FOOD COMPOSITION AND ADDITIVES

Novel Reference Gene, *High-mobility-group protein I/Y*, Used in Qualitative and Real-Time Quantitative Polymerase Chain Reaction Detection of Transgenic Rapeseed Cultivars

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With the development of transgenic crops, regulations to label the genetically modified organisms (GMOs) and their derived products have been issued in many countries. Polymerase chain reaction (PCR) methods are thought to be reliable and useful techniques for qualitative and quantitative detection of GMOs. These methods are generally needed to amplify the transgene and compare the amplified results with that of a corresponding reference gene to get the reliable results. Specific primers were developed for the rapeseed (*Brassica napus*), *high-mobility-group protein I/Y* (*HMG-I/Y*) single-copy gene and PCR cycling conditions suitable for the use of this sequence as an endogenous reference gene in both qualitative and quantitative PCR assays. Both methods were assayed with 15 different rapeseed varieties, and identical amplified products were obtained with all of them. No amplification was observed when templates were the DNA samples from the other species of *Brassica* genus or other species, such as broccoli, stem mustard, cauliflower, Chinese cabbage, cabbage, sprouts, *Arabidopsis thaliana*, carrot, tobacco, soybean, mung bean, tomato, pepper, eggplant, plum, wheat, maize, barley, rice, lupine, and sunflower. This system was specific for rapeseed. Limits of detection and quantitation in qualitative and quantitative PCR systems were about 13 pg DNA (about 10 haploid genomes) and about 1.3 pg DNA (about 1 haploid genome), respectively. To further test the feasibility of this *HMG-I/Y* gene as an endogenous reference gene, samples containing transgenic rapeseed GT73 with the inserted *glyphosate oxidoreductase* (*GOX*) gene were quantitated. These demonstrated that the endogenous PCR detection systems were applicable to the qualitative and quantitative detection of transgenic rapeseed.

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In recent years, agricultural production problems have been solved with genetically modified (GM) plants, adopting modern biotechnology, which has been used successfully in the United States and several other countries to create new crops with beneficial traits for improving agricultural production and addressing problems of malnutrition. For example, in the People's Republic of China, Bt cotton farmers reduced pesticide use by an average of 49.9 kg per hectare per season in 1999, based on surveys in provinces with a series of bollworm attacks (1).

The planting areas of GM crops had increased by 40 times since 1996 and up to about 67.7 million hectares in the world until 2003 (1). The most popular GM crops for field trials are

soybeans, tomatoes, maize, potatoes, wheat, cotton, sugar beets, oilseed rape, cucumbers, melons, alfalfa, lettuce, sunflowers, rice, and tobacco. Since 1997 in China, 6 GM crops were approved for commercialization, such as virus-resistant sweet pepper, cauliflower mosaic virus (CMV)-resistant tomato, shelf-life altered tomato, colored altered petunia, and 2 kinds of insect-resistant cottons (2). In addition, GM soybean, maize and rapeseed are being imported from other countries. Because these modified foods do not gain worldwide acceptance, many countries, including China, have established related regulations to label the GMOs and their derived products.

In conjunction with these labeling policies, the polymerase chain reaction (PCR) is a highly specific and sensitive method for detecting small amounts of nucleic acid to identify the presence of GMOs (3). The PCR method is used not only for identification of GM products, but also for quantitation (4). Among the PCR detection methods, real-time PCR is considered an easy-to-use, accurate, highly specific quantitative assay (4, 5). A unique feature of this PCR technique is that the amplification of target DNA sequence can be followed during the whole reaction by indirect monitoring of the product formation. GMO detection by the PCR method relies on parallel amplification of the exogenous gene and an endogenous reference gene that provides a control both for the lack of inhibition and for the ability to amplify the target DNA in the sample. The amplification of transgenic specific sequences is relative to an endogenous reference gene that gives an estimation of the total amount of target DNA in the sample. The amount of GMO is calculated as a fraction of total plant-specific DNA (4, 6, 7). In general, a reference gene should be species-specific, with low and exact copy number (to obtain high sensitivity, a single copy number reference gene is the best), and no allelic variation among cultivars in the same species.

For this purpose, much effort has been made to obtain a reference gene. Some genes are now used as reference genes for GMO detection, for example, the *invertase I* (7, 8), 10 kilodalton (kD) *zein* and *hmg-A* genes (7, 9–12) in maize, *lectin* (7, 9, 13) and β-*actin* (14) genes in soybean, and *cruciferin* (14) and *BnACCg8* gene in rapeseeds (7). However, the real-time PCR product of *BnAccg*8 gene is not specific, and some real-time PCR methods such as SYBR® Green I cannot be used with this reference gene (7). Moreover, the copy number of *BnAccg*8 in rapeseeds has not been confirmed (15). As to the *cruciferin* gene, Hernández et al. (7) proved that *cruciferin* was not suitable as endogenous gene.

To obtain a novel endogenous gene for qualitative and quantitative detection of transgenic rapeseed, we used a rapeseed-specific (*B. napus*), single-copy endogenous gene, *high-mobility-group protein I/Y* (*HMG-I/Y*, GenBank accession No. AF127919) and its PCR detection systems. The *HMG-I/Y* gene is one of high-mobility-group (HMG) protein genes (16). We used qualitative and real-time quantitative PCR assay to validate its species specificity, to test the limits of detection (LOD) and quantitation (LOQ), and to set up a reliable qualitative and quantitative system for GM rapeseed detection. Finally, this optimal quantitative PCR condition was used for detection of transgenic rapeseed GT73 containing *glyphosate oxidoreductase* gene (*GOX*; 17) in mixed rapeseed samples.

Experimental

Materials

Fifteen different nontransgenic cultivars of *Brassica napus*: Huyou-12, Huyou-50, An-437, Su-991246, Su-97-2126, Suyou No. 1, Zheshuang-758, Jiaxing-9308, Zhongshuang No. 6, and Hunong No. 1 were provided by the Horticulture Institute of China SAAS (Shanghai, China); PGCP-15, PGCP-23, PGCP-55, PGCP-57, and PGCP-59 were exchanged with France by the Horticulture Institute of China SAAS.

Six different nontransgenic cultivars of *Brassica*; broccoli (*B. oleracea* ssp. *italica*), stem mustard (*B. juncea* var. *zhatsai*), cauliflower (*B. oleracea* ssp. *botrytis*), Chinese cabbage (*B. campestris* ssp. *pekinensis*), cabbage (*B. oleracea* var. *capitata*), and sprouts (*B. oleracea* ssp. *gemmifera*) were collected from the local seed company in Shanghai.

Fifteen common crop cultivars from 15 different plant families, such as *Arabidopsis thaliana*, carrot (*Daucus carota*), tobacco (*Nicotiana tabacum*), soybean (*Glycine max*), mung bean (*Phaseolus aureus*), tomato (*Lycopersicon esculentum*), pepper (*Capsicum annum*), eggplant (*Solanum melongena*), plum (*Prunus domestica*), wheat (*Triticum aestivum*), maize (*Zea mays*), barley (*Hordeum vulgare*), rice

Table 1. Primer pair and fluorogenic probe

Name	Orientation	Sequence	Length, bp	Position, bp
hmg-F	Sense primer	5′-GGTCGTCCTCCTAAGGCGAAAG-3′	22	449–470
hmg-R	Antisense primer	5′-CTTCTTCGGCGGTCGTCCAC-3′	20	528–547
hmg-P	Probe	5′-HEX-CGGAGCCACTCGGTGCCGCAACTT-TAMRA-3′	24	495–518
gox-F	Sense primer	5′-CTTATCCGTCACGAAGGTCACC-3′	22	7497–7518
gox-R	Antisense primer	5′-TTGAGACGACGAAGTTCCCAAC-3′	22	7564–7585
gox-P	Probe	5′-FAM-TCCACGGTCCTTGGCGAAGTCTGCT-TAMRA-3′	25	7538–7562

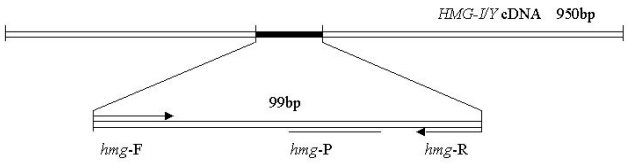

Figure 1. Positions of PCR primers and probes used for quantitative PCR analysis: Primer pair *hmg*-F/*hmg*-R, probe *hmg*-P.

(*Oryza sativa*), lupine (*Lupinus albus*), and sunflower (*Helianthus annuus*) were collected by our laboratory.

The transgenic rapeseed GT73 developed by Monsanto Co. (St. Louis, MO) and nontransgenic rapeseed were supplied by the Horticulture Institute of China SAAS. Two mixed samples, S1 and S2, with known GM rapeseed content (1 and 10% GM rapeseeds), were prepared and analyzed to determine the accuracy of the duplex TaqMan real-time PCR detection system using the *HMG-I/Y* gene as endogenous reference gene.

DNA Extraction

The plant genomic DNA samples used for qualitative and quantitative PCR detection were extracted and purified using a DNA extraction kit which was developed by Shanghai Ruifeng Agro-tech Co. Ltd (Shanghai, China).The genomic DNA for Southern blot was extracted from 10 g fresh leaves according to the cetyltrimethylammonium bromide (CTAB) protocol (Murray and Thompson; 18) as modified by Luo et al. (19). DNA concentration and quality were checked spectrophotometrically using Biophotometer (Beckman Co., Hamburg, Germany) and analyzed by 0.8% agarose gel electrophoresis in 1X Tris-borate EDTA (TBE) stained with ethidium bromide.

Oligonucleotide Primers and Probes

The oligonucleotide primers and TaqMan fluorescent dye-labeled probes were designed by the analysis results of the Primer Express V2.0 (Applied Biosystems, Foster City, CA). The corresponding nucleotide sequences of *HMG-I/Y* gene and *GOX* gene are listed in Table 1. All PCR primers and fluorogenic probes were synthesized by Shanghai Shenyou Co., Ltd. (Shanghai, China). The *HMG-I/Y* and *GOX* probes were labeled with the fluorescent reporter dye 5-hexachloro-fluorescein (HEX) and 6-carboxy-fluoroscein (FAM), respectively, on the 5′ end, and the fluorescent quencher dye 6-carboxytetramethylrhodamine (TAMRA) was located on the 3′ end of the 2 probes.

The primer pair *hmg*-F/*hmg*-R was used in the qualitative PCR detection and in the real-time PCR detection with the *hmg*-P probe. Both qualitative and real-time quantitative PCR gave rise to a 99 base pair (bp) amplification product. The *GOX* quantitative PCR with primer pair *gox*-F/*gox*-R and *gox*-P probe gave rise to an 89 bp product.

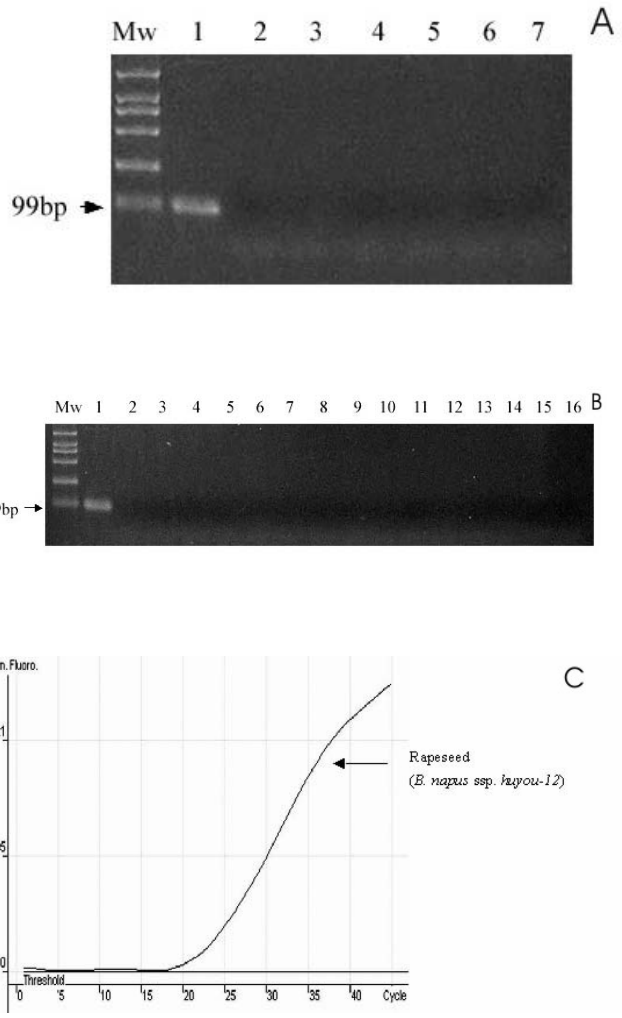

Figure 2. (A) Amplification result of template DNA from 7 species of the genus *Brassica*: lanes 1–7 correspond to rapeseed (*B. napus* ssp. *huyou-12*), broccoli (*B. oleracea* ssp. *italica*), stem mustard (*B. juncea* var. *zhatsai*), cauliflower (*B. oleracea* ssp. *botrytis*), Chinese cabbage (*B. campestris* ssp. *pekinensis*), cabbage (*B. oleracea* var. *capitata*), and sprouts (*B. oleracea* ssp. *gemmifera*). Mw: DL 2000 was used as molecular weight (MW) markers; fragments: 2000, 1000, 750, 500, 250, and 100 base pairs. (B) Amplification result of DNA from 16 different species: lanes 1–16 correspond to rapeseed, *A. thaliana*, carrot, tobacco, soybean, mung bean, tomato, pepper, eggplant, plum, wheat, maize, barley, rice, lupine, and sunflower in conventional PCR. Mw: DL 2000 was used as molecular weight (MW) markers; fragments: 2000, 1000, 750, 500, 250, and 100 base pairs. (C) In the real-time PCR, amplification result of DNA from 22 different species: rapeseed (*B. napus* ssp. *huyou-12*), broccoli (*B. oleracea* ssp. *italica*), stem mustard (*B. juncea* var. *zhatsai*), cauliflower (*B. oleracea* ssp. *botrytis*), Chinese cabbage (*B. campestris* ssp. *pekinensis*), cabbage (*B. oleracea* var. *capitata*), sprouts (*B. oleracea* ssp. *gemmifera*) and rapeseed, *A. thaliana*, carrot, tobacco, soybean, mung bean, tomato, pepper, eggplant, plum, wheat, maize, barley, rice, lupine sunflower. Only rapeseed (*B. napus* ssp. *huyou-12*) had the signal.

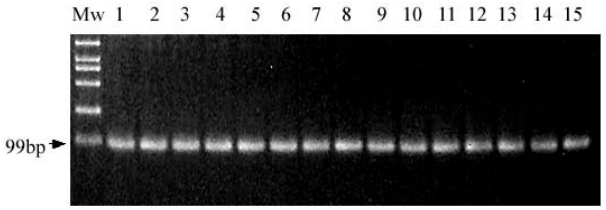

Figure 3. Amplification result of DNA from 15 cultivars of rapeseed; lanes 1–15 correspond to Huyou-12, Huyou-50, An-437, Su-991246, Su-97-2126, Suyou No. 1, Zheshuang-758, Jiaxing-9308, Zhongshuang No. 6, Hunong No. 1, PGCP-15, PGCP-23, PGCP-55, PGCP-57, and PGCP-59. Mw: DL 2000 was used as molecular weight (MW) markers; fragments: 2000, 1000, 750, 500, 250, and 100 base pairs.

PCR Conditions

Conventional PCR was performed in a PTC-100 (MJ Research, Waltham, MA) thermocycler. Each reaction mixture contained 1X PCR buffer, 0.2 mM dNTP, 500 nM each primer, and 1.5 units of *Taq* DNA polymerase; the final volume was 30 μL. All reagents were from TaKaRa Biotechnology Co., Ltd. (Dalian, China) except the primers. The amplification reaction used the following cycle conditions: denaturing of DNA at 94°C for 10 min; 35 cycles of 30 s at 94°C; 30 s at 60°C; 30 s at 72°C, and a final extension at 72°C for 5 min. Amplification products were analyzed by 2% agarose gel electrophoresis (in 0.5× TBE) and stained with ethidium bromide.

Real-time quantitative PCR was performed in a fluorometric thermal cycler (Rotor Gene 2000; Corbett Research, Melbourne, Australia) using the TaqMan system. Each reaction mixture contained 1× PCR buffer; 5mM $MgCl_2$; 0.2mM each of dATP, dCTP, and dGTP; 0.4mM dUTP; 0.2 unit uracil-*N*-glycosylase (UNG); 1.5 units *Taq* DNA polymerase; 500 nM each of *hmg*-F and *hmg*-R primers; and 300 nM fluorogenic *hmg*-P probe. The final volume of this PCR reaction was 25 μL. The amplification condition was 5 min at 50°C, 10 min at 95°C, 50 cycles of 30 s at 94°C, 45 s at 60°C, and 30 s at 72°C. Each sample was quantitated in triplicate. The PCR reactants were purchased from Gene Co., Ltd. (Shanghai, China). Results were analyzed using a sequence detection system provided by the Software ROTORGENE 4.6 (Corbett Research). With the same conditions, the reproducibility assay was performed on another fluorometric thermal cycler (PE7000, Applied Biosystems) by another analyst.

To generate a standard curve and test the LOD and LOQ in the 2 PCR systems, the extracted DNA was serially diluted to final concentrations of 13, 1.3, 0.13, 0.013, 0.0013, and 0.00013 ng/μL. The amount of DNA per reaction was 13, 1.3, 0.13, 0.013, 0.0013, and 0.00013 ng, respectively.

For quantitation of GT73 rapeseed content in the mixed rapeseed samples, the primers and probes of *HMG-I/Y* and *GOX* gene (*hmg*-F/*hmg*-R, *gox*-F/*gox*-R, *hmg*-P, and *gox*-P) were used in duplex real-time PCR analysis. The transgenic rapeseed DNA was serially diluted. The amount of total rapeseed DNA per reaction was 13 ng and the amount of transgenic rapeseed DNA per reaction was 13, 1.3, 0.13, and 0.013 ng. Each reaction mixture also contained 1X PCR buffer; 5mM $MgCl_2$; 0.2mM each of dATP, dCTP, and dGTP; 0.4 mM dUTP; 1.5 units of *Taq* DNA polymerase; 0.2 unit UNG; 500 nM *hmg*-F and *hmg*-R; 500 nM *gox*-F and *gox*-R; 300 nM *hmg*-P; and 300 nM *gox*-P probe. The final volume of the PCR reaction was 25 μL. The amplification program was 5 min at 50°C, 10 min at 95°C, 50 cycles of 30 s at 94°C, 45 s at 60°C, and 30 s at 72°C.

Southern Blot

A 12 μg amount of each DNA from 10 different non-transgenic rapeseed lines, such as Huyou-12, Huyou-50, An-437, Su-991246, Suyou No.1, Zheshuang-758, PGCP-15, PGCP-23, PGCP-55, and PGCP-57, was completely digested with *Eco*RI and *Bam*HI. Digested DNA was resolved in 0.8% agarose gel electrophoresis, and then transferred onto nitrocellulose transfer membrane, which was purchased from Gene Co., Ltd. (Shanghai, China). A 149 bp *HMG-I/Y* DNA fragment (from 454 to 602, GenBank accession No. AF127919) was used as the hybridized probe. This DNA fragment was labeled by α-[^{32}P]-dCTP using Random primer DNA labeling kit Ver. 2 from TaKaRa Biotechnology Co.) Hybridization was performed at 62 °C for 24 h, and the filter was washed at room temperature with 2X sodium saline citrate (SSC) 0.1% sodium dodecyl sulfate (SDS), 1X SSC 0.1% SDS for 10 min each, and 60°C 0.2X SSC 0.1% SDS for 30 min. DNA markers (λ DNA digested with *Hind*III and *Eco*RI) were run on the same gel (20).

Results and Discussion

Selection of Suitable Gene Fragment as Reference Gene in Qualitative and Quantitative Detection of Rapeseed Cultivars

Requirements for an endogenous reference gene include species-specific, low-copy number (to obtain high sensitivity,

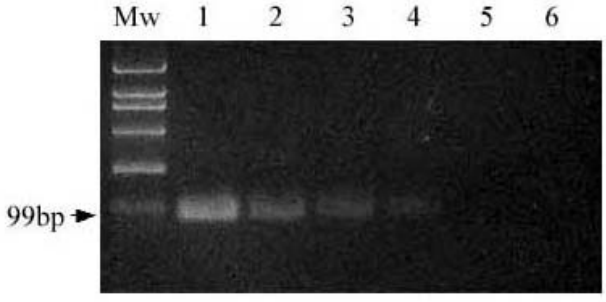

Figure 4. Amplification of serial dilutions of rapeseed DNA as PCR template: lanes 1–6 correspond to 13 ng, 1.3 ng, 130 pg, 13 pg, 1.3 pg, 0.13 pg. Mw: DL 2000 was used as molecular weight (MW) markers; fragments: 2000, 1000, 750, 500, 250, and 100 base pairs.

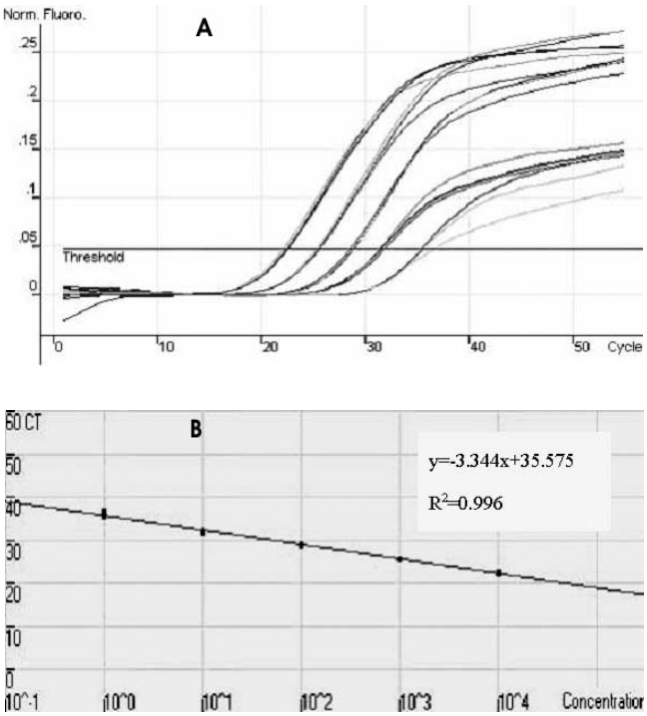

Figure 5. Real-time quantitative PCR detection: (A) amplification plot generated by serial dilution of rapeseed DNA ranging from 13 ng to 1.3 pg with the primer pair *hmg*-F/*hmg*-R and probe *hmg*-P; (B) standard curve generated from the amplification data given in (A).

a single-copy number reference gene is the best), and no allelic variation among cultivars. To select a suitable endogenous reference gene of rapeseed (*B. napus*) for quantitative and qualitative PCR amplification, we searched the public gene databank European Molecular Biology Laboratory (EMBL) for the candidate DNA sequences. After selection of several candidate genes, we chose a DNA fragment encoding high-mobility-group protein, which was one of HMG proteins, a family of the most abundant and ubiquitous nonhistone proteins common to eukaryotic organisms. This 930 bp cDNA (*HMG-I/Y*) contained a 612 bp open reading frame encoding a protein of 203 amino acids residues (16). Then we designed the specific primers and probe on this DNA sequence and tested them for species specificity, in both qualitative and real-time quantitative PCR assays. Several groups of primers and probes for tests had been synthesized, and the optimized primer pair *hmg*-F/*hmg*-R and probe *hmg*-P were selected (Table 1 and Figure 1).

Specificity of HMG-I/Y DNA Fragment

Primer pair *hmg*-F/*hmg*-R was used in conventional PCR and real-time PCR assays (*hmg*-P as TaqMan probe). This primer pair gave rise to a 99 bp amplification product, which was in a size range considered to be optimal for real-time PCR amplification. To test the species specificity of the *HMG I/Y* reference gene, we ran both conventional and real-time PCR (Taqman assay) reactions using 13 ng template DNA from 6 different plant species, i.e., *Brassica* species such as broccoli, stem mustard, cauliflower, Chinese cabbage, cabbage and sprouts, which were in the same genus and evolutionarily related to rapeseed. The electrophoresis results of qualitative PCR showed that no amplification products were observed with any of the species tested other than rapeseed (*B. napus*; Figure 2A), and no fluorescent signals were detected with any of the species tested other than rapeseed in real-time PCR (Figure 2C).

To verify that the assay did not cross-react with nontarget taxa of other genera, we selected 15 common cultivars from 15 different plant families, such as *Arabidopsis thaliana*, carrot (*Daucus carota*), tobacco (*Nicotiana tabacum*), soybean (*Glycine max*), mung bean (*Phaseolus aureus*), tomato (*Lycopersicon esculentum*), pepper (*Capsicum annum*), eggplant (*Solanum melongena*), plum (*Prunus domestica*), wheat (*Triticum aestivum*), maize (*Zea mays*), barley (*Hordeum vulgare*), rice (*Oryza sativa*), lupine (*Lupinus albus*), and sunflower (*Helianthus annuus*). Conventional and real-time PCR reactions were run with 13 ng genomic DNA of each plant as the templates. The electrophoresis results of qualitative PCR showed that no amplification products were observed with any of the species tested other than rapeseed (Figure 2B), and no fluorescent signals were detected with any of the species tested other than rapeseed in real-time PCR (Figure 2C).

Table 2. Reproducibility of the rapeseed *HMG-I/Y* gene with TaqMan assay

DNA amount, ng/reaction	Ct value			Mean	SD[a]	CV, %[b]
	1	2	3			
13	22.52	22.23	25.50	22.45	0.10	0.44
1.3	25.64	25.43	25.72	25.60	0.15	0.59
0.13	28.98	28.94	28.72	28.88	0.14	0.48
0.013	31.80	31.68	31.47	31.65	0.17	0.54
0.0013	36.74	35.55	35.80	36.03	0.63	1.74

[a] SD = Standard deviation.
[b] CV = Coefficient of variation.

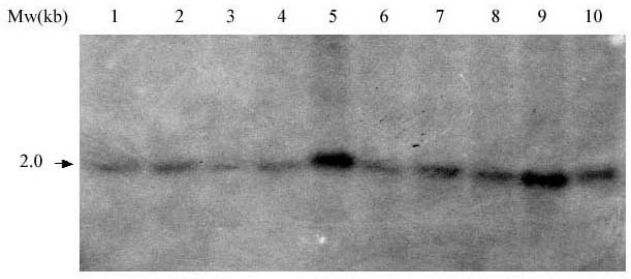

Figure 6. Southern blot of the genomic DNA of the 10 rapeseed cultivars digested with *Eco*RI: lanes 1–10 correspond to Huyou-12, Huyou-50, An-437, Su-991246, Suyou No. 1, Zheshuang-758, PGCP-15, PGCP-23, PGCP-55, and PGCP-57. Mw: Lambda DNA/*Eco*RI+*Hind*III Marker.

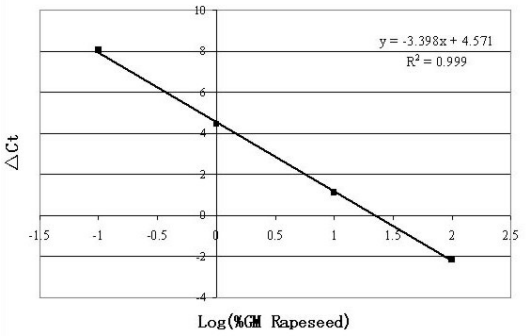

Figure 7. Standard curve for detection of GM rapeseed GT73 content in mixed samples by duplex real-time PCR system, plotting log (GM rapeseed amount) versus ΔCt (R^2 = 0.999, reaction efficiency = 0.94).

Allelic Variation of HMG-I/Y among Cultivars of Brassica napus

Identical bands were obtained with all of the 15 rapeseed varieties using 13 ng DNA as templates to run qualitative PCR (Figure 3). The real-time PCR analysis showed similar fluorescent intensity with all of the tested rapeseed cultivars (data not shown). Fifteen cultivars in the species of *B. napus* were used in the analysis of the allelic variation of *HMG-I/Y*, which included 10 Chinese cultivars, representing different ecotypes and distributed in different areas of China, and 5 French cultivars. These results showed that this *HMG-I/Y* DNA fragment had no allelic variation among the tested cultivars of *B. napus*.

LOD and LOQ

PCR reaction was conducted using the known DNA amount ranging from 13 ng to 0.13 pg. Conventional PCR allowed detection in 13 pg rapeseed genomic DNA (Figure 4). It showed that 13 pg (about 10 haploid genomes) rapeseed genomic DNA could be detected by qualitative PCR with *hmg*-F/*hmg*-R primer pair.

As to real-time PCR assay, the *HMG-I/Y* specific fluorescent signal could be detected when the amount of DNA template was lowered to 1.3 pg (about 1 haploid genome; Figure 5A). The standard curve of *HMG-I/Y* gene real-time PCR system was obtained with rapeseed DNA serial dilutions ranging from 13 ng to 0.13 pg, with each repeated in triplicate. PCR efficiency was 0.99 and the linear relationship (R^2) was 0.995 (Figure 5B). The Ct values ranged from 22.46 to 36.03, and the coefficient of variation (CV) values ranged from 0.38 to 1.42%. Additionally, the result of the reproducibility assay showed that the Ct values varied from 22.23 to 36.74 with the CV of 0.44–1.74% (Table 2). These data indicate that the CV

Table 3. Values of threshold (Ct) measurements of replicate standards from 0.1 to 100% (w/w) genetically modified (GM) rapeseed content

	Ct value for reaction				
GM content, % (w/w)	1	2	3	Mean	SD
		Total (*HMG-I/Y*)			
0.1	22.67	22.51	22.46	22.55	0.11
1	22.40	22.37	22.53	22.44	0.08
10	22.60	22.43	22.35	22.46	0.13
100	22.33	22.55	22.38	22.42	0.12
		GM rapeseed (*GOX*)			
0.1	30.53	30.51	30.80	30.61	0.16
1	26.77	26.93	27.00	26.90	0.12
10	23.70	23.38	23.66	23.58	0.17
100	20.26	20.43	20.11	20.27	0.16

Table 4. Detection of 2 samples with known genetically modified (GM) rapeseed content

Sample	GM rapeseed, %	*HMG-I/Y* Ct, mean	*GOX* Ct, mean	ΔCt	Calculated GM rapeseed, %
S1	1	23.71	28.14	4.43	1.1
S2	10	24.36	25.56	1.20	9.81

deriving from these tests was relatively small and the real-time detection system worked stably and reliably.

Only the Ct values were obtained after amplification with 1.3 pg genomic DNA slightly separated from the calculated standard curve, which indicated that quantitation was not accurate at such low DNA levels (Figure 5, Table 2). These results indicated that, in real-time PCR analysis, the rapeseed *HMG-I/Y* gene exhibited reliable amplification linearity over 5 orders of magnitude but that quantitation was not possible below 13 pg template DNA.

Copy Number of HMG-I/Y

Masek et al. (16) proved that *HMG-I/Y* was a single-copy gene in *B. napus*. We performed the Southern blot to analyze the copy number of *HMG-I/Y* in 10 rapeseed cultivars. After being digested completely with *Eco*RI, the genomic DNA of the 10 rapeseed cultivars was hybridized with a 149 bp *HMG-I/Y* DNA fragment labeled by α-[^{32}P]-dCTP. Only one DNA band with about 2.0 kbp size was detected in each tested rapeseed cultivar, which confirmed that *HMG-I/Y* gene was a single-copy gene per rapeseed haploid genome in the tested cultivars (Figure 6). Similar results were also obtained when the 10 rapeseed cultivars' genomic DNA was digested with *Bam*HI.

Quantitative Determination of GM Rapeseed Content in Mixed Rapeseeds

To prove that *HMG-I/Y* gene could be used as an endogenous reference gene for practical GM rapeseeds detection, 2 samples (S1 and S2) containing 1 and 10% GM rapeseed GT73, respectively, were quantitated by the duplex quantitative PCR with *HMG-I/Y* and *GOX* as endogenous and exogenous gene, respectively. To verify this duplex system, standard curves of these 2 genes were constructed by serial dilution of mixed rapeseed (containing GM rapeseed) DNA. Results showed that the PCR reaction efficiency of *GOX* gene (0.95) and *HMG-I/Y* gene (0.97) was similar in the duplex PCR system (data not shown). Thus, the relative quantitation method could be used in this duplex PCR system (21).

Before transgenic content was quantitated in the mixed samples, the standard curve was constructed with the ΔCt between the $Ct_{Exogenouse\ gene} - Ct_{Endougenous\ gene}$ versus Log (% GMO; 22, 23). The amount of GM rapeseed DNA was serially diluted from 13 to 0.013 ng per reaction, and the total DNA content per reaction was 13 ng per reaction; therefore, the GM rapeseed content per reaction was 100, 10, 1, and 0.1%, respectively. Then the standard curve was obtained with the ΔCt between the $Ct_{GOX} - Ct_{HMG\text{-}I/Y}$ versus Log (% GM rapeseed; Figure 7, Table 3). Based on the standard curve, 2 mixed GM rapeseed samples (S1 and S2) with known GM contents of 1 and 10% were detected by the duplex PCR system. The calculated GM contents of the 2 samples were 1.1 and 9.81%, respectively (Table 4).

All these results demonstrated that *HMG-I/Y* gene could be used as the endogenous reference gene for GM rapeseed detection, and the endogenous PCR detection systems were applicable to the qualitative and quantitative detection of transgenic rapeseeds.

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