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Detection of Transgenic Rice Containing *Cry1Ac* Gene Derived from *Bacillus thuringiensis* by PCR

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Abstract Polymerase chain reaction (PCR) method was developed for the specific detection of insect-resistant rice containing *cry1Ac* gene derived from *Bacillus thuringiensis* (*Bt*). Primers were designed from the 35S promoter, NOS terminator, *cry1Ac* gene, and sucrose phosphate synthase (SPS) for general screening of *Bt* rice. By sequencing the PCR products from the two putative kinds of *Bt* rice, we designed a specific primer from the junction region between the *cry1Ac* gene and the NOS terminator that had been inserted into *Bt* rice. The construct-specific primer was employed to amplify a 147 bp product in the two lines of *Bt* rice. No amplified products were observed from the other *Bt* crops with various *Bt* genes introduced. In qualitative PCR analysis, the limit of detection was 0.005 ng from genomic DNA of *Bt* rice. In addition, PCR analysis was performed on 64 kinds of rice presently available in the Korean market, and no *Bt* rice was detected. This method presented in this paper can be used as a highly sensitive and specific detection method of *Bt* rice.

Keywords: *Bacillus thuringiensis*, *Cry1Ac*, *Bt* rice, PCR

Introduction

Bacillus thuringiensis (*Bt*) is a ubiquitous spore-forming bacterium that produces crystalline inclusions consisting of Cry proteins exhibiting a highly specific insecticidal activity (1). *Bt* crystalline inclusions dissolve in the larval midgut, releasing one or more insecticidal Cry proteins from 27 to 140 kDa. The activated toxin interacts with midgut epithelium cells of susceptible insects and generates pores in the cell membrane. The bacterium *Bt* has been used for more than 50 years as a biological insecticide (2, 3) and is considered non-toxic to plants and animals. Recently, *Bt* crops have been altered with *cry* genes modified from *Bt* to express the crystal protein. These *Bt* crops can produce the proteins themselves and protect themselves from insects without any external *Bt* or synthetic pesticide sprays. The recent and significant progress in plant biotechnology and the reservoir of cloned *cry* genes that encode *Bt* δ-endotoxins have combined to enable the expression of this novel insecticidal protein in various crops including rice (4, 5). To date, transgenic crops with increased insect resistance attained by transferring the *Bt cry* gene, such as cotton, maize, and potato, have been commercialized but insect-resistant *Bt* rice has not been commercialized anywhere in the world (6, 7). China, Iran, and Philippine have shown advanced progress in the commercialization of *Bt* rice (8). Especially, China, the world's largest rice producer, is the pioneer of hybrid rice. Chinese researchers have developed several *Bt* rice varieties resistant to major rice pests, such as the lepidopteron insect stem borer, bacteria blight, and leaf rollers (9). *Bt* rice is not yet authorized to be grown from Korea. However, commercialization of *Bt* rice will increase customer concerns of illegal inflows into the Korean market. As a preventive measure against such inflows, the development of monitoring and detection methods of *Bt* rice is urgently needed. Polymerase chain reaction (PCR) methods are considered to be the most common DNA detection method for identifying the presence of *Bt* crops.

In this study, PCR was used for the detection of two lines of *Bt* rice and the method was validated by enzyme digestion of the PCR products.

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Materials and Methods

Samples *Bt* crops containing various *Bt* genes, maize events (Mon 810, Bt 11, Bt 176, and TC 1507) and cotton events (Event 15985 and Event 531) were provided by the Korea Food and Drug Administration (KFDA). Two varieties of *Bt* rice containing *cry1Ac* gene were purchased from local markets in China. Non-*Bt* crops were used as reference and analysis materials were provided by the Rural Development Administration.

DNA extraction Samples were ground in an electric mill. The DNeasy Plant Maxi kit (Qiagen GmbH, Hilden, Germany) was used according to the manufacturer's instructions. A ground sample (0.5 g) was mixed in a 15 mL tube with 5 mL of buffer AP1, and 10 μL of RNase A (100 mg/mL) and then the mixture was incubated for 10 min at 65°C. After incubation, 1.8 mL of buffer AP2 was added to the lysate, mixed, and incubated for 10 min on ice. The lysate was applied to the QIAshredder maxi spin column placed in a 50-mL tube and centrifuged for 5 min at 5,000×g. The flow-through fraction was transferred to a new tube and 1.5 volumes of buffer AP3 were added. After the tube was mixed, it was applied to the DNeasy maxi spin column and centrifuged for 5 min at 5,000×g, after which this column was placed in a new 50-mL tube and washed in buffer AW. After the column was washed,

genomic DNA was eluted with 500 μL of preheated (65 °C) sterile distilled water.

Polymerase chain reaction (PCR) PCR was carried out on a PCR thermal cycler (ASTEC PC808, Fukuoka, Japan). The 25-μL volume reaction mixtures contained 2.5 μL of 10× buffer (Warrington WA1 4SR; Applied Biosystems, UK), 200 nM of dNTP (Applied Biosystems), and 0.8 units of Ampli Taq Gold™ (Applied Biosystems). The concentrations of template DNA and the optimized primer pairs are shown in Table 1. PCR was performed by pre-incubation at 94°C for 5 min, followed by 40 cycles of denaturation at 94°C for 30 sec, annealing at 62°C for 30 sec, extension at 72°C for 30 sec, and terminal elongation at 72°C for 8 min.

Oligonucleotide primers Various primer pairs were designed from genetic information of other *Bt* crops such as soybean, maize, cotton, and canola, to detect and distinguish the promoter and terminator introduced in *Bt* rice with the *cry* gene. To detect the *cry* gene introduced in *Bt* rice, primers were designed on the basis of delta-endotoxin gene derived from *Bt* var. aizawai (10). We used the primers OsSPS-F and OsSPS-R to amplify sucrose phosphate synthase (SPS) as the rice endogenous gene. Oscry1Ac-F and OsNos-R2 were used to amplify the junction region of *cry1Ac* gene and NOS terminator as the specific region in *Bt* rice with *cry1Ac* gene. The sequences of the oligonucleotide primers are shown in Table 1.

Agarose gel electrophoresis PCR products were separated by gel electrophoresis and the inserted DNAs were identified from the length of the amplified DNA fragments. After PCR, 6 μL of each product was loaded onto 3% agarose gel that contained 0.5 μg/mL ethidium bromide. The 100 bp DNA ladder (Takara, Kyoto, Japan) was used as a size standard for amplified DNA fragments.

DNA Sequencings The PCR products amplified in *Bt* rice were extracted using a gel extraction kit (Qiagen). The pMD18-T vector (Takara) was used to clone the amplified DNA fragments and transformed into *Escherichia coli* strain DH5α. The selected *E. coli* containing the recombinant plasmid was cultivated and purified. The sequencing of the amplified DNA fragment in the pMD18-T vector was executed twice with ABI PRISM 3700 DNA analyzer (Perkin Elmer, Norwalk, CT, USA). The DNA sequences were analyzed with the GenBank sample.

Restriction enzyme digestion PCR products of the junction region between the *cry1Ac* gene and the NOS terminator inserted in *Bt* rice were digested with the restriction enzyme *Xho*I. The reaction mixture in 100 μL volume contained 25 μL PCR products purified by QIAquick PCR purification kit (Qiagen), 10 μL of 10× New England Biolabs (NEB, Beverly, MA, USA) reaction buffer 2 (10 mM Tris-HCl, pH 7.9, 10 mM $MgCl_2$, 50 mM NaCl, 1 mM dithiothreitol), 1× BSA (NEB), and 20 units of *Xho*I (NEB). The reaction mixture was incubated at 37°C for 6 hr and was separated by gel electrophoresis.

Results and Discussion

Identification of genes introduced in the two varieties of *Bt* rice Two varieties of *Bt* rice containing *cry1Ac* gene were confirmed by strip-based method. Seed Btk test strip (Strategic Diagnostics Inc., Newark, DE, USA) was used to detect Cry1Ac in rice. The reaction showed a red color on the strip and produced a second line as positive control (data not shown). We performed PCR analysis for a survey of a gene cassette called a 'promoter-gene-terminator', based on these results. To detect the 35S promoter from the cauliflower mosaic virus, and the NOS terminator from *Agrobacterium tumefaciens*, the GMOs were screened by PCR or real time PCR, respectively. The majority of permitted GMOs worldwide contain at least one of these two genetic elements (11, 12).

To confirm the genetic information of promoters, terminators, and *cry1Ac* gene from *Bt* used for the development of the two varieties of *Bt* rice, various primer sets were designed from nucleotide sequences of 35S promoter, NOS terminator, and *Bt* genes which had been introduced into other previously developed *Bt* crops (13, 14). PCR using these primer pairs confirmed the presence of PCR amplicons related to *Bt* rice (Fig. 1). Sequencing of the PCR products indicated that the same *cry1Ac* gene and NOS terminator were introduced into the two varieties of *Bt* rice with different promoters. On the basis of these sequencing data, the complete nucleotide sequences of *cry1Ac* gene introduced into *Bt* rice were identified. The nucleotide and deduced amino acid sequences of the insert

Table 1. Primer pairs for PCR

Primer name	Sequences(5'-3')	Target	Amplicon size (bp)
OsP35S-F	ACGTTCCAACCACGTCTTCA	P35S	95
OsP35S-R	AGGGTCTTGCGAAGGATAGT	P35S	
OsNOS-F	AAGATTGAATCCTGTTGCCG	T-nos	137
OsNOS-R1	ATAATTGCGGGACTCTAATC	T-nos	
OsSPS-F	GATCGGTTCCGCCATTAGCA	SPS	110
OsSPS-R	AACCGAGCGCGATCACTTGC	SPS	
Oscry1Ac-F	GCAGGAGTGATTATCGACAG	Cry1Ac	147
OsNOS-R2	AAGACCGGCAACAGGATTCA	T-nos	

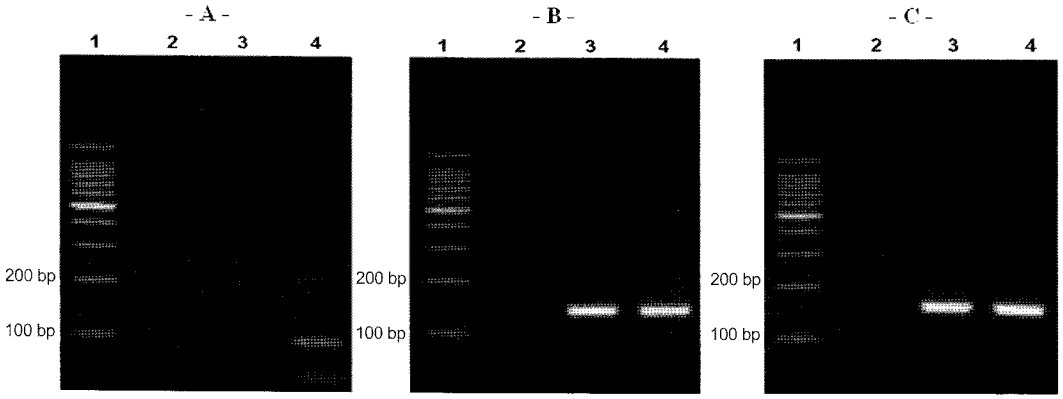

Fig. 1. PCR results of 35S promoter, NOS terminator, and *Bt* gene introduced in two varieties of *Bt* rice. Lane 1: Marker, 100 bp DNA ladder; lane 2: Non-*Bt* rice; lane 3: *Bt* rice A; lane 4: *Bt* rice B. PCR products for (A) 35S promoter, (B) NOS terminator, and (C) the junction region between *cry1Ac* gene and NOS terminator introduced in two varieties of *Bt* rice.

of *cry1Ac* gene are shown in Fig. 2. The total length of nucleotide sequences was 1,827 bp, encoding a putative protein of 609 amino acids.

Qualitative PCR analysis of *Bt* rice Qualitative PCR of *Bt* rice used primer pairs OsSPS-F/OsSPS-R and Oscry1Ac-F/OsNos-R2 for amplification of the rice endogenous and exogenous gene produced 110 bp and 147 bp products, respectively. The SPS gene is a single copy gene per rice haploid genome (15, 16). To test the specificity of the SPS gene, we performed qualitative PCR by using 50 ng of DNA from 12 different plant species: soybean, corn, wheat, buck wheat, barley, pepper, red-bean, mung bean, cucumber, sunflower, canola, and cotton. The PCR results showed no amplification products with any of the species tested except rice (Fig. 3). We performed duplex qualitative PCR to detect the inserted and endogenous genes in the *Bt* rice (Fig. 4). To test the sensitivity of this duplex PCR, we carried out PCR using genomic DNA amounts ranging from 50 ng to 0.5 pg. Qualitative PCR allowed the

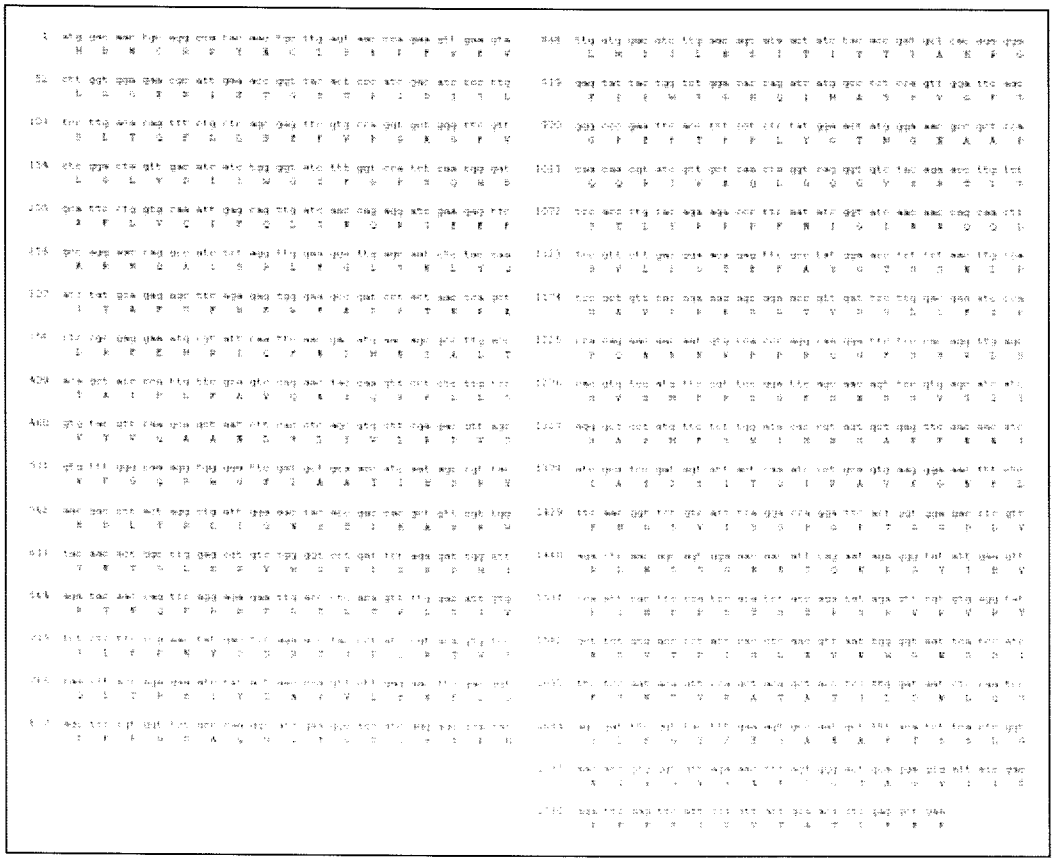

Fig. 2. The nucleotide and deduced amino acid sequences of *cry1Ac*-introduced *Bt* rice developed in China.

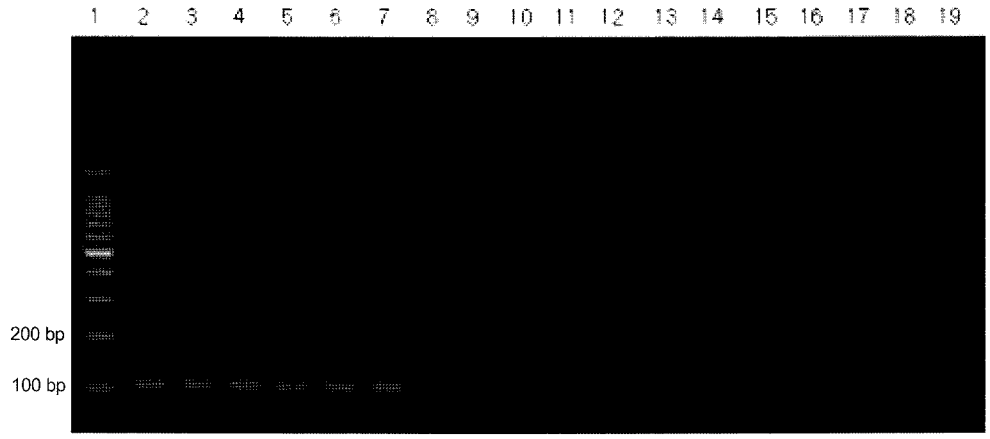

Fig. 3. PCR products amplified from genomic DNAs of 18 different plants. Lane 1: Marker, 100 bp DNA ladder; lanes 2-5: non-*Bt* rice; lanes 6-7: *Bt* rice; lane 8: soybean; lane 9: corn; lane 10: wheat; lane 11: buck wheat; lane 12: barley; lane 13: pepper; lane 14: red-bean; lane 15: mung bean; lane 16: cucumber; lane 17: sunflower; lane 18: canola; lane 19: cotton.

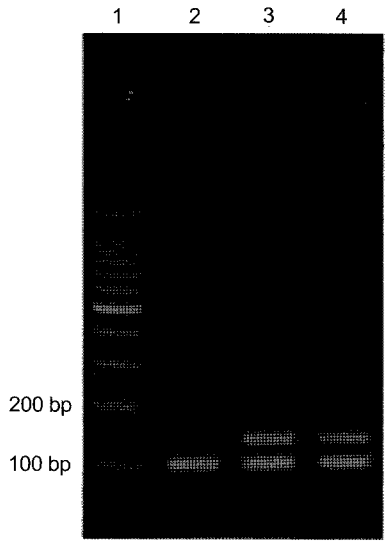

Fig. 4. PCR products amplified from *Bt* rice and non-*Bt* rice with construct-specific detection primer pair. Lane 1: Marker, 100 bp DNA ladder; lane 2: Non-*Bt* rice; lane 3: *Bt* rice A; lane 4: *Bt* rice B.

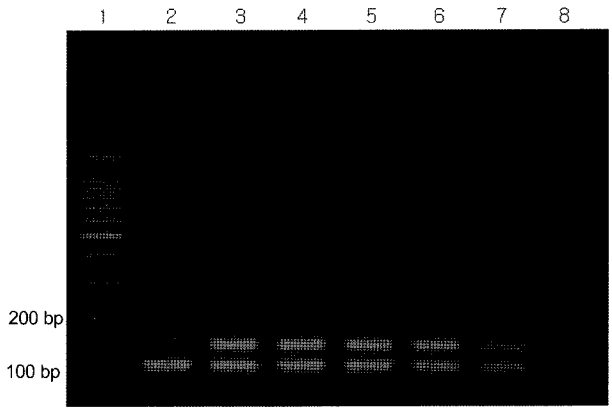

Fig. 5. Sensitivity detection of *Bt* rice using duplex qualitative PCR. Lane 1: Marker, 100 bp DNA ladder; lane 2: Negative control (non-*Bt* rice); lanes 3-8: 50, 5, 0.5, 0.05, 0.005, and 0.0005 ng of *Bt* rice.

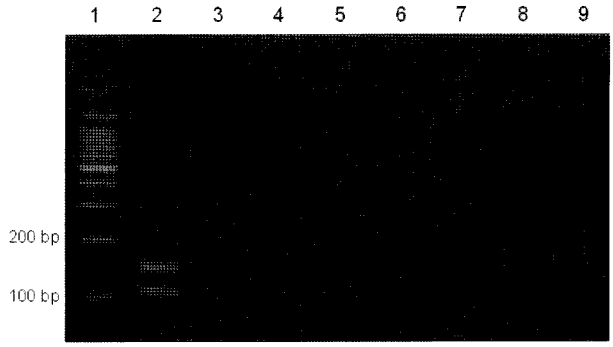

Fig. 6. PCR products amplified from genomic DNAs of *Bt* crops containing *Bt* gene. Lane 1: Marker, 100 bp DNA ladder; lane 2: *Bt* rice; lane 3: Event 15985 (cotton); lane 4: Event 531 (cotton); lane 5: Mon810 (maize); lane 6: Mon863 (maize); lane 7: Bt176 (maize); lane 8: Bt11 (maize); lane 9: TC1507 (maize).

detection of *Bt* rice in 5 pg of rice genomic DNA (Fig. 5). In addition to testing the specificity of duplex qualitative PCR, no amplified products were observed at DNA samples from several *Bt* crops containing various *Bt* genes such as *Bt* maize events (Mon 810, Bt 11, Bt 176, and TC 1507) and *Bt* cotton events (Events 15985 and 531), as the templates (Fig. 6). Therefore, this method can be effectively used for the monitoring of *Bt* rice.

Validation of PCR products by digestion of restriction enzyme Qualitative PCR of *Bt* rice using primer pair Oscry1Ac-F/OsNos-R2 for amplification produced a 147 bp product. Especially, the nucleotide sequence of the 147 bp amplicon, consisting of PCR products of the junction between *cry1Ac* gene and NOS terminator inserted in *Bt* rice, involved restriction enzyme sites such as *Kpn*I, *Sac*I, and *Xho*I. PCR products of *Bt* rice were exactly digested with the restriction enzyme *Xho*I and divided into 98 bp and 49 bp DNA fragments (Fig. 7). This digestion suggests that the accuracy of PCR results is simply evaluated on the basis of restriction enzyme digestion without any

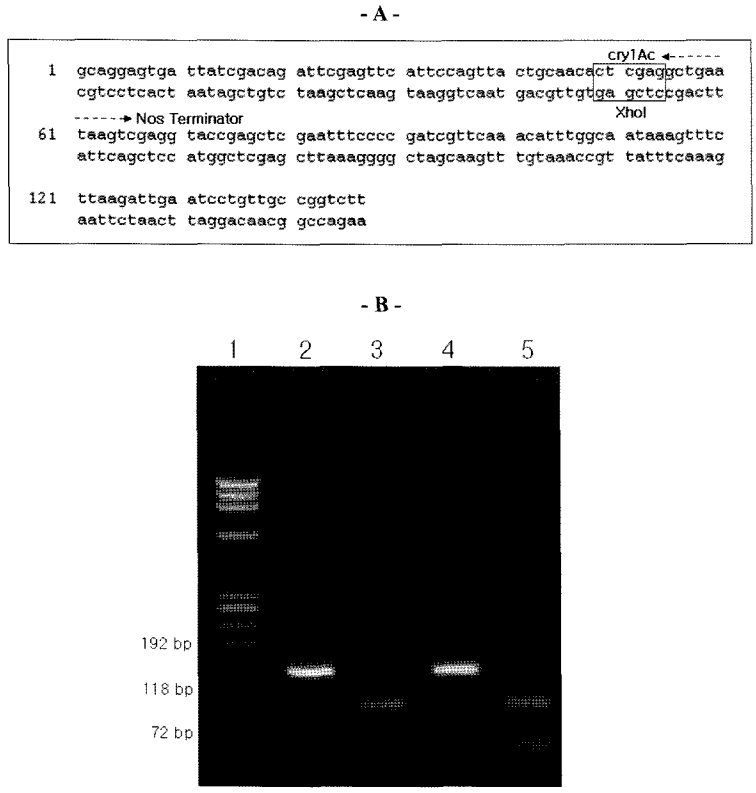

Fig 7. Nucleotide sequences (A) and PCR products (B) of *Bt* rice digested with the restriction enzyme *XhoI*. Lane 1: Marker, ØX174 DNA/*Hae*III; lane 2: PCR products of *Bt* rice A (147 bp); lane 3: 98 bp and 49 bp DNA fragments from 2; lane 4: PCR products of *Bt* rice B (147 bp); lane 5: 98 bp and 49 bp DNA fragments from 4. (A) Nucleotide sequence of 147 bp amplicon, and (B) PCR products of the junction of between *cry1Ac* gene and NOS terminator inserted in *Bt* rice.

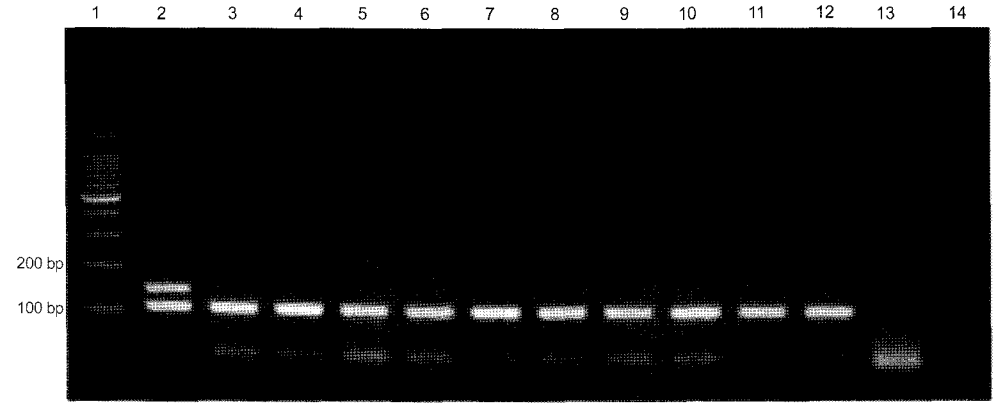

Fig. 8. PCR products amplified from 10 rice varieties (Seoul regional KFDA). Lane l: Marker, 100 bp DNA ladder; lane 2: Positive control (*Bt* rice); lanes 3-12: rice varieties; lane 13: Non-template; lane 14: Non-primers.

sequencing of PCR products.

In this study, we performed the qualitative PCR detection of two transgenic lines. Irrespective of the commercialization of these two transgenic lines, this method will be helpful to monitor *Bt* rice containing Cry1Ac. In the near future, as a preventive measure for the commercialization of *Bt* rice, we will attempt to detect other varieties of *Bt* rice.

GMO monitoring of rice placed in the domestic market Six regional KFDA offices, Seoul, Busan, Gyeong-in, Daegu, Gwangju, and Daejeon, collected 10, 7, 15, 12, 10, and 10, respectively, varieties of rice placed in the domestic market. All 64 samples collected from the six regional KFDA offices underwent PCR analysis and were confirmed to be GMO-free products (Fig. 8).

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