J. Microbiol. Biotechnol. (2007), **17**(2), 335–341

JOURNAL OF MICROBIOLOGY AND BIOTECHNOLOGY

© The Korean Society for Microbiology and Biotechnology

Qualitative and Quantitative Analysis of Genetically Modified Pepper

SONG, HEE-SUNG, JAE-HWAN KIM, DONG-HERN KIM[1], AND HAE-YEONG KIM*

Institute of Life Science & Resources and Graduate School of Biotechnology, Kyung Hee University, Suwon 446-701, Korea
[1]Division of Biosafety, National Institute of Agricultural Biotechnology, Suwon 441-100, Korea

Received: October 2, 2006
Accepted: November 26, 2006

Abstract For the development of qualitative and quantitative PCR methods of genetically modified (GM) pepper developed in Korea, a capsanthin-capsorubin synthase (CCS) gene was used as the endogenous reference gene. The primer pair ccs-F/R amplifying the pepper endogenous gene gave rise to an amplicon of 102 bp. No amplified product was observed when DNA samples from 16 different plants were used as templates. The construct-specific primer pairs amplifying the junction region of the *bar* gene and Ti7 introduced in GM pepper gave rise to an amplicon of 182 bp. Quantitative PCR assay was performed using a TaqMan probe and a standard plasmid as a reference molecule, which contained both an endogenous and event-specific sequence. For the validation of this method, the test samples containing 0.1, 1, 3, 5, and 10% GM pepper were quantified.

Key words: Capsanthin-capsorubin synthase, GMO detection, GM pepper, real-time PCR

Numerous genetically modified (GM) crops have been developed by recombinant DNA (r-DNA) techniques, and GM foods are becoming an increasingly important part of the common food supply throughout the world [7, 10]. Although the GM crops have been authorized for commercialization in many countries, numerous controversial issues still exist, such as biosafety, environment risk, and ethical concerns. Therefore, many countries have regulations to label foods that include genetically modified organisms (GMOs): Labeling systems have been introduced for GM foods in the European Union (EU), Korea, Japan, Australia, and other countries except the United States and Canada [14]. Furthermore, the following governments have announced the following threshold levels for the adventitious presence of GMOs with food products: 0.9% in the EU [18], 3% in Korea [16], and 5% in Japan [15].

*Corresponding author
Phone: 82-31-201-2660; Fax: 82-31-204-8116;
E-mail: hykim@khu.ac.kr

To confirm the level of GM content in foods on the market, the DNA-based polymerase chain reaction (PCR) technique has been most frequently used [6, 9, 11, 13, 17, 20]. Of the PCR detection methods, real-time PCR is considered as an accurate, highly specific quantitative assay [8]. This analytical method has also been adopted as the official method in Korea [1, 19].

GM pepper has not yet been introduced into the commercial market; however, herbicide-tolerant pepper is now being developed by the Rural Development Administration (RDA) in Korea [12], and the safety of the former has been estimated. Pepper is used as the main spice in Korean foods and food additives. Since GM pepper is likely to appear on the market in the near future, the development of detection methods for GM pepper is therefore required.

In this paper, we report the specific primer pairs and probes for qualitative and quantitative detection of transgenic pepper. Qualitative and quantitative PCR assays were also used to confirm its species specificity, to test the limits of detection (LOD) and quantitation (LOQ), and to set up a dependable PCR system for GM-pepper detection. Finally, this optimal quantitative PCR condition was used for detection of GM pepper.

MATERIALS AND METHODS

Materials

Herbicide-tolerant (HT) pepper and its parental cultivar *Subicho* (SC) and the seeds of 8 different non-transgenic pepper varieties (IT 100763, IT 032447, IT 032494, IT 102752, IT 104017, IT 104567, IT 113594, and IT 113733) were provided by the National Institute of Agricultural Biotechnology (NIAB) in Korea. The seeds of 16 different plants [rice (*Oryza sativa*), canola (*Brassica napus*), corn (*Zea mays*), soybean (*Glycine max*), potato (*Solanum tuberosum*), tomato (*Lycopersicon esculentum*), perilla (*Perilla frutescens*) broccoli (*Brassica oleracea* var. *italica*), sunflower (*Helianthus annuus*), spinach (*Spinacia*

oleracea), parsley (*Petroselinum crispum*), cotton (*Gossypium hirsutum*), radishes (*Raphanus sativus*), lettuce (*Lactuca saligna*), scallions (*Allium fistulosum*), and chinese cabbage (*Brassica oleracea* var. *capitata* L.)] were collected by our laboratory.

Extraction of Genomic DNA

Genomic DNA of pepper and other crops was extracted from the ground sample (about 1 g) using the DNeasy Plant Maxi kit (Qiagen GmbH, Hilden, Germany) according to the modified manufacturer's manual. The plant genomic DNA samples used for Southern blot analysis were extracted and purified from 1g of fresh leaves according to the CTAB method. The DNA concentration was determined by UV absorption at 260 nm. The quality of extracted DNAs was evaluated from the ratio of UV absorptions at 260/280 and 260/230 nm and further analyzed by 1% agarose gel electrophoresis.

Oligonucleotide Primers and Probe

Sequences of oligonucleotide primers and TaqMan probes used in this study are listed in Table 1. The oligonucleotide primers and TaqMan probes that correspond to nucleotide sequences of the endogenous reference gene *CCS* and the exogenous *bar* gene were designed with Primer Designer (Version 3.0; Scientific and Educational Software, Durham, NC, U.S.A.).

The TaqMan probe of the endogenous reference gene *CCS* and the exogenous *bar* gene were labeled with 6-carboxy-fluorescein (FAM) on the 5' end as fluorescent reporter. The fluorescent quencher dye 6-carboxytetramethylrhodamine (TAMRA) was located on the 3' end of the probes. All primers were synthesized and purified on PAGE columns by Bioneer. Co. Ltd. (Daejeon, Korea), and TaqMan probes for real-time PCR were synthesized by Applied Biosystems (Foster City, CA, U.S.A.).

Standard Plasmid as a Reference Molecule

A standard plasmid, used as a reference molecule, was constructed based on a Perfect-T cloning kit (TaKaRa, Kyoto, Japan) integrated with two PCR amplicons. The primer pair, ccsF/ccsR and batF/batR, were used to amplify the *CCS* gene and construct-specific region in GM pepper, respectively. All PCR amplicons were analyzed by 2% agarose gel electrophoresis and purified with a Gel Extraction kit (Qiagen). The PCR product was ligated into a pMD18-T vector (TaKaRa). The recombinant plasmid was transformed into *Escherichia coli* strain DH5α (Novagen, Madison, WI, U.S.A.). The sequences of the cloned DNA were analyzed with an ABIPRISM 3700 DNA analyzer (Perkin Elmer, Boston, MA, U.S.A.).

According to the above procedures, the standard plasmid pCBT for the reference molecule was constructed by the tandem combination of two amplicons obtained by using respective primer pairs for endogenous and exogenous fragments in GM pepper (Fig. 1).

On the basis of the pepper genome size of 3,061 Mb per haploid genome [3], one haploid genome molecule corresponds to 3.17 pg of pepper DNA, assuming that 965 Mb weighs 1 pg. According to the plasmid size, one plasmid molecule (2,886 bp) corresponds to 3.16 pg of plasmid DNA, considering that 965 Mb weighs 1 pg. For example, the mass of plasmid DNA containing 200,000 copies of inserted sequence is as follows.

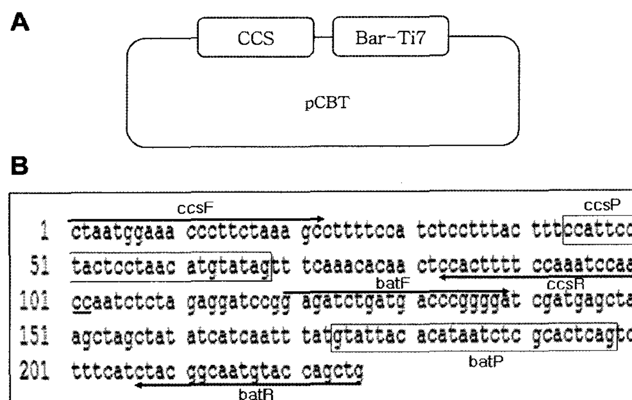

Fig. 1. Standard plasmid (pCBT) as a reference molecule.
A. Schematic diagram of pCBT; CCS stands for the a pepper endogenous gene, and Bar-Ti7 represents the a construct-specific region introduced in GM pepper with partial same DNA sequences. **B.** Nucleotide sequence of the integrated PCR amplicons in pCBT. The arrows locate primers with direction and the squared boxes indicate TaqMan probes.

Table 1. Primer pairs and fluorogenic probes for qualitative and quantitative PCR.

Name	Orientation	Sequence (5'-3')	Amplicons size (bp)	Purpose
ccs-F	Sense	CTAATGGAAACCCTTCTAAAGC	102	Qualitative duplex PCR
ccs-R	Antisense	GGTTGGATTTGGAAAAGTGG		
bat-F	Sense	AACTGGCATGACGTGGGTTT	182	
bat-R	Antisense	CAGCTGGTACATTGCCGTAG		
ccs-F	Sense	CTAATGGAAACCCTTCTAAAGC	102	Quantitative real-time PCR
ccs-R	Antisense	GGTTGGATTTGGAAAAGTGG		
ccs-P	TaqMan probe	CCATTCCTACTCCTAACATGTATAG		
bat-1F	Sense	GAGATCTGATGACCCGGGGA	107	
bat-R	Antisense	CAGCTGGTACATTGCCGTAG		
bat-P	TaqMan probe	GTATTACACATAATATCGCACTCAG'		

$$[3.16\times10^{-18}\ \text{g/copy}][200{,}000\ \text{copies}]=6.33\times10^{-13}\ \text{g}$$

Condition of Qualitative PCR

Qualitative PCR was run in a 25-µl final volume on a PCR PC 808 (ASTEC, Tokyo, Japan). Each reaction mixture contained 50 ng of sample DNA, 10× PCR buffer, dNTP mixture (each 2.5 mM), 400 nM each primer, and 0.5 units of Ex Taq DNA polymerase (TaKaRa). The amplification reaction was performed according the following cycle condition: denaturing of DNA at 94°C for 5 min, 40 cycles of 30 sec at 94°C, 30 sec at 62°C, 30 sec at 72°C, and final extension at 72°C for 7 min. The duplex PCR conditions were the same as explained above, except for the primer concentration of 400 nM ccsF/ccsR and 80 nM batF/batR instead. The amplification products were analyzed by 3% agarose gel.

Conditions of Quantitative Real-Time PCR

Real-time PCR assays with a TaqMan probe were carried out by ABI PRISM 7900 (Applied Biosystems) in a final volume of 25 µl. The PCR reaction mixture contained the following ingredients: 50 ng of DNA, 0.5 µM primer pair, 0.2 µM probe, and 12.5 µl of Universal Master Mix (Applied Biosystems). Real-time PCR reactions were run according to the following procedures: one cycle of 2 min at 50°C, 10 min at 95°C, 50 cycles of 30 s at 95°C, and 1 min at 58°C. Each sample was quantified in triplicates, and the PCR reactants were purchased from Applied Biosystems Co., Ltd. The results were analyzed using a sequence detection system provided by the Software ABI 7900. Standard curves were calibrated using five concentrations of reference molecules; *i.e.*, from 20 to 200,000 copies per reaction. No template control was prepared as a negative control for the analysis.

Determination of GMO Contents

According to the principle of standard curves optimization, best standard curves were obtained for the real-time PCR assays. Ct values were used to determine the amount of total DNA using the endogenous *ccs* gene PCR system, and the amount of transgenic DNA using the *bar* gene PCR system based on the standard curves. Sample GMO contents were determined by the ratio of transgenic DNA copy numbers to total DNA copy numbers with application of coefficient values (Cv) [11].

RESULTS AND DISCUSSION

Selection of the Pepper Endogenous Reference Gene, *CCS*, for Qualitative and Quantitative PCR Detection

The qualitative and quantitative PCR systems for GMOs depend on the endogenous reference genes. An endogenous reference gene should be species specific with a single copy number in the genome, and should exhibit low heterogeneity among cultivars [3]. To select a suitable endogenous reference gene of pepper for qualitative and quantitative PCR amplification, we searched for DNA sequences with a single copy number, and those candidate sequences were blasted in the GenBank database. We chose a capsanthin-capsorubin synthase (*CCS*) gene (GenBank No. X77289) as a pepper endogenous reference gene for qualitative and quantitative PCR detection. The primer pair ccs-F/R amplifying the pepper reference endogenous gene gave rise to an amplicon of 102 bp. No amplified product was observed, when DNA samples from 16 different plants were used as templates (Fig. 2).

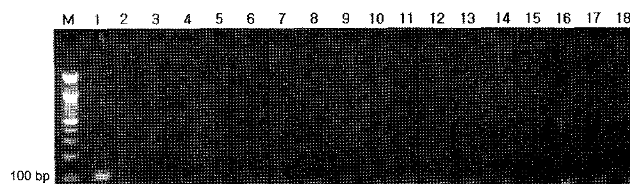

Fig. 2. Specificity analysis of the pepper endogenous *CCS* gene using qualitative PCR.

The amplification plot generated from 16 different plants species and pepper with the primer pair ccsF/R. Lane M: 100 bp DNA ladder; lanes 1–18: Pepper, Rice, Canola, Corn, Soybean, Potato, Tomato, Perilla, Broccoli, Sunflower, Spinach, Parsley, Cotton, Radishes, Lettuce, Scallions, Chinese cabbage, and no template.

Copy Number of *CCS* Gene Confirmed by Southern Blot

Targeting a single or stable low copy number DNA sequence should result in stable, real-time PCR assays. *CCS* has previously been proved as a single-copy gene in pepper [5]. Thus, to estimate the copy number of the *CCS* region in the GM-pepper genome, we performed a Southern blot analysis; the genomic DNA was digested with SpeI, XbaI, and NcoI and hybridized with the 1,274 bp DNA fragment of the *CCS* gene. A single hybridized band was detected in the SpeI-digested, and two hybridized bands were detected in the XbaI- and NcoI-digested GM-pepper genomic DNA samples, which confirmed that the *CCS* gene was a single copy gene per pepper haploid genome (data not shown).

Allelic Variation of *CCS* Gene Among Pepper Cultivars

To examine whether different pepper cultivars exhibit variation within the amplified *CCS* sequence, we carried out both qualitative and real-time PCR experiments on a fixed amount (50 ng) of DNA from 8 different pepper cultivars. As shown in Fig. 3A, the same PCR products were obtained from all of the cultivars tested in qualitative PCR. Real-time PCR performed with triplicates of DNA from these pepper cultivars exhibited similar Ct values (Fig. 3B). These results indicated that there was no main

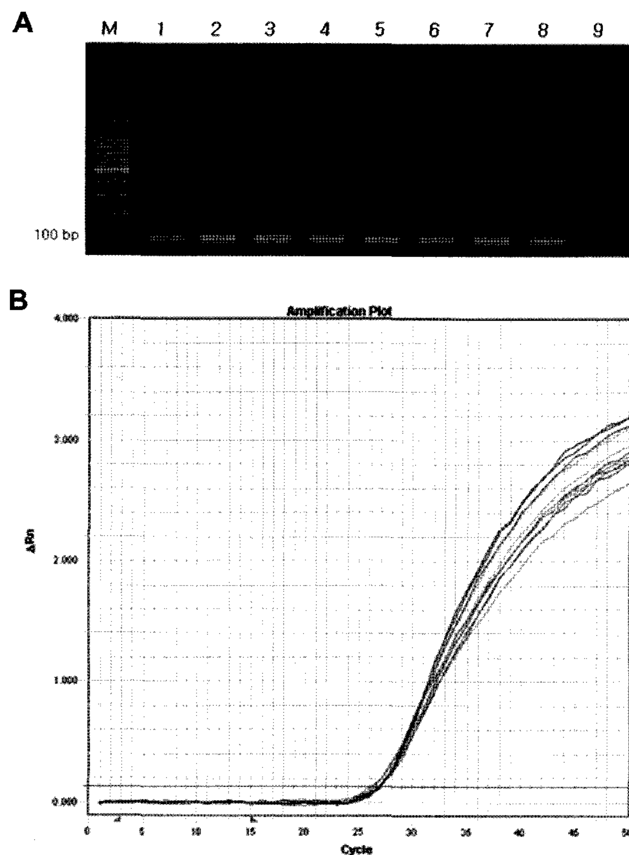

Fig. 3. Allelic variation of the *CCS* gene among pepper cultivars.
(**A**) Qualitative and (**B**) quantitative PCR results of 8 different pepper cultivars. Lane M: 100 bp DNA ladder, lanes; 1–9: IT 100763, IT 032447, IT 032494, IT 102752, IT 104017, IT 104567, IT 113594, IT 113733, and no template.

sequence difference among the different cultivars in this amplified region.

Qualitative PCR Assay of GM Pepper

The primer pairs ccsF/ccsR and batF/batR for qualitative PCR of GM pepper used to amplify the pepper endogenous gene produced 102 bp and 182 bp products, respectively. We performed duplex qualitative PCR to detect the inserted and endogenous genes in the GM pepper. To test the sensitivity of this duplex PCR, we carried out PCR using amounts of genomic DNA ranging from 50 ng to 5 pg. Qualitative PCR made it possible to detect GM pepper in 500 pg of genomic DNA (Fig. 4).

Reproducibility of Real-Time PCR

To test the reproducibility of the real-time PCR system, we generated the standard curve three times, each time with three equivalent repetitions, using five pepper DNA dilutions (ranging from 50 ng to 5 pg) as templates. In the reproducibility test, PCR products could be amplified with as little as 50 pg of pepper genomic DNA. The Ct values

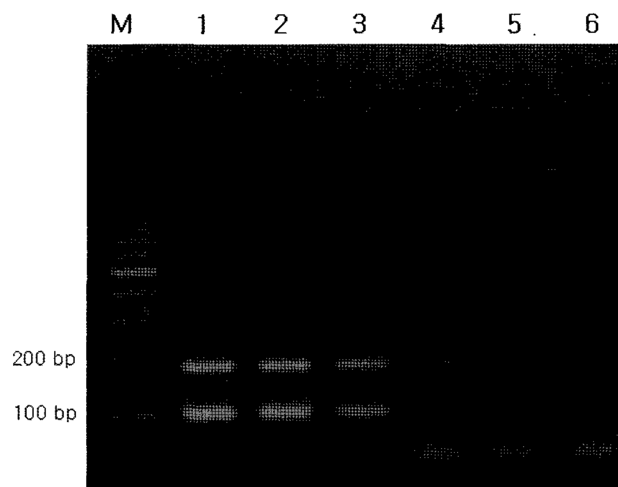

Fig. 4. Sensitivity detection of GM pepper using qualitative PCR.
Lane M: 100 bp DNA ladder; lanes 1–5: 50, 5, 0.5, 0.05, and 0.005 ng of GM pepper, Lane 6: no template.

ranged from 28.08 to 38.64, the standard deviation (SD) values ranged from 0.06 to 0.15, and the coefficient of variation (CV, %) ranged from 0.20 to 0.43 (Table 2). Because these variations are quite small, the result shows that this system worked stably and reliably.

Real-Time Quantitative PCR Assays of GM Pepper

The standard curves of the endogenous and event-specific real-time PCR assays were prepared using the standard plasmid DNA ranging from 20, 200, 2,000, 20,000, and 200,000 copies. The square regression coefficients (R^2) were 0.999 for the *CCS* and event-specific PCR assay (Figs. 5 and 6). The fine linearity between DNA quantities and fluorescence values (Ct) indicated that these assays were well-suited for quantitative measurements.

Reproducibility of the Ct values was determined, using the standard plasmid DNA dilutions, repeated three times in triplicate reactions (Table 3). The RT-PCR assay for endogenous *CCS* gene detection gave mean Ct values,

Table 2. Reproducibility of the Ct values for five levels of concentration of genomic DNA from non-GM pepper using RT-PCR.

DNA amount (ng/reaction)	Ct value for reaction			Mean	SD[a]	CV(%)[b]
	1	2	3			
50	28.15	28.05	28.05	28.08	0.06	0.20
5	31.54	31.70	31.60	31.61	0.08	0.26
0.5	35.19	35.11	35.41	35.24	0.15	0.43
0.05	38.60	38.74	38.57	38.64	0.09	0.23
0.005	–	–	–	–	–	–

[a]Standard deviation.
[b]Coefficient of variation.

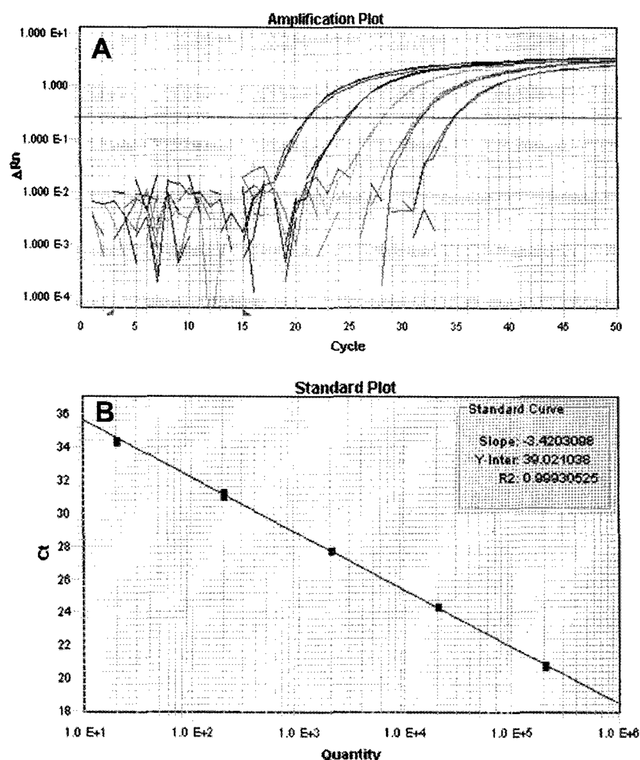

Fig. 5. Amplification plots and standard curve of quantitative real-time PCR assay for determining endogenous gene copy number in standard plasmid.

A. Amplification plots for the pepper endogenous *CCS* gene were established using 200,000, 20,000, 2,000, 200, and 20 copies of standard plasmid. **B.** Parameters of the regression line through data points are indicated within the plot.

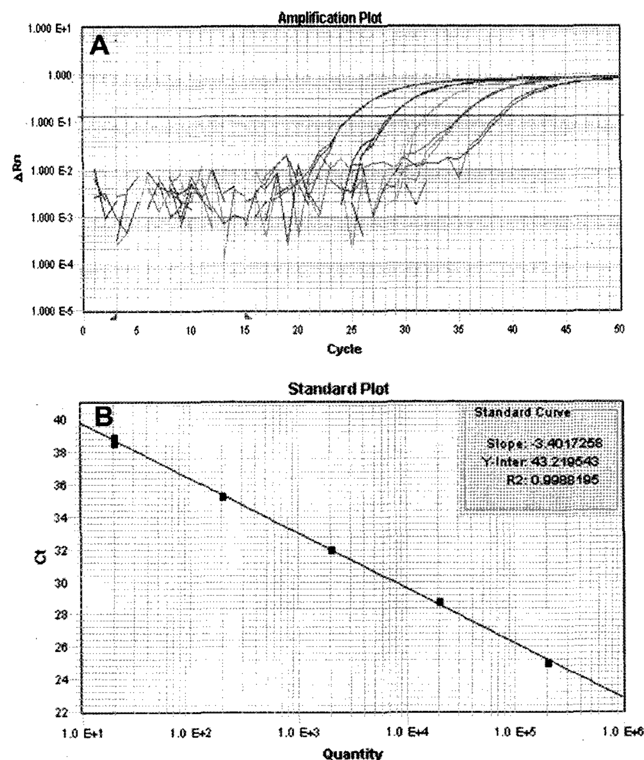

Fig. 6. Amplification plots and standard curve of quantitative real-time PCR assay for determining copy number of exogenous gene in standard plasmid.

A. Amplification plots for the GM pepper exogenous gene were established using 200,000, 20,000, 2,000, 200, and 20 copies of standard plasmid. **B.** Parameters of the regression line through data points are indicated within the plot.

ranging from 20.94 to 34.78 cycles in each standard dilution with standard deviation (SD) of 0.03–0.10. The event-specific PCR assay gene detected mean Ct values, ranging from 24.71 to 38.85 cycles in each standard dilution with SD values of 0.08–0.16. The CV values ranged from 0.14 to 0.46. All the above results indicated that the pCBT was effectively used as a standard plasmid DNA for quantification of GM pepper.

To test the accuracy of real-time PCR, samples of 10, 5, 3, 1, and 0.1% of GM pepper content were prepared by mixing the GM pepper with non-GM pepper genomic DNA. The quantitative results of the 5 samples were 10.01, 4.49, 2.71, 1.08, and 0.07%, respectively. Specifically, the true value for the 0.1% samples was corrected to 0.07%, and the bias of the 0.1% samples was -26.67%. This bias of the 0.1% samples was probably derived from the low exogenous DNA contents in the samples. The calculated mean, bias, and relative standard deviation (CV) at each mixing level are shown in Table 4. Repeated measurement of the standard DNA solution led to CV for all methods in the range of 0.7–20.83%. In addition, the bias ranged from -26.67 to 8.0%. CV and bias in similar ranges have earlier been published in connection with other quantitative GMO detection systems [2, 4, 21]. However, low concentration of the target DNA led to higher variation among the parallel runs than high concentration of that.

Table 3. Repeatability of Ct measurements of replicate standards from 200,000 to 20 copies of standard plasmid DNA.

Target copies	Ct values			Mean Ct	SD[a]	CV(%)[b]
	Mean 1	Mean 2	Mean 3			
			Event-specific assay			
200,000	24.77	24.75	24.62	24.71	0.08	0.32
20,000	28.18	28.30	28.05	28.18	0.13	0.46
2,000	31.38	31.53	31.34	31.42	0.10	0.32
200	35.17	35.23	34.92	35.11	0.16	0.46
20	38.90	38.91	38.75	38.85	0.09	0.23
			CCS assay			
200,000	20.91	20.94	20.97	20.94	0.03	0.14
20,000	24.36	24.45	24.45	24.42	0.05	0.21
2,000	27.77	27.83	27.96	27.85	0.10	0.35
2,00	31.47	31.41	31.50	31.46	0.05	0.15
20	34.79	34.80	34.75	34.78	0.03	0.08

[a]Standard deviation.
[b]Coefficient of variation.

Table 4. Accuracy statistics for quantitative method.

GM line	True value (%)	Coefficient value (Cv)	Accuracy				
			Trueness		SD[b]	Precision	
			Mean (%)	Bias[a] (%)		CV[c] (%)	Below 20 copies[d]
GM pepper	10.0	7.23	10.01	0.07	0.07	0.70	0/3
	5.0		4.49	−10.13	0.18	4.01	0/3
	3.0		2.71	−9.67	0.22	8.16	0/3
	1.0		1.08	8.00	0.18	16.97	0/3
	0.1		0.07	−26.67	0.02	20.83	1/3

[a]Bias=(mean value-true value)/true value×100.
[b]SD, standard deviation.
[c]CV, Relative standard deviation. Coefficient of variation were calculated by dividing the standard deviation by the mean value and are given in perscent.
[d]Below 20 copies; the number of experiments below 20 copies/total number of experiments.

In this study, we validated a *CCS* gene as an endogenous reference gene of pepper. We developed event-specific qualitative and quantitative real-time primers and probes for detection of GM pepper. We also constructed pCBT as a reference molecule that could easily be amplified, and it is more economical and stable than the powder of seed. As both qualitative and quantitative PCR analysis systems, our present method is expected to sensitively monitor the labeling system under the Korea GMO Labeling regulations, and have acceptable levels of accuracy and precision.

Acknowledgment

This work was supported by a grant from the BioGreen 21 Program, Rural Development Administration, Republic of Korea.

REFERENCES

1. *Guideline of Detection Methods of Genetically Modified Foods*; Korea Food and Drug Administration: Seoul, Korea, 2000.
2. Haung, H. Y. and T. M. Pan. 2004. Detection of genetically modified maize MON810 and NK603 by multiplex and real-time polymerase chain reaction methods. *J. Agric. Food Chem.* **52:** 3264–3268.
3. Hernández, m., A. Rio, T. Esteve, S. Prat, and M. Pla. 2001. A rapeseed specific gene, *acetyl-CoA carboxylase*, can be used as a reference for qualitative and real-time quantitative PCR detection of transgenes from mixed food samples. *J. Agric. Food Chem.* **49:** 3622–3627.
4. Huang, C. C. and T. M. Pan. 2005. Event-specific real-time detection and quantification of genetically modified roundup ready soybean. *J. Agric. Food Chem.* **53:** 3833–3839.
5. Huh, J. H., B. C. Kang, S. H. Nahm, S. Kim, K. S. Ha, M. H. Lee, and B. D. Kim. 2001. A candidate gene approach identified phytoene synthase as the locus for mature fruit color in red pepper (*Capsicum* spp.). *Theor. Appl. Genet.* **102:** 524–530.
6. Hupfer C., H. Hotzel, K. Sachse, F. Moreano, and K. H. Engel. 2000. PCR-based quantification of genetically modified Bt maize; single-competitive versus dual-competitive approach. *Eur. Food Res. Technol.* **212:** 95–99.
7. James, C. 2003. Preview: Global status of commercialized transgenic crops. ISAAA Briefs, 30.
8. Jung, S. J., H. J. Kim, and H. Y. Kim. 2005. Quantitative detection of *Salmonella typhimurium* contamination in milk, using real-time PCR. *J. Microbiol. Biotechnol.* **15:** 1353–1358.
9. Kim, J. H., H. S. Song, D. H. Kim, and H. Y. Kim. 2006. Quantification of genetically modified canola GT73 using TaqMan real-time PCR. *J. Microbiol. Biotechnol.* **16:** 1778–1783.
10. Kim, Y. H., S. J. Choi, H. A. Lee, and T. W. Moon. 2006. Quantitation of CP4 5-enolpyruvylshikimate-3-phosphate synthase in soybean by two-dimensional gel electrophoresis. *J. Microbiol. Biotechnol.***16:** 25–31.
11. Kuribara, H., Y. Shindo, T. Matsuoka, K. Takubo, S. Futo, N. Aoki, T. Hirao, H. Akiyama, and Y. Goda. 2002. Novel reference molecules for quantitation of genetically modified maize and soybean. *J. AOAC Int.* **85:** 1077–1089.
12. Lee, S. H., H. J. Park, S. M. Cho, H. K. Chun, D. H. Kim, and T. H. Ryu. 2004. Comparison of major nutrients and mineral contents in genetically modified herbicide-tolerant red pepper and its parental cultivars. *Food Sci. Biotechnol.* **13:** 830–833.
13. Lee, S. H., S. H. Kang, Y. H. Park, D. M. Min, and Y. M. Kim. 2006. Quantitative analysis of two genetically modified maize lines by real-time PCR. *J. Microbiol. Biotechnol.* **16:** 205–211.
14. Matsuoka, T. 2001. GMO labeling and detection methods in Japan. APEC-JIRCAS Joint Symposium and Workshop on Agricultural Biotechnology.
15. Notification No. 1775; Food and Marketing Bureau, Ministry of Agriculture, Forestry and Fisheries of Japan: Tokyo, Japan, 2000.
16. Notification No. 2000-31; Ministry of Agriculture and Forestry of Korea: Seoul, Korea, 2000

17. Permingeat, H. R., M. I. Reggiardo, and R. H. Vallejos. 2002. Detection and quantification of transgenes in grains by multiplex and real-time PCR. *J. Agric. Food Chem.* **50:** 4431–4436.
18. Regulation (EC) No. 1829/2003 of the European Parliament and of the Council.
19. *Testing Manual for Genetically Modified Agricultural Products*; National Agricultural Quality Management Services of Korea: Seoul, Korea, 2002.
20. Weng, H., L. Yang, Z. Liu, J. Ding, A. Pan, and D. Zhang. 2005. Novel reference gene, high-mobility-group protein I/Y, used in qualitative and real-time quantitative polymerase chain reaction detection of transgenic rapeseed cultivars. *J. AOAC Int.* **88:** 577–584.
21. Yang, L., S. Xu, A. Pan, C. Yin, K. Zhang, Z. Wang, Z. Zhou, and D. Zhang. 2005. Event specific qualitative and quantitative polymerase chain reaction detection of genetically modified MON863 maize based on the 5'-transgene integration sequence. *J. Agric. Food Chem.* **53:** 9312–9318.