食品藥物研究年報. 10：35-49 2019
Ann. Rept. Food Drug Res. 10 ：35-49 2019

Development of Real-Time PCR Detection Methods on Genetically Modified Apple

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ABSTRACT

The genetically modified (GM) apple, which developed by Okanagan Specialty Fruits Inc. (OSF) and called Arctic apple, has a modified characteristic of browning resistance. Arctic apple's browning resistance is achieved by suppressing the expression of polyphenol oxidase (PPO) through the method of RNA interference. This methodology can avoid the oxidation of apples by PPO catalyzed and enzymatic browning reduced. Nowadays, Arctic Golden Delicious apple event GD743 and Arctic Granny Smith apple event GS784 have been approved and legally marketed in the United States and Canada. They are mainly sold in the form of fresh bagged slices and dried apples in the United States. As OSF's Arctic apples have not yet applied for GM food registration in Taiwan, Arctic apples are still unauthorized GM foods in Taiwan. In order to avoid food safety issues caused by international non-synchronized GM food approval, this study developed GM apple PCR and real-time PCR methods to meet the needs for testing. The constructive-specific PCR and real-time PCR methods that focused on the designated region between pSR7 gene and the NOS terminator can be used to detect Arctic apples. The Taiwan Food and Drug Administration (TFDA)'s existing CaMV 35S and NOS qualitative methods are also suitable for GM apple screening as well. By further analyzing the Artic apple's transgenic genes and its flanking regions, we have confirmed the GD743's full transgenic gene sequences. In addition, we have also found two different 5' flanking junction region sequences that come from 2 different copies of the insertion fragments in GD743. One of these two fragments is located on chromosome 14 of the apple and its uniqueness can be used for GM apple testing. The transgenic sequences of 3' junction on apple chromosome 14 were further confirmed. Finally, we have developed and established the methods for detecting GD743 apple based on the event-specific levels of 5' and 3' junction.

Key words: genetically modified, apple, real-time PCR

INTRODUCTION

In 2018, the commercial planting area of genetically modified crops has reached 191.7 million hectares worldwide[1]. Genetically modified soybeans, corn, cotton and rapeseed are the main genetically modified crops. In addition, there are other genetically modified crops such as beets, alfalfa and papaya. Recently, genetically modified potatoes and apples are progressively increasingly grown across the United State. The genetically modified potatoes and apples have been approved and legally marketed in the United States. Taiwan has started the regulation of genetically modified food since 2001. Genetically modified soybeans and corn should be registered for the purpose of food usage. After the revision of the Act Governing Food Safety and Sanitation in 2014, all genetically modified food, not just soybeans and corn, need to go through the processes of registration and health risk assessment. It can be used as a food ingredient after completing the assessment and getting the registration permit from Ministry of Health and Welfare. At present, two events of genetically modified potatoes have been registered for food in Taiwan, and Taiwan Food and Drug Administration (TFDA) has started to establish its specified testing methodology and techniques. However, genetically modified apples have not yet been applied for the food registration, and no known method is used to detect them. Due to the potential risks of exposing to the unregistered genetically modified products circulating around the world market, this study will establish a genetically modified apple detection method for the official use.

Genetically modified apples were developed by Okanagan Specialty Fruits (OSF) and known as Arctic apples. It has a characteristic of browning resistance that prevents the apples from browning[2]. The browning reaction of the apple can be divided into two types: primary and secondary[3]. The primary browning is the oxidation after the phenolic compound and oxygen are catalyzed by the polyphenol oxidase (PPO). It is caused by the cell rupture due to bruising and cutting on apples. The secondary browning refers to the discoloration caused by the decomposition of fruits because of the formation of fungi and bacteria during the fruit rotten process. The fundamental modification characteristics of Arctic apples is the inhibition on the primary browning. The technology of OSF's inhibition of apple browning is derived from the research of potato browning inhibition by Commonwealth Scientific and Industrial Research Organisation (CSIRO). OSF was authorized by CSIRO to apply anti-PPO technology on genetically modified apples to reduce the browning[4]. According to the document published on the United States Department of Agriculture (USDA) Animal and Plant Quarantine Service (APHIS)[5], Arctic apples are transferred by using Agrobacterium. It contains four apple PPO family genes (*PPO2*, *GPO3*, *APO5*, *pSR7*, referred to as PGAS) carrier on GEN-03 plasmid and being transferred to different varieties of apples such as Golden Delicious, Granny Smith, Fuji and Gala. The genetically modified apples containing PGAS transgenic genes can produce small RNA to inhibits apple PPO expression by RNA interference and reduce the browning when the fruit has a cut or bruise.

At present, the US approved genetically modified products for sale are Golden Delicious apple GD743 and Granny Smith apple GS784[6],

which are mainly sold in the form of fresh bagged slices and dried apples. Fuji Apple NF872 also obtained US FDA's approval in April this year[7], and is expected to be sold in the US market soon. Although the Arctic apples are already sold in the US and Canada regions, the OSF Company has not yet applied any Arctic apples for genetically modified food registrations in Taiwan. Arctic apples are still unauthorized genetically modified foods in Taiwan. In order to avoid the public concerned about food safety and prevention of unanticipated international trade circulation, this study used the Golden Delicious apple snack sold in the United States as reference materials, applied the available knowledge to establish genetically modified apple PCR and real-time PCR test methods, and confirmed the existing CaMV 35S and NOS screening methods[8] to meet possible testing needs. In addition, this study further analyzes the transgenic gene in the flanking junction region of the apple genome, and establishes event specific detection methods for the specificity of the Arctic Golden Delicious apple GD743.

MATERIALS AND METHODS

I. Chemicals

Chloroform, isopropanol, tris (hydroxymethyl) aminomethane (Tris-base) (Merck, Germany), Hexadecyltrimethyl-ammonium bromide (CTAB) (Sigma, USA), Agarose (Lonza, USA). Chloroform was of reagent grade, others were of biological grade.

II. Sample

The non-genetically modified (NonGM) apple samples are New Zealand Granny Smith apples and Fuji apples from the consumer market. The genetically modified apple used as reference material is "Arctic Golden ApBitz Dried Apples - Apple Snack" sold by Amazon.com.

III. Equipments

PCR thermal cyclers: Applied Biosystems GeneAmp PCR System 9700 (Thermo Fisher Scientific, USA), real-time PCR: QuantStudio 12K Flex Real-Time PCR System or ViiA 7 Real-Time PCR System (Thermo Fisher Scientific, USA), NanoDrop microvolume spectrophotometers: NanoDrop ND-1000 (NanoDrop Technologies, USA), milling: Retsch MM400 (Retsch, Germany), high speed micro refrigerated centrifuge: KUBOTA-3740 (KUBOTA Corporation, Japan), mini gel electrophoresis equipment: Mupid-2 (Advance, Japan); electrophoresis gel imaging system: AlphaImager HP System (ProteinSimple, USA).

IV. DNA preparation and purification

Genomic DNA was extracted from samples using the DNeasy Mericon Food Kit (QIAGEN GmbH, Germany). QIAquick PCR purification kit and QIAquick Gel Extraction purification kit were used for DNA purification (Qiagen GmbH, Germany) following the manufacturer's instructions.

V. PCR primers, probes and reagents

The primers and probes used in this study (Table 1 and Table 2), except referring from references, were designed by Primer Express software version 3.0.1 (Thermo Fisher Scientific, USA). The setting of "Parameters" used for

Table 1. PCR primers used in this study.

Primer	Sequence 5'-3'	Specificity	Amplicon(bp)	Reference
pSR7-F	AAgCTTTTCCTTTCCACCgCATgT	*pSR7* /sense	555	(5)
NOSTERM	TATgATAATCATCgCAAgAC	TNOS /antisense		(5)
GD-nLB-1F	AgCCCgAgATAgggTTgAgT	Left border /sense	488	This study
GD-PnosT-1R	gAACCTgCgTgCAATCCATC	PNOS /antisense		This study
p-nos-F2	TTCCCCTCggTATCCAATTAgAg	PNOS /sense	1313	This study
MdG3R1	CCggCAACAggATTCAATCTTA	TNOS /antisense		This study
Primer-4	TCTCATgCTggAgTTCTTCg	*npt*II /sense	1514	(5)
Primer-6	CAgATCggACCATCACATCA	P35S /antisense		(5)
M13pUC	AgCggATAACAATTTCACACAgg	vector /sense	734	This study
35S-cr4	TCCTCTCCAAATgAAATgAACTTCC	P35S /antisense		(11)
Primer-3	TTCgCAAgACCCTTCCTCTA	P35S /sense	2055	(5)
MdG3R1	CCggCAACAggATTCAATCTTA	TNOS /antisense		This study
Primer-3	TTCgCAAgACCCTTCCTCTA	P35S /sense	936	(5)
GPO3-R 76	CCTggATCTggTTCAgTgC	*GPO3* /antisense		(5)
GD-GPO3-2F	CAACggCACggATgAAACAA	*GPO3* /sense	1263	This study
MdG3R1	CCggCAACAggATTCAATCTTA	TNOS /antisense		This study
MdG3F2	gCggTgACCCgAACCA	*pSR7* /sense	281	This study
NOSter 2-3	CgCTATATTTTgTTTTCTATCgCgT	TNOS /antisense		(12)
Nt2-FR-2F	ATACAgAgggTATAAAATCgAACCT	apple genome /sense	902	This study
Primer-1	CCAAACgTAAAACggCTTgT	PNOS /antisense		(5)
Nt2-FR-2F	ATACAgAgggTATAAAATCgAACCT	apple genome /sense	496	This study
Nt2-FR-1R	TgCAAggCTgAgAgTAACCg	apple genome /antisense		This study
MdG3F1	CAgACCCACCACgAAgACATg	*pSR7* /sense	906	This study
Chro14-1F	gggAgACgTACTTCCTCAgC	apple genome /antisense		This study
Ct2-FR-1F	ACgTCgTgACTgggAAAACC	vector /sense	503	This study
Chro14-1F	gggAgACgTACTTCCTCAgC	apple genome /antisense		This study
NOSter 2-5	gTCTTgCgATgATTATCATATAATTTCTg	TNOS /sense	790	(12)
Chro14-1F	gggAgACgTACTTCCTCAgC	apple genome /antisense		This study

a. P35S: CaMV 35S promoter; PNOS: NOS promoter, TNOS: NOS terminator.

primers and probes were generated automatically by the software design's default values. Following the manufacturer's software guide, 58 °C to 60 °C Tm was for primers; 68 °C to 70 °C Tm was for probes. The sizes of amplicon length were adjusted by request. Briefly, HPLC-grade primers were synthesized by TIB Molbiol (Berlin, Germany). The 5' end of probes was labeled with 6-carboxy-fluorescein (6-FAM), and the 3' end was labeled with a quencher dye (TAMRA, BBQ and BHQ1 by TIB or double-quenched probes ZEN/Iowa Black FQ by Integrated

Table 2. Real-time PCR primers and probes used in this study.

Primer/Probe	Sequence 5'-3'	Specificity	Amplicon (bp)	Reference
CaMV 35S promoter screening				
35S-PF	CCgACAgTggTCCCAAAgAT	P35S /sense	107	(8)
35S-PR	AgTggAgATATCACATCAATCCACTT	P35S /antisense		
35S-PP	FAM-CCCACCCACgAggAgCATCg-XT-PH (X=TAMRA)			
NOS terminator screening				
NOS-TF	gTCTTgCgATgATTATCATATAATTTCTg	TNOS /sense	151	(8)
NOS-TR	CgCTATATTTTgTTTTCTATCgCgT	TNOS /antisense		
NOS-TP	FAM- AgATgggTTTTTATgATTAgAgTC CCgCAA-TAMRA			
Construct-specific (pSR7-NOS terminator)				
MdG3F1	CAgACCCACCACgAAgACATg	*pSR7* /sense	118	This
MdG3R1	CCggCAACAggATTCAATCTTA	TNOS /antisense		study
MdG3p1	FAM- AACTTCTAC/ZEN/TCCgCg gCTCgACCC-IBFQ			
GD743 5' junction region				
5GD55F	CTCTgATTgATTgATgATgCgTgTA	apple genome /sense	115	This
5GD169R	gTgAAAAgAAAAACCACCCCAgTAC	vector /antisense		study
5GD85p	FAM- CCggTTACT/ZEN/CTCAgC CTTgCAgTTTAgACA -IBFQ			
GD743 3' junction region				
GDCh14CF	AACTTgATTTgggTgATggTTCA	vector /sense	118	This
GDCh14CR	gAATCgAATCggAgAAgATCgA	apple genome /antisense		study
3GD6551p	FAM- TCAgTgTTT/ZEN/gAATTT ACgACCgCggAA -IBFQ			
Apple internal control				
AppleF	ggAATATgAACgAAAgAgCg	ITS1 /sense	103	(9)
AppleR	ATCCgTTgCCgAgAgTCgTT	ITS1-5.8S /antisense		
AppleP Long [a]	FAM- TgCgTCgTCgTCTTCgATAAA AgTCA-BBQ			
18SrRNA				
18SrRNA 2-5'	TgTTggCCTTCgggATCggAgTA	18SrRNA /sense	111	(10)
18SrRNA 2-3'	gCTTTCgCAgTTgTTCgTCTTTCA	18SrRNA /antisense		
18SrRNA-2-Taq	FAM-TCgggggCATTCgTATTTCATAgT CAgA-BHQ1			

a. The TaqMan probe (AppleP Long) was slightly modified because of lower Tm value in original sequence.

b. FAM: 6-carboxy-fluorescein; TAMRA: 6-carboxytetramethyl-rhodamine; XT-PH (X=TAMRA): internal label quencher; /ZEN/: internal quencher; IBFQ: Iowa Black Fluorescent Quencher; BBQ: BlackBerry Quencher; BHQ1: Black Hole Quencher-1.

c. P35S: CaMV 35S promoter; TNOS: NOS terminator; ITS: internal transcribed spacer 1; 5.8S: 5.8S ribosomal RNA gene; 18SrRNA: 18S rRNA gene.

DNA Technologies, USA). FastStart Taq DNA Polymerase (Roche Diagnostics GmbH, Germany) and dNTP (PROtech Technologies Inc., Taiwan) were used for PCR. TaqMan Universal PCR Master Mix (Thermo Fisher Scientific, USA) was used for real-time PCR.

VI. PCR reactions and products analysis

PCR was performed in a total volume of 50 μL, containing 5 μL of DNA (10 ng), 2 μL of each primer (5 μM), 4.0 μL of dNTP (2.5 mM), 5 μL of 10-folds PCR reaction buffer with 20 mM $MgCl_2$ buffer solution and 0.5 μL of FastStart Taq DNA Polymerase (5U/μL). PCR was carried out under the following conditions: denaturation at 95°C for 4 min, 40 polymerization cycles (95°C for 30 sec or 1 min, the appropriate annealing temperature for 30 sec or 1 min, and 72°C for 30 sec or 1 min), a final extension of 72°C for 7 min and then cool down. The PCR products were analyzed using a 1.5% or 2.5% agarose gel electrophoresis. The sequencing of the PCR product was serviced by Genomics (New Taipei City, Taiwan).

VII. Real-time PCR and analysis

Real-time PCR was performed in a TaqMan probe system using the QuantStudio 12K Flex Real-Time PCR System (QS12K) or ViiA 7 Real-Time PCR System (ViiA7). Each reaction contains 25 μL of reagents, including 1.25 μL of 5 mM primers, 1.7 μL of 3.3 mM probe, 12.5 μL of TaqMan Universal PCR Master Mix, 5 μL of DNA (~50 ng), and 3.3 μL of H_2O. The reactions were performed using an initial incubation at 50˚C for 2 min, 95°C for 10 min, followed by 45 cycles of 95°C for 15 sec and annealing and extension at the appropriate temperatures for 1 min at 60°C (using a thermal cycler protocol in the standard mode). Real-time PCR results were analyzed using QuantStudio 12K Flex software v1.2.2 for QS12K or QuantStudio Software V1.3 for ViiA7. The threshold of Ct analysis was set at 0.064 for apple internal control; 0.128 for others detection, and the manual baseline was between 3 and 15.

VIII. Analysis flanking region of transgenic genes in GM apple genome

The 5’ and 3’ flanking region sequences of transgenic fragments identified through PCR libraries were carried out using the Universal GenomeWalker 2.0 (Takara Bio, USA) according to the manufacturer’s instructions. The PCR products amplified from the libraries were sequenced, and used the BLAST to confirm the identity of genome sequences to determine whether they were transgene or flanking in the National Center for Biotechnology Information (NCBI) genome databases.

RESULTS AND DISCUSSION

I. PCR detection method of Arctic apples

Refer to the document submitted by OSF on the USDA APHIS’s publication[6], the tissue of the apple gene transfer strain uses the pSR7-F and NOSTERM primer pair (table 1) to detect whether it contains the transgenic gene of GEN-03 plasmid. The PCR amplified region of this primer pair is between the pSR7 gene and the NOS terminator and it can be used for designing the construct-specific detection method for Arctic apple. Use pSR7-F and NOSTERM primer pair

to amplify the DNA extract from Arctic Golden Delicious apple snack (GD743), NonGM Granny Smith apples and NonGM Fuji apples. Only GD743 was amplified product of 555 bp (figure 1a). NonGM Granny Smith apples, NonGM Fuji apples and no template control (NTC) were consistent with expectations without amplification products (figure 1a). After confirmed the sequence of 555 bp amplicon, we used this sequence to develop detection method of construct-specific PCR and real-time PCR. We designed the primer pair MdG3F2 and MdG3R1 (table 1) can amplify 131 bp only in GD743 (figure 1b), others did not reveal on any product. It showed the specificity of construct-specific detection. We also found the internal control target gene which referred from reference, the primer pair Apple F and Apple R (table 1) were specificity for internal transcribed spacer (ITS) in apples[9]. All apple samples presented 103 bp products on gel and NTC did not have amplification (figure 1c). This primer pair is routinely used to confirm the endogenous control for apple product's specificity detection.

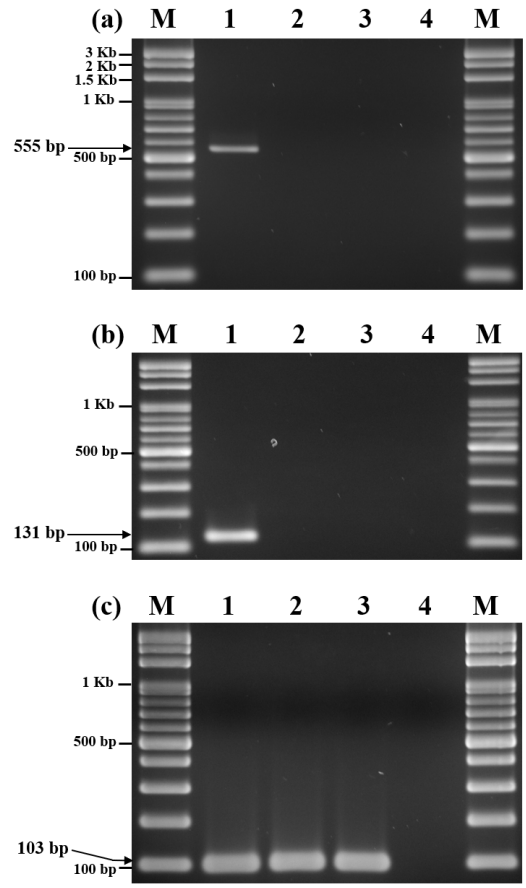

Figure 1. Electrophoresis analysis for PCR products amplified from GD743 GM apple and NonGM apples.
(a) Construct-specific PCR (between *pSR7* and NOS terminator region, primers: pSR7-F /NOSTERM, 555 bp)
(b) Construct-specific PCR (between *pSR7* and terminator region, primers: MdG3F2/ MdG3R1, 131 bp)
(c) Apple internal control gene PCR (primers: Apple F/Apple R, 103 bp)
M: 100 bp DNA ladder marker; Lane 1: GD743 Arctic Golden Delicious apple snack; Lane 2: NonGM Fuji apple; Lane 3: NonGM Granny Smith apple; Lane 4: NTC (No template control)

II. Application of real-time PCR on screening and construct-specific detection

Before the GD743 reference material has been obtained, we expected that the TFDA CaMV 35S and NOS real-time PCR method[8] could be used to screen the Arctic apples based on the structure of the GEN-03 vector. 18S gene and apple internal control gene was used to confirm the eukaryotic and apple genome in sample DNA. The TaqMan probe (AppleP Long, table 2) for detecting the internal control gene of apple was slightly modified according to the literature sequence[9], and the length of the probe sequence was increased to reach the Tm value required for the reaction. When we used above four real-

time PCR method (CaMV 35S and NOS[8], apple internal control[9], 18S[10]) to test all apple samples, all amplification plots revealed positive response in GD743 GM apple (figure 2a-d). NonGM Granny Smith apples and NonGM Fuji apples showed positive response in 18S and apple internal control testing, CaMV 35S and NOS testing were negative. The results can confirm that the methods of CaMV 35S and NOS real-time PCR are suitable to screen the GM apple containing GEN-03 transfer genes.

The 131 bp PCR product between the *pSR7* and NOS terminator can identify GD743 GM apple for construct-specific test (figure 1b). We design the TaqMan probe in this area for construct-specific real-time PCR testing. The combination of primer pair MdG3F1 and MdG3R1, and TaqMan probe MdG3p1 (table 1) can produce the best fluorescence amplification plot for GD743 testing (figure 3a). NonGM Granny Smith apples and NonGM Fuji apples showed negative results, and did not generate amplification plots. The amplification plots of apple internal control showed positive results in all apple samples (figure 3b). This real-time PCR method can detect the other GM apples if they have same sequences region between *pSR7* and NOS terminator.

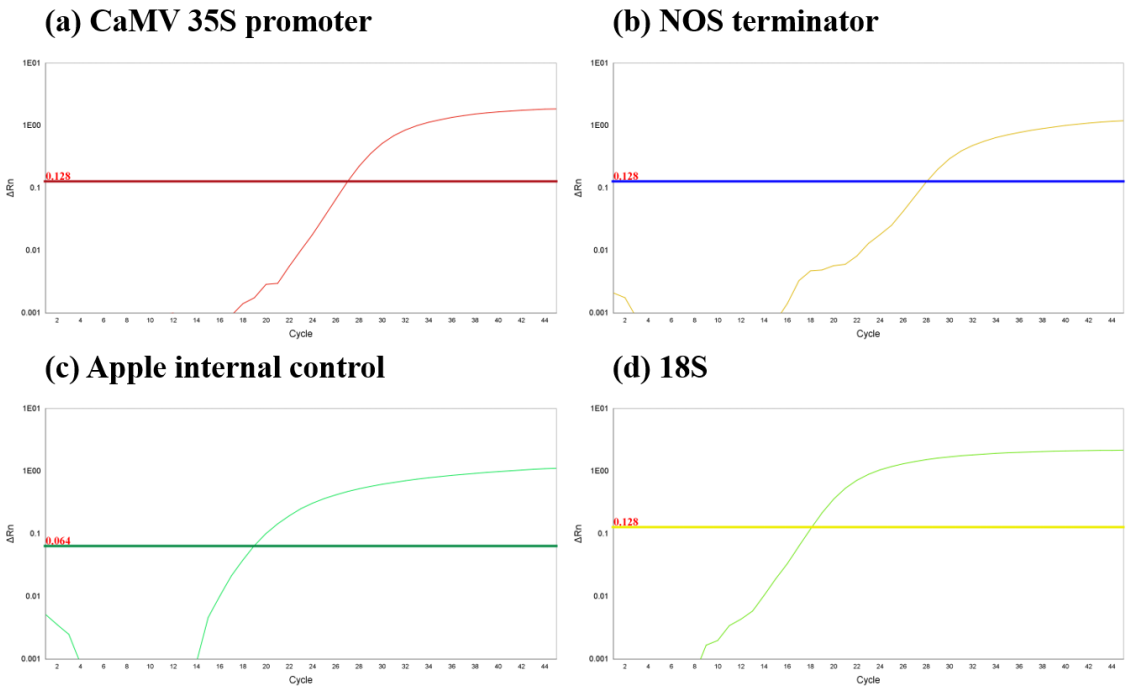

Figure 2. Real-time PCR amplification plots for GD743 GM apple screening.
(a) Real-time PCR for CaMV 35S promoter detection
(b) Real-time PCR for NOS terminator detection
(c) Real-time PCR for apple internal control detection
(d) Real-time PCR for 18S gene detection

III. Analysis of insertion gene and junction regions in GD743

In order to develop event-specific real-time PCR method for GD743 testing, we designed the PCR primer pair (table 1) to analyze the GD743 transgenic gene using the reference from the publish document of OSF from APHIS[6], search and align the nucleotide sequences at NCBI. The eight groups of PCR amplification products (fragment No.1 to No.8) covered the major regions of the transgenic genes (figure 4), the sequenced fragment is approximately 5.2 kb.

The Universal GenomeWalker 2.0 kit was used for libraries preparing to explore the 5' and 3' junction region sequences of GD743. The PCR library fragments larger than 500 bp were selected for sequence analysis. One of fragments were probably located on the 5' junction, the length of fragment No.9 is 1205 bp (figure 5a). The sequences of fragment No.9 was identified using Nucleotide BLAST at NCBI. It can be divided into 3 regions: 507 bp of unknown sequence, 210 bp of upstream of GEN-03 vector sequence, and 488 bp of fragment No.1 (figure 4). The 507 bp unknown sequence was further aligned with whole-genome shotgun contigs sequence database of *Malus domestica* at NCBI-BLAST. The full length of 507 bp was 100% identical with Golden Delicious isolate X9273 #13 chromosome 14 genome (GenBank: MJAX01000015). Two sets of PCR reaction were used to confirm the fragment No.9 located on chromosome 14 of apple. The primer pair Nt2-FR-2F and Primer-1 (table 1) was expected to amplify 920 bp product (fragment No.10), which was being amplified from the 5' junction of apple chromosome 14 genome to NOS promoter in GD743 (figure 5a). The other primer pair Nt2-FR-2F and Nt2-FR-1R was expected to amplify 496 bp product (fragment No.11) for apple chromosome 14 genome (figure 5a). To analyze GD743 and NonGM apples, only GD743 revealed 920 bp product of fragment No.10 (figure 5b). All apple DNA can amplify 496 bp product of fragment No.11 on apple genome (figure 5c). This indicates that fragment No.10 is included in the 5' flanking region of the Arctic Golden Delicious apple GD743, and we can use it to design the method of event-specific real-time PCR.

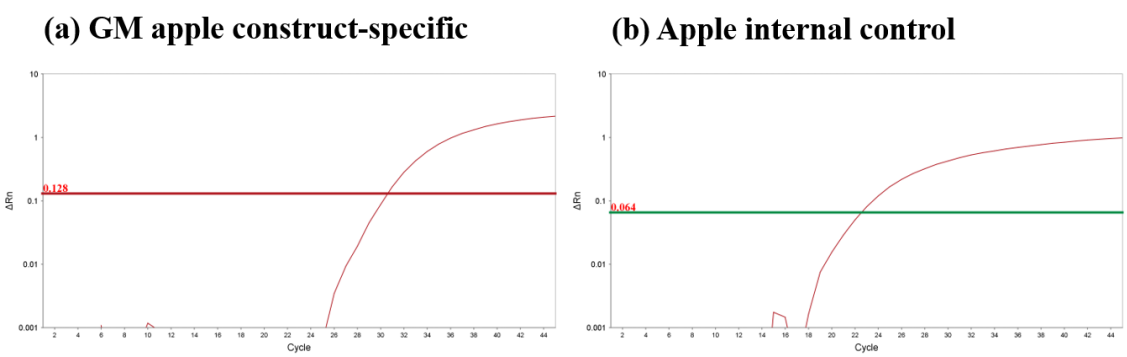

Figure 3. Real-time PCR amplification plots for GD743 GM apple detection.
(a) Real-time PCR for construct-specific (between *pSR7* and NOS terminator region, primers: MdG3F1/MdG3R1)
(b) Real-time PCR for apple internal control (primers: Apple F/Apple R)

To further quickly explore the 3'junction nucleic acid sequence of the transgenic gene located on apple chromosome 14, we designed primers directly on the upstream and downstream (± 250 bp) of previous 507 bp apple chromosome 14 nucleic acid sequence (GenBank: MJAX01000015). Those primers were PCR reacted with the primer MdG3F1 or primer NOSter 2-5 located in the *pSR7* or NOS terminator region of the transgenic gene. The primer pair MdG3F1 and Chro14-1F amplified 906 bp product (fragment No.12, figure 6a). This fragment was verified as 3'junction of GD743 after sequencing analysis, including *pSR7*, NOS terminator, GEN-03 vector sequence, and chromosome 14 genome of apple (figure 6). The 3'

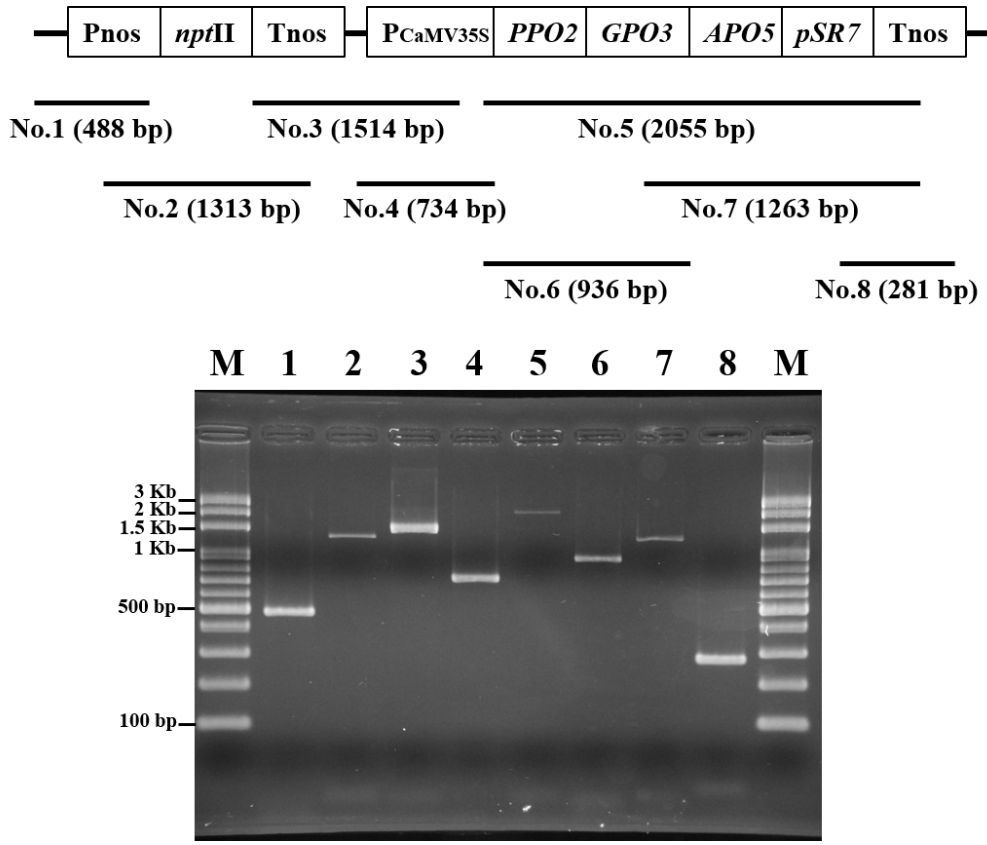

Figure 4. Electrophoresis analysis of PCR amplification from GD743 GM apple.
M: 100 bp DNA ladder marker,
Lane 1: No.1 (primers: GD-nLB-1F/GD-PnosT-1R, 488 bp)
Lane 2: No.2 (primers: p-nos-F2/MdG3R1, 1313 bp)
Lane 3: No.3 (primers: Primer-4/Primer-6. 1514 bp)
Lane 4: No.4 (primers: M13pUC/35S-cr4, 734 bp)
Lane 5: No.5 (primers: Primer-3/MdG3R1, 2055 bp)
Lane 6: No.6 (primers: Primer-3/GPO3-R 76, 936 bp)
Lane 7: No.7 (primers: GD-GPO3-2F/MdG3R1, 1263 bp)
Lane 8: No.8 (primers: MdG3F2/NOSter 2-3, 281 bp)

junction region was confirmed by PCR reaction in two different regions. The primer Ct2-FR-1F located on GEN-03 vector sequence and primer Chro14-1F located on chromosome 14 amplified 503 bp product (fragment No.13) for GD743. The primer pair NOSter 2-5 and Chro14-1F amplified 790 bp product (fragment No.14) for GD743 (figure 6a-b). NonGM Granny Smith apples, NonGM Fuji apples and NTC revealed negative results without any PCR products (figure 6b). The fragment No.13 demonstrated the insertion sequence of 3' junction on chromosome 14, this

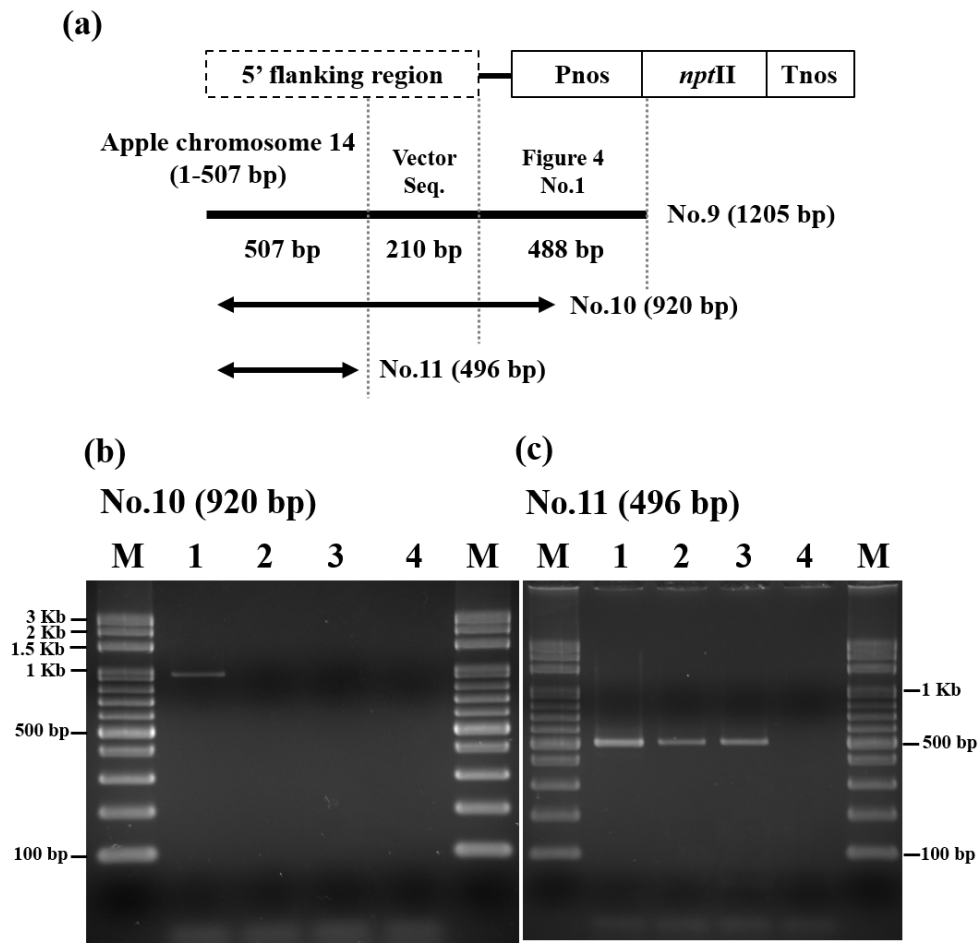

Figure 5. Analysis the 5' junction region of GD743 on chromosome 14.
(a) Schematic genetic structure for 5' junction region of GD743
(b) Electrophoresis analysis of fragment No.10 PCR amplification (primer: Nt2-FR-2F/Primer-1)
(c) Electrophoresis analysis of fragment No.11 PCR amplification (primer: Nt2-FR-2F/Nt2-FR-1R)
M: 100 bp DNA ladder marker; Lane 1: GD743 Arctic Golden Delicious apple snack; Lane 2: NonGM Fuji apple; Lane 3: NonGM Granny Smith apple; Lane 4: NTC

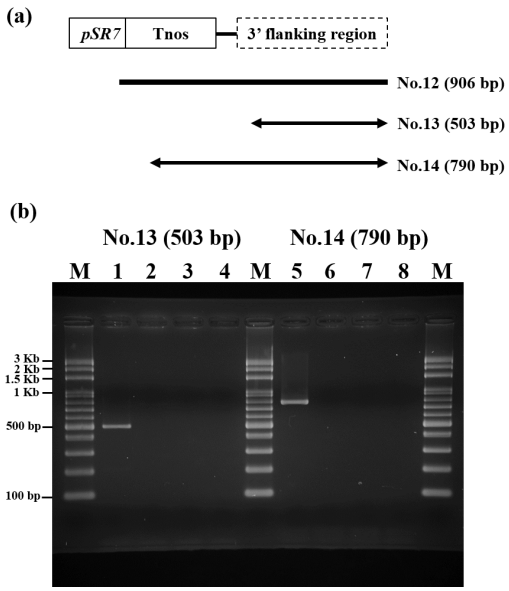

Figure 6. Analysis the 3' junction region of GD743 on chromosome 14.

(a) Schematic genetic structure for 3' junction region of GD743

(b) Electrophoresis analysis of fragment No.13 PCR amplification (primers: Ct2-FR-1F/Chro14-1F, Lane 1 to Lane 4) and fragment No.14 PCR amplification (primers: NOSter 2-5/Chro14-1F, Lane 5 to Lane 8)

M: 100 bp DNA ladder marker; Lane 1 and 5: GD743 Arctic Golden Delicious apple snack; Lane 2 and 6: NonGM Fuji apple; Lane 3 and 7: NonGM Granny Smith apple; Lane 4 and 8: NTC

will provide more information to develop another event-specific method of real-time PCR for GD743 testing.

We also found another PCR library fragment from GenomeWalker, it was also located on the 5' junction of insertion gene. The length of this fragment was 705 bp, like fragment No.10 composed of three parts: 68 bp of unknown sequence, 149 bp of upstream of GEN-03 vector sequence, and 488 bp of fragment No.1 (data not show). The 68 bp of unknown sequence was 100% identical with Golden Delicious isolate X9273 #13 chromosome 15 genome (GenBank: MJAX01000001). According to the results of southern blot from OSF[5-6], Arctic Golden Delicious apple GD743 has 2 copies of the transgenic gene. This fragment and fragment No.9 are most likely to be 2 different copies of each 5' junction. Consider 68 bp of flanking sequence was very short, we need more evidence to confirm the integrity of this transgenic fragment on chromosome 15.

IV. Establish the methods of 5' and 3' junction event-specific real-time PCR for GD743

We designed primers and TaqMan probes to establish real-time PCR assays for the 5' and 3' junction of GD743. The combination of primer 5GD55F, 5GD169R and TaqMan probe 5GD85p (table 2) had specificity for 5' junction of GD743. GD743 generated a fluorescence amplification plot (figure 7a), NonGM apple did not reveal any amplification plot. The primer pair of GDCh14CF, GDCh14CR and TaqMan probe 3GD6551p (table 2) had specificity for 3' junction of GD743. Like result of 5' junction, only GD743 showed an amplification plot (figure 7b). The amplification of apple internal control presented positive results in all apple samples (figure 7c). In this study, we newly established two event-specific real-time PCR analyses for 5' and 3' junction of GD743, which can be identified in commercially available products of Arctic Golden Delicious apple GD743. Here we used the dried apple snack as a reference material. The specificity of method had confirmed by in-house verification. If we can

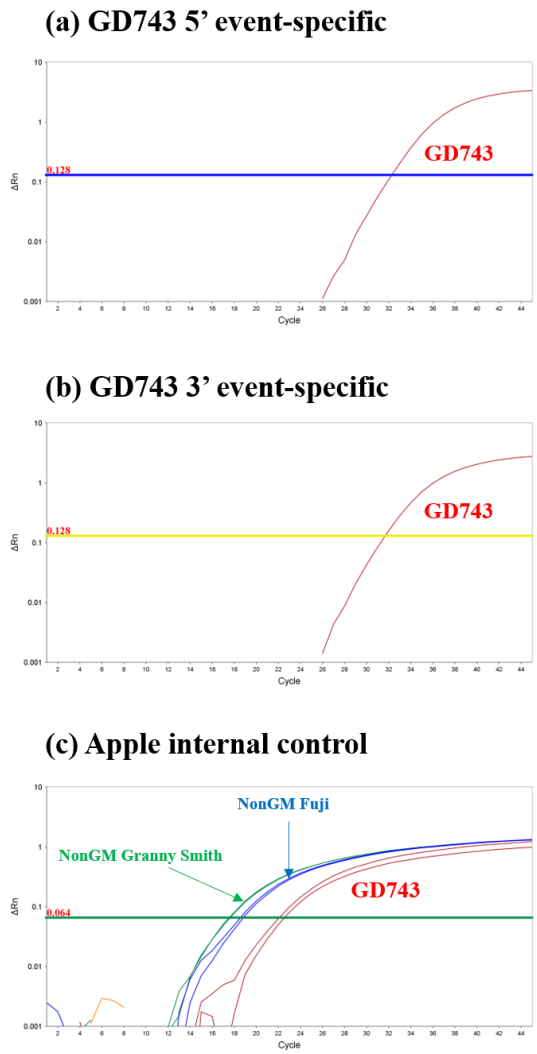

Figure 7. Amplification plots of event-specific real-time PCR for GD743 GM apple detection.
(a) Real-time PCR for GD743 event-specific detection of 5' junction
(b) Real-time PCR for GD743 event-specific detection of 3' junction
(c) Real-time PCR for apple internal control detection

acquire the certified reference materials of GD743 or other GM apples in the future, we will further use them to implement method validation.

CONCLUSION

The construct-specific PCR and real-time PCR assays developed in this study can detect the Arctic Golden Delicious apple GD743. It is expected that Arctic Granny Smith apples GS784 and Arctic Fuji apple NF872 with the same structural genes can be identified using this construct-specific method. The CaMV 35S and NOS real-time PCR method from TFDA are also suitable for GM apples screening. This study also identified the transgenic gene sequence of Arctic Golden Delicious apple GD743, and believed to find two different copies of the transgenic gene on apple genome. One of transgenic gene was confirmed transgenic into chromosome 14 and its 5' and 3' flanking sequences were also identified. We used the finding to establish the event-specific real-time PCR methods for 5' and 3' junction identification of GD743.

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基因改造蘋果Real-Time PCR檢驗方法之建立

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摘 要

Okanagan Specialty Fruits (OSF)公司所研發的基因改造蘋果，產品名為北極(Arctic)蘋果，其基改特性為抗褐變，利用RNA干擾(RNA interference)的方式抑制蘋果多酚氧化酶(polyphenol oxidase, PPO)的表現，降低PPO催化蘋果氧化形成褐色產物。目前北極金冠蘋果GD743和北極翠玉蘋果GS784已獲得美國及加拿大核准上市，主要以新鮮袋裝切片和蘋果乾的商品形式在美國販售，由於OSF公司的北極蘋果尚未向我國申請基因改造食品查驗登記，北極蘋果在台灣仍屬於未核准的基因改造食品，為了避免國際非同步許可所造成的食安議題，本研究開發基因改造蘋果PCR及real-time PCR檢驗方法以因應檢驗所需。針對pSR7基因與NOS終結子區域設計的構造特異性(construct-specific) PCR及real-time PCR檢驗方法可檢測北極蘋果，TFDA現有CaMV 35S與NOS定性方法也適用於北極蘋果的篩檢。進一步分析金冠北極蘋果GD743的轉殖基因及其跨接區域，除了確認北極金冠蘋果GD743的轉殖基因序列全長，亦找到2個不同的5’端轉殖基因片段跨接蘋果基因體區域源自GD743中2個不同的殖入片段拷貝，本研究已確認2個不同片段中的1個拷貝轉殖基因片段位於蘋果chromosome 14具特異性可做為基改蘋果檢測，並進一步確認3’端跨接蘋果chromosome 14的基因序列，運用解析出5’端及3’端跨接蘋果chromosome 14的核酸序列，建立北極金冠蘋果GD743轉殖品項特異性(event-specific) real-time PCR檢驗方法。

關鍵詞：基因改造、蘋果、real-time PCR