position to the heme ferryl-oxo in the active site is not optimal. This hypothesis is further supported by the production of the desaturated compounds **4** and **5,** that would be favored by ineffective rebound of hydroxyl radical from heme iron to the 2 position of valencene. Despite this uncoupled reaction, CYP71D51v2 has the highest k_{cat} (5.8 ± 0.8 s^{-1}) for (+)-valencene oxygenation so far reported for a native plant P450 enzyme (Table 2).

While high activity was detected *in vitro* with the recombinant enzyme, the performance of the native CYP71D51v2 in yeast-mediated bioconversion did not match the requirements for implementation of an industrial process. Our data point to several factors which concur to limit CYP71D51v2-dependent valencene bioconversion in *S. cerevisiae*. One is the toxicity of β-nootkatol and to a lesser extent of (+)-nootkatone for yeast. This toxicity is significant only at high concentration (≥100 mg L^{-1}), but is a problem for production of nootkatone in the g L^{-1} range, which would be required for an efficient industrial process. Another is the trapping of β-nootkatol in the yeast endomembranes and cell wall. Products can be recovered from the cultures by the extraction

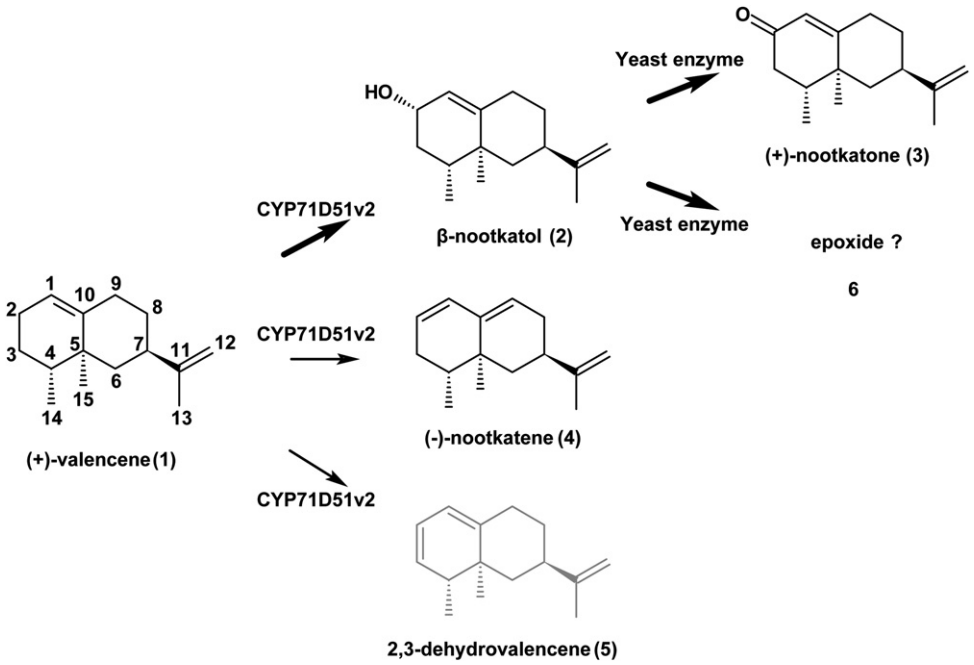

Fig. 9. Reaction of (+)-valencene bioconversion into (+)-nootkatone via β-nootkatol by the yeast expressing CYP71D51v2. Thin arrows represent the minor reactions. The grey compound and compound number 6 are suspected to be the 2,3-dehydrovalencene and an epoxide, respectively.

Table 2
Comparison of kinetic parameters of the hydroxylation of (+)-valencene by CYP71D51v2 with those reported for other sesquiterpene hydroxylases. No information is available concerning catalytic parameters of CYP71AV8.

Enzyme	Substrate	Product	K_m (μM)	k_{cat} (s^{-1})	k_{cat}/K_m (s^{-1} μM^{-1})	Reference
CYP71D51v2	(+)-valencene	β-nootkatol	52 ± 2	5.8 ± 0.8	0.15	
CYP71D55	(+)-valencene	β-nootkatol	7.4 ± 1.2	1.9 ± 0.07	0.26	Takahashi et al. (2007)
CYP71D55	Premnaspirodiene	Solavetivol	14.0 ± 1.9	2.1 ± 0.10	0.15	Takahashi et al. (2007)
CYP71D20	5-*epi*-aristolochene	Capsidiol	19.2 ± 2.9	0.5 ± 0.04	0.03	Ralston et al. (2001)

process, but the accumulation of large concentrations of β-nootkatol in the membranes is expected to alter their permeability and integrity, as well as the function of integral proteins as previously reported for other hydrocarbons which concentrate in the membranes (Sikkema et al., 1994, 1995). Membrane accumulation is thus likely to strongly contribute to β-nootkatol toxicity. Sesquiterpene alcohols and ketones in this respect differ from the acids such as artemisinic acid which are trapped onto the yeast cell-wall outer surface under acidic fermentation conditions (Ro et al., 2006), but do not accumulate in the endomembranes and show minor yeast toxicity (Ro et al., 2008). β-Nootkatol not only accumulates in yeast membranes, it also binds the CYP71D51v2 active site via direct coordination with the heme iron, as revealed by differential spectrophotometry. CYP71D51v2 affinity for β-nootkatol is in the same range as its affinity for (+)-valencene. Combined with β-nootkatol accumulation in the ER, this is expected to lead to strong inhibition of the enzyme during the bioconversion process.

Recently yeast has become a popular bioengineering tool for the production of high value natural compounds for the pharmaceutical, food and perfume industry (Trantas et al., 2009; Keasling, 2012; Brazier-Hicks and Edwards, 2013). In particular the production of sesquiterpenes, such as artemisinin or santalene, has led to extensive yeast engineering for optimizing metabolic fluxes to the production of the desired compounds (Farhi et al., 2011; Keasling, 2012; Scalcinati et al., 2012; Chen et al., 2013). So far no effective P450-based process was developed for the production of (+)-nootkatone from natural microorganisms or recombinant enzymes. The interfering factors identified in this work, might provide a more general explanation for this failure. Based on our data, a strategy can be proposed for the development of a bioproduction process from CYP71D51v2 that may also, to some extend, apply for successful exploitation of other candidate enzymes. The first step would involve enzyme improvement by directed mutagenesis, with the aim to reduce the volume of the active site and to optimize the distance between the 2-position of the substrate and ferryl-oxo oxygen donor in the active site. Such a distance adjustment would be expected to improve the coupling of the reaction and to limit the binding of the β-nootkatol product. Takahashi et al. (2007) have previously reported a 8-fold increase in k_{cat} and 5-fold increase in catalytic efficiency for (+)-valencene to β-nootkatol by HPO upon exchange of two valine and alanine residues located in the roof of the active site for a larger isoleucine (V482I/A484I). Sequence alignment with HPO (Supplementary information, Fig. S29) indicates that a valine 494 and an alanine 496 of CYP71D51v2 correspond to the valine 482 and alanine 484 residues in the active site of HPO. Mutations leading to a change of both residues to isoleucine or to another bulky hydrophobic amino acid (e.g. phenylalanine) would thus be expected to reduce the size of the active site and to increase CYP71D51v2 activity. Depending on the outcome of such experiments, a more sophisticated approach of random mutagenesis might be considered, using as a screen the

production of nootkatone detected by a synthetic RNA-switch, provided a nootkatone-specific switch can be designed (Michener and Smolke, 2012).

A second and more critical bottleneck is the P450 inhibition and toxicity resulting from the production of β-nootkatol. Several options can be proposed to solve the problem of product toxicity. A drastic improvement could be brought by the conversion of the β-nootkatol product into hydrophilic glycoside. Such a glucoside conjugate would not be trapped in the membranes and would no longer fit in the active site of CYP71D51v2. Identification of a suitable glycosyltransferase (preferably membrane-bound) for further transformation of recombinant yeast can be achieved via screening of existing recombinant enzyme collections (Caputi et al., 2008; Williams and Thorson, 2008). For the conversion of β-nootkatol into nootkatone, product extraction, enzymatic deglycosylation and further fermentation with wild-type yeast would be required. Another option to reduce product accumulation in the endomembranes would be the isolation and expression of a selective transporter of nootkatol and nootkatone. No such transporter is described so far, but such a transporter might be involved in the compartmentation of nootkatone in *Citrus* fruits. *Citrus* cells and callus cultures show a potential for the conversion of valencene into nootkatone with only minor accumulation of nootkatol (Drawert et al., 1984; del Río et al., 1991). The specialized enzyme(s) responsible for the formation of nootkatone in the pomelo or *Citrus paradisi*, one of the main natural sources of nootkatone, could also be investigated to test their ability to more directly convert (+)-valencene into (+)-nootkatone, resistance to nootkatol inhibition and/or ability to catalyze nootkatol into (+)-nootkatone conversion. It has however to be noted that incubation of *C. paradisi* cells in the presence of increasing concentrations of (+)-valencene also led to a reduced production of nootkatone (Drawert et al., 1984). Finally, plants offer another attractive option for the production of high-value sesquiterpenoids in specialized structures, allowing compartmented accumulation of otherwise toxic compounds, like glandular trichomes (Tissier, 2012), which offer in addition an important metabolic capacity and optimized fluxes for the production of isoprenoids. This option is however not compatible with the present definition and public acceptance of a "natural product" if produced from genetically modified plant.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.ymben.2013.02.003.

References

Ancian, B., Bourgeois, I., Dauphin, J.F., Shaw, A., 1997. Artifact-free pure absorption PFG-enhanced DQF-COSY spectra including a gradient pulse in the evolution period. J. Magn. Reson., Ser. A 125, 348–354.
Andersen, N.H., 1970. Biogenetic implications of the antipodal sesquiterpenes of vetiver oil. Phytochemistry 9, 145–151.
Barton, G.M., 1976. A review of yellow cedar (*Chamaecyparis nootkatensis* [D. Don] Spach) extractives and their importance to utilization. Wood Fiber Sci. 8, 172–176.
Beyeler, S., Testa, B., Perrissoud, D., 1988. Flavonoids as inhibitors of rat liver monooxygenase activities. Biochem. Pharmacol. 37, 1971–1979.
Brazier-Hicks, M., Edwards, R., 2013. Metabolic engineering of the flavone-C-glycoside pathway using polyprotein technology. Metab. Eng. 16, 11–20.
Cankar, K., van Houwelingen, A., Bosch, D., Sonke, T., Bouwmeester, H., Beekwilder, J., 2011. A chicory cytochrome P450 mono-oxygenase CYP71AV8 for the oxidation of (+)-valencene. FEBS Lett. 585, 178–182.
Caputi, L., Lim, E.-K., Bowles, D.J., 2008. Discovery of new biocatalysts for the glycosylation of terpenoid scaffolds. Chem. Eur. J. 14, 6656–6662.
Chen, Y., Daviet, L., Schalk, M., Siewers, V., Nielsen, J., 2013. Establishing a platform cell factory through engineering of yeast acetyl-CoA metabolism. Metab. Eng. 15, 48–54.
Cicero, D.O., Barbato, G., Bazzo, R., 2001. Sensitivity enhancement of a two-dimensional experiment for the measurement of heteronuclear long-range coupling constants, by a new scheme of coherence selection by gradients. J. Magn. Reson. 148, 209–213.
De Kraker, J.W., Schurink, M., Franssen, M.C.R., König, W.A., de Groot, A., Bouwmeester, H.J., 2003. Hydroxylation of sesquiterpenes by enzymes from chicory (*Cichorium intybus* L.) roots. Tetrahedron 59, 409–418.
del Río, J.A., Ortuño, A., Puig, D.G., Iborra, J.L., Sabater, F., 1991. Accumulation of the sesquiterpenes nootkatone and valencene by callus cultures of *Citrus paradisi*, *Citrus limonia* and *Citrus aurantium*. Plant Cell Rep. 10, 410–413.
del Río, J.A., Ortuño, A., Puig, D.G., Porras, I., Lidon, A.G., Sabater, F., 1992. Variations of nootkatone and valencene levels during the development of grapefruit. J. Agric. Food Chem. 40, 1488–1490.
Drawert, F., Berger, R.G., Godelmann, R., 1984. Regioselective biotransformation of valencene in cell suspension cultures of *Citrus* sp. Plant Cell Rep. 3, 37–40.
Engström, K., Widmark, A.K., Brishammar, S., Helmersson, S., 1999. Antifungal activity to *Phytophthora infestans* of sesquiterpenoids from infected potato tubers. Potato Res. 42, 43–50.
Farhi, M., Marhevka, E., Masci, T., Marcos, E., Eyal, Y., Ovadis, M., Abeliovich, H., Vainstein, A., 2011. Harnessing yeast subcellular compartments for the production of plant terpenoids. Metab. Eng. 13, 474–481.
Feng, X., Wang, J.S., Luo, J., Kong, L.Y., 2009. Two new sesquiterpene glucosides from the leaves of *Nicotiana tabacum*. J. Asian Nat. Prod. Res. 11, 675–680.
Feng, X., Wang, J.S., Luo, J., Kong, L.Y., 2010. A pair of sesquiterpene glucosides from the leaves of *Nicotiana tabacum*. J. Asian Nat. Prod. Res. 12, 252–256.
Fraatz, M.A., Berger, R.G., Zorn, H., 2009. Nootkatone—a biotechnological challenge. Appl. Microbiol. Biotechnol. 83, 35–41.
Gietz St, D., Jean, A., Woods, R.A., Schiestl, R.H., 1992. Improved method for high efficiency transformation of intact yeast cells. Nucleic Acids Res. 20, 1425.
Girhard, M., Machida, K., Itoh, M., Schmid, R.D., Arisawa, A., Urlacher, V.B., 2009. Regioselective biooxidation of (+)-valencene by recombinant *E. coli* expressing CYP109B1 from *Bacillus subtilis* in a two-liquid-phase system. Microb. Cell Fact. 8, 36.
Hall, T.A., BioEdit: A User-friendly Biological Sequence Alignment Editor and Analysis Program for Windows 95/98/NT. vol. 41, 1999, pp. 95–98.
Haring, H.G., Rijkens, F., Boelens, H., Van der Gen, A., 1972. Olfactory studies on enantiomeric eremophilane sesquiterpenoids. J. Agric. Food Chem. 20, 1018–1021.
Helander, I.M., Alakomi, H.L., Latva-Kala, K., Mattila-Sandholm, T., Pol, I., Smid, E.J., Gorris, L.G.M., Von Wright, A., 1998. Characterization of the action of selected essential oil components on Gram-negative bacteria. J. Agric. Food Chem. 46, 3590–3595.
Höfer, R., Boachon, B., Gavira, C., Ginglinger, J.-F., Larbat, R., Grec, S., Werck-Reichhart, D. Functional versatility of the CYP76 family of cytochrome P450 enzymes from *Arabidopsis thaliana*. submitted.
Huffaker, A., Kaplan, F., Vaughan, M.M., Dafoe, N.J., Ni, X., Rocca, J.R., Alborn, H.T., Teal, P.E.A., Schmelz, E.A., 2011. Novel acidic sesquiterpenoids constitute a dominant class of pathogen-induced phytoalexins in maize. Plant Physiol. 156, 2082–2097.
Jeener, J., Meier, B., Bachmann, P., Ernst, R.R., 1979. Investigation of exchange processes by two dimensional NMR spectroscopy. J. Chem. Phys. 71, 4546.
Jefcoate, C.R., 1978. Measurement of substrate and inhibitor binding to microsomal cytochrome P-450 by optical-difference spectroscopy. Methods Enzymol. 52, 258–279.
Kaspera, R., Krings, U., Nanzad, T., Berger, R.G., 2005. Bioconversion of (+)-valencene in submerged cultures of the ascomycete *Chaetomium globosum*. Appl. Microbiol. Biotechnol. 67, 477–483.
Keasling, J.D., 2012. Synthetic biology and the development of tools for metabolic engineering. Metab. Eng. 14, 189–195.
MacLeod, J.R.W.D., Buigues, N.M., 1964. Sesquiterpenes. I. Nootkatone, a new grapefruit flavor constituent. J. Food Sci. 29, 565–568.
Manter, D.K., Kelsey, R.G., Karchesy, J.J., 2007. Antimicrobial activity of extractable conifer heartwood compounds toward *Phytophthora ramorum*. J. Chem. Ecol. 33, 2133–2147.
Michener, J.K., Smolke, C.D., 2012. High-throughput enzyme evolution in *Saccharomyces cerevisiae* using a synthetic RNA switch. Metab. Eng. 14, 306–316.
Nour-Eldin, H.H., Hansen, B.G., Norholm, M.H., Jensen, J.K., Halkier, B.A., 2006. Advancing uracil-excision based cloning towards an ideal technique for cloning PCR fragments. Nucleic Acids Res. 34, e122.
Omura, T., Sato, R., 1964. The carbon monoxide-binding pigment of liver microsomes. I. evidence for its hemoprotein nature. J. Biol. Chem. 239, 2370–2378.
Ortiz de Montellano, P.R., Correia, M.A., 1995. Inhibition of cytochrome P450 enzymes. In: Ortiz de Montellano, P. (Ed.), Cytochrome P450: Structure, Mechanism, and Biochemistry, second ed. Plenum, New York, pp. 305–364.

Parella, T., Sánchez-Ferrando, F., Virgili, A., 1997. Improved sensitivity in gradient-based 1D and 2D multiplicity-edited HSQC experiments. J. Magn. Reson. 126, 274–277.

Ralston, L., Kwon, S.T., Schoenbeck, M., Ralston, J., Schenk, D.J., Coates, R.M., Chappell, J., 2001. Cloning, heterologous expression, and functional characterization of 5-*epi*-aristolochene-1,3-dihydroxylase from tobacco (*Nicotiana tabacum*). Arch. Biochem. Biophys. 393, 222–235.

Ro, D.K., Ouellet, M., Paradise, E.M., Burd, H., Eng, D., Paddon, C.J., Newman, J.D., Keasling, J.D., 2008. Induction of multiple pleiotropic drug resistance genes in yeast engineered to produce an increased level of anti-malarial drug precursor, artemisinic acid. BMC Biotechnol. 8, 83.

Ro, D.K., Paradise, E.M., Ouellet, M., Fisher, K.J., Newman, K.L., Ndungu, J.M., Ho, K.A., Eachus, R.A., Ham, T.S., Kirby, J., Chang, M.C.Y., Withers, S.T., Shiba, Y., Sarpong, R., Keasling, J.D., 2006. Production of the antimalarial drug precursor artemisinic acid in engineered yeast. Nature 440, 940–943.

Salvador, J.A.R., Clark, J.H., 2002. The allylic oxidation of unsaturated steroids by *tert*-butyl hydroperoxide using surface functionalised silica supported metal catalysts. Green Chem. 4, 352–356.

Scalcinati, G., Knuf, C., Partow, S., Chen, Y., Maury, J., Schalk, M., Daviet, L., Nielsen, J., Siewers, V., 2012. Dynamic control of gene expression in *Saccharomyces cerevisiae* engineered for the production of plant sesquitepene α-santalene in a fed-batch mode. Metab. Eng. 14, 91–103.

Schenkman, J.B., Remmer, H., Estabrook, R.W., 1967. Spectral studies of drug interaction with hepatic microsomal cytochrome. Mol. Pharmacol. 3, 113–123.

Shaffer, G.W., Eschinasi, E.H., Purzycki, K.L., Doerr, A.B., 1975. Oxidations of valencene. J. Org. Chem. 40, 2181–2185.

Sikkema, J., De Bont, J.A., Poolman, B., 1994. Interactions of cyclic hydrocarbons with biological membranes. J. Biol. Chem. 269, 8022–8028.

Sikkema, J., De Bont, J.A., Poolman, B., 1995. Mechanisms of membrane toxicity of hydrocarbons. Microbiol. Rev. 59, 201–222.

Sime, J.T., Cheetham, P.S.J., Gradley, M.L., Banister, N.E., 2000. Use of Sesquiterpenes for Inhibiting Oxidative Enzymes. US Patent no. 6054490.

Sowden, R.J., Yasmin, S., Rees, N.H., Bell, S.G., Wong, L.-L., 2005. Biotransformation of the sesquiterpene (+)-valencene by cytochrome $P450_{cam}$ and $P450_{BM-3}$. Org. Biomol. Chem. 3, 57–64.

Takahashi, S., Yeo, Y.-S., Zhao, Y., O'Maille, P.E., Greenhagen, B.T., Noel, J.P., Coates, R.M., Chappell, J., 2007. Functional characterization of premnaspirodiene oxygenase, a cytochrome P450 catalyzing regio- and stereo-specific hydroxylations of diverse sesquiterpene substrates. J. Biol. Chem. 282, 31744–31754.

Tassaneeyakul, W., Guo, L.-Q., Fukuda, K., Ohta, T., Yamazoe, Y., 2000. Inhibition selectivity of grapefruit juice components on human cytochromes P450. Arch. Biochem. Biophys. 378, 356–363.

Tissier, A., 2012. Glandular trichomes: what comes after expressed sequence tags? Plant J. 70, 51–68.

Trantas, E., Panopoulos, N., Ververidis, F., 2009. Metabolic engineering of the complete pathway leading to heterologous biosynthesis of various flavonoids and stilbenoids in *Saccharomyces cerevisiae*. Metab. Eng. 11, 355–366.

Urban, P., Mignotte, C., Kazmaier, M., Delorme, F., Pompon, D., 1997. Cloning, yeast expression, and characterization of the coupling of two distantly related *Arabidopsis thaliana* NADPH-cytochrome P450 reductases with P450 CYP73A5. J. Biol. Chem. 272, 19176–19186.

Urban, P., Werck-Reichhart, D., Teutsch, H.G., Durst, F., Regnier, S., Kazmaier, M., Pompon, D., 1994. Characterization of recombinant plant cinnamate 4-hydroxylase produced in yeast. Kinetic and spectral properties of the major plant P450 of the phenylpropanoid pathway. Eur. J. Biochem. 222, 843–850.

Vögeli, U., Chappell, J., 1988. Induction of sesquiterpene cyclase and suppression of squalene synthetase activities in plant cell cultures treated with fungal elicitor. Plant Physiol. 88, 1291–1296.

Whitehouse, C.J.C., Bell, S.G., Wong, L.-L., 2008. Desaturation of alkylbenzenes by cytochrome $P450_{BM3}$ (CYP102A1). Chem. Eur. J. 14, 10905–10908.

Williams, G.J., Thorson, J.S., 2008. A high-throughput fluorescence-based glycosyltransferase screen and its application in directed evolution. Nat. Protoc. 3, 357–362.

Wilson III, C.W., Shaw, P.E., 1978. Synthesis of nootkatone from valencene. J. Agric. Food Chem. 26, 1430–1432.

Yao, M.K., Désilets, H., Charles, M.T., Boulanger, R., Tweddell, R.J., 2003. Effect of mycorrhization on the accumulation of rishitin and solavetivone in potato plantlets challenged with *Rhizoctonia solani*. Mycorrhiza 13, 333–336.

Yokose, T., Katamoto, K., Park, S., Matsuura, H., Yoshihara, T., 2004. Anti-fungal sesquiterpenoid from the root exudate of *Solanum abutiloides*. Biosci. Biotechnol. Biochem. 68, 2640–2642.