JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

Real-Time Polymerase Chain Reaction (PCR) Quantitative Detection of *Brassica napus* Using a Locked Nucleic Acid TaqMan Probe

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Several countries have introduced mandatory labeling requirements on foods derived from genetically modified organisms. Real-time quantitative Polymerase Chain Reaction (PCR) has quickly become the method of choice in support of these regulations and requires the development of separate PCR assays targeting the transgenic sequence as well as a specific endogenous gene sequence. To develop a *Brassica napus*-specific PCR assay, partial sequences of the *acetyl-CoA carboxylase BnACCg8* gene from *B. napus* and the closely related *Brassica rapa* were determined and compared, and a region of unique nucleotide sequence was identified. Universal amplification primers were designed to either side of this region, and a locked nucleic acid TaqMan probe was designed to the *B. napus*-specific sequence. Evaluation of this primer/probe combination indicated a high level of specificity to *B. napus*: no amplification signal was observed with any other species tested, including five closely related *Brassica* species. The method was assayed with 14 different *B. napus* cultivars, and comparable amplification curves were consistently obtained for all. The assay was highly sensitive, with a limit of detection between 1 and 10 haploid copies. Practically, the method was demonstrated to be effective for the detection of processed food samples and for the quantification of Roundup Ready canola content in mixed samples.

KEYWORDS: ***Brassica napus*; canola; acetyl-coenzyme A carboxylase; endogenous gene; genetically modified; real-time PCR; locked nucleic acid**

INTRODUCTION

Canola makes up 13% of the world's oilseed supply, second only to soybean in global oilseed production (*1*). Although canola seeds are crushed for their oil, the solids (meal) are also important and used as a protein supplement in animal feeds. Canola is grown extensively in Canada, Europe, China, India, and Australia and, to a limited extent, in the United States. It is one of four principal "biotech" crops grown worldwide, occupying 6% of the total global biotech area. In Canada, 77% of the canola hectarage is planted with genetically modified (GM) varieties, establishing canola as the dominant GM crop in this country (*2*). Growers choose GM varieties for improved weed control and use of minimum tillage and better soil management practices, leading to higher returns and increased profit. However, public concern about genetically modified organisms (GMOs) has influenced regulations for their production, marketing, and use as food and feed worldwide. Nations in the European Union (EU), as well as Japan, Australia, New Zealand, Thailand, and China, have implemented mandatory labeling for foods and feed derived from transgenic plants, and several other countries are considering voluntary or mandatory labeling proposals. Although no mandatory labeling legislation has been implemented in Canada to date, nearly 75% of the canola seed, oil, and meal produced in Canada is being exported (*3*), in some cases to destinations where mandatory labeling regulations exist (e.g., the EU, Japan, and China) or where cultivation of GM canola is not permitted (e.g., the EU). Therefore, the development of reliable methods to detect, identify, and trace transgenic events has become increasingly important for government regulators, international trade organizations, and industries utilizing these products.

Polymerase Chain Reaction (PCR) is among the most powerful and useful techniques for the identification and quantification of transgenic crops and food/feed products derived from these crops, primarily due to the stable nature of the DNA molecule, as well as the extreme sensitivity of the PCR technology (*4*). Real-time PCR has quickly become the method of choice for the quantitative analysis of GM content in food and feed products, and numerous studies have demonstrated the speed, sensitivity, and specificity of this method (see, for example, ref *5* and references cited therein). Quantification of GM content by real-time PCR is determined by comparing the relative amounts of transgene and endogenous reference gene sequences that can be amplified from a sample. The amount of endogenous gene that can be amplified gives an estimation of

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10.1021/jf052036m CCC: $33.50 Published 2006 by the American Chemical Society
Published on Web 01/21/2006

the total amount of target DNA in the sample, and the amount of GM content is calculated as a fraction of total plant-specific DNA. For accurate, reliable, and sensitive quantitative results, selection of a suitable reference gene sequence is critical. Ideally, this sequence should be highly specific for the plant species to be tested, present in a low and stable copy number, and highly homogeneous among cultivars of the same species.

Some genes currently used as endogenous references for GMO detection and quantification include the maize *10-kDa zein* gene (*6*), the soybean *Le1 lectin* gene (*6*), the rice *sucrose phosphate synthase* gene (*7*), the tomato *LAT52* gene (*8*), and the cotton *Sad1* gene (*9*). Canola is a member of the genus *Brassica*, which includes a wide variety of plant species under commercial cultivation, such as broccoli, cauliflower, cabbages, kale, Brussels sprouts, wong-bok, turnips, kohlrabi, swede, rutabagas, and various mustards. This makes selection of a gene sequence specific for canola, which will not cross-react with other *Brassica* species, particularly difficult in comparison to, for example, maize (*Zea mays*), which is the only commercially grown species of the genus *Zea*. Furthermore, commercially grown GM canola consists of two genetically distinct species, *B. napus* and *B. rapa*, which differ in genome size. Because genomic size is an important determinant used in quantitative real-time PCR calculations, it is essential to be able to distinguish the two species for accurate quantitative analysis of GM content. The development of several distinct *B. napus*-specific endogenous reference systems for use in quantitative real-time PCR has been reported, targeting such sequences as the *high-mobility-group protein I/Y* (*HMG-I/Y*) gene (*10*), the *phosphoenolpyruvate carboxylase* (*pep*) gene (*11*), the *acetyl-CoA carboxylase BnACCg8* gene (*12*), and the *acyl-acyl carrier protein* (*ACP*) *thioesterase FatA* gene (*13*). However, examination of these systems revealed that there was considerable cross-reactivity with other *Brassica* species, and none were able to distinguish between *B. napus* and *B. rapa*. The difficulty in finding an appropriate endogenous reference sequence specific only to *B. napus* lies in its close genetic association to *B. rapa*: *B. napus* (AACC genome) is an amphidiploid species resulting from the interspecific hybridization of *B. rapa* (AA genome) × *B. oleracea* (CC genome) (*14*).

Here we report a quantitative real-time PCR method to specifically detect *B. napus*. Due to the high degree of sequence similarity between *B. napus* and *B. rapa*, our method utilizes "universal *Brassica* primers" that amplify a region of the *acetyl-CoA carboxylase* (ACC) *BnACCg8* gene from both species, in combination with a *B. napus*-specific TaqMan probe located within a region of sequence divergence between the two species. Because the number of differing nucleotides in this region is small, specificity could be improved by decreasing the overall length of the probe; however, this is normally restricted due to minimum melting temperature (T_m) requirements for TaqMan probes. To circumvent this limitation, several locked nucleic acid (LNA) residues were incorporated into the design of the probe so that the length could be reduced while a high T_m was maintained. LNA nucleotides contain a methylene bridge that connects the 2′-oxygen of ribose with the 4′-carbon, resulting in a locked 3′-*endo* conformation that reduces the conformational flexibility of the ribose. The incorporation of LNA(s) into a DNA oligomer results in an increased T_m due to their rigid conformational structure (*15*). Compared to DNA, oligonucleotides containing LNA(s) have an increased T_m of up to 9.6 °C per LNA nucleotide incorporated, depending on the position and number of the modified residues and the total length of the oligomer (*16*). Additionally, the introduction of LNA oligonucleotides improves the hybridization affinity for complementary sequences (*15*, *17*), which helps to enhance the specificity of a reaction.

We demonstrate that our *B. napus*-specific system, utilizing a TaqMan probe containing LNA oligonucleotides, meets the requirements for a suitable endogenous reference and that it can be used for the quantification of GM canola derived from *B. napus*.

MATERIALS AND METHODS

Materials. Certified seed of the *Brassica* species *napus* 'Westar', *rapa* 'AC Parkland', *carinata* 'Dodolla', *juncea* 'Blaze', *nigra*, and *oleracea* 'Morden Midget' was provided by Plant Gene Resources of Canada. Seeds of 15 different *B. napus* cultivars were provided by the Canadian Food Inspection Agency. The species white mustard (*Sinapis alba*), radish (*Raphanus sativus*), flax (*Linum usitatissimum*), wheat (*Triticum aestivum*), rye (*Secale cereale*), barley (*Hordeum vulgare*), oat (*Avena sativa*), corn (*Zea mays*), potato (*Solanum tuberosum*), rice (*Oryza sativa*), soybean (*Glycine max*), sunflower (*Helianthus annuus*), and peanut (*Arachis hypogaea*) were collected by our laboratory. Crude and refined canola oils were obtained from an industrial canola oil processing plant. Commercial horse feed (proprietary blend) was purchased at a local market. Non-GM canola seed was provided by Monsanto (Chesterfield, MO). Defatted and ground transgenic Roundup Ready (RR) GT73 (synonym RT73) canola was prepared by POS Pilot Plant Corp., Saskatoon, Canada, using material provided by Monsanto. The reference materials containing 0, 0.5, 1.0, 2.0, and 5.0% of GT73 canola were prepared by our laboratory for quantitative analysis of RR canola content.

DNA Extraction. Genomic DNA was extracted and purified from all plant samples using a modified Wizard (Promega) protocol (*18*). Canola oils were concentrated, and the DNA was extracted according to the method of Green et al. (*19*). In brief, oil samples were initially aqueous extracted and then concentrated using centrifugal filter devices. DNA was extracted from the concentrated aqueous extracts using a high-salt phenol−chloroform method. Following the extraction, DNA was concentrated by the addition of 3 M sodium acetate (pH 5.2) and 95% ethanol and incubated at −20 °C overnight. DNA was pelleted by centrifugation, washed with 70% ethanol, dried, and resuspended in ultrapure nuclease-free water. DNA was quantified using a NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies, Inc., Montchanin, DE), and sample purity was determined by measuring the A_{260}/A_{280} ratio. The extracts (200 ng) were further analyzed by electrophoresis on a 0.8% agarose gel containing 0.1 μg/mL ethidium bromide.

Primers and Probes. PCR primers were designed with PrimerSelect software (DNASTAR, Inc., Madison, WI), using the *B. napus BnACCg8* gene (accession number X77576) as input sequence and based on the sequence alignment generated from the two cloned and sequenced *Brassica* species (see below). The Bnacc4912/Bnacc5055 primers amplified a 144 bp product. For specific detection of GT73 canola, the RT73 primer 1/primer 2 pair amplified a 108 bp product. Primer pair hmg-F/hmg-R amplified a 99 bp product. All primers were purchased from Operon Biotechnologies, Inc. (Huntsville, AL) except for the latter pair, which was purchased from Integrated DNA Technologies, Inc. (Coralville, IA). The TaqMan LNA probe was purchased from and designed in consultation with Integrated DNA Technologies, Inc., and was labeled with 6-carboxyfluorescein (FAM) on the 5′ end and with Iowa Black FQ quencher at the 3′ end. The TaqMan RT73 probe was purchased from IT BioChem (Salt Lake City, UT) and was labeled with FAM on the 5′ end and tetramethyl-6-carboxyrhodamine (TAMRA) on the 3′ end. The TaqMan hmg-P probe was purchased from Integrated DNA Technologies Inc. and was labeled with FAM on the 5′ end and TAMRA on the 3′ end. Primer and probe sequences are listed in **Table 1**.

PCR Conditions with the LightCycler. Unless otherwise stated, real-time PCR assays were carried out using a LightCycler 1.0 (Roche, Laval, Canada). For the *B. napus*-specific endogenous gene assays, a total reaction volume of 20 μL contained 10 mM Tris, pH 8.3, 25 mM KCl, 5 mM $MgCl_2$, 0.2 μM probe, 0.4 μM each primer, 0.3 mM dNTP,

Table 1. Primer and Probe Sequences for Real-Time PCR

primer/probe	sequence (5′→ 3′)	orientation	ref
Bnacc4912F	TTCAACAATAACATGCTGGTAATA	sense primer	this study
Bnacc5055R	GTGACGCATACGTTCTATAACATC	antisense primer	this study
napusLNA	TcTgAggACTcTTa[a]	sense probe	this study
RT73 primer 1	CCATATTGACCATCATACTCATTGCT	sense primer	*13*
RT73 primer 2	GCTTATACGAAGGCAAGAAAAGGA	antisense primer	*13*
RT73 probe	TTCCCGGACATGAAGATCATCCTCCTT		*13*
hmg-F	GGTCGTCCTCCTAAGGCGAAAG	sense primer	*10*
hmg-R	CTTCTTCGGCGGTCGTCCAC	antisense primer	*10*
hmg-P probe	CGGAGCCACTCGGTGCCGCAAGTT		*10*

[a] LNA nucleotides are shown in lower case.

0.25 mg/mL BSA, and 1 unit of FastStart Taq DNA polymerase (Roche). Reactions ran under the following conditions: initial denaturation at 95 °C for 10 min; 50 cycles of 5 s at 95 °C, 60 s at 60 °C, 8 s at 72 °C with a temperature transition rate of 20 °C/s; followed by cooling at 40 °C for 30 s. For the GT73 assays, a total reaction volume of 20 μL contained 50 mM Tris, pH 8.3, 6 mM $MgCl_2$, 0.2 μM probe, 0.4 μM each primer, 0.3 mM dNTP, 0.25 mg/mL BSA, and 1 unit of FastStart Taq DNA polymerase. Reaction conditions were as follows: initial denaturation at 95 °C for 10 min; 50 cycles of 5 s at 95 °C, 8 s at 60 °C, 8 s at 72 °C with a temperature transition rate of 20 °C/s; followed by cooling at 40 °C for 30 s. For the *HMG-I/Y* assay, a total reaction volume of 20 μL contained 50 mM Tris, pH 8.3, 5 mM $MgCl_2$, 0.2 μM probe, 0.4 μM each primer, 0.3 mM dNTP, 0.25 mg/mL BSA, and 1 unit of FastStart Taq DNA polymerase (Roche). Reaction conditions were as follows: initial denaturation at 95 °C for 10 min; 50 cycles of 5 s at 95 °C, 10 s at 60 °C, 6 s at 72 °C with a temperature transition rate of 20 °C/s; followed by cooling at 40 °C for 30 s. For all reactions, fluorescence was measured during each PCR cycle at the annealing step.

PCR Conditions with the iCycler. Temperature gradients were carried out using a Bio-Rad iCycler iQ (Mississauga, Canada). For the *B. napus*-specific endogenous gene assays, a total reaction volume of 25 μL contained 10 mM Tris, pH 8.3, 25 mM KCl, 5 mM $MgCl_2$, 0.2 μM probe, 0.4 μM each primer, 0.3 mM dNTP, and 1 unit of FastStart Taq DNA polymerase. Reactions ran under the following conditions: initial denaturation at 95 °C for 10 min; 50 cycles of 20 s at 95 °C, 90 s at 55−60 °C, and 30 s at 72 °C. Fluorescence was measured during each PCR cycle at the annealing step.

Cloning, Sequencing, and Data Analysis. Amplicons generated from various primer combinations were separated by electrophoresis on agarose gels. Fragments were cut from the agarose under UV light and purified using the QIAquick Gel Extraction kit (Qiagen, Mississauga, Canada). Purified PCR fragments were ligated into the pCR 2.1-TOPO vector and cloned using the TOPO TA cloning kit (Invitrogen Life Technologies, Burlington, Canada). Fragments were sequenced at the University of Victoria, Centre for Biomedical Research. The final sequence was based on a consensus of three to four individual clones. Sequence identification and homology analysis was done using BLASTN (NCBI). Sequence alignments were compiled and analyzed using MegAlign Lasergene (DNASTAR, Inc.) sequence analysis software (accession numbers DQ173668 and DQ173669 for *B. napus* and *B. rapa*, respectively).

Determination of the Sensitivity of the *B. napus*-Specific Assay. The limit of detection (LOD) for the *B. napus*-specific assay was determined using a serial dilution of DNA extracted from 100% canola flour. Samples were diluted to give a concentration range of approximately 100 ng−0.1 pg, equivalent to approximately 87000−0.1 copies of the *B. napus* genome, based on a *C* value of 1.15 pg for *B. napus* (*20*). Each serial dilution was assayed in triplicate per real-time PCR run, and four independent runs were performed.

Preparation of Standard Curves and Determination of RR Canola Content in Samples. Serially diluted DNA extracted from 100% GT73 canola flour was used to generate eight-point standard curves for the quantification of RR content of samples derived from GT73 canola flour. Samples were serially diluted, and each of three concentrations was assayed in triplicate per PCR run with both the *B. napus*-specific and GT73 detection systems. The equation of the standard curve (logarithm of nanograms of standard versus corresponding C_t) was used to determine the amount of either RR or the endogenous gene in the samples based on the mean of the measured C_t values. Percent RR canola was determined from the ratio of the amount of RR relative to the amount of the *B. napus* endogenous gene.

Figure 1. Design of the *B. napus*-specific real-time PCR assay. The sequence represents a portion of the *BnACCg8* gene with primer sites designated by arrows and the TaqMan probe site designated by a dotted line. Boxed regions indicate sequence differences between *B. napus* and *B. rapa*. The forward and reverse primers Bnacc4912 and Bnacc5055 amplify a 144 bp amplicon in both *B. napus* and *B. rapa*, with annealing sequences for the napusLNA probe placed in a region of species sequence divergence between the primers.

RESULTS

Selection of a Suitable DNA Sequence as a *B. napus*-Specific Endogenous Reference. The development of several distinct *B. napus*-specific endogenous reference systems for use in quantitative real-time PCR has been reported (*8*−*11*), all of which were determined to be species-specific; however, we found that each of these systems allowed the amplification of several *Brassica* species, including *B. rapa* as well as the desired *B. napus*. Therefore, to select a *B. napus*-specific DNA fragment suitable for use as an endogenous reference, we sequenced and compared ∼6300 bp (bp 3456−9715) within the *BnACCg8* gene of *B. napus* and *B. rapa*. Due to the high degree of sequence similarity between these species, only limited regions could be identified in which to design species-specific primers and probe. As a result, "universal *Brassica* primers" were designed, which amplify fragments in both *B. napus* and *B. rapa*, and a *B. napus*-specific TaqMan probe was designed in a divergent sequence region to differentiate the two species. Several LNA residues

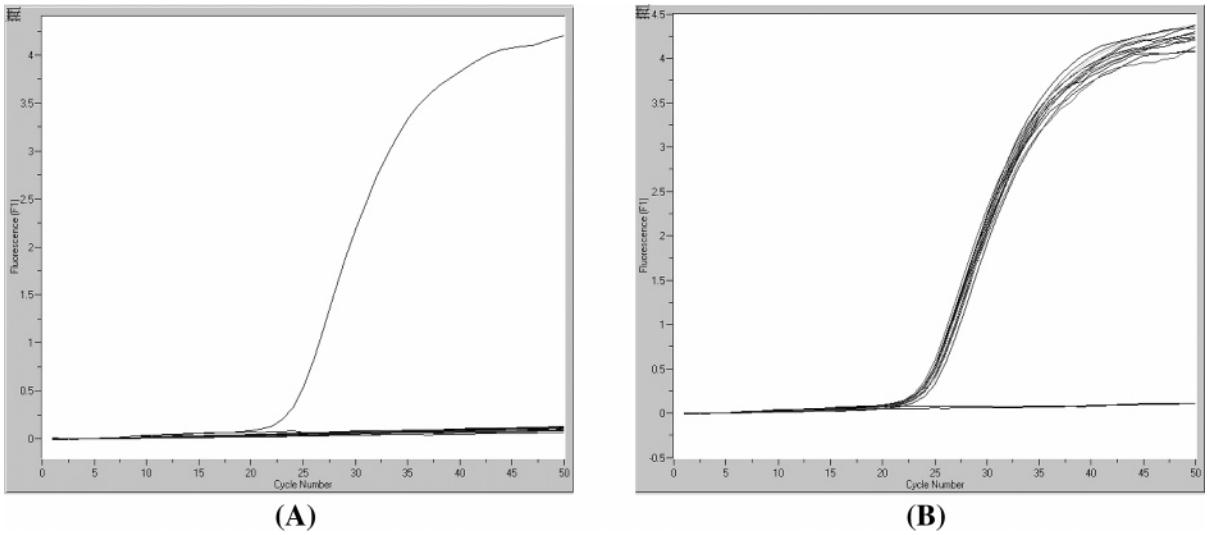

Figure 2. Species specificity and allelic variation analyses of the real-time PCR assay targeting *BnACCg8* gene sequences. (**A**) Specificity analysis: amplification plot generated from 18 different plant species (*B. rapa*, *B. nigra*, *B. oleracea*, *B. juncea*, *B. carinata*, *S. alba*, *R. sativus*, flax, wheat, rye, barley, oat, corn, potato, rice, soy, sunflower, and peanut) and *B. napus* with the Bnacc4912/Bnacc5055 primer pair and the napusLNA probe. No amplification signal was observed with any of the species tested other than *B. napus* (assayed in duplicate). (**B**) Allelic variation of the *BnACCg8* gene sequence among *B. napus* cultivars. Amplification plot generated with the Bnacc4912/Bnacc5055 primer pair and the napusLNA probe from 14 different cultivars, including SW Arrow, Cartier BX, Westar, Exceed, Hyola 401, 2473, 2273, 2153, 2663, 2393LL, AC Excel, Innovator, Sponsor, and SW RaideRR. No amplification product was observed for *B. rapa* or the no-template control.

Table 2. Amplification Data Used To Determine the LOD of the *B. napus*-Specific Real-Time PCR Assay

DNA amount (ng)	100	10	1.0	0.1	0.01	0.001	0.0001
signal rate (no. of positives)	12/12	12/12	12/12	12/12	12/12	8/12	1/12
mean concentration (ng)	108.55	10.16	0.9313	0.0873	0.0109	0.0012	
SD[a] of concentrations (ng)	1.65	0.36	0.0407	0.0057	0.0020	0.0001	
CV[b] of concentrations (%)	1.5	3.5	4.4	6.5	18.5	12.0	

[a] Standard deviation. [b] Coefficient of variation.

were incorporated into the probe, which permitted a shorter design while maintaining a high T_m. The positions of these primers and probe within the *BnACCg8* gene sequences of *B. napus* and *B. rapa* are shown in **Figure 1**.

Species Specificity of the *BnACCg8* Gene Sequence. Specificity of the *BnACCg8* target sequence was determined by real-time PCR using 50 ng of DNA from 19 different plant species. These included the six genetically related species of *Brassica* (*14*), *B. napus*, *B. nigra*, *B. oleracea*, *B. rapa*, *B. juncea*, and *B. carinata*; the closely related species *S. alba* (white mustard) and *R. sativus* (radish) (*21*); the major Canadian grain crops of flax, wheat, rye, barley, and oat; common food adulterants including corn, potato, and rice; as well as the common oil crops of soy, sunflower, and peanut. No amplification signal was observed in the real-time PCR assay (which was repeated twice) with any of the species tested other than *B. napus* (**Figure 2A**), demonstrating that the selected *BnACCg8* gene sequence is highly specific for *B. napus.* In addition, the optimal annealing temperature for the assay was determined using a Bio-Rad iCycler iQ with the six genetically related *Brassica* species. A temperature gradient from 55 to 60 °C was evaluated, and 60 °C gave optimal results; however, the reaction remained specific for *B. napus* at all temperatures (data not shown). To confirm the presence of amplifiable DNA, standard PCR was performed with DNA from the 19 different plant species using primers targeting the chloroplast tRNA-leu gene (*22*). The expected PCR product was obtained for all species tested (data not shown).

Allelic Variation of the *BnACCg8* Gene Sequence among *B. napus* Cultivars. To test for allelic variation of the amplified *BnACCg8* gene sequence among *B. napus* cultivars, four independent real-time PCR assays using 50 ng of DNA from 14 different *B. napus* cultivars were analyzed (**Figure 2B**). All cultivars gave similar C_t values, ranging from 23.84 to 24.91, indicating that the copy number of this gene is consistent and that there were no major sequence differences among the cultivars in the amplified region.

Sensitivity of the *B. napus*-Specific Real-Time PCR Assay. Serially diluted GT73 control DNA ranging from 100 ng to 0.1 pg was used to determine the LOD of the *B. napus*-specific real-time PCR assay. Each dilution was run in triplicate in four independent reactions. LOD is defined as the lowest quantity of target that can be detected reliably and quantified with a probability of ≥95% (*23*). Applying this definition, the LOD of the *B. napus*-specific real-time PCR assay was determined to be 10 pg (12/12 positive signals; **Table 2**), corresponding to 9 copies per haploid genome of *B. napus* (*20*). However, the absolute detection limit (*23*) was determined to be 1.0 pg (8/12 positive signals), corresponding to approximately 1 initial template copy. The coefficient of variation (CV) of the concentrations of each dilution was 1.5−18.5% (**Table 2**), with greater variability observed at lower concentrations. The CV values are indicative of the good robustness, reproducibility, and precision of this PCR assay. Estimated reaction efficiencies of the four runs were consistently high, ranging from 0.96 to 0.99, on the basis of the slope of the standard curve generated in each reaction (**Figure 3**).

***B. napus* Detection in Processed Foods.** Application of the *B. napus*-specific real-time PCR assay for the practical detection from material other than seed was determined using DNA

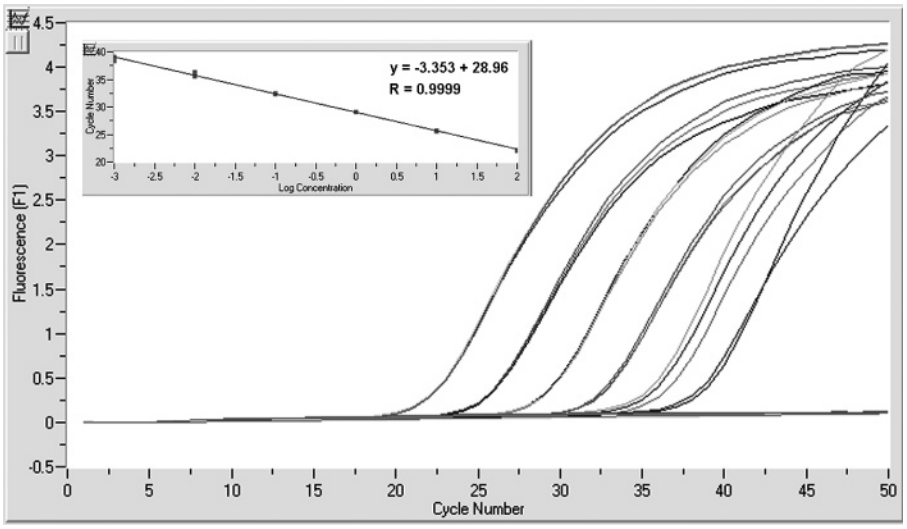

Figure 3. Limit of detection of the *B. napus*-specific real-time PCR assay. A representative amplification plot was generated by triplicate serial dilutions of *B. napus* DNA corresponding to 100, 10, 1, 0.1, 0.01, and 0.001 ng, and the standard curve (inset) was generated from the amplification data.

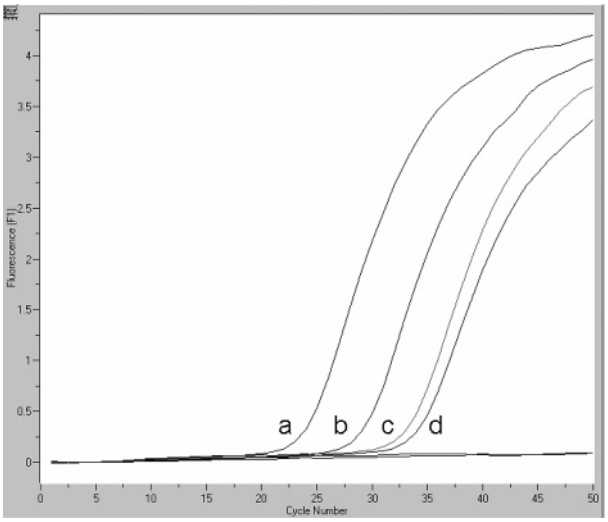

Figure 4. Detection of *B. napus* DNA from processed foods: amplification of the *BnACCg8* gene sequence in (**a**) *B. napus*, (**b**) horse feed, (**c**) refined canola oil, and (**d**) crude canola oil using the Bnacc4912/Bnacc5055 primer pair and the napusLNA probe. No amplification product was observed for *B. rapa* or the no-template control.

extracted from two major groups of processed foods derived from canola: canola oil (crude and refined) and animal feed. Amplification products from both of the oil samples as well as from horse feed were obtained (**Figure 4**). C_t values were 33.38, 34.16, and 29.14 for the refined oil, crude oil, and horse feed, respectively.

Quantification of RR Content in Transgenic Canola. RR content was quantified using the available GT73 material. Standard curves for the *BnACCg8* were generated from a 2-fold serial dilution ranging from 200 to 1.56 ng of total endogenous DNA, whereas for RR, 10−0.078 ng of DNA was used. Several test samples were made by mixing different amounts of 100% GT73 canola DNA with 100% non-GM canola DNA. Each test sample was assayed in triplicate at three different concentrations (total of nine assays) per PCR run with both the *B. napus*- and GT73-specific PCR assays. The mean PCR efficiencies of the *BnACCg8* and RR reactions were estimated at 0.97 and 0.98, respectively, on the basis of the slope of the standard curve. The mean calculated R^2 value for the *B. napus*-specific standard curve was 0.9999 (**Figure 5A**), and that of the GT73 standard curve was also 0.9999 (**Figure 5B**), indicating a good linear dynamic range for both reactions. In the quantitative analysis, experimental values of 0.46, 0.98, 1.93, and 4.74% were obtained for the test samples containing theoretical RR contents of 0.5, 1.0, 2.0, and 5%, respectively.

DISCUSSION

GM canola derived from both *B. napus* and *B. rapa* is commercially grown worldwide (*1*). Because these two species have different genome sizes, it is essential to be able to distinguish them for accurate quantitative analysis of GM content. Quantitative real-time PCR methods for GMO analysis require the identification of an endogenous reference control sequence that is species-specific, is present in low copy number, has high homogeneity among cultivars, and is sensitive and reliably detectable by PCR. We have developed a real-time PCR system using an LNA-modified TaqMan probe targeting a *B. napus BnACCg8* gene sequence that meets these criteria.

Several groups have reported the development of distinct *B. napus*-specific endogenous reference systems (*10*−*13*); in each case, however, *B. rapa* was not included in species specificity tests and, in our hands, none of these systems enabled the discrimination of *B. rapa* from *B. napus*. We determined also that the method reported by Weng et al. (*10*) allowed the amplification of the other genetically related *Brassica* species *juncea*, *nigra*, and *carinata*, with weak amplification of the genetically related species *oleracea* (**Figure 6**). Furthermore, we found that the methods reported by Zeitler et al. (*11*), Hernández et al. (*12*), and Monsanto (*13*) did not distinguish between *B. napus* and any of the aforementioned related *Brassica* species. Weng et al. (*10*) utilized the *B. napus HMG-I/Y* gene as an endogenous reference, stating that the copy number of the *B. napus BnACCg8* gene (used by Hernández et al.) has not been confirmed. However, through Southern blot analysis, Hernández et al. established that the *B. napus BnACCg8* gene was present in two to three copies per *B. napus* haploid genome. More recently, and by way of real-time PCR, Green et al. (*19*) confirmed that *BnACCg8* was present at about two copies per haploid genome in *B. napus*-derived GT73 canola. Therefore, we continued working with this low copy number gene for use as an endogenous reference and sequenced a section of ∼6300 bp in both *B. napus* and *B. rapa*, seeking a region of sequence divergence between the two species in which

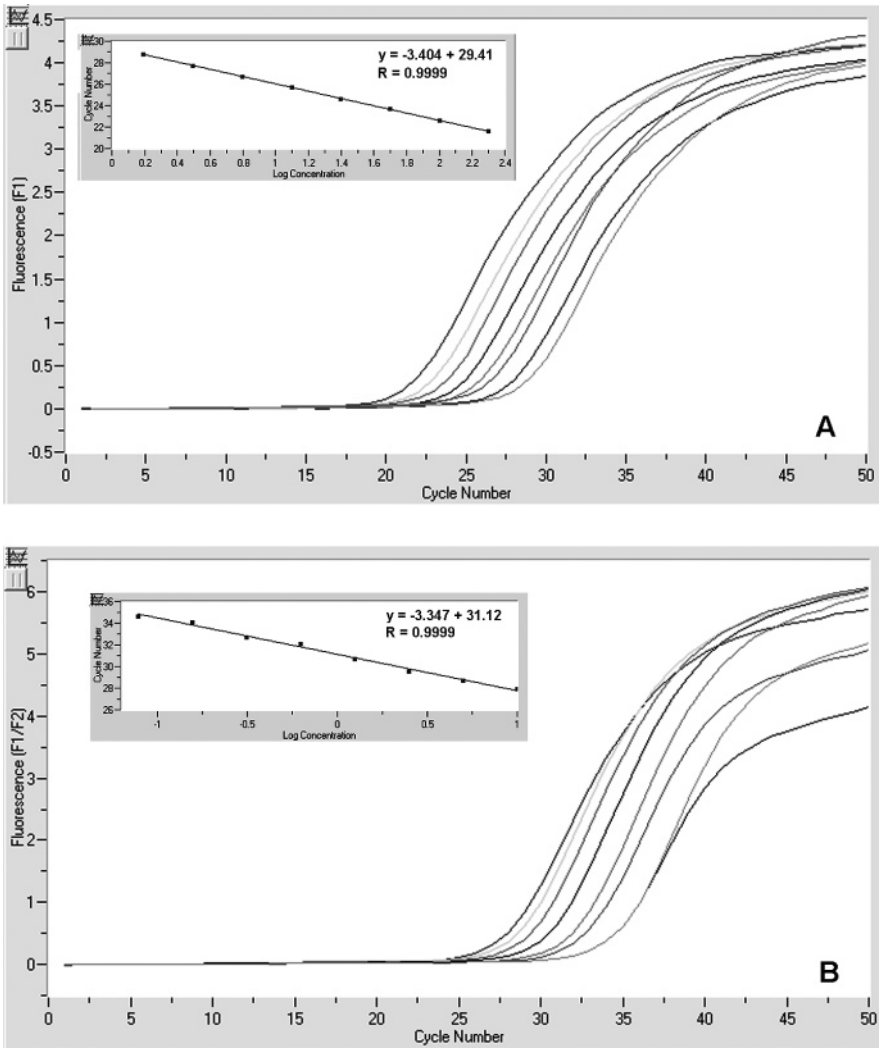

Figure 5. Standard curves of endogenous and transgenic real-time PCRs. Amplification plots were generated using known amounts of DNA and corresponding standard curves (inset) of (**A**) the *B. napus*-specific system for total *B. napus* DNA quantification with primer pair Bnacc4912/Bnacc5055 and napusLNA probe and (**B**) the GT73 system for RR quantification with RT73 primer 1 and primer 2, and RT73 probe.

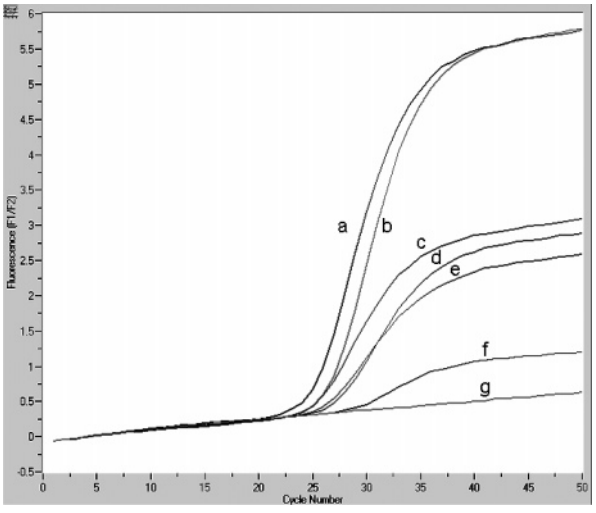

Figure 6. Specificity analysis of the hmg-F/hmg-R primer pair and the hmg-P TaqMan probe (*10*). The amplification plot was generated from the six closely related *Brassica* species [(**a**) *B. rapa*, (**b**) *B. napus*, (**c**) *B. juncea*, (**d**) *B. carinata*, (**e**) *B. nigra*, and (**f**) *B. oleracea*] and (**g**) the no-template control.

to design primers and probe. Sequencing of several *B. napus* clones identified two distinct sequences of the *BnACCg8* gene (data not shown), which also confirms the presence of at least two gene copies. Considering that *B. napus* is amphidiploid resulting from interspecific hybridization of *B. rapa* × *B. oleracea*, it is possible that in these species *BnACCg8* is/was present as a single copy, and two copies were present after hybridization. Due to the high degree of sequence similarity between *B. napus* and *B. rapa*, "universal *Brassica* primers" were designed, which amplify fragments in both species, and a *B. napus*-specific probe was designed in a divergent sequence region to differentiate the two species. The divergent region consists of a six-nucleotide insertion in the *B. napus* sequence not present in the corresponding *B. rapa* sequence (**Figure 1**). The number of differing nucleotides is small, so improving specificity by designing a short probe was desirable. There are different strategies to minimize the size of the probe while a relatively high T_m is maintained, including the incorporation of minor groove binder (MGB) ligands or LNA residues. Although the modes of action of MGBs and LNAs differ, they are functionally equivalent in terms of sensitivity and specificity in TaqMan 5′ nuclease assays (*24*). MGB ligands bind to the minor groove of the DNA helix of the target sequence, forming a highly stable nucleic acid duplex (*25*). Probes conjugated with MGBs also provide increased specificity compared with standard DNA probes (*26*). Alternatively, LNA residues may be incorporated into probes. As well as allowing reduction in probe

length, the introduction of LNA oligonucleotides improves the hybridization affinity for complementary sequences (*15*, *17*), which can help to enhance the specificity of a reaction. Goldenberg et al. (*27*) reported a similar real-time PCR assay approach, using a pair of "universal" eubacterial-conserved primers, which generated a 16S rDNA product spanning species-specific regions among several bacterial species, then using individual specific LNA/FRET probes to identify and quantify different species. Other real-time PCR applications utilizing LNA technology have been described recently, including the use of LNA primers and probes in single-nucleotide polymorphism (SNP) genotyping assays (*28*−*31*) and for the detection of bacterial pathogen genes (*24*).

Analysis of genomic DNA isolated from 19 different plant species revealed that the *B. napus* real-time PCR detection method using an LNA-modified TaqMan probe is highly specific. It is important that any real-time PCR method for quantitative GM canola analysis be unequivocally species-specific, as GM canola lines are derived from more than one species with differing genome sizes. Although canola crops are largely derived from the species *B. napus* and *B. rapa*, a more heat-tolerant canola oil quality variety from the *B. juncea* species was developed and, in 2002, introduced under contract production in Canada (*32*). This variety is expected to expand the production of canola into more hot and dry regions of Canada and other countries and will undoubtedly be used in the development of transgenic lines. Furthermore, canola can be a problematic crop concerning contamination during harvest, because it is a crop in which close weedy relatives, such as *Raphanus*, *Sinapis*, and other *Brassica* species, occur within the crop (*33*). The method developed here results in no amplification from genetically related *Brassica* species *rapa*, *juncea*, *nigra*, *oleracea*, and *carinata* or from the closely related species *S. alba* and *R. sativus*. Additionally, an amplification signal was not detected for the major Canadian grain crops of flax, wheat, rye, barley, and oat, which may occur as grain contaminants; common food adulterants including corn, potato, and rice; as well as the common oil crops of soy, sunflower, and peanut. In the selection of an appropriate endogenous reference gene, database searches alone are not sufficient; empirical testing of species specificity must also be carried out, as countless gene sequences for numerous different plant species have yet to be entered into sequence databases.

An ideal endogenous reference gene should not exhibit allelic variation among cultivars of the same species and should be present in the same number of copies in all cultivars. Fourteen different *B. napus* cultivars in four independent assays were tested, and all cultivars gave similar C_t values, indicating consistent copy number and high homogeneity among the cultivars tested. Furthermore, the real-time assay displayed a high level of sensitivity, with a practical LOD of 10 pg and an absolute LOD of 1.0 pg of *B. napus* template DNA. This sensitivity is similar to that reported for the maize *10-kDa zein* gene (*6*), the soybean *Le1 lectin* gene (*6*), the rice *sucrose phosphate synthase* gene (*7*), the tomato *LAT52* gene (*8*), and the cotton *Sad1* gene (*9*), all of which are used in quantitative GMO analysis. Given the sensitivity, and to test the practicality of the method as an endogenous reference assay for *B. napus* in processed foods derived from canola, DNA extracted from canola oil (crude and refined) and horse feed was tested. Amplification from both oil samples, as well as from horse feed, was observed, indicating that the method is effective for processed foods, even from difficult matrices which have been subjected to significant mechanical treatment, such as refined canola oil. Assay performance was further evaluated and confirmed using a different real-time thermal cycler (Bio-Rad iCycler iQ) under various annealing temperatures. At all temperatures, results were comparable to those obtained on the LightCycler, clearly demonstrating the robustness of the method.

To test the reliability and accuracy of the PCR assay targeting the *BnACCg8* gene sequence as an endogenous reference in real-time quantitative PCR, the assay was used in combination with a canola GT73-specific assay (*13*) to detect and quantify the amount of transgenic GT73 (RR) canola present in a given sample. An objective of these experiments was to check whether standard curves of sufficient quality could be generated and used for relative quantification purposes. Using the standard curves obtained, the quantitative estimates for the mixed samples corresponded well with the assigned concentrations, and overall reaction performance was excellent: slope and R values were consistently close to the theoretically optimal value, indicating high reaction efficiencies (slope), and a high correlation between the original amount of template DNA and C_t value was obtained after amplification (R), demonstrating that this assay is well suited for quantitative measurements. Furthermore, the *B. napus*-specific system is highly reproducible. In the LOD experiments, four standard curves were generated, and for each one, three replicates of each dilution were included, for a total of 12 replicate points for each dilution. Variation among replicates was determined by measuring the CV, based on calculated concentrations for each dilution, and ranged from 1.5 to 18.5%, in contrast to CV values calculated from C_t values, which ranged from 0.49 to 1.07%. In several previously reported studies (*7*−*10*), variation was calculated from the C_t values, which misrepresents true variability and should be avoided because C_t values are logarithmic units (*34*, *35*); instead, data used for variation calculations should be linear values, such as concentration or copy number.

Results of the experiments described herein demonstrate that utilization of an LNA TaqMan probe targeting a *BnACCg8* gene sequence unique to *B. napus*, together with more universal *Brassica*-specific primers, satisfies the criteria for a good endogenous reference system for the detection and/or quantification of *B. napus*: species specificity, high homogeneity among cultivars, sensitivity, reliability, and reproducibility. Additionally, these results demonstrate that employing shorter, LNA-modified probes in quantitative real-time PCR assays can effectively address the issue of specificity and discrimination between closely related species.

ACKNOWLEDGMENT

We thank Dennis Welch of POS Pilot Plant Corp. for providing defatted and ground transgenic canola.

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Received for review August 18, 2005. Revised manuscript received December 14, 2005. Accepted December 19, 2005.

JF052036M