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Multiplex PCR Detection of the GT73, MS8xRF3, and T45 Varieties of GM Canola

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Abstract A multiplex polymerase chain reaction (PCR) method was developed to simultaneously detect three varieties of genetically modified (GM) canola. The construct-specific primers were used to distinguish the following three varieties of GM canola; GT73, MS8xRF3, and T45, using multiplex PCR. The *FatA* (fatty acyl-ACP thioesterase) gene was used as an endogenous canola reference gene in the PCR detection. The primer pair Canendo-F/R containing a 105 bp amplicon was used to amplify the *FatA* gene and no amplified product was observed in any of the 15 different plants used as templates. The GT73-KHUF1/R1 primer recognized the 3'-flanking region of GT73, resulting in an amplicon of 125 bp. The Barstar-F1/MS8xRF3-R primer recognized the junction region of barstar and the NOS terminator introduced into MS8xRF3, resulting in a 162 bp amplicon, and the T45-F2/R2 primer recognized the junction region of PAT and the 35S terminator introduced into T45, resulting in an amplicon of 186 bp. This multiplex PCR allowed for the detection of construct-specific targets in a genomic DNA mixture of up to 1% GM canola containing GT73, MS8xRF3, and T45.

Keywords: multiplex PCR, GM canola, GT73, MS8xRF3, T45

Introduction

Oilseed rape (*Brassica napus* L.) is an annual plant that belongs to the genus *Brassica* in the family *Brassicaceae* and is grown as a commercial crop in 53 countries with a combined harvest of over 43 million metric tons (1). Canola is the trademarked name for low erucic acid and low glucosinolate rapeseed. Canola seed contains lower concentrations of the natural rapeseed toxins erucic acid and glucosinolate (2), in comparison to wild rapeseed. Canola is commonly used for the production of oil and its byproduct, canola grist, is often incorporated into feed products. According to the genetically modified organism (GMO) database of Agbios (Agriculture and Biotechnology Strategies Inc., Merrickville, ON, Canada), 15 varieties of GM canola were developed and approved in several countries. As of September 2006, the Korea Food and Drug Administration (KFDA) has approved six of these varieties, including GT73, MS8xRF3, T45, MS1xRF1, MS1xRF2, and Topas19/2. The GT73 variety has been genetically engineered to be tolerant to the herbicide glyphosate (3). Two genes, CP4 EPSPS (5-enolpyruvylshikimate-3-phosphate synthase) from *Agrobacterium tumefaciens* strain CP4 and the goxv247 gene (isolated from *Ochrobactrum anthropi* which codes for a modified version of the glyphosate oxidase enzyme) confer this herbicide tolerance in GT73. The MS and RF varieties are pollination-controlled, parental breeding varieties used in hybrid production. MS8 contains the bacteria-derived gene barnase and RF3 expresses the bacteria-derived gene barstar. These varieties are tolerant to phosphinothricin (glufosinate-ammonium). T45 is an open pollinated canola variety known commercially as LibertyLink® canola which is also tolerant to glufosinate-ammonium (4). Korea has implemented mandatory labeling requirements for GM foods that contain more than 3% GM crops; therefore, the development of methods for GMO detection are continuously required to monitor various GM crops.

Polymerase chain reaction (PCR) has been successfully employed to screen for the presence of GMOs (5-9). A PCR method that can distinguish one particular variety from all other varieties using primers is described as being 'gene-specific', 'construct-specific', or 'event-specific'. An event-specific PCR system was designed based on the junction between the transgenic insert and the host DNA and was regarded as the most specific approach.

The simultaneous amplification of multiple targets has routinely been performed using multiplex PCR and a useful strategy involving the optimization of multiplex PCR primer mixtures has been developed (10-13). Multiplex PCR detection methods that use gene-specific primer pairs to screen for some GM canola varieties have also been previously reported (14, 15).

In this study, construct-specific and event-specific primers were developed to simultaneously distinguish the 3 varieties of GM canola, GT73, MS8xRF3, and T45.

Materials and Methods

Canola samples Three varieties of GM canola; herbicide-tolerant GT73 from Monsanto (St. Louis, MO, USA), MS8xRF3, and T45 from Bayer Crop Science (Monheim am Rhein, Germany), as well as non-GM canola were provided by the KFDA. The canola samples were collected from oil manufacturing companies in Korea. A schematic diagram of the 3 varieties of GM canola is shown in Fig. 1.

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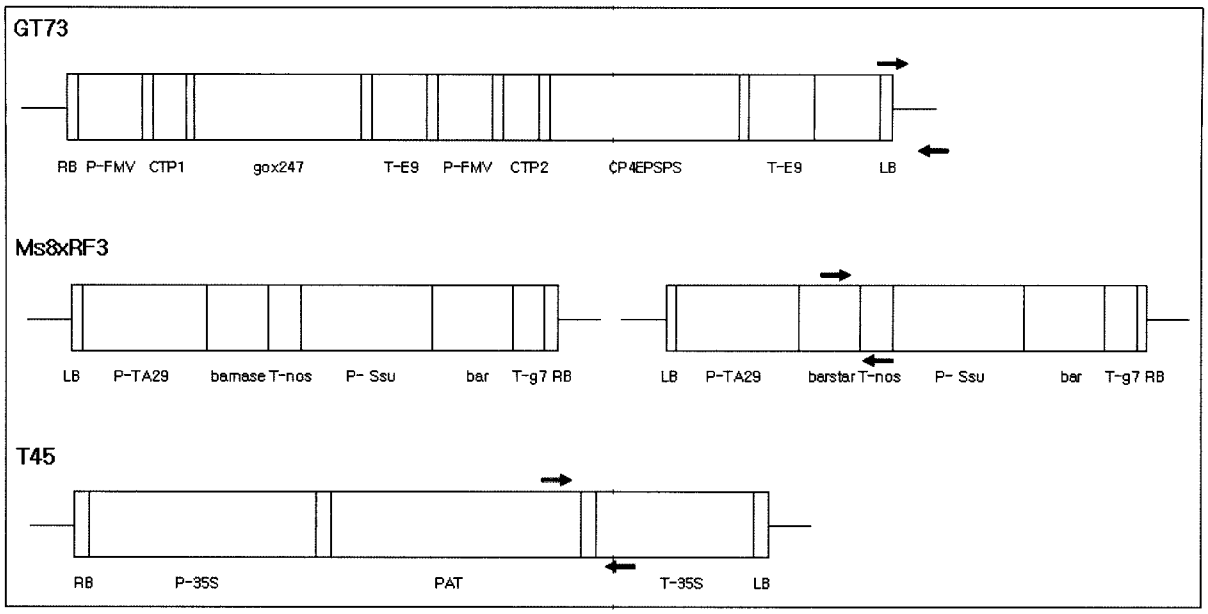

Fig. 1. Schematic diagram of 3 varieties of GM canola. The primers used for the amplification are indicated by arrows.

DNA extraction The 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 (1 g) was mixed in a 15 mL tube with 5 mL of buffer AP1 and 10 μL of RNase A (100 mg/mL). The mixture was then incubated for 15 min at 65 °C. After incubation, 1.8 mL of buffer AP2 was added to the lysate, which was then mixed and incubated on ice for 10 min. The lysate was applied to a QIAshredder mini spin column, placed in a 50 mL tube, and centrifuged for 5 min at 4,500×g. The flow-through fraction was transferred to a new tube and 1.5 volumes of buffer AP3 were added to the tube. The tube was then mixed, applied to the DNeasy maxi spin column and centrifuged for 5 min at 4,500×g. The column was then placed in a new 50 mL tube and washed with buffer AW. After the column was washed, the genomic DNA was eluted with 500 μL of preheated (65°C) sterile distilled water.

Polymerase chain reaction (PCR) PCR was carried out on a Mastercycler (Eppendorf, Hamburg, Germany). The reaction mixture in 25 μL volumes contained 2.5 μL of 10× buffer (Applied Biosystems, Foster City, CA, USA), 200 μM of each dNTP (Applied Biosystems), 1.5 mM of $MgCl_2$, 0.8 unit of Ampli Gold *Taq* DNA polymerase (Applied Biosystems) and the appropriate concentrations of the template DNA. The optimized primer pairs are shown in Table 1. The PCR conditions were pre-incubation at 94°C for 5 min, 40 cycles of denaturation at 94°C for 30 sec, annealing at 60°C for 30 sec, extension at 72°C for 30 sec and terminal elongation at 72°C for 8 min.

Oligonucleotide primers Four sets of primer pairs were designed for multiplex PCR to detect and distinguish 3 varieties of GM canola. The inserted DNA fragments and amplified regions for GT73, MS8RF3, T45, and an endogenous gene are shown in Fig. 1. The sequences of the oligonucleotide primers are shown in Table 1.

Agarose gel electrophoresis The PCR products were separated by gel electrophoresis and the sizes of the inserted DNA fragments were compared to the sizes of the amplified DNA fragments. After PCR, 10 μL of each product was loaded on a 3.5% agarose gel containing 0.5

Table 1. Primer pairs for multiplex PCR

GM crops	Primer name	Sequences (5'-3')	Target	Primer concentration (μM)	Amplicon size (bp)
FatA (endogenous gene)	Canendo-F	AAGAGATCTGAAGCATGGCG	FatA	2	105
	Canendo-R	GTCGATAATTTCACCTGCCG			
GT73	GT73-KHUF1	ATAACGCTGCGGACATCTAC	3-flanking region	5.5	125
	GT73-KHUR1	GGATGATCTTCATGTCCGGG			
MS8xRF3	Barstar-F1	CTGCGACATCACCATCATAC	Barstar	1.8	162
	MS8xRF3-R	ATCATCGCAAGACCGGCAAC	T-NOS		
T45	T45-F2	AAGCATGGTGGATGGCATGA	PAT	3	186
	T45-R2	CCCTTATCTGGGAACTACTCAC	T-35S		

μg/mL ethidium bromide. A 100 bp DNA ladder (Takara, Kyoto, Japan) was used as the size standard for the amplified DNA fragments.

DNA sequencing The PCR products of GT73, MS8xRF3, and T45 were extracted from the agarose gel using a gel extraction kit (Qiagen). The pMD18-T vector (Takara) was used to clone the amplified DNA fragments and was transformed into *Escherichia coli* strain DH5α. The selected *E. coli* containing the recombinant plasmid was grown and the plasmid was purified. The sequencing of the amplified DNA fragment in the pMD18-T vector was executed twice using an ABIPRISM 3700 DNA analyzer (Perkin Elmer, Boston, MA, USA).

Results and Discussion

Specificity and sensitivity of primer pairs for the multiplex PCR The primer pair canendo-F/R was used to amplify an endogenous canola gene (*FatA*; fatty acyl-ACP thioesterase). The primer pair gave rise to a 105 bp amplicon via PCR. We performed a qualitative PCR using 50 ng of genomic DNA from 16 different plant species in order to investigate the specificity of the intrinsic primer pair. As shown in Fig. 2, no amplicon was observed in any of the species tested except for in canola. Single PCR was used to individually asses the specificity of the designed primer pairs. The genomic DNA fragments extracted from the seeds of 3 varieties of GM canola were used as the templates. As shown in Fig. 3, the expected amplicons were specifically amplified from each target GM canola; GT73, MS8xRF3, and T45. The GT73-KHUF1/R1 primer amplified the 3'-flanking region of GT73 to a size of 125 bp, the Barstar-F1/MS8xRF3-R primer amplified the junction region of barstar (isolated from *Bacillus amyloliquefaciens*) and the NOS terminator introduced into MS8xRF3 to a size of 162 bp, and the T45-F2/R2 primer amplified the junction region of PAT and the 35S terminator introduced into T45 to a size of 196 bp. The multiplex PCR assay for some GM canola varieties, including GT73, HCN92/28, OXY235, and MS8xRF3, was previously reported (14, 15). These protocols involve using more than two primer pairs to detect one variety. These primer pairs have a limited range of application (e.g., gene-specific primers only suitable for screening). Many GM crops have been developed and commercialized. Most of them contain the same or a similar promoter, terminator, foreign gene, or a combination of two of these elements. Thus, construct- and event-specific primer pairs are chosen for the effective and reliable identification of GM canola using multiplex PCR. Figure 4 shows the result of a multiplex PCR performed under the optimized conditions. The multiplex PCR amplified two fragments corresponding to the chosen target sequences for each GM

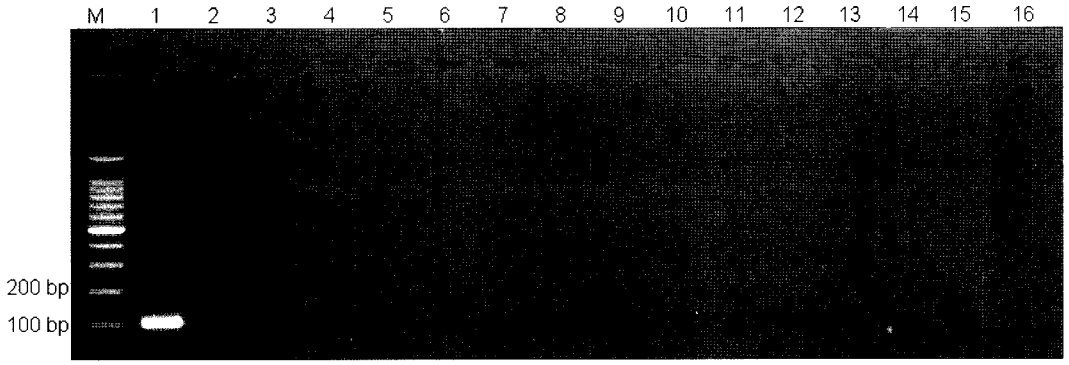

Fig. 2. PCR products amplified from the genomic DNA of 16 different plants. Lane M, marker (100 bp DNA ladder); lane 1, canola; lane 2, soybean; lane 3, maize; lane 4, cotton; lane 5, barley; lane 6, wheat; lane 7, buckwheat; lane 8, rice; lane 9, pepper; lane 10, perilla leaf; lane 11, mung bean; lane 12, red-bean; lane 13, sunflower; lane 14, cucumber; lane 15, chinese cabbage; lane 16, potato.

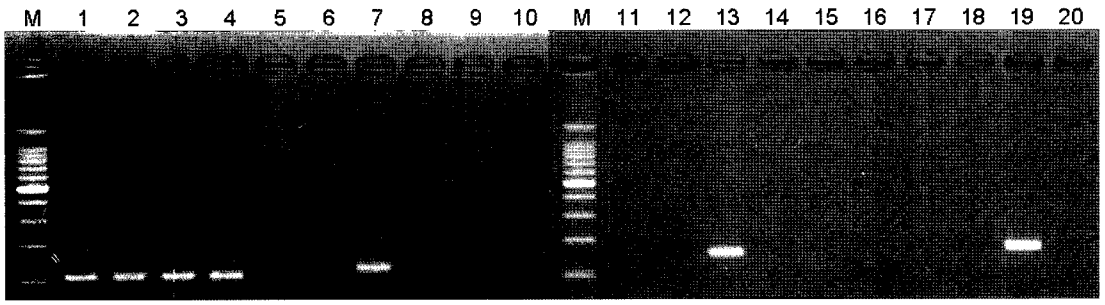

Fig. 3. Specificity of the detection primer pairs designed for the multiplex PCR. Lane M: marker (100 bp DNA ladder); lanes 1, 6, 11, and 16: canola control; lanes 2, 7, 12, and 17: GT73; lanes 3, 7, 13, and 18: MS8xRF3; lanes 4, 9, 14, and 19: T45; lanes 5, 10, 15, and 20: no template; lanes 1-5: using primer Canendo F/R (105 bp); lanes 6-10: using primer GT73-KHUF1/R1 (125 bp); lanes 11-15: using primer Barstar-F1/MS8xRF3-R (162 bp); lanes 16-20: using primer T45-F2/R2 (186 bp).

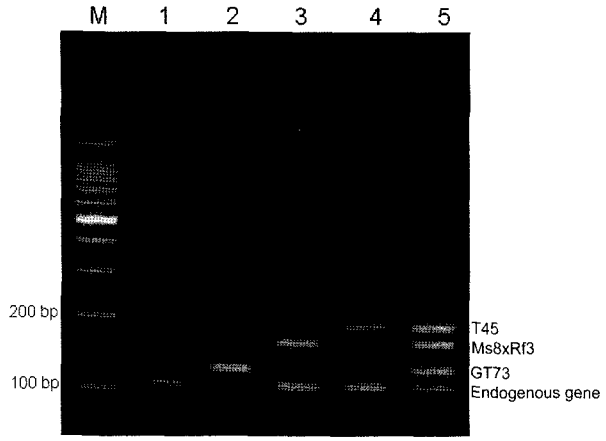

Fig. 4. Multiplex PCR products amplified from GM canola containing the *FatA* gene (an endogenous gene). Lane M, marker (100 bp DNA ladder); lane 1, the endogenous gene; lane 2, GT73 and the endogenous gene; lane 3, MS8xRF3 and the endogenous gene; lane 4, T45 and the endogenous gene; lane 5, GT73, MS8xRF3, T45, and the endogenous gene.

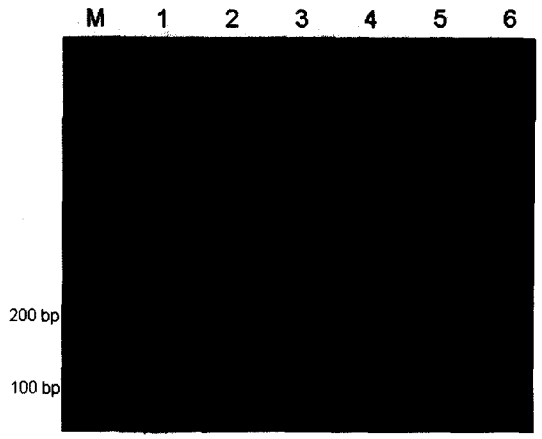

Fig. 6. Sensitivity analysis of the multiplex PCR. Lane M: marker (100 bp DNA ladder); lanes 1-5: 100, 10, 1, 0.1, and 0.01 % of a mixture of GM canola (GT73, MS8xRF3, and T45); lane 6: non-GM canola.

canola and for *FatA*, an internal control in the non-GM canola. As shown in lane 5 of Fig. 4, the intensities of the bands derived from the 4 amplicons were equivalent. The sequences of these amplicons were determined and are shown in Fig. 5. This PCR result is evidence that this method is sufficient to distinguish the 3 varieties of GM canola.

Limit of detection (LOD) of the multiplex PCR The limit of detection (LOD) is defined as the lowest amount or concentration of analyte that can be reliably detected in a sample (16). The reference materials containing 10, 0, 0.1, 0.01, and 0.001% of the GM canola mixture were prepared by mixing GM DNA from each variety of GM canola (GT73, MS8xRF3, and T45) with non-GM DNA from the control canola in order to determine the LOD value of the multiplex PCR. In the multiplex PCR assay, the LOD value was determined to be 1% of the GM canola mixture in 100 ng of genomic DNA (Fig. 6). This LOD is of significant value for the PCR method used for label monitoring in Korea.

In this study, a multiplex PCR method was developed to

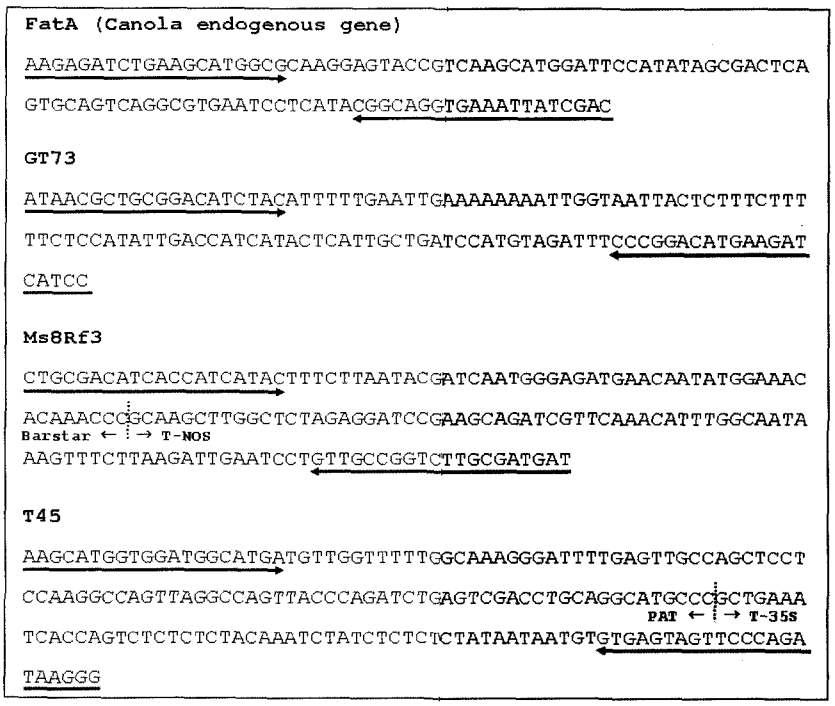

Fig. 5. Sequencing results of the multiplex PCR products. FatA, *fatty acyl-ACP thioesterase*; GT73, the 3'-flanking region of GT73; MS8xRF3, the junction region of barstar and the NOS terminator introduced in MS8xRF3; T45, the junction region of PAT and the 35S terminator introduced in T45. The primers used for the amplification are indicated by arrows.

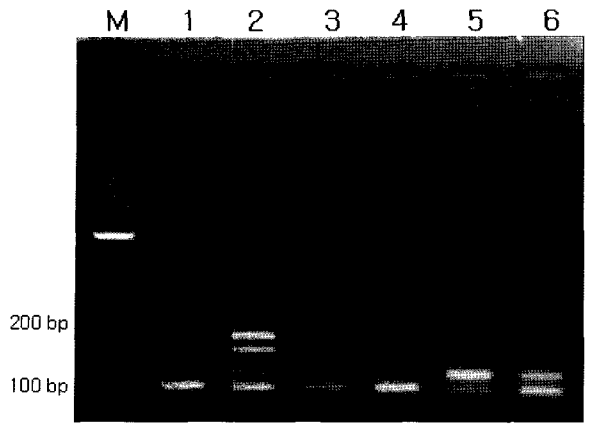

Fig. 7. Multiplex PCR of canola samples. Lane M, marker (100 bp DNA ladder); lane 1, negative control; lane 2, positive control; lanes 3-6, China (A), Korea, Canada, and China (B) canola seeds.

detect 3 varieties of GM canola, which have been 'safety-approved' in Korea. These results indicated that this detection method using multiplex PCR is, in fact, an effective qualitative PCR method to screen for the presence of GM canola in foods and feeds.

Multiplex PCR for the detection of GM canola from unknown samples Multiplex PCR was performed for GMO detection in canola seeds produced in China, Canada, and Korea. As shown in Fig. 7, canola seed A from China (lane 3) and canola seed from Korea (lane 4) are determined along with non-GM canola. Canola seed from Canada (lane 5) and canola seed B from China (lane 6) are determined along with GM canola. Canola seed B from China contained GT73 and the canola seed from Canada contained a mixture of GT73 and MS8xRF3. A single PCR was performed on all samples in order to reconfirm the precision of this analysis for tracking GM canola (Fig. 8). There were no differences in the multiplex and single PCR results. These results demonstrate that this multiplex PCR method is suitable for the detection of GM canola in raw material derived from GT73, MS8xRF3, and T45.

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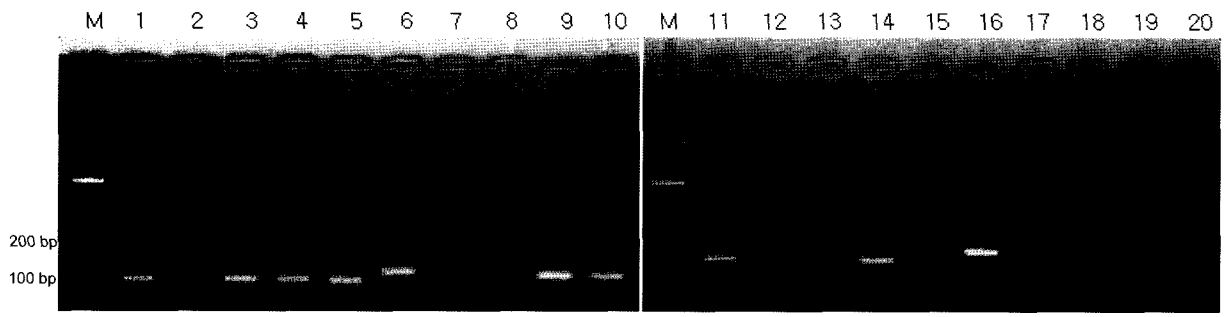

Fig. 8. Single PCR of canola samples. Lane M, marker (100 bp DNA ladder); lane 1, negative control (endogenous gene: 105 bp); lanes 2-5, canola samples 1-4 (Chinese (A), Korea, Canada, and Chinese (B) canola seeds); lane 6, positive control (GT73: 125 bp); lane 7-10, canola samples 1-4; lane 11, positive control (MS8xRF3: 162 bp); lanes 12-15, canola samples 1-4; lane 16, positive control (T45: 186 bp); lanes 17-20, canola samples 1-4.

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