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Assessment of Screening Methods for the Identification of Genetically Modified Potatoes in Raw Materials and Finished Products

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Qualitative polymerase chain reaction methods for the detection of genetically modified potatoes have been investigated that can be used for screening purposes and identification of insect-resistant and virus-resistant potatoes in food. The presence of the *nos* terminator from *Agrobacterium tumefaciens* and the antibiotic marker gene *npt*II (neomycin-phosphotransferase II) was demonstrated in three commercialized Bt-potato lines (Monsanto Co., St. Louis, MO, USA) and one noncommercial GM-potato product (high amylopectin starch, AVEBE, Veendam, The Netherlands) and allows for general screening in foods. For further identification, specific primers for the FMV promoter derived from the figwort mosaic virus, the *Cry*IIIA gene (δ-endotoxin from *Bacillus thuringiensis* subsp. *tenebrionis*), potato leafroll virus replicase gene, and the potato virus Y coat protein gene, were designed. The methods described were successfully applied to processed potato raw materials (dehydrated potato powders and flakes), starch samples, and finished products.

KEYWORDS: ***Solanum tuberosum*; potato; GMO detection; polymerase chain reaction; nested PCR; CryIII A gene; potato leafroll virus; potato virus Y; patatin**

INTRODUCTION

The potato (*Solanum tuberosum*), a major staple food and an important feedstuff, is subject to attack by many pests and pathogens. Enhancing potato's intrinsic resistance to them by transfer of resistance genes by breeding is laborious and inefficient due to the tetraploid character of the potato genome (*1*, *2*). Therefore, potato has been at the forefront of genetic engineering for the production of virus-resistant or insect-resistant crop plants by gene-transfer technology (*3*, *4*). The first approval of a genetically engineered potato for human consumption, commercialized by Monsanto Co., USA, was given in the United States in 1995 for the NewLeaf potato expressing a pest resistance. In 1996, this potato was approved in Canada. This was followed in 1999 by the approval in the United States and Canada of two new transgenic potato lines (NewLeaf Plus and NewLeaf Y potatoes), both expressing a pest resistance and a virus resistance. Consequently, substantial acreages (20 000 ha) of NewLeaf potatoes were grown in the United States in 1999. In the European Union in 1995, the Dutch company AVEBE got an approval for commerce of the Apriori and Apropos genetically modified (GM) potato cultivars with high amylopectin starch. These so-called waxy potatoes, in which amylose biosynthesis was repressed, were intended for the extraction and utilization of the modified starch for purposes including food use. In 1998, the European Commission's Scientific Committee on Plants withdrew the approval, arguing that through gene transfer from this GM potato, which contains the *npt*III gene conferring resistance to the antibiotic amikacin, antibiotic resistance could develop in humans via the gut flora (*5*). Since that time, no GM potatoes have been approved for commercialization or for human (or animal) consumption in the European Union.

Many governments now have implemented or are considering regulations for the use and labeling of genetically modified organism (GMO)-derived ingredients. To comply with the legislation, reliable and accurate methods for the identification of GMOs in raw materials and food products are required. Numerous analytical methods have been described so far, and the adoption of official or validated GMO testing methods is in its initial stage (CEN/ISO standards). Polymerase chain reaction (PCR) methods for screening of GMO crops, and for the qualitative and quantitative detection of GM soya and GM maize (*6*−*8*), are widely applied in routine analysis to assess whether a food product contains material derived from genetically modified crops. Taking into account that trade in these two commodities is dominated by only a few countries, both can be found in varying quantities in about 75% of the volumes traded globally (*9*). The food industry in Europe and elsewhere imports processed potato products from North America, including French fries, potato starch, and potato powder or flakes. One cannot objectively exclude that some of these products are of genetically modified origin; therefore, they are to be dismissed while not approved. No specific detection methods have been

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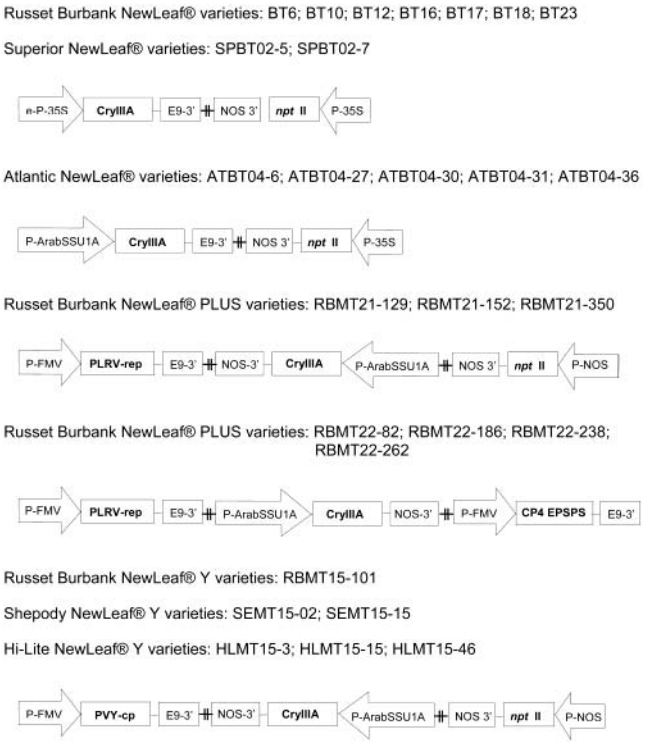

Figure 1. Schematic representation of gene organization in transgenic potatoes. The insert structure of the transgenic cultivars of "Superior" (SPBT02-5 and SPBT02-7) NewLeaf potatoes was described by AGBIOS (Agriculture & Biotechnology Strategies Inc.; www.agbios.com). According to Stone and Lavrik (*24*), the "Superior" cultivars were transformed with the same plasmid vector (PV-STBT02) as the seven "Russet Burbank" NewLeaf lines (BT6, BT10, BT12, BT16, BT17, BT18, and BT23). The PV-STBT04 plasmid vector used to transform the five "Atlantic" NewLeaf lines (ATBT04-6, ATBT04-27, ATBT04-30, ATBT04-31, and ATBT04-36) employs a different promoter (P-ArabSSU1A) for the CryIIIA gene than PV-STBT02 (e-P-35S). The construction of the "Hi-Lite" NewLeaf Y potatoes was described by Lavrik and Reding (*28*). The plus sign indicates different lengths of the plasmid vector between the construct elements.

published for commercialized transgenic potatoes so far, but such methods have been studied on model systems (*10*, *11*). This development might be hampered due to the lack of availability of GM potato reference material and the lack of detailed knowledge about the transgenic DNA sequences and the molecular structure of these GMOs, which is needed in order to select appropriate oligonucleotide primers. Though the transgenic potatoes engineered by the Monsanto Co., USA, were described in U.S. patents, their molecular characterization is difficult to reconstruct. The molecular description of a specific transgenic potato line covers many different patents, and its characterization from such a patchwork of information is hard to reconstitute. Furthermore, some transgene sequences described in the patents might be incomplete when compared to the same sequences deposited at the NIH, NCBI, Genbank Collection, Washington, DC. Consequently, the molecular characterization of Monsanto's transgenic potatoes was made possible only after a request was made to the U.S. Food and Drug Administration, under the Freedom of Information Act, for a complete description of Monsanto's transgenic potato gene constructs (*12*) (**Figure 1**).

In this paper, we describe a combination of screening and gene-specific PCR detection methods for the identification of transgenic potatoes in raw materials and processed food samples. Primer systems for the detection of universal GMO markers CaMV 35S promoter (P-35S), nos3′ terminator (nos3′), npt*II*, FMV promoter (P-FMV), and resistance genes (CryIIIa) against Colorado potato beetle (CPB) and virus (PLRV-rep, PVY-cp) were investigated.

MATERIALS AND METHODS

Reference Materials and Potato Products. A small piece of a fresh CPB-resistant NewLeaf potato (Bt-potato, Monsanto Co.), cultivated in Eastern Europe, was obtained from Carbotech AG (Basel, Switzerland). Seed potatoes, New Leaf Plus, a Russet Burbank which contains Bt and PLRV resistance, New Leaf Y, a Shepody which has Bt and virus Y resistance, standard Russet Burbank (control sample from the same seed-growing plot as the GMO varieties), and standard Shepody (control sample from the same seed-growing plot as the GMO varieties), were obtained from a local seed supplier in the United States. The two GM potato samples and the two control samples were cut into strips (not peeled), blanched, partially dried, and frozen by Nestlé USA, Dublin, OH, and then shipped frozen to the Nestlé Research Center (Lausanne, Switzerland). A test sample of a noncommercialized high amylopectin potato starch made from GM potatoes was obtained from AVEBE R&D (The Netherlands). Fresh control potatoes and finished products were purchased from local supermarkets (snacks, chips, potato purée, and French fries). Potato starch and dehydrated potato powder and flakes were obtained from different suppliers in the United States and Canada.

DNA Extraction and Purification. Homogenized samples (raw material, dehydrated potatoes, finished products),150 mg each, were incubated in 1.5 mL of hexadecyltrimethylammonium bromide (CTAB) extraction buffer [1.4 M NaCl, 20 mM EDTA, 100 mM Tris-HCl, 2% (w/v) CTAB, pH 8.0] in a thermomixer at 65 °C for 60 min. After maceration, the samples were centrifuged at 15 000*g* for 10 min. The aqueous phase was transferred into a new tube, extracted with an equal volume of chloroform, and centrifuged again at 15 000*g* for 10 min. The supernatant was added to 5 volumes of binding buffer (PB buffer from Qiagen, Hilden, Germany), mixed, and loaded onto a QIAquick column (Qiagen) over a vacuum manifold. The column was washed twice with 750 μL of PE buffer (Qiagen), and the sample was dried at 12 000*g* for 5 min and placed in a new centrifuge tube. The purified DNA was eluted by centrifugation for 5 min at 12 000*g* with 50 μL of elution buffer (10 mM Tris-HCl, pH 8.5) after 5 min of incubation and stored at −20 °C.

Potato starch samples (2 g each) were incubated in 10 mL of CTAB buffer and 200 μL of heat-stable α-amylase solution (10 mg/mL) from Sigma (No. A-4551) for 60 min at 65 °C. After maceration, the samples were centrifuged at 3000*g* for 10 min. The supernatant was added to 5 volumes of binding buffer (Qiagen), mixed, and loaded onto a QIAamp Maxi column (Qiagen) over a vacuum manifold. The column was washed twice with 10 mL of PE buffer (Qiagen), dried at 3000*g* for 5 min, and placed in a new 50-mL tube. DNA was isolated after 1 min of incubation with elution buffer at 3000*g* for 5 min. Five milliliters of PB buffer was added to the eluted DNA, mixed, and loaded on a QIAquick column (Qiagen) over a vacuum manifold. The column was washed twice with 750 μL of PE buffer (Qiagen), and the sample was dried at 12 000*g* for 5 min and placed in a new centrifuge tube. The purified DNA was eluted by centrifugation for 5 min at 12 000*g* with 50 μL of elution buffer after 5 min of incubation and stored at −20 °C.

DNA Target Sequences and Oligonucleotide Selection. All oligonucleotides designed in this study are listed in **Table 1**. They were synthesized by MWG-Biotech (Ebersberg, Germany) in HPSF quality and stored at −20 °C.

PCR and DNA Analysis. Five microliters of the isolated DNA was added to 45 μL of amplification mixture. Amplifications were carried out in 0.5-mL microcentrifuge tubes containing the reaction buffer (Promega no. M5661, Madison, WI) [50 mM KCl, 10 mM Tris-HCl (pH 9.0), 0.1% Triton X-100)], a specific magnesium chloride concentration adapted to each PCR (see **Table 2** for details), 2.0 μg/mL bovine serum albumin (Sigma No. A-8022, St. Louis, MO), 0.2 mM each of dATP, dCTP, dGTP, and dTTP nucleotides (Promega No. U1240), together with 1 bead (1.25 units) of TaqBead Hot Start DNA

Table 1. List of Oligonucleotide Primers

target	primer	sequence (5′ to 3′)	position	GenBank database accession no.
CryIIIA gene	ST01 (for)	CTACCACTAAGGATGTTATCC	38–58	X70979
	ST03 (rev)	ATGCACTCACGTAGTCCTCC	279–298	
	ST02 (rev)	TTGTATAGAAGCTCACGAGG	120–139	
PVY coat protein gene	PVY05 (for)	CAAGGCTATCACGTCCAAAA	8707–8726	X12546
	PVY06 (rev)	ACCAAACCATAAGCCCATTC	8902–8921	
	PVY07 (for)	TTTGCTTGAGTATGCTCCAC	8773–8792	
	PVY08 (rev)	TTTCTCCTATGTCGTATGCC	8863–8882	
PLRV replicase gene	PLRV01 (for)	CGATGAGGATTACGGTCTG	1639–1657	X14600
	PLRV02 (rev)	TTCTCCCCACCATAGCTGT	1874–1892	
	PLRV03 (for)	AGAGAGGCTGCAACAAATGC	1671–1680	
	PLRV04 (rev)	GCCGCTTGCCCATTTAAA	1768–1785	
Patatin gene (exon 4)	PatGa (for)	GTTGTTGCTCTCATTAGGCAC	2762–2782	X03932
	PatGb (rev)	CAGTCATGTAAGAACTTGCTGC	2880–3001	
	PatGc (for)	CTCATTAGGCACTGGCACT	2771–2789	
	PatGd (rev)	GTAAGAACTTGCTGCACTAGTC	2873–2894	
FMV genome	FMV181 (for)	GCCAAAAGCTACAGGAGATCAATG	6538–6561	X06166
	FMV366 (rev)	GCTGCTCGATGTTGACAAGATTAC	6670–6693	
35S promoter[a] (cloning vector pAVA319)	35S–1 (for)	GCTCCTACAAATGCCATCA	1214–1232	AF078810
	35S–2 (rev)	GATAGTGGGATTGTGCGTCA	1389–1408	
NOS terminator[b] (cloning vector pBin19)	NOS4 (for)	GATTGAATCCTGTTGCCGGT	1603–1622	U12540
	NOS5 (rev)	GTAACATAGATGACACCGCG	1796–1815	
	NOS1 (for)	GAATCCTGTTGCCGGTCTTG	1607–1626	
	NOS6 (rev)	CCCATCTCATAAATAACGTC	1691–1710	
npt II gene[c] (transposon Tn5)	Tn5–1 (for)	GGATCTCCTGTCATCT	1850–1865	U00004
	Tn5–2 (rev)	GATCATCCTGATCGAC	2007–2022	
Chloroplast[d] intergenic spacer between *trn* L (UAA) 3′ exon and *trn* F (GAA)	A3 (for)	GGTTCAAGTCCCTCTATCCC		
	A4 (rev)	ATTTGAACTGGTGACACGAG		

[a] Described by Pietsch et al. (*6*). [b] Kantonales Laboratorium, CH-Basel (*20*). [c] Described by Beck et al. (*21*). [d] Described by Taberlet et al. (*13*).

Table 2. Primer and $MgCl_2$ Concentrations and Cycling Conditions[a]

	primer concn (μM)	$MgCl_2$ concn (mM)	denaturation	annealing	extension	cycles
A3/A4	0.3	2.5	95 °C, 40 s	55 °C, 40 s	72 °C, 45 s	45
35S-1/35S-2	0.5	2.5	95 °C, 30 s	54 °C, 40 s	72 °C, 40 s	40
FMV181/FMV336	0.3	2.5	95 °C, 30 s	55 °C, 30 s	72 °C, 40 s	40
NOS4/NOS5	0.3	3	95 °C, 30 s	55 °C, 30 s	72 °C, 40 s	25
NOS1/NOS6	0.5	3	95 °C, 30 s	50 °C, 30 s	72 °C, 40 s	35
Tn5-1/Tn5-2	0.5	2.5	95 °C, 30 s	50 °C, 30 s	72 °C, 40 s	40
PatGa/PatGb	0.3	2.5	95 °C, 30 s	55 °C, 30 s	72 °C, 40 s	25
PatGc/PatGd	0.5	2.5	95 °C, 30 s	53 °C, 30 s	72 °C, 40 s	35
ST01/ST03	0.3	3.5	95 °C, 30 s	60 °C, 30 s	72 °C, 40 s	20
ST01/ST02	0.3/0.5	4.5	95 °C, 30 s	60 °C, 30 s	72 °C, 40 s	40
PVY05/PVY06	0.3	4	95 °C, 30 s	57 °C, 30 s	72 °C, 40 s	19
PVY07/PVY08	0.5	4	95 °C, 30 s	65 °C, 30 s	72 °C, 40 s	35
PLRV01/PLRV02	0.3	2.5	95 °C, 30 s	57 °C, 30 s	72 °C, 40 s	25
PLRV03/PLRV04	0.5	2.5	95 °C, 30 s	65 °C, 30 s	72 °C, 40 s	35

[a] Each PCR started with an initial denaturation step of 2 min at 98 °C and ended with a final extension step of 3 min at 72 °C. Cycling conditions are optimized for use of a thermocycler Trio-thermoblock (Biometra, Göttingen, Germany).

polymerase (Promega No. M5661) and 0.3 or 0.5 μM (see **Table 2** for details) of each specific primer. When a nested PCR was carried out, 2 μL from the previous PCR was added to a new 48-μL amplification mixture containing the same reaction buffer, 2.0 μg/mL bovine serum albumin, a concentration of $MgCl_2$ adapted to each specific reaction, 0.5 μM of the internal primer pair, and 1 bead (1.25 units) of TaqBead Hot Start DNA polymerase. Amplification products (15 μL) were submitted to electrophoresis on 2% Seakem LE agarose gels (FMC Bioproducts, Rockland, ME) in 1× TAE buffer [4 mM Tris−acetate; 1 mM EDTA (pH 8.3)] and made visible by staining with ethidium bromide at UV (312 nm) transillumination. The expected size of the amplified fragments was estimated by comparison with DNA fragments of known size (DNA molecular weight markers, Roche Molecular Biochemicals, Rotkreuz, Switzerland).

Restriction analysis was achieved when 15 μL of the amplification product was incubated with 10−12 U of the appropriate restriction endonucleases (*Xmn*I, *Rsa*I, or *Nsi*I) for at least 2 h at 37 °C. The whole volume of this digestion mixture was then analyzed by gel electrophoresis.

RESULTS AND DISCUSSION

DNA Assessment. All DNA-extracted potato samples were first assayed for the detection of plant-specific DNA (chloroplast PCR) according to the method described by Taberlet et al. (*13*). A fragment of 450−500 bp of the intergenic noncoding spacer region of chloroplastic DNA (cpDNA) between *trn* L (UAA) 3′exon and *trn* F (GAA) exon was amplified in all of the potato samples (data not shown). A cpDNA approach using A3/A4 primers has already been demonstrated to be feasible to assess food authenticity of unknown plant ingredients (*14*).

Patatin PCR. To assess the detection of potato DNA, a nested PCR was designed in the patatin gene, the major storage protein gene in potato tubers encoded by a multigene family (*15*, *16*). The external primer pair Pat Ga/Pat Gb, together with the internal primer pair Pat Gc/Pat Gd, were chosen in exon 4 to generate a 140- and 124-bp amplicon, respectively. Soya, maize, rapeseed, tomato, and tobacco DNA were used to check for the specificity of these primers. Potato, tomato, and tobacco DNAs generated a 124-bp amplified fragment, whereas no fragments were amplified from the soya, maize, and rapeseed samples. Therefore, these primers were found to be specific for Solanaceae, the botanical family to which tomato, tobacco, and potato are belonging.

The intense 124-bp amplified fragment was first believed to be formed by the addition of the undiscriminated external and internal amplicons. Agarose gel electrophoresis of decreasing volumes of patatin PCR products revealed that this amplicon is a single 124-bp fragment (data not shown), its intensity resulting from the strong amplification of a multicopy gene. This multicopy gene might also account for the detection of this 124-bp fragment when as little as 0.5 pg of total DNA was amplified

Table 3. Analytical Results

	DNA control	screening for transgenic DNA				identification of transgenes		
	patatin gene	P-35S CaMV	P-FMV	NOS 3′	Npt II	CryIIIA	PLRV-rep	PVY-cp
				Potato Reference Samples				
raw potato (negative control)	+	−	−	−	−	−	−	−
NewLeaf (Monsanto)	+	+	−	+	+	+	−	−
Russet Burbank (negative control)	+	−	−	−	−	−	−	−
NewLeaf Plus	+	−	+	+	+	+	+	+
Shepody (negative control)	+	−	−	−	−	−	−	−
NewLeaf Y (Monsanto)	+	−	+	+	+	+	−	+
starch (AVEBE)	+	+	−	+	+	−	nd[a]	nd
				Processed Potato Samples				
dehydrated potato powder (A)[b]	+	+	−	+	+	+	nd	nd
dehydrated potato powder (B) #1[c]	+	+	−	+	+	+	nd	nd
dehydrated potato powder (B) #2	+	+	−	+	+	+	nd	nd
dehydrated potato powder (B) #3	+	+	−	+	+	+	nd	nd
dehydrated potato powder (B) #4	+	+	−	+	+	+	nd	nd
dehydrated potato powder (B) #5	+	+	−	+	+	+	nd	nd
dehydrated potato powder (B) #6	+	+	−	+	+	+	nd	nd
dehydrated potato powder (C) #1	+	−	−	−	−	−	nd	nd
dehydrated potato powder (C) #2	+	−	−	−	−	−	nd	nd
dehydrated potato powder (C) #3	+	−	−	−	−	−	nd	nd
dehydrated potato powder (C) #4	+	−	−	−	−	−	nd	nd
dehydrated potato powder (C) #5	+	−	−	−	−	−	nd	nd
dehydrated potato powder (D)	+	+	−	+	+	+	nd	nd
dehydrated potato flakes (E)	+	−	−	−	−	−	nd	nd
dehydrated potato flakes (F)	+	−	−	nd	nd	−	nd	nd
finished product (G)	+	−	−	−	−	−	nd	nd
finished product (H)	+	−	−	−	−	−	nd	nd
finished product (I)	+	−	−	−	−	−	nd	nd
finished product (J)	+	−	−	−	−	−	nd	nd
finished product (K)	+	−	−	−	−	−	nd	nd
finished product (L)	+	−	−	−	−	−	nd	nd
starch (M)	+	−	−	−	−	−	nd	nd
starch (N)	+	−	−	−	−	−	nd	nd

[a] nd, not determined. [b] A–N: different suppliers or finished product (potato purée, snacks, chips, French fries). [c] #: different batches.

(data not shown). All potato-extracted DNAs tested with this PCR system show a very intense 124-bp amplicon (**Table 3**).

SCREENING METHODS FOR THE DETECTION OF TRANSGENIC POTATOES

Based on the presence of the promoter and the terminator or termination sequence in a chimeric gene, screening methods for the detection of transgenic potato were established. Such methods are indicative only for the presence of a transgene, and its presence should be further assessed by a specific PCR detection method.

CaMV 35S Promoter (P-35S) and Enhanced CaMV 35S Promoter (e-P-35S). The primer pair 35S-1/35S-2 (*6*) allows the amplification of a 195-bp fragment from the 35S promoter of the cauliflower mosaic virus (P-35S). In transgenic potatoes, an enhanced version of this 35S promoter (e-P-35S) is used (*17*) that contains a duplicated nucleotide sequence (positions 1116−1368 of the P-35S described in GenBank accession no. AF078810). This sequence carries a second complementary annealing site for the 35S-1 primer. Therefore, when DNA of NewLeaf potato or processed NewLeaf potato products was amplified, a 457-bp fragment together with a 195-bp fragment were detected from the e-P-35S (**Figure 2**). When digested for confirmation by the *Xmn*I restriction enzyme, the 195-bp fragments yielded two fragments of 115 and 80 bp in length, whereas the *Xmn*I restriction of the 457-bp fragment displayed a fragment of 262 bp as well as the two fragments of 115 and 80 bp (data not shown). In contrast, no amplicon was displayed when NewLeaf Y and NewLeaf Plus potato DNA samples were amplified by the 35S-1/35S-2 primers, suggesting

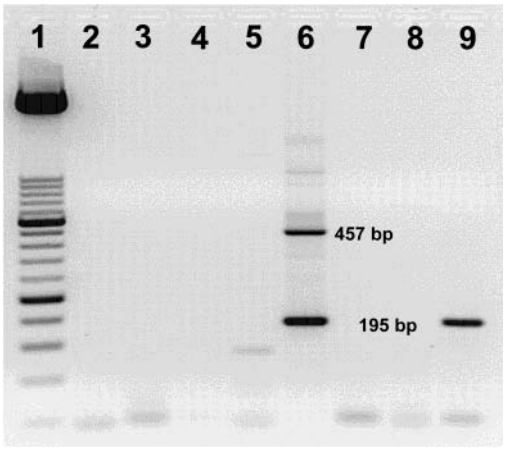

Figure 2. Amplification of CaMV 35S promoter: 15 μL of PCR products loaded per lane. Lane 1, 50-bp molecular weight marker; lane 2, negative control; lane 3, potato negative control reference; lane 4, Russet Burbank control potato; lane 5, Shepody control potato; lane 6, NewLeaf transgenic potato; lane 7, NewLeaf Plus transgenic potato; lane 8, NewLeaf Y transgenic potato; lane 9, CaMV 35S positive control (Roundup Ready soya).

that these potato strains do not contain the enhanced 35S promoter (**Table 3**).

FMV Promoter (P-FMV). NewLeaf Y and NewLeaf Plus transgenic potatoes contain the 35S promoter region of the figwort mosaic virus (P-FMV) (*18*, *19*) instead of the enhanced CaMV 35S promoter. P-FMV was detected by means of the primer pair FMV181/FMV336, amplifying a 156-bp fragment (**Table 3**). This fragment was digested by the *Nsi*I restriction

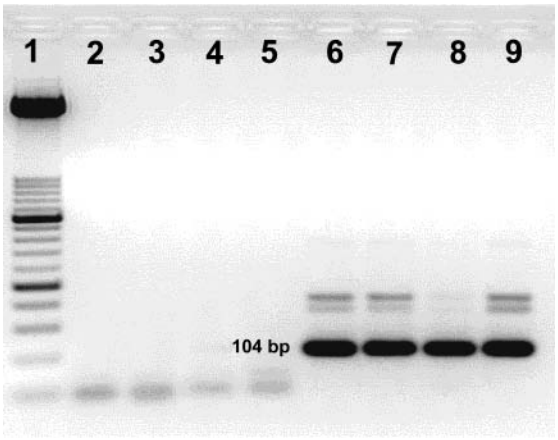

Figure 3. Amplification of NOS 3′ terminator: 15 μL of PCR products loaded per lane. Lane 1, 50-bp molecular weight marker; lane 2, negative control; lane 3, potato negative control reference; lane 4, Russet Burbank control potato; lane 5, Shepody control potato; lane 6, NewLeaf Plus transgenic potato; lane 7, NewLeaf Y transgenic potato; lane 8, dehydrated potato powder (A); lane 9, NOS 3′ positive control (plasmid pBI121). Carryover of PCR primers from the first PCR to the nested PCR generates cross-reaction between primers NOS4/NOS6 and NOS1/NOS5, amplifying fragments of 213 and 209 bp, respectively.

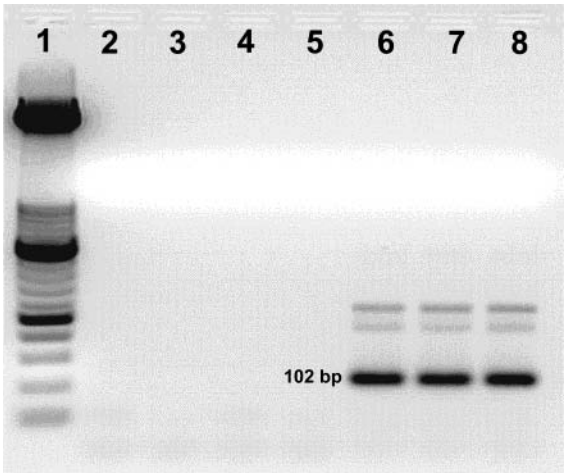

Figure 4. Amplification of CryIII A gene: 15 μL of PCR products loaded per lane. Lane 1, 50-bp molecular weight marker; lane 2, negative control; lane 3, potato negative control reference; lane 4, Russet Burbank control potato; lane 5, Shepody control potato; lane 6, NewLeaf transgenic potato; lane 7, NewLeaf Plus transgenic potato; lane 8, NewLeaf Y transgenic potato. The upper amplicon of 261 bp corresponds to the amplification product of primers ST01/ST03. These amplification products disappear when lower concentrations of DNA are amplified (data not shown).

enzyme for confirmation, yielding two fragments of 94 and 62 bp (data not shown).

NOS 3′ Terminator. Isolated from the nopaline synthase gene from *Agrobacterium tumefaciens*, the terminator of this gene (NOS 3′) is displayed by means of a nested PCR (*20*). All the transgenic DNA potato samples tested with this PCR system gave a positive amplification signal (**Table 3**). The primer pair NOS 4/NOS 5 amplified a 213-bp fragment, which was then used as a DNA template for the primer pair NOS 1/NOS 6 to generate a 104-bp amplicon. The primers NOS 4 and NOS 5 from the first amplification reaction cross-reacted with the primer pair NOS 1/NOS 6 during the nested PCR amplification to generate amplicons of 108 bp (primers NOS 4/6) (**Figure 3**). The intense amplified fragment of 104 bp might, in fact, be formed by the addition of the 108- and 104-bp fragments, their resolution on a 2% SeaKem LE agarose gel being impossible.

***Npt*II Gene.** The coding sequence for the *npt*II gene is derived from the prokaryotic transposon Tn5 (*21*). The expression of this gene confers resistance to kanamycin, and it is used as a marker gene in the construction of transgenic plants, allowing the selection of transformed cells. The purpose of inserting the *npt*II gene into potato cells with any other transgene is to have an effective method for selecting cells that contain the transgene. Therefore, the potato samples were tested for the presence of the *npt*II gene by means of the primer pair Tn5-1/Tn5-2 (*22*). A 173-bp fragment from the *npt*II gene was amplified in all potato DNA samples of transgenic origin (**Table 3**). For confirmation, this amplicon was further digested by the *Rsa*I restriction enzyme for confirmation, yielding two fragments of 134 and 39 bp (data not shown). According to the 2% SeaKem LE agarose gel resolution, this latter fragment might not be visible.

SPECIFIC METHODS FOR THE DETECTION OF TRANSGENIC POTATOES

CryIIIA Gene. The CryIIIA gene, conferring resistance to the Colorado potato beetle (CPB) pest, was genetically engineered into potato plants (*17*, *23*, *24*). A semi-nested PCR system was developed to allow the detection of this gene: the primer pair ST01/ST03 amplified a 261-bp fragment, which was then reamplified by means of the primer pair ST01/ST02 to generate a 102-bp fragment (**Figure 4**). Usually 100−200 ng of total DNA was amplified, but the CryIIIA gene was detected when a PCR was carried out with as little as 10 pg of total DNA (2.7 genome copies). NewLeaf and NewLeaf Y as well as NewLeaf Plus potatoes were positively tested for the presence of the CryIIIA gene (**Table 3**), in agreement with the previous description of these transgenic potato strains. All transgenic potato product samples tested so far generated the identical 102-bp amplicon (**Table 3**).

Potato Leafroll Virus Replicase Gene. The cDNA sequence of the PLRV-rep gene, comprising the two overlapping open reading frames ORF2a and ORF2b, was inserted to confer resistance to PLRV (*25*, *26*). The PLRV transgene coding for the replicase protein was visualized by means of a nested PCR designed in the overlapping ORF2a and ORF2b sequences. Amplification by the external primer pair PLRV01/PLRV02 was targeting a 254-bp fragment from the replicase gene. This fragment was then reamplified by means of the internal primer pair PLRV03/PLRV04, yielding a 125-bp fragment. All transgenic potato strains were assayed for the detection of the replicase gene, but only the NewLeaf Plus potato strain exhibited a positive response to the PCR (**Figure 5**). As for the CryIIIA gene, this sequence of the PLRV genome was detected after PCR of only 10 pg of total DNA (data not shown).

Potato Virus Y Coat Protein Gene. The portion of the virus genome that encodes the PVY coat protein (PVY-cp) was integrated into plants to confer resistance against PVY (*27*, *28*). The PVY-cp coding sequence was displayed by a nested PCR performed first with the external primer pairs PVY05/PVY06, yielding a 215-bp fragment. This fragment was then used as a template for the internal primer pair PVY07/PVY08, generating an amplicon of 110 bp which was still detectable when 10 pg of total DNA was amplified (data not shown). All transgenic potato strains were tested with the system which was expected to be specific for the detection of the NewLeaf Y potatoes. Though the primer pairs PVY05/PVY06 and PVY07/PVY08 were specifically designed on the PVY-cp gene, they also

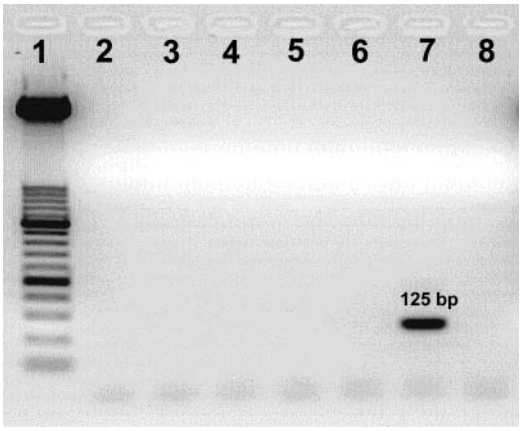

Figure 5. Amplification of PLRV replicase gene: 15 μL of PCR products loaded per lane. Lane 1, 50-bp molecular weight marker; lane 2, negative control; lane 3, potato negative control reference; lane 4, Russet Burbank control potato; lane 5, Shepody control potato; lane 6, NewLeaf transgenic potato; lane 7, NewLeaf Plus transgenic potato; lane 8, NewLeaf Y transgenic potato.

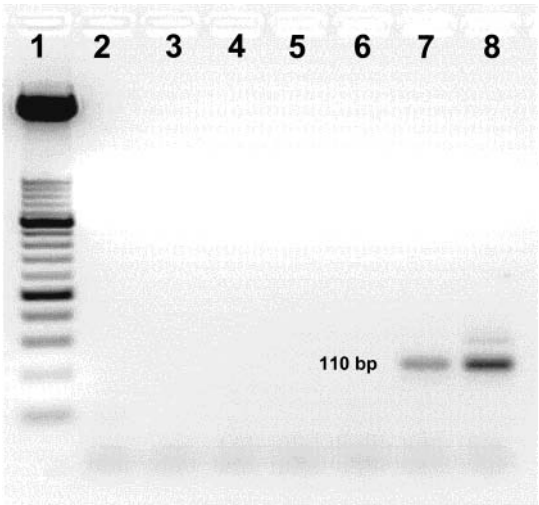

Figure 6. Amplification of PVY coat protein gene: 15 μL of PCR products loaded per lane. Lane 1, 50-bp molecular weight marker; lane 2, negative control; lane 3, potato negative control reference; lane 4, Russet Burbank control potato; lane 5, Shepody control potato; lane 6, NewLeaf transgenic potato; lane 7, NewLeaf Plus transgenic potato; lane 8, NewLeaf Y transgenic potato.

matched the NewLeaf Plus potatoes, generating an amplicon of the same size (**Figure 6**, lane 7). To exclude either a contamination of the starting material or a spill-over during gel loading, the DNA extraction and amplification were carried out on five different days by two different experimenters.

On the basis of our findings, we conclude that the spectrum of virus resistance might have been extended by combining PVY coat protein gene with PLRV replicase gene to obtain resistance to both PLRV and PVY, as stated by Mitsky et al. (*25*). Dual virus resistance was previously reported for potato plants made resistant to infection by potato virus X (PVX) and PVY by transforming the plant to express the coat proteins of the two viruses (*1*). Raising the question of a dual gene resistance, we checked whether the PVY coat protein gene and the PLRV replicase gene were cloned as a double-construct driven by a unique FMV promoter, or whether each gene was driven by its own FMV promoter. An amplification carried out with the primers FMV181 and PLRV04 generated a fragment of about 2000 bp in NewLeaf Plus potatoes only (**Figure 7**, lane 5).

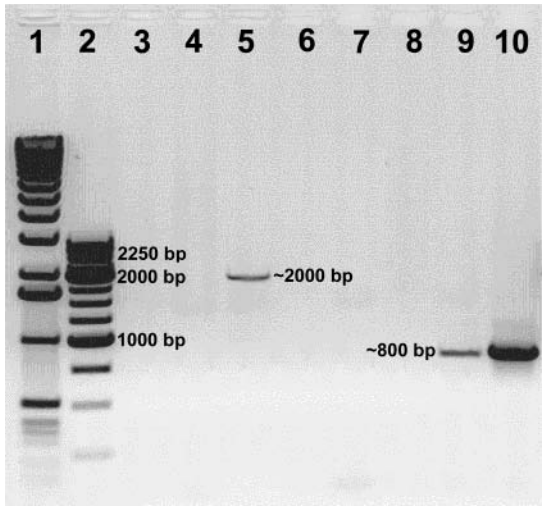

Figure 7. Dual transgene construct in NewLeaf Plus transgenic potato: 15 μL of PCR products loaded per lane. Lane 1, 0.075–12.21-kbp molecular weight marker; lane 2, 250-bp molecular weight marker; lane 3, negative control; lane 4, NewLeaf transgenic potato; lane 5, NewLeaf Plus transgenic potato; lane 6, NewLeaf Y transgenic potato; lane 7, negative control; lane 8, NewLeaf transgenic potato; lane 9, NewLeaf Plus transgenic potato; lane 10, NewLeaf Y transgenic potato. Potato DNAs were analyzed with the primer pair FMV181/PLRV04 (lanes 3–6) and FMV181/PVY06 (lanes 7–10). See text for detailed explanations.

Another PCR performed with the primers FMV181 and PVY06 amplified a fragment of about 800 bp in both potato lines (**Figure 7**, lanes 9 and 10). On the basis of these results, a construct as described in **Figure 8** is proposed. If these genes were driven by a unique promoter, then fragments of about 2800 and 4700 bp might have been expected, indicating a different gene order (P-FMV/PVY-cp/PLRV-rep/E9-3′ or P-FMV/PLRV-rep/PVY-cp/E9-3′). The sequence analysis of the 800-bp amplicon generated in NewLeaf Plus and in NewLeaf Y potatoes with the primers FMV181 and PVY06 revealed a complete sequence homology between these two DNA fragments in both potato lines, suggesting a dual virus resistance in NewLeaf Plus potato line. The transgenic potato constructs approved both in the United States and in Canada mentioned a pest resistance (CryIIIA) coupled with a unique virus resistance against either PLRV or PVY. A dual virus resistance was never mentioned or described for the approved NewLeaf Plus genetic construct. Our results revealed that the information about the insert structure of NewLeaf Plus potatoes was incomplete and that further investigation is required to check the accuracy of technical dossiers (i.e., characterization of junction or cross-border fragments) in order to develop methods to identify transgenic lines.

Potato Starch as Model Sample. Potato starch is an important ingredient for the food industry, and the detection of the transgenic origin of starch is therefore important for regulatory reasons (labeling). As a model sample of transgenic potato starch, the amylopectin potato starch from AVEBE was tested. These transgenic potatoes (waxy potatoes) (*29*) were not engineered to express any insect or virus resistance but rather to minimize the amylose content of the starch, which contained little or no amylose and consisted of branched amylopectin. The genetic modification involved antisense inhibition of the gene encoding granule-bound starch synthase I (*gbss* I), which is responsible for the amylose biosynthesis. The *gbss* I gene was fused in antisense orientation between the 35S promoter from the cauliflower mosaic virus and the nopaline synthase termina-

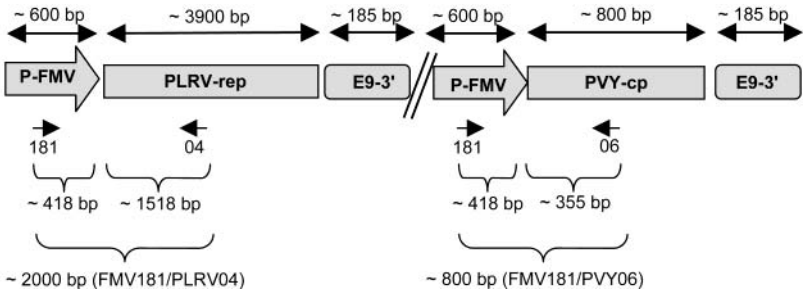

Figure 8. Schematic representation of assumed gene order in NewLeaf Plus transgenic potatoes, suggesting a dual virus resistance. Double-direction arrows indicate the size of each genetic element. A single left arrow shows the forward primer (FMV181), and single right arrow show the reverse primers (PLRV04 or PVY06). The expected sizes of the different primer arrangements are indicated below the brackets.

tor, whereas the kanamycin resistance from the *npt*II gene was used as a selectable marker. Consequently, when screening methods for the detection of transgenic potatoes were performed on DNA samples extracted from such a "waxy" potato starch, the 35S promoter, the NOS terminator, and the *npt*II gene were detected (**Table 3**). Apart from the *npt*II gene from the transposon Tn5, the plant vector used to genetically engineer these potatoes also contains the *npt*III gene. Though this gene remains under the control of a bacterial promoter, the *npt*III gene could confer resistance to amikacin, the antibiotic of value in the treatment of nosocomial infections. As it was suspected that amikacin resistance in humans could develop through horizontal gene transfer from these GM potatoes to human via the gut flora, the EU Scientific Committee on Plants concluded it was not possible to fully assess the safety of the transgenic potato lines in question (*5*).

The first method developed in this study allows the detection of the presence of potato DNA by means of a PCR amplification of the patatin gene, the major storage protein gene in potato tubers. Screening methods were investigated to monitor transgenic potatoes by amplification of the promoter sequences (P-35S, P-FMV), the termination sequence (t-NOS), or the selective marker for transformation (*npt*II gene). The specific characterization of transgenic potato lines was achieved by methods developed to detect the CryIIIA gene, conferring resistance to the Colorado potato beetle, the PLRV-rep gene, or the PVY-cp gene, conferring resistance to potato leafroll virus and potato virus Y, respectively. Because the annealing temperatures of the primer pairs and the $MgCl_2$ concentrations were optimized, the specificity and the sensitivity of the PCRs were ensured. Furthermore, none of the amplified fragments was longer than 270 bp, so analysis of degraded DNA from highly processed products was possible. The methods were validated (specificity) on a variety of different food matrices such as potato tubers, processed potato raw materials (starch, dehydrated potato powder, and flakes), and potato finished products (e.g., french fries and potato snacks), including GM-positive raw material obtained from the United States and Canada (**Table 3**).

For routine analysis, detection of all transgenic potato lines (NewLeaf, NewLeaf Plus, NewLeaf Y) can be achieved by amplification of the NOS terminator and of the *npt*II gene, whereas the PCR amplification of the CryIIIA gene allows the detection of a transgene in all lines. Screening for the 35S promoter is not sufficient for all GM potatoes because it detects only NewLeaf potatoes. The discrimination of NewLeaf Plus and NewLeaf Y potatoes from NewLeaf potatoes can be achieved by screening for the FMV promoter. Such a differentiation is further confirmed by the specific amplification of the PLRV-rep gene in NewLeaf Plus potatoes, whereas the PVY-cp gene is present in both NewLeaf Plus and NewLeaf Y potatoes.

The present methodology is an appropriate tool for checking transgenic raw potatoes and transgenic processed potato products. New GM traits of potatoes and other crops are in development and will enter the market sooner or later, whereas existing commercialized GM products will disappear (e.g., New Leaf Plus has been withdrawn from the market; R. Goodman, Monsanto Co., personal communication). Therefore, continuous monitoring of the level of GM materials and the identification of variety genotypes will be a prerequisite for the verification of non-GMO status in the supply chain, and validated detection methods are required (*30*).

ABBREVIATIONS USED

e-P-35S, cauliflower mosaic virus (CaMV) promoter with the duplicated enhancer region; cryIIIA, δ-endotoxin gene of *Bacillus thuringiensis* subsp. *tenebrionis* (Btt); E9-3′, nontranslated 3′ region of *pisum sativum* small subunit of ribulose-1,5-bisphosphate carboxylase (Rubisco); NOS 3′, 3′ nontranslated polyadenylation signal of *Agrobacterium tumefaciens* nopaline synthase (NOS) gene; *npt*II, neomycin phosphotransferase type II gene inducing kanamycin resistance; P-35S, cauliflower mosaic virus (CaMV) promoter; P-ArabSSU1A, *Arabidopsis thaliana* ribulose-1,5-bisphosphate carboxylase (Rubisco) small subunit *ats*1A promoter; P-FMV, 35S promoter region of the figwort mosaic virus; PLRV-rep, overlapping ORF2a and ORF2b sequences of the potato leafroll virus replicase gene; ORF, open reading frame; P-NOS, promoter of the nopaline synthase (NOS) gene of *Agrobacterium tumefaciens*; CP4 EPSPS, 5-enolpyruvylshikimate-3-phosphate synthase (EPSPS) gene of *Agrobacterium tumefaciens* strain CP4 inducing tolerance to the glyphosate herbicide; PVY-cp, potato virus Y coat protein gene; *gbss* I, granule-bound starch synthase I.

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