Indicated Detection of Two Unapproved Transgenic Rice Lines Contaminating Vermicelli Products

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We analyzed the DNA fragments extracted from four rice vermicelli products. The *Bacillus thuringiensis* (Bt) rice line, which has a construct similar to the GM Shanyou 63 line, was detected in some vermicelli products by identification of the junction region sequence between rice *Act1* promoter and the *Cry1Ac* gene, and that between *Cry1Ac* and *nos.* In addition, we also detected a different Bt rice line by means of the junction region sequence between the maize ubiquitin promoter and *cry1Ab* gene and that between the cauliflower mosaic virus 35S promoter and the hygromycin phosphotransferase in some vermicelli products. Accordingly, we for the first time have detected the two transgenic Bt rice lines contaminating rice vermicelli samples. Furthermore, we developed a duplex real-time polymerase chain reaction (PCR) method for the simultaneous detection of both Bt rice lines.

KEYWORDS: Genetically modified rice; Bt toxin; detection method; real-time PCR; rice vermicelli; *Bacillus thuringiensis*

INTRODUCTION

In recent years, there has been great progress in food biotechnology, including transgenic crop breeding and genetic modification for food production. In some countries, the acceptance of these genetically modified (GM) foods by consumers is still controversial, and concerns about their safety persist among the public. GM foods have been authorized for food and/or feed by many countries based on their own criteria for safety assessment. In the EU, the authorization and use of GM foods and feed is stipulated by the provisions in regulations (EC) 1829/2003 and (EC) 1830/2003 (*1*, *2*). Japan has also announced a mandatory safety assessment of GM foods and processed foods containing GM ingredients. Since April 1, 2001, any GM food that has not been authorized is prohibited from import or sale in Japan. Therefore, qualitative detection methods of regulated and unauthorized GM foods are required for unauthorized GM food regulation. We previously reported qualitative detection methods for GM maize, GM potatoes (NewLeaf Plus, NewLeaf Y), and GM papayas (Line 55-1 or its derivatives), including qualitative polymerase chain reaction (PCR) methods and a histochemical assay (*3*−*10*).

The Bt crops are GM crops in which *cry* genes derived from *Bacillus thuringiensis* (Bt) conjugated with a suitable plant expression promoter and terminator are transformed, expressing the Bt toxin protein to confer tolerance against insects. To date, Bt crops of cotton (*11*), maize (*12*), and potatoes (*13*) that have insect resistance have been commercialized in some countries, including Japan (*14*, *15*). However, no developed Bt rice has yet been authorization for food use in the European Union, Korea, and Japan (*16*−*18*).

In the present study, we analyzed DNA fragments extracted from four rice vermicelli products and detected two lines of unauthorized Bt rice harboring the Bt toxin *cry* gene, one of which has a construct similar to the previously reported GM Shanyou 63 line (*19*−*21*) and the other is an unknown Bt rice line, which has a construct similar to the Kemingdao. Furthermore, we developed a detection system to monitor these Bt rice lines using a real-time PCR method.

MATERIALS AND METHODS

Samples. One rice vermicelli sample (G) was kindly provided by Greenpeace International. Three rice vermicelli samples (A, B, and C) (imported products from China), which were suspected to be contami-

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nated with Bt rice on the basis of testing at a quarantine inspection center, were obtained through the Ministry of Health, Labor, and Welfare (MHLW) of Japan. The rice vermicelli samples (D) imported from Thailand were commercially purchased in Tokyo as the negative control.

Extraction and Purification of Genomic DNA. The samples were ground with an electric mill. DNA extraction and purification were carried out by use of the Nippon Gene GM quicker 2 kit (Nippon Gene, Toyama, Japan) according to the manufacturer's manual with the following modification: The ground samples (500 mg) were suspended in 2.1 mL of GE1 buffer, 60 μL of proteinase K (20 mg/mL), 6 μL of α-amylase, and 30 μL of RNase A (100 mg/mL) by use of a vortex mixer for 30 s and then heated at 65 °C for 30 min. A 255-μL aliquot of GE2-K buffer was added to the mixture, which was sufficiently mixed by use of a vortex mixer, followed by standing on ice for 10 min. After centrifugation at 6000*g* for 15 min at 4 °C, the collected supernatant was transferred into a fresh tube (LF tube; Prescribe Genomics Co., Tsukuba, Japan), and the mixture was centrifuged again at 13000*g* or above at 4 °C for 5 min. To 1 mL of the supernatant placed in a new LF tube were added 375 μL each of GE3 buffer and 2-propanol, and the solution was then gently mixed by being shaken 10−12 times. The mixed solution was applied onto a spin column included in the kit and centrifuged at 13 000*g* and 4 °C for 30 s to discard the eluate. This procedure was repeated until the entire eluate was loaded. The spin column was washed with 650 μL of GW buffer by centrifugation at 13 000*g* and 4 °C for 1 min. The column was transferred to a new tube, 50 μL of TE buffer was added, and the mixture was allowed to stand for 3 min at room temperature. Finally, the tube was inserted into the column and centrifuged at 13 000*g* and 4 °C for 1 min, and the eluate was then used as the DNA sample solution in the following experiments.

Polymerase Chain Reaction. The PCR reaction mixture (25 μL) in the tubes consisted of 2.5 μL of PCR buffer II (Applied Biosystems, CA), 0.16 mM dNTP, (Applied Biosystems), 1.5 mmol/L $MgCl_2$, 1.2 μmol/L 5′ and 3′ primers, and 0.8 unit of AmpliTaq Gold (Applied Biosystems). PCR was performed by preincubation at 95 °C for 10 min, followed by 45 cycles of denaturation at 95 °C for 30 s, annealing at 56 °C for 30 s, extension at 72 °C for 30 s, and terminal elongation at 72 °C for 7 min by use of the GeneAmp PCR System 9700 (Applied Biosystems).

To determine the nucleotide sequence of the transgenic construct harbored in the Bt rice, DNA fragments were amplified by PCR with a primer set of actACF3 (5′-GGG GAA TGG GGC TCT CGG ATG TAG-3′) and actACR3 (5′-GGA GAT GTC GAT GGG AGT GTA ACC-3′) for the junction region between the rice actin 1 (*Act1*) promoter sequence and the *cryIAb/cryIAc* fusion gene; a primer set of Oscry1Ac-F (5′-GCA GGA GTG ATT ATC GAC AG-3′) and OsNOS-R2 (5′-AAG ACC GGC AAC AGG ATT CA-3′) for the junction between the *cryIAb/cryIAc* fusion gene and the nopaline synthase terminator (*nos*) sequence; a primer set of Pubi-5 (5′-ATG TTG ATG CGG GTT TT-3′) and Cry1Ab-1 (5′- TCG CGG AGA GCT GGG TTA GTA-3′) for the junction region between the maize ubiquitin promoter (*Pubi*) sequence and the synthetic *cry1Ab* gene sequence; and 35S-HPH-1F (5′-ACG TTC CAA CCA CGT CTT CA-3′) and 35S-HPH-3R (5′-CAA AGT GCC GAT AAA CAT AAC GA-3′) for the selection marker genes, by use of DNA extracts prepared from rice vermicelli products as templates.

After PCR amplification, the amplified products were analyzed by agarose gel electrophoresis according to previous reports (*7*−*10*, *18*).

DNA Sequencing. The DNA fragments amplified from rice vermicelli products were extracted and purified by use of a QIAquick PCR purification kit (Qiagen GmbH, Hilden, Germany), and both strands of the DNA fragment were directly sequenced on an ABI Prism 3700 DNA analyzer (Applied Biosystems). In the case of two different amplified DNA fragments mixed together, after purification of the DNA fragments by use of a QIAquick PCR purification kit, the DNA fragments were treated with T4 polynucleotide kinase followed by T4 DNA polymerase. Each DNA fragment was then ligated with the pUC118/*Hin*cII BAP vector (TaKaRa Bio Inc., Shiga, Japan) and introduced into *Escherichia coli* DH5α. The plasmids containing the inserts were subjected to analysis to determine the nucleotide sequences on the ABI Prism 3700 DNA analyzer (Applied Biosystems). The nucleotide sequences were analyzed with the Lasergene v. 7.0 software (DNASTAR Inc.).

Table 1. Primer and Probe Sequences of Real-Time PCR Systems Developed in the Present Study

name	oligonucleotide sequence (5′–3′)	final concn (nM)
For Simultaneous Detection of Bt Rice Line (like GM63-Taq) and the Other Bt Rice Line (like NGMr-Taq)		
T51-SF	GCAGGAGTGATTATCGACAGTTC	750
OsNOS-R2	AAGACCGGCAACAGGATTCA	750
GM63-Taq	FAM-AATAAGTCGAGGTACCGAGCTCGAATTTCCC-TAMRA	150
NGMr-Taq	VIC-AATGAGAATTCGGTACCCCGACCTGCA-TAMRA	150
For Detection of Taxon-Specific Rice Reference Genes		
SPSF	TTGCGCCTGAACGGATAT	750
SPSR	CGGTTGATCTTTTCGGGATG	750
SPS-Taq	FAM-GACGCACGGACGACGGCTCGGA-TAMRA	300

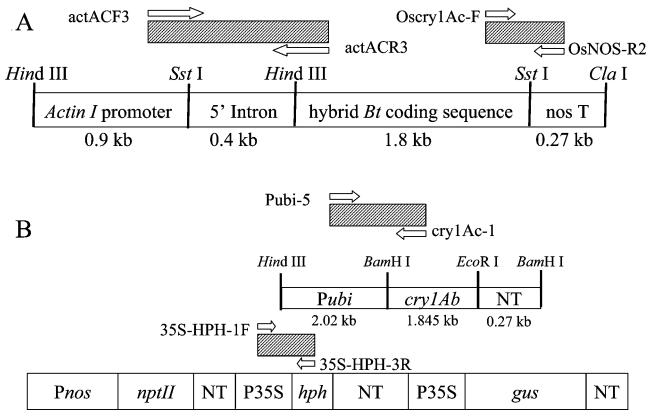

Figure 1. Diagrams of construct pFHBT1 (**A**) inserted in the GM Shanyou 63 line and construct pKUB (**B**) inserted in the Kemingdao line. The primers used in this study to generate PCR products suitable for DNA sequencing are indicated by arrows. Location of sequences taken for the alignments shown in **Figures 2**–**4** are indicated by the hatched boxes.

Real-Time PCR. Real-time PCR was performed on an ABI Prism 7900 instrument (Applied Biosystems). All reactions were run as duplicates in 96-well plates. PCR reaction mixtures were placed in a 25-μL final volume containing 50 ng of the template DNA, 12.5 μL of the universal master mix (Applied Biosystems), 0.75 μM primer pair, and two kinds of probes (150 nM) designed by the Primer Express 2.0 software (Applied Biosystems) (**Table 1**). The reaction conditions included the initiation step for 10 min at 95 °C, followed by 45 cycles of 20s at 95 °C and 1 min at 60 °C. The primer and probe sequences for the detection of the sucrose phosphate synthase (*SPS*) gene, a taxon-specific rice reference gene, were used as previously reported (*22*).

RESULTS

Identification of GM Rice Lines Contaminating Rice Vermicelli. After Greenpeace announced the contamination of rice vermicelli products by Bt rice, we began to establish a DNA extraction method from the rice vermicelli products and analyzed the nucleotide sequence of the transgene for the predicted GM Shanyou 63 line, using the rice vermicelli G sample provided by Greenpeace.

According to previously published reports, we examined the construct of the transgenic DNA sequence for the GM Shanyou 63 line as shown in **Figure 1A**. Some researchers have already reported the junction region in the construct of the transgenic DNA sequences of the GM Shanyou 63 line (*16*, *17*). We

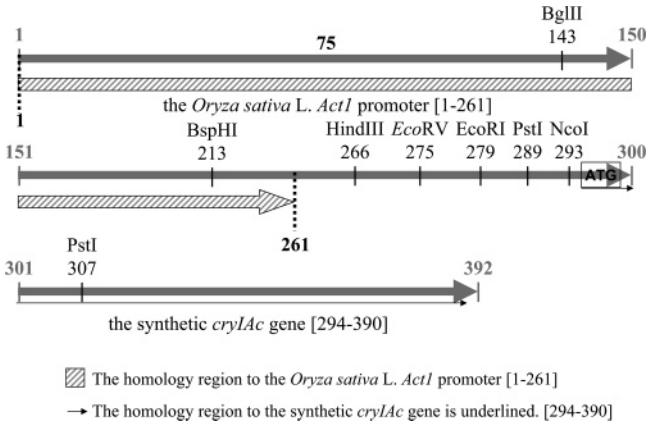

Figure 2. Nucleotide sequence alignments of PCR products generated with genomic DNAs extracted from rice vermicelli G and A samples for the junction region between the rice *Act1* promoter sequence and the *cry1Ac* gene. Restriction enzyme sites are marked.

therefore attempted to amplify some construct-specific fragments from the rice vermicelli sample DNAs using various primer pairs. Several different primer combinations targeting the presumed transgenic construct inserted in the GM Shanyou 63 were used to generate PCR products for direct DNA sequencing in the rice vermicelli samples. The nucleotide sequences of these products were analyzed by use of BLASTN. The two regions selected for alignments of the transgenic sequences with the identical GenBank sequences are shown in **Figures 2** and **3**. For the DNA fragments obtained from samples G, A, and C by use of the primer set actACF3/actACR3, as shown in **Figure 2**, the 5′ sequence part [1−261] of the amplified fragment showed 100% homology to the rice *Act1* promoter sequence reported previously (*23*, *24*). The sequence was followed by a 32-bp fragment containing multiple restriction enzyme sites. The next 96-bp region [294−390] showed 100% homology to a synthetic *cry1Ac* gene (GenBank accession number Y09787). The sequences consistent with the parts of plasmid pFHBT1 were used for the production of the GM Shanyou 63 line (*17*). In addition, we attempted to generate PCR products in the junction region between the *cry1Ab/cry1Ac* fusion gene and *nos* from the genomic DNAs extracted in all the rice vermicelli samples, using the Oscry1Ac-F/OsNOS-R2 primer pair designed by Kim et al. (*16*). The approximate expected stretched PCR products can be detected in all the rice vermicelli samples. In the PCR products from the rice vermicelli G and A samples, the direct DNA sequence was consistent with that previously reported for pFHBT1 of the GM Shanyou 63 line (*16*, *17*). However, in the PCR product sequences cloned from the rice vermicelli B sample, the junction region sequences between the *cry1Ab/cry1Ac* fusion gene and *nos* were different from that of GM Shanyou 63. These sequences are shown in **Figure 3**. Both the 5′ sequence part [1−61] and the 3′ sequence part [91−147] of the amplified fragment showed 100% homology to those previously reported for pFHBT1. The middle part [62−90] of the major amplified fragments cloned from the rice vermicelli B sample PCR products (142 bp) is slightly shorter than that (147 bp) of the GM Shanyou 63. As shown in **Figure 3**, the sites and varieties of the restriction enzyme digestion in the middle part [62−90] of the amplified fragment are different from that of the GM Shanyou 63. In rice vermicelli C sample, we obtained 91 clones from the amplified products in terms of the junction sequences between the *cry1Ab/cry1Ac* fusion gene and *nos*. Fourteen of these clones are the sequence derived from pFHBT1, and 77 of these clones are predicted to be the sequence derived from another unknown Bt rice line construct. These results suggest that the rice vermicelli C sample contains both Bt rice lines, one similar to the GM Shanyou 63 line and the other an unknown Bt rice line.

Since the unknown Bt rice line could be predicted to be the Kemingdao line, which has the *Pubi*-driven *cry1Ab* gene (**Figure 1B**) (*25*), we attempted to generate PCR products in the junction region between *Pubi* and *cry1Ab* from the genomic DNAs extracted from the rice vermicelli B samples, using the designed primer pair combinations. As shown in **Figure 4**, the 5′ sequence portion [7−451] of the amplified fragment shows 98% homology to the maize polyubiquitin gene sequence (GenBank accession number S94464). The sequence is followed by a 21-bp fragment containing multiple restriction enzyme sites. The next 365 bp region [471−836] shows 79% homology to a *B. thuringiensis cry1Ab* gene for insecticidal crystal protein gene (GenBank accession number X54939). Furthermore, as shown in **Figure 5**, the 5′ sequence part [17−136] of the amplified fragment shows 99% homology to the cauliflower mosaic virus 35S promoter (*CaMV*) sequence (GenBank accession number S51061). In addition, the 3′ sequence part [162−376] of the amplified fragment shows 100% homology to the plasmid pJR225 *E. coli* hygromycin phosphotransferase (*hph*) gene (GenBank accession number K01193). These results suggest that the junction region sequence between the *Pubi* and *cry1Ab* gene and the junction region between the *CaMV* and *hph* gene could be detected by use of the designed primer pair combinations in the rice vermicelli B and C samples, not in the rice vermicelli G and A samples.

Duplex Construct-Specific Detection of Two Unauthorized Bt Rice Lines in Rice Vermicelli. To simultaneously detect the two different Bt rice lines contaminating the rice vermicelli samples, we attempted to develop a duplex real-time PCR method. On the basis of the two-line sequence data identified from the rice vermicelli A and B samples, two probes were designed for the specific sequence of the Bt rice lines. We first confirmed that the amplification curves of SPS-Taq labeled with the FAM dye were detected in all the rice vermicelli product DNAs for evaluation of the quality of the extracted genomic DNAs. In addition, to discriminate both lines, we designed a probe for the detection of the similar construct rice line of GM Shanyou 63 labeled with the FAM and TAMRA dyes (GM63-Taq) and a probe for the detection of the unknown Bt rice line labeled with VIC and TAMRA (NGM-Taq). Consequently, one primer pair and two probes for both line detections were considered to be mixed in one reaction tube to simultaneously detect both Bt lines in the genomic DNA extracted from the rice vermicelli samples. As shown in **Figure 6**, the amplification curves of GM63-Taq labeled with the FAM dye and NGM-Taq labeled with the VIC dye were observed in rice vermicelli products A and B, respectively. The amplification curves of both GM63-Taq and NGM-Taq were observed in rice vermicelli product C, presumably contaminated with both Bt rice lines. These results suggest that rice vermicelli G and A samples primarily contain the similar construct line of GM Shanyou 63;rice vermicelli B sample primarily contains the unknown Bt rice, which has a construct similar to that of the Kemingdao line; and rice vermicelli C sample contains both Bt rice lines.

DISCUSSION

We have for the first time clarified that the unauthorized Bt rice line, which has a construct similar to that of GM Shanyou 63, is contaminating rice vermicelli samples, based on detection of the junction region sequence between rice *Act1* promoter and

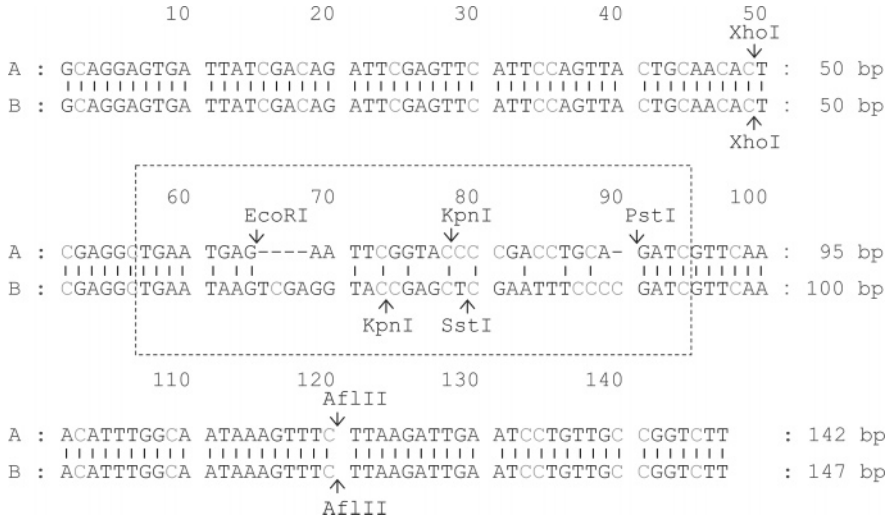

Figure 3. Nucleotide sequence alignments of PCR products generated with genomic DNAs extracted from rice vermicelli G, A, and B samples for the junction region between the *cry1Ab/cryAc* fusion gene and the *nos* sequence. *Xho*I, *Eco*RI, *Kpn*I, *Pst*I, and *Sst*I sites are denoted by arrows.

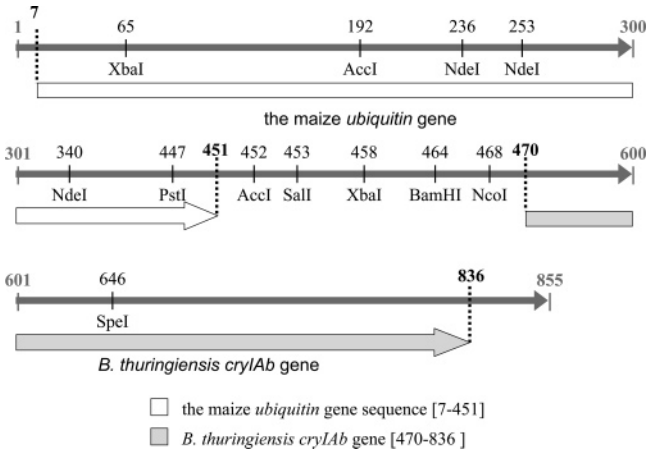

Figure 4. Nucleotide sequence alignments of PCR products generated with genomic DNAs extracted from rice vermicelli B and C samples for the junction region between the *Pubi* and *cry1Ab* genes. Restriction enzyme sites are marked.

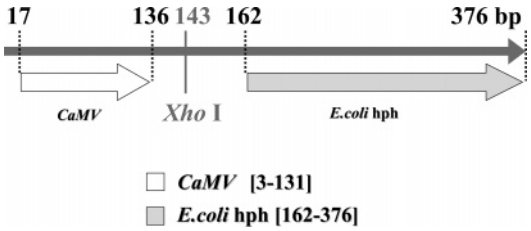

Figure 5. Nucleotide sequence alignments of PCR products generated with genomic DNAs extracted from rice vermicelli B and C samples for the junction region between the *CaMV* sequence and the *hph* gene. The *Xho*I site at position 143 is marked.

Cry1Ac gene and that between *Cry1Ac* and *nos*. We also detected a junction region sequence between *cry* and *nos* different from that of the GM Shanyou 63 on the PCR fragments in the rice vermicelli B and C samples, as shown in **Figure 3**. The presence of the different junction region indicated that the detected Bt rice line had been developed by use of a different vector, not the pFHBT that is used in GM Shanyou 63. In other words, rice vermicelli B and C samples contained another unknown, but similar, Bt rice line, not GM Shanyou 63. We also suggested that the unknown Bt rice line contaminating the rice vermicelli B and C samples may have the construct of the *Pubi*-driven *cry1Ab* gene due to the detection of the junction region between the *Pubi* and *cry1Ab* gene in them. Furthermore, the junction region between *CaMV* and the selectable marker *hph* could be detected in B and C samplea but not in G and A samples. These results suggest that the *CaMV*−*hph* sequences would be derived from the unknown Bt rice line contaminating rice vermicelli B and C samples, because the construct in the pFHBT for the GM Shanyou 63 line should not contain *CaMV*−*hph*, and removal of the sequence by segregation has been reported for the parental elite restorer rice Minghui 63 (**Figure 1B**). The presence of the DNA sequence provides the evidence that the unknown Bt rice line, which has a construct similar to that of the Kemingdao line, might be contaminating the rice vermicelli B and C samples because the *Pubi*-driven *cry1Ab* gene and the 35S promoter-driven *hph* marker gene have been used in plasmid vector pKUB for production of the Kemingdao line (**Figure 1B**) (*25*).

In China, some GM rice varieties have already been developed and tested in the field and environmental trials (*15*, *26*). The Bt rice expresseing the Bt toxin has been developed in China and approved for environmental release trials, and at least two Bt rice lines, GM Shanyou 63 and Kemingdao, entered preproduction trails in 2001 (*19*, *27*). Contamination by the GM Shanyou 63 line has been detected in Chinese rice in the European Union and Korea. However, the Kemingdao line has not yet been detected in rice and rice products. Our findings suggest that the unknown Bt rice line found in this study in rice vermicelli sample might have a construct similar to the Kemingdao line (*25*).

Furthermore, we developed a duplex real-time PCR method for the simultaneous and rapid qualitative detection of both unauthorized Bt rice lines. German researchers have already developed a real-time PCR method for detection of the GM Shanyou 63 line (*17*). They designed a reverse primer on a multiple cloning site in the junction between the *cry1Ab/cry1Ac* gene and *nos*. Therefore, the unknown Bt rice line we found in this study presumably may not be detectable by their method because a reverse primer cannot be annealed, although the GM Shanyou 63 line can be sensitively detected.

However, we cannot identify and estimate the detection limit of the real-time PCR method for both Bt rice lines because we do not have any authentic reference material for the Bt rice lines, and because transgenic sequences in processed food products usually can be degraded. Further studies are required to examine the feasibility of detecting lower levels of the two Bt lines by

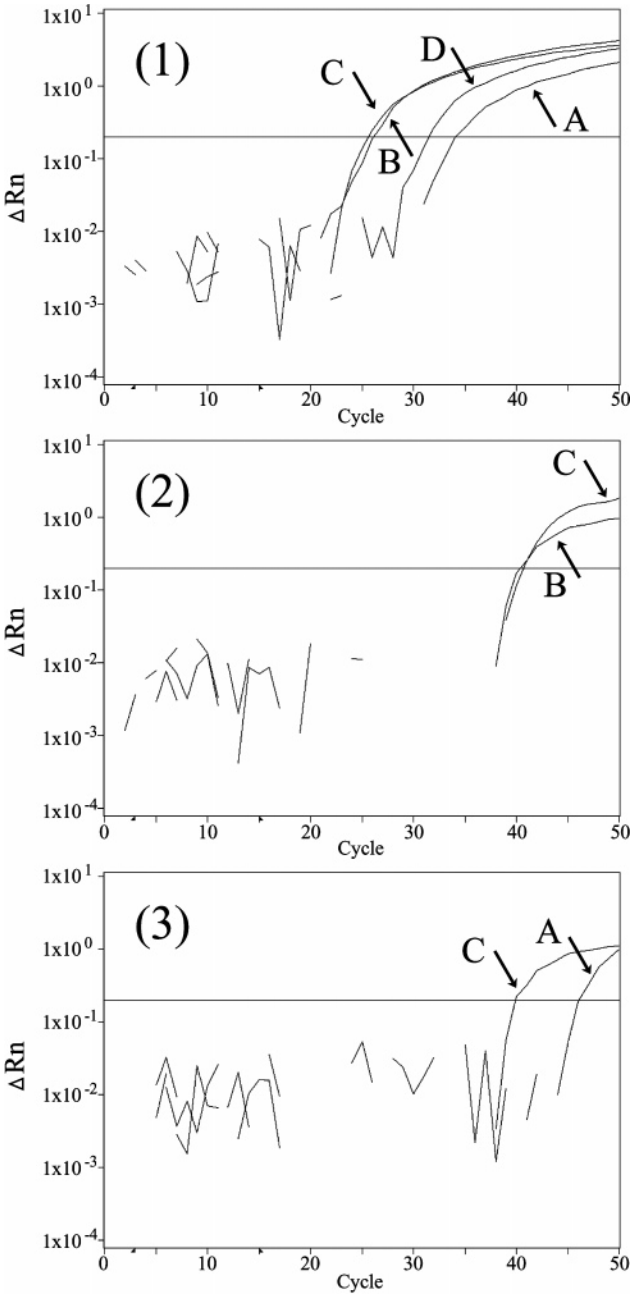

Figure 6. Amplification curves of construct-specific sequences of the two Bt rice lines using the developed real-time PCR method. (**Panel 1**) SPS gene detection in all rice vermicelli samples. (**Panel 2**) Specific detection for unknown Bt rice, which has a construct similar to that of Kemingdao in the rice vermicelli samples B and C. (**Panel 3**) Specific detection for GM unknown Bt rice, which has a construct similar to Shanyou 63 specific detection in the rice vermicelli sample. (**A**) Rice vermicelli sample A containing the Bt rice line, which has a construct similar to that of the GM Shanyou 63 line; (**B**) rice vermicelli sample B containing an unknown Bt rice line, which is similar to the Kemingdao; (**C**) rice vermicelli sample C, containing the two Bt rice lines; (**D**) rice vermicelli sample D, containing non-GM rice.

use of reference materials and to extend the applications of the developed method to more complex processed food products.

In conclusion, we detected, for the first time, two transgenic Bt rice lines contaminating rice vermicelli samples. One was the Bt rice line, which has a construct similar to that of the GM Shanyou 63 line, and the other was an unknown Bt rice line, which is similar to the Kemingdao line. In addition, we developed a duplex real-time PCR method for the simultaneous detection of both Bt rice lines. We consider this developed method to be a reasonable assay for monitoring Bt rice in processed food products.

ABBREVIATIONS USED

GM, genetically modified; Bt, *Bacillus thuringiensis*; *nos*, nopaline synthase terminator; *Pubi*, maize ubiquitin promoter; *CaMV*, cauliflower mosaic virus 35S promoter; *SPS*, sucrose phosphate synthase; *hph*, hygromycin phosphotransferase.

ACKNOWLEDGMENT

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