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Development and Comparison of Four Real-Time Polymerase Chain Reaction Systems for Specific Detection and Quantification of *Zea mays* L.

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Four real-time polymerase chain reaction systems aiming at the specific detection and quantification of maize DNA are described. They have been developed in four independent laboratories targeting different maize sequences, i.e., alcohol dehydrogenase (*Adh*1), high mobility group protein (*hmg*a), invertase A (*ivr*1), and *zein*, respectively. They were all fully specific, showing a very similar quantification accuracy along a number of distantly related maize cultivars and being either single or low copy number genes. They were highly sensitive and exhibited limits of quantification below 100 maize genomic copies. In consequence, they are considered suitable for use as maize specific endogenous reference genes in DNA analyses, including GMO quantitative tests.

KEYWORDS: ***Zea mays*; maize; corn; alcohol dehydrogenase; *Adh*1; high mobility group protein; *hmg*a; invertase A; *ivr*1; *zein*; quantitative, real-time PCR; GMO detection; endogenous reference gene**

INTRODUCTION

In Europe, the acceptance of genetically modified (GM) food by consumers is controversial, and concerns about their safety persist among public opinion. As a response, the European Union has pronounced regulations, currently under amendment, about the commercial release and the labeling of GM foods and feedstuffs (*1*−*4*). These texts state that except for products derived from GMO-fed animals and products produced with the help of GM enzymes, all products containing or derived from authorized GMO have to be labeled. An approved accidental or technically unavoidable presence of 0.9% authorized GMO per ingredient is tolerated without labeling. To comply with these regulations, the European Union supports several research programs aimed at the development and the validation of reliable, standardized, specific, and quantitative GMO detection methods in foods and feed. Twenty-three percent (15.5 million hectares) of the transgenic crops grown during 2003 was maize (*5*). The most frequent GM maize cultivars presented resistance to the European corn borer (e.g., events 176 and Bt11 from Syngenta formerly Novartis Seeds, MON810 from Monsanto, and CBH-351 from Bayer Crop Science formerly Aventis, Hoescht Schering AgrEvo), tolerance to the herbicide phosphinothricin (e.g., T25 from Bayer Crop Science), and tolerance to the herbicide glyphosate (e.g., GA21 and NK603 from Monsanto).

According to the European regulations, GMO quantification in mixed food samples by polymerase chain reaction (PCR)-based methods requires the amplification of a species specific gene to establish the ratio of GMO vs plant species specific DNA content. An adequate plant species specific real-time PCR (RTi-PCR) assay is so needed (*6*). It must not detect any other plant species and give the same quantitative result when analyzing equal amounts of DNA from different cultivars of the same species (prEN ISO 2426 and 21569). Alternative maize species specific RTi-PCR assays are imperative for accurate GMO quantification in any GM maize. Moreover, the availability of several optimized alternative systems is desirable to overcome possible modifications in one of the target genes (e.g., mutations, copy number, and new GMO affecting it). We report the development and comparison of four alternative RTi-PCR

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Table 1. Oligonucleotides

gene	accession no.	orientation	use	name	sequence	position	amplicon (bp)
Adh1	X04050	forward	RTi-PCR	*Adh-F3*	5′-CGTCGTTTCCCATCTCTTCCTCC-3′	1020	136
		reverse		*Adh-R4*	5′-CCACTCCGAGACCCTCAGTC-3′	1155	
		RTi-Probe[a]		*Adh1-MDO*	5′-AATCAGGGCTCATTTTCTCGCTCCTCA-3′	1063	
		forward	S-probe[b]	*Adh-F3*	5′-CGTCGTTTCCCATCTCTTCCTCC-3′	1020	231
		reverse		*Adh-R1*	5′-GACAGAGGAGAAACAAGGCG-3′	1250	
hmga	AJ131373	forward	RTi-PCR	*mhmg for*	5′-TTGGACTAGAAATCTCGTGCTGA-3′	719	79
		reverse		*mhmg rev*	5′-GCTACATAGGGAGCCTTGTCCT-3′	797	
		RTi-Probe[a]		*mhmg probe*	5′-CAATCCACACAAACGCACGCGTA-3′	774	
		forward	S-probe[b]	*hmgaf1*	5′-GAAATCCCTGAGCGAGTCGGTA-3′	664	228
		reverse		*hmgbr3*	5′-AGTAACAACGCAATTGAAGCATC-3′	892	
Ivr1	U16123	forward	RTi-PCR	*ivr7*	5′-CGCTCTGTACAAGCGTGC-3′	2289	104
		reverse		*ivr8*	5′-GCAAAGTGTTGTGCTTGGACC-3′	2392	
		RTi-probe[a]		*ivrp*	5′-CACGTGAGAATTTCCGTCTACTCGAGCCT-3′	2310	
		forward	S-probe[b]	*ivr7*	5′-CGCTCTGTACAAGCGTGC-3′	2289	248
		reverse		*ivr9*	5′-CCGTTTCCTAGCTCATTGTCG-3′	2536	
Zein	X07535	forward	RTi-PCR	*taqze1*	5′-GCCATTGGGTACCATGAACC-3′	117	104
		reverse		*taqze3*	5′-AGGCCAACAGTTGCTGCAG-3′	220	
		RTi-probe[a]		*taqze2*	5′-AGCTTGATGGCGTGTCCGTCCCT-3′	175	
		forward	S-probe[b]	*taqze1*	5′-GCCATTGGGTACCATGAACC-3′	117	256
		reverse		*taqze4 rev*	5′-CGAGACGGCGTCGCAG-3′	372	

[a] Dual-labeled fluorescent TaqMan probe (FAM as reporter; TAMRA as quencher). [b] Primers used for preparation of the Southern probes.

systems for maize specific quantification: three newly developed and one previously published but whose performance had still to be tested on different maize cultivars (*7*). These systems target four different genes (i.e., *Adh*1, *hmg*a, *ivr*1, and *zein*, respectively), which were chosen in compliance with the requirements defined by the CEN/TC 25/WG 11 standardization commission. The candidate genes were independently chosen and tested by four laboratories in charge of endogenous reference genes in the framework of the QPCRGMOFOOD research program. These maize specific RTi-PCR systems are currently under validation by an international collaborative trial under the auspices of the Institute for Health and Consumer Protection of the Joint Research Centre (Ispra, Italy).

The *Adh* system constitutes one of the most well-characterized gene systems in plants. Maize alcohol dehydrogenase (*Adh*, EC 1.1.1.1) is a zinc-dependent oxydoreductase acting on the alcohol group of donors in the presence of NAD^+ as the acceptor. It is a dimeric isozyme system encoded by two nonallelic genes, *Adh*1 and *Adh*2, induced during anaerobiosis and located on maize chromosomes 1 and 4, respectively. The *Adh*1 gene, interrupted by nine introns (*8*), was chosen as a potential candidate for the development of a maize specific RTi-PCR test.

The chromatin-associated high mobility group (HMG) proteins are a heterogeneous class of relatively abundant nonhistone proteins (*9*). One subgroup, the so-called HMG1 proteins, which contain the HMG box DNA binding domain, considered an architectural factor in chromatin, has also been described in plants (*10*, *11*). In maize, five different HMG1 proteins have been identified (*12*−*14*), from which the gene encoding the maize HMGa protein has been used as species specific gene in this work (*15*).

Invertase (β-fructofuranosidase, EC 3.2.1.26) is a key enzyme for carbohydrate metabolism present in all plant species. It hydrolyzes sucrose into glucose and fructose providing a source of carbon and energy. Several isoforms have been identified. They are encoded by different genes and show different biochemical properties and subcellular locations (*16*, *17*). Vacuolar and cell wall invertases cleave sucrose most efficiently between pH 4.5 and pH 5.0, thus, being called soluble acid invertases. One of the vacuolar soluble acid invertases is encoded by *ivr*1, a gene containing six intronic sequences (*18*) that was also selected as a target for maize species specific RTi-PCR assays.

Finally, the *zein* gene encoding a methionine-rich 10 kDa zein storage protein specific from maize was also chosen. According to Kirihara et al. (*19*), zein 10 kDa seems to be a low copy number gene in the corn genome as indicated by Southern blot assays. Thus, this gene could be an interesting candidate as a corn species specific marker.

MATERIALS AND METHODS

Plant Materials. DNA samples from seeds of 20 different *Zea mays* lines (M1−20) were kindly provided by Bio-GEVES (France). The seeds originated from the United States and Europe and covered as far as possible intercultivar variability (based on the most discriminating morphological traits, i.e., type of grains, precocity, plant height, and silk) (Zhang, D. Personal communication). Leaves from *Z. mays* L. cultivars W64A (CSIC) or MO17 (INRA), *Zea diploperennis* Iltis, Doebley and Guzman, *Arabidopsis thaliana* L. ecotype *Columbia*, *Brassica napus* L., *Brassica oleracea* L., *Helianthus annuus* L., *Hordeum vulgare* L., *Oryza sativa*, *Sorghum bicolor* L. Moench, *Lycopersicon esculentum* L. var. *Ailsa Craig*, and *Solanum tuberosum* L. var. *Désirée* were harvested from plants cultivated in greenhouses at the IBMB-CSIC or provided to INRA by GeneScan. The DNA extracted from *Secale cereale* L., *Triticum aestivum* L., *Panicum miliaceum* L., *Glycine max* L. Merr, *Lens esculenta* L. Moench, *Vicia faba* L., *Phaseolus vulgaris* L., and *Lupinus albus* L. was provided by GeneScan within the framework of the QPCRGMOFOOD project.

DNA Extraction. The large-scale maize genomic DNA cultivar W64 was isolated from 30 g of leaves in two laboratories either according to Dellaporta et al. (*20*) or using a CTAB-based method (*21*). Dellaporta-extracted DNA was quantified using the spectrophotometer GeneQuant RNA/DNA Calculator (Amersham Pharmacia Biotech Europe GmbH, Freiburg, Germany), and concentrations were further checked by agarose gel electrophoresis and ethidium bromide staining. The UV fluorescent emission was recorded and quantified with Quantity One software (Bio-Rad Laboratories Inc., Hercules, CA). CTAB-extracted DNA was assayed by 0.4% agarose gel electrophoresis and ethidium bromide staining, using the Vilbert−Lourmat apparatus (V99.01s) and software (Bio-profil, Bio1D V99.06), by comparison with a range of λ DNA (MBI Fermentas). All genomic DNA obtained was mixed together and used for validation studies.

Oligonucleotides. Oligonucleotide primers and probes (**Table 1**) were designed using the Primer Express v1.5 software (Applied

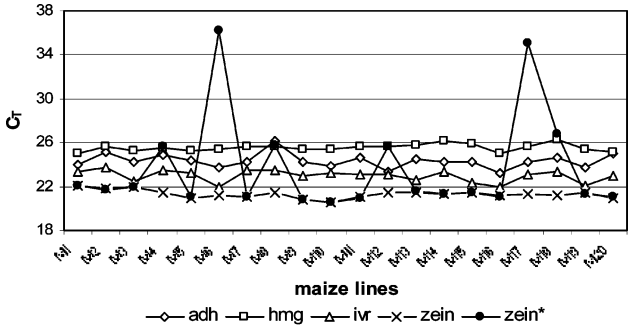

Figure 1. C_T values obtained from triplicate RTi-PCRs performed using 100 ng of genomic DNA extracted from 20 different maize lines (numbered M1–20). Reactions targeted *ivr*1 (△), *Adh*1 (◇), *hmg*a (□), *zein* (×), and *zein** previously described (*7*) (●).

Biosystems Division of Perkin-Elmer Corp., Foster City, CA) to give rise to amplicons with a size compatible with the current RTi-PCR requirements. They were purchased from MWG (Germany). TaqMan probes were labeled on the 5′-end with the fluorescent 6-carboxyfluorescein (FAM) reporter dye and with the quencher dye 6-carboxytetramethylrhodamine (TAMRA) attached to its 3′-end.

Real-Time Quantitative PCR. PCR reactions were performed in 25 μL final volume containing, in addition to 1 unit of Ampli*Taq* Gold DNA polymerase and 0.2 units of AmpErase uracil *N*-glycosylase (Applied Biosystems-Roche Molecular Systems Inc., Branchburg, NJ): for the *adh*1 gene: 2× ABI's Mastermix (ref 4304437) with 300 nM each primer and 200 nM probe; for the *hmg*a gene: 1× TaqMan buffer A (includes ROX as a passive reference dye), 4.5 mM $MgCl_2$, 400 μM each dATP, dCTP, dGTP, 800 μM dUTP, 300 nM primers, and 180 nM probe; for the *ivr*1 gene: 1× TaqMan buffer A, 6.5 mM $MgCl_2$, 200 μM each dATP, dCTP, dGTP, dUTP, 300 nM each *ivr7* and *ivr8*, and 200 nM *ivrp*; and for the *zein* gene: 1× TaqMan buffer A, 5 mM $MgCl_2$, 400 μM each dATP, dCTP, dGTP, dUTP, 100 nM each primer, and 200 nM probe.

RTi-PCR reactions were run on ABI PRISM 7700 Sequence Detection System devices (Applied Biosystems division of Perkin-Elmer Corp.) using the following standard program: 2 min at 50 °C, 10 min at 95 °C, and 45 cycles of 15 s at 95 °C and 1 min at 60 °C. The RTi-PCR products were then analyzed using the Sequence Detection System software 1.7 (Applied Biosystems). The fluorescence signals obtained were continuously measured once for each cycle at the extension step. Quantification was performed by interpolation in a standard regression curve of C_T values generated from DNA samples of known concentrations. For the generation of standard curves, purified genomic DNA was diluted to final concentrations of 100, 33.4, 11.2, 3.8, 1.2, and 0.4 ng/μL. (Thus, the amounts of DNA per reaction tube were 500, 167, 56, 19, 6, and 2 ng.)

Southern Blot. Thirteen micrograms of genomic DNA extracted from maize cultivar W64 was fully digested with *EcoRI* or *HindIII*, and equivalent amounts of sorghum DNA (i.e., 10 μg) were digested with *EcoRI*. The fragments were separated by electrophoresis on 0.9% w/v agarose gel at 60 V for 5 h 30 min and blotted onto Hybond-N+ filters (Amersham Pharmacia Biotech, Piscataway, NJ). Four independent gels were performed as described, and then, they were incubated in hybridization buffer [1% bovine serum albumin, 1 mM ethylenediaminetetraacetic acid (EDTA), 0.125 M $NaHPO_4$, pH 7.2, and 7% sodium dodecyl sulfate (SDS)] at 65 °C for 16 h with the α^{32}P-radiolabeled PCR products detailed in **Table 1**. The PCR products were labeled using a random primed DNA kit (Roche Diagnostics GmbH, Mannheim, Germany). The filters were washed three times at 65 °C for 20 min with sufficient volumes of Church wash buffer (1 mM EDTA, 0.02 M $NaHPO_4$, pH 7.2, and 1% SDS) and finally exposed to X-ray films (Kodak X-Omat AR film and intensifying screen) for 1 week.

RESULTS AND DISCUSSION

Intraspecies Variability of Zein. We first tested the performance of the published zein-based method (*7*) to accurately quantify maize from different lines. We performed RTi-PCR on 100 ng of genomic DNA extracted in parallel from 20 different maize lines. A comparison of the C_T values obtained showed strong variability in six maize lines out of 20 (M4, M6, M8, M12, M17, and M18), suggesting that they have either zein sequence or copy number differences, as compared to the other lines (**Figure 1**). We thus cloned and sequenced the zein region corresponding to the 20 maize lines. The sequences harbored significant differences that allowed us to divide them into three homology groups (**Figure 2**). The RTi-PCR zein (*7*) oligonucleotides targeted a low conserved region of the gene, indicating that the C_T differences observed among maize lines arise from the presence of mismatches within the primers and/or probe in some of the lines. Further on, alignment of the obtained sequences shows a highly conserved region at the 5′-end of the zein gene, which therefore would be a suitable target for a RTi-PCR system for maize DNA quantification, regardless of the particular line. This part of the zein gene was used as a target for a new more stable primers/probe set here described.

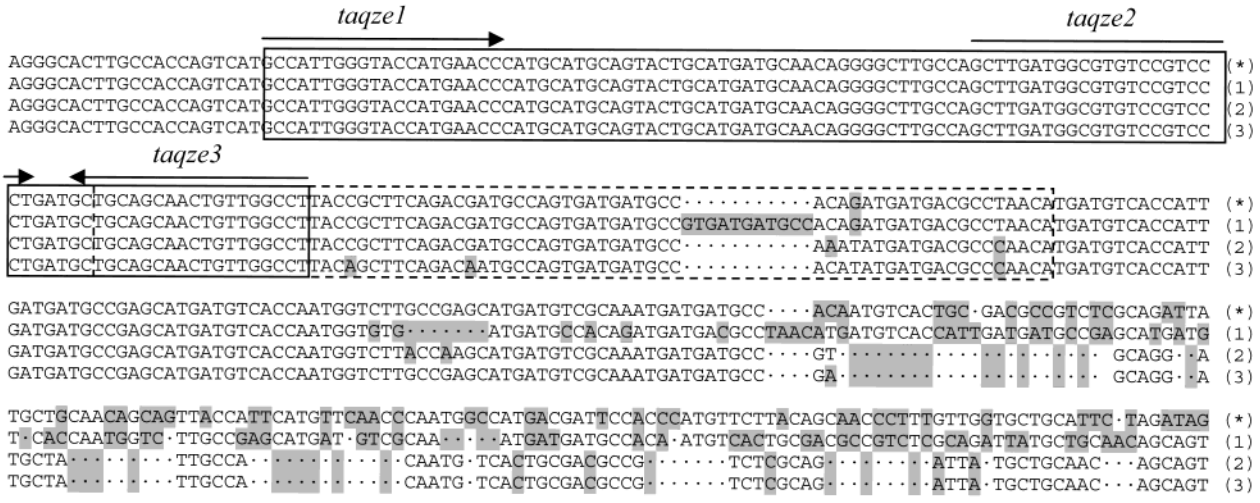

Figure 2. *Zein* sequence variability among 20 maize lines. Sequence alignment could be placed in three homology groups indicated as 1–3 in comparison with the sequence of the corresponding gene from transgenic maize line Bt176 (*). Mismatches are presented by gray shading. The previous described (*7*) zein primers/probe annealed into a nonconserved area (dashed box). However, this alignment shows a very high conserved area at the beginning of the gene, which has been used for designing a new more stable primers/probe set (boxed, with arrows indicating the orientation of primers and probe).

Table 2. Comparison of C_T Values Obtained through Maize DNA Amplifications Using the Four RTi-PCR Systems[a]

		C_T means							
amount of DNA (ng)		500	167	56	19	6	2	regression curve	R^2
genome copy number		183486	61162	20387	6796	2265	755		
RTi-PCR systems	*hmg*	31.91	30.05	28.09	26.57	24.91	22.88	$y = -1.773x + 33.606$	0.9985
	adh	30.30	28.18	26.44	24.67	22.64	21.34	$y = -1.8053x + 31.914$	0.9968
	ivr	33.81	31.64	29.88	28.24	26.66	25.29	$y = -1.691x + 35.172$	0.9945
	zein	29.97	28.17	26.46	24.92	22.90	21.50	$y = -1.7058x + 31.625$	0.9986

[a] The genome copy number (and the amount of DNA) for the different dilution levels of the standard curves are indicated.

Selection of Four Maize Species Specific RTi-PCR Systems. The stability of the target sequences—poorly studied for most genes—is a critical point in species specific RTi-PCR test development. Taking this factor into account, we simultaneously developed and optimized four new maize specific RTi-PCR systems in four independent laboratories, i.e., CSIC, INRA, GeneScan, and TEPRAL, the latter also testing the previously described system (*7*).

ADH1, HMGa, and IVR1 proteins are encoded by low copy number gene families, with enough interspecific divergence to produce specific amplification products, and together with ZEIN, had not been subjected to any genetic manipulation. Seven sets of primers and TaqMan probes were designed (**Table 1** and not shown) targeting *Adh*1 (at INRA), the *zein* coding sequences (at TEPRAL), an *hmga* intron/exon border region (at GeneScan), and three alternative *ivr*1 sequences (at CSIC), i.e., two within exons (nt 597−670 and 916−1033) and the last within an intron. The theoretical specificity of the primers and probes was checked with BLASTN tool against the GenBank database. No significant homology was observed with any plant sequence other than the targets, except particularly on the 3′-end of the *Adh*1 primers.

For *ivr*1, preliminary experiments were performed on maize line W64 and the negative set of plants to test the specificity. They showed amplification of the two exonic sequences from *S. bicolor* genomic DNA, indicating that the exon-based assays were not suitable for use as maize specific reference systems (data not shown). Conversely, the intron-based RTi-PCR system did not cross-react with any species other than *Z. diploperennis*, the probable ancestor of *Z. mays*; therefore, it was selected for further experiments.

Additionally, to discard any possible false positive result due to the lack of specificity at the 3′-end of *Adh*1 primers and also of the other two RTi-PCR systems described, preliminary end point PCR and RTi-PCR were also performed on the negative set of plants. Positive results were only obtained with the two *Zea* spp. tested.

Optimization of the Four RTi-PCR Systems. The appropriate sets of primers and probes (**Table 1**) were used to amplify *Adh*1, *zein*, *hmg*a, and *ivr*1 gene fragments of 136, 104, 79, and 104 bp, respectively. The reactions were optimized for primers, probes, and $MgCl_2$ concentrations (optimal conditions are described in the Materials and Methods section); PCR running conditions were established a priori as the standard ones (ABI PRISM 7700 sequence detection system user's manual), to facilitate both multiplexing and comparison of the four RTi-PCR systems. In the case of the *Adh*1-based system, the attempts of optimization did not show any improvement as compared to the standard conditions of the ABI's MasterMix, which was selected for further experiments.

Using the optimal conditions, six dilutions of maize genomic DNA (containing in each PCR tube: 500 ng, 183486 copies; 167 ng, 61162 copies; 56 ng, 20387 copies; 19 ng, 6796 copies; 6 ng, 2265 copies; and 2 ng, 755 copies, respectively) were assayed in quadruplicates by each of the four RTi-PCR systems in a 2-fold experiment, and standard curves were generated. **Figure 3** and **Table 2** show the mean C_T values of each dilution obtained using all RTi-PCR systems after adjusting fluorescence thresholds to give the best slope (*s*) and regression coefficient (R^2) in each set of reactions. The slope values denote very high efficiencies of the reactions, and the four R^2 values were above 0.99, showing its high linearity and, thus, suitability for use with quantification purposes.

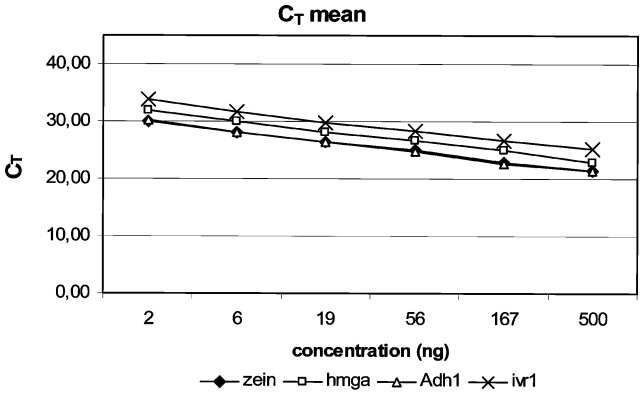

Figure 3. Standard curves generated from amplification by quadruplicate of dilutions (ranging from 2 to 500 ng) of maize DNA using the four RTi-PCR systems in a 2-fold experiment.

Table 3. Specificity

	RTi-PCR systems			
plant species	*adh*	*hmg*	*ivr*	*zein*
Z. mays (maize)	+	+	+	+
Z. diploperennis (teosinte)	+	+	+	+
S. bicolor (sorghum)	–	–	–	–
S. cereale (rye)	–	–	–	–
O. sativa (rice)	–	–	–	–
T. aestivum (wheat)	–	–	–	–
P. miliaceum (millet)	–	–	–	–
H. vulgare (barley)	–	–	–	–
B. napus (rapeseed)	–	–	–	–
A. thaliana	–	–	–	–
H. annuus (sunflower)	–	–	–	–
G. max (soybean)	–	–	–	–
L. esculenta (lentils)	–	–	–	–
V. faba (white bean)	–	–	–	–
S. tuberosum (potato)	–	–	–	–
L. esculentum (tomato)	–	–	–	–
P. aureus (mung bean)	–	–	–	–
L. albus (lupine)	–	–	–	–

Specificity. The specificity of the four RTi-PCR systems was assayed using 100 ng of genomic DNA from 17 different plant species (**Table 3**). These included dicotyledon species (*A. thaliana*, lentils, mung bean, rapeseed, soybean, sunflower, white bean, potato, tomato, and lupine) and monocotyledon species

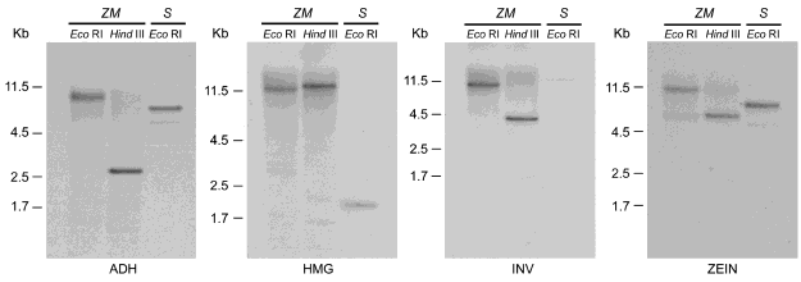

Figure 4. Southern blot analysis of genomic DNA from *Z. mays* (Zm) and *S. bicolor* (S); digested with *Hind*III (*Hind*) or *Eco*RI (*Eco*). Maize genomic DNA was digested with *Hind*III and *Eco*RI, and sorghum DNA was digested with *Eco*RI. ^{32}P-labeled PCR products from *Adh*1, *hmg*a, *ivr*1, and *zein* genes were used as probes.

(millet, rice, rye, sorghum, and wheat). Maize (*Z. mays*) and the maize ancestor teosinte (*Z. diploperennis*) were used as positive controls. The four RTi-PCR systems only detected genomic DNA from the two *Zea* species tested. Therefore, the four systems were considered appropriate for use in maize DNA specific quantitative assays.

Performance of the RTi-PCR Systems on Different Maize Cultivars. RTi-PCR systems suitable for species specific DNA quantification must exhibit identical efficiencies in the analysis of different cultivars of a particular species, thus avoiding over- or underestimation of DNA depending upon the particular cultivar to be tested (which is frequently unknown). One hundred nanograms of genomic DNA extracted from 20 different highly divergent maize lines was analyzed using the four RTi-PCR systems and also the previously described *zein* system (*7*). The 20 DNA samples were prepared and quantified in one laboratory, and identical aliquots of each sample were transmitted to the four developer laboratories, which analyzed in triplicate each maize DNA sample. **Figure 1** shows the mean C_T values obtained by each RTi-PCR system and each maize cultivar. The described zein system (*7*), as reported above, shows some strong differences in the C_T values among the different lines.

For each RTi-PCR system developed within this study, the differences observed among C_T values were below the following: *ivr*1, 1.82 cycles; *Adh*1, 2.90 cycles; *hmg*a, 1.14 cycles; and *zein*, 1.49 cycles. The slight variability observed through the four RTi-PCR systems could be attributed to an error associated to the dilution of the DNA delivered or differences in the fluorescence emission of the reporters. In consequence, the four developed RTi-PCR methods could be considered adequate for quantification of maize DNA from unknown variety because all get very stable C_T values among different corn lines.

Copy Number of the Target Sequences. Single copy genes are in general associated with low rates of mutation and changes in the copy number among the different cultivars. Targeting a low copy number gene should make RTi-PCR assays less dependent on this type of putative intercultivar variation. We thus analyzed the copy number of our four target genes by Southern blot experiments, in which two 13 μg aliquots of maize DNA were digested with *Eco*RI and *Hind*III, respectively, and probed with four short PCR fragments (228−256 bp) that included the four RTi-PCR target sequences. Equivalent amounts of *Eco*RI-digested *S. bicolor* genomic DNA were included to further assess the species specificity of the PCR fragments. *Adh*1, *hmg*a, and *ivr*1 probes revealed single hybridizing bands in both, *Eco*RI- and *Hind*III-digested maize DNAs, which is indicative of these sequences being either unique or present in very low copy numbers within the haploid maize genome (**Figure 4**). Conversely, two bands hybridized with the *zein* sequence, indicating the presence of at least two copies per haploid genome.

Even RTi-PCR reactions did not show any relevant cross-reactivity with sorghum DNA (**Table 3**), and clear hybridization bands with this DNA were observed in the four Southern blots, *ivr*1 sequences being the ones exhibiting minor levels of cross-reaction. These results confirmed that the stringency conditions applied to the Southern analyses were weak enough to allow the detection of any copy of the genes, even subjected to a certain level of mutation.

Limits of Detection (LOD) and Quantification (LOQ). We then assessed the sensitivity of the developed RTi-PCR assays by performing PCR reactions on decreasing amounts of maize genomic DNA that had been spectrophotometrically quantified. According to Arumuganathan and Earle (*22*), 2.595 pg of maize genomic DNA was considered one haploid genome copy. Three to four replicates were performed for each reaction containing 50000 to 1000 initial genome copies; and at least 10 replicates were performed for each RTi-PCR containing 100 to 1 initial template copies. All reactions consistently detected down to 100 genome molecules, and one single molecule could be detected at least in 15% of the replicates. These results are consistent with statistical studies that considered the error associated with serial dilution processes.

The LOD is defined as the lowest copy number that exhibits positive results in 95% of the replicates. The four RTi-PCR systems showed LOD values of 30 (*ivr*1), 10 (*zein*), four (*Adh*1), and one genome copy for the *hmg*a system. The LOQ was determined as the lowest copy number that exhibited linear correlation with the C_T, with R^2 values above 0.99 and nonoverlapping SD among contiguous DNA dilutions. The different LOQ ranged from 100 (*ivr*1) and 100 (*zein*) to 40 (*Adh*1) and 10 (*hmg*a) maize genome copies.

Thus, the four developed RTi-PCR systems are shown to be suitable for use on maize DNA of unknown cultivars with detection, identification, and quantification purposes. The four methods proved to be specific, sensitive, and reliable to be used as endogenous reference controls for GMO analysis. The systems are evaluated in ring trials and will be proposed for standardization.

ACKNOWLEDGMENT

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LITERATURE CITED

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