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A real-time PCR assay for the relative quantification of the tetrahydrocannabinolic acid (THCA) synthase gene in herbal *Cannabis* samples

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ABSTRACT

In this study, we wanted to investigate whether or not the tetrahydrocannabinolic acid (THCA) synthase gene, which codes for the enzyme involved in the biosynthesis of THCA, influences the production and storage of tetrahydrocannabinol (THC) in a dose-dependent manner. THCA is actually decarboxylated to produce THC, the main psychoactive component in the *Cannabis* plant.

Assuming as the research hypothesis a correlation between the gene copy number and the production of THC, gene quantification could be useful in forensics in order to complement or replace chemical analysis for the identification and classification of seized *Cannabis* samples, thus distinguishing the drug-type from the fibre-type varieties.

A real-time PCR assay for the relative quantification of the THCA synthase gene was then validated on *Cannabis* samples; some were seized from the illegal drug market and others were derived from experimental cultivation. In order to determine the gene copy number to compare high *vs.* low potency plants, we chose the ΔΔCt method for TaqMan reactions. The assay enabled single plants with zero, one, and two copies of the gene to be distinguished.

As a result of this first part of the research on the THCA synthase gene (the second part will cover a study of gene expression), we found no correlation between THCA synthase gene copy number and the content of THC in the herbal *Cannabis* samples tested.

1. Introduction

Cannabis sativa L. contains more than 420 chemical substances including at least 61 cannabinoids; the narcotic compounds [1] within (-9-tetrahydrocannabinol (THC) are responsible for the main psychoactive effects. Since the 1980s, the use of faster and more controllable methods of plant growth under optimal growing conditions in combination with the breeding of new high-performance varieties has resulted in increased yields of flower buds and increased levels of THC. Given that THC is thought to be directly derived from cannabigerolic acid (CBGA) via tetrahydrocannabinolic acid (THCA) in all *Cannabis* strains [2–5], and that the conversion into THCA is catalysed by the THCA synthase enzyme, in this study, we wanted to investigate whether or not the THCA synthase gene, which codes for the enzyme [6–9], influences the production and storage of THC in a dose-dependent manner.

This research hypothesis aimed to explore the genetic differences between high- and low-THC cannabis strains, starting from the idea of finding an alternative method to chemical analysis to examine forensic samples of *Cannabis* in order to determine the THC content. Assuming a correlation between the gene copy number and the production of THC, gene quantification could therefore be useful in forensics to distinguish the psychoactive power of seized *Cannabis* samples. In this study, a real-time PCR assay for the relative quantification of the gene copy number of the THCA synthase gene was validated on *Cannabis* samples received by our forensic laboratory.

This is the first of a two-part research study concerning the THCA synthase gene in *Cannabis*; the second part, concerning the study of THCA synthase gene expression by the reverse transcriptase polymerase chain reaction (RT-PCR) in real time, did not show, for similar classes of samples, a constant correlation between the gene copy number and the gene expression data.

2. Materials and methods

Genetic investigations using the real-time PCR technique were performed after the chemical analysis of 18 *Cannabis* samples (Table 1).

Each sample was from a single plant, nine of which were seized as marijuana from the illegal drug market and the others, which were dried, were obtained from the experimental cultivation of declared potency *Cannabis* variety seeds. The

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Table 1
The 18 single-plant samples tested by a real-time PCR assay.

ID	THC (%)	DNA		Origin
		ng/μl	260/280	
CS 1	23.3	662.1	1.57	Marijuana seizure
CS 2	0.3	559.7	1.61	Marijuana seizure
CS 3	4	254.4	1.52	Marijuana seizure
CS 4	15	650.9	1.80	Marijuana seizure
CS 5	20	311.1	1.68	Marijuana seizure
CS 6	20	722.9	1.86	Marijuana seizure
CS 7	11	917.2	1.76	Marijuana seizure
CS 8	14	986.0	1.77	Marijuana seizure
CS 9	16	778.0	1.69	Marijuana seizure
CE 10	2.4	361.0	1.52	Dried plant
CE 11	2.5	725.5	1.65	Dried plant
CE 12	0.7	1104.2	1.80	Dried plant
CE 13	20	1060.4	1.62	Dried plant
CE 14	4.9	1353.9	1.57	Dried plant
CE 15	3.1	2661.0	1.37	Dried plant
CE 16	0.6	878.5	1.72	Dried plant
CE 17	0.3	1142.7	1.65	Dried plant
CE 18	0.3	2306.6	1.37	Dried plant

experimental cultivation, authorised by the Ministry of Health, was carried out in a sunny place during the spring–summer 2007.

2.1. Chemical experiments

All solvents and chemicals were of analytical grade. Reference standard solutions of THC, CBD and CBN were obtained from Promochem Lgc (Teddington, UK) and α-colestane was obtained from Sigma–Aldrich (Seelze, Germany).

The samples were first prepared by grinding them into a fine powder using a mortar and pestle. Following this, 0.08–0.10 g of each sample was extracted using 4 ml of internal standard/extracting solution (1 mg/ml of α-colestane) at room temperature for 15 min, and then the extract was sonicated for about 10 min.

After this, a 1 μl aliquot of each extract was injected into the gas chromatograph coupled to a mass spectrometer (GC/MS). Gas chromatography coupled to mass spectrometry (GC/MS) analysis was performed using a ThermoElectron Focus gas chromatograph coupled to a quadrupole DSQ™.

Chromatographic separation was performed on a fused silica-capillary 30 m column with a 0.32 mm i.d. and a 0.25 μm film thickness (Zebron, Phenomenex, Torrance, CA, USA). The gas chromatography parameters were: an initial temperature of 100 °C, a first ramp with a 25 °C/min slope, leading to 250 °C with a 5 min hold time, a second ramp with a 10 °C/min slope, leading to 280 °C with a 3 min hold time. The inlet temperature was maintained at 250 °C and the MS transfer line at 270 °C. Detection was performed on positive ions in the range of 50–650 *m/z* and the THC results are shown in Table 1.

2.2. Genetic experiments

DNA was extracted using a modified CTAB (hexadecyl trimethyl-ammonium bromide) method [10] and its concentration and quality were estimated using a spectrophotometer (Biophotometer Eppendorf); the results are shown in Table 1.

In order to quantify the THCA synthase gene (our target gene) copy number, we used the endogenous control real-time PCR detection technique [11–13]. This approach uses a quantitatively constant endogenous control gene in all samples to be investigated [14] in order to normalise the target gene copy number tested [15,16].

Table 2
Primer and probe sequences specifically designed on the conservative region of the THCA synthase gene.

THCA forward	5′-TTT CAG AAT CAG CAA TTC-3′
THCA reverse	5′-CAG TGT ACC AAA GTT CAT AC-3′
THCA probe	5′-FAM-CCC TCA TCG AGC TGG AAT-TAMRA-3′

2.2.1. Target gene primers and probe design

We investigated a conservative region in the THCA synthase gene sequence in order to design the primers and probes to allow the amplification of all variants of the target gene.

A search of GenBank (http://www.ncbi.nlm.nih.gov) showed 21 available sequences for the THCA synthase gene (accession numbers: AB212829, AB212830, AB212831, AB212832, AB212833, AB212834, AB212835, AB212836, AB212837, AB212838, AB212839, AB212840, AB212841, AB183699, AB183700, AB183701, AB183702, AB183703, AB183704, AB183705). All these sequences are given in GenBank as coding sequences (CDS) and are therefore considered as sequences of functioning genes.

These sequences, aligned using Clustal-W software (available at http://www.ebi.ac.uk/Tools/clustalw2/index.html), revealed high nucleotide variability, except for one conservative region (Fig. 1) on which was designed the primer and probe sequences (Table 2) using Primer Express Software v 2.0 (Applied Biosystems, Foster City, CA), according to the conditions of the TaqMan® reaction [17].

2.2.2. Endogenous control gene primers and probe design

After excluding the existence of a suitable endogenous control gene specific for *Cannabis* by the GenBank and literature searches, we chose the sequence of the chalcone synthase gene that codes for a key enzyme in the biosynthesis of flavonoids for this study. These are ubiquitous compounds with many functions in plants (pigmentation of flowers, protection against UV light, plant pathogen defence, etc.). The chalcone synthase gene was judged to be suitable because of its constant copy number (generally in a single copy) in different plants [18–20].

The available sequence of chalcone synthase in GenBank (accession number AY082343) was for an mRNA; therefore, using a nucleotide Blast search (http://www.ncbi.nlm.nih.gov/blast/Blast.cgi) on the homologue sequence database, we found an intron (at 180 bp from ATG) in the genomic DNA region that was excluded from the preceding region.

Endogenous control gene primers and probes were then designed on the sequence following the 180 bp intron region using the cited Primer Express Software v 2.0 (Applied Biosystems); the primers and probe design process took into account the efficiency of the PCR reaction for the target as well as the endogenous control gene through the selection of similar features such as primer and probe length, amplicon size and annealing temperature (Table 3).

In order to check the chalcone synthase gene as the endogenous control in our *Cannabis* samples, we performed the following steps: RNase digestion of a final 10 μg/ml DNA solution (extracted samples: CS 1, CS 4, CS 5, CS 6) at 37 °C for 30 min to obtain a better quantification of DNA in the spectrophotometer; dilution of the DNA solutions to the same 50 μg/μl concentration; preparation of a real-time PCR reaction (10× HotMaster Taq Buffer with 25 mM Mg^{2+} (5Prime, Eppendorf), 0.2 mM dNTPs and 2.5 mM per primer, 1 U HotMaster Taq DNA Polymerase (5Prime, Eppendorf) and 50 ng of extracted DNA at a final volume of 20 μl; pre-incubation at 95 °C for 3 min, followed by 40 cycles of 95 °C for 30 s, 55 °C for 1 min, 72 °C for 1 min, and a subsequent final extension at 72 °C for 7 min).

Fig. 1. Conservative region (signed by asterisks) of the THCA synthase gene sequences from the GenBank after alignment. In green (in gray, for black and white print): specific region where primer and probe were designed. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)