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A Single Nucleotide Polymorphism (SNP839) in the *adh1* Reference Gene Affects the Quantitation of Genetically Modified Maize (*Zea mays* L.)

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The real-time PCR methods recommended in the European Union for the quantitation of genetically modified (GM) maize events NK603, GA21, and MON 863 measure the number of copies of the GM event in relation to those of the maize-specific *adh1* reference gene. The study reported here revealed that the targeted 70 base pair *adh1* region exhibits a single nucleotide polymorphism (SNP839) that hampers the binding of the reverse primer used in the *adh1* detection method. Partial fragments of the *adh1-A* and *adh1-F* allele were cloned. By allele-specific real-time PCR, it was shown that SNP839 corresponds to a common allelic polymorphism in maize. As a result, the quantitation of the GM maize events mentioned is positively or negatively biased, depending on the *adh1* genotype of sample and calibrant. Therefore, it is proposed to revise the quantitative detection methods for NK603, GA21, and MON 863 maize.

KEYWORDS: ***adh1*; alcohol dehydrogenase; genetically modified organism; genotype; GMO; maize; real-time PCR; single nucleotide polymorphism; SNP; *Zea mays***

INTRODUCTION

The presence of genetically modified organisms (GMOs) in food and feed products is subject to regulation in the European Union (EU) and elsewhere, which, in some jurisdictions, includes a requirement to label products containing above-threshold GMO concentrations. As part of the authorization process for GMOs in Europe, Regulations (EC) 1829/2003 and 641/2004 specify that an analytical method for GMO analysis shall be submitted by the applicant (*1, 2*). Furthermore, this method shall be validated by the Community Reference Laboratory (CRL) for GM Food and Feed, established at the Institute for Health and Consumer Protection of the Joint Research Centre. The preferred approach for GMO quantitation is event-specific real-time PCR, and the DNA copy number measured for the GM event is expressed in relation to that of a plant taxon-specific endogenous reference gene (*3*). For maize, in contrast to soybean, no uniformity exists on the employed reference gene systems. In the CRL-validated quantitative methods for different maize GM events (*4*), the measured GM DNA copy numbers are related to endogenous genes encoding the high mobility group I protein (*hmg*), alcohol dehydrogenase 1 (*adh1*), zein, invertase (*ivr1*), or starch synthase IIb (*zssIIb*), which are all single- or low-copy genes (*5, 6*). For the *adh1* reference gene, there are two different detection methods, which target a sequence in the 5′-untranslated region of the gene (called Zm*adh1* method) or in the junction between the second intron and the third exon (called the *adh1* method), both regions being separated by roughly 1 kilo base pair (kb). The *adh1* gene of maize has been intensively studied from an evolutionary point of view (*7, 8*), and several alleles, particularly *adh1-S* and *adh1-F*, have been studied in great detail (*9*). Among the current CRL-validated quantitative methods, the *adh1* method is exclusively used for the NK603, GA21, and MON 863 events, three GMOs for which EU authorization was requested by Monsanto in 2004 and which have more recently been used in various stacked events (*4*).

The study presented here was initiated by the observation that two maize samples containing approximately 1% (m/m) NK603 maize contained strongly differing *adh1* copy numbers when measured by real-time PCR using genomic DNA from NK603 seeds for calibration. One sample was a Certified Reference Material (ERM-BF415d) and the other an unknown sample investigated in the frame of proficiency testing (GeM-Su03, organized by FAPAS, York, U.K.). As a result of their deviating *adh1* contents, the calculated GM percentage differed strongly between both samples. In contrast to *adh1*, the amounts of three other reference genes, Zm*adh1*, *hmg*, and *zssIIb*, were comparable in both samples. Further investigations showed that the unusually high *adh1* value measured in the CRM could be traced back to the non-GMO comparator variety used in the processing of this CRM (*10*). In the latter variety, RX670, the measured *adh1* amount was approximately 2.5 times higher than that in the NK603 variety. Alarmed by this finding, the

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Table 1. Oligonucleotides Used in the Study [The Sequences of the Primers and Probes Used in the Validated Quantitative Detection Methods Can Be Retrieved from the CRL Website (*4*)]

target[a]	code	intended use	type	sequence 5′−3′[b]	amplicon (bp)
upstream *hmg*	P-063	cloning	forward	5′-gattcccctctcctggtcga-3′	351
downstream *hmg*	P-064	cloning	reverse	5′-caacacatggttcagtaagcatacg-3′	
upstream Zm*adh1*	P-738	cloning	forward	5′-atcAAGCTTccacggaccacggctatg-3′	322
downstream Zm*adh1*	P-739	cloning	reverse	5′-atcAAGCTTcggacctttgcacttgatcac-3′	
upstream *adh1*	P-740	cloning	forward	5′-aGGATCCaatcagccatcccatttgtgat-3′	256
downstream *adh1*	P-741	cloning	reverse	5′-aGGATCCaaagaccaccaaccatacccataa-3′	
upstream *zssIIb*	P-742	cloning	forward	5′-atCTGCAGgctgtggattctgcttcac-3′	235
downstream *zssIIb*	P-743	cloning	reverse	5′-atCTGCAGatgcctatgctttccaccg-3′	
upstream NK603	P-744	cloning	forward	5′-atTCTAGAcccgactctcttctcaagca-3′	161
downstream NK603	P-745	cloning	reverse	5′-atTCTAGAtactagtctgttatggttcgag-3′	
adh1	P-411	quantitation (*adh1-A* specific)	reverse	5′-ccagcctc**a**tggccaaag-3′	
adh1	P-733	quantitation (*adh1-F* specific)	reverse	5′-ccagcctc**g**tggccaaag-3′	
between Zm*adh1* and *adh1*	P-771	quantitation (conserved)	forward	5′-gtgtggtccatccgacagtct-3′	146−148[c]
between Zm*adh1* and *adh1*	P-772	quantitation (conserved)	reverse	5′-attcaaagaaagaaacgcctcctt-3′	
between Zm*adh1* and *adh1*	P-773	quantitation (*adh1-A* specific)	probe	5′-FAM-catcatacgatatt**g**agcaaa**g**atctatcttccctgttc-3′	
between Zm*adh1* and *adh1*	P-774	quantitation (*adh1-F* specific)	probe	5′-FAM-tgaacacatcatacgat**ct**at**g**gagcaaa**a**atctatc-3′	
downstream *adh1*	P-784	quantitation (conserved)	reverse	5′-gcacatatatagtcagcaacatagtgaagta-3′	

[a] *hmg*, Zm*adh1*, *adh1*, *zssIIb*, and NK603 refer to the specific sequence fragments targeted in the recommended GMO quantitation methods. [b] Capital letters refer to restriction enzyme binding sites added for cloning purposes; underlined, bold letters are discriminative nucleotides. [c] Sizes are for *adh1-A* and *adh1-F*, respectively.

performance of the *adh1* detection method was investigated in-depth in comparison with the validated methods for other maize reference genes. The study focused on NK603 maize, but also affects the quantitative GA21 and MON 863 maize detection methods, which all use *adh1* as the preferred reference gene system (*4*).

MATERIALS AND METHODS

Plant Materials. The maize plant materials analyzed here are the seed powders used at IRMM for the processing of various maize GMO matrix CRMs. For ERM-BF415, these base seed powders corresponded to the non-GM variety RX670 and the NK603 hybrid variety DKC 57-40. RX670 was also used as the comparator variety in the processing of the GMO CRMs ERM-BF414, BF416, and BF417, containing GA21 (GMO variety RX740RR), MON 863 (variety TP5504-DP), and MON 863 × MON 810 (variety TP6705-BG), respectively. Other non-GMO and GMO maize varieties analyzed included Bahia and Garona Bt-176 (ERM-BF411), Pelican and NX 3707 Bt11 (ERM-BF412), and DK 512 and DK 513 (MON 810; ERM-BF413), respectively.

DNA Extraction. Genomic DNA was extracted from maize CRMs according to either a modified CTAB method based on the validated protocol for NK603 (*11*), but using a 100 mg sample intake and a final ethanol precipitation, or the commercial GeneSpin extraction method (GeneScan GmbH, Freiburg, Germany), using 200 mg of sample. The results reported were independent of the extraction method used. Fresh leaf samples were first ground in liquid nitrogen using the TissueLyser (Qiagen, Hilden, Germany), after which the DNA was extracted with the DNeasy plant mini kit (Qiagen). Plasmid DNA was purified from overnight bacterial cultures in LB supplemented with ampicillin (100 μg/mL) by the Qiaprep plasmid mini kit (Qiagen). DNA quantitation was done on a FluoStar Galaxy reader (BMG Labtech GmbH, Offenburg, Germany) using the Picogreen dsDNA quantitation kit (Molecular Probes−Invitrogen, Carlsbad, CA) or on a Nanodrop ND-1000 spectrophotometer (NanoDrop Technologies, Wilmington, DE) for genomic DNA and plasmid DNA, respectively. Each of these methods was a reliable DNA quantitation method for the respective DNA source.

Plasmid Construction. The multitarget plasmid pIRMM-0086, containing the CRL-validated target sequences for the detection of *adh1*, Zm*adh1*, *hmg*, *zssIIb*, and NK603, was constructed stepwise following PCR amplification of the respective fragments with Platinum *Pfx* DNA polymerase (Invitrogen, Carlsbad, CA) using the primers shown in **Table 1**, each tagged with a different restriction enzyme recognition site. The template for PCR was NK603 seed powder (variety DKC 57-40), except for the *adh1* fragment, which was amplified from RX670. Prior to T/A cloning into pCR2.1 (Invitrogen), the PCR products were dA-tailed with Platinum Taq DNA polymerase (Invitrogen). Following sequence confirmation of the cloned inserts, the fragments were excised at the flanking restriction sites and sequentially ligated into pUC18 vector. Each of the five target amplicons was embedded in between 21 and 163 base pairs (bp) of flanking maize DNA to avoid primer binding artifacts due to the adjacent sequences. The final 4052 bp vector pIRMM-0086, containing all five target sequences in the same orientation, was analyzed by restriction mapping and sequencing using the M13 and *adh1*/Zm*adh1* detection primers.

Real-Time PCR Analysis. Real-time PCR was performed on an ABI 7900HT apparatus (Applied Biosystems, Foster City, CA) in 96-well format according to the validated methods for different GMOs (primers and probes, see ref *4*). Each reaction was run in triplicate during 40 or 45 cycles in a total volume of 50 μL and contained 1× TaqMan Universal PCR Mastermix (Applied Biosystems) and oligonucleotides at the following concentrations: 150 nM primers and 50 nM probe for NK603; 300 nM *adh1*, *hmg*, or Zm*adh1* primers with, respectively, 50, 160, or 200 nM probe; 500 nM primers and 200 nM probe for *zssIIb*. All oligonucleotides used in the detection methods were obtained from Applied Biosystems. Quantitation was based on calibration curves prepared by serially diluting plasmid DNA in low TE buffer (1 mM Tris-HCl, 0.01 mM EDTA, pH 8.0), covering from 500 to 500000 copies/reaction in six standards. The amount of genomic DNA in each test reaction was approximately 50 ng.

***Adh1* Gene Cloning and Sequence Analysis.** Qualitative PCR was performed in a total reaction volume of 50 μL with 100 ng of genomic DNA and containing 1× *Pfx* amplification buffer (Invitrogen), 1.5 mM $MgSO_4$, 0.2 mM dNTPs, 500 nM primers (Thermo Fisher Scientific, Ulm, Germany), and 1.25 untis of Platinum *Pfx* DNA polymerase (Invitrogen). Amplification was done in a GeneAmp PCR system 9700 (Applied Biosystems), starting with a 2 min denaturation at 94 °C, then 35 cycles of 30 s at 94 °C, 30 s at 56 °C, 90 s at 72 °C, and a final 7 min extension step at 72 °C. Twenty microliters of the reaction products was mixed with 10× loading dye (Invitrogen) and run through a 1% (m/v) agarose gel, which was stained with ethidium bromide. Bands of the expected size were cut out from the gel, and the PCR products were purified with QiaExII (Qiagen) and dA-tailed with Platinum Taq DNA polymerase (Invitrogen) for 5 min at 72 °C. The final amplicons were cloned into pCR2.1 vector using the TOPO TA cloning kit (Invitrogen), transformed into One Shot TOP10 chemically competent *Escherichia coli* (Invitrogen), and selected by plating onto LB agar plates containing X-gal and 100 μg/mL ampicillin or 50 μg/mL kanamycin. Prescreening of colonies was done by picking bacterial cells from a single colony, briefly boiling them in 100 μL of water, and using 5 μL of disrupted cells in a 20 μL PCR reaction using Platinum Taq DNA polymerase and M13 forward and reverse primers. Sequence analysis of purified plasmid DNA was performed on an ABI Prism 3130 Genetic Analyzer (Applied Biosystems) with POP-7 polymer in a 36 cm capillary array using the BigDye Terminator v1.1

Table 2. Average Performance of Real-Time PCR Methods for Maize Using the pIRMM-0086 Calibrant

target[a]	efficiency (%)	linearity (R^2)	intercept
adh1 (13)	96	0.999	42.39
Zm*adh1* (10)	94	0.999	42.51
hmg (6)	95	1.000	41.93
Zssllb (4)	90	1.000	44.28
NK603 (3)	88	0.999	45.21

[a] Number of independent experiments is shown in parentheses.

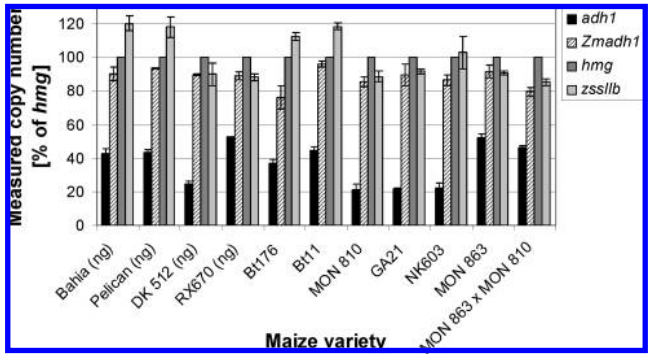

Figure 1. Copy number quantitation of different reference genes in non-GM (ng) and GM maize seed powders by real-time PCR using the plasmid DNA calibrant pIRMM-0086 ($n = 3$). The GM powders analyzed are indicated by the GM event (see Materials and Methods for the variety names).

Ready Reaction Cycle Sequencing kit (Applied Biosystems). Primers used for sequencing of both DNA strands included both the M13 forward and reverse vector primers and the *adh1*/Zm*adh1* detection method primers. The overlapping sequences obtained from three independent colonies per maize variety were aligned using Clone Manager Suite 7 (Sci-Ed Software, Cary, NC) and compared to GenBank sequences using NCBI's BLASTN program. The resulting plasmids pIRMM-0087 and pIRMM-0088 contained the partial sequence of the *adh1-A* and *adh1-F* alleles, respectively.

RESULTS

Quantitative Comparison of Different Maize Reference Genes. Experiments in our laboratory had shown that the *adh1* quantity measured in seeds of some maize varieties was unexplainably high (see Introduction). To comprehend the extent of the phenomenon observed, the relative copy numbers of *adh1*, Zm*adh1*, *hmg*, and *zssIIb*, four reference gene targets employed in CRL-validated GMO detection methods (*4*), were investigated in several non-GM and GM maize varieties. A multitarget plasmid, pIRMM-0086, was constructed for use as a calibrant in real-time PCR. This would ensure that the measured copy numbers reflected the genome copy numbers for every reference gene target (*12, 13*). All cloned sequences in this plasmid were derived from the NK603 variety, except for the *adh1* region. The *adh1* fragment was amplified from the non-GM maize variety RX670, as this was believed to be a more reliable source of this target fragment on the basis of the results obtained so far. All cloned target sequences were efficiently quantified using the respective CRL-validated methods (**Table 2**). **Figure 1** shows the comparative results of real-time PCR measurements, calibrated with pIRMM-0086, for the four reference genes in four non-GM and seven GM maize varieties. Whereas the measured quantities of Zm*adh1*, *hmg*, and *zssIIb* were roughly similar in all DNA samples (±25%), the results obtained for *adh1* were remarkable: the *adh1* level detected reached only half that of *hmg* in seven non-GM and GM varieties, and 4 times less *adh1* compared to *hmg* was measured in DK 512, MON 810, GA21, and NK603. For NK603 and its non-GM comparator line, the measured *adh1*/*hmg* ratios were 22 ± 3 and 53 ± 3%, respectively. These results were in line with the observations made earlier using the genomic DNA from NK603 seeds for calibration to measure DNA from RX670: 2.5 times more *adh1* was measured in the latter variety compared to the genomic calibrant (see Introduction). It was preliminary concluded that the variable *adh1* copy numbers among different maize varieties suggested the occurrence of a genetic polymorphism near the *adh1* target sequence.

Cloning of the *adh1* Gene. To find out if sequence heterogeneity in the *adh1* region was affecting the quantitative results, a contiguous 1255 bp sequence covering the Zm*adh1* and the *adh1* target regions together with 106 bp of upstream DNA and 121 bp of downstream DNA, respectively, was amplified from both the RX670 (non-GMO) and NK603 seed powders using primers P-738 and P-741 (**Table 1**). The sequence amplified encompassed approximately 300 bp of the *adh1* promoter, the first 3 exons and 2 introns, and ended within intron 3. Sequence analysis of three selected colonies revealed the presence of a single *adh1* sequence in the NK603 line, which appeared identical to the known sequence of the *adh1-F* allele (GenBank entry AF123535). In the non-GM variety, the same *adh1-F* allele sequence was present in two colonies, whereas a different sequence, identical to GenBank's *adh1-A* allele (AY691949) was found in one other colony. Both allelic sequences differ at 13 positions in the cloned region, 9 of which are base substitutions and 4 are insertions/deletions (indels), composed of 2 groups of 2 adjacent base pairs (**Figure 2**). Ten of the 13 polymorphisms are somewhat clustered in the middle of the sequence, that is, between the regions targeted by the two *adh1* detection methods, and corresponding with the first *adh1* intron. One remaining single nucleotide polymorphism (SNP) was present in the Zm*adh1* region, but outside the oligonucleotide binding sites, another one was located downstream of the *adh1* detection target, and the last one was positioned in the middle of the binding site of the reverse primer (P-411) for *adh1* amplification (position −10 relative to the 3′-end of the primer). The P-411 *adh1* reverse primer sequence recommended in the validated *adh1* detection method matches the *adh1-A* allele sequence, whereas the *F* allele has a T/C base substitution at this position (**Figure 3**). This SNP was called SNP839 (relative to the translational start codon).

***Adh1* Polymorphism in Maize.** To determine the *adh1*genotype of maize varieties and reveal the genomic polymorphism for this gene in maize, two allele-specific real-time PCR assays were designed targeting the most polymorphic region in the cloned *adh1* gene fragment. The *adh1-A* and *adh1-F* assays were composed of the same forward and reverse primer (P-771 and P-772) and two different probes (P-773 and P-774) recognizing either the *F* or the *A* allele based on four discriminative nucleotides in the central part of the probe (**Figure 2**; **Table 1**). The calibrant employed in the allele-specific real-time PCR assays was either the plasmid pIRMM-0087, harboring the *adh1-A* fragment, or pIRMM-0088, containing the corresponding fragment of *adh1-F*. The specificity of the methods for one or the other allele was shown by the absence of a signal when the alternative plasmid DNA template was tested even at high copy numbers (approximately 5×10^5 copies/reaction). For the NK603 variety, in which the *F* allele was detected following amplification and cloning, a total of 24 bacterial colonies containing the *adh1* fragment were screened for either allele, and all were found to contain only the *adh1-F* allele. This confirmed the sequencing results and indicated that

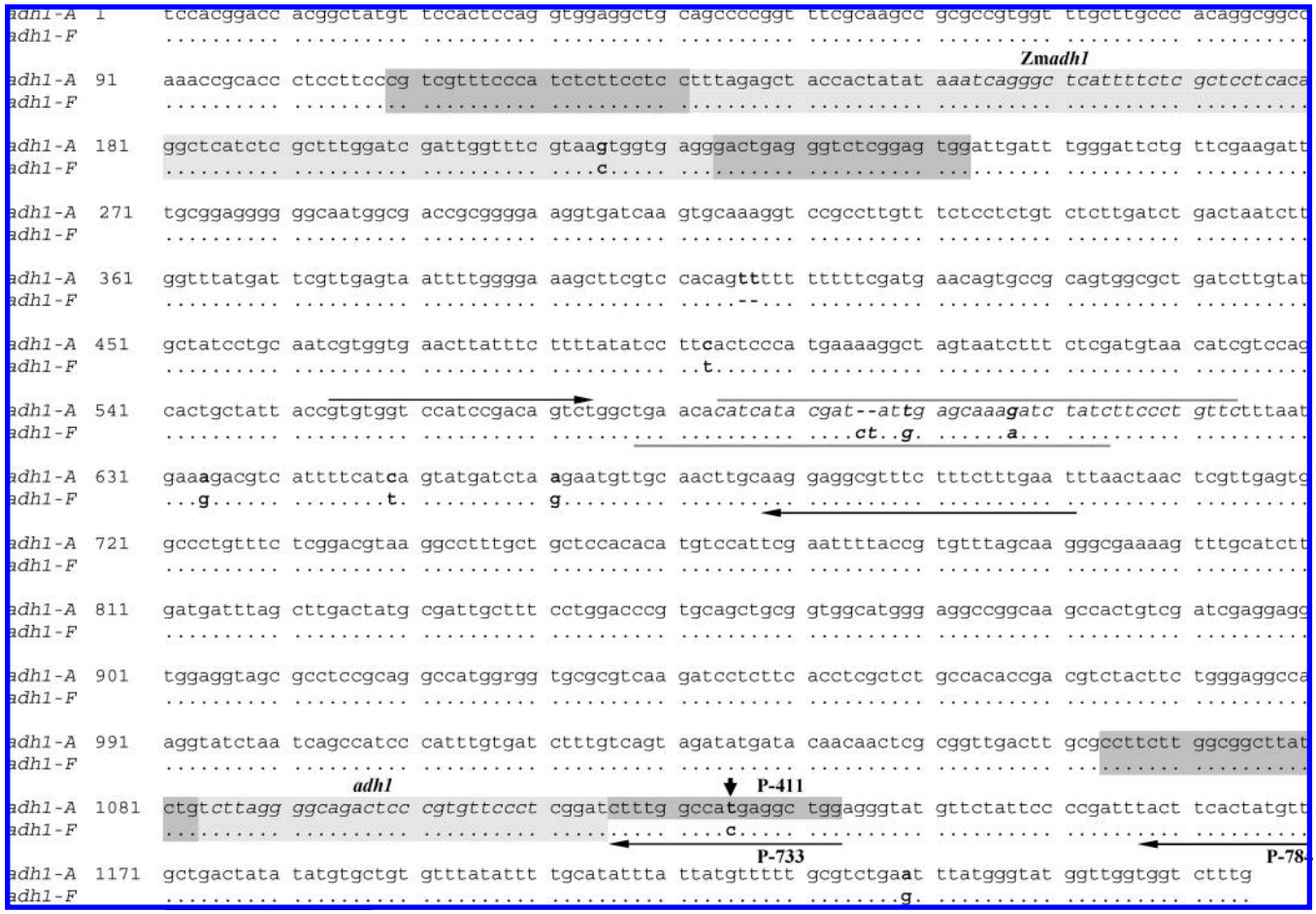

Figure 2. Nucleotide sequences of cloned maize *adh1* alleles. Identical nucleotides are denoted with a dot in the *adh1-F* sequence, and polymorphic nucleotides are in bold. The amplified target regions in the recommended detection methods for Zm*adh1* and *adh1* are in gray boxes, with the primer binding sites in darker gray and the probe binding sites in italics. Additional primers and probes, such as those used for allelic discrimination (in the middle of the sequence shown), are identified by arrows and gray lines, respectively. The short vertical arrow points to SNP839, which affects *adh1* quantitation.

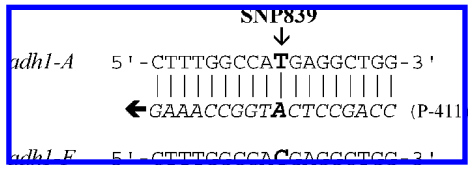

Figure 3. Binding specificity of the *adh1* reverse primer P-411 (shown in italics) on the *adh1* genomic sequence. The primer completely matches the *adh1-A* sequence, but reveals a nucleotide mismatch with the *adh1-F* sequence at SNP839.

NK603 was homozygous *FF* for the *adh1* gene. Using the same allele-specific real-time PCR methods, the powders from several other maize varieties were screened. The results, shown in **Table 3**, revealed that, besides NK603, also the non-GM variety DK 512 and the GM varieties of MON 810 and GA21 exclusively bore the *adh1-F* allele. All other varieties tested harbored both alleles, with the ratio between their measured copy numbers (*adh1-A*/*adh1-F*) being close to 1 (1.11 ± 0.14).

From the results, it cannot be excluded that other alleles, in addition to *F* and *A*, occur in these varieties. Indeed, whereas the *adh1-F* assay exclusively amplifies the *F* allele, the *adh1-A* probe cannot discriminate the *A* allele from two other *adh1* alleles (*S* and *Cm*), the sequences of which can be retrieved from GenBank (X04049 and M32984, respectively). Whereas the occurrence of the *A* allele in the RX670 variety was shown through sequence analysis, it was beyond the scope of this study to further investigate if the second allele in the other six heterozygous varieties was *A* or *S* or *Cm*. The two latter alleles are furthermore identical to *adh1-A* at the position of SNP839. In any case, the results indicated that polymorphism at the *adh1* gene is common in maize.

Table 3. *Adh1*-Allele-Specific Real-Time PCR Analysis of Different Maize Varieties, Calibrated with Plasmid DNA Bearing either the *A* Allele (pIRMM-0087) or the *F* Allele (pIRMM-0088) [($n = 1$); PCR Efficiency in Both Assays was 95% and $R^2 > 0.998$]

maize variety	*adh1-A* (10^3 cp)	*adh1-F* (10^3 cp)	*Adh1*-genotype[a]
Bahia (non-GMO)	52	51	heterozygous
Pelican (non-GMO)	59	47	heterozygous
DK 512 (non-GMO)	0	62	FF
RX670 (non-GMO)	42	40	AF[b]
Bt176	45	47	heterozygous
Bt11	67	50	heterozygous
MON 810	0	70	FF
GA21	0	76	FF
NK603	0	75	FF[b]
MON 863	49	45	heterozygous
MON 863 × MON 810	42	39	heterozygous

[a] Inferred from allele-specific real-time PCR (see text). [b] Determined by nucleotide sequencing.

A Single Nucleotide Mismatch in the Reverse Primer Binding Site. To investigate if SNP839 affected amplification of the nonmatching *adh1-F* allele, DNA dilution series of pIRMM-0087 and pIRMM-0088, bearing either *adh1* allele,

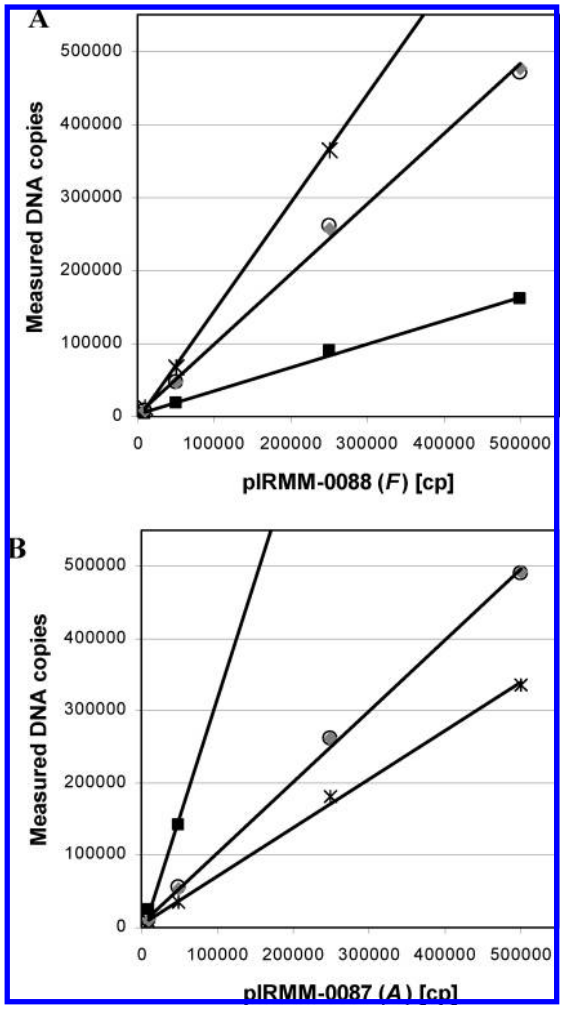

Figure 4. Quantitation of the effect of SNP839 by real-time PCR using cloned *adh1* allele fragments. In **A**, the *adh1-A*-containing plasmid pIRMM-0087 was used for calibration, and DNA of pIRMM-0088, containing *adh1-F*, was quantified; in **B**, pIRMM-0088 was used as calibrant (*adh1-F*), and pIRMM-0087 (*adh1-A*) was quantified. In both cases, a serial dilution of plasmid DNA, ranging from 1×10^4 to 5×10^5 copies/reaction, was analyzed for Zm*adh1* (○) and for *adh1*, using the reverse primers P-411 (■, *adh1-A* specific; validated method), P-733 (∗, *adh1-F* specific) or P-784 (gray diamonds, consensus primer).

were alternatively used as calibrant and unknown sample in real-time PCR analyses. A new reverse primer (P-733), specific for the *F* allele, was designed, and its real-time PCR performance was compared to that of P-411 (*A*-specific). P-733 differed from P-411 only at the central polymorphic nucleotide discriminating both alleles in this region (**Table 1**). With the Zm*adh1* detection method targeting a conserved region in both plasmid inserts, a linear 1:1 correlation ($R^2 = 0.999$) was found between input and output DNA copy numbers using the other plasmid for calibration (**Figure 4**, open circles). The result was expected and confirmed that equal plasmid DNA quantities were compared in these experiments. Clear differences were, however, seen with the *adh1* method using the reverse primer P-411: when calibration was performed with pIRMM-0087, containing the *A* allele, only 37 ± 5% (cp/cp) (mean ± *s* for four serial DNA dilutions) of the input DNA copy number of pIRMM-0088, containing the alternative (*F*) allele, was measured (**Figure 4A**, black squares). Similarly, calibration with pIRMM-0088 (*adh1-F*) in the variant *adh1* method with the reverse primer matching the *F* allele (P-733) resulted in the detection of only 72 ± 6% (cp/cp) of the nonmatching *A* allele copy numbers in pIRMM-0087 (**Figure 4B**, asterisks). Furthermore, the pIRMM-0088 (*adh1-F*) calibrant strongly overestimated the copy number of pIRMM-0087 (*adh1-A*) samples in the *adh1* (P-411) method (**Figure 4B**, black rectangles). The latter condition resembled the experiments that formed the basis for the study presented here, in which an elevated *adh1* content was measured in the non-GM RX670 variety (genotype *AF*) using the genomic NK603 calibrant (*FF*) (see Introduction). In both cases, the P-411 reverse primer amplified the nonmatching *F*-allele-bearing calibrant less efficiently, producing higher Ct values and, hence, lower quantitative values for the reference gene, than those obtained on similar DNA amounts from samples harboring the matching *A* allele in addition to the *F* allele (heterozygote). Finally, using a reverse primer binding to a conserved sequence further downstream of the binding site of the *adh1* reverse primer, P-784 (see **Figure 3**), both *adh1* alleles were comparably detected irrespective of the calibrant used, and the results were similar to those obtained for Zm*adh1* (**Figure 4**, gray diamonds). The same consensus reverse primer in combination with the forward primer and probe of the validated *adh1* method also detected similar copy numbers as measured for Zm*adh1* on genomic DNA from the RX670 and NK603 varieties (data not shown). The results obtained using alternative *adh1* reverse primers clearly proved that the single nucleotide polymorphism near the binding site of P-411 caused the aberrant quantitative results for *adh1* observed.

DISCUSSION

In this study we have discovered a systematic error in currently recommended event-specific methods for the quantitation of the maize events GA21, NK603, and MON 863 in the EU. The bias is caused by a sequence polymorphism in the endogenous reference gene used as a denominator in the calculation of the GM percentage. Inaccurate measurement results, particularly around the threshold value for labeling, may affect the legal requirement whether or not to inform the consumer of the presence of GMOs in a food or feed product, following EU legislation EC 1829/2003 (*1*). The consequences of the incorrect *adh1* amplification for the calculation of the GM percentage of a GM maize sample depend on the *adh1* genotype of both the maize sample and the calibrant used. Because the *adh1-F* allele is less efficiently amplified compared to the *A* allele when using the validated *adh1* method, use of a calibrant bearing the *F* allele to measure a sample containing the *A* allele will result in overestimated *adh1* quantities, hence reducing the GM percentage. The opposite will be true if the calibrant contains the *A* allele and the sample the *F* allele. Simulations showed that a 1% (m/m) GM maize sample was quantified as being from 0.3 to 1.6% GM using a genomic calibrant purified from CRMs such as ERM-BF414 (GA21) or BF415 (NK603). These CRMs were processed from non-GM and GM varieties containing the *AF* and *FF* genotype, respectively. With a plasmid calibrant, the calculated GM copy number ratio could even range from 0.2 to 2.8% for the same 1% (m/m) sample material, where a value around 0.5% is expected, taking into account the hybrid nature of commercial (2*n*) maize seeds. Such a plasmid calibrant, containing *adh1* as reference standard, is currently not commercially available. As the genetic makeup of the maize varieties processed into any sample to be analyzed is mostly unknown and may be heterogeneous as well, that is, could be composed of different *adh1* genotypes, any quantitative result generated on them using the recommended GMO detection methods for GA21, NK603, and MON 863 will be affected by a systematic measurement bias. It should be stressed that

the observed bias is not due to an intrinsic characteristic of the calibrant used, be it a CRM or a plasmid, but is caused by an inaccurately designed analytical method as such.

The purpose of the study presented here was to explore the cause of the biased *adh1* quantitation. It was shown that a single nucleotide polymorphism, SNP839, located in the middle of the binding region of the reverse primer used for the quantitation of the *adh1* reference gene strongly affected the measurement results. In the detection of SNPs in, for example, human disease screening, one of the primers used for allelic discrimination is generally designed such that the polymorphic nucleotide is at its terminal 3′-end, or close to the 3′-end (*14*). Primer mismatches further away from the extension end of the primer, such as was the case in P-411 where the mismatch was at position −10, are generally believed not to contribute significantly to the measurement result (*15*). For that reason the initial focus of the investigations to explain the aberrant results obtained had been dedicated to other aspects, such as PCR inhibition, oligonucleotide depletion by aspecific binding, seed heterogeneity, or technical performance of the PCR instrument. It was, however, observed that raising the primer annealing temperature from 60 to 64 °C increased the severity of the deviations for *adh1*. Such an effect is likely to be seen in the case of primer−template mismatches. Bru et al. (*16*) reported recently that a primer mismatch at −10 from the 3′-end of a primer of the same length as P-411 caused a reduction of 1 log of the measured bacterial gene copy number, an effect surpassing even our observations.

The investigations presented here exploited a plasmid carrying several target sequences for calibrating the real-time PCR measurements. It has been shown previously that the use of cloned plasmid GMO target sequences for calibration can produce accurate quantitative results (*12, 13*). Plasmid CRMs are increasingly available from reference material producers such as the Institute for Reference Materials and Measurements (IRMM). The suitability of plasmids or other types of DNA for calibration is systematically assessed by IRMM in case they are used for the characterization of reference materials in terms of relative copy numbers (*17*). The assessment of the suitability of the here employed plasmids to obtain unbiased (true) results was, however, not the subject of this study. Plasmids containing different GMO targets in addition to a single taxon-specific sequence have also been described, including some targeting one or more of the GMO events described herein in addition to the *ivr1* or *zssIIb* reference targets (*18, 19*). The use of cloned fragments for the quantitative comparison of different reference gene methods was, however, not reported before. A plasmid, bearing the embedded sequences for four common maize reference gene targets, in addition to that for the NK603 event, was used to analyze their relative quantities in different maize varieties. Together with further unreported results on other maize varieties, the screening revealed the wide occurrence of the observed polymorphism at the *adh1* target region.

Numerous in-depth studies have explored the complex origin and high degree of diversity of the maize genome, which has evolved from a tetraploid to one approaching a diploid genome through multiple rearrangements and gene losses (*7, 8*). The *adh1* locus itself is embedded in massive amounts of repetitive DNA encompassing several classes of retrotransposons (*20, 21*), and mutant alleles have been discovered following tissue culture (*22*) or mutagenesis treatments (*23*). The described polymorphisms in the *adh1* sequence were already reported in the 1980s (*9*) and correspond to diverse isozyme patterns observed already in the 1960s (*24*). Additionally, naturally occurring maize lines have been described in which the *adh1* locus was duplicated, resulting in an increased stability of the *adh1* heterodimer, hence providing an element for positive selection, which might further thwart the GM quantitation (*24, 25*). It is, therefore, unfortunate that the target method for *adh1* detection, provided by the GM seed producer to the CRL for GM Food and Feed, had been chosen within a known polymorphic and evolutionary unstable region. For GA21, it should be noted that this event recently changed ownership and that the current recommended detection method is now targeting the more conserved Zm*adh1* region instead of *adh1* (*4*).

The discovery reported here stresses the uttermost importance of the careful design and thorough testing of analytical methods for GMO detection. The current legislation clearly demands applicants for GMO authorization under EC 1829/2003 to justify how and why the proposed primer pair has been selected and to provide experimental results from testing the method with different varieties (*2*). A stronger emphasis should also be given to homology searches to show the absence of polymorphisms within the targeted sequences during the method validation process. It is evident from the study presented here that the method validation should focus not only on the specific GM event but also on the reference gene used for the relative expression of the GMO fraction.

In general, the results of this study plead for an in-depth comparison of the quantitative methods targeting different endogenous genes in several species. This should lead to the harmonization of taxon-specific reference systems used in GMO testing. The need for harmonized reference gene systems is even more pertinent at a time when GMOs bearing two or more stacked events are increasingly submitted for regulatory approval. Indeed, small variations may appear between the quantitative results obtained with different reference gene methods, as observed in this study for *hmg*, Zm*adh1*, and *zssIIb*. At one moment, one may face the situation that a food product contains one of the stacked GMO events at a measured copy number fraction above the threshold for labeling, whereas another event may have been detected below the threshold, because both validated methods targeted different reference gene sequences. During the first Global Conference on GMO Analysis in Como in June 2008, the need for studies on the reliability of different endogenous reference gene systems for GM quantitation was recognized. It is hoped that any initiative on this front would encompass the wide genomic variability existing in the crops investigated in order not to miss such single nucleotide polymorphisms as the one reported here.

ABBREVIATIONS USED

bp, base pairs; CRM, Certified Reference Material; Ct, cycle threshold; ERM, European Reference Material; GM(O), genetically modified (organism); *n*, number of subsamples (replicates); PCR, Polymerase Chain Reaction; *s*, standard deviation.

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