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Qualitative and Quantitative PCR Methods for Detection of Three Lines of Genetically Modified Potatoes

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Qualitative and quantitative polymerase chain reaction (PCR) methods have been developed for the detection of genetically modified (GM) potatoes. The combination of specific primers for amplification of the promoter region of *Cry3A* gene, potato leafroll virus replicase gene, and potato virus Y coat protein gene allows to identify each line of NewLeaf, NewLeaf Y, and NewLeaf Plus GM potatoes. Multiplex PCR method was also established for the simple and rapid detection of the three lines of GM potato in a mixture sample. For further quantitative detection, the realtime PCR method has been developed. This method features the use of a standard plasmid as a reference molecule. Standard plasmid contains both a specific region of the transgene *Cry3A* and an endogenous *UDP-glucose pyrophosphorylase* gene of the potato. The test samples containing 0.5, 1, 3, and 5% GM potatos were quantified by this method. At the 3.0% level of each line of GM potato, the relative standard deviations ranged from 6.0 to 19.6%. This result shows that the above PCR methods are applicable to detect GM potatoes quantitatively as well as qualitatively.

KEYWORDS: GM potato; multiplex PCR; realtime PCR; *Cry3A* gene; potato leafroll virus; potato virus Y

INTRODUCTION

In recent years, agricultural enterprises in the USA, Canada, and European Union (EU) have developed new plant varieties by adopting modern biotechnology including genetic transformation. Examples of such crops are corn, cotton, soybean, canola, and potato. Growers in the United States as well as other parts of the world, notably Canada and Argentina, rapidly accepted these crops. Rice and wheat crops are currently under development (*1*).

Potato (*Solanum tuberosum*) is one of the most important crops and is susceptible to many pathogens and pests. Due to its tetraploid nature, potato improvement against these diseases through breeding is rather arduous. Thus, it has actively involved genetic engineering in the improvement of potato. Engineering potato for resistance to viruses started in 1990 in the USA with the potato cultivar Russet Burbank.The potato was genetically transformed with coat protein genes from both PVX (potato virus X) and PVY (potato virus Y), and the transgenic plants were resistant to those viruses (*2*). Further transgenic potato lines were generated for broad-range protection against viruses using PLRV (potato leafroll virus) movement protein gene (*3*). Resistance against the Colorado potato beetle (CPB) was introduced in 1993 by an expression of the insecticidal Cry protein gene from *Bacillus thuringiensis* (Bt) (*4*).

Agribiotech crops are produced by introducing novel bits of DNA, which code for production of specific proteins into plants, resulting in the expression of new characteristics. The new DNA and protein can be found to varying degrees in many parts of these plants, including seeds and grain and certain processed fractions and final foods prepared from them. Methods of detecting and measuring DNA and protein are available and are used extensively in medical diagnostics. However, the task of analyzing the myriad of final food products is overwhelming, costly, and impractical (*5*). GM products contain an additional trait that is encoded by an introduced gene. Thus, raw material and processed products derived from GM crops might be identified by testing for the presence of an introduced DNA or by detecting expression of novel protein encoded by the introduced DNA. PCR methods have already been used for the screening of GM crops, such as GM soybean and GM maize, both quantitatively and qualitatively (*6*−*9*).

As labeling regulations on GMOs in Korea became effective in 2001, the development of qualitative and quantitative analytical methods was required for the implementation of relevant rules and the methods needed to approve practical application. Specific detection of GMOs in food has been successful using the PCR method worldwide and has settled as the working system. Recently, three lines of GM potatoes were developed by the Monsanto Company in the USA. Along with other GM crops that are already on the market, it has become necessary to detect these GMOs in food materials for labeling

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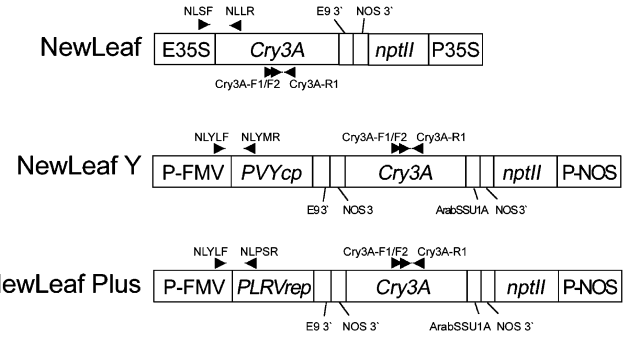

Figure 1. Schematic diagram of PCR strategy for detection of three GM potato lines. The foreign genes are shown in the squared box. The arrows and arrowheads indicate the location and direction of each primer, respectively.

in Korea. The GM potato lines include insect-resistant NewLeaf, insect- and virus-resistant NewLeaf Plus, and NewLeaf Y. All the NewLeaf series potatoes were Bt-protected by the introduction of the *Cry 3A* gene while NewLeaf Plus and NewLeaf Y had additional PVY coat protein gene and PLRV replicase genes, respectively, for resistance to viral infection (*10*).

In this study, we designed sets of specific primers for the detection of three GM potato lines in raw materials and processed foods and confirmed the specificity of the primers for each specific line. Qualitative multiplex PCR method was also established for simpler and quicker detection and a new quantification method, based on a realtime PCR, was developed using a new reference molecule for three GM potato lines.

MATERIALS AND METHODS

Potato and Other Crops. Four lines of potato powders (*Solanum tuberosum* L) were kindly provided by Monsanto Company (St. Louis, MO). Two lines of NewLeaf, Russet Burbank (RBBT 6) and Superior (SPBT 02−5) are resistant to the Colorado potato beetle while the NewLeaf Plus line, Russet Burbank (RBMT 21−350) is resistant to both the Colorado potato beetle and the potato leafroll virus. The NewLeaf Y line, Shepody (SEMT 15−15) is resistant to potato virus Y as well as to the Colorado potato beetle. Conventional non-GM potato variety Sumi, tomato (*Lycopersicon esculetum*) variety Seokwang, and pepper (*Capsicum annuum*) variety Nokkwang were used for comparison in designing specific primers.

DNA Extraction. Potato and other crop samples were lyophilized and ground into a fine powder by using an electric mill (Fritsch pulverizette 14, Germany). Genomic DNA was extracted from the powders obtained from fine grinding of samples (1 g each) using the DNeasy Plant Maxi kit (Qiagen, Hilden, Gemany) according to manufacturers instructions with minor modifications (*11*). The quality of the extracted DNA was monitored by a UV spectrophotometer DU650 (Beckmann Coulter Inc., Fullerton, CA.) and an agarose gel electrophoresis. The concentration of dsDNA was determined using PicoGreen dye assay (*12*).

Primers and Probes. The primers and probes used in this study were designed by using Primer Express software version 1.5 (Applied Biosystems, Foster City, CA). The position of primers and probes is shown in **Figure 1**, and the sequences are listed in **Table 1**. The primer pair of NLSF and NLLR was designed to amplify a region containing both E35S and *Cry3A* gene based on the deposited sequences (GeneBank Accession No. AF078810 and X70979) for detection of the NewLeaf potato. The NLYLF primer was used for detection of the promoter region of figwort mosaic virus (FMV) that was inserted into NewLeaf Y and NewLeaf Plus potatoes (GeneBank Accession No. X06166 and U. S. Patent 5,463,175) (*13*, *14*). The NLYMR primer was used to amplify the coat protein gene of PVY in NewLeaf Y and the NLPSR primer was used for replicase gene of PLRV in NewLeaf Plus potatoes (GeneBank Accession No. X68222 and D00530, and U.S. Patent 5,510,253) (*15*, *16*). The Cry3A primer pairs were designed based on the *Cry3A* gene sequence (GeneBank Accession No. X70979) and the UGP primer pairs were based on the sequence of *UDP-glucose pyrophosphorylase* (UGP) gene (GeneBank Accession No. U20345) (*17*). All the primers were synthesized by CoreBioSystem (Seoul, Korea) and all the probes were labeled with 6-carboxy-fluorecein and 6-carboxyteramethyl-rhodamine at the 5′ and 3′ ends, respectively. These were synthesized by Applied Biosystems.

Qualitative PCR. All the amplifications, except realtime PCR, were carried out in 50-μL volume reactions, with 100 ng of genomic DNA, 10mM Tris-HCl (pH 8.3), 50 mM KCl, 1.5 mM $MgCl_2$, 0.2 μM of each dNTP, 0.3 μM of each primer, and 2.5 units of HotStarTaq DNA polymerase (Qiagen), in the GeneAmp PCR System 9700 (Applied Biosystems). The multiplex PCR conditions were the same as described above, except the modified primer concentration as 0.8 μM of NLSF, NLLR, NLYMR primers, 0.4 μM of NLYLF primer and 0.1 μM of NLPSR primer, were used instead. The multiplex PCR was performed in a Peltier Thermal Cycler (MJ Research, Watertown, MA) with 40 cycles of 94 °C for 30 s, 62 °C for 30 s, and 72 °C for 30 s, and followed by a final extension at 72 °C for 10 min. A 10-μL aliquot of the PCR reaction was analyzed on 2% (w/v) agarose gel.

Quantitative PCR. Realtime PCR was set up using a 25-μL volume, with a 100 ng sample of DNA added to 12.5 μL Universal Master Mix (Applied Biosystems), 0.5 μM primer pair, and 0.2 μM probe. The PCR consisted of uracil-N-glycosylase treatment at 50 °C for 2 min, denaturation at 95 °C for 10 min, and 40 cycles of denaturation at 95 °C for 30 s, annealing and extension at 59 °C for 1 min.

Five concentrations (2×10, 3×10^2, 4×10^3, 7×10^4, 1×10^6 copies per reaction, respectively) of standard plasmid DNA were used as reference molecules for the preparation of a standard curve. Salmon testis DNA (5 ng/μL) was used as no-template control (NTC). All the realtime PCR was carried out in triple-replication by using three reaction wells for each template DNA (i.e., NTC, standard plasmid DNA, and sample DNA).

Analysis of Quantitative PCR. Standard curves were obtained for the real-time PCR according to the methods described by Kuribara et al. (*9*). GMO amounts (%) were calculated by the ratios of copy numbers of the transgene DNA and an endogenous gene in GM potato samples with application of a conversion factor (C_f), according to the method described previously (*9*).

Standard Plasmid as Reference Molecule. Standard plasmid as a reference molecule was constructed based on pCR2.1 vector (Invitrogen, Carlsbad, CA) with the integration of the PCR amplicons for an endogenous gene and a foreign gene. The PCR amplicons were obtained by using primer pairs UGP-F2/UGP-R2 for *UGP*, an endogenous gene, and Cry3A-F2/Cry3A-R1 for *Cry3A*, a foreign gene common in all the three lines of GM potato, respectively. Connection of the UGP and Cry3A amplicons was done by PCR with the following primers: UGP-Cry3A-5′ (5′-ctcctgctgatgccggcccgggctgtggccatccgcagtttactcag-3′) and Cry3A-UGP-3′ (5′-ctgagtaaactgcggatggccacagcccgggggccggcatcagcaggag-3′). The first PCR was carried out in a 25-μL reaction containing a 2.5-μL 10× reaction buffer, 0.2 μM of each dNTP, 1U *pfu* DNA polymerase (Stratagene, La Jolla, CA), 0.5 μM of each primer pair UGP-Cry3A-5′/UGP-R2 or Cry3A-F2/ Cry3A-UGP-3′, and 25 ng of genomic DNA from NewLeaf potato as a template. The GeneAmp PCR system 9700 (Applied Biosystems) was used for PCR (5 min at 95 °C, and 35 cycles of 30 s at 95 °C, 30 s at 60 °C, and 30 s at 72 °C, followed by a final extension at 72 °C for 7 min). The second PCR was carried out in a 50-μL volume containing 1 μL of the first PCR products serving as template DNA, 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 1.5 mM $MgCl_2$, 0.2 μM of each dNTP, 0.3 μM of each of primer UGP-F2 and Cry3A-R1, and 2.5 units of HotStarTaq DNA polymerase (Qiagen), in the GeneAmp PCR System 9700. The second PCR products were ligated into pCR2.1 plasmid vector using TOPO TA cloning kit (Invitrogen). The cloned DNA was confirmed by *Eco*RI digestion (Roche Applied Science, Mannheim, Germany) and sequencing analysis.

The cloned DNA was purified with the Qiagen Plasmid Midi kit (Qiagen). Following purification, the plasmid DNA was digested with the *Sma*I restriction enzyme (Roche), and separated on 1% agarose gel. The linearized plasmid DNA was purified by the QIAquick Gel Extraction kit (Qiagen) and its concentration was determined by the

Table 1. List of Primers and TaqMan Probes Used in This Study

target	name	sequence (5′ → 3′)	specifity	length (bp)
		Primer for qualitative PCR		
NewLeaf	NLS F	CCC ACT ATC CTT CGC AAG ACC	35S promoter	526
	NLL R	CGT AAC CGG AGA TAG CAA AGC	Cry3A gene	
NewLeaf Y	NLYL F	TGG TGC AGA ATT GTT AGG CG	FMV promoter	365
	NLYM R	GGA TGC TGC TTT GCT CTG C	PVY coat protein gene	
NewLeaf Plus	NLYL F	TGG TGC AGA ATT GTT AGG CG	FMV promoter	430
	NLPS R	CAG AGT AAT CCC CAC TCG AGG	PLRV replicase gene	
UGP	UGP–F1	GCT GAG GGA AGC GAG ACT GA	UGP gene	131
	UGP–R1	CAA TCC TTC TTG GGC CTA CCT	UGP gene	
Cry3A	Cry3A–F1	TGT GGC CAT CCG CAG TTT A	Cry3A gene	121
	Cry3A–R1	CAA GAG ACT GCG CCA ACG T	Cry3A gene	
		Primer and TaqMan probe for quantitative PCR		
UGP	UGP–F2	CTC TCC ATA CTC TCT GCT CCT CG	UGP gene	111
	UGP–R2	CGG CAT CAG CAG GAG AAA G	UGP gene	
	UGP–Taq	(FAM)-TCA CAA TCT TCT TCT CTG CTA TGG TCA CTG CT-(TAMRA)	UGP gene	
Cry3A	Cry3A–F2	CCG CAG TTT ACT CAG GCG TC	Cry3A gene	112
	Cry3A–R1	CAA GAG ACT GCG CCA ACG T	Cry3A gene	
	Cry3A-MGB	(FAM)-CGA TCA GAC GAT GAG GCC A	Cry3A gene	

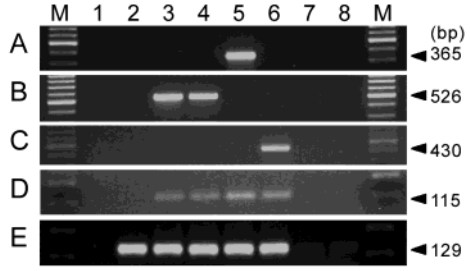

Figure 2. Specificity of the primer pairs designed for the three lines of GM potatoes. The numbers on the top indicate template DNA used in each lane: 1, no template control; 2, non-GM potato; 3, NewLeaf (Suprior); 4, NewLeaf (Russet Burbank); 5, NewLeaf Y; 6, NewLeaf Plus; 7, tomato; 8, pepper; M, 100 bp ladder. The primers used in each panel are shown on the left: **A**, NewLeaf Y; **B**, NewLeaf; **C**, NewLeaf Plus; **D**, Cry3A; E, UGPase. The size of fragment in bp is shown on the right.

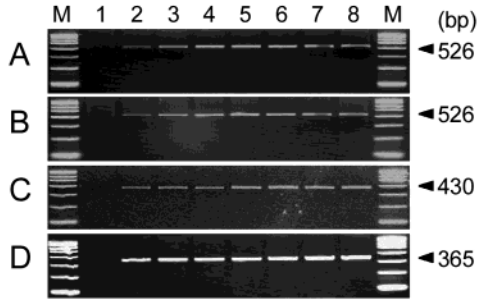

Figure 3. Sensitivity of the primer pairs designed for the three lines of GM potatoes. PCR products were amplified from each GM potato line DNA containing various amounts of GM potato genomic DNA. Lane: **A**, NeuLeaf Russet Burbank (RBBT6); **B**, NeuLeaf Superior (SPBT02-5); **C**, NeuLeaf Plus; **D**, NeuLeaf Y. Lane: M, 100 bp ladder marker; 1, amplification of non GM potato; 2–8, amplification of each GM potato containing 0.01, 0.05, 0.1, 0.5, 1, 3, and 5%, respectively. The size of each PCR fragment is shown in bp on the right.

UV spectrophotometer DU-650 (Beckmann Coulter Inc.). The standard plasmid was then serially diluted with salmon testes DNA solution (5 ng/μL, Sigma Chemcals Co., St. Louis, MO) to 2×10, 3×10^2, 4×10^3, 7×10^4, and 1×10^6 copies per 2.5 μL solution for use in realtime PCR.

Sequence Analysis. All PCR products produced in this study were subcloned into TA cloning vectors (Invitrogen). DNA sequencing was performed using the ABI PRISM 310 Genetic Analyzer (AppliedBiosystems) and the BigDye Terminator Cycle Sequencing Ready Kit (AppliedBiosystems).

RESULTS AND DISCUSSION

Specificity and Sensitivity of the Primer Pairs Designed for GM Potatoes. New primer pairs were designed to screen and identify three transgenic potato lines with inceased specificity of PCR (**Figure 1**). Each primer pair was successful to differentiate the single line from others (**Figure 2**). As shown in **Figure 2**, parts **A**−**C**, each primer pair was specific to NewLeaf, NewLeaf Y, or NewLeaf Plus potato, respectively, and no amplification was observed from non-GM potato, tomato, or pepper. This specificity is basically attributable to specific primer pairs designed to amplify the region containing both a trait gene and a regulatory gene, such as a promoter. It is not only practical to detect GM potatoes from other potatoes and other *Solanaceae* crops, but is also distinguishable from false positives caused by potatoes infected by PVY or PLRV. The *Cry3A* gene, conferring resistance to the Colorado potato beetle, is common to all the three lines of GM potatoes. PCR, with the primer pair for the *Cry3A* gene, was able to differentiate transgenic lines from non-GM lines tested (**Figure 1**, **Figure 2D**). The primer pair of UGP-F1/-R1 for intrinsic *UGP* gene was successful to amplify a 130-bp fragment from potato DNA, whereas no fragment was amplified from non-potato crops, such as tomato and pepper.

To determine the sensitivity of the PCR, the DNA mixture was prepared with each of three GM potato lines and non-GM potato line at various levels such as 0.01, 0.05, 0.1, 0.5, 1, 3, and 5%. The amplified fragment was detected from all the levels tested. The lowest level was 0.01% (**Figure 3 A**−**D**) and the amount of DNA required was as little as 10 pg (2.7 genome copies) for amplification. This sensitivity would be acceptable to ensure verification of non-GMO materials and to monitor the reliability of the labeling system.

Detection of GM Potato Lines by Multiplex PCR. The specific PCR bands derived from the three lines of GM potatoes were distinguishable from each other on the basis of the expected length produced by multiplex PCR. Three lines of GM potatoes were mixed in an equal weight (33% each) and DNA was extracted from this mixture. Multiplex PCR yielded three bands with different lengths that corresponded to the expected length

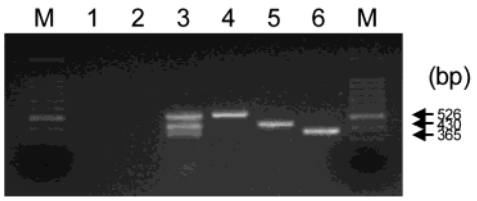

Figure 4. Detection of 3 GM potato lines from mixed sample by a multiplex PCR. The lanes 1–3 are the result of multiplex PCR with mixture of specific primers; lane 1, no template DNA; lane 2, non-GM potato; lane 3, mixed sample containing 3 GM potato lines. The lanes 4–6 show respective single PCR product amplified with respective single primer pair; lane 4, NewLeaf primers; lane 5, NewLeaf Plus primers; lane 6, NewLeaf Y primers. The size of each PCR fragment is shown in bp on the right.

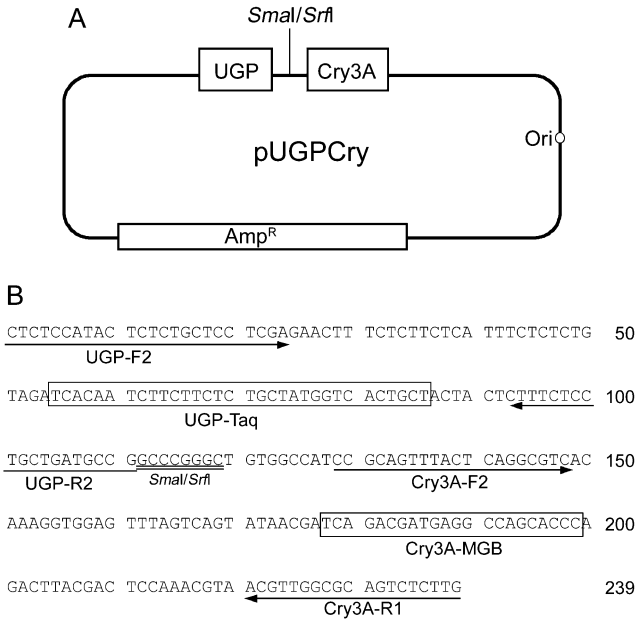

Figure 5. Standard plasmid pUGPCry as reference molecule. (**A**) Schematic diagram of pUGPCry. *SmaI*/*SrfI* indicates a restriction site; Amp, ampicilin resistance gene. (**B**) Sequence of the UGP and Cry3A region in pUGPCry. The arrows locate primers with direction and the squared boxes indicate TaqMan probes.

for NewLeaf (526 bp), NewLeaf Y (365 bp), and NewLeaf Plus (430 bp) potatoes, respectively (**Figure 4**). This multiplex PCR method was useful to detect each transgene in the mixed lines tested.

Standard Plasmid as Reference Molecule for Real-Time PCR. We have developed a highly sensitive and quantitative real-time PCR assay for the accurate measurement of GM potato lines. For real-time PCR, a plasmid pUGPCry for reference molecule was constructed by the tandem integration of two amplicons obtained by using respective primer pairs for *UGP*, an endogenous and single copy gene in potatoes, and *Cry3A*, a foreign gene common in all three lines of GM potato (**Figure 5A**). The sequence of the integrated fragments in (pUGPCry) are shown in **Figure 5B**. For calibration of the GM potato-specific real-time PCR, six levels of concentration of the reference molecule were set to 0, 2×10, 3×10^2, 4×10^3, 7×10^4, and 1×10^6 copies per reaction. The range from 2×10 to 1×10^6 copies was decided based on the genome size of tetraploid potatoes and the amount of genomic DNA template (100 ng) to be used in PCR (*18*−*20*). The range was supposed to be sufficient to quantify in a range of 3 orders of magnitude (0.5−100%) and could be used for quantitative detection of the threshold values of labeling regulations, for example, EU (1%),

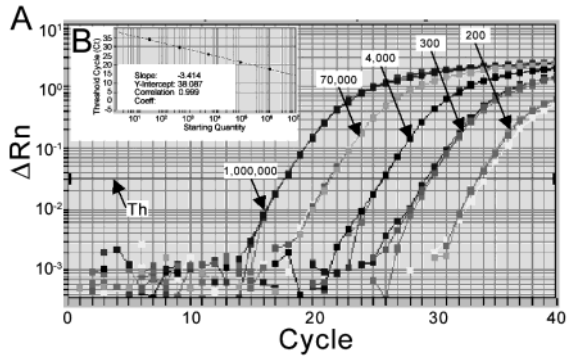

Figure 6. Amplification plots and standard curves for real-time PCR. (**A**) Amplification curves (6 doses, 0–1×10^6 copies of pUGPCry) were generated for detection of GM potato lines (0.5 μM Cry3A–F2/R1 primers and 0.2 μM Cry3A-MGB probe), and each curve corresponds to three replicates. The horizontal line indicates the threshold line determined for drawing up the standard curve according to the method described previously (11). (**B**) Parameters of the regression line through data points are indicated within the plot. The slope of repression line (−3.296) is close to theoretical value of −3.322.

Table 2. Repeatability and Reproducibility of pUGPCry

	copy no.			
target	true value	mean	RSDr	RSD_R[a]
UGP	20	23.2	31.4	7.4
	300	271.1	13.0	8.9
	4000	3817.4	8.2	6.6
	70000	70568.4	4.9	4.2
	1000000	1041889.0	4.0	4.3
Cry3A	20	23.1	21.8	3.6
	300	257.2	8.1	7.4
	4000	3858.0	5.0	4.4
	70000	76000.9	1.6	1.6
	1000000	1024092.2	7.0	6.2

[a] RSD (relative standard deviation) values were calculated by dividing the standard deviation by mean value, and given in %.

Korea (3%), and Japan (5%). With six levels of the reference molecule concentration, the linearity of the standard curve for *Cry3A*-specific quantification was calculated and was highly linear ($R^2 = 0.999$, **Figure 6**).

Repeatability standard deviation (RSDr) and reproducibility standard deviation (RSD_R) of the reference molecule pUGPCry were calculated by three sets of replication, respectively. The RSDr values ranged from 1.6 to 31.4% (**Table 2**). Larger RSDr values were expected at lower concentrations of reference molecule. The RSD_R values ranged from 1.6 to 8.9%. Variation within this range was not significantly large. Therefore, the above results show that the pUGPCry was useful as reference molecule for quantification of GM potatoes.

As previous work has reported, the advantages of using standard plasmid as reference molecule are as follows: first, standard plasmid can be supplied in unlimited quantities with consistent quality; second, the dilution procedure does not contribute greatly to the overall error rate; third, single plasmid can be used for many GM lines as long as the single plasmid contains every line- specific PCR amplicons derived from various GM lines (9). In this study, the use of the plasmid DNA as a reference molecule provided a stable standard curve for GM potatoes tested.

Measurement of Conversion Factors. The conversion factor (C_f) is a ratio between the copy numbers of the introduced DNA and an endogenous gene in each GM potato line. The GMO

Table 3. Conversion Factor for PCR Systems of Each GM Potato Line

target	mean	SD[a]	RSD[b]
NewLeaf (Superior)	0.15	0.02	12.69
NewLeaf (Russet Burbank)	0.21	0.02	8.46
NewLeaf Plus	0.65	0.01	2.18
NewLeaf Y	1.52	0.16	10.2

[a] SD = standard deviation. [b] RSD = relative standard deviation. Experiments were performed five times.

content (%) of unknown sample can be calculated by the following formula: copy number of a foreign DNA in unknown sample DNA/copy number of an endogenous DNA in unknown sample DNA $\times$ 1/ C_f $\times$ 100. We determined the C_f of each line by triple-replication, and a mean value was decided as C_f. **Table 3** shows the C_f of GM lines tested, and the values are 0.15, 0.21, 0.65, and 1.52 for NewLeaf (two lines), NewLeaf Plus, and NewLeaf Y, respectively. These results reflect that the C_f is proportional to the copy number of trasgenes in the genome. The *Cry3A* gene was integrated into 1−3 loci depending on the GM potato line, for example, a single copy in the genome of NewLeaf (RBBT 02-26 and SPBT 02-05), two copies at different loci in NewLeaf Plus (RBMT 21-350), and five copies at three loci in NewLeaf Y (SEMT 15-15) (D. Y. Kim, Monsanto Korea Co., personal communication). The ideal C_f of tetraploid GM potato for a single copy transgene per genome will be 0.25. The discrepancy between experimental and theoretical values could be basically attributable to different PCR efficiencies.

Accuracy and Precision of Quantitative PCR Methods. To evaluate the accuracy and precision of the real-time PCR method, interlaboratory tests or proficiency tests were performed with various GM line mixtures. Due to the lack of homogenized test materials, the mixtures that contained DNA of each GM line and DNA of non-GM potato variety Sumi at different ratios were prepared. Before mixing the DNAs, we adjusted the copy number of UGP gene of each GM line and non-GM potatoes after measuring the copy number with real-time PCR using standard plasmid. Test DNA samples containing 0.5, 1.0, 3.0, or 5.0% of genomic DNA of each GM potato line were used for evaluation of this real-time PCR method.

As shown in **Table 4**, accuracy of the method was measured as bias (%) of the experimental mean value from the theoretical value. As shown in **Table 4**, accuracy of the method was measured as bias (%) of the experimental mean value from the theoretical value. In this study, the bias of NewLeaf lines was the lowest among the GM lines tested ranged from −15.8 to 11.7, except for the 0.5% sample (about 30%). The mean values at 1.0 and 3.0% of NewLeaf lines were especially close to their true values. However, the bias of NewLeaf Plus and NewLeaf Y was all minus ranging from −14.1 to 25.5, indicating underestimation of these lines. The bias range found in this study is also slightly wider than one previously reported (−0.7.7−21.6) from maize and soybean samples (*9*). This result implies that the accuracy of this measurement needs to be improved by reducing the differences between mean values and true values. Evaluation of precision is shown as relative standard deviations (RSDs). The RSDs at the level of 1.0, 3.0, and 5.0%, which are the thresholds of unintentional mixing level in EU, Korea, and Japan, respectively, ranged from 3.8 to 20.2%. RSDs within the same range as above have been published earlier in GM soybean and maize detection systems (*9*). Thus, application of this real-time PCR method for GM potato might be useful, but requires additional validation with homogenized test materials.

Table 4. Accuracy and Precision Statistics for Quantitative Methods

		accuracy			
		mean	bias	precision	
GM line	true value	GMO %	true value %	SD[a]	RSD[b]
NewLeaf (Superior)	0.5	0.65	30.8	0.09	14.13
	1.0	1.07	6.6	0.14	13.58
	3.0	2.91	–3.0	0.34	11.85
	5.0	4.56	–8.9	0.75	16.49
NewLeaf (Russet Burbank)	0.5	0.67	33.2	0.10	15.04
	1.0	1.12	11.7	0.11	10.17
	3.0	2.93	–2.2	0.52	17.67
	5.0	4.21	–15.8	0.58	13.85
NewLeaf Plus	0.5	0.37	–25.1	0.10	26.76
	1.0	0.78	–22.0	0.16	20.24
	3.0	2.23	–25.5	0.08	3.78
	5.0	3.89	–22.2	0.69	17.77
NewLeaf Y	0.5	0.43	–14.1	0.08	18.14
	1.0	0.80	–19.6	0.05	5.81
	3.0	2.52	–15.9	0.44	17.54
	5.0	3.76	–24.8	0.19	4.98

[a] SD = standard deviation [b] RSD = relative standard deviation. Experiments were performed three times with three lots.

Currently, our detection system was based on a single ingredient sample. Quantification of the three different lines, if present in mixture, is possible by using a line-specific quantitation system, as published previously (*9*). Even if GM lines present in unequal amount in an impartially mixed sample need to be quantified, it is still possible to quantify each line separately by either of the above systems mentioned. Then, the sum of the values will represent a total amount of GM lines in the sample. Therefore, detection of a single line at a time seems to be a theoretically ideal method. Application of multiplex detection system to either sample (equal or unequal amount), however, appears to be difficult, because using more than one fluorescent dye of probe at a time possibly influences each other to competition between GM lines, resulting in an incorrect value.

ABBREVIATIONS USED

E35S, cauliflower mosaic virus (CaMV) 35S promoter with duplicated enhanced region; Cry3A, Cry3A delta-endotoxin gene from *Bacillus thuringiensis* Subsp. Tenebrionis; E9 3′, 3′ polyadenylation signal of *pisum sativum* ribulose-1,5-bisphosphate carboxylase (Rubisco); NOS 3′, 3′ nontranslated polyadenylation signal of *Agrobacterium tumefaciens* nopaline synthase (NOS) gene; nptII, gene encoding for neomycin phosphotransferase II; P35S, cauliflower mosaic virus 35S promoter; P-FMV, promoter region from figwort mosaic virus; PVYcp, coat protein gene from potato virus Y (PVY); ArabSSI1A, Arabidopsis ribulose-1,5-bisphosphate carboxylase small subunit (rbcS) promoter; P-NOS, promoter of *Agrobacterium tumefaciens* nopaline synthase gene (NOS); PLRVrep, replicase gene from potato leafroll virus (PLRV). UGP, UDP-glusoce pyrophosphorylase gene from *Solanum tuberosum*; C_f, conversion factor.

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