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Engineering yeasts as platform organisms for cannabinoid biosynthesis

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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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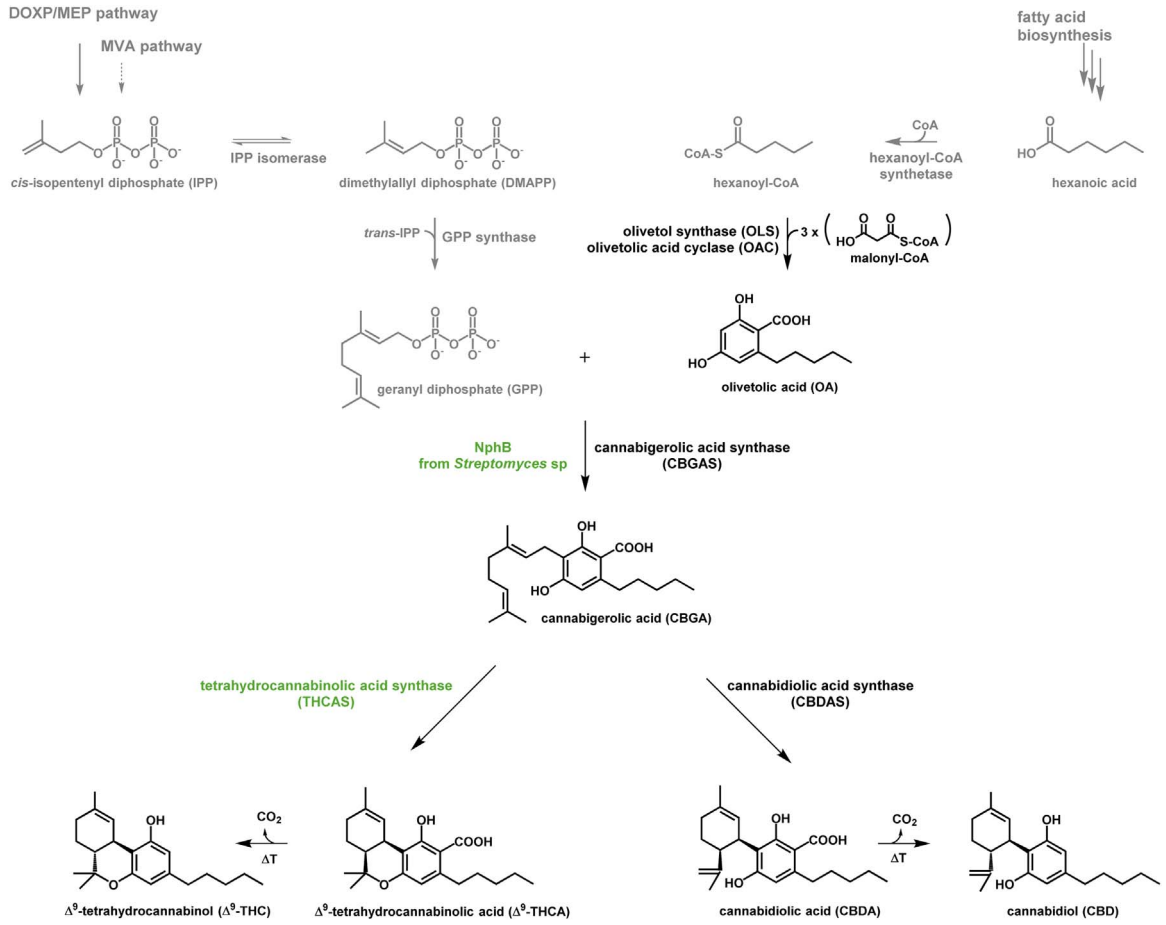

Fig. 1. Biosynthetic pathway of cannabinoids in *C. sativa.* The precursors geranyl diphosphate (GPP) and olivetolic acid (OA) are converted to the central intermediate of the cannabinoid pathway cannabigerolic acid (CBGA). Subsequently CBGA is further converted by two different oxidoreductases tetrahydrocannabinolic 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