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Optimization of Δ^9-tetrahydrocannabinolic acid synthase production in *Komagataella phaffii* via post-translational bottleneck identification

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ABSTRACT

Δ^9-Tetrahydrocannabinolic acid (THCA) is a secondary natural product from the plant *Cannabis sativa* L. with therapeutic indications like analgesics for cancer pain or reducing spasticity associated with multiple sclerosis. Here, we investigated the influence of the co-expression of 12 helper protein genes from *Komagataella phaffii* (formerly *Pichia pastoris*) on the functional expression of the Δ^9-tetrahydrocannabinolic acid synthase (THCAS) heterologously expressed in *K. phaffii* by screening 21 clones of each transformation. Our findings substantiate the necessity of a suitable screening system when interfering with the secretory network of *K. phaffii*. We found that co-production of the chaperones CNE1p and Kar2p, the foldase PDI1p, the UPR-activator Hac1p as well as the FAD synthetase FAD1p enhanced THCAS activity levels within the *K. phaffii* cells. The strongest influence showed co-expression of *Hac1s* - increasing the volumetric THCAS activities 4.1-fold on average. We also combined co-production of Hac1p with the other beneficial helper proteins to further enhance THCAS activity levels. An optimized strain overexpressing *Hac1s*, *FAD1* and *CNE1* was isolated that showed 20-fold increased volumetric, intracellular THCAS activity compared to the starting strain. We used this strain for a whole cell bioconversion of cannabigerolic acid (CBGA) to THCA. After 8 h of incubation at 37 °C, the cells produced 3.05 g L^{-1} THCA corresponding to 12.5% g_{THCA} $g_{CDW}{}^{-1}$.

1. Introduction

Cannabis sativa L. and its cannabinoids have gained increasing pharmaceutical importance in the last decades. Its most prominent cannabinoid, the major psychoactive Δ^9-tetrahydrocannabinol (THC), is nowadays used in several indications for treatment of chemotherapy-associated nausea and vomiting, AIDS-related loss of appetite as well as pain and muscle spasms in multiple sclerosis (Carlini, 2004; Pertwee, 2006). *In planta,* the acidic precursor of THC, namely Δ^9-tetrahydrocannabinolic acid (THCA), is produced by the Δ^9-tetrahydrocannabinolic acid synthase (THCAS) *via* oxidative cyclization from the precursor cannabigerolic acid (CBGA) and is decarboxylated upon heat induction. While THCA does not elicit psychoactive effects in humans, it is also currently examined for its immunomodulatory, anti-inflammatory, neuroprotective and anti-neoplastic effects (Moreno-Sanz, 2016).

Due to legal restrictions and drawbacks in chemical synthesis or agricultural production of cannabinoids for clinical use, researchers currently try to develop a controlled cannabinoid production platform in a heterologous host. Essential steps and recent advances towards development of such host were reviewed by Carvalho et al. (2017). The latest achievements and potentials of developing *Komagataella phaffii* (formerly *Pichia pastoris*) as a microorganism suitable for systems metabolic engineering have also been summarized (Schwarzhans et al., 2017). Additionally, the functional expression of *thcas* and *nphB* in *K. phaffii* has been reported as a major step towards this production in a heterologous host (Zirpel et al., 2017, 2015).

However, the THCAS possesses several structural features that might present obstacles in yielding high amounts of functionally active protein in the heterologous yeast. The enzyme contains a disulfide bond

Abbreviations: CBGA, cannabigerolic acid; THC, Δ^9-tetrahydrocannabinol; THCA, Δ^9-tetrahydrocannabinolic acid; THCAS, Δ^9-tetrahydrocannabinolic acid synthase; CNE1p, calnexin-like protein; PDI1p, protein disulfide isomerase; Ero1p, endoplasmic reticulum oxidoreductin 1; CPR5p, ER-resident peptidyl-prolyl-*cis-trans*-isomerase; Sec53p, phosphomannomutase; FAD, flavin adenine dinucleotide; FAD1p, FAD synthetase; FMN1p, riboflavin kinase; Yap1p, basic leucine zipper transcription factor; Kar2p, chaperone of the endoplasmic reticulum lumen; Lhs1p, chaperone of the endoplasmic reticulum lumen; Hac1p, basic leucine zipper transcription factor; *Hac1s*, spliced gene version of basic leucine zipper transcription factor; UPR, unfolded protein response; ROS, reactive oxygen species; GCN, gene copy number

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(C37, C99) and at least six *N*-glycosylation sites (N65, N89, N168, N329, N467, N499), as well as a bi-covalently attached flavin adenine dinucleotide (FAD) cofactor moiety (H114, C176) in its active site (Shoyama et al., 2012). Functional expression of *thcas* in *K. phaffii* was reported first by Taura et al. (2007) and experimental data indicates that THCAS is produced in a strongly glycosylated form, although glycosylation was necessary rather for correct folding than for functionality as the THCAS was still active after EndoH deglycosylation. Furthermore, addition of riboflavin to the cultivation medium yielded higher THCAS activities in the supernatant of the expression cultures, pointing to an insufficient FAD-cofactor pool (Taura et al., 2007). Higher volumetric THCAS activities were achieved when expression cultivation was performed at lower temperatures (10 °C–15 °C), again indicating folding issues in general. Compared to the wild-type strain not expressing the enzyme, a high degree of flocculation in the THCAS-overexpressing cells could be observed, which might be due to excessive generation of reactive oxygen species (ROS) upon excessive and/or incorrect protein folding (Delic et al., 2012; Zirpel et al., 2015).

We therefore wanted to identify bottlenecks during folding of the THCAS polypeptide chain by co-expressing different helper protein genes. There are many reviews about the impact of post-translational modifications of proteins, the involved helper proteins and the cell mechanisms on recombinant protein yields (Puxbaum et al., 2015; Schröder, 2008). An overview of the potential bottlenecks targeted in this study is shown in Fig. 1. After translocation of the nascent THCAS into the endoplasmic reticulum (ER), chaperones, co-chaperones and foldases prevent the polypeptide chain from aggregation and ensure proper folding. The ER-resident, essential Hsp70 chaperone Kar2p plays a major role by binding to hydrophobic patches of the unfolded polypeptide chains facilitating their transport through the ER lumen and preventing their agglomeration (Hale et al., 2010). The chaperone activity of Kar2p is regulated by its co-chaperones Sil1p and Lhs1p which also act as nucleotide exchange factors responsible for the regeneration of Kar2p ATPase cycles (Hale et al., 2010; Steel et al., 2004). The co-overexpression of Kar2p and Lhs1p has been successfully used to increase the levels of correctly folded recombinant proteins (Damasceno et al., 2007; de Ruijter et al., 2016). The chaperone CNE1p, the integral ER-membrane-bound calnexin-like protein, is responsible for correct folding and quality control of glycoproteins within the ER (Parlati et al., 1995). Co-expression of the respective *CNE1* was used to increase levels of a secreted target protein in *Hansenula polymorpha* as well as *K. phaffii* (Gu et al., 2015; Klabunde et al., 2007). Furthermore, foldases such as protein disulfide isomerase (PDI1p) and peptidyl-prolyl-*cis-trans* isomerase (PPIase/CPR5p) catalyze rate-limiting steps of protein folding. The major task of PDI1p is the correct arrangement of disulfide bonds of the polypeptide by an iterative cycle, but also a chaperone-like function can be assigned to the isomerase (Buck et al., 2007). PPIase catalyze the isomerization of peptidyl-prolyl bonds to facilitate folding of the peptide chain, however recently investigated PDI-PPIase interactions also substantiate the role in ER-chaperone/foldase partnerships. Co-overexpression of *PDI1* and *CPR5* has been utilized to enhance recombinant protein production in yeasts (de Ruijter et al., 2016; Inan et al., 2006). Likewise, a PDI1p-producing *K. phaffii* strain was used for the secretion of recombinant berberine-bridge-enzyme-like enzymes, which share high sequence and structural homology to the THCAS (Daniel et al., 2016). Another aspect to consider for THCAS production is the cofactor and precursor availability during processing of the nascent protein. Overproduction of Sec53p, a phosphomannomutase responsible for guanosine diphosphate mannose synthesis and involved in ER quality control, has been successfully realized previously (Gasser et al., 2007; Gu et al., 2015; Kepes and Schekman, 1988). The availability of bi-covalently bound cofactor FAD was investigated by co-overexpression of *FAD1*, coding for the FAD synthetase (FAD1p) which produces FAD from flavin mononucleotide (FMN) and co-overexpression of *FMN1*, the FMN synthase (FMN1p) which in return synthesizes FMN from riboflavin (Tu, 2000). While *FAD1* and *FMN1* are already upregulated in *K. phaffii* when growing on methanol, most of the FAD moieties presumably end up in the alcohol oxidase as well as endoplasmic thiol oxidase Ero1p (Rußmayer et al., 2015). However, recombinant protein overexpression is often encompassed by oxidative stress due to production of ROS as well as induction of the unfolded-protein-response (UPR). The regeneration of PDI1p during its iterative cycle of disulfide

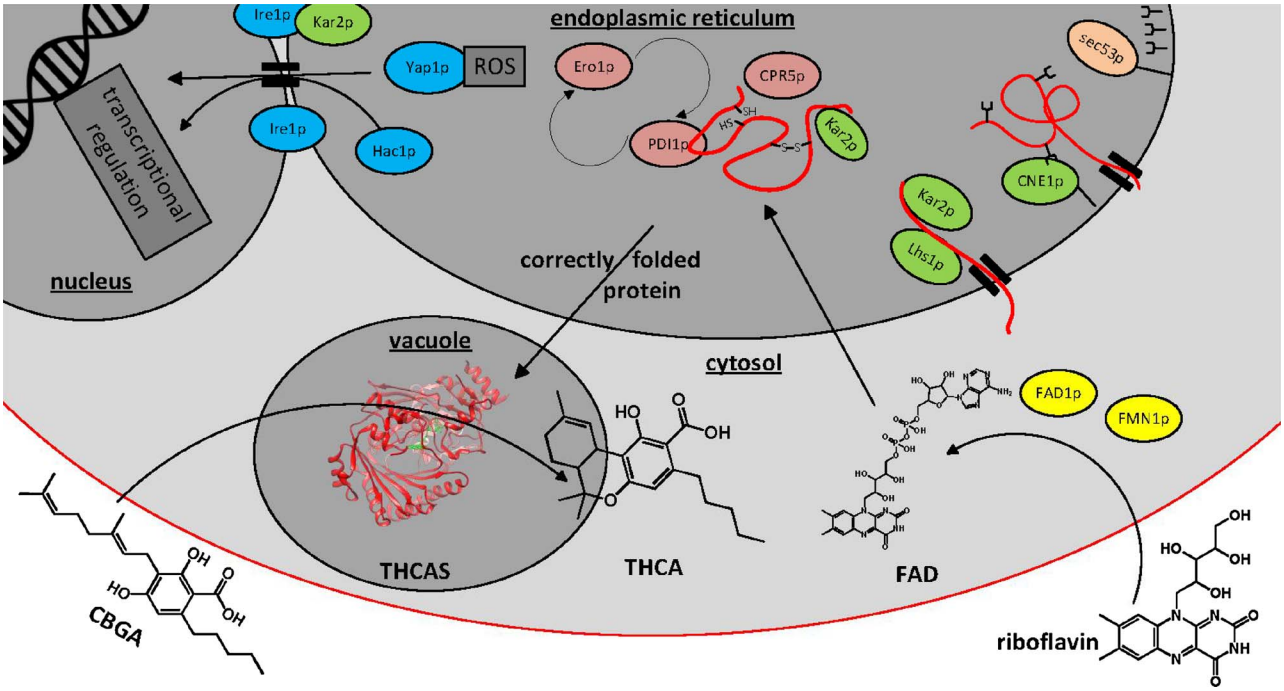

Fig. 1. Schematic overview of investigated bottlenecks during folding of functional THCAS. The THCAS (PDB: 3VTE) is targeted into the vacuole by using a PEP4 signal peptide (Zirpel et al., 2015). Proteins involved in folding and ER quality control (CNE1p, Lhs1p, Kar2p, PDIp, CPR5p, Ero1p), cofactor availability (FAD1p, FMN1p, Sec53p), reactive oxygen species (ROS) control (Yap1p) and unfolded protein response (Hac1p) were co-produced to enhance correctly folded THCAS. Riboflavin was added to the culture medium. After enzyme production, cells were used for a whole cell conversion of CBGA to THCA.