^{2}(2)=22.51, p<.01 ; 豆腐 χ^2(2)=31.36, p<.01）。多重比較の結果，「遺伝子組換えである」と表示された商品は，3 商品とも価格提示群，ベネフィット提示群で統制群よりも選択率が有意に高かった。

さらに，性差の検討したところ，豆腐の価格提示群において男性の方が女性よりも遺伝子組換え表示商品の選択率が有意に高かった（χ^2(1)=5.56, p<.05）。しかし，その他の群およびその他の商品において有意な男女差は見られなかった。また年代差の比較では，どの商品どの群においても年代差は見られなかった。

(4)実験 2

学童保育条件と大学サークル条件とに分けた課題が,商品の栄養への関心に関係したかどうかを確認した。

パッケージ表示注視の有無を問う質問紙の栄養表示と成分表示の項目を Fisher の直接法により検定を行ったが，有意な差は見られなかった(x^2(1)=0.65,*n.s.*, x^2(1)=0.17,*n.s.*)。

実験刺激中、せんべいのカテゴリーは遺伝子組み替えの表示が「遺伝子組み換えでない」，「不分別」，非表示の 3 種類であった。学童保育条件と大学サークル条件でパッケージの注視時間と回数が変化するかを調べるために,被験者間因子を各条件，被験者内因子を遺伝子表示とした。遺伝子表示(不分別，非表示,遺伝子組み換えなし)を独立変数，商品を見る総時間，栄養表示注視回数，栄養表示注視時間，成分表示注視回数，成分表示注視時間を従属変数として,二要因分散分析を行った。

表示ごとの成分表示への注視時間において，有意な交互作用は見られなかった(F(2.16)=0.601,*n.s.*)。また，対象ごとの主効果も見られなかった(F(1.8)=0.515,*n.s.*)

表示ごとの成分表示への注視回数において，有意な交互作用は見られなかった(F(2.2)=0.355,*n.s.*)。また，対象ごとの主効果も見られなかった(F=(1.8)=0.194,*n.s.*)

表示ごとの栄養表示への注視時間において，有意な交互作用は見られなかった(F(1.8)=1.24,*n.s.*)。また，対象ごとの主効果も見られなかった(F(2.16)=0.116,*n.s.*)

表示ごとの栄養表示への注視回数において，有意な交互作用は見られなかった(F(2.2)=0.154,*n.s.*)。また，対象ごとの主効果も見られなかった(F(1.8)=0.504,*n.s.*)。

(5)フォーカス・グループインタビュー

a.消費者へのヒヤリング結果

食品のリスク一般に対する関心としては、多くの人は程度の差はあるが普段の食事に心がけ、健康な生活を送るようにこころがけている。大きな傾向としては、栄養のバランスを気にする、脂肪分やカロリー/炭水化物等を抑え体型維持に努める、持病や体調が悪いために食事を心がけるなどといった意見があげられた。

子供のいる人は、その子の年齢により添加物への意識が異なっていた。

新しい食品技術として認識されているものは主に遺伝子組み換え技術である。遺伝子組み換え食品に対しては懐疑的な態度を示す人が多く、「遺伝子組み換え」という言葉や遺伝子組み換え食品は市場に浸透しているものの、その実態や将来への身体への影響などの情報が一般の消費者にはほとんど到達していない状況が明らかになった。

新しい食品技術についてどう思うかという問に対しては、遺伝子組み換えが最も想起されやすかったが、品種改良や養殖、ゼロカロリー食品などが挙げられた。

遺伝子組み換えについては安全性の面で漠然とした不安感を感じている人が多く、特に新しい技術だけに長期間摂取することによる将来的な身体への影響がまだ明らかになっていないことからリスクがわからないこと自体がリスクであると感じられる傾向が認められた。そのため印象は否定的なものが多く、購入を控える人が多かった。

新しい技術なので、良いものなのではないかと感じている人も一人いた。また、積極的には肯定的な態度ではないものの、売られているということは一定の安全性を持つ食品なのではないか、検証されているのではないかと感じている人もみられた。

遺伝子組み換え食品の利益としては、「虫がつきにくい／病気になりにくい」などの安全面でのメリットの他に、「年中つくれる／長持ちしそう」などの保管機能、「生産原価が安い」といった経済的メリットがあげられた。

一方、遺伝子組換え食品のリスクとして捉えられているのは、「人間の遺伝子にも影響がありそう／将来的な発ガン性が気になる」などの健康面でのリスクの他に、「何がいいのか悪いのかわからない／わからないことが怖い」などリスクがわからないことにリスクを感じるといった意見があがった。

遺伝子組換え食品の安全性については、ほとんどの人は安全性に疑問を抱いていた。

この問題に関して行政の規制が出来ていると感じる人は、日本人の食に対する関心の高さゆえに他の国に比べてきちんとした管理がなされていると認識していた。

一方、行政の規制が不十分と感じる点としては、製造に関与していない故の知識不足の可能性があげられている。「きちんとした説明ができていない」ことも行政の知識不足を疑う要因となっている。

b.事業者対象のヒヤリング結果

A社は、会社の方針として遺伝子組み換え食品を扱っていない。安全性が立証されていないというのがその理由である。

遺伝子組み換え食品の消費者に対するメリットとしては、次のような点があげられた。

①食料の安定生産＝安定供給、⇒ 食料不足の解決

②単位面積当たりの収穫量アップ ⇒ コストダウン

③除草剤耐性のものは農薬不要 ⇒ 残留農薬のリスクなし

④味を変える、形を変える、栄養価をアップさせるなど

遺伝子組み換え食品の一般消費者にとってのデメリットとしては、まず、安全性に対する潜在的な不安が指摘された。具体的な事例が報告されているわけではないが、遺伝子組み換えのものを食べると体のホルモンバランスが崩れるのではないか、将来生まれる子供に悪影響を及ぼすのではないかといった漠然とした不安を持つ人が少なくない。

そのほかのデメリットとしては、製造業であるF社の方から、次の2点があげられた。

①安全性審査をきちんと受けていない遺伝子組み換え食品がバラまかれる可能性がある

②生態系の破壊

一般消費者への情報伝達について、企業として何らかの情報伝達を行っているのは製造業のみである。F社は、遺伝子組み換えは、通常の交配による品種改良も同じであり、安全性試験を通っていてむしろ安全であるという見解である。その上で、お客様から問い合わせがあれば答えているし、話す機会があれば、その安全性を伝えているとしている。しかし、現在の国民の理解は不充分であり、さらなる啓蒙が必要性を感じている。

他は、自社から積極的に遺伝子組み換え食品についての情報伝達は行っていない。そういう状況の中で気にしているのは、法律の整備面や行政からの情報が不充分だということである。

製造業においても、遺伝子組み換え食品を使っていると報告しているので、それに関連しての工場立ち入り検査などはない。ただ、敷地内に遺伝子組み換え品が発育していないかのチェックがあるだけとのことで、それに対して、特に不満とか要望はない。

行政からの直接的な指導・監督はないが、今後国がどう取り組むのか、法整備の方向性含めて関心は高い。

不分別等の表示に関しては賛否両論みられる。賛成意見は、情報過多になると迷うので、今の表示でいいというものである。

反対意見は、例えばたばこのように、遺伝子組み換えだとどんな問題があるのかまで表

記すべきというものである。ただし、現時点では、どういう問題があるのか不明確なので、そういう段階で、使用の有無のみを表示することが問題だとされた。また、表示するメリットがあるのか懸念する声もある。

そのほか、小売業の2社からは、「不分別」という表示はよくわからない方もいるのでは、あるいは、商品のトレーサビリティという観点では、責任感がないという印象をもつといった意見がある。

遺伝子組み換えであるかどうかが仕入れに影響するは1社、4社は仕入れには影響しないとしている。1社は商品によっては非組み換え品に限定している。

遺伝子組み換えを使った商品を仕入れないとしているのは小売業のA社で、安全性が証明されていないことがその理由である。A社では、遺伝子組み換え以外にも、合成着色料など、有害とは決まっていなくても、安全だともいえないものは扱わないという社内規定になっている。

他の小売業2社と卸売業は、遺伝子組み換えがどうかは仕入れに全く影響しないとしている。メーカーが責任をもって作っている商品であり、現時点では、安全でないという発表がないことが取り扱う理由である。

E社も、PBブランドの冷凍コーンに関しては、非組み換えを使っているが、問屋という立場では品揃えの中で組み換え商品も取り扱っている。

D．考察

アイカメラを用いた実験の結果、遺伝子組換え食品の表示は注視時間が短く、読まれていない可能性が高いことが示唆された。この結果は、主婦群と学生群で差はなかった(実験1)。

表示をスクリーン上に投影せず、自然な状況で表示を読ませた実験2においても、遺伝子組換えの表示には特に注目されているという結果は得られなかった。教示による差異も見られなかった。ただし、手元で商品を見るという実験状況は、キャリブレーション等の実験実施上の技術上の問題があり、非接触型のアイカメラを用いる方がより良いと考えられる。

遺伝子組換え食品について、どのような情報が読まれているかを、情報探索法を用いて調査した結果、個人があらかじめ持っている態度によって、探索される情報に違いがあることが示唆された。また、探索される情報は、安全性や環境影響に関する者が多く、これらの論点に対する人びとの関心の高さが示唆される。

個人の健康の要因、すなわちアレルギーの保持の有無は、遺伝子組換え食品の選択に影響を及ぼさなかった。

他方、遺伝子組換え食品のベネットが提示されると選択率が高くなることが示された。

消費者や食品企業等の利害関係者へのフォーカス・グループインタビューの結果から、新しい食品技術として、最も名前が挙がったのは遺伝子組み換え技術であり、その名前の浸透度は高かった。また店頭でも目にしている人がほとんどであり、市場にも浸透している様子が伺えた。しかし、遺伝子組み換えがどんな技術なのかわからない、新しい技術であるため長期間の摂食による身体への将来的な影響や遺伝子レベルへの影響などが実証されていない、と感じられておりまたそうした説明情報もほとんどないことに不安が感じられていた。

遺伝子組み換え食品の安全性に対しては安全性が低いと考えている人が多く、外食など自分で選択できない場面は仕方がないとあきらめているものの、遺伝子組み換え食品をできるだけ購入しないようにしている人がほとんどである。

E．結論

アイカメラを用いた心理学実験によって、遺伝子組換え食品の表示はあまり注目されていないことが明らかになった。

情報提供についての複数の調査(インターネット調査およびフォ

ーカス・グループインタビュー)から、遺伝子組換え食品については安全性についての情報が最も探索されていることがわかった。他方、提供する情報を操作した調査結果から、ベネフィットについての情報が提示されると、組み換え食品の受容が高まることが明らかになった。

また、22年度以降、イギリス・スイスの2大学と研究内容を調整しながら研究を進めることができた。今後は先方からのデータと比較検討し、成果の発表につなげていく。

F.健康危険情報

該当なし

G. 研究発表

1. 論文発表

なし

2. 学会発表

内藤岳・吉川肇子・芳賀繁(2011)
栄養表示注視に関する研究
日本心理学会第75回大会(予定)

H. 知的財産権の出願・登録状況

1. 特許取得

なし

2. 実用新案登録

なし

3. その他

なし

該当なし

研究成果の刊行に関する一覧表

書籍

著者氏名	論文タイトル名	書籍全体の編集者名	書籍名	出版社名	出版地	出版年	ページ
吉川肇子	健康リスク・コミュニケーションの手引き	吉川肇子	健康リスク・コミュニケーションの手引き	ナカニシヤ出版	京都	2009	全204
吉川肇子，矢守克也，杉浦淳吉	クロスロード・ネクスト：ゲームで学ぶリスク・コミュニケーション		クロスロード・ネクスト：ゲームで学ぶリスク・コミュニケーション	ナカニシヤ出版	京都	2009	全223
手島玲子	新開発食品の安全性 1. 遺伝子組換え食品とは	米谷民雄	食品中の化学物質と安全性	日本食品衛生協会	東京	2009	132-137
手島玲子	遺伝子組換え農作物のアレルゲン性評価		食品安全ハンドブック	丸善	東京	2010	574-577

雑誌

発表者氏名	論文タイトル名	発表誌名	巻名	ページ	出版年
Akiyama H, Sakata K, Kondo K, Tanaka A, Liu MS, Oguchi T, Furui S, Kitta K, Hino A, Teshima R.	Individual Detection of Genetically Modified Maize Varieties in Non-Identity Preserved Maize Samples.	J. Agric. Food Chem.	56	1977-1983	2008
Shimizu E, Kato H, Nakagawa Y, Kodama T, Futo S, Minegishi Y, Watanabe Y, Akiyama H, Teshima R, Furui S, Hino A, Kitta K.	Development of Screening Method for Genetically Modified Soybean by Plasmid Based Quantitative-Competitive PCR.	J. Agric. Food Chem.	56	5521-5527	2008
手島玲子	遺伝子組換え食品.	食品衛生学雑誌	49 (4)	J269–J274	2008
Akiyama H, Nakamura F, Yamada C, Nakamura K, Nakajima O, Kawakami H, Harikai N, Furui S, Kitta K, Teshima R.	A screening method for the detection of the 35S promoter and the nopaline synthase terminator in genetically modified organisms in a real-time multiplex polymerase chain reaction using high-resolution melting-curve analysis.	Biol Pharm Bull.	32	1824-1829	2009
Harikai N, Saito S, Abe M, Kondo K, Kitta K, Akiyama H, Teshima R, Kinoshita K.	Optical detection of specific genes for genetically modified soybean and maize using multiplex PCR coupled with primer extension on a plastic plate.	Biosci Biotechnol Biochem.	73(8)	1886-1889	2009
Oguchi T, Onishi M, Minegishi Y, Kurosawa Y, Kasahara M, Akiyama H, Teshima R, Futo S, Furui S, Hino A, Kitta K.	Development of quantitative duplex real-time PCR method for screening analysis of genetically modified maize.	Shokuhin Eiseigaku Zasshi.	50	117-125	2009
T. Oguchi, M. Onishi, Y. Chikagawa, T. Kodama, E. Suzuki, M. Kasahara, H.Akiyama, R. Teshima, S. Futo, A. Hino, S. Furui, K. Kitta.	Investigation of residual DNAs in sugar from sugar beet (beta vulgaris L.).	日本食品衛生学会誌	50 (1)	41-46	2009
穐山浩、佐々木伸大、大木果林、中村文美、坂田こずえ、中村公亮、大森清美、中島安基江、古井聡、橘田和美、小関良宏、手島玲子	PCR法を用いた米加工品の安全性未審査遺伝子組換え米の検知法	日本食品化学会誌	16	147-151	2009

発表者氏名	論文タイトル名	発表誌名	巻名	ページ	出版年
Nakamura K, Akiyama H, Yamada C, Satoh R, Makiyama D, Sakata K, Kawakami H, Mano J, Kitta K, Teshima R.	Novel method to detect a construct-specific sequence of the acetolactate synthase gene in genetically-modified flax CDC Triffid (FP967).	Biol Pharm Bull.	33	532-534	2010
Nakajima O, Koyano S, Akiyama H, Sawada J, Teshima R.	Confirmation of a predicted lack of IgE binding to Cry3Bb1 from genetically modified (GM) crops.	Regul. Toxicol. Pharmacol.	56 (3)	306-11	2010
Oguchi, T., Onishi, M., Mano, J., Akiyama, H., Teshima, R., Futo, S., Furui, S., Kitta, K.	Development of multiplex PCR method for simultaneous detection of four events of genetically modified maize DAS-59122-7, MIR604, MON863 and MON88017	Food Hygiene and Safety Science (Shokuhin Eiseigaku Zasshi),	51	92-100	2010
Harikai, N., Akiyama, H., Kondo, K., Kitta, K., Teshima, R., Yoshida, Y.	A novel chromogenic method for determining the genetically modified soybean content in soybean powder with primer extension	Jpn. J. Food Chem. Safety	17	110-115	2010
Nakamura, K., Yamada, C., Akiyama, H., Takabatake, R., Kitagawa, M., Kitta, K., Kawakami, H., Teshima, R	Evaluation of tomato DNA fragmentation and PCR amplicon size for detection of tomato DNA in processed products	Jpn. J. Food Chem. Safety	17	123-128	2010
Mano, J., Yanaka, Y., Akiyama, H., Teshima, R., Furui, S., Kitta, K.	Improvement of polymerase chain reaction-based Bt11 maize detection method by reduction of non-specific amplification	Food Hygiene and Safety Science (Shokuhin Eiseigaku Zasshi)	51	32-36	2010
Akiyama, H., Sakata, K., Spiegelhalter, F., Furui, S., Nakashima, A., Kitta, K., Teshima, R.,	Interlaboratory Validation of an Event-Specific Real time Polymerase Chain Reaction Detection Method for Genetically Modified DAS59132 maize,	Food Hygiene and Safety Science (Shokuhin Eiseigaku Zasshi),	51	65-70	2010
Takabatake, R., Onishi, M., Koiwa, T., Futo, S., Minegishi, Y., Akiyama, H., Teshima, R., Furui, S., Kitta, K.	Establishment and Evaluation of Event-Specific Quantitative PCR Method for Genetically Modified Soybean MON89788	Food Hygiene and Safety Science (Shokuhin Eiseigaku Zasshi)	51	242-246	2010
Takabatake, R., Futo, S., Minegishi, Y., Watai, M., Sawada, C., Nakamura, K., Akiyama, H., Teshima, R., Furui, S., Hino, A., Kitta, K.	Evaluation of quantitative PCR methods for genetically modified maize (MON863, NK603, TC1507 and T25)	Food Sci. Technol. Res.	16	421-430	2010
Kodama T., Kasahara M., Minegishi Y., Futo S., Sawada C., Watai M., Akiyama, H., Teshima, R., Kurosawa, Y., Furui, S., Hino, A., Kitta, K.	Interlaboratory Study of Qualitative PCR Method for Roundup Ready Soybean	J AOAC Int.	94(1)	224-231	2010
Akiyama, H., Makiyama, D., Nakamura, K., Sasaki, N., Minegishi, Y., Mano, J., Kitta, K., Ozeki, Y., Teshima, R.	Novel Detection System for the Genetically Modified Canola (Brassica rapa) Line RT73	Anal Chem.	82	9909-9916	2010

発表者氏名	論文タイトル名	発表誌名	巻名	ページ	出版年
清水えり、布藤聡、増渕友子、峯岸恭孝、笠原正輝、穐山浩、手島玲子、日野明寛、真野潤一、古井聡、橘田和美	リアルタイムPCRによるDNA検査に好適なポリプロピレンチューブの選択方法	食品衛生学雑誌	51	43-47	2010
清木興介、織田浩司、柴原裕亮、蒲生玲子、有馬優美、酒井信夫、中村厚、安達玲子、塩見一雄、穐山浩、手島玲子	加工食品中の甲殻類タンパク質定量検査法における標準品調製法の検討	食品衛生学雑誌	51	133-138	2010
峰松和彦、中村公亮、穐山浩、張替直輝、中島治、橘田和美、手島玲子、飯塚太由	コンニャク製粉含有コメ粉からのコメDNA抽出精製法の検討	食品衛生学雑誌	51	247-252	2010
手島玲子，中村亮介	食品中のアレルゲンの予測	食品衛生学雑誌	52	1-9	2011
Takabatake, R., Akiyama, H., Sakata, K., Onishi, M., Koiwa, T., Futo, S., Minegishi, Y., Teshima, R., Furui, S., Kitta, K.	Development and Evaluation of Event-Specific Quantitative PCR Method for Genetically Modified Soybean A2704-12	Food Hygiene and Safety Science (Shokuhin Eiseigaku Zasshi)	52(2)	100-107	2011
Akiyama, H., Sakata, K., Makiyama, D., Nakamura, K., Teshima, R., Nakashima, A., Ogawa, A., Yamagishi, T., Futo, S., Mano, J., Oguchi, T., Kitta, K.	Inter-laboratory Study of DNA Extraction from Multiple Ground Samples, Multiplex Real-Time PCR and Multiplex Qualitative PCR for Individual Kernel Detection System of Genetically Modified Maize	J AOAC Int.	in press		2011

JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

J. Agric. Food Chem. 2008, 56, 1977–1983 1977

Individual Detection of Genetically Modified Maize Varieties in Non-Identity-Preserved Maize Samples

HIROSHI AKIYAMA,*,† KOZUE SAKATA,† KAZUNARI KONDO,† ASAKO TANAKA,§ MING S. LIU,# TAICHI OGUCHI,⊥ SATOSHI FURUI,⊥ KAZUMI KITTA,⊥ AKIHIRO HINO,⊥ AND REIKO TESHIMA†

National Institute of Health Sciences, 1-18-1 Kamiyoga, Setagaya-ku, Tokyo 158-8501, Japan; Daiichi Pure Chemicals Company, Ltd., 3-13-5 Nihombashi, Chuo-ku, Tokyo 103-0027, Japan; eGene, Inc., 17841 Fitch, Irvine, California 92614; and National Food Research Institute, 2-1-12 Kannondai, Tsukuba, Ibaraki 305-8642, Japan

In many countries, the labeling of grains and feed- and foodstuffs is mandatory if the genetically modified organism (GMO) content exceeds a certain level of approved GM varieties. The GMO content in a maize sample containing the combined-trait (stacked) GM maize as determined by the currently available methodology is likely to be overestimated. However, there has been little information in the literature on the mixing level and varieties of stacked GM maize in real sample grains. For the first time, the GMO content of non-identity-preserved (non-IP) maize samples imported from the United States has been successfully determined by using a previously developed individual kernel detection system coupled to a multiplex qualitative PCR method followed by multichannel capillary gel electrophoresis system analysis. To clarify the GMO content in the maize samples imported from the United States, determine how many stacked GM traits are contained therein, and which GM trait varieties frequently appeared in 2005, the GMO content (percent) on a kernel basis and the varieties of the GM kernels in the non-IP maize samples imported from the United States were investigated using the individual kernel analysis system. The average (± standard deviation) of the GMO contents on a kernel basis in five non-IP sample lots was determined to be 51.0 ± 21.6%, the percentage of a single GM trait grains was 39%, and the percentage of the stacked GM trait grains was 12%. The MON810 grains and NK603 grains were the most frequent varieties in the single GM traits. The most frequent stacked GM traits were the MON810 × NK603 grains. In addition, the present study would provide the answer and impact for the quantification of GM maize content in the GM maize kernels on labeling regulation.

KEYWORDS: Combined-trait genetically modified maize; multiplex real-time PCR; multiplex qualitative PCR; capillary gel electrophoresis

INTRODUCTION

Genetically modified (GM) crops developed by recombinant DNA (r-DNA) technology are grown in the United States, Canada, and several other countries and are widely consumed worldwide as food and feed. Over the past two decades, the production of GM crops, especially maize and soybeans, has increased in the United States (*1*). Recently, the production of combined-trait (stacked) products of GM maize, in which two or more different characteristic traits are inserted, has also increased in the United States due to their enhanced production efficiency.

Under these circumstances, there has been increasing interest and concerns about this technology among consumers, and perception gaps have emerged between consumers and scientist/authorities. Numerous opinions have been expressed, but the arguments surrounding GM crops and their processed foods generally focus on two topics: safety and labeling (*2–5*). In terms of this, many countries and international organizations have been discussing new labeling systems focusing on product information intended for the general public (*6–9*). Consequently, the labeling of grains, feed, and foodstuff is mandatory if the genetically modified organism (GMO) content exceeds a certain level of approved GM varieties. For instance, the European Union, Japan, and Korea have set threshold values of 0.9, 5, and 3%, respectively, of the GMO material in a non-GM

* Corresponding author (telephone +81-3-3700-9397; fax +81-3-3707-6950; e-mail akiyama@nihs.go.jp).
† National Institute of Health Sciences.
§ Daiichi Pure Chemicals Co., Ltd.
# eGene, Inc.
⊥ National Food Research Institute.

10.1021/jf0727239 CCC: $40.75
Published on Web 02/26/2008

background as the basis for labeling (*10–12*). In Japan, non-GM crops are segregated as non-GM material and imported from the United States by an identity-preserved (IP) handling system that requires document certification from U.S. farms to Japanese processing traders. However, the unintentional mixing of GM products in non-GM materials is inevitable. Accordingly, the enforcement of these threshold values has created a demand for the development of reliable GMO analysis methods.

Most of the developed analytical methods for the detection of GMO in foods are based on the Polymerase Chain Reaction (PCR) for the analysis of complex food matrices (*13–27*).

Furthermore, many real-time PCR systems have been developed to identify and quantify the GM maize, GM soybeans, and GM varieties of other agricultural commodities (*28–35*). The threshold levels for the unintentional mixing of GM materials and non-GM materials required for the labeling system are based on a weight per weight (w/w) percentage. The GM percentages calculated using the current quantitative PCR methods are calculated by converting the relative copy numbers between a specific rDNA sequence and a taxon-specific DNA sequence into a w/w percentage using appropriate reference materials. The GMO percentage in a maize sample containing the stacked GM maize as determined by the currently available methodology is likely to be overestimated as compared to the actual w/w percentage of GM maize in the sample because the relative copy numbers are calculated on a haploid basis (*36*). However, there has been little information on the determination of stacked GM maize. To solve this problem, we previously developed a rapid and simple detection system that delivers informative results by the individual kernel analysis of grain samples that could potentially contain stacked GM maize kernels. The detection system includes the developed multiplex qualitative PCR method for simultaneously detecting eight GM maize events. Some researchers have also developed multiplex PCR methods as simultaneous detection methods of multi-GM maize events using capillary gel electrophoresis (CGE) (*37, 38*). The combined use of multiplex PCR and CGE is a rapid and prospective method for multiple samples. In particular, the automated multichannel CGE could be a beneficial tool for the high-throughput analyses of amplified DNA fragments (*39*).

The present study was designed to clarify the GMO content of non-IP maize samples that may contain GM maize, investigate how many stacked GM traits are contained therein, and determine which GM trait varieties frequently appeared in 2005. We investigated the GMO content (percent) on a kernel basis and the varieties of GM kernels in the non-IP maize samples imported from the United States using the previously developed individual kernel analysis system, including the multiplex real-time PCR method (*36*), and coupled it to the multiplex qualitative PCR method (*27*) followed by multichannel CGE system analysis.

EXPERIMENTAL PROCEDURES

Maize Samples. The nongenetically modified (non-GM) maize grain and non-IP maize samples were obtained from the Ministry of Health, Labour and Welfare (MHLW) in Japan. Event 176 and Bt11 seeds were kindly provided by Syngenta (formerly Novartis Seeds). The T25 maize seeds were kindly provided by Bayer Crop Science. The TC1507 seeds were kindly provided by Pioneer Hi-Bred International, Inc. Furthermore, the MON810 seeds, MON863 seeds, GA21 seeds, NK603 seeds, and six stacked maize seeds (MON863 × NK603, MON810 × GA21, MON810 × T25, MON863 × MON810 and MON863 × MON810 × NK603) were kindly provided by the Monsanto Co., and TC1507 × NK603 was kindly provided by Pioneer Hi-Bred International, Inc., for the positive controls of the GM maize.

Oligonucleotide Primers and Probes for Multiplex Real-Time PCR Method. Sets of primer pairs and Taq-Man probes for the construct-specific and universal GM quantification were those described in our previous paper (*36*). The SSIIb-3 system (SSIIb 3-5′ and SSIIb 3-3′ with SSIIb−TaqV) was used as the primer and probe for the detection of the taxon-specific gene encoding the maize starch synthase IIb gene sequence (SSIIb) in the multiplex real-time PCR method, whereas the p35S-1 system (P35S 1-5′ and P35S 1-3′ with P35S-Taq) and GA21-3 system (GA21 3-5′ and GA21 3-3′ with GA21-Taq) were used for the multiplex real-time PCR method. All sets of primer pairs and the Taq-Man probes (p35S-Taq and GA21-Taq) for the detection of the cauliflower mosaic virus (CaMV) 35s promoter sequence (p35S) and GA21 specific sequence, respectively, were purchased from Fasmac Co., Ltd. (Kanagawa, Japan). SSIIb−TaqV, which is labeled with VIC and TAMRA at the 5′ and 3′ ends, was synthesized by Applied Biosystems (St. Louis, MO) and used as a probe for the detection of SSIIb. The target sequence of the p35S-1 system to detect the 35S promoter region derived from CaMV is widely found in the recombinant DNA of almost all GM events with the exception of GA21. The GA21-3 system was designed to detect the construct-specific sequence GM maize event GA21 (*36*).

Grinding of Individual Maize Kernels. One hundred and eighty kernels from each of the five non-IP maize samples (total of 900 kernels for 5 lots) were randomly sampled. To remove the broken pieces of the other kernels in order to avoid their contamination, the nonground kernels were washed with 1% sodium dodecyl sulfate (SDS), rinsed three times with distilled water in a beaker, and dried at 40 °C for 40 min in the incubation box (DO-300A, AS ONE Co., Osaka, Japan) before they were ground. Each maize kernel and metal corn as a disruption medium (MC0316MZ, Yasui Kikai Co.) were placed in a sample tube (ST-0350MZ, Yasui Kikai Co.), and the tube was closed using the attached cap (ST-0350MZ, Yasui Kikai Co.). Twenty-four sample tubes were arrayed in the tube holder (type SH-123, Yasui Kikai Co.). Two tube holders can be accommodated in a multibead shocker (model MB601NIHS, Yasui Kikai Co. Osaka, Japan; http://www.yasuikikai.co.jp/company/e_index.html) at a time. The maize kernels were ground by vigorously shaking the tubes and the metal corns using a multibead shocker at 2500 rpm for 1 min and then repeated for 1 min after the tube holder was reversed (*36*).

DNA Extraction from Each Maize Kernel Using DNeasy 96 Plant Kit. Genomic DNA extraction from the finely ground individual powders was performed using the DNeasy 96 Plant kit (Qiagen GmbH, Hilden, Germany) according to the manufacturer's protocol with the following modification. Buffer AP1 (preheated to 65 °C) and RNase A (final concentration = 100 μg/mL) were combined to make the working solution. One milliliter of the working solution was added to each sample tube containing the ground maize powder, which is described under Grinding of Individual Maize Kernels. The tubes were capped and incubated for 30 min at 65 °C (inverted 10 times at intervals of 10 min). A 170 μL aliquot of buffer AP2 solution was then added to each solution. The tubes were sealed to avoid leakage during shaking and then vigorously shaken for 15 s. The tubes were next incubated for 10 min at −20 °C and then centrifuged for 20 min at 3000 rpm using a Metalfuge (MBG100, Yasui Kikai Co.). A 400 μL aliquot of each supernatant was carefully transferred to new microtubes. The collection microtubes were centrifuged for 5 min at 12000 rpm. Each supernatant was carefully transferred to new microtubes, and 1.5 volumes (typically 600 μL) of buffer AP3/E was added to each sample. One milliliter of each sample was carefully transferred to the DNeasy 96 plates. The DNeasy 96 plate was sealed with tape. The plate was aspirated until each DNeasy membrane was dry. After removal of the tape, 800 μL of buffer AW was carefully added to each sample. The plate was again sealed with the tape and aspirated until each DNeasy membrane was dry. The washing was repeated three times. A 800 μL aliquot of 100% ethanol was then added to each sample. The plate was aspirated for 15 min to dry each DNeasy membrane. After removal of the tape, the DNeasy 96 plate was placed in the correct orientation on a rack of elution microtubes, and then 75 μL of distilled water (DW) (preheated to 65 °C) was added to each sample. The plate was resealed and incubated for 5 min at room temperature and then aspirated until each DNeasy membrane was dry.

Multiplex Real-Time PCR Conditions. To simultaneously detect the genomic DNAs from the individual GM maize kernels along with the confirmation of the validity of the extracted genomic DNAs, multiplex real-time PCR analyses were performed according to a previous paper (*36*). The amplification curves of the target sequence were monitored using a fluorescent dye, which was used to label the designed oligonucleotide probes, using the ABI PRISM 7900HT sequence detection system (Applied Biosystems). The reaction volume of 25 μL contained 2.5 μL of the sample genomic DNA solution, 12.5 μL of Universal Master Mix (Applied Biosystems), 0.5 μM primer pair, and 0.2 μM probe (except for the case of p35S, 0.1 μM probe). The PCR step-cycle program was as follows: 2 min at 50 °C and 95 °C for 10 min followed by 45 cycles of 30 s at 95 °C and 1.5 min at 59 °C.

If the amplification curves for the GMO detection could be clearly observed after 15 cycles, we considered the sample to be positive for GMOs; otherwise, it was considered to be negative, because we adopted the clarity of the amplification curves after 15 cycles of the real-time PCR as the threshold for the discrimination of the GM or non-GM maize kernel in a previous study (*36*). In this study, the "GM Maize Detection Plasmid Set - ColE1/TE -" (Nippon Gene Co., Tokyo, Japan) was used as the positive control. This plasmid set contained six concentrations of the reference plasmid pMul5, into which is inserted the amplification products of p35S, GA21, and SSIIb, diluted with the TE buffer (pH 8.0) including 5 ng/μL of the ColE1 plasmid (*31, 36*).The ColE1 plasmid contained none of the amplification GM products and was used as the negative control. The positive controls were prepared using the two concentrations of the plasmid set with 250000 and 1500 copies per plate. In the negative control, the ColE1 plasmid was also used as the nontemplate control for the analysis. In the reaction plate, the real-time PCR was performed in duplicate using two reaction vessels for the no-template control as the negative control and positive control (two concentrations of the plasmid set). The other 90 reaction vessels were used for the genomic DNA samples extracted from the single maize kernels.

Multiple Qualitative PCR Conditions. To identify which GM trait varieties are contained in the genomic DNAs extracted from individual kernels, a multiple qualitative PCR detection was performed according to our previously reported method with some modifications (*27*). The reaction mixture for the PCR was prepared in a 96-well plate. The reaction volume of 25 μL contained 25 ng of the genomic DNA, 0.2 mmol/L dNTP, 1.5 mmol/L $MgCl_2$, 0.2 μmol/L of the 5′ and 3′ primers, 1.25 units of AmpliTaq Gold DNA polymerase (Applied Biosystems), and 15 primers at the following concentrations: 0.2 μmol/L for M810 1-5′, NK603 1-5′ M863 1-5′, Btl1 1-5′, and CryIA 1-3′; 0.1 μmol/L for T25 2-5′, T25 2-3′, GA21 1-5′, GA21 1-3′, TC1507 1-5′, and TC1507 1-3′; 0.05 μmol/L for Event 176 1-5′; and 0.045 μmol/L for SSIIb 1-5′ and SSII 1-3′. The reactions were buffered with PCR buffer II (Applied Biosystems) and amplified in a thermal cycler, the Silver 96 well GeneAmp PCR System 9700 (Applied Biosystems) in 9600 mode, according to the following PCR step-cycle program: preincubation at 95 °C for 10 min, 10 cycles consisting of denaturation at 95 °C for 0.5 min, annealing at 65 °C for 1 min, and extension at 72 °C for 1 min; 27 cycles consisting of denaturation at 95 °C for 0.5 min, annealing at 65 °C for 1 min, and extension at 72 °C for 1 min; followed by a final extension at 72 °C for 7 min. The cycle was repeated 40 times followed by a final extension at 72 °C for 7 min.

Capillary Gel Electrophoresis. The PCR amplified products of multiple samples were analyzed using an automated multicapillary electrophoresis system, HDA-GT12 system (*39*) (eGene Inc., Irvine, CA), with a 12-capillary gel cartridge (GCK5000F), to rapidly and simultaneously resolve the samples. The HDA-GT12 system is an automated DNA fragment analyzer, capable of the rapid and simultaneous analysis of 12 samples at a high resolution, and allows hands-free sample analysis from a 96-well plate. Samples are electrokinetically injected into capillary channels and separated with internal calibration markers (CM: CM1 lower, CM2 upper) in every run for automatic lane alignment in an array of 13 cm long fused silica capillary columns. Ethidium bromide, included in the GCK5000F gel cartridge, is used as an intercalator dye, which intensely fluoresces in the presence of dsDNA using a green light-emitting diode (peak wavelength = 525 nm) as the excitation source. BioCalculator, software integrated in the system, controls the scheduling and separation and calculates the size of the DNA fragments on the basis of the relative migration time of each peak of the reference DNA fragments.

The 96-well plate after PCR amplification was placed in the HDA system. After the Biocalculator's AL400 method was selected, aliquots (a few nanoliters) of the PCR products in the 96-well plate were automatically injected into the capillary channels at 8000 V for 20 s and separated at 5000 V for 400 s under ambient temperature with the calibration markers, CM1 and CM2 (50 and 1000 bp DNA fragments, respectively). The separations were aligned by CM1 and CM2 and estimation of the DNA fragment size based on the registered data, which refers to PCR amplification products from each of the eight GM event seeds estimated by the Biocalculator.

Table 1. GMO Content on a Kernel Basis in Five Non-IP Maize Samples

non-IP maize sample lot	kernel number non-GM	kernel number GM	GMO content (%)
A	41	139	77.2
	total = 180		
B	53	127	70.6
	total = 180		
C	106	74	41.1
	total = 180		
D	129	51	28.3
	total = 180		
E	112	68	37.8
	total = 180		
av	441	459	51.0
	total = 900		

RESULTS

GMO Content in Non-IP Maize Samples Using Multiplex Real-Time PCR Method. We randomly sampled 180 kernels from each of the five non-IP maize samples and performed single-kernel analyses using the multiplex real-time PCR method. The multiplex real-time PCR method allows us to individually discriminate GM maize or non-GM maize and simultaneously evaluate the quality of the extracted genomic DNA for PCR in one run. As shown in **Table 1**, the GMO contents on a kernel basis in the five non-IP maize samples were 77.2, 70.6, 41.1, 28.3, and 37.8%, respectively, and their average ± standard deviation was 51.0 ± 21.6%. This result indicates that the non-IP maize samples are highly contaminated with GM maize, showing a highly variable GMO content. **Figure 1** shows the end-point analyses of the multiplex real-time PCR. Considering the amplification curves and the end-point analyses, we could clearly discriminate the non-GM kernels and GM kernels on the basis of the results of the amplification curves for all but 2 of a total of 900 kernels.

GM Trait Analysis of GM Grains Using Multiplex Qualitative PCR Method. Next, we attempted to identify, using the multiplex qualitative PCR detection method (*27*) coupled to the multichannel CGE, the HDA system, the traits of which are present in the genomic DNA from each kernel and whether these are present as single traits or stacked genes. **Figure 2A** shows a typical electropherogram of amplification products from eight reference single GM seeds. As expected, each GM trait of the eight reference single GM seeds was clearly separated on the electropherogram. In addition, six reference stacked seeds (GA21 × MON810, NK603 × MON863, NK603 × MON810, MON863 × MON810, NK603 × MON863 ×

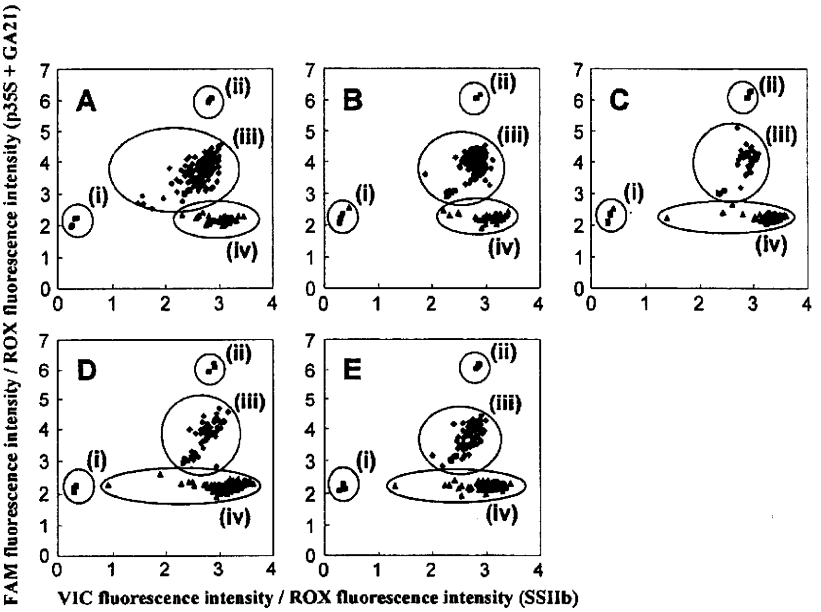

Figure 1. End-point analyses of the five non-IP maize samples and reference plasmids using the multiplex real-time PCR. A–E show the result of each non-IP maize sample lot: (i) nontemplate control (ColE1 plasmid) as the negative control; (ii) amplification of 250000 copies of reference plasmid as the positive control; (iii) amplification of genomic DNA extracted from GM maize kernels; (iv) amplification of genomic DNA from non-GM maize kernels.

MON810, and NK603 × TC1507) were also identified by the simultaneous detection of the corresponding GM traits. The electropherogram of the NK603 × MON810 reference seed is shown in **Figure 2B**.

The genomic DNAs extracted from 900 kernels of 5 non-IP maize grain samples were individually analyzed using the multiplex qualitative PCR detection method with the multichannel CGE of the HDA system. **Figure 3** shows typical electropherograms of the amplification products from the genomic DNAs extracted from individual non-IP maize grain. As shown in **Table 2**, although the GMO content of each non-IP maize sample widely varied, the tendency of the contaminated GM trait variety population appears to be similar among the samples. The populations of the non-GM grains, single GM trait grains, and stacked trait grains in the 900 kernels are shown in **Table 2**. The percentage of the GM grains is 51%, and, in the GM grains, the percentage of the single GM trait grains is 39% and that of the stacked GM trait grains is 12%. These results show that the ratio of the stacked GM trait grain population was comparatively high in the GM grains of the non-IP maize samples in 2005. The percentages of the single GM trait variety population and that of the stacked GM trait variety population also are shown in **Table 2**. The MON810 grains (55.7%) and NK603 grains (21.1%) were mainly detected in single GM traits, followed by the Bt11 grains (9.4%), TC1507 grains (4.6%), and MON863 (4.3%). Most of the detected stacked GM traits were MON810 × NK603 (87.6%), although MON810 × T25 grains (5.6%) and MON863 × NK603 grains (5.6%) were also present. In addition, the MON810 × MON863 grain was also detected, although only in a single grain. These results showed that MON810 and NK603 were most frequently detected among the single GM traits and that MON810 × NK603 was the most frequently detected stacked GM trait.

DISCUSSION

According to the Japanese GM labeling system, foods are classified into three categories as follows: (1) If GM materials are intentionally used in foods, they should, without exception, be mandatorily labeled with the phrase "GM Materials Used". (2) If the raw materials for these foods are not segregated from the GM materials, they should be mandatorily labeled with "GM Ingredient Not Segregated". (3) To be labeled "Non-GM"(volunteer system), the processor must manage their raw materials with "identity-preserved" (IP) from the U.S. farm for processing in Japan. However, some unintentional mixing of GM products in non-GM materials is inevitable and could be acceptable only when proper confirmation of the IP handling has been conducted. Consequently, the threshold level for the unintentional mixing of the GM constituents (soybean and maize) is 5% in Japan. The maize and soybean imported from the United States are segregated as non-GM by the IP handling system for the GM labeling system. Crop samples segregated as non-GM by the IP handling system are monitored to assess the validity of the GM labeling at the quarantine inspection centers using the real-time PCR method. If a sample's GM content is over 5%, the material's labeling would be corrected with guidance from the Japanese Ministry of Health, Labour and Welfare. Therefore, the definition of the threshold values is a very important issue and remains controversial in Japan.

To date, several GM maize events have been authorized for import into Japan. The GM traits include resistance to feeding damage by the European corn borer [Event 176 and Bt11 from Syngenta (formerly Novartis Seeds) and MON810 from Monsanto Co.] resistance to corn rootworm (e.g., MON863 from Monsanto Co.), tolerance to the herbicide phosphinothricin (PPT) (e.g., T25 from Bayer Crop Science), resistance to the European corn borer (TC1507 from Pioneer Hi-Bred International, Inc., Mycogen Seeds/Dow Agro Sciences LLC), and tolerance to the herbicide glyphosate (e.g., GA21 and NK603, Monsanto Co.). Furthermore, seven stacked maize products (MON863 × NK603, MON810 × NK603, MON810 × GA21, MON810 × T25, TC1507 × NK603, MON863 × MON810, and MON863 × MON810 × NK603) have already been authorized in Japan. If a stacked GM maize variety is present among the grains, the measured GM percentage might be overestimated by the measurement method. The GM percentage

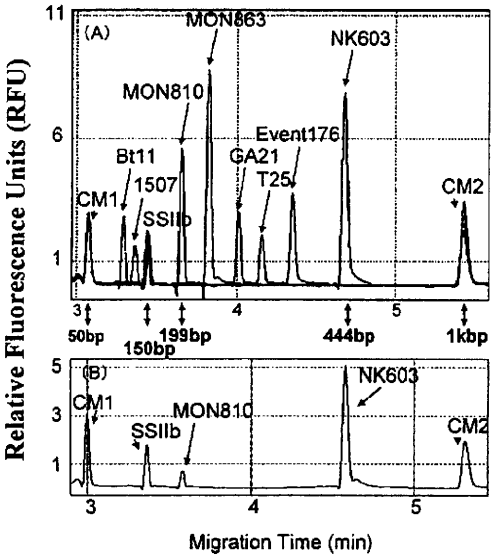

Figure 2. Typical electropherograms of amplification products from eight reference single GM seeds (**A**) and MON810 × NK603 reference seed (**B**). The PCR amplified products of multiple samples were analyzed by an automated multicapillary electrophoresis system, HDA-GT12 system (eGene, Inc.) using a 12 capillary gel cartridge (GCK5000F). (**A**) SSIIb represents the amplification product from the maize taxon specific gene; *SSIIb* gene (150 bp), Bt11 (110 bp), TC1507 (131 bp), MON810 (199 bp), MON863 (199 bp), GA21 (270 bp), T25 (311 bp) Event 176 (343 bp), and NK603 (444 bp) represent the PCR amplification product from each GM line, respectively. (**B**) Arrowheads indicate the PCR amplification product from the respective reference GM event seed. The 96-well plate after PCR amplification was placed in the HDA system. Aliquots (a few nanoliters) of the PCR products (25 μL) were automatically injected into the capillary channels at 8000 V for 20 s and separated at 5000 V for 400 s at ambient temperature with calibration markers, CM1 (50 bp) and CM2 (1000 bp). Estimation of the DNA fragments size based on the registered data, which refers to PCR amplification products from each of the eight GM event seeds, was performed by the Biocalculator.

can be expressed on a kernel basis, which is based on discrimination of the GM or non-GM for single kernels, or an a haploid genome basis. Therefore, we have previously developed a rapid and simple detection system that delivers informative results on the basis of a single-kernel analysis of grain samples and could potentially contain stacked trait GM maize kernels.

In this study, we determined the GMO content of the non-IP maize samples on a kernel basis. As expected, the results indicate that GMOs are present at a high level in the range from 30 to 80%. We used the multiplex qualitative PCR detection method coupled to the HDA multichannel CGE system. Consequently, we showed that, among an analyzed total of 900 kernels, the percentage of GM grains is 51%, the percentage of single GM trait grains is 39%, and that of the stacked GM trait grains is 12% in the GM grains. This result showed that the stacked GM traits, as well as the single GM traits, are present at high levels in the non-IP maize samples imported from the United States in 2005. This evidence implies that the measured GMO percent in the maize sample using the conventional real-time PCR method might be overestimated because the target sequences of a stacked GM maize variety are doubly or triply quantified on the basis of the determination using the haploid. Furthermore, this evidence suggests that only the individual kernel testing as shown in the present study provides informative

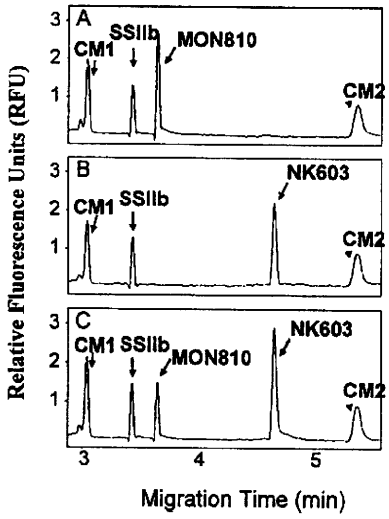

Figure 3. Typical electropherograms of amplification products from genomic DNAs extracted from typical individual maize kernels. **A**–**C** represent typical electrophregrams of amplification products from three individual maize kernels. **A**, **B**, and **C** are identified as MON810, NK603, and NON810 × NK603, respectively, in the GM trait analysis. Arrowheads indicate the identified PCR amplification products from the maize taxon specific gene, *SSIIb* gene, MON810 kernel, and NK603 kernel. SSIIb represents the identified PCR amplification product from the *SSIIb* gene. MON810 and NK603 represent the identified PCR amplification products from the MON810 kernel and NK603 kernel, respectively. CM1 (50 bp) and CM2 (1000 bp) are calibration markers.

Table 2. Population of Individual Kernels in GM Grains of Non-IP Maize Samples

	kernel number						
	nonsegregated sample lot						
GM trait	A	B	C	D	E	total	percentage
Bt11	10	10	7	2	4	33	9.4
1507	2	8	0	2	4	16	4.6
MON810	36	36	51	34	39	196	55.7
MON863	7	3	3	1	1	15	4.3
GA21	2	1	2	1	0	6	1.7
T25	8	1	0	1	2	12	3.4
NK603	33	22	7	3	9	74	21.1
single GM	98	81	70	44	59	352	39.0
MON810 × NK603	36	43	3	6	6	94	87.6
MON810 × T25	2	2	0	0	2	6	5.6
MON810 × MON863	1	0	0	0	0	1	0.9
MON863 × NK603	2	1	1	1	1	6	5.6
stack GM	41	46	4	7	9	107	12.0
non-GM	41	53	106	129	112	441	49.0
						900	100

and traceable results for the labeling regulation on a weight per weight basis.

We also clarified that MON810 grains (55.7%) and NK603 grains (21.1%) were mainly detected in the single GM trait variety, followed by the Bt11 grains (9.4%), TC1507 grains (4.6%), and MON863 (4.3%). The MON810 × NK603 grains (87.6%) were detected in the stacked GM trait variety, followed by the MON810 × T25 grains (5.6%) and the MON863 × NK603 grains (5.6%). The National Agricultural Statistics Service (NASS) reported that the single-trait GM maize planted area and the stacked GM trait variety planted area in the United States during 2005 were 52 and 9%, respectively (*40*). We

estimated that the present multiplex qualitative PCR analyses can almost cover all of the GM maize events cultivated in 2005, because it has been reported that other authorized GM maize events such as DBT41 and DDL25 are not cultivated anymore. Considering this report and these results, these results are reasonable and probably reflect the total GM grain mixing.

It would be necessary to clarify the acceptable uncertainty in consideration of the level of a risk and method applicability by a statistical approach to consider how many kernels to test as the threshold for when a declaration is made that GM or non-GM is 5%. Therefore, we analyzed them using SeedGalc7.1 software provided by the International Seed Testing Association (ISTA;www. seedtest.org) to model the proposed testing plan. The proposed testing plan has two stage tests for the individual kernel analysis. The testing plan scheme uses 90 kernels for the first screen. If there are 2 or more GM kernels in the first 90 kernel testing, another set of 90 kernels is tested. If the total GM kernels combining the two tests (180 kernels) is 9, the lot sample is determined to be a non-GM sample. In this case, the analytical data showed that the plan can keep producer confidence levels at 98.9% (and producer risk at 1.1%), although the consumer confidence level is at 83.4% (and consumer risk at 16.6%). For the feasibility of testing individual maize kernels and the hazard of GM grains, we consider that it would be a practical way to sequentially determine the percent GMO using the conventional real-time PCR assay as a screening monitor in the first step and the proposed testing plan composed of two stages for individual maize kernel testing using the present detection system as the second step.

In conclusion, we were the first to successfully determine the GMO content in non-IP maize samples imported from the United States using the single-kernel detection system that we had previously developed and coupled it to the multiplex qualitative PCR method followed by the multichannel CGE system analysis. In addition, we analyzed the GM trait varieties in GM maize kernels using the multichannel CGE system analysis. The total time needed to perform the GMO content analysis and the varieties of GM kernels for 180 maize kernels is 3 days for a sample, although the GMO content analysis would take only 2 days. The present study would provide the answer and impact for the quantification of the GM maize content in GM maize kernels on the labeling regulation. It will be necessary to clarify the acceptable uncertainty for considering the level of risk and the applicability of the detection method.

ACKNOWLEDGMENT

We are very grateful to the Monsanto Co., USA, and Pioneer Hi-Bred International, Inc., for providing the reference materials. We thank Dr. Doris Dixon and Hiroo Wakimori for providing many useful suggestions. We thank Dr. Takahiro Watanabe for teaching us the capillary gel electrophoresis analysis technique and Dr. Tamio Maitani and Dr. Satoshi Futo for providing many useful suggestions.

LITERATURE CITED

(1) James, C. Global status of commercialized transgenic crops: ISAAA Briefs, 2003.

(2) Losey, J. E.; Rayor, L. S.; Carter, M. E. Transgenic pollen harms monarch larvae. *Nature* **1999**, *399*, 214.

(3) Pimentel, D. S.; Raven, P. H. Bt corn pollen impacts on nontarget Lepidoptera: assessment of effects in nature. *Proc. Natl. Acad. Sci. U.S.A.* **2000**, *97*, 8198–8199.

(4) SAP Report 2000–07 March 12, 2001, Report of FIFRA Scientific Advisory Panel Meeting (http://www.epa.gov/scipoly/sap/2000/october/octoberfinal.pdf).

(5) Masood, E. Food scientist in GMO row defends 'premature' warning. *Nature* **1999**, *398*, 98.

(6) Regulation (EC) No. 258/97 of the European Parliament and of the Council of 27 January, 1997, *Off. J. Eur. Communities* **1997**, *L043*, 1–7.

(7) Regulation (EC) No 1829/2003 of the European Parliament and of the Council of 22 September 2003 on genetically modified food and feed. *Off. J. Eur. Communities*, available from http://europa.eu.int/eurlex/pri/en/oj/dat/2003/l_268/l_26820031018en0001002-3.pdf.

(8) Regulation EC No 1830/2003 of the European Parliament and of the Council concerns the traceability and labelling of genetically modified organisms and the traceability and labelling of food and feed products produced from genetically modified organisms and amending Directive 2001/18/EC. *Off. J. Eur. Communities*, available from http://eur-lex.europa.eu/LexUriServ/site/en/oj/2003/l_268/l_26820031018en00240028.pdf.

(9) Notification No. 517 of the Ministry of Agriculture, Forestry and Fisheries (Japan) of March 31, 2000.

(10) Commission Regulation (EC) No. 49/2000 of January 10. *Off. J. Eur. Communities* **2000**, *L 6*, 13–14.

(11) Notification No. 79 of March 15, 2000, Department of Food Safety, the Ministry of Health, Labour and Welfare of Japan.

(12) Notification No. 2000-31 of April 22, 2000, Ministry of Agriculture and Forestry of Korea.

(13) Hardegger, M.; Brodmann, P.; Herrmann, A. Quantitative detection of the 35S promoter and the NOS terminator using quantitative competitive PCR. *Eur. Food Res. Technol.* **1999**, *209*, 83–87.

(14) Jankiewicz, A.; Broll, H.; Zagon, J. The official method for the detection of genetically modified soybeans (German Food Act LMBG §35): a semi-quantitative study of sensitivity limits with glyphosate-tolerant soybeans (Roundup Ready) and insect-resistant Bt maize (Maximizer). *Eur. Food Res. Technol.* **1999**, *209*, 77–82.

(15) Vollenhofer, S.; Burg, K.; Schmidt, J.; Kroath, H. Genetically modified organisms in food-screening and specific detection by polymerase chain reaction. *J. Agric. Food Chem.* **1999**, *47*, 5038–5043.

(16) Hupfer, C.; Hotzel, H.; Sachse, K.; Engel, K. H. Detection of the genetic modification in heat-treated products of Bt maize by polymerase chain reaction. *Z. Lebensm. Unters. Forsch.* **1998**, *206*, 203–207.

(17) Matsuoka, T.; Kawashima, Y.; Akiyama, H.; Miura, H.; Goda, Y.; Sebata, T.; Isshiki, K.; Toyoda, M.; Hino, A. A detection method for recombinant DNA from genetically modified soybeans and processed foods containing them. *J. Food Hyg. Soc. Jpn.* **1999**, *40*, 149–157.

(18) Matsuoka, T.; Kawashima, Y.; Akiyama, H.; Miura, H.; Goda, Y.; Kusakabe, Y.; Isshiki, K.; Toyoda, M.; Hino, A. A method of detecting recombinant DNAs from four lines of genetically modified maize. *J. Food Hyg. Soc. Jpn.* **2000**, *41*, 137–143.

(19) Matsuoka, T.; Kuribara, H.; Akiyama, H.; Miura, H.; Goda, Y.; Kusakabe, Y.; Isshiki, K.; Toyoda, M.; Hino, A. A multiplex PCR method of detecting recombinant DNAs from five lines of genetically modified maize. *J. Food Hyg. Soc. Jpn.* **2001**, *42*, 24–32.

(20) Matsuoka, T.; Kuribara, H.; Takubo, K.; Akiyama, H.; Miura, H.; Goda, Y.; Kusakabe, Y.; Isshiki, K.; Toyoda, M.; Hino, A. Detection of recombinant DNA segments introduced to genetically modified maize (*Zea mays*). *J. Agric. Food Chem.* **2002**, *50*, 2100–2109.

(21) Akiyama, H.; Sugimoto, K.; Matsumoto, M.; Isuzugawa, K.; Shibuya, M.; Goda, Y.; Toyoda, M. A detection method of recombinant DNA from genetically modified potato (NewLeaf Plus Potato) and detection of NewLeaf Plus Potato in snack. *J. Food Hyg. Soc. Jpn.* **2002**, *43*, 24–29.

(22) Akiyama, H.; Watanabe, T.; Wakui, C.; Chiba, Y.; Shibuya, M.; Goda, Y.; Toyoda, M. A detection method of recombinant DNA from genetically modified potato (NewLeaf Y Potato). *J. Food Hyg. Soc. Jpn.* **2002**, *43*, 301–305.

(23) Watanabe, T.; Kasama, K.; Wakui, C.; Shibuya, M.; Matsuki, A.; Akiyama, H.; Maitani, T. Laboratory-performance study of the notified methods to detect genetically modified maize (CBH351) and potato (NewLeaf Plus and NewLeaf Y). *J. Food Hyg. Soc. Jpn.* **2003**, *44*, 281–288.

(24) Watanabe, T.; Kuribara, H.; Mishima, T.; Kikuchi, H.; Kodama, T.; Futo, S.; Kasama, K.; Toyota, A.; Nouno, M.; Saita, A.; Takahashi, K.; Hino, A.; Akiyama, H.; Maitani, T.; Kubo, M. New qualitative detection methods of genetically modified potatoes. *Biol. Pharm. Bul.* **2004**, *27*, 1333–1339.

(25) Studer, E.; Rhyner, C.; Luthy, J.; Hubner, P. Quantitative competitive PCR for the detection of genetically modified soybean and maize. *Z. Lebensm. Unters. Forsch.* **1998**, *207*, 207–213.

(26) Hupfer, C.; Hotzel, H.; Sachse, K.; Moreano, F.; Engel, K. H. PCR-based quantification of genetically modified Bt maize: single-competitive versus dual-competitive approach. *Eur. Food Res. Technol.* **2000**, *212*, 95–99.

(27) Onishi, M.; Matsuoka, T.; Kodama, T.; Kashiwaba, K.; Futo, S.; Akiyama, H.; Maitani, T.; Furui, S.; Oguchi, T.; Hino, A. Development of a multiplex polymerase chain reaction method for simultaneous detection of eight events of genetically modified maize. *J. Argic. Food Chem.* **2005**, *53*, 9713–9721.

(28) Vaitilingom, M.; Pijnenburg, H.; Gendre, F.; Brignon, P. Real-time quantitative PCR detection of genetically modified Maximizer maize and Roundup Ready soybean in some representative foods. *J. Agric. Food Chem.* **1999**, *47*, 5261–5266.

(29) Kuribara, H.; Shindo, Y.; Matsuoka, T.; Takubo, K.; Futo, S.; Aoki, N.; Hirao, T.; Akiyama, H.; Goda, Y.; Toyoda, M.; Hino, A. Novel reference molecules for quantitation of genetically modified maize and soybean. *J. AOAC Int.* **2002**, *85*, 1077–1089.

(30) Shindo, Y.; Kuribara, H.; Matsuoka, T.; Futo, S.; Sawada, C.; Shono, J.; Akiyama, H.; Goda, Y.; Toyoda, M.; Hino, A. Validation of real-time PCR analyses for line-specific quantitation of genetically modified maize and soybean using new reference molecules. *J. AOAC Int.* **2002**, *85*, 1119–1126.

(31) Yoshimura, T.; Kuribara, H.; Matsuoka, T.; Kodama, T.; Shigematsu, M.; Watanabe, T.; Akiyama, H.; Maitani, T.; Furui, S.; Hino, A. Applicability of the quantification of genetically modified organisms to foods processed from maize and soy. *J. Argic. Food Chem.* **2005**, *53*, 2052–2059.

(32) Hubner, P.; Waiblinger, H. U.; Pietsch, K.; Brodmann, P. Validation of PCR methods for quantitation of genetically modified plants in food. *J. AOAC Int.* **2001**, *84*, 1855–1864.

(33) Hubner, P.; Studer, E.; Luthy, J. Quantitation of genetically modified organisms in food. *Nat. Biotechnol.* **1999**, *17*, 1137–1138.

(34) Lipp, M.; Brodmann, P.; Pietsch, K.; Pauwels, J.; Anklam, E.; Borchers, T.; Braunschweiger, G.; Busch, U.; Eklund, E.; Eriksen, F. D.; Fagan, J.; Fellinger, A.; Gaugitsch, H.; Hayes, D.; Hertel, C.; Hortner, H.; Joudrier, P.; Kruse, L.; Meyer, R.; Miraglia, M.; Muller, W.; Phillipp, P.; Popping, B.; Rentsch, R.; Wurtz, A.; et al. IUPAC collaborative trial study of a method to detect genetically modified soy beans and maize in dried powder. *J. AOAC Int.* **1999**, *82*, 923–928.

(35) Huang, H. Y.; Pan, T. M. Detection of genetically modified maize MON810 and NK603 by multiplex and real-time polymerase chain reaction methods. *J. Agric. Food Chem.* **2004**, *52*, 3264–3268.

(36) Akiyama, H.; Watanabe, T.; Wakabayashi, K.; Nakade, S.; Yasui, S.; Sakata, K.; Chiba, R.; Spiegelhalter, F.; Hino, A.; Maitani, T. Quantitative detection system for maize sample containing combined-trait genetically modified maize. *Anal. Chem.* **2005**, *77*, 7421–7428.

(37) Garcia-Canas, V.; Gonzalez, R.; Cifuentes, A. Sensitive and simultaneous analysis of five transgenic maizes using multiplex polymerase chain reaction, capillary gel electrophoresis, and laser-induced fluorescence. *Electrophoresis* **2004**, *25*, 2219–2226.

(38) Nadal, A.; Coll, A.; La Paz, J. L.; Esteve, T.; Pla, M. A new PCR-CGE (size and color) method for simultaneous detection of genetically modified maize events. *Electrophoresis* **2006**, *27*, 3879–3888.

(39) Liu, M. S.; Amirkhanian, V. D. A multichannel electrophoresis system for high-resolution, high-throughput genotyping. *Am. Biotechnol. Lab.* **2004**, *22*, 8–19.

(40) Acreage, No. 06.30.06, 2006, National Agricultural Statistics Service (NASS), Agricultural Statistics Board, U.S. Department of Agriculture.

Received for review September 13, 2007. Revised manuscript received January 15, 2008. Accepted January 16, 2008. This study was supported in part by a grant from the Ministry of Health, Labour and Welfare of Japan.

JF0727239

JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

J. Agric. Food Chem. 2008, 56, 5521–5527 5521

Development of a Screening Method for Genetically Modified Soybean by Plasmid-Based Quantitative Competitive Polymerase Chain Reaction

ERI SHIMIZU,[†] HISASHI KATO,[‡] YUKI NAKAGAWA,[‡] TAKASHI KODAMA,[§] SATOSHI FUTO,[†] YASUTAKA MINEGISHI,[∥] TAKAHIRO WATANABE,[⊥] HIROSHI AKIYAMA,[⊥] REIKO TESHIMA,[⊥] SATOSHI FURUI,[#] AKIHIRO HINO,[#,▽] AND KAZUMI KITTA*[,#]

Fasmac Co., Ltd., 5-1-3 Midorigaoka, Atsugi, Kanagawa 243-0041, Showa Sangyo Co., Ltd., 2-20-2 Hinode, Funabashi, Chiba 273-0015, Food and Agricultural Materials Inspection Center, 2-1 Shintoshin, Chuo, Saitama 330-9731, Nippon Gene Co., Ltd., 1-8-7 Toiya-machi, Toyama 930-0834, National Institute of Health Sciences, 1-18-1 Kamiyoga, Setagaya, Tokyo 158-8501, and National Food Research Institute, 2-1-12 Kannondai, Tsukuba, Ibaraki 305-8642, Japan

A novel type of quantitative competitive polymerase chain reaction (QC-PCR) system for the detection and quantification of the Roundup Ready soybean (RRS) was developed. This system was designed based on the advantage of a fully validated real-time PCR method used for the quantification of RRS in Japan. A plasmid was constructed as a competitor plasmid for the detection and quantification of genetically modified soy, RRS. The plasmid contained the construct-specific sequence of RRS and the taxon-specific sequence of lectin1 (Le1), and both had 21 bp oligonucleotide insertion in the sequences. The plasmid DNA was used as a reference molecule instead of ground seeds, which enabled us to precisely and stably adjust the copy number of targets. The present study demonstrated that the novel plasmid-based QC-PCR method could be a simple and feasible alternative to the real-time PCR method used for the quantification of genetically modified organism contents.

KEYWORDS: Roundup Ready soybean (RRS); plasmid-based QC-PCR (PQC-PCR); genetically modified organism (GMO)

INTRODUCTION

Since the first commercialization of genetically modified (GM) crops developed through recombinant DNA technologies, GM crop acreage has kept on increasing over the world. Along with the expansion of GM crop production, the global area of approved GM crops reached 102 million hectares in 2006 (*1*). To enable consumers to make informed choices, the governments of some countries require food products and ingredients to be labeled if the contents of GM organisms (GMO) in food products exceed a certain threshold level of adventitious contamination, such as 0.9, 3, and 5% in the European Union (*2*), Korea (*3*), and Japan (*4*), respectively. For the affirmation of the labeling authenticity, the development of accurate, reliable, and rapid methods for the detection of GMOs has been highly demanded.

Methods based on polymerase chain reaction (PCR) are suitable for the specific and sensitive detection of DNA from GM crops as described for Roundup Ready soybean (RRS) and several lines of GM maize (*5–8*). For GMO screening, qualitative PCR methods are practical and can determine whether the content of GMO in a sample exceeds certain detectable limits (*7*). For the enforcement of threshold values, quantitative analytical methods are required to obtain more precise numerical information.

Recently, real-time PCR has been a first choice for the quantitative analysis of GMO due to its specificity and sensitivity (*5–13*). However, real-time PCR instruments and reagents are quite expensive and not always preferable for every laboratory. A quantitative competitive polymerase chain reaction (QC-PCR) method, in which a target DNA is coamplified together with a competitor DNA carrying the same primer binding sites, has proven to be a feasible method (*14*). The QC-PCR method has also been studied for the quantification of GMO in foods, and a single-QC-PCR was proposed as a simple quantitative screening method to survey the 1% threshold limit for GMOs in food (*15, 16*). Usually, a competitive plasmid used

* To whom correspondence should be addressed. Tel: +81-29-838-7369. Fax: +81-29-838-7996. E-mail: kaz@affrc.go.jp.
[†] Fasmac Co., Ltd.
[‡] Showa Sangyo Co., Ltd.
[§] Food and Agricultural Materials Inspection Center.
[∥] Nippon Gene Co., Ltd.
[⊥] National Institute of Health Sciences.
[#] National Food Research Institute.
[▽] Current address: Food Safety Commission Secretariat, Cabinet Office, 2-13-10 Nagata-cho, Chiyoda, Tokyo 100-8989, Japan.

10.1021/jf073348n CCC: $40.75
Published on Web 06/18/2008

for QC-PCR contains an insertion or deletion in the sequence, large enough to allow separation from the target DNA. In the QC-PCR method, each sample is "titrated" with a competitor; that is, accretive known contents of a competitor are added to aliquots containing a constant content of target followed by PCR and electrophoresis. At an equivalence point where the same band densities are densitometrically acquired, the quantities of target and competitor are determined to be equal. After further modifications, dual competitive-PCR (DC-PCR) was developed for the quantitative analysis of heat-treated and/or processed foods (*13, 17, 18*). In the DC-PCR, target sequences, that is, endogenous and recombinant sequences, are coamplified with respective competitive plasmid DNAs, and the ratio of these two target sequences allows one to calculate the relative proportion of a GMO in foods. Furthermore, in a recently developed high-throughput double quantitative competitive PCR (HT-DCPCR), PCR products were detected by a biluminometric hybridization assay performed in a microtiter plate, instead of electrophoresis (*19*). In these above-mentioned methods, ground seeds were used as reference materials for the calibration of competitor plasmid to quantify the GMO contents. However, it is difficult to maintain the constant quality of reference materials because the reference materials are prepared from agricultural products in which the quality of DNA extract could be affected by many factors such as variety, growing area, production year, and so on.

The objective of this study was to develop a novel QC-PCR method using plasmid DNA as a reference molecule instead of ground seeds for the quantification of GMOs. In this report, we described the development of the method as compared with the data obtained by a fully validated real-time PCR method, which has not only been adopted as a Japanese official method but has also been adopted and published in the Annex of ISO 21570 (*7, 20*). In addition, analyses using two PCR machines and three kinds of analytical instrument systems were performed to assess the applicability of the proposed method.

MATERIALS AND METHODS

Materials. Genuine seeds of GM soy variety AG3301, a progeny of RRS developed by Monsanto (St. Louis, MO), were provided by the developer to the Ministry of Agriculture, Forestry and Fisheries (MAFF) to comply with the regulations under Food Sanitation Law and Japanese Agricultural Standard Law regarding mandatory labeling of GM foods. Certified reference materials (CRMs) produced by the Institute for Reference Materials and Measurement (IRMM) containing 1 and 5% RRS were purchased from Sigma-Aldrich (St. Louis, MO).

Oligonucleotide Primers and Probes. Oligonucleotide primers were synthesized and purified on a reversed-phase column by Fasmac (Kanagawa, Japan). Each oligonucleotide was diluted to an appropriate concentration with the appropriate volume of TE buffer. Primer sequences used to construct a competitor plasmid and to perform plasmid-based QC-PCR (PQC-PCR) are listed in **Table 1**. TaqMan probes for the quantification of RRS and soybean endogenous gene, lectin1 (Le1), have been described previously (*7*). These probes were labeled with 6-carboxy-fluorescein (FAM) and 6-carboxytetramethil-rhodamin (TAMRA) at the 5′- and 3′-ends, respectively, and were synthesized by Applied Biosystems (Foster City, CA).

Preparation of Samples and DNA Extraction. Dry seeds were ground with the P-14 speed rotor mill (Fritsch, Ibar-Oberstein, Germany). Ground seeds were lyophilized for 24 h in the FDU-540 freeze drier (Tokyo Rikakikai, Tokyo, Japan) and stored at −20 °C until use. The five levels of simulated GM-mixture samples containing 0.5, 1, 3, 5, and 10% (w/w) of RRS in non-GM soy were made from the ground seeds. DNA extraction was performed using DNeasy Plant Maxi Kit (Qiagen, Hilden, Germany) with slight modifications as described previously (*7*). The DNA concentrations of solutions were determined by measuring UV absorbance at 260 nm, and the quality was evaluated by the absorbance ratios at 260/280 nm and 260/230 nm; the absorbance ratio at 260/280 nm was between 1.7 and 2.0, and that at 260/230 nm was >1.7. The homogeneity of these simulated GM-mixture samples was confirmed by a real-time PCR method as described elsewhere (*7*).

Table 1. Primer Sequences

no.	name	sequences (5′ −3′)
		for construction of competitor plasmid
1	QTso-F	ATAGGCGCCGCCCTCTACTCCACCCCCA
2	QTsi-1	ACATTCAGACGTCAGTGCTAATTTCTTTGTCCC
3	QTsi-2	TTAGCACTGACGTCTGAATGTCCGGTAGCGTTGCC
4	QTsi-3-3	GGGTTGACGCCCATCTGCAAGCCTTTTT
5	QTsi-4-1	AGATGGGCGTCAACCCTTTAGGATTTCAGCATCAGTGG
6	QTso-R	GCAGATGTCAAAGGATTGGGAGGACTTGTCGCCGGGAATG
7	QTsi-F	CATTCCCGGCGACAAGTCCTCCCAATCCTTTGACATCTGC
8	QTsi-R2	ATAGGCGCCTCGATTTCTCTCTTGGTGACAGG
		for PQC-PCR system
9	Le1n 02-5′	GCCCTCTACTCCACCCCCA
10	Le1n 02-3′	GCCCATCTGCAAGCCTTTTT
11	RRS 01-5′	CCTTTAGGATTTCAGCATCAGTGG
12	RRS 01-3′	GACTTGTCGCCGGGAATG

Real-Time PCR. The experimental procedure of real-time PCR followed the established method (*9*), also known as a Japanese official method for GM detection (*21*). One of the main features of this analysis method was to use plasmid DNAs as reference materials. Briefly, a 25 μL reaction solution contained 50 ng of sample DNA, 12.5 μL of Universal Master Mix (Applied Biosystems), 0.5 μM primer pair (Fasmac), and 0.2 μM probe (Applied Biosystems). The real-time PCR reactions were performed with the ABI PRISM 7700 (Applied Biosystems) according to the following step-cycle program: preincubation at 50 °C for 2 min and 95 °C for 10 min, 40 cycles consisting of denaturation at 95 °C for 30 s, annealing, and extension at 59 °C for 1 min. Calibration curves were drawn for RRS and Le1 using the GM Soy Detection Plasmid set-ColE1/TE- (Nippon Gene, Tokyo, Japan), which contains five different concentrations of reference molecule, that is, 20, 125, 1500, 20000, and 250000 copies per 2.5 μL. The copy number of each target sequence was obtained based on the calibration curve. Then, the GM contents of the test samples were calculated using a conversion factor (Cf) value described below (*7*).

Calculation of Cf and GM Contents (%). The ratio of the copy number of recombinant DNA to the taxon-specific sequence of genuine seed was calculated by formula 1 and defined as Cf. The GMO content (%) was calculated by formula 2.

$$\text{Cf} = \text{copy number of recombinant DNA sequence in the DNA extracted from genuine GM seed/copy number of taxon-specific sequence in the DNA extracted from genuine GM seed} \quad (1)$$

$$\text{GM content (\%)} = \text{copy number of recombinant DNA sequence in sample/copy number of taxon-specific sequence in sample/Cf} \times 100 \quad (2)$$

Construction of the Competitor Plasmid. The construction of a competitor plasmid for PQC-PCR system was performed with slight modifications of the overlap extension method described elsewhere (*7*) and followed the schematic diagram shown in **Figure 1**. PCR templates used in the reactions **A**−**D** in **Figure 1** were the following: **A**, reference molecule contained in GM Soy Detection Plasmid set-ColE1/TE-; **B**, same as for **A**; **C**, subcloned plasmid that contained 21 bp insertion in the RRS sequence; and **D**, reference molecule contained in GM Maize Detection Plasmid set-ColE1/TE- (Nippon Gene). The integrated fragment was ligated into pCR2.1 plasmid vector (Invitrogen, Carlsbad, CA) by using TOPO TA Cloning kit (Invitrogen). The cell of *Escherichia coli* strain TOP10 (Invitrogen) was transformed with the plasmid. The plasmid was extracted and purified with the GFX Micro Plasmid Prep Kit (GE Healthcare Bio-Sciences, Piscataway, NJ). The sequence of the inserted fragment was confirmed. After sequencing, the fragment was ligated into pUC19 plasmid vector. The sequence of

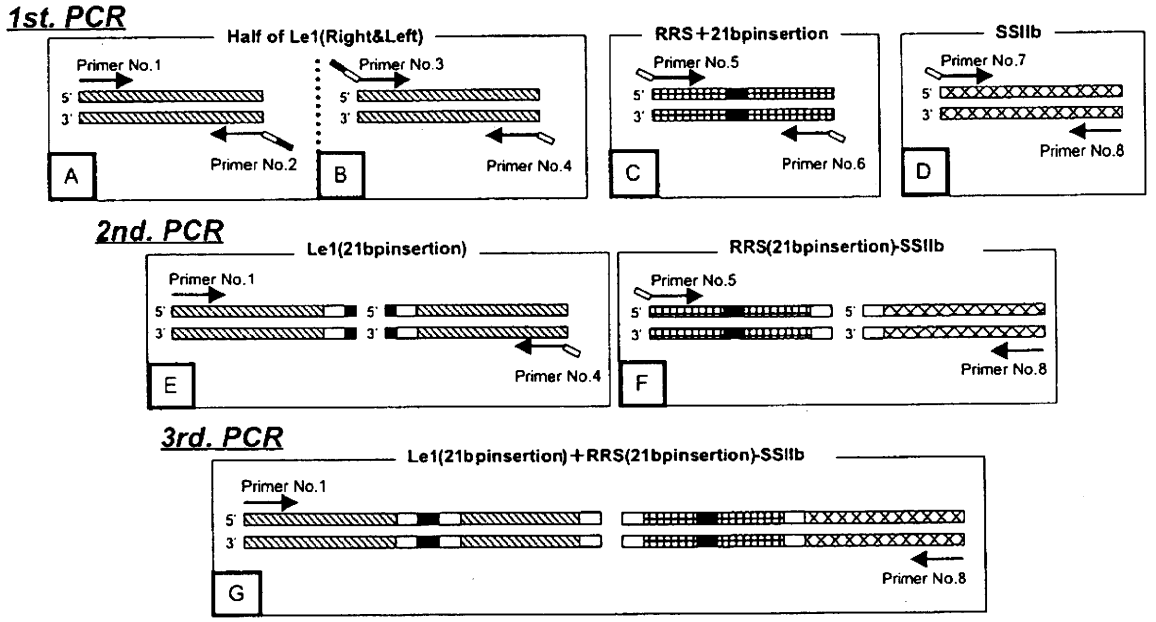

Figure 1. Scheme for the construction of competitor plasmid for PQC-PCR system. PCRs A, B, C, and D were performed using primer pair nos. 1 and 2, 3 and 4, 5 and 6, and 7 and 8, respectively, and of which primers 2−7 were tailed (**A**−**D**). The resultant amplicons from PCRs A and B and C and D were mixed and PCRs E and F were performed, respectively (**E** and **F**). The connected amplicons were used as templates for the third PCR (**G**). Finally, the integrated fragment was ligated into a plasmid vector.

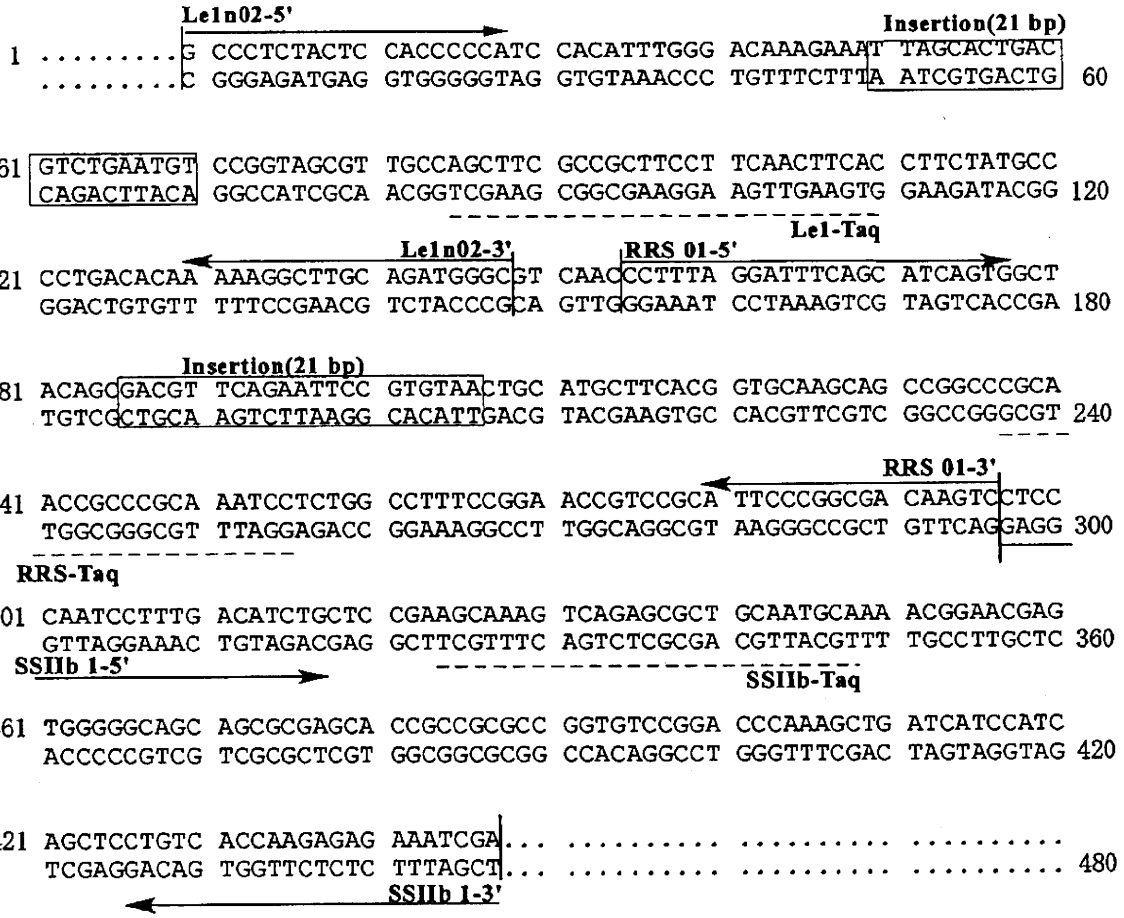

Figure 2. Sequence of integrated fragments inserted into pQCS. Arrowed lines indicate the sequences of primers for the detection of Le1, RRS, and SSIIb. The sequences of 21 bp insertions into Le1 and RRS are boxed, respectively. Dotted lines indicate the sites of TaqMan probes.

the competitor plasmid, pQCS, for GM-soy is shown in **Figure 2**.

Calibration of Competitor Plasmid. The plasmid, pQCS, was extracted and purified with the Qiagen plasmid Giga kit (Qiagen) and diluted with ColE1/TE buffer (Nippon Gene) to appropriate concentrations. To perform precise quantification, we made three sets of pQCS, that is, competitor plasmid sets H, M, and L, and each competitor plasmid set consisted of five different concentrations of pQCS (**Table 2**).

The copy numbers of the plasmid in each set were decided based on our experimental data, aiming to quantify samples containing RRS

Table 2. Competitor Plasmid Sets for PQC-PCR System

a. Competitor Plasmid Sets and Copy Numbers

competitor plasmid set	numbers of competitor plasmid/reaction tube				
H	56000	28000	14000	7000	3500
M	2800	1400	700	350	175
L	560	280	140	70	35

b. Combination of Competitor Plasmid Sets for Quantification of RRS

sample	Le1	RRS
100% RRS	H	H
3.0−10.0% RRS	H	M
0.5−3.0% RRS	H	L

Table 3. Cf of the PQC-PCR System[a]

	Gene Amp 9700			PTC-200		
analytical instrument	average	SD	RSD (%)	average	SD	RSD (%)
system 1	0.98	0.04	3.7	0.98	0.05	5.1
system 2	1.01	0.08	7.5	0.97	0.04	4.1
system 3	1.03	0.07	7.0	0.99	0.05	4.6

[a] System 1, Molecular Imager FX and Quantity One; system 2, Light-Capture (ATTO) and CS Analyzer; system 3, Printgraph (ATTO) and Image J; SD, standard deviation; and RSD, relative standard deviation.

Table 4. Quantification of RRS Content in Five Simulated Mixture Samples by a Real-Time PCR System[a]

		precision	
mixing level (%)	average	SD	RSD (%)
0.5	0.56	0.03	5.7
1	1.11	0.05	4.6
3	3.13	0.18	5.6
5	4.55	0.21	4.7
10	10.61	0.47	4.4

[a] Cf = 1.04; ABI PRISM 7700.

approximately 1 and 5%. The copy numbers of the competitor plasmid in each set were quantified and confirmed by real-time PCR. The sequence of Le1 constructed into the plasmid enabled us to quantify the copy numbers of the plasmid using the calibration curve obtained by the GM Soy Detection Plasmid set-ColE1/TE-, which also contains the sequence of Le1.

PQC-PCR. The genomic DNA extracted from ground seeds was coamplified with the competitor plasmid in a single PCR tube, and all reactions were conducted twice. Each reaction mixture contained 50 ng of sample DNA, 2 μL of the competitor plasmid (contents ranging from 35 to 56000 copy numbers per reaction as shown in **Table 2**), 2.5 μL of PCR bufferII (Applied Biosystems), 0.2 mM dNTP, 1.5 mM $MgCl_2$, 0.625 U of AmpliTaq Gold polymerase (Applied Biosystems), and 0.25 mM each primer pair (**Table 1**) for a final volume of 25 μL. To examine the applicability for PQC-PCR, we demonstrated the PCR reaction with two kinds of PCR equipment, that is, the GeneAmp 9700 (Applied Biosystems) and the PTC-200 DNA engine (MJ Research, Watertown, MA), with the following PCR step-cycle program: 10 min at 95 °C, 40 cycles, 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.

Analysis of PCR Products. *Electrophoresis.* After the PCR amplification, 4 μL of each PCR product was electrophoresed at a constant voltage of 100 V on 5% (w/v) agarose gel Takara LO3 (Takara Bio, Shiga, Japan) containing 0.5 mg/mL ethidium bromide (Sigma) in TAE [40 mM Tris-HCl, 40 mM acetic acid, and 1 mM EDTA (pH 8.0)]. A 100 bp ladder (New England Biolabs, Beverly, MA) was used for size control of the amplified fragments.

Analysis of Amplified Fragments. To examine the applicability for PQC-PCR, analytical instruments were used as follows: system 1, the Molecular Imager FX system (Bio-Rad Laboratories, Hercules, CA) and the Quantity One (Bio-Rad); system 2, the ATTO Light-Capture Cold CCD Camera System type AE6972 (ATTO, Tokyo, Japan) and the CS Analyzer (ATTO); and system 3, the CCD camera Printgraph-CX type AE6911 (ATTO) and the NIH Image (public domain image processing and analysis program). The relative band intensities were determined with these three kinds of image analysis software. The copy numbers of competitor plasmid and target should be the same at the equivalence point as depicted in **Figure 3**. Precisely, the decimal logarithm of the copy number of pQCS was plotted on the x-axis, and that of the intensity ratio of pQCS to target was plotted on the y-axis. The intersection between the calibration curve and the y-axis was defined to be the equivalence point, and the copy number of target was determined. The copy numbers of Le1 and RRS were determined, and Cf value was calculated with formula 1. The GM content in a sample was calculated with formula 2 using an obtained Cf value.

RESULTS AND DISCUSSION

Measurement of Cf. Initially, we obtained a conversion factor required to calculate GM contents for RRS. The 100% RRS sample was subjected to real-time PCR. To determine the Cf value, the copy numbers of recombinant DNA and taxon-specific sequence were separately measured, and all of the measurements were repeated three times. The resultant Cf was 1.04 ± 0.03, which was the average of triplicate PCR reactions with the ABI PRISM 7700, similar to the value calculated by our previous report (*22*) and also close to the theoretical Cf value of 1.00.

Because the measurement principle of real-time PCR is different from that of PQC-PCR, the Cf value for PQC-PCR was also independently measured. The same 100% RRS sample was subjected to PQC-PCR using a competitor plasmid set H. Competitor plasmid pQCS, having target sequences of Le1 and RRS together on the same plasmid, enabled us to ignore the dilution error caused by diluting the plasmid. If Le1 and RRS were constructed on separate plasmids, the dilution error of each plasmid had to be considered. The reaction was performed on the GeneAmp 9700 and the PTC-200 DNA engine. Then, the amplified fragments were analyzed using three types of analytical systems consisting of a photographic device and software, which resulted in six independent Cf values as shown in **Table 3**. These six independent Cf values were analyzed by one-way analysis of variance and the obtained P value was 0.74. This result demonstrated that there was no significant difference. In all measurements, the coefficients of determination (R^2) for standard curves were between 0.98 and 1.00. All of the obtained Cf values were close to the theoretical Cf value of 1.00 as well as that of real-time PCR. Therefore, both of the systems were suggested to work well to quantify the targets, although the measurement principles of real-time PCR and PQC-PCR are different. In addition, because Cf values for PQC-PCR, obtained irrespective of the combination of PCR machines and analytical systems, were all close to the theoretical Cf value, the variance of Cf values caused by machine and analytical system could be considered negligible. The average Cf value of 0.99 was used to calculate the following measurements.

Measurement of GM Contents (%). First, five samples of dried ground seeds with different mass fractions of RRS, that is, 0.5, 1, 3, 5, and 10% (w/w), were subjected to real-time PCR. The obtained Cf value (1.04) was used to calculate GM contents (%), and triplicate PCR reactions for each sample were performed with the ABI PRISM 7700 (**Table 4**).

Next, PQC-PCR was performed to determine the GM contents of the same simulated RRS samples. Competitor plasmid set L was used to measure the copy number of RRS in 0.5−3% of

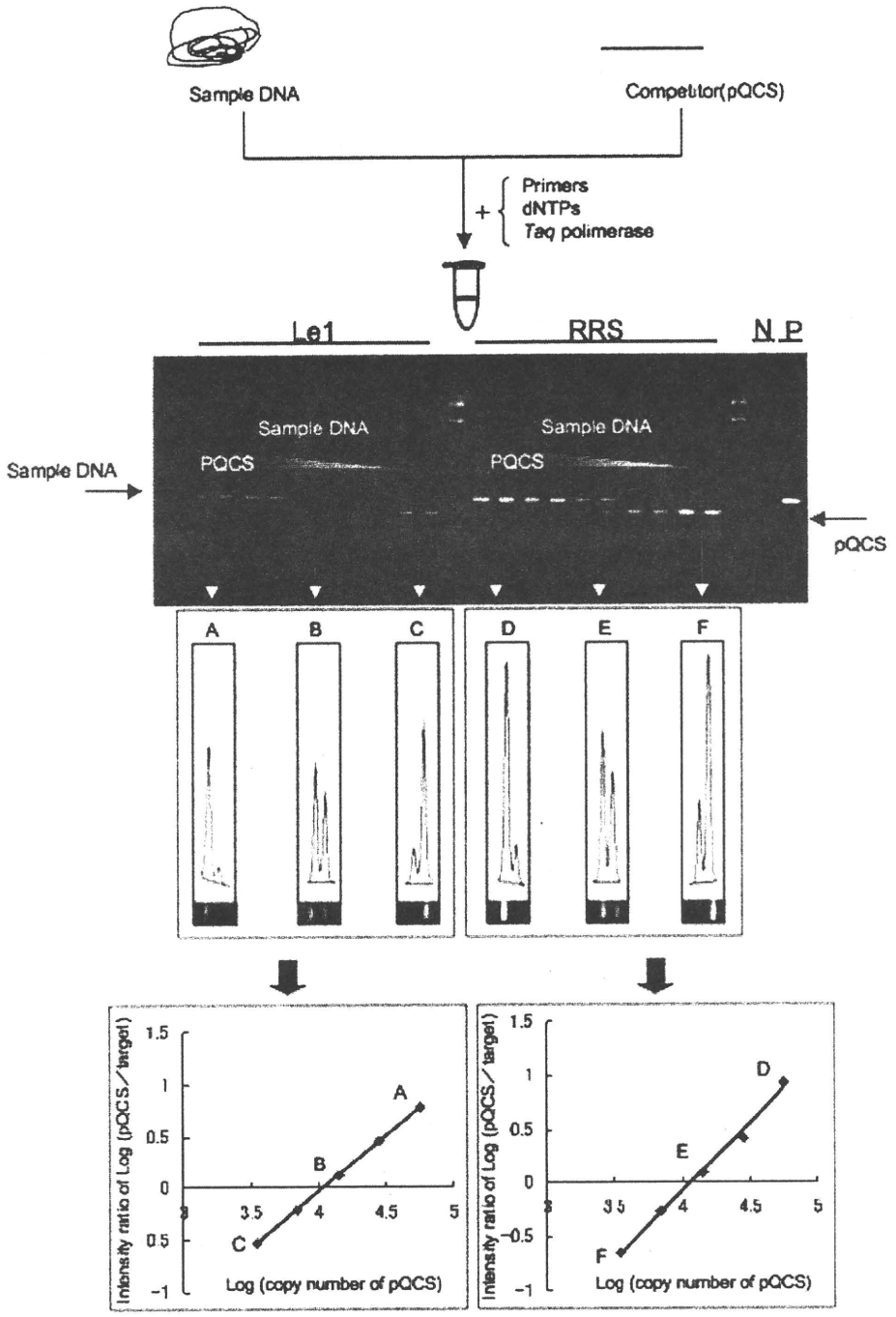

Figure 3. Principle of GMO quantification by the PQC-PCR system. Sample DNA and competitor were coamplified in the same reaction tube. The amount of DNA was constant in all PCR tubes. After PCR, the amplification products were separated by agarose gel electrophoresis on which the amplified competitor sequence could be distinguished from the amplified target sequence by size. At the equivalence point, the starting concentrations of target and competitor were equal. N, negative control (no primer); P, positive control (pQCS). Negative control (no DNA) was performed but not shown.

simulated RRS mixtures. Competitor plasmid set M was used for RRS in 3−10% samples, and competitor plasmid set H was used to determine the copy number of Le1 in all samples. The reaction was performed on the GeneAmp 9700 and the PTC-200 DNA engine in triplicate, and the amplified fragments were analyzed with three analytical systems as shown in **Table 5**. As intended, the samples in ranges of 0.5−3 and 3−10% were quantified well with each combination of PCR and analytical instruments with RSDs below 16.5%. The measurements of simulated samples obtained with PQC-PCR were compared with those obtained with real-time PCR (**Figure 4**). It was found that the GM contents obtained by both methods were almost equivalent, and the regression line between the methods was $y = 0.9810x + 0.0294$ ($R^2 = 0.9959$).

These results demonstrated that the PQC-PCR method would give almost equivalent values obtained with real-time PCR. Also, both of the real-time PCR and the PQC-PCR methods required about 2.5 h to complete the measurements. In addition, introduction of another technique, such as capillary electrophoresis into the PQC-PCR system, would reduce the analysis time.

PQC-PCR Measurement of IRMM-CRM. The CRMs containing 1 and 5% of RRS produced by IRMM were used to examine the PQC-PCR method. PQC-PCR was performed on

Table 5. Quantification of RRS Content in Five Simulated Mixture Samples by a PQC-PCR System[a]

	Gene Amp 9700			PTC-200		
mixing level (%)	average	SD	RSD (%)	average	SD	RSD (%)
			system 1			
0.50	0.54	0.08	14.3	0.55	0.02	4.2
1	1.10	0.11	10.4	1.06	0.05	5.2
3	3.17	0.02	0.8	2.95	0.24	8.0
3	2.64	0.31	3.14	0.49	11.6	15.5
5	4.24	0.22	5.1	4.59	0.07	1.6
10	10.15	1.29	12.7	10.16	0.47	4.6
			system 2			
0.50	0.64	0.08	13.0	0.57	0.05	8.7
1	1.16	0.04	3.0	1.13	0.08	7.4
3	3.08	0.22	7.3	3.11	0.34	10.9
3	2.70	0.33	12.1	3.37	0.22	6.5
5	4.59	0.42	9.1	4.71	0.16	3.4
10	10.19	0.33	3.2	10.86	0.57	5.3
			system 3			
0.50	0.60	0.10	16.5	0.60	0.05	8.4
1	1.06	0.14	12.8	1.10	0.06	5.5
3	3.14	0.15	4.8	3.14	0.31	9.8
3	2.85	0.13	4.4	3.54	0.12	3.2
5	4.61	0.44	9.5	4.78	0.32	6.6
10	10.29	0.53	5.2	10.85	0.29	2.7

[a] System 1, Molecular Imager FX and Quantity One; system 2, Light-Capture (ATTO) and CS Analyzer; and system 3, Printgraph (ATTO) & Image J; and Cf = 0.99.

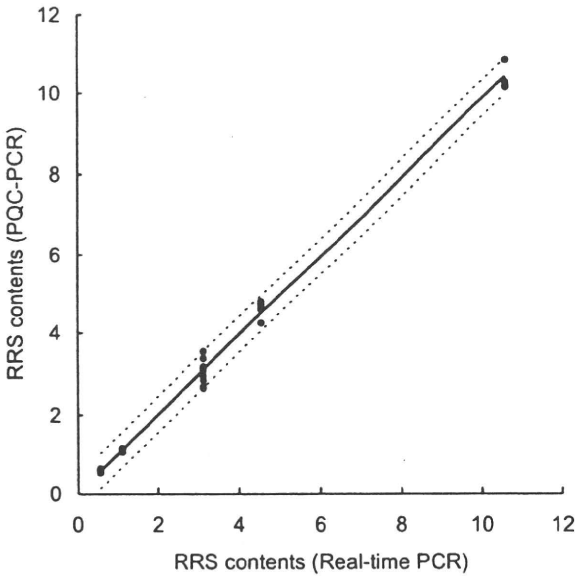

Figure 4. Correlation between RRS contents acquired by PQC-PCR and real-time PCR. RRS contents acquired by real-time PCR shown in **Table 4** are plotted on the *x*-axis, and those by PQC-PCR shown in **Table 5** are plotted on the *y*-axis. $y = 0.9810x + 0.0294$ ($R^2 = 0.9959$). The prediction interval for individual *y* is shown with dotted lines. The maximum and minimum values of standard uncertainties were calculated to be 0.23 and 0.22, respectively. For PQC-PCR, GeneAmp 9700 and PTC-200 DNA engine were used. For real-time PCR, ABI PRISM 7700 was used.

the GeneAmp 9700 and the PTC-200 DNA engine in triplicate, and the amplified fragments were analyzed with three analytical systems as shown in **Table 6**. The measured value for each of 1 and 5% RRS was in the range of the respective certified value. These results suggested that the PQC-PCR method could accurately measure GM contents and thus be useful for practical use.

Table 6. Quantification of RRS Contents in IRMM CRM by a PQC-PCR System[a]

		Gene Amp 9700			PTC-200		
sample (%)	analytical instrument	average	SD	RSD (%)	average	SD	RSD (%)
1 ± 0.16	system 1	0.92	0.01	0.6	1.00	0.08	8.4
	system 2	0.89	0.07	7.9	1.00	14.0	14.0
	system 3	0.92	0.05	5.1	1.04	0.17	16.0
5 ± 0.53	system 1	4.92	0.50	10.1	4.75	0.22	4.5
	system 2	5.07	0.23	4.6	4.74	0.38	8.0
	system 3	4.87	0.30	6.1	4.72	0.20	4.3

[a] System 1, Molecular Imager FX and Quantity One; system 2, Light-Capture (ATTO) and CS Analyzer; system 3, Printgraph (ATTO) and Image J; and Cf = 0.99.

ABBREVIATIONS USED

QC-PCR, quantitative competitive polymerase chain reaction; GM, genetically modified; RRS, Roundup Ready soybean; Le1, lectin 1; PQC-PCR, plasmid-based QC-PCR; GMO, genetically modified organism; MAFF, Ministry of Agriculture, Forestry and Fisheries; Cf, conversion factor; RSD, relative standard deviation; SD, standard deviation; CRM, certified reference materials; IRMM, Institute for Reference Materials and Measurements.

ACKNOWLEDGMENT

This study was supported in part by a grant from the Ministry of Agriculture, Forestry and Fisheries of Japan for the Research Project, "Assurance of Safe Use of Genetically Modified Organisms", and by a grant from the Ministry of Health, Labour and Welfare of Japan.

LITERATURE CITED

(1) James, C. Review—Global status of commercialized biotech/GM crops: 2006. *ISAAA Briefs* **2006**, *35*.
(2) Regulation (EC) No. 1829/2003 of the European Parliament and of the Council of 22 September 2003 on genetically modified food and feed. *Off. J. Eur. Union* **2003**, L268/1−L268/23.
(3) Notification No. 2000-31; Ministry of Agriculture and Forestry of Korea: Seoul, Korea, 2000.
(4) Notification No. 1775; Food and Marketing Bureau, Ministry of Agriculture, Forestry and Fisheries of Japan: Tokyo, Japan, 2000.
(5) Matsuoka, T.; Kawashima, Y.; Akiyama, H.; Miura, H.; Goda, Y.; Kusakabe, Y.; Isshiki, K.; Toyoda, M.; Hino, A. A method of detecting recombinant DNAs from four lines of genetically modified maize. *J. Food Hyg. Soc. Jpn.* **2000**, *41*, 137–143.
(6) Matsuoka, T.; Kuribara, K.; Takubo, H.; Akiyama, H.; Miura, H.; Goda, Y.; Kusakabe, Y.; Isshiki, K.; Toyoda, M.; Hino, A. Detection of recombinant DNA segments introduced to genetically modified maize (*Zea mays*). *J. Agric. Food Chem.* **2002**, *50*, 2100–2109.
(7) Kuribara, H.; Shindo, Y.; Matsuoka, T.; Takubo, K.; Futo, S.; Aoki, N.; Hirao, T.; Akiyama, H.; Goda, Y.; Toyoda, M.; Hino, A. Novel reference molecules for quantitation of genetically modified maize and soybean. *J. AOAC Int.* **2002**, *85*, 1077–1089.
(8) Shindo, Y.; Kuribara, H.; Matsuoka, T.; Futo, S.; Sawada, C.; Shono, J.; Akiyama, H.; Goda, Y.; Toyoda, M.; Hino, A. Validation of real-time PCR analyses for line-specific quantitation of genetically modifed maize and soybean using new reference molecules. *J. AOAC Int.* **2002**, *85*, 1119–1126.
(9) Terry, C. F.; Harris, N. Event-specific detection of Roundup Ready soya using two different real time PCR detection chemistries. *Eur. Food Res. Technol.* **2001**, *213*, 425–431.
(10) Permingeat, H. R.; Reggiardo, M. I.; Vallejos, R. H. Detection and quantification of transgenes in grains by multiplex and real-time PCR. *J. Agric. Food. Chem.* **2002**, *50*, 4431–4436.