

ANALYSIS OF GENES FROM CANNABINOID BIOSYNTHETIC PATHWAY

STIASNA KLARA¹, PRESINSZKA MARIA¹, VYHNANEK TOMAS¹, TROJAN VACLAV¹, MRKVICOVA EVA², HRIVNA LUDEK³, HAVEL LADISLAV¹

¹Department of Plant Biology

²Department of Animal Nutrition and Forage Production

³Department of Food Technology

Mendel University in Brno

Zemedelska 1, 613 00 Brno

CZECH REPUBLIC

klara.stiasna@mendelu.cz

Abstract: Cannabis, or hemp, (*Cannabis sativa* L.) has been grown for thousands of years all around the world for its valuable traits in fabric making industry and traditional medicine. Today it is still considered as an important crop and medicinal plant. The most studied cannabinoids, secondary metabolites of genus *Cannabis*, are Δ -9-tetrahydrocannabinol (THC) and cannabidiol (CBD). Ratio between THC and CBD content is relevant marker in differentiation of “fiber-type” and “drug-type”. Biosynthesis of THC and CBD is catalyzed by enzymes tetrahydrocannabinolic acid synthase and cannabidiolic acid synthase. Sequence heterogeneity of genes encoding these enzymes in six varieties of industrial hemp, namely ‘Finola’, ‘Tiborszálási’, ‘Tisza’, ‘Kompolti’, ‘Kompolti hybrid TC’ and ‘Carmagnola’, was studied. Partial sequences of *cannabidiolic acid synthase* gene with numerous indels and single nucleotide polymorphisms were detected. Similar situation was observed in full-length *tetrahydrocannabinolic acid synthase* sequences. According to PCR marker, three tested varieties were indicated as potentially rich in THC content, what will be verified by HPLC in future.

Key Words: PCR, CBDA synthase, THCA synthase, *Cannabis* sp.

INTRODUCTION

Cannabis (*Cannabis sativa* L.) has been grown worldwide for thousands of years for its valuable properties, like fiber and oil content, and for medicinal purposes and as an intoxicant (Small, Cronquist 1976, Kojoma et al. 2005). Despite its negative reputation in recent years due to drug abuse, it still remains an extremely important agricultural crop plant, particularly as a source of fiber (Gilmore, Peakall 2002). For industrial purposes hemp, or “fiber-type” cannabis, with low or no Δ -9-tetrahydrocannabinol (THC) content and low THC : cannabidiol (CBD) ratio, is used, while “drug-type” marijuana with higher THC content and high THC : CBD ratio is used for its psychoactive potency (Alghanim, Almirall 2003, Staginnus et al. 2014).

Cannabinoids are terpenophenolic secondary metabolites produced in the sessile and stalked trichomes by cannabis plants. More than 100 cannabinoids have been discovered and studied until now. The most interesting and the most studied compounds of this class are THC and CBD (ElSohly, Slade 2005, Happyana et al. 2013, Onofri et al. 2015). The synthesis of cannabinoids is catalyzed by a series of synthase enzymes. The final step in previously mentioned cannabinoid synthesis consists in conversion of cannabigerolic acid (CBGA) into tetrahydrocannabinolic acid (THCA) by THCA synthase. THCA is then decarboxylated to THC. Alternatively, CBGA is converted into cannabidiolic acid (CBDA) by CBDA synthase followed by decarboxylation to CBD (Taura et al. 1995, Sirikantaramas et al. 2004, Rotherham, Harbison 2011).

The aim of this analysis was to study sequence heterogeneity of the key enzymes (THCA synthase and CBDA synthase) in cannabinoid biosynthetic pathway in hemp varieties primarily considered as industrial or “non-drug” type.

MATERIAL AND METHODS

Plant material and DNA extraction

Six varieties of industrial hemp of various proveniences were used in experiments (see Table 1). Samples originated from harvest year 2014 and were provided by Hempoint, Ltd., Czech Republic. All varieties were grown in fields in Jihlava, Czech Republic. Dried shredded leaves were used, except in case of ‘Carmagnola’ where DNA was extracted from the supplied seeds.

Total genomic DNA was isolated from 0.025 g of plant material, which was homogenized using mortar and pestle with addition of liquid nitrogen. DNA isolation was performed using DNeasy Plant Mini Kit (Qiagen, GE). Concentration and purity of obtained DNA was measured by spectrophotometer Picopet 1.0 (Picodrop, UK).

Table 1 Overview of analyzed varieties

Marking	Variety	Isolation matrix	Origin
TE1	Finola	dried leaves	Finland
TE2	Tiborszálási	dried leaves	Hungary
TE3	Tisza	dried leaves	Hungary
TE4	Kompolti	dried leaves	Hungary
TE5	Kompolti hybrid TC	dried leaves	Hungary
TE6	Carmagnola	seeds	Italy

PCR amplification

DNA was amplified using polymerase chain reaction (PCR) with specific primers (Table 2) for partial sequence of the gene *CBDA synthase* (Onofri et al. 2015), for partial sequence for gene *THCA synthase* (Staginnus et al. 2014) and complete sequence of the coding region of the gene *THCA synthase* (Kojoma et al. 2005).

PCR was performed in a total volume of 25 µl under conditions: *CBDA synthase* – preheating at 95°C for 10 min, 40 cycles at 95°C for 30 s, 57°C for 30 s, and 72°C for 1 min with a final extension at 72°C for 10 min; partial *THCA synthase* – 96°C for 2 min, then 35 cycles of 94°C for 20 s, 62°C for 30 s, 72°C for 1 min followed by final extension of 72°C for 5 min (Staginnus et al. 2014); complete *THCA synthase* – 95°C for 2 min, 30 cycles at 95°C for 30 s, 50°C for 30 s, 72°C for 2 min with a final extension at 72°C for 10 min (Kojoma et al. 2005).

Control electrophoresis in 1.5% agarose gel with Tris-acetate-EDTA buffer (TAE) stained with ethidium bromide was performed to verify presence and size of PCR products. Gels were photographed under UV light. PCR products were directly sequenced in Macrogen company (NL). Sequences were analyzed using BioEdit Sequence Alignment Editor 7.2.5 (Hall 1999).

Table 2 Primers used for sequencing of the *CBDA synthase* and *THCA synthase* genes

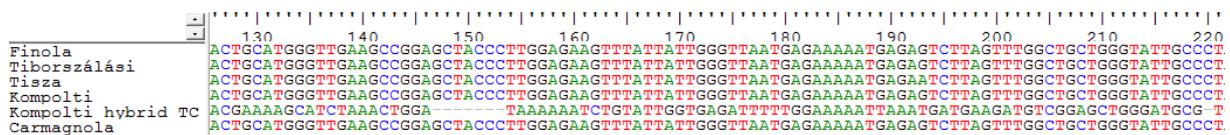
Primer	Gene	Sequence 5'-3'
CBDAS_F	partial <i>CBDA synthase</i>	AAGAAAGTTGGCTTGCAG
CBDAS_R		ATCCAGTTTAGATGCTTTTCGT
THCAS1_F	partial <i>THCA synthase</i>	CCTGAATTCGACAATACAAAATCTTAGATTCAT
THCAS1_R		ACTGAATATAGTAGACTTTGATGGGACAGCAACC
THCAS2_F	complete <i>THCA synthase</i>	TGAAGAAAAAAAATGAATTGCTCAGCATTTC
THCAS2_R		TCTATTTAAAGATAATTAATGATGATGCGGTGG

RESULTS AND DISCUSSION

Partial *CBDA synthase* sequences

Genomic DNA was amplified with *CBDA synthase* specific primer pair covering coding region of this gene and sequences with length slightly above 1 kb were obtained. Partial *CBDA synthase* sequences of all six tested genotypes were obtained and significant number of single nucleotide polymorphisms and indels were observed, in contrary to Onofri et al. (2015), who detected only little heterogeneity in *CBDA synthase* sequences. Especially in variety ‘Kompolti hybrid TC’, three-way-cross hybrid where two selections of Chinese origin ‘Kinai Kétlaki’ (dioecious) and ‘Kinai Egylaki’ (monoecious), and ‘Kompolti’ were combined. Also bands of PCR products from this variety were weaker than others on the agarose gel, what could be caused by less complementarity with used primers. The rest of studied varieties showed high percentage of identity 87.62–97.05%.

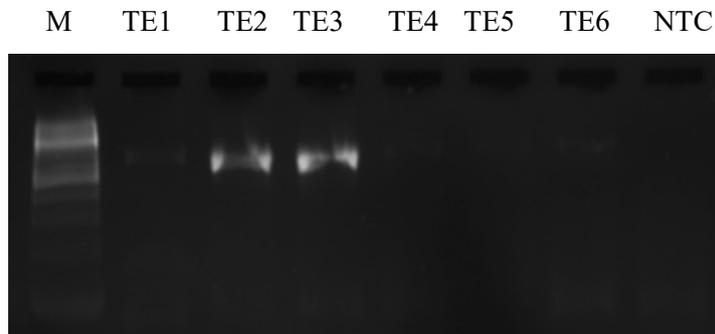
Figure 1 Demonstration of the partial *CBDA synthase* sequence with indels and single nucleotide polymorphisms



Partial and full-length *THCA synthase* sequences

Using primers designed by Staginnus et al. (2014), who developed a molecular method to discriminate potentially THC-rich plants, three partial *THCA synthase* sequences were obtained. Figure 2 shows electrophoresis of PCR products using these primers. It is possible to see PCR products for varieties ‘Tiborszálási’ and ‘Tisza’ and a weak PCR product for variety ‘Finola’. According to these authors, positive PCR product with expected length (589 bp) marks accessions with high THC-content. This statement will be verified by HPLC as the used varieties are supposed to be “fiber-type”, i.e. the THC content should be below 0.2% in the European Union (Mechtler et al. 2004).

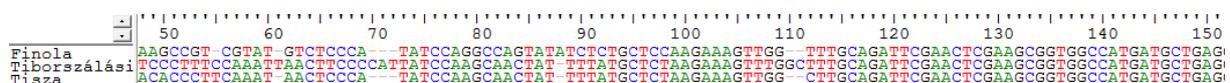
Figure 2 Detection of PCR products of the *THCA synthase* gene



Legend: M –size marker, TE1–Finola, TE2–Tiborszálási, TE3–Tisza, TE4–Kompolti, TE5–Kompolti hybrid TC, TE6–Carmagnola, NTC–negative template control

In Figure 3 it is possible to see section of the partial *THCA synthase* gene. Only a few single nucleotide polymorphisms and indels were detected in variety ‘Finola’, varieties ‘Tiborszálási’ and ‘Tisza’ were identical.

Figure 3 Demonstration of the partial *THCA synthase* sequence with indels and single nucleotide polymorphisms



Full-length sequences for five samples up to 1.6 kb were gained by sequencing the PCR products, sequencing of the sixth variety ‘Finola’ was not successful, using primers by Kojoma et al. (2005), which were designed to cover the whole coding region. Variety ‘Tisza’ showed large sequence divergence including several indels comparing to the rest of tested varieties (Figure 4).

Yoshikai et al. (2001) claims that *CBDA synthase* gene and *THCA synthase* gene are very similar with homology 87.9% which corresponds to results observed in our study.

Figure 4 Demonstration of the full-length THCA synthase sequence with indels and single nucleotide polymorphisms

	300	310	320	330	340	350	360	370	380	390																																																																																																																																																																																																																																		
Tiborszálási	A	C	T	A	T	T	T	A	T	G	C	T	C	T	A	A	G	A	A	A	G	G	T	T	G	G	C	T	T	G	C	A	G	A	T	T	C	G	A	A	C	T	C	G	A	A	G	C	G	G	T	G	G	C	C	A	T	G	A	T	G	C	T	G	A	G	G	G	T	A	T	G	T	C	C	T	A	C	A	T	T	T	C	T	C	A	A	C	T	C	C	C	A	T	T	C	C	C	A	A	G	A	A	A	A	G	T	T	G	G	T	T	G	C	A	A	A	T	T	C	C	A	A	G	C	G	G	G	G	C	C	T	G	A	T	G	C	T	G	A	G	G	G	T	T	G	T	C	C	T	A	C	A	T	T	T	C	T	C	A	A	G	T	T	C	C	C	A	T	T	C	T	G	G	C	T	C	C	A	A	G	A	A	A	G	T	T	G	G	T	T	G	C	A	A	A	T	T	C	C	A	A	G	C	G	G	G	C	C	T	G	A	T	G	C	T	G	A	G	G	G	T	T	G	T	C	C	T	A	C	A	T	T	T	C	T
Tisza	A	G	T	A	T	T	C	T	C	T	G	G	C	T	C	C	A	A	G	A	A	A	G	T	T	G	G	T	T	G	C	A	A	A	T	T	C	C	A	A	G	C	G	G	G	G	C	C	T	G	A	T	G	C	T	G	A	G	G	G	T	T	G	T	C	C	T	A	C	A	T	T	T	C	T																																																																																																																																																																	
Kompolti	A	G	T	A	T	T	C	T	C	T	G	G	C	T	C	C	A	A	G	A	A	A	G	T	T	G	G	T	T	G	C	A	A	A	T	T	C	C	A	A	G	C	G	G	G	C	C	T	G	A	T	G	C	T	G	A	G	G	G	T	T	G	T	C	C	T	A	C	A	T	T	T	C	T																																																																																																																																																																		
Kompolti hybrid TC	A	G	T	A	T	T	C	T	C	T	G	G	C	T	C	C	A	A	G	A	A	A	G	T	T	G	G	T	T	G	C	A	A	A	T	T	C	C	A	A	G	C	G	G	G	C	C	T	G	A	T	G	C	T	G	A	G	G	G	T	T	G	T	C	C	T	A	C	A	T	T	T	C	T																																																																																																																																																																		
Carmagnola	A	G	T	A	T	T	C	T	C	T	G	G	C	T	C	C	A	A	G	A	A	A	G	T	T	G	G	T	T	G	C	A	A	A	T	T	C	C	A	A	G	C	G	G	G	C	C	T	G	A	T	G	C	T	G	A	G	G	G	T	T	G	T	C	C	T	A	C	A	T	T	T	C	T																																																																																																																																																																		

CONCLUSION

Six partial *CBDA synthase* sequences were gained using direct sequencing of PCR products. Significant differences between ‘Kompolti hybrid TC’ and the rest of tested varieties were observed. Using the same method, five full-length THCA synthase sequences were obtained with major divergence in variety ‘Tisza’.

PCR markers for differentiation of the “drug-type” and “fiber-type” cannabis were tested. Three varieties of industrial hemp were marked as potentially rich in THC content. These results will be verified in HPLC.

In future, quantitative PCR will be performed to determine expression of genes involved in biosynthetic pathway of cannabinoids in various “drug-type” and “fiber-type” varieties. Assays with male-specific markers will be performed to identify sex of the plants with molecular biology approaches.

ACKNOWLEDGEMENT

This research was financially supported by the IGA FA MENDELU No. TP 4/2015. The authors thank to Hana Gabrielová for providing research material and Ana Cunha for technical support.

REFERENCES

- Alghanim H. J., Almirall J. R. 2003. Development of Microsatellite Markers in *Cannabis sativa* for DNA Typing and Genetic Relatedness Analyses. *Analytical and Bioanalytical Chemistry*, 376(8): 1225–1233.
- Gilmore S., Peakall R. 2002. Isolation of Microsatellite Markers in *Cannabis sativa* L. (Marijuana). *Molecular Ecology Notes*, 3(1): 105–107.
- ElSohly M. A., Slade D. 2005. Chemical Constituents of Marijuana: The Complex Mixture of Natural Cannabinoids. *Life Sciences*, 78(5): 539–548.
- Hall T. A. 1999. BioEdit: A User-Friendly Biological Sequence Alignment Editor and Analysis Program for Windows 95/98/NT. *Nucleic Acids Symposium*, 41: 95–98.
- Happyana N., Agnolet S., Muntendam R., Van Dam A., Schneider B., Kayser O. 2013. Analysis of Cannabinoids in Laser-Microdissected Trichomes of Medicinal *Cannabis sativa* Using LCMS and Cryogenic NMR. *Phytochemistry*, 2013(87): 51–59.
- Kojoma M., Seki H., Yoshida S., Muranaka T. 2005. DNA Polymorphisms in the Tetrahydrocannabinolic Acid (THCA) Synthase Gene in “Drug-Type” and “Fiber-Type” *Cannabis sativa* L. *Forensic Science International*, 159(2006): 132–140.
- Mechtler K., Bailer J., de Hueber K. 2004. Variations of Δ^9 -THC Content in Single Plants of Hemp Varieties. *Industrial Crops & Products*, 19(1): 19–24.
- Onofri C., de Meijer E., Mandolino G. 2015. Sequence Heterogeneity of Cannabidiolic and Tetrahydrocannabinolic Acid-Synthase in *Cannabis sativa* L. and its Relationship with Chemical Phenotype. *Phytochemistry*, 2015(116): 57–68.
- Rotherham D., Harbison S. A. 2011. Differentiation of Drug and Non-Drug *Cannabis* Using a Single Nucleotide Polymorphism (SNP) Assay. *Forensic Science International*, 207(2011): 193–197.

- Sirikantaramas S., Morimoto S., Shoyama Y., Ishikawa Y., Wada Y., Shoyama Y., Taura F. 2004. The Gene Controlling Marijuana Psychoactivity. *Journal of Biological Chemistry*, 279(38): 39767–39774.
- Small E., Cronquist A. 1976. A Practical and Natural taxonomy for *Cannabis*. *Taxon*, 25(4): 405–435.
- Staginnus C., Zörntlein S., de Meijer E. 2014. A PCR Marker Linked to a THCA Synthase Polymorphism is a Reliable Tool to Discriminate Potentially THC-Rich Plants of *Cannabis sativa* L. *Journal of Forensics Sciences*, 59(4): 919–926.
- Taura F., Morimoto S., Shoyama Y., Mechoulam R. 1995. First Direct Evidence for the Mechanism of Δ^1 -tetrahydrocannabinolic Acid Biosynthesis. *Journal of the American Chemical Society*, 117(38): 9766–9767.
- Yoshikai K., Taura T., Morimoto S., Shoyama Y. 2001. DNA Encoding Cannabidiolate Synthase. Japanese patent JP 2000-78979A.