The identification of tetrahydrocannabinolic acid synthase in the non-aqueous secretions of the storage cavities from *Cannabis sativa* glandular trichomes

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INTRODUCTION

Cannabis sativa L. is an important herbaceous species cultivated since ancient times due to its unique medicinal and recreational properties, but also as a source of valuable seed oil and high quality fibre. Cannabinoids represent the unique class of terpenophenolics, which largely contribute to the pharmacological properties of this species. More than 100 cannabinoids is known, but Δ^9-tetrahydrocannabinolic acid (THCA), cannabidiolic acid (CBDA) and cannabichromenic acid (CBCA) are produced in the largest quantities in this plant (Fig. 1). Cannabinoids are synthesized in glandular trichomes present mainly on female flowers and their main reservoirs are storage cavities of these hair-like structures (Fig. 2) [1].

Tetrahydrocannabinolic acid synthase (THCA synthase) catalyses the unique oxidative cyclization of cannabigerolic acid (CBGA) into THCA (Fig. 1), which is a direct precursor of the mind-affecting compound Δ^9-tetrahydrocannabinol (THC). Thus, it is considered to be the key enzyme controlling the psychoactive properties of *Cannabis sativa.* The THCA synthase gene consists of a 1635-nucleotide open reading frame, encoding a 545-amino acid polypeptide and theoretical mass 62 kDa. However, the reported mass value obtained by SDS-PAGE of the purified enzyme was higher indicating that THCA synthase undergoes post-translational modifications, e.g. glycosylation. The first 28 amino acid residues constitute the signal peptide for which secretory pathway was predicted [2]. It has already been suggested that THCA synthase is secreted into storage cavities of glandular trichomes, where it might also synthesize the final product – THCA [3]. However, no direct evidence on protein level has yet been presented.

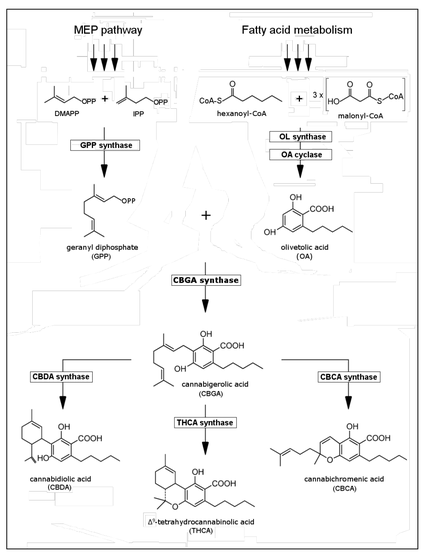

Fig. 1. The biosynthetic pathway of major cannabinoids. The precursors of cannabinoids originate from two distinct biosynthetic pathways: the polyketide pathway, giving rise to olivetolic acid (OA) and the plastidal 2-C-methyl-D-erythritol 4-phosphate (MEP) pathway, leading to the synthesis of geranyl diphosphate (GPP). DMAPP - dimethylallyl pyrophosphate, IPP - isopentenyl pyrophosphate

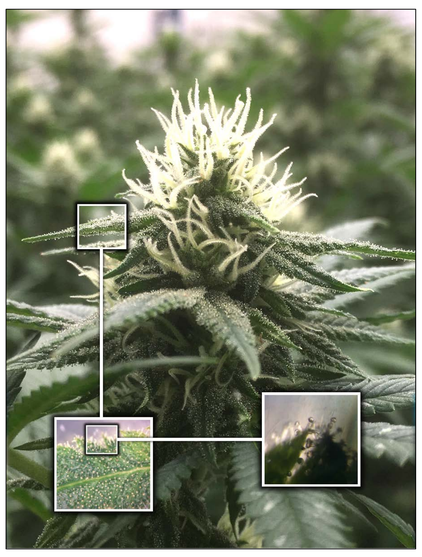

Fig. 2. Female cannabis plant at the 7th week of flowering stage. Leaves containing glandular trichomes (marked in squares) were used for experiments.

METHODS

Leaves containing glandular trichomes (Fig. 2) of drug type *Cannabis sativa* L. at the 7th week of flowering stage were used for experiments. Secretions from trichome storage cavities were extracted using thin capillary attached to the cell micromanipulator TransferMan 2 (Eppendorf, Germany) (Fig. 3). After microsuction of every 10 storage cavities the collected material was transferred to vials containing 1,5 ml of ice-cold acetone. Samples containing secretions obtained from 100, 200, 400, 800 trichomes were centrifuged (16 000 g, 10 min, 4° C) and the supernatant was removed. The remaining pellet was dissolved in 5 µl of SDS sample buffer and submitted for SDS PAGE (Fig. 4). The Coomassie-stained protein bands were excised and digested with trypsin following the protocol described by Shevchenko *et al.* [4]. After digestion peptides were extracted from the gel slices and subjected to nanoLC-MS/MS analysis using Orbitrap Elite™ Hybrid Ion Trap-Orbitrap Mass Spectrometer coupled to UltiMate™ 3000 RSLCnano System (Thermo Fisher Scientific, USA). Prior analysis a blank-MS-run was acquired to identify contaminations from the LC-column. Histone H3/H4 and elongation factor 1 were identified and removed from protein lists. Simultaneously, the sample containing secretions collected from 1000 storage cavities was submitted to western blot analysis using antibody specific for THCA synthase.

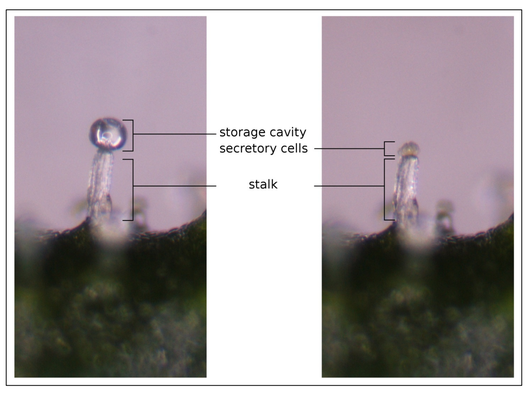

Fig. 3. A representative image of the trichome before and after microsuction using cell manipulator. The developed technique enabled precise extraction of oily secretions without disrupting secretory cells.

RESULTS

Due to detection limit of Coomassie staining, protein bands were visualized only for samples containing 400 and 800 secretions (Fig. 4). However, the THCA synthase was clearly identified by mass spectrometry only from the sample containing 800 trichomes. A few cellular proteins were concomitantly identified, but appeared to be of very low abundance (Tab. 1). To confirm the identification of THCA synthase in storage cavitory secretions western blot analysis was also applied. Using antibody specific for THCA synthase we were able to detect a signal below 70 kDa, corresponding to the protein band on the SDS gel identified as THCA synthase (Fig. 5).

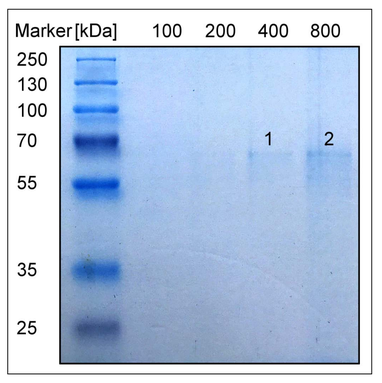

Fig. 4. SDS-PAGE gel of the samples prepared from 100, 200, 400 and 800 secretions of trichome storage cavities. Bands 1 and 2 were subjected to LC-MS/MS analysis.

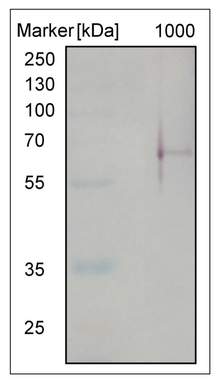

Fig. 5. Western blot analysis of the sample prepared from 1000 secretions of trichome storage cavities.

Tab. 1. List of proteins identified with at least two unique peptides from excised gel bands 1 and 2. THCA synthase was clearly identified only from the sample containing material extracted from 800 secretory cavities.

Protein name`	Organism	Score	Sequence coverage[%]	Unique peptides	Peptides	PSMs	MW [kDa]	Calc. pI	Accession
Tetrahydrocannabinolic acid synthase	***Cannabis sativa***	**1121**	**34**	**10**	**20**	**52**	**61.9**	**8.95**	**Q8GTB6**
(+)-alpha-pinene synthase, chloroplastic	*Cannabis sativa*	117	6	4	4	4	71.8	6.30	A7IZZ2
(-)-limonene synthase, chloroplastic	*Cannabis sativa*	87	6	3	3	3	72.3	6.92	A7IZZ1
NAD-dependent malic enzyme 1,	*A. thaliana*	66	3	2	2	2	69.6	5.45	Q9SIU0
Protein disulfide isomerase-like 1-1	*A. thaliana*	82	2	2	2	2	55.6	4.92	Q9XI01
ATP-citrate synthase beta chain protein 1	*Oryza sativa*	639	26	2	13	20	66.0	7.68	Q93VT8
Phosphoglycerate kinase, cytosolic	*N.tabacum*	206	10	2	3	4	42.3	5.97	Q42962

CONCLUSIONS AND FUTURE PROPSPECTS

In this study for the first time the extracellular presence of THCA synthase in the non-aqueous secretions of storage cavities from glandular trichomes was confirmed on the protein level both by mass spectrometry and western blot analysis. However, the identification of the enzyme was only possible when a large number of secretions was analyzed.

The subsequent experiments will concern monitoring of the THCA synthase abundance over flowering period and development of the assay tests to examine the THCA synthase activity in the non-aqueous environment. In the next steps we will also conduct similar analysis on the CBDA-rich non-drug type cannabis plants (hemp) to investigate the potential extracellular localization of the CBDA synthase.

REFERENCES

1. Andre, C.M., Hausman, J.-F., and Guerriero, G. (2016). Cannabis sativa: The Plant of the Thousand and One Molecules. Front. Plant Sci. 7.
2. Sirikantaramas, S., Morimoto, S., Shoyama, Y., Ishikawa, Y., Wada, Y., Shoyama, Y., and Taura, F. (2004). The Gene Controlling Marijuana Psychoactivity: MOLECULAR CLONING AND HETEROLOGOUS EXPRESSION OF Δ1 -TETRAHYDROCANNABINOLIC ACID SYNTHASE FROM CANNABIS SATIVA L. J. Biol. Chem. 279, 39767–39774.
3. Sirikantaramas, S. (2005). Tetrahydrocannabinolic Acid Synthase, the Enzyme Controlling Marijuana Psychoactivity, is Secreted into the Storage Cavity of the Glandular Trichomes. Plant Cell Physiol. 46, 1578–1582.
4. Shevchenko, A., Wilm, M., Vorm, O., and Mann, M. (1996). Mass Spectrometric Sequencing of Proteins from Silver-Stained Polyacrylamide Gels. Anal. Chem. 68, 850–858.