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DEVELOPMENT OF NICOTIANA-SPECIFIC MOLECULAR MARKERS AND THEIR APPLICATION IN A LOOP-MEDIATED ISOTHERMAL AMPLIFICATION ASSAY

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Abstract

An effective identification method for detecting illegal goods involving raw tobacco material is crucial for tobacco monopolies to conduct surveillance. We developed *Nicotiana*-specific molecular markers to determine whether seized goods contain raw tobacco material. The sequence data for genes related to the nicotine metabolism pathway and genomic data from the public Solanaceae database were used to establish *Nicotiana*-specific molecular markers. These markers were determined by experimentally verifying 17 types of nontobacco plant material and 91 types of tobacco material belonging to 11 sections of 3 subgenera. Two reliable *Nicotiana*-specific markers, Ntsp027 and Ntsp151, were selected from among the 209 newly developed markers. The results indicated that the primers corresponding to these two markers can amplify the target fragments in the 91 types of *Nicotiana* material without amplification of any PCR products in the 17 types of non-*Nicotiana* material. Furthermore, utilizing the marker Ntsp151, we verified the efficacy of the loop-mediated isothermal amplification (LAMP) assay in authenticating tobacco material. The identification of 21 tea-cigarette products via the combination of GC–MS, a *Nicotiana*-specific molecular marker and LAMP methods underscores the utility of *Nicotiana*-specific DNA markers in determining whether illegal goods contain raw tobacco material. Our results indicate an impressive accuracy rate of 100%, which is consistent with the reliability assessment, underscoring the accuracy of these markers in effectively identifying tobacco material. Our findings can significantly augment the capacity for surveillance and anticounterfeiting efforts by aiding the fight against illicit trade and ensuring the integrity of all tobacco-related products in the market.

Keywords: LAMP. Molecular marker. Nicotine. Tobacco monopoly.

1. Introduction

Tobacco is a cash crop that belongs to the genus *Nicotiana* in the Solanaceae family (Xu et al. 2017). Its leaves contain a signature alkaloid commonly known as nicotine, which underpins the revenue of the tobacco industry. Although tobacco plants are under government regulation in China, numerous illegal operations involving raw tobacco material driven by profitable commercial interests have emerged. The counterfeiting and smuggling of tobacco not only has an adverse effect on the development of the tobacco

industry but also poses new challenges for the identification and inspection of suspected raw tobacco material.

To date, numerous studies have identified and analyzed tobacco cultivars at the molecular level using markers such as AFLP (Ren and Timko 2001, Sarcevic et al. 2013), RAPD (Zhang et al. 2005, Sun et al. 2020), SSR (Moon et al. 2008, Gholizadeh et al. 2012, Zhang et al. 2013), ISSR (Li et al. 2008, Denduangboripant et al. 2012, Qi et al. 2012), DArT (Maryan et al. 2012, Lu et al. 2013), and SNPs (Zhang et al. 2017), or combinations thereof, to perform genetic diversity assessments and fingerprint mapping. Additionally, limited study has reported the rapid identification of tobacco cultivars using cured tobacco leaves with SSR, SCAR, and RAPD markers (Sun et al. 2020). However, no study has investigated the seizure/detection of illegal tobacco-derived goods/operations based on the identification of raw tobacco material at the molecular level. In China, the domestic tobacco industry has primarily adopted sensory and chemical inspection methods for the detection and identification of illicit goods. However, both methods have certain drawbacks. Regarding the sensory inspection method (SBQTS 1992, pp. 1–10, 2000, pp. 1–5, AQSIQ 2005, pp. 1–2), there is a high probability of inaccuracy because it relies on the subjective and unreliable physical senses and assessment experience level of individual human inspectors. According to the State Tobacco Monopoly Administration (STMA) (2018a, pp. 1–14, 2018b, pp. 1–9, 2018c, pp. 1–7), the main objective of the chemical inspection method is to ascertain whether a sample is from a tobacco monopoly product by determining whether it contains nicotine. However, nicotine is not unique to tobacco and can be detected in numerous nontobacco (especially Solanaceae) plant products. Therefore, this method is beset with a high risk of false positives.

In certain cases, these limitations have resulted in unsuccessful lawsuits and have generated intense opinions among the public, both on the internet and more broadly in society. Thus, there is an urgent need to develop a feasible way to identify raw tobacco material reliably and effectively. Molecular markers, due to their stability, cost-effectiveness, and ease of use, are promising tools for a variety of relevant applications, including genome mapping, gene tagging, genetic diversity analysis, and forensic investigations. The development of genome sequencing technology and bioinformatics, as well as the acquisition of considerable plant (especially Solanaceae) genome data, has laid a solid theoretical and technical foundation for the identification and inspection of suspected raw tobacco material using a *Nicotiana*-specific molecular marker. Therefore, the aim of our study was to develop a simple yet reliable method that accurately distinguishes between tobacco and nontobacco material in seized/illegal goods with improved scientific methods, accuracy, and authority.

2. Material and Methods

Plant Materials

A total of 108 types of plant material were collected, 91 and 17 of which were tobacco (Table 1) and nontobacco (Table 2), respectively. Among the 91 types of tobacco material, 83 were from wild tobacco species, belonging to 11 sections of three tobacco subgenera, and eight were four different types of cultivated tobacco material. Among the 17 types of nontobacco material, one, two, two, and four were, respectively, material from the Brassicaceae, Fabaceae*, Camellia,* and Poaceae taxa, and eight belonged to seven genera of the Solanaceae. The 108 types of plant material were selected by a comprehensive consideration of factors such as experimental verification of the *Nicotiana* specificity of the developed marker and whether the plant material present in the suspect/illegal goods contained nicotine/raw tobacco material. The different types of plant material were also selected based on their genetic diversity and geographical representativeness.

Tea-Cigarette Material

Tea-cigarettes are made of cut leaves derived from tea, flowers, and Chinese herbs using fragrance conditioning as the flavoring and cigarette paper, filter rods, and tipping paper as excipients. These products have tea profiles on the packaging, imitate cigarettes in appearance, and can be smoked by

burning. Tea-cigarette products are also known as flower smokes, herb smokes, and a quit-smoking panacea. The State Tobacco Monopoly Administration of China defines tea-cigarettes as an infringing product containing raw tobacco material.

Table 1. Detailed information of 91 *Nicotiana* materials.

Wild Species			
Sub-genus	Section	Species	Accessions
Rustica	*Paniculatae*	*N.benavedesii*	M001
		N.cordifolia	M002
		N.glauca	M003
		N.glauca	M004
		N.knightiana	M005
		N.paniculata	M006
		N.paniculata	M007
		N.raimondii	M008
		N.solanifolia	M009
	Rusticae	*N.rustica*	M010
Tabacum	*Tomentosae*	*N.glutinosa*	M011
		N.glutinosa	M012
		N.otophora	M013
		N.otophora	M014
		N.setchellii	M015
		N.tomemtosa	M016
		N.tomemtosiformis	M017
Petunioides	*Undulatae*	*N.undulata*	M018
		N.undulata	M019
		N.wigandioides	M020
	Alatae	*N.alata*	M021
		N.bonariensis	M022
		N.forgetiana	M023
		N.langsdorffii	M024
		N.langsdorffii	M025
		N.longiflora	M026
		N.longiflora	M027
		N.longiflora	M028
		N.plumbaginifolia	M029
		N.plumbaginifolia	M030
		N.plumbaginifolia	M031
		N.sylvestris	M032
		N.sylvestris	M033
		N.sylvestris	M034
	Nudicaulisae	*N.nudicaulis*	M035
	Repandae	*N.repanda*	M036
		N.repanda	M037
		N.stocktonii	M038
		N.stocktonii	M039
		N.stocktonii	M040
	Noctiflorae	*N.noctiflora*	M041
		N.noctiflora	M042
		N.petunioides	M043
	Acuminatae	*N.acuminata*	M044
		N.attenuata	M045
		N.corymbosa	M046
		N.pauciflora	M047
	Bigelovianae	*N.bigelovii*	M048
		N.clavelandii	M049
	Suaveolensae	*N.africana*	M050
		N.amplxicaulis	M051
		N.amplxicaulis	M052
		N.benthamiana	M053

Table 1. Continued.

	N.cavicola	M054
	N. debneyi	M055
	N.excelsior	M056
	N.excelsior	M057
	N.goodspeedii	M058
	N.gossei	M059
	N.linearis	M060
	N.miersii	M061
	N.maritima	M062
	N.megalosiphon	M063
	N.megalosiphon	M064
	N.occidentalis	M065
	N.occidentalis	M066
	N.occidentalis	M067
	N.occidentalis	M068
	N.rosulata	M069
	N.rosulata	M070
	N.rosulata	M071
	N.rotundifolia	M072
	N.rotundifolia	M073
	N.suaveolens	M074
	N.suaveolens	M075
	N.suaveolens	M076
	N.umbratica	M077
	N.velutina	M078
	N.velutina	M079
	N.velutina	M080
	N.acuminata	M081
	N.acuminata	M082
	N.benthamiana	M083
Cultivated Species		
Names	Types	Accessions
K326	Flue-cured	M084
Honghua dajinyuan HD	Flue-cured	M085
TN90	Burley	M086
Burley 21	Burley	M087
Basma Xanthi	Oriental	M088
Sumsun NN	Oriental	M089
Beinhart1000-1	Cigar	M090
Florida301	Cigar	M091

Table 2. Information of 17 non-tobacco materials.

Family	Genus	Name	Accessions
Leguminosae	*Arachis* L.	*Arachis hypogaea* L.	M092
	Vicia L.	*Vicia faba* L.	M093
Poaceae	*Zea* L.	*Zea mays* L.	M094
	Triticum L.	*Triticum aestivum* L.	M095
	Hordeum L.	*Hordeum vulgare* L.	M096
	Oryza L.	*Oryza sativa* L.	M097
Solanaceae	*Capsicum* L.	*Capsicum annuum* L.	M098
	Solanum L.	*Solanum melongena* L.	M099
	Solanum L.	*Solanum tuberosum* L.	M100
	Lycopersicon L.	*Solanum lycopersicum* L.	M101
	Petunia Juss.	*Petunia hybrida* Vil.	M102
	Datura L.	*Datura stramonium* L.	M103
	Lycium L.	*Lycium chinense* Mill.	M104
	Cestrum L.	*Cestrum purpureum*	M105
Brassicaceae	*Brassica* L.	*Brassica napus* L.	M106
Theaceae Mirb.	*Camellia* L.	*Camellia sinensis* (L.) O.Kuntze	M107
	Camellia L.	*Camellia sinensis* var. assamica	M108

The 21 tested tea-cigarette products utilized in this study were collected from actual cases of seizures from nine manufacturers. The raw material listed on the product packages were mainly tea, flowers, and honey, and none of them listed tobacco components. Details of the samples tested are included in Table 3.

Table 3. Detail information of the 21 tea-cigarette products.

Manufacturer	Product Name	Raw materials listed on the package	Accessions
Zining Technology Co., Ltd Henan, China	Tieguanyin·Yunyan	Tea, chrysanthemum, rose, peppermint, edible flavor	TM01
Chawang Biotechnology Co., Ltd, Anhui, China	Baicha Chayan·Tujiamei	Tea, chrysanthemum, honey, peppermint, edible flavor	TM02
	Pu'ercha Chayan·Chazhilong	Tea, chrysanthemum, honey, peppermint, edible flavor	TM03
Guzaowei Smoking Cessation Product Technology Co., Ltd, Henan, China	Meicha Chayan·Xiang'amei	Tea, chrysanthemum, honey, peppermint, edible flavor	TM04
Huangling Biotechnology Co., Ltd, Henan, China	Fenghuang Shuixian·Mingcun Huangling	Tea, chrysanthemum, honey, peppermint, edible flavor	TM05
	Longjing Meigui·Mingcun Huangling	Tea, chrysanthemum, honey, peppermint, edible flavor	TM06
	Jinsi Meigui·Sifangcha	Tea, chrysanthemum, honey, peppermint, edible flavor	TM07
Heshengcha Biotechnology Co., Ltd, Henan, China	Molihuacha·Baoyangcha	Jasmine tea, chrysanthemum, peppermint, honey	TM08
	Tieguanyin·Hesheng	Tea, chrysanthemum, honey, peppermint	TM09
	Longjing·Baoyangcha	Tea, chrysanthemum, honey, peppermint	TM10
	Huangjinya·Baoyangcha	Tea, chrysanthemum, honey, peppermint	TM11
	Guihua Tea·Baoyangcha	Tea, chrysanthemum, peppermint, honey	TM12
	Meigui Dahongpao·Hesheng	Tea, rose, chrysanthemum, honey	TM13
	Kafeicha·Chashui·Yanyuan	Tea, coffee concentrate, chrysanthemum, peppermint, honey	TM14
	Pibacha·Hesheng	Tea, Pipa concentrate, chrysanthemum, peppermint, honey	TM15
	Yingtaohuacha·Hesheng	Tea, cherry concentrate chrysanthemum, peppermint, honey	TM16
Chawang Smoking Cessation Biotechnology Co., Ltd, Anhui, China	Chama Gudao·Chawang	Tea, chrysanthemum, honey, peppermint	TM17
	Chang'anming·Chawang	Tea, chrysanthemum, peppermint, honey mel	TM18
Pinxin Trading Co., Ltd, Yunnan, China	Pu'er Chenxiang·Chatu	Tea, agarwood, jujube, honey	TM19
Bencaotang Smoking Cessation Research Center, Yunnan, China	Qingfei Jieyanling·Bencaotang	Tea, wormwood leaves, wild chrysanthemum, honeysuckle, licorice, peppermint, loquat leaves, orange, Chuanbei	TM20
Hongsheng Smoking Cessation Products Co., Ltd, Shanghai, China	Luohanguo Qingfeiling·Hongsheng	Ginseng leaves, tea, chrysanthemum, mulberry leaves, peppermint, perilla, Momordica grosvenori, honeysuckle, wormwood leaf	TM21

Controls

There were four positive controls (CK+), namely, a standard cigarette smoke sample (CK+01), standard flue-cured tobacco (CK+02), standard cigarette sensory sample (Ziyunyan, CK+03), and multi-flue-cured tobacco (CK+04) as an internal standard. Additionally, there were four negative controls (CK-), namely, chrysanthemum tea (CK-01), rose tea (CK-02), black tea (CK-03), and green tea (CK-04), all of which are commercially available.

Genome Data

The data included 46 genome sequences related to nicotine synthesis, transport, transformation, and metabolism that were downloaded from the National Center for Biotechnology Information (NCBI) public database (https://www.ncbi.nlm.nih.gov/) (Xu et al. 2-17) in FASTA file format. Among the sequences, there were 32 structural genes and 14 transcription factors; the details are included in Table 4. The genomic data for tomato, potato, and pepper plants were downloaded from the Solanaceae database (https://www.sgn.cornell.edu/) in FASTA file format. Published tobacco genomic data were downloaded from https://www.sgn.cornell.edu/organism/Nicotiana_tabacum/genome (Wang and Bennetzen 2015), while unpublished tobacco whole-genomic data (Honghua Dajinyuan, Yunyan 87, Beinhart1000-1, Yunshai No. 1 and Huanghuayan G366, etc.) and resequencing data (10-15× genome resequencing data for 369 core tobacco resources) were provided by the Yunnan Academy of Tobacco Agricultural Sciences.

Development and Identification of Primers

Primers for the downloaded gene sequences obtained from 46 nicotine metabolism pathways were designed using Primer3 software (http://www.frodo.wi.mit.edu). In principle, three pairs of primers were designed for the DNA sequence of each gene. A 500 bp nucleic acid sequence was cut at the beginning, end, and middle of each gene for primer development. If the DNA sequence of the gene was large (more than 3 kb), 2~3 primer pairs could be appropriately added in the middle of the gene sequence. Ultimately, 3~6 pairs of primers were designed for each gene. The relevant parameters for primer design were established according to a previously described method by Tong et al. (2015).

The obtained primers were subjected to preliminary *Nicotiana* specificity analysis using bioinformatics. This was achieved by combining published tobacco genomic data (including those not yet published from the Chinese tobacco industry), Solanaceae genomic data (excluding tobacco data), and genomic data currently published in public databases such as the NCBI and European Molecular Biology Laboratory (EMBL) databases. All the developed primer sequences were subjected to sequence alignment with the genomic data mentioned above. The alignment results were as follows: 1) the primer sequences were completely aligned with the tobacco genome sequence (forward and reverse identification). %, Forward & Reverse Cvg. The forward and reverse primer sequences were 100% aligned, and the BLAST result was unique. 2) The primer sequences could not be aligned with known genome sequences except for that of tobacco. The primers that met the abovementioned requirements were candidate *Nicotiana*-specific markers.

The genomic DNA samples of the 108 types of test material were amplified via PCR using candidate *Nicotiana*-specific markers, and these markers were validated via the PCR amplification product results.

PCR Amplification and Product Analysis

Genomic DNA Extraction

The genomic DNA of the test material was extracted and purified using the filter column method (DNeasy Plant Mini Kit, QIAGEN, Shanghai, China) according to the manufacturer's instructions.

PCR Amplification System

The volume of the amplification system was 20 µL, which included 2.0 µL of 10 × Buffer (10 mmol/L Tris-Cl, pH 8.4; 50 mmol/L KCl; 1.5 mmol/L $MgCl_2$); 200 µmol/L dNTPs (Takara Biotechnology Co. Ltd., Dalian, China); 0.5 µmol/L upstream and downstream primers; 0.75 U of rTaq polymerase (Takara, Dalian, Liaoning, China); 30~50 ng of template DNA; and ddH_2O q.s. The PCR procedure was as follows: predenaturation at 95 °C for 5 min; 30 cycles of denaturation at 95 °C for 30 s, renaturation at 60 °C for 30 s, extension at 72 °C for 30 s, extension at 72 °C for 5 min; and storage at 4 °C.

Table 4. Information of the nicotine metabolism pathways in *Nicotiana tabacum* L.

Abbr. Name	Description	NCBI Accession
	Structural genes	
ODC	ornithine decarboxylase	AB031066.1
ODC1		AF321138.1
ODC2		AF233849.1
SAMS	S-adenosylmethionine synthase	AB006187.1
BYJ6	*BYJ6*	AB005879.2
ADC	arginine decarboxylase	AF321137.1
ADC1	arginine decarboxylase 1 (*ADC1*) gene	AB005880.1
ADC2	arginine decarboxylase 2 (*ADC2*) gene	AF127241.1
PMT	putrescine N-Methyltransferase	D28506.1
PMT1	putrescine N-methyltransferase 1	NM_001325108.1
PMT2	putrescine N-methyltransferase 2	AB004323.2
A662	*A622* mRNA for isoflavone reductase-like protein	D28505.1
A662L	*A622L* mRNA for isoflavone reductase-like protein	AB445396.1
MPO	methylputrescine oxidase	DQ873385.1
MPO1	*NtMPO1* mRNA for N-methylputrescine oxidase	AB289456.1
MPO2	*NtMPO2* mRNA for N-methylputrescine oxidase	AB289457.1
QPT	quinolinate phosphoribosyl transferase	AB038494.1
MATE	multi antimicrobial extrusion family protein	AB286961.1
MATE1	*NtMATE1* gene for multi antimicrobial extrusion family protein	AB286963.1
MATE2	*NtMATE2* mRNA for multi antimicrobial extrusion family protein	AB286962.1
NUP1	nicotine uptake permease 1	GU174267.1
NUP2	nicotine uptake permease 2	GU174268.1
BBLa	*BBLa* mRNA for berberine bridge enzyme-like protein	AB604219.1
BBLb	berberine bridge enzyme like protein (T440 gene)	AM851017.1
BBLc	*BBLc* mRNA for berberine bridge enzyme-like protein	AB604220.1
BBLd	*BBLd* mRNA for berberine bridge enzyme-like protein	AB604221.1
SPDS	spermidine synthase	AF321139.1
AO	KR3B.001A05	DW001381.1
QS	*PR48*	AF154657.1
SAMDC	S-adenosylmethionine decarboxylase	U91924.1
JAT1	putative *MATE* transporter (T401 gene)	AM991692.1
EFa	elongation factor-1 alpha	D63396.1
	Transcription Factors	
NtERF189	*Nicotiana tabacum ERF189* mRNA for ethylene response factor 189	AB827951.1
NtERF115	*Nicotiana tabacum ERF115* mRNA for ethylene response factor 115	AB828149.1
NtERF221	*Nicotiana tabacum* ethylene-responsive transcription factor 1-like	XM_016622819.1
NtERF104	*Nicotiana tabacum* ethylene-responsive transcription factor 2-like	XM_016625526.1
NtERF179	*Nicotiana tabacum ERF179* mRNA for ethylene response factor 179	AB828150.1
NtERF17	*Nicotiana attenuata* ethylene-responsive transcription factor 1-like	XM_019377263.1
NtERF168	*Nicotiana tabacum ERF168* mRNA for ethylene response factor 168	AB828151.1
NtMYC2a	*Nicotiana tabacum MYC2a* transcription factor	HM466974.1
NtMYC2b	*Nicotiana tabacum MYC2b* transcription factor	HM466975.1
NtMYC2c	*Nicotiana tabacum MYC2c* transcription factor	HM466976.1
NtERF10	*Nicotiana tabacum* ethylene-responsive transcription factor 2-like	XM_016623277.1
NtERF91	*Nicotiana tabacum ERF91* mRNA for ethylene response factor 91	AB828153.1
NtERF32	*Nicotiana tomentosiformis* ethylene-responsive transcription factor 2	XM_009608650.3
ERFSNV	*Nicotiana tomentosiformis* AP2-like ethylene-responsive transcription factor AIL5	XM_009591706.3

PCR Amplification Product Analysis

A one-sixth volume of 6 × loading buffer was added to the PCR amplification products. Using 2.5 μL of a 6% nondenaturing polyacrylamide gel (nondenaturing PAGE, 550 V, 2.5 h), electrophoresis separation was performed using a DYY-8 electrophoresis apparatus (Beijing Liuyi Factory, Beijing, China). Following electrophoresis, the gel was subjected to silver staining, such as fixation, silver staining, rinsing, color development, and rinsing, as previously described (Huang et al. 2018). Finally, the gel was photographed and processed with the non-PAGE film data.

Rapid Genomic DNA Extraction for the LAMP Assay

Chelex-100 resin (Bio-Rad Laboratories Co., Ltd., Shanghai, China) was used for rapid and easy preparation of genomic DNA from plant material. Briefly, 10~15 mg of plant material was placed in an Eppendorf tube, after which 5% Chelex-100 resin solution was added. After grinding with a plastic pestle, protease K (Takara) and RNase A (Takara) were added, and the samples were vortexed for 30 s. Thereafter, the sample was boiled for 5 min and vortexed for another 30 s. After centrifuging at 12,000 rpm, the supernatant was collected as a template for the loop-mediated isothermal amplification (LAMP) assay.

LAMP to Identify Tobacco Materials

The Ntsp151 sequence was used to design specific LAMP primers using the software Primer Explore V5 (http://primerexplorer.jp/lampv5e/index.html). The LAMP primers (Table 5) used included forward inner primer (FIP), backward inner primer (BIP), two outer primers (F3 and B3), and loop primer B (LoopB). The LAMP mixture was prepared with 1× Bst2.0 DNA polymerase buffer (New England Biolabs Ltd., Beijing, China), 6 mmol/L $MgSO_4$ (New England Biolabs Ltd.), 0.5 mmol/L dNTP (Takara), 0.1 mmol/L F3 primer, 0.1 mmol/L B3 primer, 8 mmol/L FIP primer, 8 mmol/L BIP primer, 0.2 mmol/L LoopB primer, 8 U of Bst2.0 DNA polymerase (New England Biolabs Ltd.), and 2 µL of sample DNA (with a final reaction volume of 25 µL). To visualize the results, 1 µL of diluted SYBR Green I (Sigma Co., Ltd., Chengdu, China) was mixed with the LAMP reaction product, resulting in a fluorescent green color for the positive reaction as it was intercalated into the nucleic acid; conversely, the negative reaction remained orange, indicating no amplification.

Table 5. Primers for LAMP and qPCR.

Name	Seq (5'-3')
F3	TTGGCTATGGAATTTATCACAT
B3	AGCCGCTTTCAAAATCCG
FIP (F1c+F2)	ACCCGAGCCATCCTCTTCTTCTATATTCCTTTTTCTTGGCACATT
BIP (B1c+B2)	TACGACTACGCCTCGCTGTTAGGCTCAATTTTCCCCACT
LoopB (LB)	GCTTAGGCATGTTTGAGCCAATT
Ntsp151-qF1	CTCGCTGTTACTCTAGCC
Ntsp151-qR1	ACTTACGAGACCGCAGA

To optimize the LAMP reaction conditions, the reaction mixtures were incubated at 60, 61, 62, 63, 64, or 65 °C to determine the optimal reaction temperature. LAMP was also performed for 15, 30, 45, 60, 75, and 90 min to determine the optimal incubation time. In addition, the LAMP products were subjected to gel electrophoresis, as a positive reaction results in the production of a typical ladder-like electrophoresis pattern.

Ninety-one tobacco samples and 17 types of nontobacco samples were chosen for the specificity of the LAMP reaction. Moreover, qPCR was performed using the primers Ntsp151-qF1 and Ntsp151-qR1 to verify the LAMP results.

Moreover, the LAMP assay was tested on 21 tea-cigarette samples to verify whether they contained raw tobacco material derived from plants.

Fluorescence Intensity Measurement

The fluorescence intensity of the LAMP products was measured using a fluorescence measurement device (Gene-8C, Allsheng, Hangzhou, China).

Detection of Nicotine Content

The nicotine content in the main smoke stream of test tea-cigarette samples was determined based on the characteristic components of tobacco via alkaloid-gas chromatography–mass spectrometry (GC–MS) and gas chromatography-tandem mass spectrometry methods: YC/T 559-2018 (STMA 2018a, pp. 1–14).

3. Results

Analysis of Nicotiana*-specific Markers*

Two hundred and nine qualified primer pairs were designed for 46 full-length DNA sequences related to tobacco nicotine synthesis, transport, transformation, and metabolism; 3~6 pairs of primers were obtained for each gene development, with an average of 4.5 (209/46) pairs. The DNA sequences of all the markers are available upon request.

The 209 pairs of primer sequences were aligned with genomic data (including that of tobacco) published in public databases and unpublished tobacco genomic data from the Chinese tobacco industry. Finally, five pairs of candidate *Nicotiana*-specific primers were obtained. The results revealed an extremely low acquisition rate of 2.39% for the candidate *Nicotiana*-specific primers.

Verification of Nicotiana*-specific Markers*

Five pairs of candidate *Nicotiana*-specific primers were validated using the 108 types of tested material; ultimately, two pairs of primers with *Nicotiana* specificity were obtained (Table 6). The primer Ntsp027 amplified a specific 303 bp band in the 91 types of tobacco material (certain tobacco material had nonspecific PCR amplification products larger than 303 bp); however, no PCR amplification products were observed for the 17 types of nontobacco plant material (Figure 1). The Ntsp151 primer amplified a specific band of 300 bp in the 91 types of tobacco material (certain tobacco material have nonspecific PCR amplification products larger than 300 bp), with no PCR amplification products in the 17 types of nontobacco plant material (Figure 2). The abovementioned primer pairs effectively amplified target PCR products in the 91 types of tobacco material, with no PCR products detected in the 17 types of nontobacco material, indicating that the two primer pairs were specific to *Nicotiana*.

Table 6. Information of *Nicotiana*-specific markers.

Name	F-Seq	R-Seq	Tm ℃	Amplicon bp	NCBI No.
Ntsp027	GTTGTTCGCTTCCCTGATGT	AACCAAAGCAAGCGAAATGT	60	303	AF321137.1
Ntsp151	ATTTGGCTTTGGCTATGGAA	CGGAGACAAGAGACCCAAGT	60	300	AB827951.1

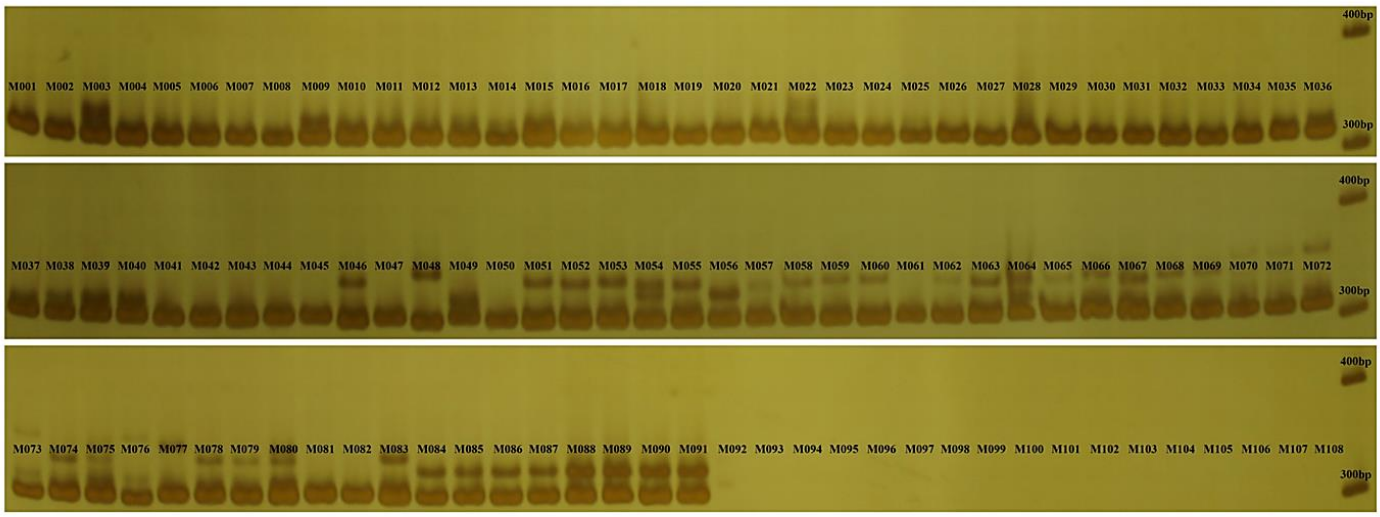

Figure 1. PCR amplification using primers corresponding to the *Nicotiana*-specific marker Ntsp027 in 108 tested materials. Accession nos. M001–M091 were 91 *Nicotiana* materials and M092–M108 were 17 non-*Nicotiana* materials. The rightmost lane is 400 bp DNA marker.

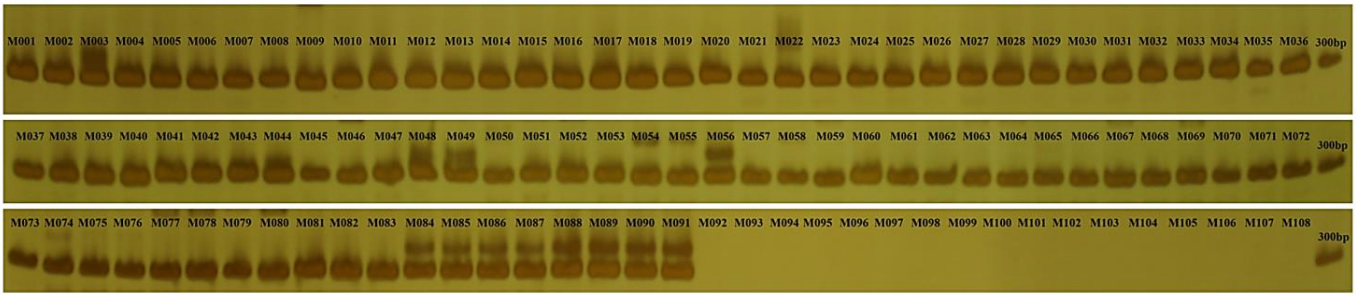

Figure 2. PCR amplification using primers corresponding to of the *Nicotiana*-specific marker Ntsp151 in 108 tested materials. M001–M091 were 91 *Nicotiana* materials and M092–M108 were 17 non-*Nicotiana* materials. The rightmost lane is 300 bp DNA marker.

Identification of Nicotiana*-specific Markers for Tea-Cigarettes*

To identify whether tea-cigarette products contain raw tobacco material, such as cut tobacco, tobacco pieces, or cut stems, the genomic DNA samples of 21 tea-cigarette product material, four positive controls (CK+), and four negative controls (CK-) were amplified via PCR using the tobacco genus-specific markers Ntsp027 and Ntsp151 (Figure 3). For Ntsp027 (Figure 3a), a specific 303 bp band was amplified from the four positive controls (CK+), while no PCR products were amplified from the four negative controls. Only TM20 and TM21 amplified a unique band of 303 bp in the 21 tea-cigarette products; moreover, no PCR products were amplified in the remaining 19 tea-cigarette products. It was consequently inferred that of the 21 tested tea-cigarette products, only TM20 and TM21 contained raw tobacco material. Similarly, based on the tobacco genus-specific marker Ntsp151 (Figure 3b), a 300 bp band was amplified from the four positive controls (CK+), no PCR products were amplified from the four negative controls, a 300 bp band was amplified from TM20 and TM21 in the 21 tea-cigarette products, and no PCR products were amplified from the remaining 19 tea-cigarette products. Therefore, of the 21 tested tea-cigarette products, only TM20 and TM21 contained raw tobacco material.

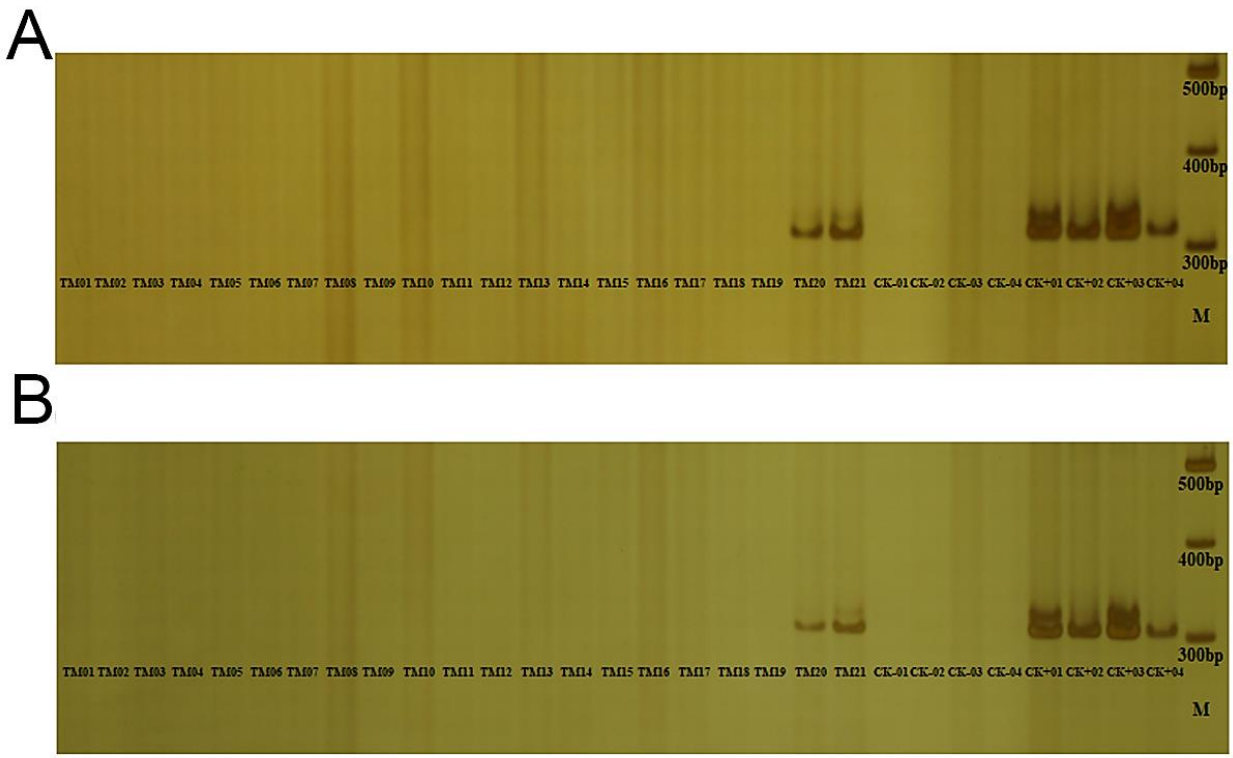

Figure 3. PCR amplification results obtained using A - primers corresponding to the Nicotiana-specific marker Ntsp027 and B - primers corresponding to of the Nicotiana-specific marker Ntsp151 in 21 test tea-cigarettes. TM01–TM21 are 21 types of tea-cigarette products, CK+01–04 are four positive control samples, and CK-01–04 are four negative control samples. The rightmost lane is 500 bp DNA marker.

The Utility of the LAMP Assay in Verifying Tobacco Material

Six temperature levels were used to evaluate the optimal LAMP temperature using five LAMP primers, which were designed based on the Ntsp151 sequence. As shown in Figure 4, all the reaction temperatures manifested a change in color from orange to light green (Figure 4a), which was visible to the naked eye and was accompanied by a typical ladder-like electrophoresis pattern (Figure 4b). However, the fluorescence intensity measurements showed that the optimum reaction temperature was 63 °C (Figure 4c).

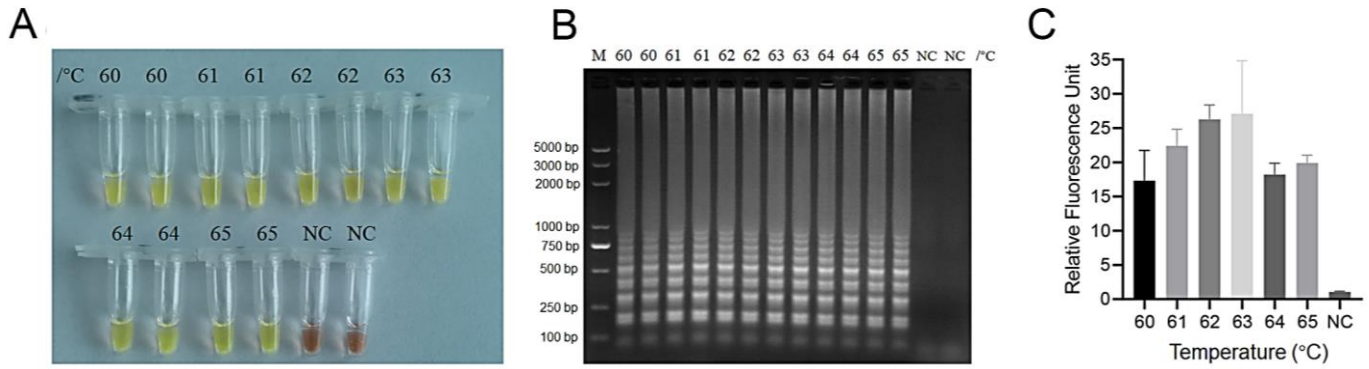

Figure 4. LAMP assay with a temperature gradient from 60 °C to 65 °C. A - Visualization of color changes and B - agarose gel electrophoresis analysis of the LAMP products corresponding to Ntsp151 sequence. C - Fluorescence intensity measurement of LAMP products. NC, negative control; LAMP, loop-mediated isothermal amplification.

Both a color change and a ladder-like electrophoresis pattern were observed in the reaction products incubated from 45 min to 90 min, while short incubation periods of 15 min or 30 min did not result in any amplification products (Figure 5a and b). These findings implied that at least 45 min is required for the incubation time of the LAMP reaction. Measurement of the fluorescence intensity indicated that an incubation time of 60 min was optimal for the LAMP reaction (Figure 5c).

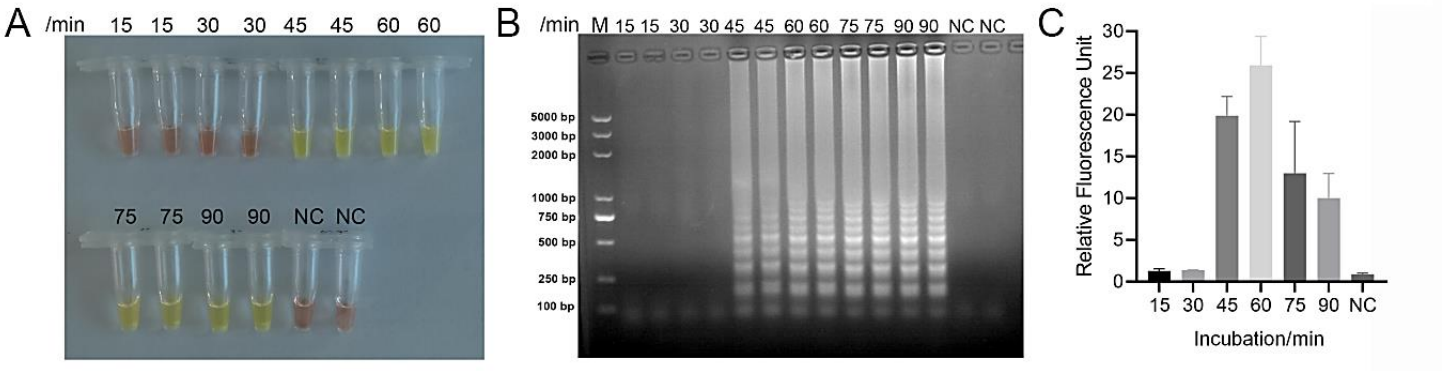

Figure 5. Optimization of incubation time for LAMP assay corresponding to Ntsp151 sequence. A - Visualization of color changes and B - agarose gel electrophoresis analysis of the LAMP products. C - Fluorescence intensity measurement of LAMP products. NC, negative control. LAMP, loop-mediated isothermal amplification.

Seventeen types of nontobacco plant material were subjected to the LAMP reaction to test the specificity of this method. As shown in Figure 6, no amplification products were detected with genomic DNA isolated from the 17 types of non-tobacco plant material, while the positive controls exhibited visible color changes (Figure 6a and b). In addition, 88 of the 91 types of tobacco plant material generated amplification products in the LAMP assay (Table 7), for a detection rate of 96.7%. This result was comparable to the qPCR results, which showed a percentage of 100%. The LAMP assay therefore served as a rapid, easy, and efficient method to distinguish between tobacco and nontobacco plant material.

Table 7. LAMP results and qPCR results of 91 tobacco materials.

Tobacco Samples	N	LAMP		qPCR	
		Positive	Negative	Positive	Negative
Rustica	10	10	0	10	0
Tabacum	7	7	0	7	0
Petunioides	66	63	3	66	0
Cultivated varieties	8	8	0	8	0

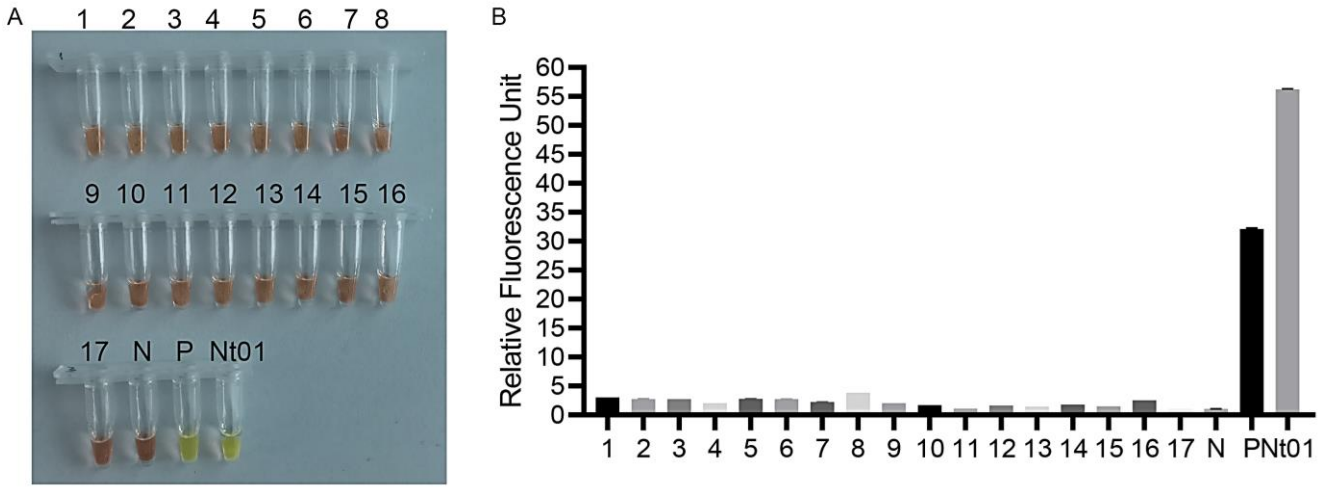

Figure 6. LAMP reaction results of 17 non-tobacco materials obtained using LAMP primers corresponding to Ntsp151 sequence. A - Visualization of color changes and B - fluorescence intensity measurement of LAMP products. N, negative control; P, positive control; Nt01, tobacco material sample; LAMP, loop-mediated isothermal amplification.

In the LAMP assay, the tea-cigarette samples TM20 and TM21 displayed positive results, while the other 19 samples showed no amplification (Figure 7), which was in line with the PCR results obtained using the tobacco genus-specific markers (Figure 2). These results indicate that the LAMP reaction can be adopted as a feasible and easier alternative to PCR for the identification of cigarette samples.

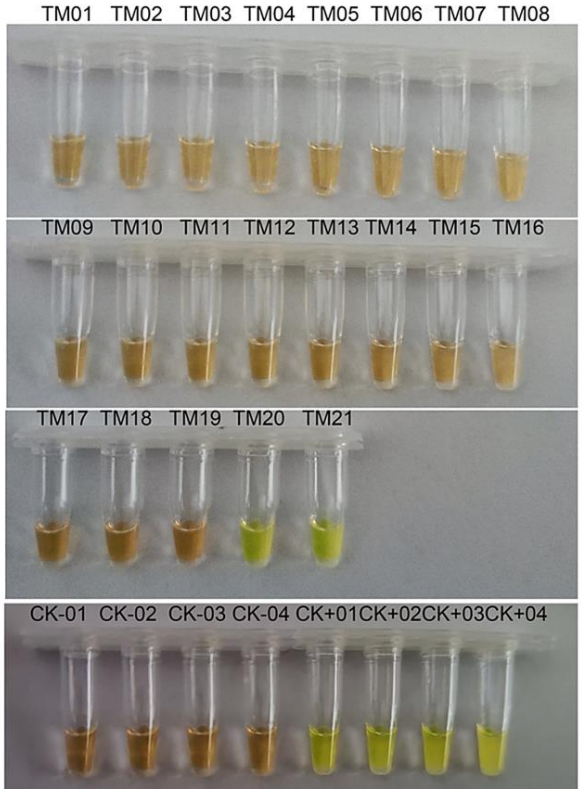

Figure 7. LAMP assay to detect tobacco raw materials in the 21 tea-cigarette samples using LAMP primers corresponding to Ntsp151 sequence. LAMP, loop-mediated isothermal amplification.

Analysis of the Nicotine Content in Tea-Cigarettes

In addition to the direct use of raw tobacco material in tea-cigarette products, there is a possibility of artificial addition of exogenous nicotine (chemically synthesized nicotine). To verify whether the source of nicotine in tea-cigarettes is generated from raw tobacco material or from the addition of exogenous nicotine, GC–MS was used to determine the nicotine content in the main smoke stream of the tested tea-cigarette products. Among the 21 tea-cigarette products, nicotinic components were detected in the main smoke stream of three products, namely, TM04, TM20, and TM21, the contents of which were 0.92 cig.$^{-1}$, 0.94 mg cig.$^{-1}$, and 0.83 mg cig.$^{-1}$, respectively (Table 8). These findings matched those of previous studies regarding the nicotine content of the main smoke streams among common marketed cigarettes (AQSIQ 2005, pp. 1–2).

Table 8. Nicotine content information of 21 tea-cigarette materials.

Sample Name	Nicotine Content mg cig.$^{-1}$	Accessions
Tieguanyin·Yunyan	0.00	TM01
Baicha Chayan·Tujiamei	0.00	TM02
Pu'ercha Chayan·Chazhilong	0.00	TM03
Meicha Chayan·Xiang'amei	0.92	TM04
Fenghuang Shuixian·Mingcun Huangling	0.00	TM05
Longjing Meigui·Mingcun Huangling	0.00	TM06
Jinsi Meigui·Sifangcha	0.00	TM07
Molihuacha·Baoyangcha	0.00	TM08
Tie Guanyin·Hesheng	0.00	TM09
Longjing·Baoyangcha	0.00	TM10
Huangjinya·Baoyangcha	0.00	TM11
Guihua Tea·Baoyangcha	0.00	TM12
Meigui Dahongpao·Hesheng	0.00	TM13
Kafeicha·Chashui·Yanyuan	0.00	TM14
Pibacha·Hesheng	0.00	TM15
Yingtaohuacha·Hesheng	0.00	TM16
Chama Gudao·Chawang	0.00	TM17
Chang'anming·Chawang	0.00	TM18
Pu'er Chenxiang·Chatu	0.00	TM19
Qingfei Jieyanling·Bencaotang	0.94	TM20
Luohanguo Qingfeiling·Hongsheng	0.83	TM21

4. Discussion

Most Solanaceae plants, especially those belonging to the genus *Nicotiana*, contain a special alkaloid, namely, nicotine (Ye et al. 2022). Therefore, DNA sequences of genes involved in nicotine metabolism pathways serve as crucial markers for distinguishing Solanaceae plants from others. Using bioinformatics, forty-six DNA genome sequences were aligned with genomic data from the US NCBI, EMBL, Plant Genome and Systems Biology Bank (PGSB), Solanaceae Genome Data (Sol Genomics Net-work) and unpublished tobacco genome data from the Chinese tobacco industry. In addition to tobacco, other nightshade plants, tea leaves, and even a small number of microorganisms were present in the BLASTn analysis, the results of which were consistent with the identification (Ident. %) and coverage (Cvg. %) to ≥75%. Only nine nicotine-related DNA sequences, including three genes, NtADC, NtA662, and Nt ERF189, could be aligned into the tobacco genome data. A strict BLASTn analysis (Ident. & Cvg. ≥95%) was performed with alignment results still containing tobacco, other Solanaceae plants, and microorganisms. However, the number of genes that were aligned only to the tobacco genome decreased to five. Finally, the structural gene NtADC, which encodes *Nicotiana tabacum* arginine decarboxylase, and the transcription factor gene NtERF189, which encodes *Nicotiana tabacum* ERF189 mRNA for ethylene response Factor 189, were shown to be *Nicotiana* specific following the experimental verification with the 108 types of tested material with candidate *Nicotiana*-specific markers. Therefore, the markers developed based on the DNA sequences of the above two genes were *Nicotiana* specific.

The two *Nicotiana*-specific markers developed herein should theoretically amplify specific target PCR products in *Nicotiana* plants. Moreover, they should not amplify specific target PCR products or produce any PCR amplification products in other organisms (especially plants) except tobacco. To achieve the specificity of the above two markers in *Nicotiana* and eliminate false-positives to the greatest extent possible, the key work of the present study focused on bioinformatics analysis of extensive genomic data, experimental material selection, and marker development of nicotine-related genes; of these, rational selection of experimental material was paramount. The representativeness and quantity of the selected types of tobacco material, as well as nontobacco material commonly used in cigarette-making samples, should be fully considered in this case. The specific methods used were as follows: first, nicotine-related gene markers that were strictly aligned with a large number of published and unpublished genomic data were developed via bioinformatics; second, as many types of *Nicotiana* material as possible with the maximum complete coverage were selected (83 wild species in 11 sections of three *Nicotiana* subgenera and 8 cultivated tobacco cultivars in four types); and for non-nicotine plant material, nine nontobacco material belonging to eight genera in four plant families humans commonly used in daily life (primary crops) and in the irregularities of involved cases were selected. Finally, an optimized plant tissue genome extraction kit was used to exclude microbial DNA; additionally, the annealing temperature during PCR amplification was increased to promote specific PCR amplification. Following the abovementioned bioinformatics analysis, screening, experimental optimization, and verification, two markers that can effectively enable tobacco and nontobacco materials to be distinguished were obtained.

To further verify the effect of two *Nicotiana*-specific markers in the reported cases, we combined two *Nicotiana*-specific markers and GC–MS methods to detect 21 tea-cigarette products collected from actual cases. The definition of a cigarette is currently clearly enumerated in the relevant national standards in China: it refers to tobacco products made of cut tobacco wrapped in cigarette paper and smoked by burning (AQSIQ 2015, p. 1). Its core element is cut tobacco, that is, raw tobacco material derived from plants. The results for the analysis of 21 tea-cigarette products using molecular markers specific to the tobacco genus (Ntsp027 and Ntsp151) showed that two tea-cigarette products, namely, TM20 (Qingfei Jieyanling·Bencaotang) and TM21 (Luohanguo Qingfeiling·Hongsheng), contained raw tobacco material derived from plants. Therefore, the two samples can be classified as cigarettes because they meet the definition of "cigarette products". Therefore, these two "tea-cigarette" products should be strictly regulated by the tobacco monopoly in accordance with the tobacco monopoly law. The remaining 19 tea-cigarette products did not contain "tobacco products" and therefore should be considered "nontobacco products" under the law of the regulatory authorities of market supervision.

Generally, the chemical method (continuous flow method) (STMA 2002, pp. 1–3) is used to determine whether the material to be tested contains total plant alkali components (calculated based on nicotine content). This method is integral to tobacco anti-counterfeiting and anti-smuggling operations in China. Specifically, if the samples to be tested are judged to be tobacco material, they are considered "tobacco products". However, as this method determines the total amount of a class of alkaloids rather than the characteristic substances of nicotinic tobacco, there is a possibility of false-positive results. In the present study, nicotine components in the main smoke stream of the 21 tested tea-cigarette products were characterized using GC–MS. Nicotine components were detected in three tea-cigarette products, namely, TM04 (Meicha Chayan·Xiang'amei), TM20 (Qingfei Jieyanling·Bencaotang), and TM21 (Luohanguo Qingfeiling·Hongsheng), indicating that the three tea-cigarette products are nicotine-containing material. Additionally, the source of nicotine might be plant-derived raw material, that is, tobacco plants, nicotine-containing Solanaceae plants, or exogenously added nicotine, which are chemically synthesized nicotine or tobacco extracts. Therefore, whether the source is a "tobacco product" cannot be accurately determined solely based on the presence of nicotine components in the material tested. Consequently, nicotine detection based on the GC–MS method cannot be used to scientifically characterize the above three tea-cigarette products. To overcome the defect of inaccuracy in the GC–MS method, we used the novel method of tobacco genus-specific markers to accurately identify 21 tea-cigarette products at the genomic DNA level. The results indicated that only two tea-cigarette products, TM20 (Qingfei Jieyanling·Bencaotang) and TM21 (Luohanguo Qingfeiling·Hongsheng), could be classified as "tobacco products" rather than the three tea-cigarette products detected with the GC–MS method. This indicates the scientific rigor and precision of

being able to distinguish "tobacco products" and "nontobacco products" in anticounterfeiting and antimasking operations in the tobacco industry. Moreover, this technique enables the inaccuracy of the GC–MS method to be avoided.

Loop-mediated isothermal amplification (LAMP) technology was designed with the aim of improving the sensitivity and specificity of nucleic acid amplification (Notomi et al. 2000). As the LAMP reaction can be completed within a short time span without thermal cycling, it has been widely applied in nucleic acid analysis (Fu et al. 2011). For example, LAMP assays have been developed to identify genetic modification events in maize (Chen et al. 2011). The LAMP assay has also been used for the authentication of plant species, such as the medicinal plant *Catharanthus roseus* (L.) (Chaudhary et al. 2012). In the present study, the LAMP reaction was applied to identify tobacco material, and an incubation time as short as 45 min was sufficient to render a visual change. Moreover, the LAMP results showed a high detection rate of 96.7%, which confirmed its reliability for further application. Additionally, the LAMP results were consistent with the PCR results obtained when using the molecular markers for testing 21 tea-cigarette samples to determine whether they contained raw tobacco material derived from plants. Therefore, LAMP amplification is productive, efficient, and optimal for the authentication of tobacco plant material based on tobacco-specific markers.

5. Conclusions

For the first time, samples obtained from illegal goods/operations in the tobacco industry were identified and detected at the molecular level (DNA). Although there are no relevant prior studies that serve as a benchmark for comparison, either in China or globally, our method has advantages over existing sensory and chemical detection methods. Since the markers and PCR amplification product sequences used in the present method are *Nicotiana*-specific and the abovementioned DNA sequences are well conserved and have high stability in vivo, this method enables the influence of external factors, including human activity, environmental influence, instruments, and equipment, to be effectively avoided. Therefore, the present study established a scientific, efficient, simple, and highly operable method for detecting tobacco and nontobacco material. Furthermore, the *Nicotiana*-specific marker Ntsp151 was developed into a colorimetric loop-mediated isothermal amplification kit for the rapid, low-cost, real-time molecular testing of raw tobacco material in seized illegal products. In the present study, we developed tobacco-specific molecular (DNA) markers that were applied in the identification and detection of raw tobacco material. This approach could compensate for the defects in anticounterfeiting and antimasking work in the tobacco industry and improve the scientific rigor and accuracy of these methods while enhancing the methods of identifying and detecting the raw material incorporated in tobacco manufacture.

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