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TECHNICAL NOTE

CRIMINALISTICS

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Investigations into the Hypothesis of Transgenic Cannabis*

ABSTRACT: The unusual concentration of cannabinoids recently found in marijuana samples submitted to the forensic laboratory for chemical analysis prompted an investigation into whether genetic modifications have been made to the DNA of *Cannabis sativa* L. to increase its potency. Traditional methods for the detection of genetically modified organisms (GMO) were used to analyze herbal cannabis preparations. Our analyses support the hypothesis that marijuana samples submitted to forensic laboratories and characterized by an abnormal level of Δ^9-THC are the product of breeding selection rather than of transgenic modifications. Further, this research has shown a risk of false positive results associated with the poor quality of the seized samples and probably due to the contamination by other transgenic vegetable products. On the other hand, based on these data, a conclusive distinction between the hypothesis of GMO plant contamination and the other of genetic modification of cannabis cannot be made requiring further studies on comparative chemical and genetic analyses to find out an explanation for the recently detected increased potency of cannabis.

KEYWORDS: forensic science, cannabis, tetrahydrocannabinol, delta-9-tetrahydrocannabinol, marijuana, transgenic, potency

The amount of the main active constituent of *Cannabis sativa* L., Δ^9-tetrahydrocannabinol (Δ^9-THC), naturally depends on the influence that factors such as genotype, plant age, and environment have on the biosynthesis of cannabinoids, modulating the conversion of the precursor cannabigerol (CBG) into the three components: tetrahydrocannabinol (THC), cannabidiol (CBD), and cannabichrome (1–3).

Marijuana, the herbal form of cannabis, is traditionally known to have a lower level of Δ^9-THC (very rarely exceeding 5%) in comparison with other resin-type cannabis preparations such as hashish (generally up to 20%) and hash oil (sometimes even above 50%) (4–7).

Significantly higher THC concentrations in cannabis derivatives, particularly in marijuana, were recently documented in the literature (8,9) and repeatedly detected in our forensic laboratory analyses.

Since the 1990s, it has been noticed (10,11) that innovative and advanced cultivation techniques of *C. sativa* L. have increased the potency of marijuana, creating novel varieties such as, for instance, "sinsemilla" (from the Spanish "sin semilla"—without seeds—which is obtained from unpollinated female plants) and growing plants from selected seeds via intensive indoor methods to reach THC concentrations as high as 20–30%.

On the other hand, developments in genetic research over the few last decades have increased the technical feasibility of bioengineering the cannabis plant to increase its natural THC production levels. In fact, strategies (based on available genetic and molecular tools not yet exploited in this context) have been proposed (12) for bioengineering, the metabolism of plant glandular tissues specialized in the production and accumulation of chemicals. Δ^9-THC is a mixed type (terpenoid and polyketide) product of specific glandular tissues (the epidermale trichomes, which accumulate essential oils and resins) that is synthesized by *C. sativa* L. The hypothesis put forward and tested in this paper was that about the origin of high-potency cannabis from bioengineering processes.

The use of bioengineering techniques to increase either or both the tissue density of glandular trichomes and their metabolism could lead to significantly higher levels of THC without noticeably altering other characteristics of the product profile. Regarding the possibilities for metabolism modification, recent genetic research (13) has identified and cloned the tetrahydrocannabinolic acid-synthase (THCA-synthase) enzyme, responsible for the production of THC from the CBG precursor, which was later shown (14) to be synthesized by secretory cells in the storage cavity of the glandular trichomes.

In this study, we screened for the possible transgenic origin of the seized cannabis preparations initially put forward for chemical analyses.

Materials and Methods

We carried out genetic analyses routinely employed for the screening of genetically modified organisms (GMO) on 38 cannabis samples: 13 marijuana and 16 hashish samples of unknown origin confiscated from the illegal drug market and nine dried plants from an experimental indoor cultivation of high-potency varieties seeds from the Netherlands (Tables 1 and 2).

The seized cannabis samples were split into aliquots before the analyses, which were all repeated in two different laboratories for both chemical and genetic analyses.

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TABLE 1—*Pool of hashish samples tested for transgenic sequences.*

Decoder	Type	THC %	260/280	Origin
1	Hashish	13	1.193	Seized
2	Hashish	18.4	1.191	Seized
3	Hashish	11	1.173	Seized
4	Hashish	13	1.140	Seized
5	Hashish	12.1	1.095	Seized
6	Hashish	11.1	1.237	Seized
7	Hashish	9.8	1.306	Seized
8	Hashish	22	1.178	Seized
13	Hashish	11.6	1.711	Seized
14	Hashish	20	1.266	Seized
15	Hashish	5.2	1.470	Seized
16	Hashish	9.3	1.367	Seized
17	Hashish	2.6	1.383	Seized
18	Hashish	10.4	1.155	Seized
19	Hashish	35	1.723	Seized
20	Hashish	6.4	1.469	Seized

THC, tetrahydrocannabinol.

TABLE 2—*Pool of herbal cannabis samples tested for transgenic sequences.*

Decoder	Type	THC %	260/280	Origin
9	Marijuana	0.3	1.421	Seized
10	Marijuana	23.3	1.588	Seized
11	Marijuana	0.4	1.493	Seized
12	Marijuana	8.2	1.750	Seized
21	Marijuana	4–5	1.599	Seized
22	Marijuana	4–5	1.732	Seized
23	Marijuana	4–5	1.863	Seized
24	Marijuana	15	1.729	Seized
25	Marijuana	11	1.742	Seized
26	Marijuana	16	1.660	Seized
27	Marijuana	14	1.691	Seized
28	Marijuana	20	1.587	Seized
29	Marijuana	20	1.862	Seized
30	Dried plant	23	1.525	Experimental
31	Dried plant	20	1.651	Experimental
32	Dried plant	0.7	1.792	Experimental
33	Dried plant	20	1.624	Experimental
34	Dried plant	4.9	1.572	Experimental
35	Dried plant	3.1	1.378	Experimental
36	Dried plant	0.6	1.729	Experimental
37	Dried plant	0.3	1.653	Experimental
38	Dried plant	0.3	1.372	Experimental

THC, tetrahydrocannabinol.

Chemical Analyses

All solvents and chemicals were of analytical grade. Reference standard solutions THC, CBD, and cannabinol were obtained from Promochem Lgc (Teddington, UK) and α-colestane was obtained from Sigma-Aldrich (Seelze, Germany). Samples of marijuana were first prepared by grinding to a fine powder using a mortar and pestle; 0.08–0.10 g of each sample was then extracted with 4 mL of internal standard/extracting solution (1 mg/mL of α-colestane) at room temperature for 15 min and then sonicated for about 10 min. One microliter aliquot of each extract was injected into the gas chromatograph coupled to mass spectrometry (GC-MS). GC-MS analyses were performed with a Thermo Electron Focus Dual-Stage Quadrupole (DSQ) GC-MS system (Thermo Electron, (Waltham, MA). The chromatography separation was performed on a fused silica-capillary column 30 m with 0.32 mm i.d. and 0.25 μm film thickness (Zebron; Phenomenex, Torrance, CA). The GC parameters were as follows: 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; and a second ramp with a 10°C/min slope, leading to 280°C with a 3 min hold time. The inlet temperature was settled at 250°C and the MS transfer line at 270°C. The detection was performed in positive ion in the range 50–650 *m/z*.

Genetic Analyses

A modified Wizard method was used to extract the DNA from 300 mg (dry weight) of each of the confiscated cannabis specimens; this large amount of dry material was chosen because of the poor quality of most samples (street samples). After DNA purification via chromatographic columns (Wizard; Promega, Madison, WI), spectrophotometric analysis was used to evaluate its characteristics. An acceptable quantity of DNA (ranging from 300 to 700 ng/μL) was obtained, although of poor quality, as can be expected for "street samples," in line with the common results for herbarium plants. There were no differences in the preparation of hashish and herbal cannabis samples.

The amplificability of the extracted marijuana DNA was first verified using the GeneAmp PCR System 2700 amplifier (Applied Biosystems, Foster City, CA) with random amplified polymorphic DNA (RAPD) Operon A4 and Operon A9 (15), the presence of which had already been confirmed in *C. sativa* species (16). The DNA was then amplified with specific primers for transgenic sequences (17,18) such as GUS, nptII, NOS, and CaMV35S (Table 3). PCR solutions were prepared for each sample using the following: 10× Buffer (Invitrogen (LifeTech), Grand Island, NY) 2 μL, 50 mM $MgCl_2$ 0.6 μL, 10 mM dNTPs 0.2 μL, Platinum Taq polymerase (Invitrogen) 0.2 μL, 10 μM primer RAPD 0.4 μL, 10 ng/μL DNA 20 ng, final volume 20 μL. PCR conditions were for each reaction: a first cycle at 94°C for 2 min, 36°C for 1 min and 72°C for 2 min followed by 46 cycles at 94°C for 1 min, 36°C for 1 min, 72°C for 2 min with a subsequent final extension at 72°C for 7 min. The PCR products were analyzed by agarose gel electrophoresis, using as positive controls transgenic maize and soy known to be positive for homologous tested transgenic sequences.

Results and Discussion

Analytical methods for the detection of GMO fall into two broad categories: the tracing of transgenic proteins (e.g., the ELISA technique) and the tracing of transgenic nucleotide sequences or vectors. The first type of method requires prior knowledge of the target protein, which was obviously not available for the

TABLE 3—*Primers of transgenic sequences.*

Primer	Sequence 5′–3′	Amplicon Size (bp)
NOSFZ1	**GAATCCTGTTGCCGGTCTTGCGA**	146
NOSFZ2	TCGCGTATTAAATGTATAATTGCGGGACTC	
NPTFZ1	**ACCTGTCGGGTGCCCTGAATGAACTGC**	195
NPTFZ2	GCCATGATGGATACTTTCTCGGCAGGAGC	
GUSF	**TCCGTAGAAACCCAACC**	100
GUSR	GCTAGCCTTGTCCATTG	
W35SF	**CCTACAAATGCCATCATTGCG**	207
W35SR	GGGTCTTGCGAAGGATAGTG	
35SFZ1	**CCGACAGTGGTCCCAAAGATGGAC**	162
35SFZ2	ATATAGAGGAAGGGTCTTGCGAAG	
CaMV3	**GTCTTGCGAAGGATAGTGGGA**	80
CaMV4	CACGTCTTCAAAGCAAGTGGA	

confiscated samples. Hence, to verify whether or not genetic manipulations had been performed on the samples, a wide-range screening technique for some of the transgenic vectors most commonly used in agricultural crops, such as the beta-glucuronidase (GUS) reporter gene, the neomycin-phosphotransferase type II (npt II) marker gene, the nopaline synthase (NOS) terminator gene from *Agrobacterium tumefaciens* and the cauliflower mosaic virus (CaMV) 35S promoter sequence, was performed.

Negative results were obtained in all samples using the GUS gene primers and the NPTFZ1/NPTFZ2 and NOSFZ1/NOSFZ2 primer pairs (data not shown).

Three different regions of the promoter 35S sequence, identified by primer pairs 35SFZ1/35SFZ2, W35SF/W35SR, and CaMV3/CaMV4, were amplified in the DNA from the marijuana sample with the highest Δ^9-THC level (23.3%), but this evidence was not confirmed in other marijuana samples containing similarly high THC levels. Specific amplification products were obtained for each primer set (Fig. 1). The amplification product was then tested to exclude the presence of viral contamination by CaMV virus, a natural vector of sequence 35S, by investigating the viral-specific genomic regions other than 35S (19), which were negative for the presence of this virus.

The electrophoretic bands of the amplification product that tested positive were then excised, purified, and sequenced using the ABI 310 capillary sequencer (Applied Biosystems), in triplicate. Similarities between sequences in the international nucleotide nonredundant data banks division were detected using the BLAST program (20) on network servers. The sequences were annotated using BLAST analysis, with commercial vectors used in transgenic crops and containing the 35S sequence, such as pLH7500, pXCS-HAStrep, pUC19-35S-FLAG-RBS, pSAT4A-35SP-MCS-35ST, and many others. Independent of their THC contents, all of the other cannabis specimens tested negative for the considered vectors, including the 35S promoter (data not shown); this was most likely a false positive result, probably due to contamination at the origin of the marijuana sample. In fact, seized cannabis preparations are almost always of unknown origin and are damaged and carry defects, so that it is extremely difficult, if possible at all, to distinguish cannabis DNA from the DNA of possible contaminants (e.g., other genetically modified plants). Results of our investigations allow then to exclude the hypothesis of artificial genetic modifications for high-potency cannabis samples even considering that the number of transgenic vectors in commercial use is larger then that used in this study.

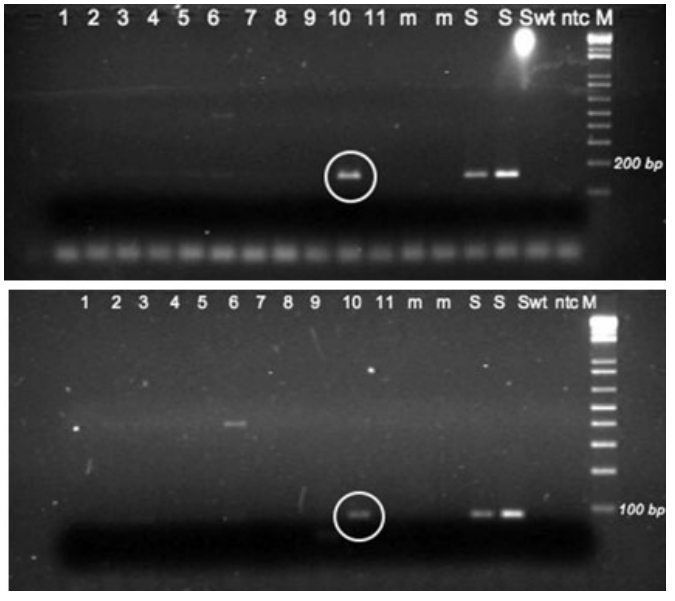

FIG. 1—*(Top) Clear 162 bp band in marijuana sample 10 (THC 23.3.%) and feeble 162 bp band in hashish samples 1–6 (THC 13%, 18.4%, 11%, 13%, 12.1%, and 11.1%, respectively) using primer pair 35SFZ1/35SFZ2. Clear bands on the right (S, S) are positive controls for 35SFZ1/35SFZ2 (Soy RoundUp Ready; Monsanto, St. Louis, MO). (Bottom) Clear 80 bp in marijuana sample 10 (THC 23.3%) using primer pair CaMV3/CaMV4.*

Promoter 35S of CaMV, commonly observed in several genetically modified crops, was found by genetic screening in one marijuana sample with a concentration of 23% Δ^9-THC and was initially thought to be one of the possible explanations for the peculiar levels of cannabinoids in the samples but the occurrence of CaMV infection in the plant was excluded by an investigation of a viral-specific genomic regions other than 35S, which tested negative. The amplification product was finally sequenced, confirming the presence of the 35S sequence and showing similarities with several other synthetic vectors that also contain the 35S sequence. The other samples, especially those of the marijuana type, showed high THC levels (also >10%) without the presence of the 35S promoter.

Our analyses support the hypothesis that marijuana samples submitted to forensic laboratories and characterized by an abnormal level of Δ^9-THC (up to 23%) are the product of breeding selection rather than of transgenic modifications. Further, the solitary positive result reveals the contamination by other transgenic vegetable products; perhaps a cover crop used to conceal the cannabis cultivation. We could indirectly exclude a laboratory contamination whereas the analyses, giving the same results, were performed twice, by different hands for each of the two independent laboratories, from aliquots of the same starting material. High levels of quality control and decontamination of exterior of the plant, possible only in case of fresh material (e.g., plants from seized crops), should rule out potential sample contamination.

However, based on these data, a conclusive distinction between the hypothesis of GMO plant contamination and the other of genetic modification of cannabis cannot be made. Further studies based on comparative chemical and genetic analyses are finally needed to find out an explanation for the recently detected increased potency of cannabis.

Highly potent types of cannabis raise indeed new concerns about public health and legislation. The availability of a more "concentrated" form of this drug might cause an increase in the total amount of THC consumed, with foreseeable consequences on public health. It has been proposed, for instance, that cannabis use could be one cause of psychotic disorders, according to a dose–response relationship (21). It has also been reported (22) that cannabis has effects on the brain reward circuits. Even if a consensus has not yet been reached, it has been proposed that cannabis consumption may lead to addiction, even acting as a promoter of several addictive behaviors. The availability of increased-potency cannabis would amplify this problem.

Furthermore, in many countries, cannabis use is socially accepted because of its perceived "lightness" and to the lower criminal impact in economic terms. Because different cannabis products are priced differently, reflecting both potency and variety, it can be argued that the introduction of modified or selected high-potency cannabis would increase the market demand, questioning both of the above assumptions and urging governments to adapt the relevant legislation. On the other hand, however, new legislation should also consider that this new breed of marijuana could lead to a drastic increase in the efficiency of cannabinoid-based medical therapies, which are still at the experimental stage (23).

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