Forensic Science International 231 (2013) 208–212

Contents lists available at SciVerse ScienceDirect

Forensic Science International

journal homepage: www.elsevier.com/locate/forsciint

Analysis of *THCA* synthase gene expression in cannabis: A preliminary study by real-time quantitative PCR

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ARTICLE INFO

Article history:
Received 16 September 2012
Received in revised form 9 April 2013
Accepted 19 May 2013
Available online 20 June 2013

Keywords:
Forensic science
Cannabis
Tetrahydrocannabinolic acid
Tetrahydrocannabinol
THCA synthase gene
Real-time PCR
Potency
RNA

ABSTRACT

In this paper we describe analyses performed by Real-Time Reverse-Transcriptase Polymerase Chain Reaction (real-time RT-PCR) on RNA of 12 samples, carried out for forensic purposes to investigate a correlation between tetrahydrocannabinol (THC) concentration in *Cannabis* and the tetrahydrocannabinol acid synthase (THCAS) gene expression.

Samples were obtained from an experimental cultivation of declared potency *Cannabis* variety seeds and from seizures. The Rubisco gene and the 26S ribosomal RNA gene were used as internal control genes for their constant expression and stability.

As results we found minor gene expression in samples from leaves of young plants.

Further, grouping results for cannabis samples with similar characteristics, we have found an increased relative expression in samples with the highest percentage of THC coming from seized sample and adult plants.

1. Introduction

Genetic research on cannabis (*Cannabis sativa*) that has been accomplished until now for forensic applications has been essentially focused on analytical tools to distinguish fibre-type from drug-type varieties and to establish genetic relationships between drug-type varieties. Seized cannabis samples have been studied by investigating DNA molecular markers, such as AFLP and STR that are commonly used in biological research for identification purposes [1–4].

Definitive conclusions characterised by high levels of reproducibility and reliability have not yet been reached on these genetic markers; there are indeed limitations resulting from innovative cultivation techniques and breeding selection procedures that create novel and more potent varieties [5,6].

New investigative approaches have been suggested by another line of genetic research on cannabis that identified the sequence of tetrahydrocannabinolic acid-synthase (THCA-synthase) enzyme [7,8], responsible for the production of THC from the CBG precursor. Following this line, in a previous study [9] we investigated whether the THCA synthase gene, which codes for the enzyme [7,8,10,11], influences the production and storage of THC in a dose-dependent manner. By setting up real-time quantitative PCR assays (ΔΔCt method) on 18 herbal cannabis samples, the relative quantification of the *THCAS* gene (which is present in a variable copy number from one to another cannabis plant) did not reveal a correlation between the gene copy number and the THC content [9].

We then decided (as second step of the research) to investigate the *THCAS* gene expression using the Real-Time Reverse-Transcriptase Polymerase Chain Reaction (real-time RT-PCR) to explore differences between high- and low-THC *Cannabis* strains and consequently variations in the psychoactive power of *Cannabis* plants.

We analysed in this study 12 samples among the 18 of the previous study and to compare the THCAS gene expression among samples we used as endogenous genes the Rubisco gene and 26S gene for their constant expression and stability.

As results we found minor gene expression in samples from leaves of young plants even if not grown under controlled experimental conditions.

Gene expression analyses have also been complemented by chemical analyses of main cannabinoids.

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http://dx.doi.org/10.1016/j.forsciint.2013.05.019

Table 1
The 12 single-plant samples tested by a real-time PCR assay. The percentage values of tetrahydrocannabinol (THC), cannabidiol (CBD) and cannabinol (CBN) were directly quantified using an internal standard. The percentage values of cannabigerol (CBG), cannabichromene (CBC) and olivetol were estimated as a ratio with THC. From the biosynthetic pathway: Olivetol → CBG → THC or CBD or CBC.

Sample ID (mg)	THC	CBD	CBN	CBG/THC (wt^{-1})	RNA		Origin
					ng/μL	$A_{260/280}$	
CS 7 P161 (26)	6.7	–	–	185.6 (7.13)	120.3	1.12	Marijuana seizure (leaves and tops)
CS 8 P162 (26)	6.4	–	–	708 (27.23)	219.4	1.31	Marijuana seizure (leaves and tops)
CS 9 P166 (26)	11.7	–	–	396 (15.23)	103.6	1.04	Marijuana seizure (leaves and tops)
CE 10 P10 (28.3)	2.41	0.05	0.3	47.8 (1.68)	402.5	1.87	Frozen young plant (leaves) indoor
CE 11 P7 (28)	2.53	1.16	0.16	28.2 (1.007)	714.1	1.92	Frozen young plant (leaves) indoor
CE 12 P2 (26.3)	0.74	0.7	0.07	0.0	514.0	1.91	Frozen young plant (leaves) indoor
CE 13 P170 (26)	5.5	17.5	2.3	240 (9.23)	235.3	1.81	Frozen adult plant (leaves and tops)
CE 14 P1 (23.6)	0.95	0.46	0.16	0.0	377.2	1.97	Frozen adult plant (leaves and tops)
CE 15 P5 (28.6)	0.72	0.17	0.22	5.4 (0.18)	225.8	1.68	Frozen adult plant (leaves and tops)
CE 16 P3 (23.6)	0.59	0.13	0.019	0.0	548.1	1.82	Frozen young plant (leaves) outdoor
CE 17 P4 (27.3)	0.26	0.07	0.02	0.0	602.5	1.95	Frozen young plant (leaves) outdoor
CE 18 P8 (26)	0.39	0.0	0.02	3.6 (0.16)	555.8	1.91	Frozen young plant (leaves) outdoor

2. Materials and methods

Gene expression investigations using real-time PCR were performed after the chemical analysis of 12 *Cannabis Sativa* samples, aliquots of which were intended for the *THCAS* gene expression study (Table 1). Each sample was from a single plant, three of which were seized as dried from the illegal drug market and the others, which were frozen at −20 °C, were obtained from an experimental cultivation of declared potency *Cannabis* variety seeds. The experimental cultivation, authorised by the Italian Ministry of Health, was carried out in a sunny place during the spring-summer period for the outdoor cultivation and in a grow box for indoor cultivation.

2.1. Chemical experiments

All solvents and chemicals were of analytical grade. Reference standard solutions of THC, CBD and CBN were obtained from PromochemLgc (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 a gas chromatograph (ThermoElectron Focus) coupled to a mass spectrometer (DSQ™quadrupole).

Chromatographic separation was performed on a fused silica-capillary 30-metre 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*. The results of chemical analyses are shown in Table 1.

2.2. Gene expression experiments

RNA was extracted using a SpectrumTMPlant Total RNA kit (Sigma–Aldrich) and its solution additionally purified by a DNA digestion with 5 U of Amplification Grade DNase I (Sigma–Aldrich). RNA concentration and quality were then estimated using a spectrophotometer (Biophotometer, Eppendorf). The results are shown in Table 1.

THCAS gene expression was analysed by real-time RT-PCR using a 'one-step' protocol [12] with a relative quantification data analysis (ΔΔCt method) [13] (Fig. 1).

Primers and probe sequences, shown in Table 2, were specifically designed to a conserved region of the *THCAS* gene as reported in a previous study [9].

In order to select one or more endogenous reference genes in *Cannabis sativa*, we took inspiration from the endogenous reference genes reported in several plant gene expression studies [14–20], thus evaluating the presence of similar genes in the *Cannabis sativa* genome through the consultation of the GenBank database (http://www.ncbi.nlm.nih.gov/genbank/). We finally selected the *Cannabis sativa rbcL* (*Rubisco*) gene (accession number: AJ402933) and the 26S rRNA gene (accession number: EU002151) [21].

Expression studies were performed for both genes.

Primers and probes for the endogenous reference genes were then designed using the Primer ExpressSoftware v2.0 (Applied Biosystems, Foster City, CA); the primer and probe design process took into account the efficiency of the PCR reaction for the target as well as the endogenous reference genes through the selection of similar features, such as primers and probe length, amplicon size and annealing temperature (Table 3).

Reaction mixture was prepared as follows: 10× HotMaster Taq Buffer with 25 mM Mg^{2+} (5Prime, Eppendorf), 0.2 mM dNTPs, 2.5 mM of each primer, 1 U HotMaster Taq DNA Polymerase (5Prime, Eppendorf) and 50 ng of extracted DNA at a final volume of 20 μL. Amplification reactions were performed on a GeneAmp PCR System 2700 thermal cycler (Applied Biosystems, Foster City, CA) as follows: pre-incubation at 95 °C for 3 min, then 40 cycles of 95 °C for 30 s, 55 °C for 1 min, 72 °C for 1 min, and a final extension at 72 °C for 7 min. The amplicons were then electrophoresed on 4% agarose gels and the band sizes resulted were all in expected dimensions. The optimisation of primer and probe concentrations was performed on the sample CS 7 by following the TaqMan® One-Step RT-PCR Master M ix protocol.

Real-time RT-PCR was performed using an ABI PRISM 7000 Sequence Detection System (Applied Biosystems, Foster City, CA), and reaction mixtures were set up as follows: 1× TaqMan Master Mix without AmpErase uracil-N-glycosylase (Applied Biosystems), 1× Multiscribe and RNase Inhibitor Mix, 50/300/900 nM of each primer in variable combinations, 250 nM of TaqMan probe, 100 ng of total extracted RNA at a final volume of 25 μL. The thermal cycling programme included: an initial incubation of 30 min at 48 °C, an AmpliTaq Gold activation step of 10 min at 95 °C, 40 cycles at 95 °C for 15 s and 55 °C for 1 min. Each PCR run contained a negative amplification control (without RNA template) and was performed twice.

The optimal *Rubisco* and 26S rRNA primer concentrations, obtained from the optimisation procedures, were 900 nM for each primer. The optimal *THCAS* primer and probe concentrations were 100 nM, 900 nM and 300 nM for probe, forward and

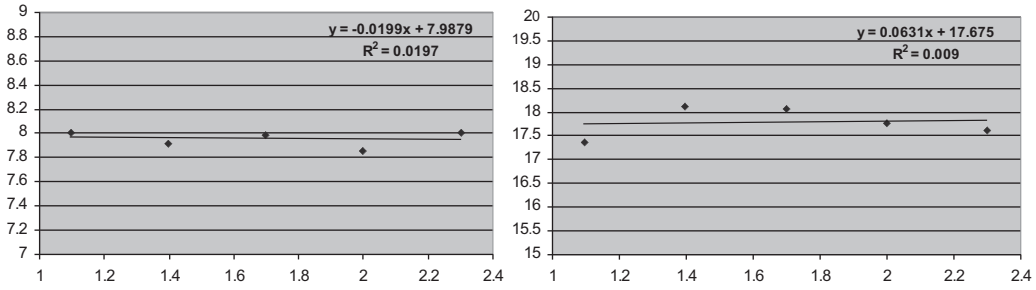

Fig. 1. Validation test: (a) *Rubisco*; (b) 26S.