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Short communication

A real-time polymerase chain reaction (PCR) method for the identification of *Nicotiana tabacum* in tobacco products

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ABSTRACT

A simple and specific real-time PCR assay based on TaqMan® technology has been developed for the identification of cultured tobacco (*Nicotiana tabacum*) in various commodities such as cigars, cigarettes and reconstituted tobacco. The TaqMan® assay targets a sequence of the putrescine N-methyltransferase gene family encoding an enzyme that plays a crucial role in the biosynthesis of nicotine. To reduce the possibility of false negatives, universal plant chloroplast primers were also used in a separate real-time PCR reaction to give indication if DNA is amplifiable in the matrix. The TaqMan® assay successfully identified tobacco in over 40 commercial tobacco products, while negative results were obtained from the assay for DNA extracted from a variety of other botanical products. In our study, two commercial DNA isolation kits were used, namely, the Qiagen DNeasy® Plant Mini kit and the Qiagen Gentra® Puregene® kit. They produced good quality DNAs in sufficient quantities for real-time PCR analysis. In a few cases, an additional purification step with the Promega DNA IQ™ system had to be implemented to obtain amplifiable DNA.

1. Introduction

At our Laboratory, tobacco products are analyzed for the purpose of tariff classification. Tobacco is usually characterized by the presence of nicotine, neophytadiene, tar (Layten and Nielsen, 1999) and vitamin E (Abbasi et al., 2007). Additionally, microscopic fiber analysis may reveal horseshoe-shaped cell fragments typical of tobacco (McCrone and Delly, 1973). These methods are adequate but in certain cases, especially reconstituted tobacco, it could be a challenge to confirm the presence of tobacco. Reconstituted tobacco is a paper-like material composed mostly or entirely of tobacco. It is primarily manufactured by the paper making process (Layten and Nielsen, 1999) which involves chemical and heat treatments such that nicotine along with other constituents could be removed and tobacco morphology is significantly affected. Therefore, an additional method for analyzing reconstituted tobacco was required.

Numerous species-specific real-time polymerase chain reaction (PCR) assays have been developed (Valasek and Repa, 2005). For the identification of *Nicotiana tabacum* (cultured tobacco) in various commodities, we targeted a sequence of the putrescine N-methyltransferase (PMT) gene which codes for a key enzyme involved in nicotine biosynthesis. In *N. tabacum*'s genome, PMT is encoded by five genes: PMT1a, PMT1b, PMT2, PMT3 and PMT4 (Riechers and Timko, 1999). The PMT2, PMT3 and PMT4 genes share a great deal of similarities with the three PMT genes found in *N. sylvestris*. The PMT1a and PMT1b genes are more similar to the PMT genes found in *N. tomentosiformis* and *N. otophora*, respectively. Limited information is available regarding the PMT gene sequences of these two *Nicotiana* species. However, to the best of our knowledge, these species are not widely cultivated (Layten and Nielsen, 1999) and are not of concern for our purpose. *N. sylvestris*, on the other hand, is grown as an ornamental plant and is readily available. Therefore, we focused on the PMT1a and PMT1b which are the gene sequences that vary between *N. sylvestris* and *N. tabacum*. Only the PMT1a (accession no. AF126810) gene was investigated for its suitability as a marker since PMT1b was not found in public databases. In this paper, we describe a real-time PCR method which detects a short sequence (77 bp) within PMT1a for the identification of tobacco. Since DNA in processed tobacco has undergone degradation to some extent (Rossi et al., 2001), our assay design was targeted at short sequences which are more likely to amplify than larger fragments.

For successful PCR, high quality template DNAs are required. For plant material, abundant secondary metabolites, polyphenols and polysaccharides tend to be co-extracted along with DNA (Varma et al., 2007). In this study, we used two commercial DNA extraction kits, namely, the Qiagen DNeasy® Plant Mini kit and the Qiagen Gentra Puregene® kit. The former makes use of the DNA properties of a silica membrane inside a spin column, while the

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latter involves the use of a series of buffered solutions. They are not as labor-intensive and do not require the use of dangerous chemicals like other commonly used methods (Varma et al., 2007).

2. Materials and methods

2.1. Plant materials

Genomic tobacco DNA from low alkaloid Burley 21 tobacco (LA21) was obtained from J.G. Jelesko. Both unprocessed and processed tobacco leaves were provided by Philip Morris Products SA (Neuchâtel, Switzerland) and from Imperial Tobacco Canada Ltd. (Montreal, Canada), respectively. Cigars, cigarettes, reconstituted tobacco and herbal cigarettes were purchased from local reputable smoke shops. Dehydrated leaves of Bay leaf (*Laurus nobilis*), cilantro (*Coriandrum sativum*), tea (*Camellia sinensis*), mint (*Mentha* sp.), oregano (*Origanum vulgare*), parsley (*Petroselinum crispum*), rosemary (*Rosmarinus officinalis*), tarragon (*Artemisia dracunculus*), and thyme (*Thymus vulgaris*) as well as fresh leaves from eggplant (*Solanum melongena*) and tomato (*Solanum lycopersicum*) were purchased from a local supermarket.

2.2. Sample preparation for real-time PCR

Samples with high moisture content such as fresh non-tobacco leaves were dehydrated for 48 h in a desiccator cabinet prior to DNA extraction. Plant materials were pulverized for 2 min at 30 Hz using a Retch® universal mixer-mill disrupter model number MM301 (ThermoFisher Scientific, Ottawa, Canada). During method development, DNA extraction from 20 mg of pulverized sample was carried out in triplicate using the DNeasy® Plant Mini kit (Qiagen, Mississauga, Canada) and the Gentra Puregene® kit (Qiagen) according to the instructions supplied by the manufacturer. For sample analysis, DNA extractions were performed in duplicates. The Puregene® protocol was used throughout our study. In the event that an additional purification step was required, 40 μL of extract was processed according to the DNA IQ™ System (Promega, Madison, WI) protocol for DNA isolation from liquid samples. The system uses paramagnetic resin for DNA isolation (Promega, 2006). All purified DNA solutions were stored at −20 °C until real-time PCR analysis.

2.3. DNA quality check

Thermal cycling conditions used with universal plant chloroplast primers CP03-F and CP03-R are described in Table 1. Amplifications were carried out in a 7500 real-time PCR system (Applied Biosystems, Foster City, CA) with Power SYBR® Green Master Mix (Applied Biosystems).

2.4. Tobacco identification

The PMT1 TaqMan® assay primers and probe (Table 1) were supplied by Applied Biosystems. A 25 μL real-time PCR reaction contained 12.5 μL of TaqMan® Gene Expression Master Mix (Applied Biosystems), 2.5 μL (6.0 μM) of each primer, 2.5 μL (1.25 μM) of the probe and 5.0 μL of extract. The amplifications were performed in a 7500 real-time PCR system using the following conditions: 95 °C for 10 min followed by 45 cycles of 95 °C for 15 s and 60 °C for 60 s. Eight serial diluted concentrations (600, 150, 37.5, 9.375, 2.344, 0.586, 0.146 and 0.037 ng/μL) of LA21 DNA, prepared in triplicates, were diluted by 1× TE buffer (Promega) and used to generate a standard curve. The real-time PCR was carried out in triplicate. To determine the limit of detection, four serial diluted concentrations (0.146, 0.037, 0.009 and 0.002 ng/μL) of LA21 DNA, prepared in 10 replicates, were diluted by 1× TE buffer (Promega).

3. Results and discussion

3.1. DNA extraction

Readily amplifiable DNA was extracted rapidly (approximately 1 h) and easily with the DNeasy® kit. Most samples could be used directly for real-time PCR. In the case of herbal cigarettes, chloroplast DNA was not amplified indicating the presence of PCR inhibitors in the DNA extracts. These samples are a complex mixture of plant materials such as wild lettuce (*Lactusa virosa*), catnip (*Nepeta* sp.), passion flower (*Passiflora* sp.), skull cap (*Scutellaria* sp.) and mint. Inhibition was minimized by implementing an additional purification step with the DNA IQ™ system. The DNeasy® kit was useful because of its simplicity and speed.

The Puregene® protocol was used whenever applicable due to its lower cost, although it requires more time (3 h vs. 1 h) compared to DNeasy® to process a sample. In most cases, real-time PCR reactions were inhibited. Reliable and reproducible amplification was achieved by a simple 1:50 dilution of the extracts with 1× TE buffer. Similar to DNeasy®, PCR inhibitors were not completely removed from herbal cigarette extracts and were further purified using the DNA IQ™ system to achieve amplification. In a few cases (i.e. thyme, mint, rosemary and oregano), the DNeasy® kit was used because amplification was not achieved using the Puregene® kit in combination with DNA IQ®.

Table 1
Description of primers and probes used for real-time PCR[a].

Use	Name	Sequence (5′–3′)	Sense/antisense	Expected amplicon size (bp)[b]	Thermal cycling conditions
Quality Check[c] (Chloroplast)	CP03-F	CGGACGAGAATAAAGATAGAGT	Sense	124	Watanabe et al. (2006)
	CP03-R	TTTTGGGGATAGAGGGACTTGA	Antisense		
Identification[d] (PMT1 TaqMan®)	PMT1-Fwd	CAGGGACGAAGTGAGCAGTTAA	Sense	77[e]	This work
	PMT1-Rev	GCTTCACCTGCAAAATTCGATTA	Antisense		
	PMT1-Prb	TTCGCATATTATCAGACGCA	TaqMan® Probe[f]		

[a] PMT1 TaqMan® assay primers and probe were designed using Primer Express® 3.0 (Applied Biosystems). Each oligonucleotide sequence was submitted to a BLAST search to verify specificity.

[b] Amplicons were electrophoresed to verify that they had the expected size. Gel electrophoresis was carried out in 2.2% FlashGel® DNA Cassettes (Lonza, Rockland, ME).

[c] Cycle threshold (C_T) values <40 were considered as positive. C_T values >40 were considered negative since non-specific amplification (e.g. primer dimers) were observed between C_T 40–45 in the reagent blanks.

[d] The limit of detection was 0.037 ng/μL (C_T 39.89 ± 0.58). Fluorescence signals were recorded for all ten replicates at concentrations of 0.037 ng/μL and above. Results were considered positive if fluorescence was recorded at C_T values <40. In all cases, C_T values were <40.

[e] PCR products from all of the different types of tobacco (Burley, Flue-cured and Oriental) were submitted to sequencing analysis. Results confirmed that there was no off-target amplification.

[f] Labeled on the 5′ end with 6-FAM reporter dye and with a minor groove binder/non-fluorescent quencher (MGBNFQ) on the 3′ end.