JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

J. Agric. Food Chem. **2002,** *50,* 4431−4436 **4431**

Detection and Quantification of Transgenes in Grains by Multiplex and Real-Time PCR

HUGO R. PERMINGEAT, MARTÍN I. REGGIARDO, AND RUBÉN H. VALLEJOS*

Centro de Estudios Fotosintéticos y Bioquímicos (CEFOBI), (CONICET, Fundación M Lillo; Universidad Nacional de Rosario), Suipacha 531, 2000 Rosario, Argentina

Multiplex PCR reactions were developed for detecting simultaneously the *CryIA(b)* and *pat* genes from events 176, MON810, BT11, and T25 of transgenic maize, using only two pairs of primers, one for the *CryIA(b)* gene and the other for the *pat* gene. The Roundup Ready soybean can be precisely detected by a multiplex PCR reaction using known primers, amplifying fragments of the *NOS* and the *epsps* sequences simultaneously. Transgenic events such as Roundup Ready soybean and GA21 maize, among others, can be quantified by real-time PCR using a pair of primers and a probe specifically designed for annealing to the *NOS* ending region. As an alternative to amplifying an endogenous gene, the addition of a foreign gene in a percentage equal to the required level of detection, in a parallel reaction, is proposed. The use of hexane to homogenize large flour samples is suggested.

KEYWORDS: GMOs; real-time PCR; maize; soybean; CryIA(b), PAT

INTRODUCTION

In the year 2000, production of transgenic crops reached 44.2 million ha, and they were grown mostly in the U.S. (30.3 million ha), Argentina (10 million ha), and Canada (3 million ha). The main transgenic crops are soybean (26 million ha), maize (9.8 million ha), and cotton (5.4 million ha), and the principal traits are herbicide and insect tolerance (*1*). International markets and regulations of some countries require detection and/or identification and quantification of genetically modified organisms (GMOs) that may be present in grains and foodstuff. Labeling may be required by some parties. For instance, the European Community (EC) requires labeling of foodstuffs containing GMOs and is asking for sampling and testing for GMOs of unlabeled imports. EC allows up to 1% of adventitious presence of authorized GMOs in imported food (EC regulations 258/97, 1139/98, and 49/2000).

Presently, the polymerase chain reaction (PCR) and immunological methods are used for analysis of GMOs. PCR is the screening method of choice. Most of the authorized events for commercialization are detected by conventional (end point) PCR using primers that recognize a region of the *CaMV 35S* promoter (*2*). It is important to have other available primers to confirm the results, and especially to identify the event that is present in the sample. In the case of Roundup Ready soybean, different pairs of primers have been published, including for the NOS fragment and the coding region of the transgene (*3*). A nesting method may be used for confirmation (*4, 5*). In the case of maize, different events are being commercialized. For instance, three of them contain a synthetic *CryIA(b)* gene (176, MON810, and BT11), three contain a synthetic *pat* gene (T14, BT11, and T25), and another contains a modified *epsps* gene (GA21). Primers for identifying the Maximizer maize (event 176 from Syngenta) have been described in the literature (*6, 7*), including a nesting method for conventional PCR (*8*). The *CryIA(b)* synthetic gene sequence of the event MON810 is not available, even though specific primers for identifying it have been published. They combine the first 21 nucleotides from the coding region with the upstream regulatory region of the *hsp70* intron 1 (*7*), or amplify a region of the junction between the 35S promoter of the transgene with the plant genome (*9*). The event BT11 from Syngenta contains a different synthetic *CryIA(b)* gene. It may be identified by using specific primers that anneal in the promoter or intron and in the *CryIA(b)* gene (*7*) or in the selectable marker gene (*pat*) (*9*). The same strategy is used to identify the event T25 (*7*) or GA21 (*10*).

More recently, real-time PCR (RTPCR) procedures for detecting and quantifying the events Maximizer 176 maize and Roundup Ready soybean in food was reported by Vaïtilingom et al (*11*). Fluorogenic probes (TaqMan) to detect the endogenous genes (zeins and lectins) and the transgenes (synthetic *CryIA(b)* and *epsps*) were used successfully. Two picograms of transgenic DNA could be detected per gram of the starting sample. Later, Trapmann et al (*12*) published sequences of primers and a probe based on the detection of *CaMV 35S* promoter. After the characterization of the transgene insertion in Roundup Ready soybean, three new detection and quantification approaches were reported (*13*−*15*).

In this paper we describe three multiplex PCR reactions (two for maize and one for soybean) to detect GMOs in grains, and the quantification of the *NOS* fragment by RTPCR. Besides, we recommend the use of hexane to simplify the homogeniza-

* To whom correspondence should be sent. Tel: +54 341 437-1955. Fax: +54 341 437-0044. E-mail: cefobi@arnet.com.ar.

10.1021/jf020081d CCC: $22.00
Published on Web 07/04/2002

Table 1. Primer Sequences and Amplification Products

primer	gene	orientation	sequence	ampl. prod. (bp)	ref.
1F	*CryIA(b)*	sense	5′- ATG GAC AAC AAC CCC AAC ATC –3′	204	this paper
1R		antisense	5′- AAA GAT ACC CCA GAT GAT GTC –3′		
2F	*pat*	sense	5′- GAA GGC TAG GAA CGC TTA CG –3′	262	this paper
2R		antisense	5′- GCC AAA AAC CAA CAT CAT GC –3′		
3F	*35S-epsps*	sense	5′-CCA CTG ACG TAA GGG ATG ACG-3′	447	(*16*)
3R		antisense	5′-CAT GAA GGA CCG GTG GGA GAT –3′		
4F	*NOS*	sense	5′-TTA AGA TTG AAT CCT GTT GCC G-3′	192	(*3*)
4R		antisense	5′-TAA TTT ATC CTA GTT TGC GCG C-3′		
5F[a]		sense	5′- GTA ATG CAT GAC GTT ATT TAT GAG A –3′	104	this paper
P1		antisense	5′- TGC GGG ACT CTA ATC ATA AAA ACC CA –3′		

[a] This primer has to be combined with primers 4R and P1 for a RTPCR analysis.

tion of flour samples, and we discuss different alternatives to check for the presence of inhibitors in the DNA isolated for amplification.

MATERIALS AND METHODS

Grains from four transgenic maize events (insect resistant MON810 from Monsanto, 176 and BT11 from Syngenta, and herbicide resistant T25 from Agrevo), from one transgenic soybean event (Roundup Ready, 40.3.2), and from nontransgenic maize and soybean, were ground to flour with a blender (Romer Mill, Series II) and used for the analyses. These seeds were obtained from Pioneer Hybrid, Pergamino, Argentina (events MON810 and T25), Nidera (Venado Tuerto, Argentina) (events 176 and 40-3-2, and nontransgenic soybean), Cargill, Pergamino, Argentina (nontransgenic maize), and Syngenta (Venado Tuerto, Argentina) (event BT11).

A 200-mg portion of flour was used as starting material to isolate the DNA by the CTAB method reported by Lipp et al (*2*). After precipitation, the pellet was dissolved in 50 μL of deionized destilated water. A small aliquot were loaded in 1% (w/v) agarose gel and electrophoresed at 40 mA for 1 h to check the integrity of DNA. The samples, including the DNA for the RTPCR standard curve, were quantified fluorometrically in triplicate in a Perkin-Elmer (Norwalk, CT) 650-40 fluorescence spectrophotometer using the H33258 dye (Hoesch Sigma-Aldrich, St. Louis, MO) as described by Ausebel et al. (*16*).

Conventional PCR was carried out in a PTC-96 (MJ Research, Waltham, MA) or a Sontec (Buenos Aires, Argentina) thermocycler in a final volume of 25 μL, containing different concentrations of $MgCl_2$ according to the fragment to amplify (1.5 mM for *CryIA(b)* and for the multiplex *epsps* and *NOS*; 2 mM for *pat* and the multiplex *zein* and *35S*; and 3 mM for the multiplex *Bt* and *pat*), dNTP (0.2 mM for single or 0.3 mM for multiplex reactions), primers (0.4 μM for single and 0.25 μM for multiplex reactions), 1.25 units of Taq Polymerase and 1× PCR buffer. All reagents were from Promega (Madison, WI) except for the primers which were synthesized by Biosynthesis Inc. (Lewisville, TX). Conditions for amplification were as follows: denaturing of DNA at 95 °C for 5 min, 40 cycles of 30 s at 95 °C, 30 s at 60 °C, 40 s at 72 °C, and a final extension at 72 °C for 3 min, unless otherwise stated. Amplification products were electrophoresed in 2% agarose gels for 1 h at 40 mA, and stained with EtBr for visualization.

Real-time PCR assays were carried out in a Perkin-Elmer AB5700 SDS (Applied Biosystems, Foster, CA) using the TaqMan system in a final volume of 25 μL. The reaction mixture included 4.5 mM $MgCl_2$, 0.4 mM each of dATP, dCTP, and dGTP, 0.8 mM dUTP (Applied Biosystems), 0.3 μM forward and reverse primers, 0.3 units of AmpErase Uracil *N*-glycosylase (Applied Biosystems), 1.25 units of Taq Polymerase (Hot-Start from Quigen, Valencia, CA, or Platinum from Invitrogen, Carlsbad, CA), 1× PCR buffer (from Quiagen or Invitrogen), and 0.25 μM fluorogenic probe (IDT, Coralville, IA). Probes were labeled with the fluorescent reporter dye 6-carboxyfluorescein (FAM) at the 5′-end, and the fluorescent quencher dye 6-carboxytetramethylrhodamine (TAMRA) at the 3′-end. Conditions for amplification were 2 min at 50 °C, 10 min at 95 °C, and 45 cycles of 15 s at 95 °C and 60 s at 60 °C. Each sample, including all the controls and points from the standard curves, were quantified in triplicates. Results were analyzed using a sequence detection system provided by P. E. Applied Biosystems.

Target DNA of maize (100 ng) or soybean (42 ng) was added to each PCR tube, representing 36 697 and 37 168 copies of the respective genomes (1 c maize: 2.75 pg; 1 c soybean: 1.13 pg) (*17*).

Safety. Laboratory procedures and manipulation of transgenic samples were performed according to good laboratory practices and the Argentine Regulatory Office (CONABIA) normatives. All the transgenic events used are approved for commercialization in Argentina.

RESULTS AND DISCUSSION

One pair of primers for amplifying a region of the synthetic *CryIA(b)* genes present in maize 176, BT11, and MON810 was designed (**Table 1**, primers 1F and R). The sequence corresponding to the synthetic *CryIA(b)* gene from event 176 (U.S. patent 5,625,136) was used for the design. **Figure 1A** shows the amplification of a fragment of 204 bp, not only in target DNA from event 176 but also from events BT11 and MON810. Because the sequence of the latter is not available, the corresponding amplicon was sequenced (**Figure 2**, GenBank AF465640). The amplicon sequence translates into the first 68 amino acids of the native *CryIA(b)* polypeptide (**Figure 2**, DNA AF059670, protein ID AAC64003). When it was compared with the corresponding DNA fragment from the event 176 (U.S. patent 5,625,136) a nucleotide homology of 89% was found, resulting in 28% of the codons changed.

The synthetic *pat* gene (U.S. patent 5,276,268) present in events T25 and BT11, conferring resistance to the herbicide ammonium glufosinate, was detected by the primers 2F and R described in **Table 1**. They amplify a fragment of 262 bp corresponding to the mentioned transgene, as shown in **Figure 1B**. In the four events 0.1% of transgenic DNA was clearly detected (**Figure 1A**, **B**).

The primers described above for detecting the *CryIA(b)* and *pat* genes can be used in a multiplex PCR reaction as shown in **Figure 1C**, where 0.5% of each of the Bt events DNA are simultaneously detected with 0.5% of T25 DNA. This multiplex reaction was optimized combining the four primers at different concentrations (1F and R at 0.25 μM, 2F at 0.067 μM and 2R at 0.125 μM), adding 3 mM $MgCl_2$, and setting the annealing temperature at 58 °C. The sensibility in this multiplex reaction was 0.5%. Thus, only one reaction is needed to detect any of the four events of transgenic maize (i.e., 176, BT11, MON810, and T25) that may be found in commercial grains.

Primers to identify Bt maize were previously reported to be used in single-end-point PCR (*6*−*10*). In our hands, one pair of these primers that anneal to the *CryIA(b)* coding region (*8*) from event 176 poorly detect the event MON810, amplifying

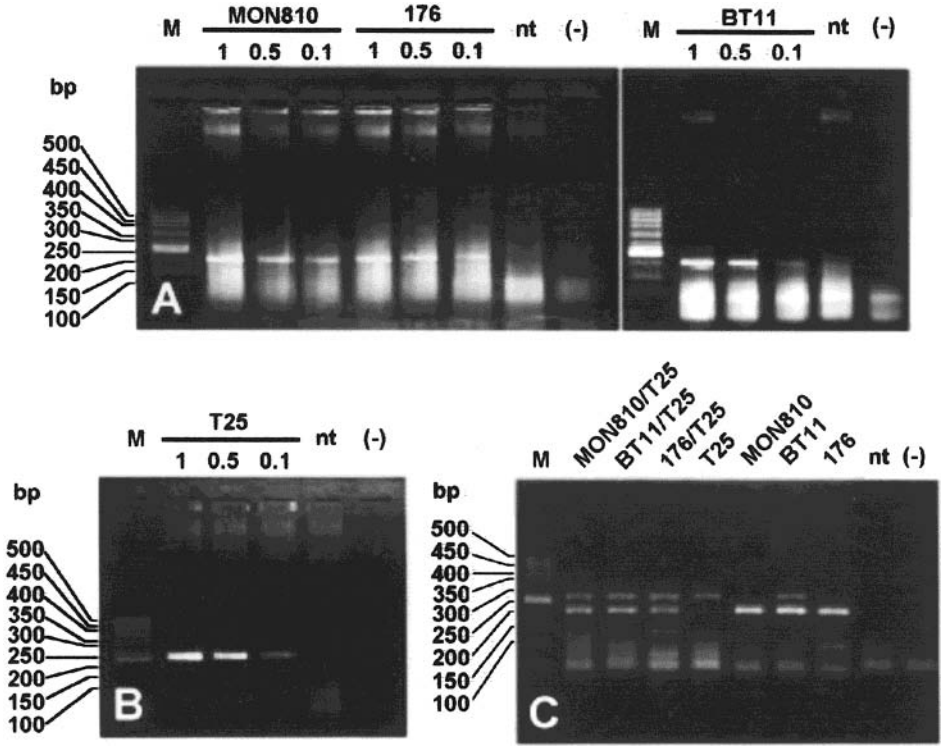

Figure 1. Single and multiplex PCR amplification of the fragments BT and PAT. Gel electrophoresis of the amplification products of PCR corresponding to the 204 bp-BT (A) and 262 bp-PAT (B) genes. Different amounts (1, 0.5, and 0.1%) of transgenic DNA in 50 ng total DNA were tested for each transgenic event (MON810, 176, BT11, and T25). The multiplex reaction (C) amplified both fragments simultaneously; nt, nontransgenic DNA; (–), no template control; M, 50 bp-ladder.

```
  1 - ATGGACAACAACCCCAACATCAACGAGTGCATCCCGTACAACTGCCTCAGCAACCCTGAG
    - M  D  N  N  P  N  I  N  E  C  I  P  Y  N  C  L  S  N  P  E
 61 - GTCGAGGTGCTCGGCGGTGAGCGCATCGAGACCGGTTACACCCCCATCGACATCTCCCTC
    - V  E  V  L  G  G  E  R  I  E  T  G  Y  T  P  I  D  I  S  L
121 - TCCCTCACGCAGTTCCTGCTCAGCGAGTTCGTGCCAGGCGCTGGCTTCGTCCTGGGCCTC
    - S  L  T  Q  F  L  L  S  E  F  V  P  G  A  G  F  V  L  G  L
181 - GTGGACATCATCTGGGGTATCTTT
    - V  D  I  I  W  G  I  F
```

Figure 2. Sequence and peptide translation of the amplicon generated by primers 1F and R, corresponding to the BT fragment present in event MON810 of transgenic maize. The sequence underlined corresponds to the primers used for amplification.

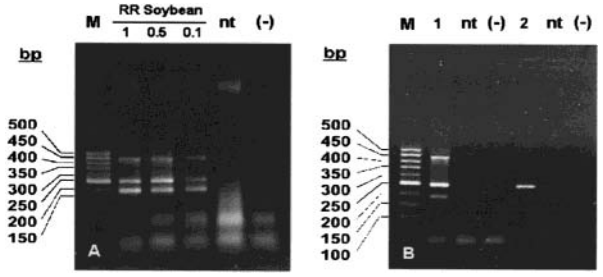

Figure 3. Multiplex PCR reaction of fragments *epsps* and *NOS* present in Roundup Ready soybean. Gel electrophoresis of the amplification products of PCR corresponding to: (A) a multiplex PCR reaction of *epsps* and *NOS* fragments, generating bands of 447 bp and 192 bp, respectively. Different amounts (1, 0.5, and 0.1%) of transgenic DNA in 34 ng of total DNA were tested for the transgenic event (Roundup Ready soybean). (B) Lane 1, is equal to panel A, lane 1; Lane 2, forward primer from *NOS* and reverse primer from *epsps* were used; nt, nontransgenic DNA; (–), no template control; M, 50 bp-ladder.

the expected fragment of 420 bp when PCR conditions are modified (data not shown). We report in this paper a new pair of primers to detect the mentioned *CryIA(b)* coding regions present in transgenic maize. Besides, they may be used with other pair of primers in a multiplex reaction to detect the *pat* gene present in other transgenic events. The use of the multiplex to detect and identify transgenic events in samples containing maize was recently reported by Matsuoka et al (*10*). They developed a multiplex PCR to identify 5 different transgenic maizes, with a sensibility of 0.5% of each GMO.

Another multiplex PCR reaction was set to detect precisely and simultaneously the *epsps* and *nos* fragments, both corresponding to the transcriptional unit of the introduced gene in Roundup Ready soybean, using the primers 3F and R, and 4F and R (*3, 18*). They amplify fragments of 447 and 192 bp respectively, as shown in **Figure 3A**. A third band of 252 bp appeared consistently in this reaction. This amplicon was generated by the *nos*-forward primer and the *epsps*-reverse primer as shown in **Figure 3B**. Sequencing the 252 bp amplicons from the multiplex (**Figure 3B**, lane 1) and from lane 2 (**Figure 3B**) gave identical results (GenBank AF465641) confirming that they correspond to a part of a truncated second copy of the transgene at 3′ of the first transcriptional unit, as previously described by Windels et al (*19*). The sensibility in this multiplex reaction is consistently 0.1%.

Different strategies to detect transgenic soybean by conventional PCR have been described; for instance, an amplification of fragments of the *35S* promoter (*2*−*4*), the *NOS* ending (*2, 3*), and the junction of 35S promoter and *epsps* coding region (*4, 5*). The multiplex proposed in this paper leads to more accurate results, because three fragments of the same transcriptional unit are amplifying simultaneously.

A pair of primers and an internal hybridization fluorogenic probe for detecting the *NOS* fragment, present in the Roundup Ready soybean DNA and in GA21 maize, and in other transgenic crops that will appear in the market soon, were designed for quantitative real-time PCR (**Table 1**, primers 5F

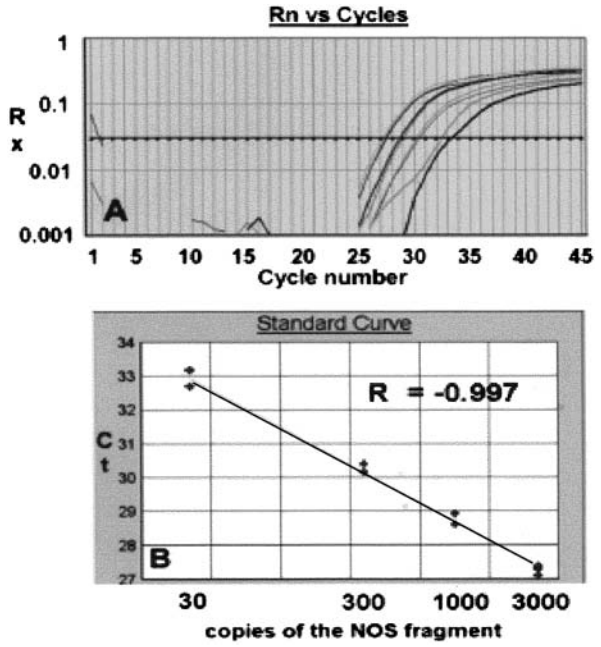

Figure 4. Real-time PCR of the *NOS* fragment from Roundup Ready soybean, using TaqMan. Amplification plot (A) and standard curve (B) generated by 3000, 1000, 300, and 30 copies of the NOS fragment present in transgenic soybean DNA, when using TaqMan detection system, showing an R^2 value of 0.997 and a slope of −3.62.

and 4R, probe P1). A standard curve with 100% Roundup Ready soybean was constructed by serial dilution to represent from 3000 to 30 copies of the transgene in a background of 34 ng total DNA (i.e., 30 000 copies). The DNA was equalized to 34 ng with nontransgenic soybean DNA. This curve revealed a correlation (R^2) of −0.997 and a slope of −3.62 (**Figure 4**). The lowest amount of transgenic DNA (30 copies, corresponding to 0.1% of the total DNA) was observed after 33 cycles (**Figure 4B**).

Vaïtilingom et al. (*11*) designed primers and fluorogenic probes for real-time PCR (TaqMan) for the *epsps* gene from Roundup Ready soybean and for the *CryIA(b)* gene present in the maize event 176. Later, Trapamann (*12*) published a pair of primers and a probe to quantify the presence of the 35S promoter. Three recent reports (*13*−*15*) describing the quantification of transgenic soybean by real-time PCR suggest the amplification of the junction region of the transgene and the plant DNA. Our approach, based on the quantification of the *NOS* ending by real-time PCR, may be used in a generic and reliable way to quantify the presence of Roundup Ready soybean and GA21 maize, and other transgenic crops that may appear in the market.

However, quantification of transgenes introduced into the maize genome may be uncertain. It should be noted that in a real-time PCR the number of copies of the transgene is determined and divided into the total copies of DNA, giving a percentage. One cause of uncertainty in quantifying transgenes present in maize grain samples may be the fact that the transgene copy number depends on the event(s) present in them. For instance, one copy of the 35S promoter is present in events T25 and MON810, two copies are in the event BT11, and 2−5 copies are in event 176 (*20*). Moreover, if the hybrid commercial seeds are hemizygous (only one parental line is transgenic) such as in the event MON810 (*21*), the grains harvested are a mix of homozygous (2 copies of the transgene per diploid genome), hemizygous (1 copy of the transgene per diploid genome), and

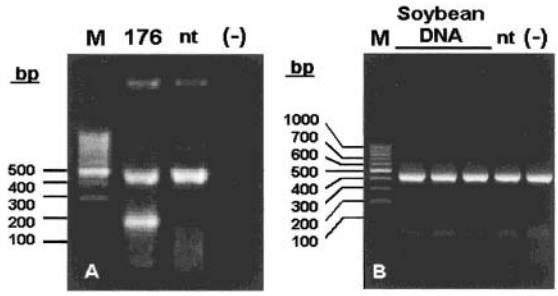

Figure 5. Evaluation of isolated DNA quality (A). Amplification products of multiplex PCR reaction of the endogenous *Zein* gene and the *CaMV 35S* promoter using imbalanced primers. The sample contains 0.5% of 176 maize DNA. (B) Amplification products of the *epsps* gene when transgenic soybean DNA was added to the reaction mixture in order to achieve an equivalent of 0.5% in each tube; M, 100 bp ladder; nt, nontransgenic DNA from maize in A and soybean in B; (–), no template DNA.

nontransgenic grains. Care should also be taken with the source of transgenic DNA for the standard curve. Using a plasmid (*15*) or DNA from hemizygous seeds of the event MON810 (*21*) is more accurate than the reference material prepared with event 176 (maize powder IRMM 411, Fluka). The latter one is useful to quantify samples containing only the event 176 since underestimation of the transgene copy number may occur if grains from the other events are present in the sample.

To evaluate the quality of DNA in conventional PCR or in quantitative RTPCR the amplification of an endogenous gene such as lectin in soybean or zein in maize has been suggested (*4*−*6*, *8*, *19*, *22*). This method may have a disadvantage at the time of detecting the presence of inhibitors in the DNA sample because the endogenous gene could be detected even when the amplification is partially inhibited, while low levels of the transgenes may not. In other words, with 100 ng of maize DNA in the PCR tube, almost 37 000 copies of a single-copy endogenous gene are present as target for amplification, while 37 copies of the transgene will have to be detected if the required level of detection is 0.1%.

An alternative is to lower the concentration of the endogenous gene primers. For instance, we successfully used a multiplex reaction for detecting zein and 35S promoter where zein primers are added at 0.08 μM while keeping in 0.35 μM the primers for 35S promoter as shown in **Figure 5A**. The lower concentration of primers for zein limits its amplification. If an inhibitor is present it may be detected. In addition, a slight increase of the detection limit of the transgene is achieved (data not shown), in agreement with Matsuoka et al. (*10*). This is probably explained by diminishing competition for the limiting reagents.

Another way to confirm that a negative result is not a false negative induced by inhibitors is by adding an amount of transgenic DNA (corresponding to the required level of detection) to the cocktail in a parallel experiment. If the transgene is not detected, it suggests that the purity of DNA of that sample may be inadequate. For instance, three different soybean DNA samples that gave negative PCR results for the *epsps* gene (not shown) were analyzed using a PCR reaction mixture containing the equivalent of 0.5% Roundup Ready soybean DNA in each tube. The results shown in **Figure 5B** indicate that the DNAs were amplifiable and the samples were free of inhibitors, confirming that they were negatives.

Finally, a third way to evaluate the presence of inhibitors is to add as an internal control, to a parallel reaction mixture, an amount of transgenic DNA from a different crop (e.g., adding

Table 2. Detection of 35S Promoter in DNA from Maize Flour and Soybean DNA[a]

sample	Ct	Ct SD	copy number 35S	35S average	total	% GMO
	34.37		38.48			
maize 0.1% MON810	34.25	0.52	40.97	44.23	37000	0.12 ± 0.02
	33.78		53.24			
	30.46		335.91			
maize 1% MON810	30.57	0.11	315.71	336.12	37000	0.91 ± 0.04
	30.35		356.75			
	30.64		303.50			
soybean 0.5% Roundup Ready[b]	30.35	0.21	357.41	346.63	74000	0.53 ± 0.05
	30.24		378.97			

[a] Ct, cycle where the generated fluorescence reaches the treshhold determined for the experiment; SD= standard deviation; 35S = copies of the 35S fragment detected according to the standard curve; % GMO = quantification of GMO in the sample. To calculate this percentage, 37 000 genomes per 100 ng of maize DNA and 42 ng of soybean DNA were estimated (Soybean genome is homozygous for the transgene –2*x*-, while MON810 maize is hemizygous –1x-). Maize samples were prepared by mixing the appropriate amount of MON810 and nontransgenic flour. [b] The DNA from soybean used as additive was also analyzed as unknown tubes in the amplification for the 35S detection.

DNA from transgenic soybean to maize DNA samples) equivalent to the sensibility level required. This is also attractive in real-time PCR. In one experiment, the equivalent of 0.5% transgenic soybean DNA was added to the reaction mixture, and two maize samples, containing 1 and 0.1% of transgenic DNA from MON810, were analyzed for the *epsps* gene by real-time PCR (*11*). Both samples showed the same Ct at any threshold when amplifying the internal control. Moreover, there was no difference in the Ct between the samples and the nontemplate control (i.e., water), indicating that there were no inhibitors in the DNA (data not shown). The samples were then analyzed to quantify the 35S promoter using the TaqMan detection system described by Trapmman et al. (*12*). The results gave values of 0.91 ± 0.04 and 0.12 ± 0.02% for maize samples containing 1 and 0.1% GMO, respectively, while the estimated amount of transgenic DNA in soybean used as internal control was 0.53 ± 0.05% (**Table 2**).

Homogenization of the sample before the DNA isolation is a critical point to consider. Homogenization of flour is a difficult process, especially when the sample is as large as 1 kg or more. One way to solve this problem is to isolate the DNA from the entire sample. This procedure is efficient, but not very practical because of the large volume of extraction buffer required. Trapman et al (*12*) used a protocol involving an aqueous suspension, subsequent freeze-drying, grinding, and repeated homogenization for preparing reference materials (Fluka). The technique led to a very homogeneous material but it contributed to DNA degradation as indicated by gel electrophoresis. Grinding and dissolving the DNA at low temperatures (4 °C) may solve, in part, this problem (*12*). Later on, to further improve the DNA quality, a dry-mixing of flour was developed to prepare the third generation of reference materials (*21*).

Homogenization of the sample in hexane is a practical alternative for routine sample mixing. Moreover, the addition of an organic solvent to the flour has the advantage of extracting oil, making the DNA extraction easier. This is more critical in soybean flour.

In one experiment, two maize samples (1 kg) containing 0.5 and 0.1% (w/w) of transgenic MON810 grains were analyzed. After stirring them during 30 min in a 5-L vessel with 2.5 L of hexane, the solvent was discarded, and the flours were dried at 40 °C overnight. Ten DNA extractions from each sample were performed. Total DNA was quantified fluorometrically, and 35S promotor sequences were quantified by real-time PCR (*12*). The results indicate that all the aliquots assayed reach the threshold in almost the same cycle (data not shown). The percentages of variation obtained were 24 and 13.2 for 0.1 and 0.5% GMO, respectively. These values are among those obtained by Trapmann (*12*) when checking the IRCC soybean standards.

Continuous efforts are being made toward the improvement of methods for detecting and identifying GMOs in grains and foodstuff, considering the requirements for international trade and new safety regulations. Here, we suggest new primers for multiplex conventional PCR and for real-time PCR, and discuss methods to control the quality of DNA and to homogenize large samples of flours that may be useful.

ACKNOWLEDGMENT

We thank Bolsa de Comercio de Rosario for giving access to the ABI Prism 5700 RT-PCR device and to Silvio Di Vanni and César Olsina for their technical assistance. R.H.V. is a member of the Research Career from CONICET.

LITERATURE CITED

(1) James, C. Global status of Commercialised Transgenic Crops: 2000. ISAAA Briefs, no. 23; ISAAA: Ithaca, NY, 2001.

(2) Lipp, M.; Brodmann, P.; Pietsch, K.; Pauwels, J.; Anklam, E. IUPAC collaborative trial study of a method to detect genetically modified soybeans and maize in dried powder. *J. AOAC Int.* **1999**, *82*, 923−928.

(3) Shirai, N.; Momma, K.; Ozawa, S.; Hashimoto, W.; Kito, M.; Utsumi, S.; Murata, K. Safety assessment of genetically engineered food: Detection and monitoring of glyphosate-tolerant soybeans. *Biosci. Biotechnol. Biochem.* **1998**, *62*, 1461−1464.

(4) Meyer, R.; Jaccaud, E. Detection of genetically modified soya in processed food products: development and validation of a PCR assay for the specific detection of Glyphosate-tolerant soybeans. *Proc. Euro Food Chem. IX Conf.* **1997**, *1*, 23−28.

(5) Koppel, E.; Stadler, M.; Luthy, J.; Hubner, P. Sensitive nachweismethode fur die gentechnisch veranderte sojabohne Roundup Ready. *Mitt. Geb. Lebensmittelunters. Hyg.* **1997**, *88*, 164−175.

(6) Hupfer, C.; Hotzel, H.; Sachse, K.; Engel, K. H. Detection of the genetic modification in heat-treated products of Bt maize by polymerase chain reaction. *Z. Lebensm.-Unters. Forsch. A* **1998**, *206*, 203−207.

(7) Lih-Ching, C.; Yen-Ling, C.; Jei-Hwa, Y.; Yang-Chih Shih, D. Detection of four types of genetically modified maize by polymerase chain reaction and immuno-kit methods. *J. Food Drug Anal.* **2001**, *9*, 50−57.

(8) Studer, E.; Dahinder, I.; Lüthy J.; Hübner, P. Nachweis des gentechnisch veränderten Maximizer Mais mitels der polymerase-kettenreaktion (PCR). *Mitt. Geb. Lebensmittelunters. Hyg.* **1997**, *88, 515−524*.

(9) ISO/TC34 WG7 N 26. Detection of genetically modified organisms and derived products. Qualitative nucleic acid based methods. Anexes. AFNOR: Saint Denis, La Plaine Cedex, France; **2001**, pp 47−50.

(10) Matsuoka, T.; Kuribara, H.; Akiyama, H.; Miura, H.; Goda, Y.; Kusakabe, Y.; Isshiki, K.; Toyoda, M.; Hino, A. A multiplex PCR method of detecting recombinant DNAs from five lines of genetically modified maize. *J. Food Hyg. Soc. Jpn.* **2001**, *42*, 24−32.

(11) Vaïtilingom, M.; Pijnenburg, H.; Gendre, F.; Brignon, P. Real-time quantitative PCR detection of genetically modified Maximizer maize and Roundup Ready soybean in some representative foods. *J. Agric. Food Chem.* **1999**, *47*, 5261−5266.

(12) Trapmann, S.; Le Guern, L.; Kramer, G. N.; Schimmel, H.; Pauwels, J.; Anklam, E.; Van den Eede, G.; Brodmann, P. The certification of a new set of reference materials of soya powder with different mass fractions of Roundup Ready soya (IRMM-410S). Institute for Reference Materials and Measurements, European Commission Joint Research Centre EUR...EN *B-2440 GEEL*, 2000. http://www.irmm.jrc.be/rm/irmm-410S.pdf.

(13) Berdal, K. G.; Holst-Jensen, A. Roundup Ready soybean event-specific real-time quantitative PCR assay and estimation of the practical detection and quantification limits in GMO analyses. *Eur. Food Res. Technol.* **2001**, *213*, 432−438.

(14) Terry, C.; Harris, N. Event-specific detection of Roundup Ready Soya using two different real time PCR detection chemistries. *Eur. Food Res. Technol.* **2001**, *213*, 425−431.

(15) Taverniers, I.; Windels, P.; Van Bockstaele, E.; De Loose, M. Use of cloned DNA fragments for event-specific quantification of genetically modified organisms in pure and mixed food products. *Eur. Food Res. Technol.* **2001**, *213*, 417−424.

(16) Ausubel, F. M.; Brent, R.; Kingston, R.E.; Moore, D. D.; Seidman, J. G.; Smith, J. A.; Struhl, K. *Current Protocols in Molecular Biology*. John Wiley & Sons: New York, 1994.

(17) Bennet, E. D.; Leitch, I. J. Plant DNA C-values Database. (release 1.0 September 2001). Royal Botanic Gardens: Kew, U.K., 2001. http:/rbgkew.org.uk/cval/homepage.html.

(18) Gachet, E.; Martin, G. G.; Vigneau, F.; Meyer, G. Detection of genetically modified organisms (GMOs) by PCR: a brief review of methodologies available. *Trends Food Sci. Technol.* **1999**, *9*, 380−388.

(19) Windels, P.; Taverniers, I.; Depicker, A.; Van Bockstaele, E.; De Loose, M. Characterisation of the Roundup Ready soybean insert. *Eur. Food Res. Technol.* **2001**, *213* (2), 107−112.

(20) Agriculture and Biotechnology Strategies (Canada) Inc. (AGBIOS). GMO Database, 2001. http://www.agbios.com/default.asp.

(21) Trapmann, S.; Le Guern, L.; Prokisch, P.; Robouch, P.; Kramer, G.N.; Schimmel, H.; Pauwels, J.; Querci, M.; Van den Eede, G; Anklam, E. The Certification of Reference Materials of Dry Mixed Maize Powder with different Mass Fractions of MON810 Maize. Certified Reference Materials IRMM-413. 2001. http://www.irmm.jrc.be/rm/irmm-413.pdf.

(22) Hurst, C. D.; Knight, A.; Bruce, I. J. PCR detection of genetically modified soya and maize in foodstuffs. *Mol. Breed.* **1999**, *5*, 579−586.

Received for review January 24, 2002. Revised manuscript received May 6, 2002. Accepted May 6, 2002.

JF020081D