表2　ジャガイモ検体の各機関における吸光度比およびDNA濃度(シリカゲル膜タイプキット法)

		O. D. 260/280					DNA濃度(ng/μL)抽出				
		Non G	PLUS 0.1%	PLUS 1%	Y 0.1%	Y 1%	Non G	PLUS 0.1%	PLUS 1%	Y 0.1%	Y 1%
A	抽出A	1.830	1.810	1.630	1.780	1.620	68.5	81.5	65.0	70.5	69.5
	抽出B	1.820	1.780	1.720	1.710	1.800	87.5	80.0	73.0	70.0	59.5
B	抽出A	1.691	1.373	1.612	1.613	1.695	68.5	57.0	54.0	50.0	50.0
	抽出B	1.704	1.507	1.500	1.606	1.396	60.5	53.5	54.0	53.0	63.5
C	抽出A	1.541	1.600	1.711	1.663	1.687	65.5	56.0	77.0	79.0	70.0
	抽出B	1.824	1.720	1.710	1.682	1.750	83.0	70.5	53.0	74.0	45.5
D	抽出A	1.583	1.508	1.521	1.537	1.670	91.0	96.5	106.5	83.0	88.5
	抽出B	1.436	1.427	1.634	1.411	1.552	72.5	83.5	76.0	79.0	104.0
E	抽出A	1.738	1.671	1.709	1.676	1.659	53.0	66.0	47.0	62.0	73.0
	抽出B	1.696	1.306	1.771	1.672	1.678	59.0	64.0	43.0	54.0	50.0
F	抽出A	1.730	1.710	1.770	1.630	1.540	142.0	85.5	102.1	104.5	129.5
	抽出B	1.790	1.790	1.760	1.830	1.800	77.0	73.7	69.7	59.6	63.1
G	抽出A	1.530	1.470	1.520	1.460	1.470	59.0	62.0	61.0	51.0	61.0
	抽出B	1.500	1.550	1.480	1.490	1.490	52.0	54.0	50.0	61.0	45.0
H	抽出A	1.090	0.970	1.090	−0.230	1.100	50.3	35.0	49.8	-3.000	42.0
	抽出B	1.090	1.000	0.980	1.130	1.150	49.8	36.5	36.8	40.5	48.5
I	抽出A	1.850	1.850	1.840	1.810	1.860	111.0	95.5	90.0	109.5	103.0
	抽出B	1.850	1.850	1.800	1.880	1.860	100.0	82.5	116.0	82.5	72.5
J	抽出A	1.710	1.770	1.830	1.760	1.780	97.7	89.2	87.8	70.9	78.5
	抽出B	1.740	1.760	1.810	1.840	1.700	114.2	80.3	100.4	92.8	89.5
K	抽出A	1.676	1.622	1.634	1.644	1.743	57.0	73.0	67.0	74.0	61.0
	抽出B	1.733	1.774	1.688	1.667	1.667	52.0	55.0	54.0	60.0	70.0
L	抽出A	1.672	1.979	1.650	1.837	1.804	163.0	94.0	115.5	118.5	138.0
	抽出B	1.921	1.837	1.696	1.832	1.713	121.0	118.5	97.5	114.5	137.0
M	抽出A	1.763	1.765	1.718	1.768	1.737	100.5	75.0	73.0	72.5	82.5
	抽出B	1.757	1.745	1.744	1.756	1.724	97.5	82.0	78.5	75.5	100.0
N	抽出A	1.680	1.790	1.670	1.780	1.760	48.0	51.0	37.5	48.0	55.5
	抽出B	1.680	1.700	1.690	1.690	1.750	48.0	51.0	33.0	40.5	42.0
平均値		1.665	1.630	1.639	1.672	1.648	80.3	71.5	70.3	72.2	74.7

に大きな差は認められなかった。

表2には，ジャガイモ検体について，各機関におけるDNA抽出液の吸光度比，およびそのDNA濃度についての結果を示す。ジャガイモからのDNA抽出法はシリカゲル膜タイプキットを用いた方法のみが指定されており，当該抽出法を用いたDNA抽出の結果は1機関(機関H)を除き機関間で大差がなく，良好な精製度および収量であった。またNL-PとNL-Yの各検体間で明確な差違は認められなかった。さらに，トウモロコシとジャガイモから抽出されたDNAの収量ならびに精製度を比較した場合，収量，精製度ともにジャガイモがトウモロコシに比べ若干劣る結果となった。これはジャガイモ試料中に存在するデンプンならびに他の多糖類等によりDNA抽出が阻害されたためと推察された。

3　トウモロコシ検体における試験の調査成績

表3に示すように，トウモロコシ試料を対象と

表5　各機関ごとの判定の正答率

実施機関	トウモロコシ正答率(%)	ジャガイモ正答率(%)
A	100	100
B	100	100
C	100	100
D	100	100
E	100	100
F	100	100
G	100	100
H	100	100
I	66.7	100
J	100	60
K	100	80
L	100	80
M	100	100
N	100	100

表6　トウモロコシ検体のDNA抽出法，プライマーおよびPCR増幅装置の種類

	試料のDNA抽出法		PCR増幅装置および増幅条件					
			プライマー				PCR増幅装置	
	CTAB法	シリカゲル膜タイプキット法	メーカー名	グレード			メーカ名	型式
				脱塩	カートリッジ精製	HPLC精製		
A	−	○	アマシャムファルマシアバイオテク	○	−	−	アプライドバイオシステムズジャパン	GeneAmp PCR System9700
B	−	○	アマシャムファルマシアバイオテク	○	−	−	アプライドバイオシステムズジャパン	GeneAmp PCR System9700
C	−	○	北海道システム・サイエンス	−	−	○	アプライドバイオシステムズジャパン	GeneAmp PCR System2400
D	−	○	ファスマック	○	−	○	アプライドバイオシステムズジャパン	GeneAmp PCR System9600
E	−	○	グライナージャパン	−	○	−	アプライドバイオシステムズジャパン	GeneAmp PCR System9700
F	−	○	ニッポンジーン(GMO用)	−	−	?	宝酒造	TP240
G	−	○	宝酒造	−	○	−	アプライドバイオシステムズジャパン	GeneAmp PCR System2400
H	○	−	インビトロジェン	○	−	−	宝酒造	TP3000
I	−	○	東亞合成	−	○	−	アプライドバイオシステムズジャパン	GeneAmp PCR System9700
J	−	○	ニッポンジーン	−	○	−	アプライドバイオシステムズジャパン	GeneAmp PCR System9700
K	○	−	アマシャムファルマシアバイオテク	−	○	−	アプライドバイオシステムズジャパン	GeneAmp9700
L	−	○	ファスマック・ニポンジーン(GMO用)	○	−	?	アプライドバイオシステムズジャパン	GeneAmp PCR System9700
M	−	○	ニッポンジーン(GMO用)	−	−	?	宝酒造	TP3000
N	○	−	北海道システム・サイエンス	−	○	−	アプライドバイオシステムズジャパン	PCR System9700

全機関で正しくNL-Y陽性と判定された。しかし，NL-Y0.1％疑似混入試料については1機関2試料についてNL-Y検出用プライマー対で増幅産物が検出されず，最終的に1機関で誤ってNL-Y陰性と判定された。さらにNL-Y 0.1％疑似混入試料についてNL-P検出用プライマー対を用いた試験で1機関2試料において増幅産物が確認された。しかし，続くNL-P確認用プライマー対を用いた試

表3　トウモロコシ検体の個別測定結果

プライマーの種類		測定試料					
		Non G		CBH 351			
				0.1%		1%	
		+	−	+	−	+	−
対照	Zein	28/28	0/28	28/28	0/28	28/28	0/28
CBH検出	CaM-Cry9C	4/28	24/28	28/28	0/28	28/28	0/28
CBH確認	Cry9C-35Ster	2/4	2/4	28/28	0/28	28/28	0/28

表4　ジャガイモ検体の個別測定結果

プライマーの種類		測定試料									
		Non G		NewLeaf Plus				NewLeaf Y			
				0.1%		1%		0.1%		1%	
		+	−	+	−	+	−	+	−	+	−
対照	PSS	28/28	0/28	28/28	0/28	28/28	0/28	28/28	0/28	28/28	0/28
Plus検出	p-FMV-PLRV	2/28	26/28	28/28	0/28	28/28	0/28	2/28	26/28	0/28	28/28
Plus確認	PLRV5-PLRV3	0/2	2/2	21/28	7/28	28/28	0/28	0/2	2/2	−	−
Y検出	p-FMV-PVY	0/28	28/28	0/28	28/28	0/28	28/28	26/28	2/28	27/28	1/28
Y確認	PVY5-PVY3	−	−	−	−	−	−	26/26	0/26	27/27	0/27

した試験の結果，全試料で対照プライマー対を用いた試験で増幅産物が確認された。0.1%ならびに1.0%疑似混入試料については，14機関で試験に供された各2検体(計28検体)すべてが陽性と正しく判定された。しかし，0%疑似混入試料(non-GM検体)については，28検体中4検体においてCBH検出用プライマー対を用いた試験で増幅産物が確認され，続く確認用プライマー対を用いた試験においても，2検体について増幅産物が確認された。

4　ジャガイモ検体を対象とした試験

表4に示すように，ジャガイモ試料を対象とした試験の結果，全試料について対照プライマー対を用いた試験で増幅産物が確認された。0%疑似混入試料(non-GM)についてNL-Y検出用プライマー対を用いた試験で増幅産物が確認された試料はなかったが，NL-P検出用プライマー対を用いた試験で1機関2試料について増幅産物が確認された。しかしこれら2試料についてNY-P確認用プライマー対を用いた試験を行ったところ増幅産物は検出されず，最終的に全機関でNL-PおよびNL-Y陰性と正しく判定された。

NL-P 1.0%疑似混入試料については，28試料すべてで検出用プライマー対および確認用プライマー対で増幅産物が確認され，全機関で正しくNL-P陽性と判定された。しかしながら，NL-P0.1%疑似混入試料については，NL-P検出用プライマー対では28試料すべてで増幅産物が確認されていたにもかかわらず，NL-P確認用プライマー対では4機関7試料で増幅産物が検出されず，最終的に3機関で誤ってNL-P陰性と判定された。なお，NL-P 0.1，1.0%疑似混入試料ではともに，NL-Y検出用プライマー対で増幅産物が確認された試料はなかった。

NL-Y 1.0%疑似混入試料については，1試料を除く27試料でNL-Y検出用プライマー対およびNL-Y確認用プライマー対で増幅産物が確認され，

表7　アガロースゲル電気泳動条件

	アガロースゲル電気泳動条件						
	電気泳動装置				染色方法		
	型　式	メーカー名	ゲル濃度	泳動時間	前染め	後染め 染色時間	後染め 脱色時間
A	Gel Mate	東洋紡績	2%	35分	−	20分	3分
B	Gel Mate	東洋紡績	2%	45～50分	−	30分	30分
C	Mupid	アドバンスバイオ	2%	20分	○	−	−
D	Mupid-21	アドバンスバイオ	3%	40分	−	30分	10分
E	Mupid-21	アドバンスバイオ	1.5%	25分	○	−	−
F	Mupid	アドバンスバイオ	2%	30分	−	10分	10分
G	i-Mupid j	アドバンスバイオ	2%	40分	−	15分	5分
H	Mupid-21	アドバンスバイオ	3%	100V25分	−	1時間	水道水でゆすぐ程度
I	Mupid	アドバンスバイオ	3%	40分	−	40分	水道水にて洗浄のみ
J	Mupid-3	アドバンスバイオ	2%	20～30分	−	15分	0分
K	Mupid Ⅱ	アドバンスバイオ	2.5%	約30分	○	−	−
L	Mupid-3	アドバンスバイオ	2%	15分	○	−	−
M	Mupid-21	アドバンスバイオ	2%	40分	−	30分	なし
N	Mupid-21	アドバンスバイオ	2.5%	約30分	−	約5分	約15分

表8　ゲルイメージ解析装置の種類

	ゲルイメージ解析			
	ゲルイメージ解析装置		インスタントカメラ	
	型　式	メーカー名	型　式	メーカー名
A	AE-6911CX	アトー	−	−
B	Printgraph/Image Saver AE-6905C	アトー	−	−
C	−	−	DS-300	フナコシ
D	Bio Doc-It System	UVP LABORATORY PRODUCTS		
E	−	−	DS-300L	フナコシ
F	−	−	DS-300	フナコシ
G	FAS-Ⅲ	東洋紡績	−	−
H	Epi-Light UV FA1100	アイシンコスモス研究所	−	−
I	−	−	RB67SD	MAMIYA
J	EDAS120	Kodak	−	−
K	プリントグラフ AE-6911CX	アトー	−	−
L	AE-6905H	アトー	−	−
M	−	−	M-085	コスモバイオ
N	Epi-Light UV FA500	アイシンコスモス研究所	−	−

験においては増幅産物が検出されず，この試料については最終的にNL-P陰性と正しく判定された。なお，NL-Y 1.0%疑似混入試料では，NL-P検出用プライマー対で増幅産物が確認された試料はなかった。

5　調査項目のまとめおよび考察

機関ごとの判定の正答率を表5に，またアンケート結果をまとめ，トウモロコシ検体のDNA抽出法，プライマーおよびPCR増幅装置の種類を表6

表9　実験区域および測定機器の共用の有無

	実験区域				遠心機			ピペット類			PCR装置	電気泳動装置	ゲルイメージ解析装置
	抽出操作	PCR反応液調製	電気泳動	ゲルイメージ解析	マイクロチューブ用	15 mL遠心管用	50 mL遠心管用	DNA抽出用	PCR用	電気泳動用			
A	独立	独立	共用	共用	専用	専用	専用	専用	専用	専用	専用	専用	専用
B	独立	独立	共用	共用	専用	専用	専用	専用	専用	専用	専用	専用	専用
C	共用	共用	共用	共用	共用	共用	共用	共用	共用	共用	共用	共用	共用
D	独立	独立	独立	独立	専用	共用	共用	専用	専用	専用	共用	共用	専用
E	共用	共用	独立	独立	専用	共用	共用	専用	専用	専用	専用	専用	共用
F	共用	共用	共用	共用	専用	共用	共用	専用	専用	専用	専用	専用	専用
G	共用	共用	独立	独立	専用	専用	専用	専用	専用	専用	専用	専用	共用
H	共用	共用	独立	独立	専用	なし	専用	専用	専用	専用	専用	専用	共用
I	独立	独立	共用	共用	共用	共用	共用	共用	共用	共用	共用	共用	共用
J	独立	独立	共用	共用	専用	専用	専用	専用	専用	専用	専用	専用	専用
K	共用	共用	共用	共用	専用	共用	専用	専用	専用	専用	専用	専用	専用
L	独立	独立	独立	独立	専用	専用	専用	専用	専用	専用	専用	専用	専用
M	共用	共用	共用	共用	共用	共用	共用	共用	共用	共用	共用	共用	共用
N	共用	共用	独立	独立	共用	共用	共用	専用	専用	専用	専用	専用	共用

に，アガロースゲル電気泳動条件を表7に，ゲルイメージ解析装置の種類を表8に，実験区域および測定機器の共用の有無を表9に示した。

トウモロコシ0％試料において判定が誤って陽性となった機関(機関Ⅰ)についてアンケート調査結果を調べたところ，電気泳動ならびにゲルイメージ解析を実施する検査実施環境や，遠心機，ピペット類，PCR装置といった広範な使用機器，器具の他の試験との共有があることが明らかとなった。このことから，誤って陽性と判定された原因は，検査実施環境やピペット類等を共用することによるコンタミネーションにあると考えられ，定性PCR法において正しい結果を得るためには，検査実施環境の区分化ならびに機器，器具類の占有化が必須と考えられた。

一方ジャガイモ試料においてnon-GM検体ならびにNL-Y 0.1％疑似混入試料についてNL-P検出用プライマー対を用いた試験で，同一機関(機関L)のそれぞれ2試料ついて誤って増幅バンドが検出された。しかし，確認用プライマー対により増幅バンドが得られていないことおよび調査結果では検査実施環境の区分化やピペット類等の占有は行われていることから，電気泳動時に他試料の増幅産物がコンタミネーションして検出された可能性が高いものと考えられた。NL-P 0.1％疑似混入試料については，NL-P検出用プライマー対では28試料すべてで増幅産物が確認されていたにもかかわらず，NL-P確認用プライマー対では4機関7試料で増幅産物が検出されず，最終的に3機関で誤って陰性と判定された。これらの判定は先に考察した検出用プライマー対と確認用プライマー対における増幅効率の差違を反映した結果に起因していると考えられた。また陰性とした3機関のうち，0.1％ NL-Yでも誤って陰性と判定1機関

(機関J)は，表7より電気泳動後のエチジウムブロミドによる染色時間がそれ15分と，検査方法に示されている時間に比べ明らかに短く，また染色後の脱染色操作も十分でないことが明らかであった。したがってこの機関では電気泳動において遺伝子増幅産物の染色が不適切であったため，増幅産物を可視化することができなかった可能性が推察された。以上の考察から，当検査方法を用いて0.1%混入率付近の検体を検査した場合，電気泳動条件や染色条件が，判定に影響を与えることから，通知法に沿った操作を行うことの重要性が示された。

またNL-Y 1.0%疑似混入試料において陰性と判定された1試料については，本来は実施の必要のないNL-Y確認用プライマーを用いたPCRが実施され，その結果，増幅産物が確認されていたことから，PCRまたは電気泳動操作において何らかの誤りがあったのではないかと推察された。

Ⅳ　ま　と　め

今回の試験では国立医薬品食品研究所で調製した疑似混入試料を14の協力機関に一斉に配布し，同時期に分析を行った。各機関で得られた分析結果を集計解析したところ，トウモロコシ，ジャガイモ試料ともおおむね添加GM試料にそれぞれ対応した正しい判定が得られた。これらの結果から，当該検査方法について外部精度管理の実施が可能であると判断された。さらに，同時に調査した検査条件に関するアンケート結果を考慮すると，当該検査の実施に際しては，検査区域，使用機器な

らびに器具の専有化が重要であり，加えて検査条件の適正化により検査機関間に認められるばらつきの低減が可能と考えられた。

謝　辞

本稿を終えるにあたり，食品衛生外部精度管理調査研究の推進に対し貴重なご意見ならびにご指導を賜りました当時厚生労働省医薬局食品保健部監視安全課の各担当官殿に深謝いたします。なお本研究は，平成13年度厚生労働科学研究補助金により実施しました。また本研究にご協力いただいた検査機関諸氏に深謝いたします。

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New Qualitative Detection Methods of Genetically Modified Potatoes

Takahiro WATANABE,*[,a] Hideo KURIBARA,[b,i] Takashi MISHIMA,[c] Hiroyuki KIKUCHI,[a] Misao KUBO,[b] Takashi KODAMA,[b,i] Satoshi FUTO,[d] Kikuko KASAMA,[e] Akie TOYOTA,[f] Masanori NOUNO,[g] Ayako SAITA,[g] Kunihiko TAKAHASHI,[h] Akihiro HINO,[i] Hiroshi AKIYAMA,[a] and Tamio MAITANI[a]

[a] *National Institute of Health Sciences; 1–18–1 Kamiyoga, Setagaya-ku, Tokyo 158–8501, Japan:* [b] *Center for Food Quality, Labeling and Consumer Services; 2–1 Shintoshin, Chuo-ku, Saitama, Saitama 330–9731, Japan:* [c] *Japan Food Research Laboratories; 2–3 Bunkyo, Chitose, Hokkaido 066–0052, Japan:* [d] *FASMAC Co., Ltd.; 5–1–3 Midorigaoka, Atsugi, Kanagawa 243–0041, Japan:* [e] *Hatano Research Institute, Food and Drug Safety Center; 729–5 Ochiai, Hadano, Kanagawa 257–8523, Japan:* [f] *Hiroshima Prefectural Institute of Public Health and Environment; 1–6–29 Minami-cho, Minami-ku, Hiroshima 734–0007, Japan:* [g] *Center for Inspection of Imported Foods and Infectious Diseases Yokohama Quarantine Station; 107–8 Nagahama, Kanazawa-ku, Yokohama, Kanagawa 236–0011, Japan:* [h] *Saitama Prefectural Institute of Public Health; 639–1 Kamiokubo, Sakura-ku, Saitama 338–0824, Japan: and* [i] *National Food Research Institute; 2–1–12 Kannondai, Tsukuba, Ibaraki 305–8642, Japan.*

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In Japan, 8 lines of genetically modified (GM) potato (2 lines of NewLeaf® potato; NL, 3 lines of NewLeaf Plus® potato; NLP, and 3 lines of NewLeaf Y® potato; NLY) have already been authorized as safe for use in foods and feeds. We have developed polymerase chain reaction (PCR) methods for the qualitative detection of the GM potatoes for the screening and the identification of NL, NLP and NLY. The gene encoding uridine diphosphate (UDP)-glucose pyrophosphorylase (UGPase) was used as a taxon specific gene. We designed the primer pair to detect the cryIIIA genes as a screening method for GM potatoes because the gene should be inserted in all 8 lines of the GM potatoes. For identification of NL, NLP and NLY, we further designed three specific primer pairs for the different recombinant DNAs (r-DNA) specifically introduced into NL, NLP, or NLY. In addition, to identify the 3 lines of NLY that have been introduced with the same r-DNA, the three line-specific primer pairs for the border sequence between the r-DNA and genomic DNA of NLY 3 lines were designed. Six lines of GM potato used as the test material were specifically identified using the each primer pair under the same PCR condition. The detection limits of all the GM potatoes should be approximately 0.1%. Furthermore, the specificity and reproducibility of the methods were confirmed in a six-laboratory collaborative study.

Key words genetically modified potato; detection method; NewLeaf; NewLeaf Plus; NewLeaf Y; uridine diphosphate (UDP)-glucose pyrophosphorylase (UGPase)

Many kinds of genetically modified (GM) crops, which include GM soy, maize, rapeseed, cotton and potato, have already been developed and the cultivated acreage of these crops has continued to grow year by year. It was reported that the global area of GM crops for 2003 was 67.7 million hectares with a growth rate of 15% compared to that in 2002. This growth is estimated to rapidly increase, since the planting has been spread all over the world in addition to the nations such as United States (U.S.) and Canada.[1] On the other hand, public concern has been raised in terms of food safety and environmental effects of the GM crops. Especially, consumers are concerned about the negative effects of GM food on their health by their consumption and scientific information has been strongly required.[2] Therefore, many governments have now been considering regulations for the use and implementing a labeling system for GM crops as food and feed. Thus, new labeling systems have been introduced for GM foods in the European Union (EU), Australia, Korea, Japan and other countries.

The commercialization of fifty-five lines of safety-assessed GM crops including soy, maize, potato, rapeseed, cotton and sugar beet, have already been approved by the Ministry of Health, Labour and Welfare (HMLW) in Japan. To monitor the labeling system, it is necessary to develop reliable and practical methods for the detection and identification of GM crops. The polymerase chain reaction (PCR) is one of the widely used systems for the quantitative or qualitative detection of GM crops and we also have previously reported PCR methods for the detection of GM soy, maize, papaya and potatoes.[3—10]

The tetraploid cultivated potato (*Solanum tuberosum*) is one of the world's four major crops and an important feedstuff, but it is easily infected by many kinds of pests and pathogens.[11] Therefore, molecular biology techniques has been attempted to improve the potato varieties which ended with the breeding of GM potatoes commercialized by the Monsanto Co. (St. Louis, MO, U.S.A.). In Japan, two lines of NewLeaf® potato (NL), three lines of NewLeaf Plus® potato (NLP) and three lines of NewLeaf Y® potato (NLY) have been approved by HMLW for human food consumption by July 2003. The information provided by the Monsanto Co. showed that (a) two lines of NL (Bt-6 and SPBT02-05 line) were transformed with the same plasmid vector, PV-STBT02, which contains cryIIIA derived from *Bacillus thuringiensis* subsp. *tenebrionis*, providing of the trait of Colorado potato beetle (CPB) resistance, (b) three lines of NLY (RBMT15-101, SEMT15-15 and SEMT15-02 line) were transformed with the same plasmid vector, PV-STBT02, which contains cryIIIA, Potato virus Y coat protein (PVYcp) gene providing of the traits of CPB and PVY resistance, (c) two of three lines of NLP (RBMT21-129 and RBMT21-350 line) were transformed with the same plasmid vector, PV-STMT21, which contains cryIIIA, the potato leaf role virus replicase (PLRVrep) gene, providing of the traits of CPB and PLRV

* To whom correspondence should be addressed. e-mail: tawata@nihs.go.jp

resistance, and (d) the other one (RBMT22-82) was transformed with the plasmid vector, PV-STMT22, which contains cryIIIA, PLRVrep, and CP4-epsps genes, providing of the traits of CPB, PLRV resistance and herbicide tolerance, respectively. The detection methods based on PCR for GM potatoes have been reported.[9,10,12] To verify the labeling and identify of the lines, however, further reliable and practicable detection methods for the GM potatoes are required.

In the present study, we designed seven primer pairs for screening, construct-specific, and line-specific detection and developed the condition of PCR to perform the test under the same conditions using all the primer pairs. To design the line-specific primer pairs, we focused on the junction sequences between the recombinant DNA (r-DNA) and potato genomic DNA as the target sequence to distinguish the specific one from the GM limes that was transformed with the same plasmid vector. The specificities and sensitivities were examined using reference materials provided by the Monsanto Company. The specificity and reproducibility of the methods were then confirmed by an inter-laboratory study.

MATERIALS AND METHODS

Reference Potato Materials and Other Crops Freeze-dried powder of six lines of GM potato (NL potatoes; Bt-6 and SPBT02-05, NLP potato; RBMT21-350, NLY potatoes; RBMT15-101, SEMT15-02 and SEMT15-15 line) and three varieties of non-GM potato (Shepody, Russet Burbank and Superior), which were conventional varieties used for the transformation, were kindly provided by the Monsanto Co. through the Department of Food Safety, Pharmaceutical and Food Safety Bureau of MHLW. Japanese conventional potato varieties (Dansyaku and May queen), egg plant (Solanum melongena), tomato (Lycopersicum esculentum), maize (Zea mays), soy (Glycine max) and wheat (Triticum aestivum) were purchased from a local market.

Preparation of Test Samples Dehydrated Japanese conventional potatoes, the provided powders of the non-GM potato varieties and GM potatoes were ground with the 0.2 mm sieve ring using an Ultra-Centrifugal Mill ZM100 (Retsch GmbH, Haan Germany) and freeze-dried for 24 h using an FD-81 freeze dryer (Tokyo Rikakikai Co., Ltd., Tokyo, Japan). To evaluate our screening and construct-specific methods, three mixing levels of the potato-powder samples containing 0, 0.1 and 1.0% of each NL-Bt-6, NLP-RBMT21-350, and NLY-SEMT15-15 lines were prepared, according to a previous study.[13] In this study, we selected the Bt-6, RBMT21-350, and SEMT15-15 lines as the representative line for each NL, NLP and NLY by referring to the acreage in the U.S. in 2000 and 2001. Six mixing levels of potato-genomic DNA samples containing 0, 0.05, 0.1, 0.5, 1.0, and 5.0% of each SEMT15-15, SEMT15-02, and RBMT15-101 line were also prepared to evaluate our line-specific methods. Because the amount of the three lines of NLY, which was provided by Monsanto, was not enough to prepare the powder samples, the genomic DNA samples were used for the evaluation. For the preparation of both the powder and genomic DNA samples, Dansyaku was used as a non-GM material. Other crops were also well ground by the Ultra-Centrifugal Mill ZM100 or Grindomix GM 100 (Retsch) and used for the test samples.

Extraction and Purification of Genomic DNA Genomic DNAs were extracted from soy and maize using a silica-gel membrane-type kit (DNeasy Plant Maxi; QIAGEN GmbH, Hilden, Germany) according to a previous study.[6] Genomic DNAs were extracted from the other crops including potatoes using a silica-gel membrane-type kit (DNeasy Plant Mini; QIAGEN) according to a previous study.[9] The DNA concentration in the solutions was determined by measuring the UV absorption at 260 nm using an ND-1000 spectrophotometer (NanoDrop Technologies Inc., Rockland, DE). The purity of the extracted DNA was evaluated using the ratio of 260/280 nm and the ratio was between 1.7 and 2.0 for most of the test samples. The extracted DNA was diluted with an appropriate volume of DW to a final concentration of 10 ng/μl, and stored at -20 °C until used. These DNA samples were used for the subsequent PCR analysis.

Oligonucleotide Primers Eight primer pairs, in which the primer pair to detect a potato taxon specific gene was included, were designed for the qualitative detection of the GM potatoes (Table 1). The primer pair UGPase 01-5′/UGPase 01-3′ to detect a gene encoding uridine diphosphate (UDP)-glucose pyrophosphorylase (UGPase) that was reported as a single copy gene of potato,[14] was designed on the first exon by referring to GenBank Accession No. U20345 which generated a 111 bp amplified fragment.

The cryIIIA gene from *Bacillus thuringiensis* should be introduced into all 8 lines of the GM potatoes conferring resistance to the CPB pest.[15] The primer pair CryIIIA 01-5′/CryIIIA 01-3′ to detect this gene as screening for the GM potatoes was designed by referring to GenBank Accession No. X70979 in order to generate a 117 bp amplified fragment. To specifically detect NL, the NL construct-specific primer pair NL 01-5′/NL 01-3′ was designed in the junction between the enhanced 35S promoter (e-p-35S) and cryIIIA by referring to GenBank Accession No. AF078810 (e-p-35S), and the primer pair generated an amplified fragment of 113 bp. In previous studies,[9] p-FMV02-5′/PLRV 01-3′ had been designed in the junction between e-p-35S and cryIIIA for the construct-specific detection of NLP and generated a 234 bp amplification fragment. Moreover, p-FMV05-5′/PVY 02-3′ had been designed in the junction between the 35S promoter sequence of the figwort mosaic virus (P-FMV) and PVYcp for the construct-specific detection of NLY and generated a 225 bp amplified fragment.[10] NLP and NLY were specifically detected using these two primer pairs, respectively, but the efficiencies of the PCR should be low because the amplified fragments were weakly detected in the potato-powder samples containing 0.1% of both the NLP and NLY.[13] In this study, we aimed to develop more reliable and practical detection methods by re-designing the primer pairs for the construct-specific detection of NLP and NLY. The primer pairs NLP 01-5′/NLP 01-3′ and NLY 01-5′/NLY 01-3′ were designed in the internal sequences of the PCR products generated by the primer pairs, p-FMV02-5′/PLRV 01-3′ and p-FMV05-5′/PVY 02-3′, to increase the PCR efficiencies while keeping the specificities. These primer pairs generate a 125 bp and 123 bp of amplified fragments, respectively.

To identify three lines of NLY that have been transformed with the same plasmid vector, the NLY line-specific primers (NLY15-01-5′/NLY15-01-3′; NLY15-15 line specific,

NLY02-01-5′/NLY02-01-3′; NLY15-02 line specific and NLY101-01-5′/NLY101-01-3′; NLY15-101 line specific) were designed in each of the border sequences between r-DNA and the genomic DNA by referring to the DNA sequence information described in the safety assessment document submitted from the developer. These primer pairs generate 164 bp, 86 bp and 150 bp amplified fragments, respectively. The locations of each targeted sequence are shown in Fig. 1.

The primers were synthesized and purified on a reversed-phase column by FASMAC Co., Ltd., (Atsugi, Japan), then diluted with an appropriate volume of DW to a final concentration of 50 μmol/l, and stored at −20 °C until used. The sequences of the designed oligonucleotides in this study are listed in Table 1.

PCR Conditions The reaction mixture for PCR was prepared in a PCR reaction tube. The reaction volume of 25 μl contained 25 ng of genomic DNA, 0.2 mmol/l dNTP, 1.5 mmol/l $MgCl_2$, 0.5 μmol/l of 5′ and 3′ primers and 0.625-units AmpliTaq Gold (Applied Biosystems, Foster City, CA, U.S.A.). The reactions were buffered with PCR buffer II (Applied Biosystems) and amplified in a thermal cycler (GeneAmp PCR System 9700; Applied Biosystems) according to the following PCR step-cycle program: pre-incubation at 95 °C for 10 min, denaturation at 95 °C for 0.5 min, annealing at 60 °C for 0.5 min, and extension at 72 °C for 0.5 min. The cycle was repeated 40 times followed by a final extension at 72 °C for 7 min.

Agarose Gel Electrophoresis After PCR amplification, agarose gel electrophoresis of the PCR product was carried out according to previous studies.[5,9] The amplification products (7.5 μl) of each specific PCR were submitted for electrophoresis at a constant voltage (100 V) on a 3% TAKARA L03 agarose gel (TAKARA Co., Ltd., Tokyo, Japan) in the TAE (40 mmol/l Tris–HCl, 40 mmol/l acetic acid, and 1 mmol/l EDTA, pH 8.0) buffer solution. After electrophore-

Table 1. List of Primers

	Name	Sequence (5′→3′)	Specificity		Amplicon
A:	UGPase 01-5′ UGPase 01-3′	5′-CTC TCC ATA CTC TCT GCT CCT CG-3′ 5′-CGG CAT CAG CAG GAG AAA G-3′	Potato *UGPase*/sense Potato *UGPase*/anti-sense	Potato	111 bp
B:	CryIIIA 01-5′ CryIIIA 01-3′	5′-GAA AGC CTA CAA GCT GCA ATC TG-3′ 5′-TCA GGT GTC ACG TAG ATA GTA G-3′	*CryIIIA*/sense *CryIIIA*/anti-sense	*CryIIIA*	117 bp
C:	NL 01-5′ NL 01-3′	5′-CCT TCG CAA GAC CCT TCC TC-3′ 5′-CGG TGT TGT TGT CTG CAG TCA-3′	p35S/sense *CryIIIA*/anti-sense	NeaLeaf	113 bp
D:	NLP 01-5′ NLP 01-3′	5′-CCC ATT TGA AGG ACA CAG AAA CA-3′ 5′-AGC GGC ATA TGC GGT AAA TC-3′	pFMV-*GmHsp*/sense *PLRV-rep*/anti-sense	NewLeaf Plus	125 bp
E:	NLY 01-5′ NLY 01-3′	5′-CAA AAT CCC AGT ATC AAA ATT CTT-3′ 5′-TGG TTT TGT ATC TTT CTT GTT GCT TC-3′	*GmHsp*/sense *PVY-cp*/anti-sense	NewLeaf Y (3 lines)	123 bp
F:	NLY15-01-5′ NLY15-01-3′	5′-AAC GCT GCG GAC ATC TAA ATT CA-3′ 5′-TAC CGT TAC CAC TAG CTA CAC T-3′	pNOS/sense Potato genomic DNA/anti-sense	NewLeaf Y (SEMT15-15)	164 bp
G:	NLY02-01-5′ NLY02-01-3′	5′-TGA AAT TCG ACT AAT TAC AAG TTG A-3′ 5′-GCA TCG ATC GTG AAG TTT CTC AT-3′	Potato genomic DNA/sense pNOS/anti-sense	NewLeaf Y (SEMT15-02)	86 bp
H:	NLY101-01-5′ NLY101-01-3′	5′-ATG GCT CGT ACC TTG TTG ATT G-3′ 5′-AGG TCA AAG TTA AAA TGA AAC ATG-3′	pFMV/sense Potato genomic DNA/anti-sense	NewLeaf Y (RBMT15-101)	150 bp

A; for confirmation of validity of the DNA extracted from potato for PCR, B; for screening detection of GM potatoes, C; for detection of NL, D; for detection of NLP, E; for detection of NLY, F; for identification of NLY-SEMT15-15 line, G; for identification of NLY-SEMT15-02 line, H; for identification of NLY-RBMT15-101 line.

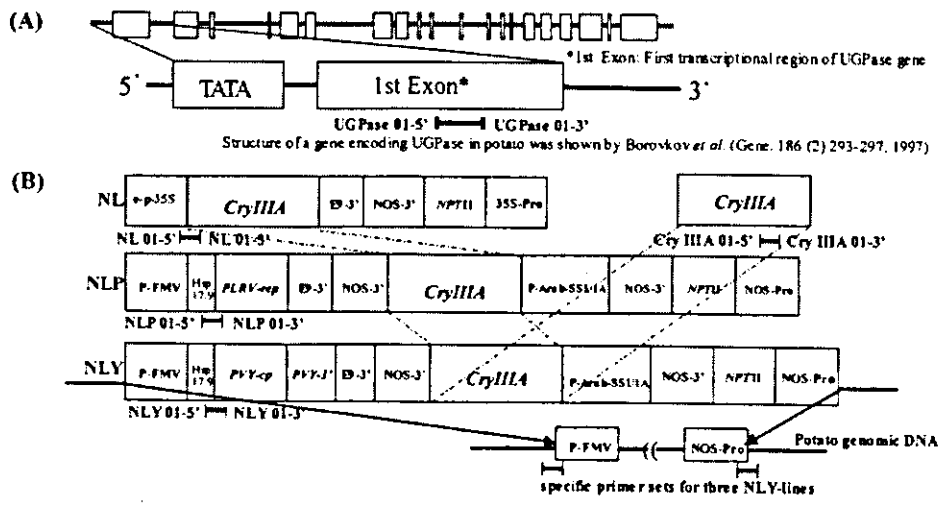

Fig. 1. Schematic Diagrams of PCR Primers Designed for Three Kinds of Genetically Modified Potatoes

(A) The structure of a gene encoding UGPase is shown. The primer pair was designed on the first exon of UGPase. (B) Schematic representation of recombinant DNA (r-DNA) introduced into three kinds of genetically modified potatoes (NL, NLP and NLY). The primer pairs were designed in the junction between the integrated r-DNA and potato genomic DNA for the specific detection of the 3 lines of NLY. Further information on the location of primers is shown in Table 1.

sis, the gel was stained in DW containing 0.5 μg/ml ethidium bromide for 30 min and then washed in DW for 30 min. The gel was photographed using a Chemi-lumi Image Analyzer (Chemi-lumi Image Analyzer with "Diana" system as the analytical software, Raytest, Germany).

Inter-Laboratory Studies Inter-laboratory studies, in which 6 laboratories participated, were organized by the National Institute of Health and Sciences (NIHS) to verify the detection methods. We prepared 24 separate tubes containing 200 mg test-samples for two Japanese varieties, and the 6 lines of GM potato described above. The homogeneities of the samples were confirmed by the methods using specific primer pairs with the 3 tubes selected randomly for each sample at the NIHS. The blind samples were designed as a pair of blind duplicates including 0 and 100% NL-Bt-6, NL-SPBT02-05, NLP-RBMT21-350, NLY-RBMT15-101, NLY-SEMT15-02, and NLY-SENT15-15. The blank samples, two Japanese conventional varieties, were used to confirm whether the tests were performed correctly without false-positive results. A total of 16 tubes containing blind samples, a solution of 8 primer pairs (5 μmol/l each), and the experimental protocol were supplied to the 6 labs from the NIHS.

RESULTS AND DISCUSSION

Evaluation of the Validity of Extracted DNA for PCR To avoid a false-negative result, it is important to confirm the validity of the extracted DNA for PCR. Therefore, the primer pair, UGPase 01-5'/UGPase 01-3', was designed as the analytical control to evaluate the validity of the potato DNA for PCR. We performed the PCR with DNAs extracted from 11 kinds of potatoes including the GM and non-GM potatoes to examine the conservation of the UGPase gene among the potato varieties. As shown in Fig. 2A, all of the potato DNAs generated a 111-bp amplified fragment with almost the same intensity, whereas no fragments were amplified from the tomato, egg plant, maize, wheat and soy DNAs. These results suggested that PCR using the primer pair should specifically detect the DNA extracted from potato. The method to identify the potato DNA has been reported by Jaceaud *et al.*[12] They designed the primer pairs to detect the patatine gene which is encoded by a multigene family. In their method, however, the amplified fragments were generated from the potato, tomato and tobacco using the primer pair for identification of the patatine gene. In contrast, we showed that PCR using the designed primer pair, UGPase 01-5'/UGPase 01-3', generated no amplified fragments in the tomato and egg plant which belong to the Solanaceae taxonomic family (Fig. 2A, lanes 13 and 14). These results suggest that potato DNA should be more stably detected with high specificity and the validity of the extracted potato DNA for PCR would be assessed by PCR using the UGPase 01-5'/UGPase 01-3' primer pair.

Specificity of Screening Method for the Detection of GM Potatoes Screening methods for the detection of the GM crops including soy and maize have already been reported.[3,6] The DNA sequences of the 35S promoter and the terminator sequence of the nopaline synthase gene from *Agrobacterium tumefaciens* (NOS terminator), which were commonly and frequently used to regulate the expression of a gene in the various GM crops, were selected to design the

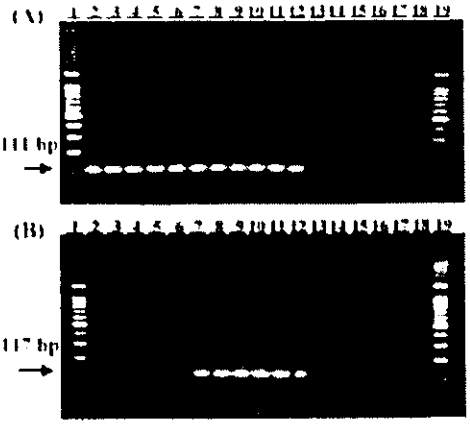

Fig. 2. Specificity of Potato and GM Potato Specific Primer Pairs

Arrowheads indicate the expected PCR amplified fragments. The primer pairs UGPase 01-5'/UGPase 01-3' (A) and CryIIIA 01-5'/CryIIIA 01-3' (B). Lanes 1 and 19, 100-bp ladder size standard; lane 2, non-GM potato (Russet Burbank); lane 3, non-GM potato (Superior); lane 4, non-GM potato (Shepody); lane 5, non-GM potato (Danshaku); lane 6, non-GM potato (May queen); lane 7, NL (SPBT02-05); lane 8, NL (Bt-6); lane 9, NLP (RBMT21-350); lane 10, NLY (SEMT15-15); lane 11, NLY (RBMT15-101); lane 12, NLY (SEMT15-02); lane 13, tomato; lane 14, egg plant; lane 15, maize; lane 16, soy; lane 17, wheat; lane 18, negative control (no template DNA).

primer pairs for the screening detection. The primer pair designed for the sequence of the NOS terminator could be used to detect GM potatoes for the screening purpose because the DNA sequence of the NOS terminator was commonly introduced in the NL, NLP and NLY (Fig. 1). However, the GM potatoes and the other GM crops, such as GM maize and GM soybean, should be undistinguishable using the primer pair for the NOS terminator because GM maize and GM soybean could generally contain the NOS terminator. On the other hand, a gene encoding cryIIIA should be introduced into all lines of NL, NLP and NLY and it has not been reported that the cryIIIA gene was introduced in the other GM crops. Therefore, the CryIIIA 01-5'/CryIIIA 01-3' primer pair was designed on the cryIIIA gene for the screening detection of the GM potatoes. An amplified fragment (117 bp) was specifically detected from all 6 lines of GM potato used in this study (Fig. 2B). In contrast, no amplified product was detected when the DNAs extracted from tomato, egg plant, maize, wheat, soy, and non-GM potatoes were used as the template DNA. Two lines of NLP (RBMT21-129 and RBMT22-82) were not provided from the developer and could not be used for this study, but the same results would be expected, because the same target sequence should be introduced into the two lines according to the information described in the safety assessment document.

Specificity of Construct-Specific Methods for NL, NLP and NLY In NL, the e-p-35Ssequence[13] is used to regulate the expression of the cryIIIA gene, whereas the riblose-1,5-bisphosphate carboxylase small subunit atsIA promoter (P-Arab-SSU1A)[5] is used to control the cryIIIA gene expression in NLP and NLY. Therefore, the construct-specific primer pair, NL 01-5'/NL 01-3', was designed in the junction between e-p-35S and cryIIIA to specifically detect NL. As shown in Fig. 3A, two lines of NL, which were transformed with the same plasmid vector PV-STBT02, generated 113 bp amplified fragments by PCR. On the other hand, no fragment was detected in any DNAs including the non-GM and other GM potatoes. These results suggest that the 2 lines of NL should be specifically detected using the NL 01-5'/NL 01-3'

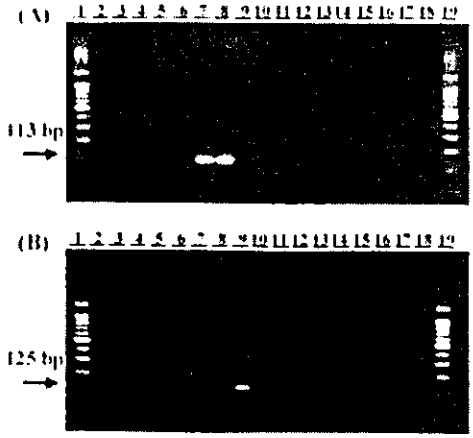

Fig. 3. Specificity of NL and NLP Construct-Specific Primer Pairs

Arrowheads indicate the expected PCR amplified fragments. The primer pairs NL 01-5′/NL 01-3′ (A) and NLP 01-5′/NLP 01-3′ (B). See footnotes for Fig. 2.

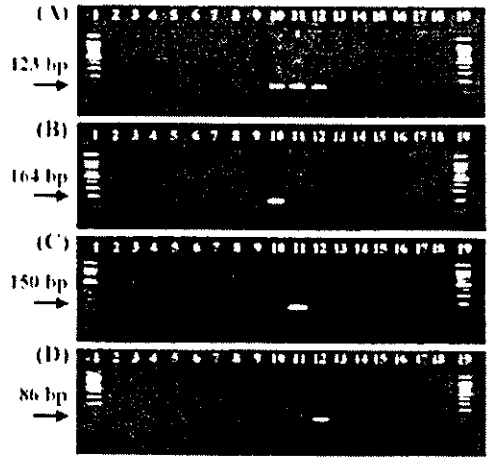

Fig. 4. Specificity of NLY Construct- and Line-Specific Primer Pairs

Arrowheads indicate the expected PCR amplified fragments. The primer pairs NLY 01-5′/NLY 01-3′ (A), NLY15 01-5′/NLY15 01-3′ (B), NLY101 01-5′/NLY101 01-3′ (C) and NLY02 01-5′/NLY02 01-3′ (D). See footnotes for Fig. 2.

primer pair.

NLP and NLY contain the 35S promoter sequence of the Figwort mosaic virus (P-FMV),[19] and P-FMV regulates the expression of PLRVrep in NLP and that of PVYcp in NLY. If the primer pairs were designed in the gene encoding PLRVrep or PVYcp for the detection of NLP and NLY, false-positive results might be obtained in the potatoes infected by the viruses. To avoid the false-positive results that might be caused in such samples, primer pairs were designed in the specific sequence available only at the junction region derived from different organisms. Therefore, the region between the leader sequence of heatshock protein isolated from Glycine max (Hsp 17.9)[20] and the sequence of the PLRVrep was selected to design the NLP construct-specific primer pair, NLP 01-5′/NLP 01-3′. To design the NLY construct-specific primer pair, NLY 01-5′/NLY 01-3′, the region between Hsp 17.9 and PVYcp was selected as the target sequence. An amplified fragment (125 bp) was obtained from only NLP using the primer pair, NLP 01-5′/ NLP 01-3′ (Fig. 3B). Furthermore, an amplified fragment (123 bp) was generated from only 3 lines of NLY during the specificity examination of the primer pair, NLY 01-5′ NLY 01-3′ (Fig. 4A). For assessment of the screening method, two lines of NLP could not be used for the examination, but the same results would be obtained because the same target sequence should be introduced into the other two lines of NLP.

Specificity of Line-Specific Methods for NLY In Japan, two lines of NLY (NLY-SEMT15-15 and RBMT15-101 line) were approved in April 2003 and the NLY-SEMT15-02 line was approved in July 2003. Therefore, it was necessary to identify the unapproved NLY-SEMT15-02 line until the safety assessment was finished. As shown in Figs. 3A and 4A, the same size PCR-amplified fragment was obtained from the three lines of NLY, which were transformed with the same plasmid vector, using the construct-specific methods. To identify the three lines, the specific region of each NLY should be selected to design the primer pair. Therefore, the border sequences between the r-DNA and potato genomic DNAs were selected as the NLY line-specific region. The NLY line-specific primers, NLY15-01-5′/NLY15-01-3′, NLY 02-01-5′/NLY02-01-3′ and NLY101-01-5′ NLY101-01-3′, were specifically designed on the NLY15-15, NLY15-02 and NLY15-101 line, respectively, and PCR using the corresponding primer pairs generated a 164 bp, 86 bp and 150 bp of amplified fragment from the specific lines, respectively (Figs. 4B, C, D). These results suggested that the border sequence between the integrated r-DNA and plant genomic DNA should be critical for examining the line-specific detection methods. The line-specific methods for GM potatoes have not been reported yet, though the line-specific methods for other GM crops have been reported.[21,22] When some GM crops, which contain the same r-DNA, might be bred in the near future, these findings will help to develop the detection method with high specificity and reliability.

Sensitivities of the Methods We examined the sensitivities of the screening and construct-specific detection methods with the potato-powder mixing samples containing 0, 0.1 and 1.0% of the NL-Bt-6, NLP-RBBT21-350 and NLY-SEMT15-15 line, respectively. Twenty-five ng of extracted genomic DNAs from the samples were amplified by PCR, and all of the target sequences were clearly detected in the 0.1% and 1.0% powder samples (Fig. 5A). It has been reported that the amount of unreplicated haploid genome (*i.e.*, the 1C value) of potato is 0.88 pg.[23] On the basis of the 1C value, 28.4 copies of the haploid GM potato genome were calculated to be present in the reaction tube prepared for a 0.1% potato-powder sample, since 25 pg of genomic DNA derived from each GM potato should be contained in the reaction tube. Furthermore, one or more copies of the target sequences are presented in each tetraploid genome of the NL-Bt-6, NLP-RBBT21-350 and NLY-SEMT15-15 line according to the information provided by Monsanto. Therefore, at least, 7.1 copies of the target sequences should be contained in the reaction tube. Theoretically, a copy of the target sequence could be amplified by PCR. In fact, the specific amplified fragments were detected in the 0.05% samples prepared by twice diluting the DNAs extracted from the 0.1% powder samples with non-GM potato DNA (data not shown). The potato-powder samples with a concentration less than 0.1% were not used for the examination since it was difficult to correctly prepare the samples. However, GM-potatoes might be detected in the lower concentrated samples using screening and construct-specific detection methods. We further ex-

amined the sensitivity of the NLY line-specific detection methods using potato-DNA samples containing 0, 0.05, 0.1, 0.5, 1.0 and 5.0% DNAs extracted from the three lines of NLY. As shown in Fig. 5B, by using the NLY-SEMT15-15 and RBMT15-101 line-specific methods, the specific amplified fragments were detected in the 0.05% sample. On the other hand, the specific amplified fragment for the NLY-SEMT15-02 was detected in the 0.5% sample. These results suggested that the detection limits of NLY-SEMT15-15, RBMT15-101 should be 0.05%, whereas the detection limit of the NLY-SEMT15-02 line-specific method was 0.5%. The sensitivity of the PCR methods would be affected by various factors including the copy numbers of the target sequence, physical character of the primer pair and PCR conditions. For the line-specific methods, the copy number should not be correlated with the sensitivity because a single target sequence is presented in each line. To increase the sensitivity of the NLY-SEMT15-02 line-specific method, we would have to increase the amount of DNA used for the template, to change the PCR condition suitable for the method, or to redesign the primer pair.

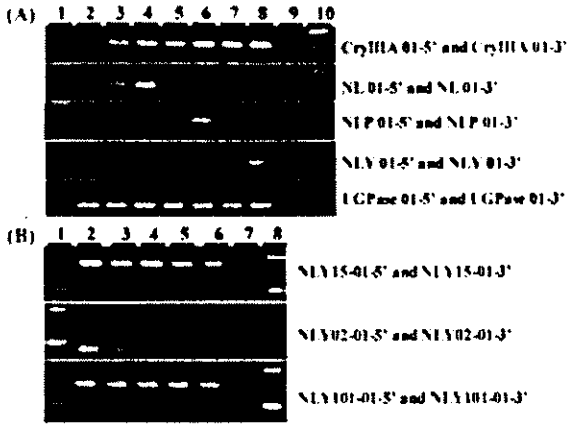

Fig. 5. Sensitivity of the PCR Methods Using the Specific Primer Pairs

Genomic DNAs extracted from the mixed samples of potato powder containing the 0.1 or 1.0% of some GM potatoes (A) or mixed samples of genomic DNAs containing various amounts of genomic DNAs extracted from some GM potatoes (B) were amplified. (A) Lanes 1 and 10, 100- and 200-bp size standard bands of ladder marker; lane 2, non-GM potato; lanes 3 and 4, potato containing 0.1 or 1.0% of NL; lanes 5 and 6, potato containing 0.1 or 1.0% of NLP; lanes 7 and 8, potato containing 0.1 or 1.0% of NLY; lane 9, negative control (no template DNA). (B) Lanes 1 and 8, 100 and 200 bp size standard bands of ladder marker; lanes 2 to 7, genomic DNAs containing 5.0, 1.0, 0.5, 0.1, 0.05% of genomic DNAs extracted from NLY-SEMT15-15, SEMT15-02, or RBMT 15-101, respectively; lane 7, negative control (DNA extracted from non-GM potato was used as template).

Inter-laboratory Study To confirm the specificity and reproducibility of the methods, we conducted inter-laboratory studies using 100% pure non-GM and GM potato-powder materials as blind samples. The blind samples were prepared as blind duplicates including two Japanese conventional varieties and 6 lines of GM potatoes (NL-Bt-6, NL-SPBT02-05, NLP-RBMT21-350, NLY-RBMT15-101, NLY-SEMT15-15 and NLY-SEMT15-02). All participants received the protocol, primer solutions, and 16 blind-sample tubes containing different potato powders. Six laboratories participated in the studies and analyzed a total of 96 samples. The methodology of the experiment was designed as described below. For the screening of the GM potato, a first PCR was performed to detect the potato taxon specific gene and cryIIIA gene. When the screening result was positive, a second PCR was performed to identify NL. If the sample was NL-negative, a third PCR was performed to identify NLY. A fourth PCR was performed for the NLY positive samples to identify the line of NLY. On the other hand, the NLY negative-samples was identified as NLP by performing an NLP construct-specific PCR. Typical results reported by a participant are shown in Table 2. The results showed that the test was correctly performed following the designed flow of the experiment, and no false-negative and -positive results were

Table 2. Typical Results of Inter-laboratory Study

Targets	Primer sets	Sample															
		A	B	C	D	E	F	G	H	I	J	K	L	M	N	O	P
UGPase	UGPase 01-5' UGPase 01-3'	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
CryIIIA	CryIIIA 01-5' CryIIIA 01-3'	+	+	+	+	+	−	−	−	−	+	+	+	−	+	+	+
NL	NL 01-5' NL 01-3'	+	−	−	−	−	/	/	+		+	−	−	/	+	−	−
NLP	NLP 01-5' NLP 01-3'	/	+	/		/		/	/	/	/	+	/	/	/	/	/
NLY	NLY 01-5' NLY 01-3'		−	+	+	+		/	/	/	/	/	+	/	/	+	+
SEMT 15-02	NLY02-01-5' NLY02-01-3'	/	/	+	−	−	/		/	/	/	/	−		/	+	−
SEMT 15-15	NLY15-01-5' NLY15-01-3'	/	/	−	+	−	/	/			/	/	−			−	+
RBMT 15-101	NLY101-01-5' NLY101-01-3'	/	/	−		+	/	/	/			/	+	/		/	/
Judgments		NL	NLP	15-02	15-15	15-101	Negative	Negative	NL	Negative	NL	NLP	15-101	Negative	NL	15-02	15-15

+: positive, −: negative, /: no test shown. A first PCR was performed with UGPase 01-5'/UGPase 01-3' and CryIIIA 01-5'/CryIIIA 01-3'. When the result was positive, a second PCR was performed with NL 01-5'/NL 01-3'. If the PCR product was not obtained from the second PCR, a third PCR was performed with NLY 01-5'/NLY 01-3'. A forth PCR was performed for the NLY-positive samples with NLY15 01-5'/NLY15 01-3', NLY101 01-5'/NLY101 01-3' and NLY02 01-5'/NLY02 01-3', respectively. On the other hand, the NLY-negative samples were identified by the PCR with NLP 01-5'/NLP 01-3'. Two Japanese conventional cultivars were judged as negative.

Table 3. Judgments and Percentage of Correct Results for All Test Samples in Inter-laboratory Study

Laboratory	Judgement						
	NL	NLP	NLP15-15	NLY15-02	NLY15-101	Dansyaku	Mayqeen
I	+ + + + / + + + +	+ + / + +	+ + / + +	+ + / + +	+ + / + +	− − / − −	− − / − −
II	+ + + + / + + + +	+ + / + +	+ + / + +	+ + / + +	+ + / + +	− − / − −	− − / − −
III	+ + + + / + + + +	+ + / + +	+ + / + +	+ + / + +	+ + / + +	− − / − −	− − / − −
IV	+ + + + / + + + +	+ + / + +	+ + / + +	+ + / + +	+ + / + +	− − / − −	− − / − −
V	+ + + + / + + + +	+ + / + +	+ + / + +	+ + / + +	+ + / + +	− − / − −	− − / − −
VI	+ + + + / + + + +	+ + / + +	+ + / + +	+ + / + +	+ + / + +	− − / − −	− − / − −
Percentage of correct results	100	100	100	100	100	100	100

+ + / + +: both of two positive samples were judged correctly, − − / − −: both of two negative samples were judged correctly. For NL, + + + + / + + + + showed that all of four positive samples were judged correctly, because NL samples were included the two lines of NL as blind duplicates, which can not be identified using the construct-specific method.

reported. In all results reported from the six laboratories, specific amplified fragments were completely detected in all test samples and no misjudgments were reported from any laboratories (Table 3). These results suggest that the proposed methods should be specific and reproducible for the detection of each line of GM potatoes.

In summary, detection methods using PCR were developed for screening and identifying eight lines of GM potato approved in Japan for human consumption. The highly specific PCR methods reported in this study are simple and useful for identifying GM potatoes.

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「ダイオキシン類等の化学物質の食品及び生体試料検査における
信頼性確保と生体曝露モニタリング法の確立に関する研究」

（平成16年度）

研究成果に関する刊行物

学会

ベビーフード中ダイオキシン類の分析および摂取量評価

斉藤貢一[1]，大村厚子[2]，竹熊美貴子[2]，伊藤里恵[1]，井之上浩一[1]，松木容彦[3]，中澤裕之[1]
[1]星薬科大学・薬品分析化学教室，[2]埼玉県衛生研究所・生体影響担当，[3]（社）日本食品衛生協会・食品衛生研究所

【目的】　乳幼児が食品から摂取するダイオキシン量を評価するために，いわゆるベビーフードのモデル食品群を提案した。更に，実際に市販されているベビーフードの各食品群におけるダイオキシン類残留分析を行うと共に，ダイオキシン類摂取量の新たな評価法を考案して，乳幼児が市販ベビーフードから摂取するダイオキシン類の摂取量評価を試みた。
【方法】　ベビーフードを大まかに6種類の食品群（菓子，野菜，フルーツ，魚，肉，乳製品）に分類してそれぞれのダイオキシン分析を行い，総摂取量の推定量を検討した。ダイオキシン分析は，公定法（食品中のダイオキシン類及びコプラナーPCBの測定方法暫定ガイドライン）に準じて行い，毒性等量(TEQ)の算出に際しては WHO-TEF(1998)を用いた。体重当たりの摂取量算出に際しては，調査対象としたベビーフードの適用月齢が 5～10 ヶ月（平均 7.5 ヶ月）であったこと，また，7.5 ヶ月の乳幼児の体重がおよそ 8kg であったことから，これを基準として適用した。
【結果】　乳幼児（月齢 7.5 ヶ月）の体重当たりの 1 日摂取量は，0.061 pg TEQ/kgbw /day（ND=0 で算出）および 1.2 pg TEQ/kgbw /day（ND=定量下限値×1/2 で算出）となった。この値は我が国がダイオキシン摂取量に対して定めた耐容 1 日摂取量（TDI：Tolerable Daily Intake）の 4pg を十分に下回っていたことから，市販ベビーフードからのダイオキシン摂取量は直ちに問題になる量ではないと考えられる。

Assessment of dietary intake of dioxins from commercial baby foods

Koichi SAITO[1], Atsuko OHMURA[2], Mikiko TAKEKUMA[2], Rie ITO[1], Koichi INOUE[1], Yasuhiko MATSUKI[3] and Hiroyuki NAKAZAWA[1]
[1]Department of Analytical Chemistry, Faculty of Pharmaceutical Sciences, Hoshi University, Japan
[2]Biological Effect Research Group, Saitama Institute of Public Health, Japan
[3]Institute of Food Hygiene, Japan Food Hygiene Association, Japan

As for the assessment of dietary intake of dioxins from baby foods, we proposed a model food group of the baby foods. We analyzed dioxins which contaminated in commercial baby foods, and we also designed an original method for the evaluation of the intake amount of baby foods. Furthermore, assessment of dietary intake of dioxins from commercial baby foods was attempted. The baby foods were roughly classified in six food groups (cake, vegetable, fruit, fish, meat, and dairy products), and each group was analyzed, followed by estimation of the total amount of dioxins. The dioxin analysis was carried out based on the Japanese official method. When the amount of intake per each body weight was calculated, the weight of a baby was assumed to be 8kg. Because the average age of babies for the baby foods was 7.5 months old (5-10 months) and, the weight of the baby of 7.5 months old was reported to be about 8kg. The amount of the daily intake of dioxins for a baby (7.5 months old) was 0.061 pg TEQ/kg bw /day (ND = 0) or 1.2 pg TEQ/kg bw /day (ND = LOQ × 1/2). These values were below the amount of TDI (Tolerable Daily Intake:4 pg TEQ/kg bw /day) which is regulated in Japan. Therefore, we think that the amount of the daily dioxin intake from commercial baby foods is not a serious problem at present.

The Proficiency Testing of Determination of Dioxins in Food

Rieko Matsuda[1], Tomoaki Tsutsumi[1], Masatake Toyoda[2], Tamio Maitani[1]

[1]National Institute of Health Sciences, Tokyo
[2]Jissen Womens University, Hino

Introduction

Food intake is the main route of human dioxin exposure, making the determination of dioxins in food indispensable for risk assessment and risk management of dioxins. The uncertainty of analytical results, however, can be very great because of the low concentration of the analytes and complicated cleanup procedures. The risk assessment of dioxins based on analytical results also suffers from a similar degree of uncertainty. The Ministry of Health, Labor and Welfare of Japan has published "Guideline for the Determination of Dioxins in Food" to standardize the analytical procedures. The guideline contains the quality assurance procedures to obtain reliable analytical results and recommends participation in the relevant proficiency testing scheme. The proficiency testing provides the fair evaluation of the analytical results. The central science laboratory in England and the food and drug safety center in Japan offer the proficiency testing on food. The National Institute of Health Sciences of Japan (NIHS) also has carried out proficiency testing of dioxins in food since 1998 to assure the quality of analytical results for dioxins. In this presentation we will show the results of 5 rounds of proficiency testing.

Methods and Materials

Samples The samples used in the proficiency testing are listed in Table 1. Table 1 also shows the number of participants and the TEQ of each sample.

BCR CRM607 and BCR RM 534 were prepared by the European Commission's Institute for Reference Materials and Measurements. Eleven certified value for PCDDs and PCDFs were given to CRM607. Eleven values were assigned for PCDDs and PCDFs in RM534 although not certified. CARP-1 was prepared by the National Research Council of Canada. Eighteen concentrations are certified, including PCBs. The custom-prepared standard solutions containing native PCDDs,

PCDFs and PCBs were prepared by Wellington Laboratories (Canada).

Other samples (freeze-dried fish and freeze-dried spinach) were prepared by the Japan Food Research Laboratories. The homogeneity of the samples was verified by the Japan Food Research Laboratories and the NIHS.

Analytical methods All participants determined dioxins by HRGC/HRMS as stipulated in the "Guideline for the Determination of Dioxins in Food".

Statistical analysis The mean and the standard deviation (SD) of the concentrations reported for each compound from the participants were calculated. There was the possibility of outlyers, but the application of tests for outlyers such as the Grubbs test was not advisable due to the small number of participants. The robust mean and the robust SD were then calculated using algorithm A[1]. The RSDs of TEQ in Table 1 were calculated from the robust mean and the robust SD. Examples of the statistical results are shown in Table 2. One participant reported a very high concentration of OCDD. This outlying high value lead to the high mean (2.85 pg/g) and the large SD (6.33 pg/g). The robust mean and SD of the same data were 0.67 pg/g and 0.25 pg/g, respectively, after the effect of the outlyer was eliminated. The z-Score of each participant was calculated using the robust mean and robust SD. The techniques of participants who gave a z-score of more than 3 or less than -3 were regarded as unsatisfactory, and review of their analytical procedures was recommended.

Results and Discussion

Year 1998 A CRM was used to verify the trueness of the results. The participants used the same standard solution, provided by the NIHS. The mean values of the results reported for two isomers were out of the confidence intervals of the certified values. All the results reported by two participants fell within the 95% confidence interval of the certified value. The other 4 participants reported results outside the 95% confidence interval but *the number of the outlying results was only 1-3. Reproducibility calculated from the 6 participants was 2.8-48 % RSD for each isomer and 6.6 % RSD for total TEQ.

Year 1999 The same CRM was used to compare the results with those in 1998. Many reports suggested that fish is the main route of dioxin intake, making the reliability of analysis of dioxins in fish crucial[2]. CARP-1 was then included in the proficiency testing. One plausible reason for poor reproducibility was the difference

among the standard solutions used by the participants. Mixed standard solutions of PCDDs, PCDFs and PCBs were used to estimate the variation in standard solutions among the participants.

For 6 isomers, the mean of participants was outside the confidence intervals of the certified values. The reproducibility for CRM607 (TEQ) was 11% RSD and larger than that in 1998. The decline in analytical performance probably arose from the difference between standard solutions. In 1998, all participants performed the determinations using the same standard solution. In 1999, each participant used their own standard solution. The number of participants increased to 15 in 1999, and inexperienced laboratories were included. This explains the increase in RSD.

The difference in the mean of the reported value for the mixed standard solution sample and the stated concentration was below 10%. The reproducibility of the standard solution sample was 8-15 RSD %. Bavel reported the RSDs of reported values of participants in proficiency testing in which a standard solution was used[3]. The RSDs after removing the outlyers were, with one exception, 10-17%. These results are similar to ours. The analysis of the solution required no cleanup procedure and the results were expected to represent the variability of the standard solutions of participants. According to the manufacturer's statement, the range of standard solution concentration is ± 5%, corresponding to an RSD of 2.9%. The higher reproducibility suggested other causes, such as the change in the concentration of the internal standards due to unsuitable storage conditions.

The mean of the reported values for CARP-1 was within the confidence interval of the certified value. The reproducibility of TEQ was 8.0% RSD. The TEQ of CARP-1 was 79 pg/g and was fairly large compared with the CRM607 (3.3). The large TEQ of CARP-1 led to its small reproducibility RSD.

Year 2000 Another RM and a standard solution with different isomer concentrations were used. The mean of the reported value for the RM was lower than the reference value for all compounds with reference values. The reproducibility of RM534 (TEQ) was 18% RSD. The reason for this poor reproducibility was not clear. The bias and reproducibility of the mixed standard solution sample were comparable to those in 1999. Differences in the standard solution used by the participants could not explain the large negative bias or large RSD.

Year 2001 As mentioned above, dioxin intake from marine fish is of great concern, and the use was requested of samples from wild polluted marine fish. The TEQ of CARP-1 is higher than that of wild fish, so it did not seem appropriate for proficiency testing aiming at the assurance of quality for analysis of common foods. Because no appropriate samples made of marine fish were available, we attempted the preparation of our own samples. Since 1998, no vegetable samples had been used in the proficiency testing, in spite of public concern about the contamination of leaf vegetables by dioxins.[4] For assurance of the performance of the vegetable analysis, a sample made of spinach was also prepared. Both samples were confirmed to be homogeneous and were thus suitable for proficiency testing. The reproducibilities of TEQ for the fish sample and spinach sample were 10% and 30%, respectively. The TEQ of the spinach sample was quite low (0.34 pg/g) at 1/20 of that of the fish sample. The large RSD was not extraordinary taking the low TEQ into consideration.

Year 2002 Another marine fish sample was prepared from grey mullet. Grey mullet contain more fat than sea bass and require further cleanup procedures. The results are likely to represent the actual analytical performance. The reproducibility was 7.1% RSD and comparable to the result of CARP-1.

The results of 5 rounds of proficiency testing revealed several problems with the determination of dioxins in foods. The variability of the standard solution is of major importance. Periodical confirmation of the validity of the standard by the use of CRM or by participation in proficiency testing is strongly recommended.

Although the TEQ of sea bass or grey mullet samples was about 1/10 of that of CARP-1, the reproducibility RSDs were comparable. These results show that repeated participation in proficiency testing improves the analytical skills of the laboratories. It is clear that for proficiency testing, the use of samples representing actual foods is preferable. Our attempted production of samples led to sufficiently homogeneous samples of fish and vegetables that could be prepared by freeze-drying. This technique opens the possibility of preparing samples from a variety of foods, leading to enhanced the effectiveness of proficiency testing.

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