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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.

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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, 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.)

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Table 3
Primer and probe sequences of the internal control gene (chalcone synthase).

Chalcone forward	5′-GAT TAC CAG CTC ACT AAG TT-3′
Chalcone reverse	5′-GAT ACA TCA TCA AAC GTT T-3′
Chalcone probe	5′-JOE-TTG GGC CTT AGA CCA TCA GT-TAMRA-3′

The constant copy number of this gene in *Cannabis* was then verified by real-time PCR whose theory states that a gene is present in the same amount in different samples when amplification curves start at about the same Ct having loaded the same quantity of DNA for the different samples.

The amplification curves showed that all of the examined DNA samples overlapped (approximately ±1 Ct), meaning that the same quantity (constant copy number) of the chalcone synthase gene was present in all of the tested *Cannabis* samples.

2.2.3. Optimisation of primer and probe concentrations for real-time PCR

After validation of primer amplificability on six randomly selected *Cannabis* samples, optimisation reactions to determine the appropriate primers and probe concentrations were performed using the manual TaqMan® Universal PCR Master Mix.

The PCR reaction solutions and conditions were the same for both the target and the endogenous gene. The machine amplifier was the Real-Time PCR 7000 Sequence Detection System (Applied Biosystems, Foster City, CA). The PCR analyses, performed twice for each primer couple concentration, included an internal negative control (without DNA). The reactions were carried out using the DNA template of sample CS 2.

The Ct values obtained for each combination of primer concentration showed that the best result for amplification was a final primer concentration of 300 nM for both the chalcone synthase and the THCA synthase genes.

Unsatisfactory results for the primer combinations in the multiplex reaction (which should have revealed a reduction of the ΔRn with no influence on the Ct obtained from the singleplex reactions) meant that we had to proceed with separate real-time amplifications.

These were finally performed with a 125 μM probe concentration using the following real-time PCR reaction: 2× TaqMan Universal PCR Master Mix (Applied Biosystems, Foster City, CA), 300 nM of each primer, 125 nM TaqMan probe and 50 ng extracted DNA at a final volume of 25 μl; for the cycle: 95 °C for 15 s, 55 °C for 1 min, for 35 cycles including 50 °C for 2 min for UNG and 95 °C for 10 min for AmpliTaq Gold activation.

2.2.4. Validation of amplification efficiency of the target and endogenous control genes

A validation test was then performed by progressively diluting the sample and monitoring variations in the ΔCt in the amplification reactions of the THCA and chalcone synthase primers and probes. The ΔCt values obtained were reported on a graph with the log of the different DNA dilutions (200, 100, 50, 25 and 12.5 ng/μl of DNA of the same sample CS 2) on the *x*-axis and the ΔCt values obtained on the *y*-axis, respectively. In order to use the ΔΔCt, the absolute value of the regression line should be <0.1 (User Bulletin #2, Applied Biosystems).

The PCR solutions and conditions were as indicated in the previous paragraph. The efficiency of the target (THCA) and endogenous (CHALC) genes were approximately the same, as shown in the graph (Fig. 2) by the straight angle = 0.0997, meaning that the ΔΔCt method could be used.

3. Results and discussion

This validation study on *Cannabis* was designed as the first part of a research project exploring the genetic differences between high- and low-THC *Cannabis* strains, and to find an analytical method other than a chemical assay to examine and classify samples seized on the illegal drug market.

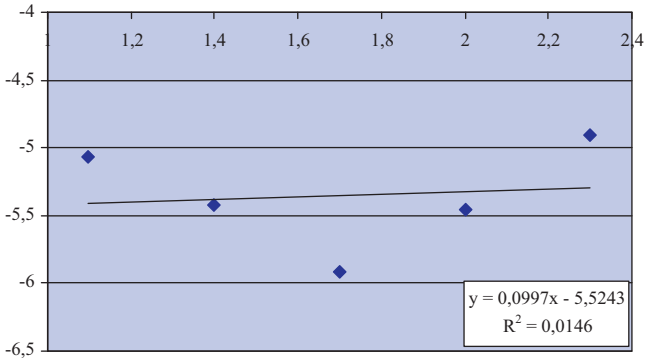

Fig. 2. Validation test.

Since the THCA synthase gene coding for the enzyme is involved in the synthesis of THC [6–9], we wanted to see if there might be a correlation between the number of copies of the enzyme gene and the amount of THC produced in the samples analysed. If so, this measurement could be useful in forensics to quantify the psychoactive power of seized samples based solely on genetic analysis, which does not suffer from, in contrast to chemical analysis, the degradation of THC due to aging or poor storage of the sample.

We then proceeded with the estimation of the copy number of the THCA synthase gene using the ΔΔCt method for the TaqMan reactions [21–23]. In this regard, all the THCA synthase gene sequences available in GeneBank were given as coding sequences (CDS) and were therefore considered as sequences of functioning genes coding for a catalytically active enzyme. To date, it is not known whether the genome of *Cannabis* contains other, non-functional (pseudogenes) THCA synthase genes; however, for the purpose of this first part of the research, this information does not affect the results of the study.

To allow for the amplification of all currently known 21 THCA synthase gene coding variants available in GeneBank we have chosen, to design primers and probes, a conservative region.

Aliquots of all 18 samples, each from a single plant (seized or experimental), were first analysed by the GC/MS technique to determine the THC content within the range of 0.4–23.3% (see Table 1).

The DNA extracted from each sample was run, in duplicate, in a singleplex TaqMan assay to compare the level of DNA in the samples to that of the endogenous control gene in order to normalise all reaction variations due to initial template DNA concentration differences. For the endogenous control gene, after excluding the existence of a suitable endogenous control gene specific for *Cannabis* by a GenBank and literature search, we selected the chalcone synthase sequence which is well conserved, constant and generally in a single copy in different plants [18–20]. In all 18 samples, amplification curves of the chalcone synthase gene started around the same Ct, meaning that this gene has a constant copy number and, whether if single or a multiple number, it is a good candidate to be used as a normaliser.

The THCA synthase gene copy number was then calculated for each sample by comparing each unknown sample to a known lowest-copy control sample included in each assay. The results obtained by the ΔΔCt method (Table 4 and the bar chart in Fig. 3) showed that most of the analysed samples presented a relative quantity of the THCA synthase gene that was quite constant, except for samples CS 2, CE 11 and CE 16, which showed a target gene copy number much higher than the others. The lowest values of the target gene (THCA synthase) copy number were observed in samples CS 1 and CS 5.

Quantification of the THCA synthase gene using the real-time PCR technique revealed a gene copy number that was independent of the chemical phenotype. Thus, the correlation between the quantity of the THCA synthase gene and the production of THC appeared to be random and variable in high and low potency varieties of *Cannabis*.

Samples CS 1 (23.3% of THC in the chemical analyses) and CS 5 (20% of THC), for instance, showed a relative quantity of THCA synthase that was clearly inferior with respect to the other samples; on the other hand, the low potency samples CE 11 (2.5% of THC), CE 16 (0.6% of THC) and CS 2 (0.3% THC) showed a gene copy number greater than most of the other samples.

These results conclusively excluded a correlation between the chemically tested THC content in our samples and the THCA synthase gene copy number, as demonstrated by the highest potency *Cannabis* samples, in particular.

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Table 4
Analyses of the 18 marijuana samples using the ΔΔCt method.

ID	THCA Mean Ct	CHALC Mean Ct	ΔCt THCA–CHALC[a]	ΔΔCt $\Delta Ct - \Delta Ct_{CS\ 5}$[b]	TCHA quantification relative to sample 5 (CS 5)[c]
CS 1	23.445 ± 0.030	24.600 ± 0.326	−1.155 ± 0.327377	−0.415 ± 0.327377	1.333 (1.063–1.673)
CS 2	19.875 ± 0.066	24.305 ± 0.161	−4.43 ± 0.174003	−3.69 ± 0.174003	12.906 (11.440–14.560)
CS 3	22.090 ± 0.041	25.175 ± 0.079	−3.085 ± 0.089006	−2.345 ± 0.089006	5.0806 (4.777–5.404)
CS 4	20.810 ± 0.011	24.315 ± 0.079	−3.505 ± 0.079762	−2.765 ± 0.079762	6.7975 (6.432–7.184)
CS 5	22.450 ± 0.012	23.190 ± 0.068	−0.74 ± 0.069051	0 ± 0.069051	1 (0.953–1.049)
CS 6	23.435 ± 0.518	25.470 ± 0.428	−2.035 ± 0.671943	−1.295 ± 0.671943	2.4538 (1.540–3.909)
CS 7	25.725 ± 0.021	28.460 ± 0.015	−2.735 ± 0.025807	−1.995 ± 0.025807	3.9862 (4.058107–3.91549)
CS 8	25.730 ± 0.030	28.710 ± 0.041	−2.98 ± 0.050804	−2.24 ± 0.050804	4.724 (4.893286–4.560514)
CS 9	25.845 ± 0.064	28.115 ± 0.165	−2.27 ± 0.176977	−1.53 ± 0.176977	2.8879 (3.264761–2.554468)
CE 10	24.145 ± 0.003	26.235 ± 0.077	−2.09 ± 0.077058	−1.35 ± 0.077058	2.5491 (2.688979–2.416538)
CE 11	22.680 ± 0.056	27.650 ± 0.075	−4.97 ± 0.0936	−4.23 ± 0.0936	18.765 (20.02319–17.58654)
CE 12	26.940 ± 0.327	29.525 ± 0.008	−2.585 ± 0.327098	−1.845 ± 0.327098	3.5925 (4.506783–2.863743)
CE 13	27.500 ± 0.015	30.495 ± 1.337	−2.995 ± 1.337084	−2.255 ± 1.337084	4.7733 (12.05938–1.889384)
CE 14	28.450 ± 0.892	31.060 ± 0.086	−2.61 ± 0.896136	−1.87 ± 0.896136	3.6553 (6.802835–1.964094)
CE 15	28.580 ± 0.253	31.295 ± 0.348	−2.715 ± 0.430248	−1.975 ± 0.430248	3.9313 (5.297265–2.91754)
CE 16	23.940 ± 0.410	29.440 ± 0.250	−5.5 ± 0.480208	−4.76 ± 0.480208	27.096 (37.79722–19.42431)
CE 17	26.665 ± 0.039	29.045 ± 0.008	−2.38 ± 0.039812	−1.64 ± 0.039812	3.1167 (3.203862–3.031828)
CE 18	26.855 ± 0.203	29.545 ± 0.260	−2.69 ± 0.329862	−1.95 ± 0.329862	3.8637 (4.856315–3.074044)

[a] ΔCt = CHALC Ct values (−) THCA Ct values. Standard deviation of the odds: $s = \sqrt{s_1^2 + s_2^2}$, where s = standard deviation; S_1 = standard deviation of THCA values; S_2 = standard deviation of CHALC.
[b] ΔΔCt = calibrator ΔCt (−) target ΔCt. ΔΔCt standard deviation = ΔCt standard deviation. In our case, the calibrator was the sample containing the lowest THCA gene copy number (ID CS 5).
[c] The relative (with respect to the calibrator sample ID 5) quantity of the THCA gene copy number was calculated by $2^{-\Delta\Delta Ct}$ with ΔΔCt + s and ΔΔCt − s, where s = standard deviation of the ΔΔCt value.

The fact that the quantity of the THCA synthase gene did not influence the production of THC by the plant could be explained by the different THCA synthase gene expressions through different regulation mechanisms (transcriptional, post-transcriptional, post-translational in *Cannabis* varieties with high *vs.* low potencies).

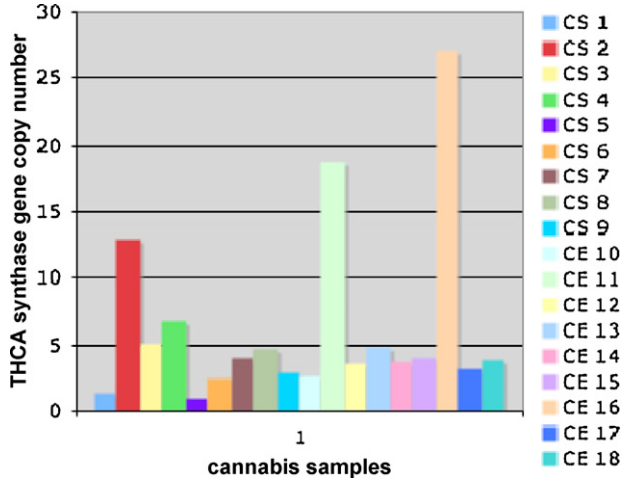

Fig. 3. Bar-chart showing the relative (with respect to the calibrator sample ID 5) quantity of the THCA gene copy number in all the 18 cannabis samples.

In the second part of this research, addressed to the study of expression of the THCA synthase gene by reverse-transcriptase RT-PCR, the RNA of 12 cannabis samples (from CS7 to CE18) was investigated using the Rubisco gene and the 26S ribosomal RNA gene as internal control genes for constant expression. The results did not show a definite correlation between the chemical concentration of THC and THCA synthase gene expression, even if the samples with the highest percentage of THC also showed increased relative expression; on the other hand, for similar classes of *Cannabis* samples, the expression of the THCA synthase have shown a correlation with the analysed part and with the maturity of the plant.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.forsciint.2011.10.041.

References

[1] C. Nora Chiang, G. Barnett, Marijuana pharmacokinetics and pharmacodynamics, in: Cocaine, Marijuana Designer Drugs: Chemistry, Pharmacology, and Behavior, CRC Press, Boca Raton, FL, 1989, pp. 113–125.
[2] E.P.M. de Meijer, M. Bagatta, A. Carboni, P. Crucitti, V.M.C. Moliterni, P. Ranalli, G. Mandolino, The inheritance of chemical phenotype in *Cannabis Sativa* L., Genetics 163 (2003) 335–346.
[3] G.A. Thakur, R.I. Duclos Jr., A. Makriyannis, Natural cannabinoids: templates for drug discovery, Life Sci. 78 (2005) 454–466.
[4] K.W. Hillig, P.G. Mahlberg, PG, A chemotaxonomic analysis of cannabinoids variation in Cannabis (Cannabaceae), Am. J. Bot. 91 (2004) 966–975.
[5] T.J. Raharjo, W.T. Chang, M.C. Verberne, A.M. Peltenburg-Looman, H.J. Linthorst, R. Verpoorte, Cloning and over-expression of a cDNA encoding a polyketide synthase from *Cannabis sativa*, Plant Physiol. Biochem. 42 (2004) 277–291.
[6] S. Sirikantaramas, S. Morimoto, Y. Shoyama, Y. Ishikawa, Y. Wada, Y. Shoyama, F. Taura, The gene controlling marijuana psychoactivity, J. Biol. Chem. 279 (2004) 39767–39774.
[7] S. Sirikantaramas, F. Taura, Y. Tanaka, Y. Ishikawa, S. Morimoto, Y. Shoyama, Tetrahydrocannabinolic acid synthase, the enzyme controlling marijuana psychoactivity, is secreted into the storage cavity of the glandular trichomes, Plant Cell Physiol. 46 (2005) 1578–1582.
[8] M. Kojoma, H. Seki, S. Yoshida, T. Muranaka, DNA polymorphisms in the tetrahydrocannabinolic acid (THCA) synthase gene in 'drug-type' and 'fiber-type' *Cannabis sativa* L., Forensic Sci. Int. 2 (2006) 132–140.
[9] F. Taura, E. Dono, S. Sirikantaramas, K. Yoshimura, Y. Shoyama, S. Morimoto, Production of Delta(1)-tetrahydrocannabinolic acid by the biosynthetic enzyme secreted from transgenic *Pichia pastoris*, Biochem. Biophys. Res. Commun. 28 (2007) 675–680.
[10] J.J. Doyle, J.L. Doyle, A rapid DNA isolation procedure for small quantities of fresh leaf tissue, Phytochem. Bull. 19 (1987) 11–15.
[11] A. Gentle, F. Anastasopoulos, N.A. McBrien, High resolution semi-quantitative real-time PCR without the use of a standard curve, Biotechniques 31 (2001) 502–550.
[12] L. Raeymaekers, Basic principles of quantitative. PCR, Mol. Biotechnol. 15 (2000) 115–122.
[13] P. Song, C.Q. Cai, M. Skokut, B.D. Kosegi, J.F. Petrolino, Quantitative real-time PCR as a screening tool for estimating transgene copy number in WHISKERS™-derived transgenic maize, Plant Cell Rep. 20 (2002) 948–954.
[14] B. Bubner, I.T. Baldwin, Use of real-time PCR for determining copy number and zygosity in transgenic plants, Plant Cell Rep. 23 (2004) 263–271.
[15] K.J. Livak, T.D. Schmittgen, Analysis of relative gene expression data using real-time quantitative PCR and the 2-ΔΔCt method, Methods 25 (2001) 402–408.
[16] M.A. Schmidt, W.A. Parrott, Quantitative detection of transgenes in soybean [*Glycine max* (L.) Merrill] and peanut (*Arachis hypogea* L.) by real-time polymerase chain reaction, Plant Cell Rep. 20 (2001) 422–428.
[17] K.J. Livak, S.A.J. Flood, J. Marmaro, W. Giusti, K. Deetz, Oligonucleotides with fluorescent dyes at opposite ends provide a quenched probe system useful for detecting PCR product and nucleic acid hybridization, PCR Methods Appl. 4 (1995) 357–362.
[18] A. Hermann, W. Schulz, K. Hahlbrock, Two alleles of the single-copy chalcone synthase gene in parsley differ by a transposon-like element, Mol. Gen. Genet. MGG 212 (1988) 93–98.
[19] R.L. Feinbaum, F.M. Ausubel, Transcriptional regulation of the Arabidopsis thaliana chalcone synthase gene, Mol. Cell. Biol. 8 (1988) 1985–1992.
[20] R.E. Koes, C.E. Spelt, J.N.M. Mil, A.G.M. Gerats, The chalcone synthase multigene family of *Petunia hybrida*: sequence homology, chromosomal localization and evolutionary aspects, Plant Mol. Biol. 10 (1987) 159–169.
[21] C.A. Heid, J. Stevens, K.J. Livak, et al., Real time quantitative PCR, Genome Res. 6 (1996) 986–994.
[22] J. Wilhelm, A. Pingoud, M. Hahn, Real-time PCR-based method for estimation of genome sizes, Nucleic Acids Res. 31 (2003) e56.
[23] D.J. Ingham, S. Beer, S. Money, G. Hansen, Quantitative real-time PCR assay for determining transgene copy number in transformed plants, Biotechniques 31 (2001) 132–140.

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