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ARTICLE

Comparison of Five Endogenous Reference Genes for Specific PCR Detection and Quantification of *Brassica napus*

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Five previously reported *Brassica napus* endogenous reference genes, including acetyl-CoA carboxylase gene (*BnACCg8*), phosphoenolpyruvate carboxylase (*PEP*), oleoyl hydrolase gene (*FatA*), high-mobility-group protein I/Y gene (*HMG-I/Y*) and cruciferin A gene (*CruA*), were analyzed for their PCR specificity between *B. napus* and other species and the quantification stability among different *B. napus* cultivars. PCR and sequencing results indicated that none of these systems was species-specific as required by the genetically modified organism labeling policy. When these genes were employed in real-time PCR, *BnACCg8* and *HMG-I/Y* systems showed relatively greater heterogeneity among 10 different cultivars. The sequencing results showed that the single nucleotide polymorphism in the primer binding sites was the potential source of the instability in the *HMG-I/Y* system. The bias of *BnACCg8* was thought to be associated with the inconsistent copy number of this gene.

KEYWORDS: Endogenous reference gene; genetically modified organism; rapeseed; real-time PCR; *BnACCg8*; *PEP*; *FatA*; *HMG-I/Y*; *CruA*

INTRODUCTION

Rapeseed (*Brassica napus*) is one of the most popular genetically modified (GM) crops. To date, 15 GM rapeseed transgenic events have been approved for cultivation in the world (*1*).

Although GM crops have been marketed and consumed in many countries for years, controversy still exists with regard to their safety (*2*). In order to protect consumers' rights to information, labeling regulations have been established in more than 30 countries and regions (*3*−*7*).

In order to clarify the definition of GMO content, the EU states that the percentage of GMO should be expressed as GM-DNA copy numbers in relation to target taxon-specific DNA copy numbers calculated in terms of the haploid genome (*2*). According to this provision, both methods for the transgenic event and the host genome should have a high degree of specificity and stability, as well as the ability to quantitatively determine or ascertain the components from mixed samples.

A large number of *B. napus*-related species are widely planted, making this crop more complex than most. The *Brassica* species contains three basic cultivated species, including *Brassica rapa* (genome AA), *Brassica nigra* (genome BB) and *Brassica oleracea* (genome CC). *Brassica juncea* (genome AABB), *B. napus* (genome AACC) and *Brassica carinata* (genome BBCC) are synthesized species from the three basic species (*8*) . Some of these crops are similar in morphology and composition. Farmers and distributors often use the same name to refer to different *Brassica* crops. In Europe, North America and China, rapeseed usually refers to *B. napus* (genome AACC). However, in India, the major rapeseed species is *B. juncea* (genome AABB). In some other regions, *B. rapa* (genome AA) and *B. carinata* (genome BBCC) are also cultivated for vegetable oil and called "rapeseed" or "oilseed" (*9*) . According to the requirements of the EU, these different related varieties must be calculated separately in GMO detection and quantification (*2*) .

In the past, event-specific detection methods for most of the important rapeseed transgenic events have been established (*10*−*20*). Accordingly, several rapeseed endogenous reference gene quantification systems have also been reported for the specific PCR detection and quantification of *B. napus* genome DNA. Acetyl-CoA carboxylase gene (*BnACCg8*) was the first reference gene used for the species-specific detection and quantification of *B. napus* (*21*). In 2002, in a validation study of real-time PCR methods for the quantification of transgenic contaminations, the gene coding for phosphoenolpyruvate carboxylase (*PEP*) was used as a reference for the quantification of total genomic *B. napus* DNA (*22*). Oleoyl hydrolase gene (*FatA*) was recommended as the endogenous reference gene in an official procedure for quantification of the GM canola RT73 of Monsanto (*10*, *17*, *19*, *23*). High-mobility-group protein I/Y gene (*HMG-I/Y*) was another widely used reference gene (*15*, *16*, *18*, *20*, *23*, *24*). After modification, *HMG-I/Y* is also the only reference gene for detection of GM *B. napus* in all related national standards of China (*25*−*30*). Beside these four systems, cruciferin A gene (*CruA*) is the only one used, verified and recommended by the Community Reference Laboratory for GM Food and Feed (CRL-GMFF) for all *B. napus* transgenic events up to now (*11*−*14*). Although these methods have been widely used

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Table 1. Primers and Fluorogenic Probes Used in This Research

gene	accession no.	PCR system	primer name	orientation	sequence	position	amplicon (bp)	ref
BnACCg8	X77576	qualitative	acc3	forward primer	GAGAATGAGGAGGACCAAGCTC	9560−9581	196	*21*
			acc2	reverse primer	GGCGCAGCATCGGCT	9741−9755		
			acc4	reverse primer	ACATCCTCCTGACTGGCACC	9961−9980	421	this research
		quantitative	acc1	forward primer	GGTGAGCTGTATAATCGAGCGA	9652−9673	104	*21*
			acc2	reverse primer	GGCGCAGCATCGGCT	9741−9755		
			accp[a]	probe	AACACCTATTAGACATTCGTTC-CATTGGTCGA	9685−9716		
PEP	D13987	quantitative	pep-F	forward primer	CAGTTCTTGGAGCCGCTTGAG	3638−3658	140	*22*
			pep-R	reverse primer	TGACGGATGTCGAGCTTCACA	3757−3777		
			pep-P[a]	probe	ACAGACCTACAGCCGATGGAA-GCCTGC	3690−3716		
FatA	AJ294419	quantitative	FatA-F	forward primer	GGTCTCTCAGCAAGTGGGTGAT	1689−1710	76	*10*
			FatA-R	reverse primer	TCGTCCCGAACTTCATCTGTAA	1743−1764		
			FatA-P[a]	probe	ATGAACCAAGACACAAGGCG-GCTTCA	1712−1737		
HMG-I/Y	AF127919	qualitative	HMG-F	forward primer	TCCTTCCGTTTCCTCGCC	421−438	206	*25−30*
			HMG-R	reverse primer	TTCCACGCCCTCTCCGCT	609−626		
		quantitative	hmg-F	forward primer	GGTCGTCCTCCTAAGGCGAAAG	449−470	99	*24*
			hmg-R	reverse primer	CTTCTTCGGCGGTCGTCCAC	528−547		
			hmg-P[a]	probe	CGGAGCCACTCGGTGCCG-CAACTT	495−518		
CruA	X14555	quantitative	MDB510	forward primer	GGCCAGGGTTTCCGTGAT	1408−1425	101	*11−14*
			MDB511	reverse primer	CCGTCGTTGTAGAACCATTGG	1488−1508		
			TM003[a]	probe	AGTCCTTATGTGCTCCACTTT-CTGGTGCA	1427−1455		

[a] Probes labeled with 5′-6-carboxy-fluorescein (FAM) and 3′-Black Hole Quencher 1 (BHQ1).

and have shown good quantitative accuracy in research and practical applications, no systematic analysis has determined whether all of the reported *B. napus* endogenous reference systems can fully meet the needs of the quantitative requirements.

In previous works, we were often puzzled about the choice of endogenous reference gene; we also found that some systems cross-reacted in non-*B. napus* species. Taking all these into account, we believe that systemic recomparison and analysis are particularly important. In this study, five reported endogenous reference genes were compared and analyzed for their PCR specificity between *B. napus* and other species and for quantification stability among different *B. napus* cultivars.

MATERIALS AND METHODS

Plant Materials. Seeds of GM *B. napus* Ms8×Rf3, Oxy-235 and Topas 19/2 were provided by Bayer CropScience Co. (Monheim, Germany). Seeds of GM rapeseed RT73 were provided by Monsanto Co. (St. Louis, MO).

Nontransgenic seeds of *B. napus* (cv. Huayou No. 15, cv. Qinyou, cv. Zheyouyou No. 2, cv. Zhongshuang No. 6, cv. Zhongshuang No. 9 and cv. Zhongyou No. 821), *B. rapa* (ssp. *chenensis* cv. Hualiangzao 5, ssp. *chinensis* var. *purpurea* cv. Shiyuehong and ssp. *chinensis* var. *rosularis*), *B. nigra*, *B. oleracea* (cv. Niuxin, ssp. *albaglabra* cv. Hong Kong and ssp. *alboglabra* cv. Tianjin), *B. juncea* (ssp. *czernajew* var. *tumida* cv. Simianshan, var. *tsatsai* cv. Da and var. *capitata* cv. Darou), *B. carinata*, *Arabidopsis thaliana*, *Arachis hypogaea*, *Capsicum minimum*, *Glycine max*, *Gossypium hirsutum*, *Helianthus annuus*, *Lycopericon esculentum*, *Nicotiana tabacum*, *Oryza sativa*, *Raphanus sativus*, *Sesamum indicum*, *Sinapis alba*, *Solanum melongena*, *Solanum tuberosum*, *Vigna unguiculata* ssp. *sesquipedalis* and *Zea mays* were collected by our laboratory (Wuhan, China).

Seeds of *B. napus* were sown and cultivated in a greenhouse at 22 °C. One month after seeds were sown, the fresh leaves were collected for DNA extraction.

DNA Extraction. Genomic DNA was extracted and purified from young leaves of *B. napus* following a cetyltrimethylammonium bromide (CTAB)-based protocol (*31*).

For the remaining samples, genomic DNA was extracted and purified from seeds with the DNA Extraction Kit for GMO Detection Ver. 2.0 (Takara, Shiga, Japan).

DNA concentrations were estimated with a spectrophotometer Lambda 25 (Perkin-Elmer, Ames, IA) and further calculated with Quality One software (Bio-Rad, Hercules, CA) after agarose gel electrophoresis with ethidium bromide staining.

Primers and Probes. The primers and probes used in this research were synthesized by Sangon (Shanghai, China) and are listed in **Table 1**.

The sequences of the primers and probes were kept in agreement with those of the original reports. However, the 5′ ends of all TaqMan fluorescent probes were labeled with the fluorescent reporter 6-carboxy-fluorescein (FAM), and the 3′ ends were labeled with the fluorescent quencher Black Hole Quencher 1 (BHQ1) to provide uniformity on the real-time PCR machine settings.

Qualitative PCR Conditions. In the qualitative PCR assay, 20 ng of genomic DNA was used as template in a volume of 50 μL. The reaction mixture contained 1× PCR buffer (10 mM Tris·HCl, pH 8.3, 50 mM KCl), 200 μM of each dNTP, 1.5 mM $MgCl_2$, 100 nM of each primer, and 2 units Hot-Start Taq (Takara, Shiga, Japan). The PCR amplifications were carried out on a DNA Engine Dyad Peltier Thermal Cycler (Bio-Rad, Hercules, CA) using the following program: a 94 °C initial denaturation step for 2 min; 35 cycles of 15 s at 94 °C (denaturation), 30 s at 60 °C (annealing), 30 s at 72 °C (extension); and termination with an extension at 72 °C for 2 min. The PCR products were electrophoresed on 2% agarose gels and stained with ethidium bromide for visualization.

Quantitative PCR Conditions. The real-time quantitative PCR reactions were performed on a DNA Engine Opticon 2 Continuous Fluorescence Detector (MJ Research, Waltham, MA). Fluorescence signals ware monitored and analyzed using the software MJ Opticon Monitor, Version 3.1 (Bio-Rad, Hercules, CA).

In the real-time PCR assay, 20 ng of genomic DNA was used as template in a volume of 20 μL. The reaction mixtures were prepared according to the previous reports. Chemicals in some reaction mixtures were slightly modified to standardize the reaction conditions.

All real-time PCR reactions were carried out with the same program as follows: a predigest step of 50 °C for 2 min; a 95 °C initial denaturation and UNG deactivation step for 10 min; 50 cycles of 15 s at 94 °C (denaturation)

Table 2. Species-Specificity Analysis of Five Endogenous Reference Genes[a]

		gene						
		BnACCg8		*PEP*	*FatA*	*HMG-I/Y*		*CruA*
sample name	scientific name	acc3/ acc2	acc1/ acc2	pep-F/ pep-R	FatA-F/ FatA-R	HMG-F/ HMG-R	hmg-F/ hmg-R	MDB510/ MDB511
thale cress	*A. thaliana*	N	N	N	P^S	N	N	P^S
peanut	*A. hypogaea*	N	N	N	N	N	N	N
Ethiopian mustard	*B. carinata*	N	N	P	P	P	P	P
tuber mustard	*B. juncea* ssp. *czernajew* var. *tumida* cv. Simianshan	P	P	P	P	P	P	P
stem mustard	*B. juncea* var. *tsatsai* cv. Da	P	P	P	P	P	P	P
leaf mustard	*B. juncea* var. *capitata* cv. Darou	P	P	P	P	P	P	P
rapeseed Huyou No. 15	*B. napus* cv. Huyou No. 15	P	P	P	P	P	P	P
rapeseed MS8×RF3	*B. napus* cv. MS8×RF3	P	P	P	P	P	P	P
rapeseed OXY-235	*B. napus* cv. OXY-235	P	P	P	P	P	P	P
rapeseed Qinyou	*B. napus* cv. Qinyou	P	P	P	P	P	P	P
rapeseed RT73	*B. napus* cv. RT73	P^S	P	P^S	P^S	P^S	P	P^S
rapeseed Topas 19/2	*B. napus* cv. Topas 19/2	P	P	P	P	P	P	P
rapeseed Zheyouyou No. 2	*B. napus* cv. Zheyouyou No. 2	P	P	P	P	P^S	P	P
rapeseed Zhongshuang No. 6	*B. napus* cv. Zhongshuang No. 6	P	P	P	P	P	P	P
rapeseed Zhongshuang No. 9	*B. napus* cv. Zhongshuang No. 9	P^S	P	P	P	P^S	P	P
rapeseed Zhongyou No. 821	*B. napus* cv. Zhongyou No. 821	P^S	P	P	P	P^S	P	P
black mustard	*B. nigra*	N	N	P^S	P^S	N	P^S	P^S
cabbage	*B. oleracea* cv. Niuxin	N	N	P	P	P	P	P
Chinese kale	*B. oleracea* ssp. *albaglabra* cv. Hongkong	N	N	P	P	P	P	P
white Chinese kale	*B. oleracea* ssp. *alboglabra* cv. Tianjin	N	N	P^S	P^S	P^S	P	P^S
Chinese cabbage	*B. rapa* ssp. *chenensis* cv. Hualiangzao 5	P^S	P	P^S	P^S	P^S	P	P^S
red *Brassica* sprouts	*B. rapa* ssp. *chinensis* var. *purpurea* cv. Shiyuehong	P	P	P	P	P	P	P
black cabbage	*B. rapa* ssp. *chinensis* var. *rosularis*	P	P	P	P	P	P	P
chiles	*C. minimum*	N	N	N	N	N	N	N
soybean	*G. max*	N	N	N	N	N	N	N
cotton	*G. hirsutum*	N	N	N	N	N	N	N
sunflower	*H. annuus*	N	N	N	N	N	N	N
tomato	*L. esculentum*	N	N	N	N	N	N	N
tobacco	*N. tabacum*	N	N	N	N	N	N	N
rice	*O. sativa*	N	N	N	N	N	N	N
radish	*R. sativus*	P^S	N	P^S	P^S	N	N	P^S
sesame	*S. indicum*	N	N	N	N	N	N	N
white mustard	*S. alba*	N	N	P^S	P^S	N	P^S	P^S
eggplant	*S. melongena*	N	N	N	N	N	N	N
potato	*S. tuberosum*	N	N	N	N	N	N	N
cowpea	*V. unguiculata* ssp. *sesquipedalis*	N	N	N	N	N	N	N
corn	*Z. mays*	N	N	N	N	N	N	N

[a] P: Positive. N: Negative. Superscript S: PCR products were sequenced.

and 1 min at 60 °C (annealing and extension). Fluorescence was measured after annealing and extension.

RESULTS

Interspecies Specificity and Intraspecies Stability in Qualitative PCR. To verify that the assay did not cross-react with untargeted taxa of other genera, the qualitative PCR assays were performed with 7 primer pairs (**Table 1**) for 5 endogenous reference genes using genomic DNA from 37 different plants species and cultivars. These primer pairs were originally designed for qualitative detection or real-time systems. The samples in this research included 3 *B. rapa* (genome AA) cultivars, 1 *B. nigra* (genome BB) cultivar, 3 *B. oleracea* (genome CC) cultivars, 3 *B. juncea* (genome AABB) cultivars, 10 *B. napus* (genome AACC) cultivars, 1 *B. carinata* (genome BBCC) cultivar and 16 non-*Brassica* cultivars, as shown in **Table 2**. All PCR reactions were carried out in quadruplicate and repeated three times.

Consistent with previous reports, all of the primer pairs worked well with all cultivars of *B. napus*. However, none of these 7 PCR systems was found to be *B. napus*-specific (**Table 2**).

As shown in **Table 2**, *FatA* and *CruA* systems had the same results in this test. Their fragments could be amplified from the genomes of all *Brassica* species and even from all tested distant *Cruciferae* samples, including *A. thaliana*, *R. sativus* and *S. alba*. The *PEP* system exhibited a performance similar to that of *FatA* and *CruA*, except for the result of the experimental plant *A. thaliana*. For *HMG-I/Y*, the experiments using different primer pairs showed different results. With the primers for real-time PCR, *HMG-I/Y* fragments could be found in *S. alba*. Aside from this exception, the amplification of the *HMG-I/Y* system was restricted in the *Brassica* species with either primer set. Additionally, the *HMG-I/Y* system reacted in all *Brassica* species just like *PEP*, *FatA* and *CruA*. In comparison, *BnACCg8* systems had relatively higher specificity. Except for the *R. sativus*, the amplification of *BnACCg8* systems was restricted in the *Brassica* species, while its fragments could not be detected in *B. nigra*, *B. carinata* or *B. oleracea* using either primer pair unlike the other four genes.

Thus, *BnACCg8* was A-genome-specific in *Brassica* species and the other four systems were not specific for any *Brassica* species. The PCR result of the three synthesized *Brassica* species also supported this assumption.

To confirm the PCR results and determine more information about the endogenous reference genes, the PCR products amplified from non-*B. napus* species and *B. napus* cv. RT73 were

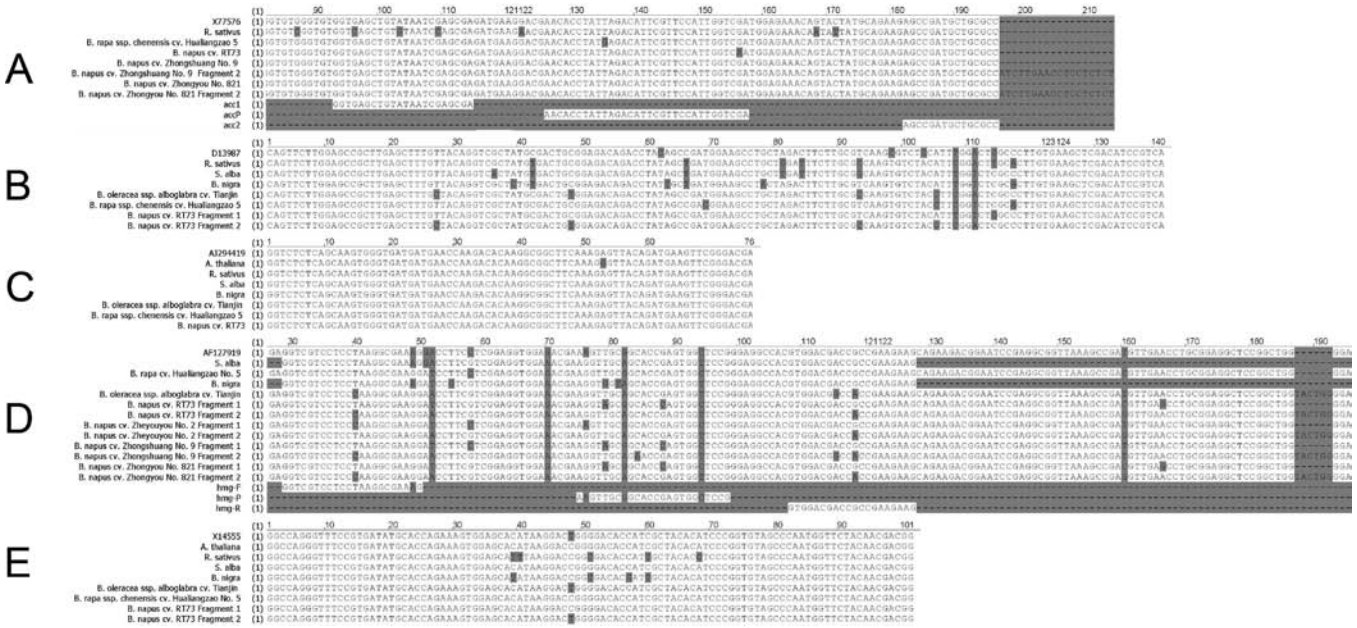

Figure 1. Alignment of endogenous reference gene fragments. **A**: *BnACCg8* gene fragments. **B**: *PEP* gene fragments. **C**: *FatA* gene fragments. **D**: *HMG-I/Y* gene fragments. **E**: *CruA* gene fragments. X77576, D13987, AJ294419, AF127919 and X14555: Sequences from GenBank with such accession numbers. acc1/accP/acc2 and hmg-F/hmg-P/hmg-R: Primers/probes used in this research.

Table 3. Comparison of Ct Values Obtained from 10 *B. napus* Cultivars Using Five Endogenous Reference Gene Real-Time PCR Systems

	mean Ct value					mean Ct value of all genes	ΔCt				
PCR template	*BnACCg8*	*PEP*	*FatA*	*HMG-I/Y*	*CruA*		*BnACCg8*	*PEP*	*FatA*	*HMG-I/Y*	*CruA*
B. napus cv. Huyou No. 15	25.224	23.223	23.244	24.888	24.078	24.131	−1.093	0.909	0.887	−0.756	0.053
B. napus cv. Zheyouyou No. 2	26.831	23.765	23.666	24.254	24.122	24.528	−2.303	0.763	0.862	0.273	0.406
B. napus cv. Qinyou	23.946	22.683	22.550	23.244	22.940	23.073	−0.874	0.390	0.522	−0.171	0.132
B. napus cv. Zhongshuang No. 6	24.223	22.880	22.508	24.208	23.622	23.488	−0.734	0.608	0.980	−0.720	−0.134
B. napus cv. Zhongshuang No. 9	25.321	23.381	23.750	27.530	24.470	24.890	−0.431	1.510	1.140	−2.640	0.420
B. napus cv. Zhongyou No. 821	24.519	22.831	23.271	26.562	23.407	24.118	−0.401	1.287	0.847	−2.444	0.711
B. napus cv. MS8×RF3	26.384	22.912	22.899	25.193	23.344	24.146	−2.238	1.234	1.248	−1.047	0.803
B. napus cv. OXY-235	26.427	22.970	23.023	24.592	23.671	24.137	−2.290	1.167	1.114	−0.455	0.465
B. napus cv. RT73	26.395	23.070	23.063	25.460	23.713	24.340	−2.055	1.270	1.277	−1.120	0.628
B. napus cv. Topas19/2	25.911	23.028	23.071	24.684	23.184	23.976	−1.935	0.948	0.905	−0.709	0.791
mean	25.518	23.074	23.104	25.062	23.655						
std deviation							0.800	0.348	0.226	0.920	0.323

recovered and sequenced as shown in **Table 2** and **Figure 1**. Single nucleotide polymorphism (SNP) sites were found in a wide range of gene fragments. Generally speaking, sequences from different cultivars of the same species were relatively more homologous than those from different species. With this information, a phylogenetic tree can be mapped, showing these data to be in line with the genetic and evolutionary relationships of these species. Based on these analyses, the interspecies nonspecific amplifications were real and sound.

Intraspecies Stability in Real-Time PCR. According to the EU requirement, the endogenous reference genes and the corresponding quantification methods must be stable in their analysis of different cultivars of *B. napus*. To assess the potential variation of these genes, genomic DNA extracted from 10 different *B. napus* cultivars was analyzed using 5 previously reported endogenous reference gene quantification systems.

The DNA samples were prepared, quantified and diluted to a concentration of 100 ng/μL. Each sample was used as PCR template for five endogenous reference systems at the same time. The reactions were run in quadruplicate, and the whole assay was repeated four times. The mean Ct values of each gene and each cultivar were calculated as listed in **Table 3**.

Because the stability of the specific endogenous reference system was to be analyzed in different cultivars, the genome copy number homogeneity of these samples was very important. However, in practice, it was impossible to keep copies of the PCR templates exactly the same as in theory. First, the genome sizes of these 10 *B. napus* cultivars were uncertain and may vary. Second, the DNA purity and quantification after extraction were not completely accurate and consistent. To facilitate the comparison and analysis of the different endogenous reference systems in different cultivars, the Ct data were normalized by the mean of all Ct values of all different endogenous reference genes obtained from the same template (**Table 3**).

The standard deviation (SD) of the normalized data from 10 different cultivars of each gene was calculated. Based on these data, the stability of these 5 endogenous reference systems was compared and analyzed. As shown in **Table 3**, the *BnACCg8* and *HMG-I*/*Y* systems had relatively greater variability among these 10 cultivars than the other three genes. Moreover, if these 10 cultivars were divided into Chinese and Canadian cultivars, it would be found that the variability of *BnACCg8* resulted primarily from the Chinese cultivars. However, *HMG-I*/*Y* showed a range of variation in both Chinese and Canadian cultivars.

To isolate the binding sites of the real-time PCR primers and probes of the *BnACCg8* and *HMG-I/Y* systems and to investigate the causes of data variations, primer pairs acc2/acc4 and HMG-F/HMG-R were used for PCR amplification. The PCR products from some cultivars were cloned and sequenced. As shown in **Figure 1**, the *BnACCg8* gene fragments from a selection of *B. napus* cultivars had identical sizes and very similar sequences (**Figure 1A**). However, the cloned *HMG-I/Y* gene fragments were highly variable (**Figure 1D**). Each PCR product from *B. napus* showed at least two fragments that differed in length. Also, SNP sites were found in all primer/probe binding sites and in all tested varieties. Some mismatchings were even close to the end of the primer/probe. This kind of DNA variation is usually considered to be related to the instability of real-time PCR data.

DISCUSSION

Event-specific quantification and endogenous reference systems constitute the basis of the GMO quantitative detection technology and labeling policy. The event-specific PCR system targets a simple, unique and single-origin sequence. An endogenous reference system calculates the host genome. In theory, there is a wide selection of genes that could be used as endogenous references, only if this gene has high specificity between species and low heterogeneity among cultivars. In truth, however, almost all crops have a number of related species. In these species, most of the genes are highly homologous. Moreover, after long-term cultivation and breeding under artificial selection, different cultivars of a specific species have a huge variation on the level of both morphology and gene. In this research, based on the relationship information between *B. napus* and other *Brassica* species, we analyzed and compared five previously reported endogenous reference genes of *B. napus*.

Under normal circumstances, species specificity is a basic requirement for endogenous reference genes. Otherwise, it is not possible to determine the target taxon-specific DNA copy numbers from mixed samples. In recent years, *BnACCg8*, *PEP*, *FatA*, *HMG I/Y* and *CruA* have been used as endogenous reference genes on various occasions. However, this study found that none of them could meet this basic requirement of being species-specific. All of the systems have the problem of cross-reaction. In fact, using such methods yielded total copy numbers of *Brassica* species rather than those of only *B. napus*. *BnACCg8* was relatively better, targeting only A-genome species including *B. rapa*, *B. juncea* and *B. juncea*. However, these three A-genome species are the most important oil crops worldwide. To date, the transgenic events T45 and RT73 have been introduced into the *B. rapa* from transgenic *B. napus* through traditional interspecific cross-breeding (*32*, *33*). We can foresee that the transformation and exchange of the transgenic events among *Brassica* species or even between *Brassica* and non-*Brassica* species will be more frequent in the future. In that case, both the existing event-specific assays and endogenous reference gene systems would be unable to distinguish either the transgenic events or the different host species, as required by law. Considering the origin of *B. napus*, we do not know whether an endogenous reference gene could be found to distinguish *B. napus* from *B. rapa* and *B. oleracea*. In conclusion, under current conditions, one should be very careful to give a percentage of GMO using these reference systems before the real source material of the samples could be determined with certainty.

Both *BnACCg8* and *HMG-I/Y* have been studied and declared to be *B. napus*-specific (*21*, *24*), unlike the outcome from our observations. However, in the report of Hernández et al., the experimental material did not contain any other *Brassica* species except *B. oleracea* and *B. napus*. Therefore, the conclusion of previous study should be that no amplification products were observed with DNA samples from other C-genome *Brassica* species, which was consistent with our findings. For *HMG-I/Y*, although Weng et al. had researched the features and applications of this gene (*24*), our conclusions were still inconsistent with theirs. All three basic *Brassica* species were used in both studies. *HMG-I/Y* fragments were found in all tested species in the present study, whereas Weng et al. did not observe amplicon in *B. rapa* and *B. oleracea* which are parent species of *B. napus* (*24*).

A reliable endogenous reference gene should have a constant copy number in different cultivars of the target species. Generally speaking, a lower copy number would lead to a less putative intercultivar variation on both copy number and sequence. Single-copy genes are often the best choice for endogenous reference genes for this reason. However, based on the results of the species-specificity analysis of these five genes, a very direct inference would show that there were at least two copies in a haploid *B. napus* genome (except for *BnACCg8*) because they could be found in both *B. rapa* and *B. oleracea*. This assumption was verified by the results of sequencing. Two different sequences of *PEP*, *HMG I/Y* and *CruA* could be found in the cloning fragments from *B. napus* in this study (**Figure 1**). However, in previous reports, the copy number of *HMG-I/Y* was estimated to be 1, which was inconsistent with our conjecture and results.

Low heterogeneity among cultivars is another basic requirement for the endogenous reference gene. In the real-time PCR analysis of this research, *BnACCg8* and *HMG-I/Y* were found to have significant allelic variation in Ct numbers. Sequencing data revealed that the SNP sites in the primer sequence were likely the cause of the observed inconsistency of *HMG-I/Y* (**Figure 1D**). However, no SNP was found in the primer/probe site of *BnACCg8* (**Figure 1A**). The bias might derive from the unstable copy number of the *BnACCg8* gene, as previously noted (*21*). In previous research, only one cultivar has been used to establish the standard curve (*21*, *24*). In that case, such nuances between different cultivars would not be found. However, because PCR is an exponential amplification procedure, these nuances could result in significant errors in the quantification of the original template. In our previous work, quantitative errors have also been noted using some endogenous reference genes when the sample included some specific rapeseeds from China and Canada. This is the first time such errors and sources of error have been specifically analyzed. In this work, we used a different method to analyze the data variation. In comparison with the line graph used previously (*34*), we normalized the Ct values to eliminate errors not caused by the methods themselves; thus, we provided more accurate quantitative results.

In conclusion, these five endogenous reference genes cannot fully meet the legal requirements of being specific between different species and stable among cultivars. However, the urgency of GMO detection work requires use of the existing system at the present time. We suggest that the composition of the sample should be determined through other means, insofar as possible, before a rapeseed sample is analyzed by PCR. Although we found that only some of the genes had problems in quantification of some cultivars, the other systems may have as yet unidentified problems because we did not test all existing cultivars. We suggest using more than one endogenous reference gene for the calculation of the target taxon-specific DNA copy numbers and analyzing the source of the bias when these systems yield obviously different data.

We hope that this research will benefit safety assessments and the detection and control of GM *B. napus* and other species.

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