Journal of Biotechnology 259 (2017) 204–212

Contents lists available at ScienceDirect

Journal of Biotechnology

journal homepage: www.elsevier.com/locate/jbiotec

Engineering yeasts as platform organisms for cannabinoid biosynthesis

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ARTICLE INFO

Keywords:
NphB
THCA synthase
CBGA
THCA
Saccharomyces cerevisiae
Komagataella phaffii

ABSTRACT

Δ^9-tetrahydrocannabinolic acid (THCA) is a plant derived secondary natural product from the plant *Cannabis sativa* L. The discovery of the human endocannabinoid system in the late 1980s resulted in a growing number of known physiological functions of both synthetic and plant derived cannabinoids. Thus, manifold therapeutic indications of cannabinoids currently comprise a significant area of research. Here we reconstituted the final biosynthetic cannabinoid pathway in yeasts. The use of the soluble prenyltransferase NphB from *Streptomyces* sp. strain CL190 enables the replacement of the native transmembrane prenyltransferase cannabigerolic acid synthase from *C. sativa*. In addition to the desired product cannabigerolic acid, NphB catalyzes an *O*-prenylation leading to 2-*O*-geranyl olivetolic acid. We show for the first time that the bacterial prenyltransferase and the final enzyme of the cannabinoid pathway tetrahydrocannabinolic acid synthase can both be actively expressed in the yeasts *Saccharomyces cerevisiae* and *Komagataella phaffii* simultaneously. While enzyme activities in *S. cerevisiae* were insufficient to produce THCA from olivetolic acid and geranyl diphosphate, genomic multi-copy integrations of the enzyme's coding sequences in *K. phaffii* resulted in successful synthesis of THCA from olivetolic acid and geranyl diphosphate. This study is an important step toward total biosynthesis of valuable cannabinoids and derivatives and demonstrates the potential for developing a sustainable and secure yeast bio-manufacturing platform.

1. Introduction

Cannabis sativa L. (hemp, marijuana; Cannabaceae) is well known for the biosynthesis of Δ^9-tetrahydrocannabinolic acid (THCA) and related cannabinoids. The plant has been used for more than 3500 years in ethnomedicine and was used as legal herbal medicine before being banned as illicit drug beginning of last century. THCA is the pharmacologically active constituent whose structure was first elucidated in 1964 (Gaoni and Mechoulam, 1964) and tested in various pharmacological assays as potential drug target to treat symptoms of different diseases like tremor in multiple sclerosis, vomiting during anti-neoplastic chemotherapy, posttraumatic stress and more (Gaoni and Mechoulam, 1964; Ligresti, 2006). Besides THCA more than 100 other cannabinoids were described so far (Mehmedic et al., 2010) that are biosynthesized mainly in trichomes located on leaves and with high density on flower buds of *C. sativa* (Happyana et al., 2013; Sirikantaramas et al., 2005). Cannabinoids are terpenophenolics with mixed biosynthetic origins. Biosynthetically, cannabinoids are prenylated polyketides derived from the polyketide and MEP pathway delivering an alkylresorcinolic acid (predominantly olivetolic acid; OA) and a monoterpene moiety (predominantly geranyl diphosphate; GPP), respectively (Fig. 1). OA is biosynthesized by two type III polyketide synthases called olivetol synthase and olivetolic acid cyclase (Gagne et al., 2012). Biosynthetic precursor of the first committed metabolite towards a high diversity of cannabinoids is cannabigerolic acid (CBGA) being formed by a C-C Friedel-Craft alkylation of OA at position C3. *In planta*, this reaction is performed by the integral membrane protein cannabigerolic acid synthase (CBGAS), which is presumably located in the membranes of the plastids (Eisenreich et al., 2001; Fellermeier and Zenk, 1998). Finally, THCA and cannabidiolic acid (CBDA) are produced from CBGA via oxidative cyclization catalyzed by the tetrahydrocannabinolic acid synthase (THCAS) or the cannabidiolic acid synthase (CBDAS), respectively (Fig. 1). On the contrary, these enzymes are exported into the secretory cavities of the glandular trichomes, suggesting a transport mechanism of CBGA through the membranes by either active transport or diffusion (Sirikantaramas et al., 2005; Taura et al., 2007).

Engineering microbial strains for the production of secondary

Abbreviations: CBDA, Cannabidiolic acid; CBGA, Cannabigerolic acid; CBGAS, Cannabigerolic acid synthase; 2-*O*-GOA, 2-*O*-Geranyl olivetolic acid; GPP, Geranyl diphosphate; MEP, 2-C-Methyl-D-erythritol 4-phosphate; OA, Olivetolic acid; THCA, Δ^9-tetrahydrocannabinolic acid; THCAS, Δ^9-tetrahydrocannabinolic acid synthase

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http://dx.doi.org/10.1016/j.jbiotec.2017.07.008
Received 5 May 2017; Received in revised form 7 June 2017; Accepted 5 July 2017
Available online 08 July 2017
0168-1656/

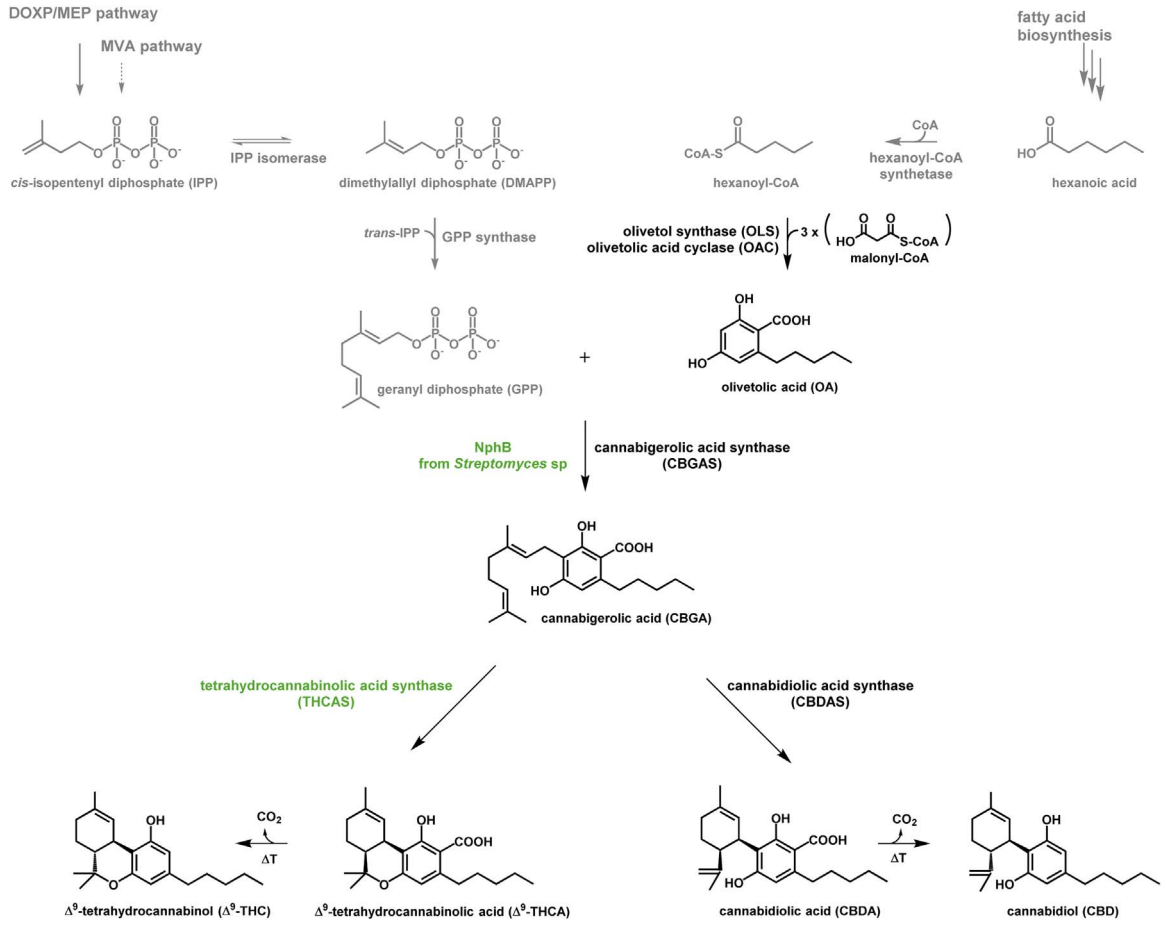

Fig. 1. Biosynthetic pathway of cannabinoids in *C. sativa.* The precursors geranyl diphosphate (GPP) and olivetolic acid (OA) are converted to the central intermediate of the cannabinoid pathway cannabigerolic acid (CBGA). Subsequently CBGA is further converted by two different oxidoreductases tetrahydrocannabinolic acid synthase (THCAS) and cannabidiolic acid synthase (CBDAS) to the acidic forms of THC and CBD accumulating in the glandular trichomes. Heterologously expressed enzymes of this study are highlighted in green. Intermediates of the primary metabolism are displayed in grey. MEP, 2-C-methyl-D-erythritol 4- phosphate; DOXP, 1 deoxy-D-xylulose-5 phosphate; MVA, mevalonate; IPP, *cis*-isopentenyl diphosphate; DMAPP, dimethylallyl diphosphate; AAE1, hexanoyl-CoA synthetase; OLS, olivetol synthase; OAC, olivetolic acid cyclase; CBGAS, cannabigerolic acid synthase; NphB, aromatic prenyltransferase from *Streptomyces* sp. strain CL190; Δ^9-THCA, Δ^9-tetrahydrocannabinolic acid; Δ^9-THC, Δ^9-tetrahydrocannabinol; CBDA, cannabidiolic acid; CBD, cannabidiol; modified from Degenhardt et al. (Degenhardt et al., 2017).

natural products provides an interesting alternative to chemical synthesis or plant based production. The reconstitution of the cannabinoid pathway in a microbial system offers the possibility of the modulation of formed products by incorporation of different starter units, by tailoring involved enzymes or by elongation of the biosynthetic pathway resulting in non-natural cannabinoids with altered pharmacological properties. However, genetic reconstruction of biosynthetic routes raises many engineering challenges either on the level of genetic pathway assembly or regulation networks. Yeast, especially *Saccharomyces cerevisiae*, is one of the major platform organisms, and geneticists and biotechnologists have demonstrated in an impressive way how various plant pathways leading to dihydroartemisinine, (Paddon and Keasling, 2014) thebaine, (Thodey et al., 2014) or resveratrol (Li et al., 2016) can be successfully reconstructed. Nevertheless, *Komagataella phaffii (*formerly *Pichia pastoris)* often shows better protein production rates and could therefore present an interesting alternative production host for plant derived pharmacologically active metabolites. Secondary natural product pathways of plants in particular, present a number of challenges to microorganisms due to complex and branched pathways, unsuitable catalytic properties of the involved enzymes, compartmentalization, and specific regulation mechanisms in different organs. For example, THCAS is directed into the secretory pathway and is a soluble enzyme in *C. sativa*. In contrast, CBGAS is an integral membrane protein most likely localized in plastids of the plant. Since the functional production of the THCAS as cytosolic protein in a prokaryotic host failed (Zirpel et al., 2015), the reconstitution of the cannabinoid pathway also comprises a compartmentation of the different biosynthetic enzymes. Feeding experiments of *thcas* expressing yeasts with CBGA showed that THCA was formed. Thus, a transport of the intermediate product CBGA through membranes seems not to be limited (Zirpel et al., 2015).

Besides expressing sufficient amounts of catalytically slow enzymes of secondary metabolism, the application of integral membrane proteins (like CBGAS) in a heterologous biosynthetic pathway is challenging due to problems of correct protein folding and incorrect organelle localization accompanied with additional compartmentation issues. To circumvent these unpredictable barriers we alternatively chose the soluble aromatic prenyltransferase NphB from *Streptomyces* sp. strain CL190 (Bonitz et al., 2011; Kuzuyama et al., 2005) to replace CBGAS. NphB catalyzes the transfer of geranyl moieties to various aromatic acceptor molecules. In previous studies it was shown that NphB is capable to prenylate dihydroxy naphthalenes, several flavonoids and polyketides particularly olivetol, the decarboxylated form of OA, at the C2 and C4 position (Kumano et al., 2008). Beside the formation of C-geranylation NphB catalyzes the formation of O-prenyl linkage to the aromatic substrates. In contrast to the promiscuous specificity towards the aromatic substrates NphB only accepts GPP as prenyl donor. But regardless its relaxed substrate specificity, NphB often shows a high regioselectivity for the prenyl group transfer. In conclusion, this makes NphB a promising candidate for the substitution of plant derived

membrane bound prenyltransferases.

We previously described the functional intracellular production of THCAS in *S. cerevisiae* and *K. phaffii* using a signal peptide from the vacuolar protease, proteinase A (Zirpel et al., 2015). In continuation of our research to develop yeasts as platform organisms for cannabinoid biosynthesis we established NphB as alternative biocatalyst for production of CBGA. Subsequently, we coupled the expression of the prenyltransferase to THCAS in *K. phaffii* resulting in the successful production of THCA from OA and GPP. Thereby, 82 ± 4.6 pmol L^{-1} OD^{-1} h^{-1} THCA were produced.

2. Materials and methods

2.1. Screening of K. phaffii transformants for high NphB activities

Colonies of PP2_HC transformed with *Pme*I-linearized pAX_NphB vector were transferred to a sterile 48-round-well Biolector® plate (m2p-labs, Baesweiler, Germany) containing 800 µL BMGY medium (15 g L^{-1} glycerol, 10 g L^{-1} yeast extract, 20 g L^{-1} peptone, 5 g L^{-1} casamino acids, 13.8 g L^{-1} yeast nitrogen base, 100 mM Bis-Tris buffer pH 5.8, 0.4 mg L^{-1} biotin) per well. Precultures were incubated using the Biolector® (m2p-labs, Baesweiler, Germany) at 1200 rpm and 28 °C for 22 h for complete use-up of glycerol. From precultures 10 µL were transferred by an attached pipetting robot Sias Xantus (Tecan Group Ltd., Männedorf, Switzerland) to fresh BMGY medium. After 18 h of incubation, feeding of cultures with 8 µL nutrient solution (200 g L^{-1} glycerol, 1.5% (v/v) ammonium hydroxide, 25% (v/v) methanol) every 2 h was started and continued for 32 h. Cells were harvested by centrifugation of 650 µL of culture and resuspension of the pellet in 500 µL assay buffer (50 mM Tris-HCl buffer pH 7.5, 10% (w/v) glycerol, 100 mM sodium chloride). Cell lysis was performed as described previously (Zirpel et al., 2015). Lysate supernatant was used for NphB activity assay (1 mM GPP, 1 mM OA, 5 mM magnesium chloride, 37 °C, 1100 rpm, 4 h).

2.2. Expression of nphB and thcas in S. cerevisiae

The expression of SC_TT2AN and SC_N as well as the respective strains expressing only NphB (SC_N) or THCAS (SC_T, SC_T2) was performed with two precultures followed by one main culture. After inoculation of synthetic mineral salt medium without leucine (6.7 g L^{-1} YNB without amino acids, 1.6 g L^{-1} drop out supplements without leucine, 20 g L^{-1} fructose) the cells were incubated overnight at 30 °C and 200 rpm. The first preculture was used for the inoculation of the second preculture which was incubated at 30 °C and 200 rpm for 12 h 100 mL complex medium (20 g L^{-1} yeast extract, 40 g L^{-1} peptone, 80 mg L^{-1} adenine hemisulfate, 40 g L^{-1} fructose, 5 g L^{-1} galactose; 100 mM potassium citrate buffer pH 5.5) in 1 L baffled flasks were inoculated with OD_{600} of 0.5 for main culture. Cultures were incubated at 15 °C and 200 rpm over 168 h. Samples were taken every 24 h for measurement of optical density (600 nm) and activity assays.

Cell culture volumes correlating with an OD_{600} of 125 were harvested by centrifugation (2000 × *g*, 4 °C, 10 min). Supernatants were discarded and cells resuspended in 500 µL assay buffer (50 mM Tris-HCl buffer pH 7.5, 10% (w/v) glycerol, 100 mM sodium chloride). Cell suspension was transferred to 0.5 mL tubes and filled with 0.4–0.6 mm glass beads. Cells were lysed by vortexing at maximum speed at 4 °C for 30 min. Cell lysate was centrifuged and supernatant used for NphB activity assays (1 mM GPP, 1 mM OA, 5 mM magnesium chloride, 37 °C, 1100 rpm, 4 h) and THCAS assays (0.3 mM CBGA, 37 °C, 1100 rpm, 4 h).

2.3. Expression of nphB and thcas in K. phaffii

Clones of PP2_HC pAX_N C1 and C23 as well as the respective control strains expressing only NphB (PP2_EV pAX_N) or THCAS (PP2_HC) were used for inoculation of 100 mL BMGY medium in baffled 1 L shaking flasks. Cultures were incubated at 200 rpm and 30 °C overnight. Cells were harvested and used for inoculation of 50 mL BMMY (1% (v/v) methanol, 10 g L^{-1} yeast extract, 20 g L^{-1} peptone, 5 g L^{-1} casamino acids, 13.8 g L^{-1} yeast nitrogen base, 100 mM Bis-Tris buffer pH 5.8, 0.4 mg L^{-1} biotin) at an OD_{600} of 10. Cultivation was conducted at 160 rpm and 15 °C for 96 h and 500 µL methanol was added every 24 h after sampling. Samples were taken for measurement of optical density (600 nm), NphB activity assays (described above) and THCAS activity assays. Cell lysis was performed as described previously (Zirpel et al., 2015), but 2 mL of cell culture were harvested and cell pellets resuspended in 500 µL assay buffer (50 mM Tris-HCl buffer pH 7.5, 10% (w/v) glycerol, 100 mM sodium chloride). For measurement of THCAS activity, lysate supernatant was incubated with 0.3 mM CBGA for 4 h at 37 °C and 1100 rpm.

2.4. HPLC-ESI–MS/MS

Activity assays were stopped by addition of 0.1 assay-volumes trichloroacetic acid (*K. phaffii*) or formic acid (*S. cerevisiae*) and 2.9 assay-volumes acetonitrile (ACN) followed by incubation on ice for 15 min. Supernatants were filtered (0.45 µm, Nylon) after centrifugation (13,100 *g*, 4 °C, 20 min) and analyzed by HPLC-DAD (Agilent 1260 Infinity HPLC, Waldbronn, Germany). Separation of compounds from *S. cerevisiae* extracts was performed on a EC 100/2 Nucleoshell RP18 2.7 µm column (Macherey Nagel, Düren, Germany) (0.8 mL min^{-1}, 40 °C, 35 % (v/v) H_2O with 0.1% (v/v) formic acid (FA), 65% (v/v) ACN). Separation of compounds from *K. phaffii* extracts was performed on a EC 100/2 Nucleoshell RP18 2.7 µm column (Macherey Nagel, Düren, Germany) (0.8 mL min^{-1}, 40 °C, 39 % (v/v) H_2O with 0.1% (v/v) FA, 61% (v/v) ACN). Quantification of products was based on integrated peak areas of the UV-chromatograms at 225 nm. Standard curves were generated for CBGA and THCA and the CBGA standard curve was used to estimate the concentration of the side-product 2-*O*-GOA. The identity of all compounds was confirmed by comparing mass and tandem mass spectra of each sample with coeluting standards analyzed by Bruker compact™ ESI-Q-TOF (Bruker, Bremen, Germany) using positive ionization mode.

2.5. Preparative RP-HPLC

The isolation of 2-*O*-GOA was done by extraction with ethyl acetate. The purification was performed on a Nucleodur C18 HTec 5 µm (250 × 10 mm) column (Macherey Nagel, Düren, Germany) using an isocratic gradient (4.0 mL min^{-1}, 40 °C, 35 % (v/v) H_2O, 65% (v/v) ACN). The separated peak was detected at 225 nm and fractionated with a FC-1 Dynamax fraction collector (Zinsser Analytik, Frankfurt am Main, Germany). After removal of solvent a lyophilisation was performed. Obtained compound was submitted for ^1H NMR measurement.

3. Results

3.1. NphB is able to produce CBGA

In a first approach, the heterologous expression of the native CBGA forming enzyme CBGAS from *C. sativa* (Page and Boubakir, 2014) was investigated in *S. cerevisiae*, but no functionally active protein was obtained. To circumvent the problems expressing the integral membrane protein CBGAS, we tried to use the soluble aromatic prenyltransferase NphB from *Streptomyces* sp. strain CL190 strain. Previous studies indicated that NphB is able to prenylate OA (Kuzuyama et al., 2005). Thus, *S. cerevisiae* CEN·PK cells carrying the coding sequence of NphB under the control of the GAL1 promoter (SC_N) were used to express the soluble prenyltransferase. HPLC analysis of the activity assay with GPP and OA revealed the formation of two products with *m/z*of 361.23. The peak with a retention time of 1.6 min was identical to the standard

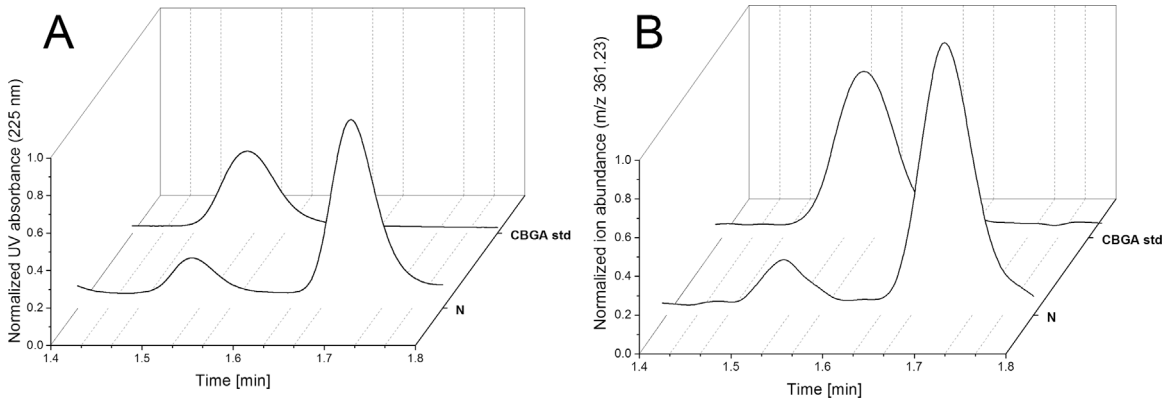

Fig. 2. LC–MS analysis of cannabinoids produced with cell lysates of *S. cerevisiae* expressing *nphB*. The yeast lysates were incubated with 1 mM GPP and OA, respectively. (A) HPLC-UV chromatograms at 225 nm of the standard CBGA and the assay products. (B) Extracted ion chromatograms (EIC) of *m/z* 361.23 (CBGA) of the standard CBGA and the assay products. N − assay of SC_N lysates.

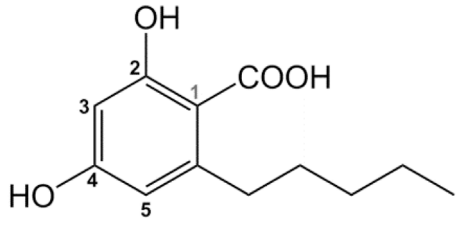

Fig. 3. Possible geranylation sites of OA by NphB. The aromatic prenyltransferase NphB is able to catalyze carbon–carbon and carbon-oxygen based geranylation of hydroxyl-containing aromatic acceptors. Possible *O*-geranylation can take place at positions 2 and 4. C-C prenylation is possible at positions 3 (CBGA) and 5.

compound of CBGA (Fig. 2) indicating that NphB is indeed able to prenylate OA to CBGA. The second peak at 1.7 min showed the same *m/z* of 361.23 but a different MS^2 spectrum (Supporting Fig. S1), suggesting that NphB prenylates OA at a different position. According to Kuzuyama et al. (2005) and Kumano et al. (2010) NphB catalyzes carbon–carbon and carbon-oxygen-based geranylation of many hydroxyl-containing aromatic acceptors. Possible prenylation sites of OA by NphB are shown in Fig. 3. For further elucidation of the unknown compound the corresponding peak was isolated via preparative RP-HPLC followed by NMR analysis (Supplementary information). That suggested that NphB is able to geranylate OA preferentially at the 2-*O* position resulting in 2-*O*-geranyl olivetolic acid (2-*O*-GOA). Other prenylation products with *m/z* of 361.23 could not be detected via HPLC–MS.

3.2. Whole cell assay of S. cerevisiae

Based on these results we tested whether *S. cerevisiae* is able to take up OA and GPP from the medium to set up a whole cell assay. Initial tests suggested that OA concentrations up to 5 mM are not toxic to *S. cerevisiae* cells (data not shown). SC_N cells were cultivated in complex medium for 72 h at 15 °C and 200 rpm. The cells were harvested and resuspended in assay buffer. Cells were either (a) lysed for a subsequent NphB activity assay to determine if NphB is functionally expressed or (b) directly supplemented with 3 mM OA and 3 mM GPP. Finally, the cells of the whole cell assay were extracted using ethyl acetate to determine CBGA formation upon uptake of OA and GPP.

The results indicate that although NphB is functionally expressed, the substrate uptake by whole cells is not occurring or insufficient under tested conditions as cultivation of cells in the presence of OA and GPP did not yield in CBGA or 2-*O*-GOA formation (Supporting Fig. S2).

3.3. Fusion of NphB and THCAS

We showed that yeast extracts expressing *nphB* catalyze the prenylation of OA by GPP to form CBGA, the substrate of the THCAS to form THCA. Based on these results we tried to express both enzymes simultaneously in *S. cerevisiae*. In a first approach, separation of the two coding sequences of THCAS and NphB was achieved by a viral T2A sequence that enables the eukaryotic expression of both enzymes driven by a single promoter (Beekwilder et al., 2014). The construct was designed with upstream *thcas* and downstream *nphB* as it is unknown whether the function of the N-terminal leader peptide of THCAS would be negatively affected by an additional proline upon co-translational cleavage of the T2A peptide sequence.

The yeast strain SC was transformed with pDionysos_TT2AN and cultivated for 168 h. Samples for OD_{600} measurement and activity assays were taken every 24 h. Product formation was analyzed after supplementing OA and GPP to the cell lysates. Results depicted in Fig. 4 and Supporting Fig. S3 show production of CBGA and 2-*O*-GOA but no THCA was formed. The low CBGA production rate of 2.5 pmol L^{-1} OD^{-1} h^{-1} indicates that the CBGA concentration might not be sufficiently high for a subsequent oxidative cyclization by THCAS. Enzyme activities of SC_N lysates supplemented with OA and GPP indicate that expression rates of separately expressed *nphB* is higher than co-expressed with *thcas* (Fig. 4). The highest CBGA production rate of SC_N was detectable 144 h after induction (12 pmol L^{-1} OD^{-1} h^{-1}, Fig. 4). The highest possible THCA production rate of SC_TT2AN lysates supplemented with CBGA was detectable 120 h after induction (126 pmol L^{-1} OD^{-1} h^{-1}, Fig. 4) whereas the highest THCA production rate of SC_T was already reached 96 h after induction (427 pmol L^{-1} OD^{-1} h^{-1}, Supporting Fig. S4).

The expression of *nphB* and *thcas* driven by a single *Gal1* promoter did not result in THCA formation. On this account we tested the bidirectional Gal10/Gal1 promoter system of yeast since the repeated application of identical promoter sequences in the same system might lead to instability of constructs caused by the highly active homologous recombination machinery of yeast (Siddiqui et al., 2012). The construct pDio2_THCAS was cloned in order to test whether *thcas* is functionally expressed under pGal10. The yeast strain SC was transformed with the desired plasmid and cultivated for 168 h. Samples for OD_{600} measurement and activity assay were taken every 24 h. Enzyme activities of SC_T2 lysates supplemented with CBGA show that the highest THCA production rate was obtained 72 h after induction (272 pmol L^{-1} OD^{-1} h^{-1} (Supporting Fig. S4). A comparison of SC_T and SC_T2 indicates that *thcas* expressed using pGal1 is more active than *thcas* expressed under pGal10. THCAS expression under pGal1 resulted, 72 h after induction, in a THCA production rate of 348 pmol L^{-1} OD^{-1} h^{-1}.

Based on the results we decided to use pGal1 for *nphB* expression.

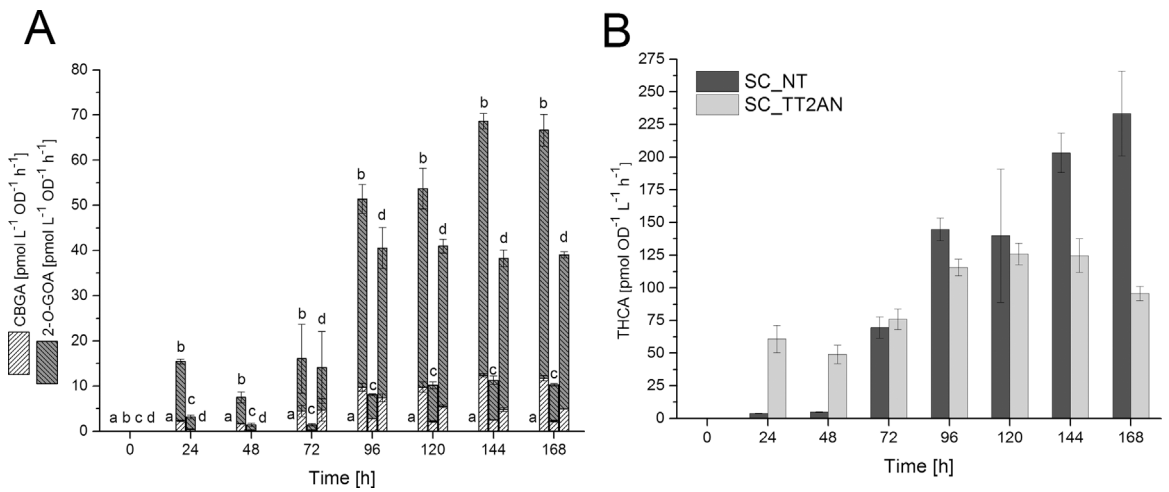

Fig. 4. Shaking flask cultivation of *S. cerevisiae* cells and analysis of product formation. (A) Comparison of cells expressing only *thcas* (SC_T, (a)), only *nphB* (SC_N, (b)) or *nphB* and *thcas* ((SC_TT2AN, (c)), (SC_NT, (d)); (B) THCAS activities in (c) and (d); Expression cultures were inoculated at OD_{600} of 0.5 and incubated for 168 h at 15 °C, 200 rpm in 1 L baffled shaking flasks (10% filling volume); cell lysate supernatants were incubated with either 1 mM OA, 1 mM GPP, 5 mM $MgCl_2$ at 37 °C for 4 h to determine CBGA, 2-*O*-GOA and THCA formation (A) or with 0.3 mM CBGA at 37 °C for 4 h to determine the highest possible THCA production under the tested conditions (B); assays were analyzed via HPLC–MS and product formation normalized on cell culture OD_{600}, cell culture volume and assay incubation time. Data points are calculated from biological triplicates each analyzed in technical duplicates.

The yeast strain SC was transformed with pDio2_NT and cultivated for 168 h. Samples for OD_{600} measurements and activity assays were taken every 24 h. Product formation was analyzed after supplementing OA and GPP to the cell lysates. Results shown in Fig. 4 indicate that CBGA and 2-*O*-GOA were formed, but no THCA. The highest CBGA production rate was obtained 96 h after induction (8 pmol L^{-1} OD^{-1} h^{-1}, Fig. 4). The formation of 2-*O*-GOA is constant between 96 and 168 h after induction (Supporting Fig. S5). The highest possible THCA production rate of SC_NT lysates supplemented with CBGA was detectable 168 h after induction (233 pmol L^{-1} OD^{-1} h^{-1}, Fig. 4).

Neither expression of *thcas* and *nphB* driven by the same promoter nor the expression of both enzymes using a bidirectional Gal10/Gal1 promoter system led to formation of sufficient amounts of CBGA which could serve as substrate for THCAS. To test if higher expression levels of *nphB* and *thcas* will lead to the formation of THCA we switched to *K. phaffii* as expression host.

3.4. Screening of different K. phaffii strains for highest cannabinoid formation

In order to improve *nphB* expression we tried to make use of *K. phaffii*'s ability to express proteins at high levels upon multi-copy integration of the coding sequences into its genome. Therefore we used the previously reported strain PP2_HC (Zirpel et al., 2015) which is already able to functionally produce THCAS intracellularly at high levels and transformed it with high amounts of linearized vector DNA pAX_NphB containing the coding sequence of *nphB*. Subsequently, 24 transformants were checked via colony PCR for successful integration of the vector DNA and screened in a 48-well format for their product formation capabilities (CBGA, 2-*G*-GOA, THCA) upon supplementation of OA and GPP to the cell lysates (Fig. 5). The results indicate a high phenotypic variety supposedly due to varying copy numbers of pAX_NphB leading to product formations from 20 to 280 pmol L^{-1} OD^{-1} h^{-1}. For a more detailed elucidation of THCA production capabilities of *K. phaffii*, clone C23 (high THCA production) and clone C1 (low THCA production) were cultivated at a larger scale in a shaking flask experiment.

3.5. Time dependent expression in K. phaffii

K. phaffii clones C1 and C23 expressing both *nphB* and *thcas* as well as control strains expressing only one of the enzymes were incubated in shaking flasks to investigate enzyme activities over time (Fig. 6).

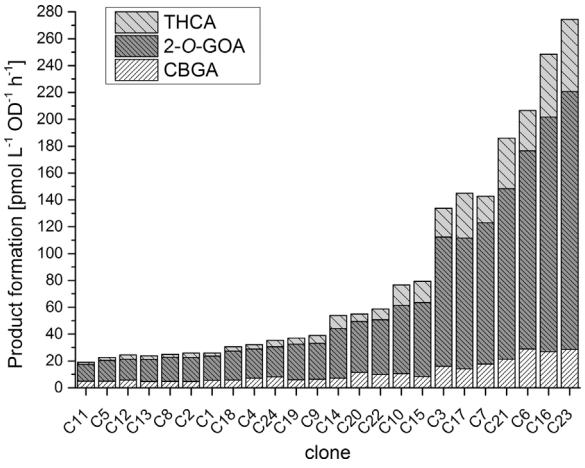

Fig. 5. Screening of 24 different *K. phaffii* transformants expressing *nphB* and *thcas* (PP2_NT). Expression cultures were grown for 32 h at 28 °C in deep-well plates; cell lysate supernatants were incubated with 1 mM OA, 1 mM GPP, 5 mM $MgCl_2$ at 37 °C for 4 h; assays were analyzed via HPLC–MS and product formation normalized on cell culture OD_{600}, cell culture volume and assay incubation time.

Cultivation conditions were adapted to optimal *thcas* expression conditions as reported before (Zirpel et al., 2015). As expected, NphB was functionally expressed and accumulation of CBGA and 2-*O*-GOA occurred in lysates of cells lacking THCAS (PP2_N, Fig. 7). THCA is only produced from GPP and OA in strains expressing both *nphB* and *thcas* (PP2_NT). Furthermore, the side-product 2-*O*-GOA is not accepted as a substrate by the THCAS. Finally, the performed screening for strains with higher NphB activities yielded a strain with a 5-fold increased production rate. PP2_NT C23 was able to produce 615 pmol L^{-1} OD^{-1} h^{-1} whereas clone C1 only produced 125 pmol L^{-1} OD^{-1} h^{-1} (Fig. 5). Similar results are obtained for the production of THCA which was 6-fold increased with clone C23 compared to C1.

4. Discussion

4.1. Replacement of the native plant prenyltransferase

In view of the difficulties of implementing an integral membrane protein, like CBGAS, into a heterologous production strain for cannabinoids we decided to replace CBGAS by NphB, a soluble

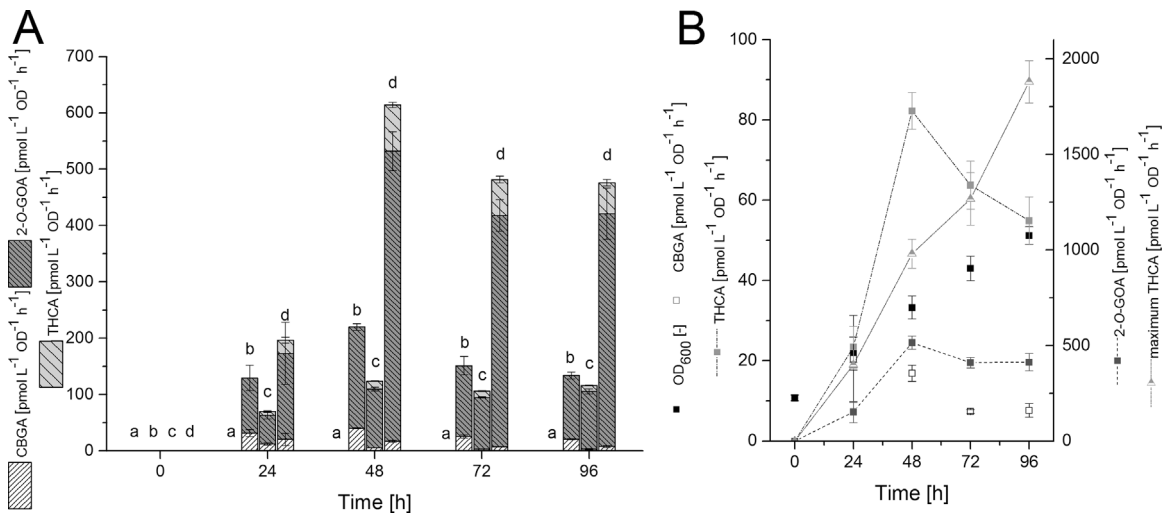

Fig. 6. Shaking flask cultivation of *K. phaffii* cells and analysis of product formation. (A) Comparison of cells expressing only *thcas* (PP2_HC, (a)), only *nphB* (PP2_N, (b)) or *nphB* and *thcas* (PP2_NT clone C1 (c), PP2_NT clone C23 (d)); (B) Cultivation of PP2_NT clone C23; expression cultures were inoculated at OD_{600} of 10 and incubated for 96 h at 15 °C, 160 rpm in 500 mL baffled shaking flasks (10% filling volume); cell lysate supernatants were incubated with either 1 mM OA, 1 mM GPP, 5 mM $MgCl_2$ at 37 °C for 4 h to determine CBGA, 2-*O*-GOA and THCA formation or with 0.3 mM CBGA at 37 °C for 4 h to determine the highest possible THCA production under the tested conditions; assays were analyzed via HPLC–MS and product formation normalized on cell culture OD_{600}, cell culture volume and assay incubation time. Data points are calculated from biological triplicates each analyzed in technical duplicates.

prenyltransferase from *Streptomyces* sp. strain CL190. It was described previously that OA might serve as prenyl acceptor molecule for NphB (Kuzuyama et al., 2005). Here we show for the first time that NphB is able to catalyze the formation of CBGA. Nevertheless, a major side-product formation (~85%) was detected resulting in the formation of an *O*-prenylated product, 2-*O*-GOA (Figs. 2 and 8), which presents a bottleneck in the coupling of both *nphB* and *thcas* in the same host. In future, a rational protein engineering approach based on the crystal structure of NphB together with homology modelling and substrate docking studies might enable the improvement of the specificity towards CBGA and higher specific activities.

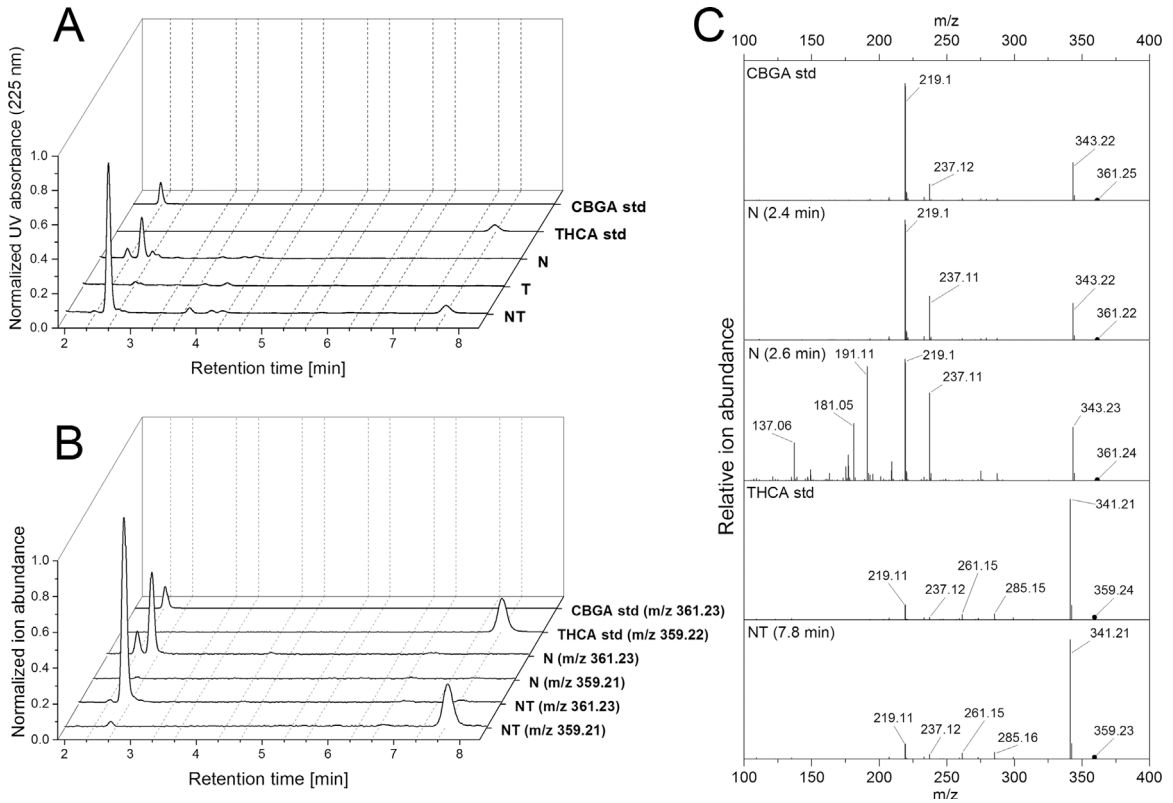

Fig. 7. LC–MS analysis of cannabinoids produced with extracts of *K. phaffii* expressing both *nphB* and/or *thcas*. The yeast extracts were incubated with 1 mM GPP and OA, respectively. (A) HPLC-UV chromatograms at 225 nm of the standards CBGA, THCA and the assay products. (B) Extracted ion chromatograms (EIC) of *m/z* 361.23 (CBGA) and *m/z* 359.21 (THCA) of the standards CBGA, THCA and the assay products. (C) Tandem mass spectra of CBGA and THCA standards and the product peaks of chromatograms in (A). 2-*O*-GOA (RT 2.6 min) was identified by NMR. All standards were also analyzed by NMR (Supplementary information). N − assay of lysed yeast expressing only NphB; T − assay of lysed yeast expressing only THCAS; NT − assay of lysed yeast expressing both NphB and THCAS.

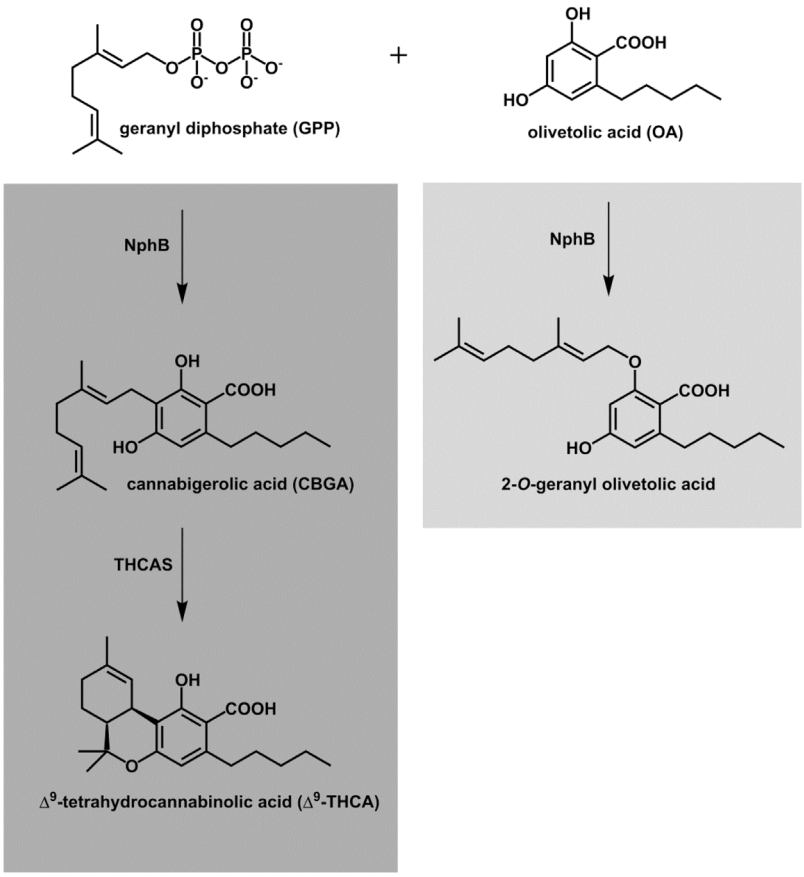

Fig. 8. Reconstitution of the final biosynthetic cannabinoid pathway of *C. sativa* in yeast. The main route resulting in THCA is dark grey shaded. The formation of the side product 2-*O*-GOA is highlighted in light grey. NphB – soluble prenyltransferase from a naphterpin biosynthetic gene cluster from *Streptomyces* sp. strain CL190, THCAS – tetrahydrocannabinolic acid synthase from *C. sativa*.

4.2. Expression capacity limitations of S. cerevisiae

S. cerevisiae is a well-established and well characterized host for the implementation of heterologous biosynthetic pathways. For example baker's yeast served as platform organism for the complete biosynthesis of opioids published by the group of Smolke (Galanie et al., 2015). Our studies show that *S. cerevisiae* is able to produce all expressed coding sequences in a functional and active manner but the expression levels are not sufficient to couple different enzyme activities in order to set up a whole new biosynthetic pathway. The first attempt to combine coding sequences of *thcas* and *nphB* separated by a T2A sequence under the control of a single promoter did not result in the formation of the final product THCA, although both enzymes were active. Based on these results we decided to use a bidirectional promoter system consisting of pGal1 and pGal10, which are both classified as strong inducible promoters in yeast (Partow et al., 2010). A comparison of the enzyme activities of SC_T and SC_T2 lysates indicated that pGal1 is a stronger promoter than pGal10. These results are also supported by West et al. (1987) and Cartwright et al. (1994). One reason that the two promoter strategy yields higher enzyme activities compared to the T2A-single-promoter system might be difficulties during cleavage of the T2A fusion proteins.

4.3. Reconstitution of the late cannabinoid pathway

Based on the results of the whole cell assay, yeast cell lysates have to be tested for activity rather than intact cells. However, taking the total biosynthetic pathway into consideration, whole cell catalysis might still be feasible upon expression of olivetolic acid cyclase (Gagne et al., 2012) and olivetol synthase (Taura et al., 2009) as well as GPP synthase (Marks et al., 2009) while feeding hexanoate/malonate.

Contrary to *S. cerevisiae*, *K. phaffii* was able to form THCA from OA and GPP (Supporting Fig. S5). The quantity of produced 2-*O*-GOA correlates with the produced intermediate CBGA and thus with the amount of functionally active NphB within the cell. The produced amounts of CBGA and 2-*O*-GOA of strain *K. phaffii* C19 (Fig. 5), based on activity measurements, are comparable to the ones in SC_NT (Fig. 4A, 168 h) and in strain C19 the produced amount of CBGA is sufficient for the formed amount of THCAS to catalyze the formation of THCA. Therefore, rather the enzyme expression level of *thcas* than *nphB* is the crucial factor that needs to be optimized in the first place. However, if baker's yeast should be used in future as platform organism for the reconstitution of the whole biosynthetic pathway both enzyme activities need to be improved by increasing the expression levels or the specific enzyme activities upon protein engineering.

In contrast, the *K. phaffii* results show (Fig. 6B) that at the tested conditions NphB activity levels are limiting the THCA production as the cell lysates after 48 h of cultivation contain enough THCAS to produce 34-fold more THCA (maximum THCA production in the THCAS assay compared to THCA production in NphB/THCAS-coupled assay after 96 h of cell growth) if NphB would supply enough CBGA as substrate. The highest specific production rates were obtained after 48 h of cultivation presumably due to no further increase in cytosolic *nphB* expression levels. However, we cannot rule out that substrates, intermediates and products are metabolized by other yeast enzymes. On the contrary, THCAS levels are still increasing over time (see Fig. 6B) which corresponds to the results reported previously (Zirpel et al., 2015). Consistent observations regarding maximum activities can be made for all strains expressing *nphB*. While THCAS is targeted into the vacuole in a strain lacking the vacuolar proteinase A, presumably facilitating accumulation of the protein in the vacuole, cytosolic NphB turnover might present an additional bottleneck for increased enzyme levels. Besides, we chose assay conditions for the coupled enzyme reaction which are more favorable for NphB rather than THCAS (pH 7.5, 37 °C). However, considering a whole metabolic process, a more acidic pH in the cytosol (Valli et al., 2005) might reduce the enzyme activity of

NphB within the cell. On the contrary, THCAS has a lower pH optimum at 4.5 that fits with the vacuolar localization of the enzyme in yeast. Additionally, substrate limitations that might occur as the result of the different compartmentation of the biosynthetic enzymes or the assumed low polarity of the substrates pose further challenges in the whole cell process.

Taken all this into consideration NphB levels have to be improved extensively. One excellent possibility offers the post-transformational vector amplification in *K. phaffii* that subsequently enables to increase gene copy numbers inside the cell (Aw and Polizzi, 2016). This might be even more important with regard to the complete biosynthesis of cannabinoids as it allows belated elimination of catalytic bottlenecks.

5. Conclusion

Our study shows that the coupling of both enzymes, NphB and THCAS, was successful using *K. phaffii* as expression host. While *K. phaffii* has so far mainly been used for excessive secreted protein production, purification and characterization, it proves its potential in metabolic engineering when linear secondary biosynthetic pathways should be integrated into a heterologous host. We effectively generated and screened gene multi-copy clones in a multi-well plate experiment and characterized the best expressing strain regarding its capability to produce THCA from OA and GPP. The cell lysate was able to produce $82 \pm 4.6\ \text{pmol}\ \text{L}^{-1}\ \text{OD}^{-1}\ \text{h}^{-1}$ THCA (Fig. 6A), but NphB activities were limiting in this strain and a considerable amount of side-product 2-*O*-GOA was produced. Overcoming NphB activity and product specificity issues by means of directed mutagenesis will be a major task, as − assuming the same expression and specific activity of a NphB mutant producing only CBGA − it would be possible to produce 34-fold more THCA within this yeast system conferring to THCA production rates in the hundred milligrams per liter and hour scale. While product titers and rates are still low in the generated heterologous systems, we hereby present a possibility to circumvent the troublesome expression of the integral membrane-bound CBGAS from *C. sativa* and demonstrate the potential for developing a sustainable and secure cannabinoid producing yeast platform.

6. Author contributions

F.S. and O.K. coordinated and supervised the study; C.M. synthesized GPP and supported analytical measurements; F.D. performed the studies in *S. cerevisiae;* B.Z. performed the studies in *K. phaffii*. All authors analyzed the data and contributed to write the manuscript.

Acknowledgments

The authors are thankful to the thesis students for their excellent help during the laboratory work: Julia Grühn, Martin Obholzer, Christian Staubach and Martin Kares. We would also like to thank Jan Schonert (CCB, TU Dortmund University, Germany) for performing NMR measurements. The CEN·PK2-1C strain was a kind gift from Peter Kötter (Goethe University Frankfurt, Germany). Studies were conducted with the permission of No. 4584989 issued by the Federal Institute for Drugs and Medical Devices (BfArM), Germany. Funding: B.Z. and F.D. were financially supported by the Ministry of Innovation, Science and Research of the German Federal State of North Rhine-Westphalia (NRW) and by TU Dortmund University through a scholarship from the CLIB-Graduate Cluster Industrial Biotechnology (CLIB^{2021}).

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.jbiotec.2017.07.008.

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