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ARTICLE

Assessment of Primer/Template Mismatch Effects on Real-Time PCR Amplification of Target Taxa for GMO Quantification

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GMO quantification, based on real-time PCR, relies on the amplification of an event-specific transgene assay and a species-specific reference assay. The uniformity of the nucleotide sequences targeted by both assays across various transgenic varieties is an important prerequisite for correct quantification. Single nucleotide polymorphisms (SNPs) frequently occur in the maize genome and might lead to nucleotide variation in regions used to design primers and probes for reference assays. Further, they may affect the annealing of the primer to the template and reduce the efficiency of DNA amplification. We assessed the effect of a minor DNA template modification, such as a single base pair mismatch in the primer attachment site, on real-time PCR quantification. A model system was used based on the introduction of artificial mismatches between the forward primer and the DNA template in the reference assay targeting the maize starch synthase (*SSIIb*) gene. The results show that the presence of a mismatch between the primer and the DNA template causes partial to complete failure of the amplification of the initial DNA template depending on the type and location of the nucleotide mismatch. With this study, we show that the presence of a primer/template mismatch affects the estimated total DNA quantity to a varying degree.

KEYWORDS: genetically modified organism; GMO; maize; primer/template mismatch; quantification; real-time PCR

INTRODUCTION

The release and use of genetically modified organisms (GMOs) are regulated in the European Union (EU) and worldwide. In the EU, the traceability and labeling legislation currently applied established a labeling threshold level of 0.9% for the presence of genetically modified (GM) material in food and feed products (*1*, *2*). As part of the authorization dossier of any new GMO, an analytical method for GMO analysis has to be provided (*1*,*3*). The method is evaluated and validated by the Community Reference Laboratory (CRL). The European Commission (EC) recommends to express a relative (%) GMO content on the basis of haploid genome equivalents (HGE) of the GM target relative to the taxon or species target (*4*). The recommended % GMO unit is calculated as the quantity of GM target divided by the total quantity of the species-specific target, that is, reference sequence (*4*). Therefore, generally, a GMO content is measured at the DNA level, and real-time polymerase chain reaction (real-time PCR) is the accepted and widely used technique for this purpose.

Real-time PCR GMO quantification is based on two separate amplification assays: an event-specific assay amplifying the target GMO event and a reference assay amplifying a plant-taxon-specific region. The latter is thus used to estimate the number of haploid genome equivalents of the plant species or ingredient. Real-time PCR amplification plots show a lag phase, an exponential phase, and a plateau phase. Quantification occurs during the exponential phase of amplification when the fluorescence signal exceeds the detection threshold value, commonly referred to as crossing threshold cycle (C_T). At an appropriate point in the exponential phase of amplification, C_T values are measured for both the event-specific and the reference assay. Quantification can be done using a direct comparison of C_T values (ΔC_T method) or by calculating the absolute DNA target quantities, derived from a calibration curve consisting of calibrants or standards in absolute DNA target quantities (standard curve method) (*5*−*7*).

Globally, maize (*Zea maize*) is an economically important crop. To date, several GM maize events have been developed and are used for food and feed production (www.agbios.com/dbase.php). Maize is the second major biotech crop after soybean with a cultivation area of 37.3 million hectares representing 30% of the global biotech area in 2008 (*8*).

The maize genome is highly diverse, and variations at the nucleotide level are commonly observed between different maize inbred lines (*9*). Single base pair substitutions occur more frequently than insertions/deletions (indels) of one or more nucleotides. Indeed, Vroh Bi et al. (*10*) reported that SNPs and indels occur in maize coding genes every 73 and 309 bp, respectively. Bhattramakki et al. (*11*) identified 655 indels by sequencing 502 loci across eight maize inbred lines. Single base indels accounted for more than half of the identified indels and

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occurred with a frequency of 44.28 indel per 10 kb followed by two- and three-nucleotide indels, which occurred with a frequency of 11.22 and 10.48 indels per 10 kb, respectively (*11*). The analysis of the sequence diversity in 21 loci distributed along maize chromosome 1 showed that two randomly selected sequences have, on average, one single base pair substitution every 104 bp (*12*). More detailed studies on single nucleotide polymorphisms (SNPs) present in 18 maize genes in 36 maize inbred lines, representing the genetic diversity in the U.S.'s elite maize breeding pool, demonstrated the high rate of nucleotide variation in maize: 1 polymorphism per 31 bp in noncoding regions and 1 per 124 bp in coding regions (*13*).

SNPs may interfere with GMO quantification. If a SNP occurs in the sequence targeted by the primers used for the quantitative real-time PCR assay, a mismatch with the DNA template will be formed. This mismatch will destabilize the primer−DNA template duplex and will lead to a less efficient amplification of the PCR product (*14*−*17*), resulting in either a decrease in the estimated template quantity or even a complete failure of the amplification. Consequently, the inaccurate estimation of the DNA template quantity amplified by the reference assay will lead to inaccurate quantification of the GMO content. If the mismatch occurs in the reference gene sequence, this will likely lead to an overestimation of the GMO content, while if it occurs in the event-specific sequence, this will lead to an underestimation. As allelic variation is more likely to occur in endogenous genomic sequences than in transgenic sequences, GMO content overestimation may occur more often than GMO content underestimation. This, in turn, might affect the labeling of the product, especially in cases where the GMO content is near the labeling threshold of 0.9%.

In this paper, we evaluate the effect of single base pair substitutions on the real-time PCR amplification in a reference assay commonly used for GMO quantification in maize. Our choice for single base pair substitutions arises from the fact that they represent the most frequent sequence variants, compared to multiple base pair substitutions or indels, in the maize genome. In principle, two approaches could be followed: (1) testing a series of DNA templates (genomic DNA or cloned fragments) carrying different single base pair substitutions in the primer attachment site, in combination with a fixed primer pair, or (2) testing a fixed DNA template in combination with a series of primers carrying single base substitutions. Bru et al. (*18*) showed that a single mismatch carried either by the template sequence or by the primer sequence will have a similar effect on the real-time PCR. For two reasons, we choose to mimick the presence of single base pair substitutions in the genomic DNA template by replacing base pairs at different positions of the forward primer targeting the maize reference assay, *SSIIb*, while using a fixed reverse primer. First, a series of maize genomic DNA carrying different single base pair substitutions specifically located in the primer attachment site of the *SSIIb* sequence are not available to conduct our study. Second, using a single common genomic DNA extract as input for all reactions allows direct comparison of quantitative data and circumvents the need for any additional normalization for the template quantity input across such a series of independent DNA extracts.

A set of plasmid DNA calibrator solutions, each containing a precisely known number of DNA target copies, were used to establish real-time standard curves for absolute quantification. We quantified the *SSIIb* gene in two types of biological material, the conventional B73 maize inbred line and the certified reference material (CRM) 5% Mon810, using 16 different primer combinations representing various types and positions of the base pair mismatch in the primer attachment site. The effects of the mismatch type and position on the measured C_T values and estimated DNA template quantities were assessed.

Table 1. Sequence of the Forward Primers (the substituted base is indicated in bold)

no.	forward primer sequence 5′-3′
1	CTC CCA ATC CTT TGA CAT CTG C
2	C**A**C CCA ATC CTT TGA CAT CTG C
3	CTC CCA ATC C**A**T TGA CAT CTG C
4	CTC CCA ATC CTT TGA CAT C**A**G C
5	C**C**C CCA ATC CTT TGA CAT CTG C
6	CTC CCA ATC C**C**T TGA CAT CTG C
7	CTC CCA ATC CTT TGA CAT C**C**G C
8	C**G**C CCA ATC CTT TGA CAT CTG C
9	CTC CCA ATC C**G**T TGA CAT CTG C
10	CTC CCA ATC CTT TGA CAT C**G**G C
11	CTC CCA ATC CTT TGA CAT CT**T** C
12	CTC CCA ATC CTT TGA CAT CT**A** C
13	CTC CCA ATC CTT TGA CAT CT**C** C
14	CTC CCA ATC CTT TGA CAT CTG **T**
15	CTC CCA ATC CTT TGA CAT CTG **A**
16	CTC CCA ATC CTT TGA CAT CTG **G**

MATERIALS AND METHODS

Materials. Grains from maize inbred line B73 and the certified reference material (CRM) powder containing 5% mixture of transgenic event Mon810 and conventional maize variety were used. The 5% Mon810 is commonly used in GMO analysis to generate standard curves. B73 is frequently used in breeding programs. These two independent biological materials are equivalent with respect to quantification of the reference genes.

Methods. *DNA Extraction*. B73 grains were ground to fine powder using a mixer mill (Retsch MM301) for 1 min at 30 Hz. DNA was isolated from 100 mg of each sample by means of DNeasy Plant Mini Kit (Westburg, The Netherlands) according to the manufacturer's protocol with the incubation time of the sample in lysis buffer extended to 30 min. DNA extracted from five samples for each matrix was pooled. The DNA concentration was measured by means of GeneQuant spectrophotometer (Amersham Pharmacia Biotech, The Netherlands).

Oligonucleotides. A primer pair amplifying a 151 bp fragment of maize starch synthase (*SSIIb*) was used for real-time PCR (*19*). A single base at different positions in the forward primer was substituted, resulting in 15 alternative primers containing a single base pair mismatch in their sequence. In primers 2−10, the thymine (T) at positions 3, 12, and 21 starting from the 3′ end was substituted either by adenine (A), guanine (G), or cytosine (C) (**Table 1**). In primers 11−13, the 3′ penultimate base (position 2; G) was substituted by either C, T, or A. In primers 14−16, the 3′ ultimate base (position 1; C) was substituted by either G, T, or A (**Table 1**).

All reactions were performed with the same reverse primer (5′-TCG ATT TCT CTC TTG GTG ACA GG-3′) and probe (5′-FAM-AGC AAA GTC AGA GCG CTG CAA TGC A-TAMRA-3′).

TaqMan Real-Time PCR. All reactions were performed in a total volume of 25 μL using 96-well microwell plates and an ABI Prism 7000 high-throughput sequence detection system (Applied Biosystems). The mixture contained 1× GMO MasterMix (Diagenode, Luik, Belguim), 300 nM of each primer, 200 nM of *SSIIb*-Taq probe, and 37 ng of genomic DNA (approximately 13 700 maize genome copies per reaction on the basis of the maize genome size) (*20*). Thermal conditions were as follows: 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 real-time PCR data were processed using ABI Prism 7000 SDS software 1.0 (Applied Biosystems).

Standard curves were prepared using plasmid DNA containing the *SSIIb* fragment (Nippongene) in quantities of 20, 125, 1500, 20 000, and 250 000 copies per reaction. Each standard was run in triplicate.

In a first set of experiments, using primers 1−10 (**Table 1**), four and three independent runs were carried out for B73 and 5% Mon810, respectively. In a second set of experiments, using primers 1, 4, 7, and 10−16, three independent runs were carried out for both B73 and 5%

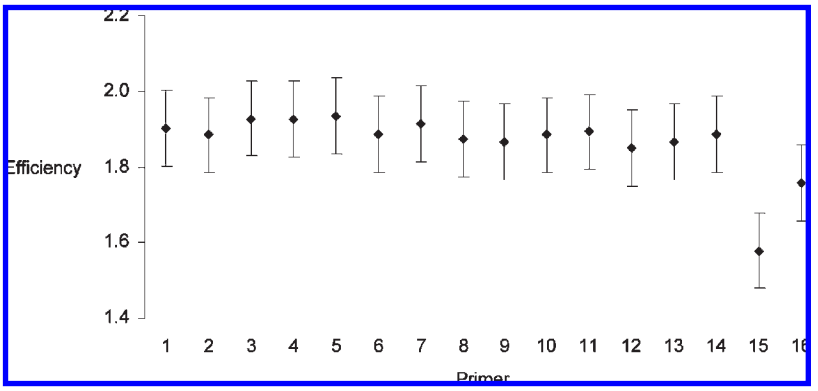

Figure 1. Comparison of the primer pairs' amplification efficiencies. Vertical bars denote error bars.

Mon810. Within each run, the reactions were performed in triplicate for each primer combination with either B73 or 5% Mon810. Absolute quantities of target DNA were obtained by interpolating the measured C_T values to the generated standard curve. Relative quantities, used to generate **Figures 3** and **5**, were determined relatively to the *SSIIb* quantity obtained with the standard primers. This *SSIIb* quantity was set as 100%. For instance, using primer 2 (where a T was replaced by an A at position 21), the obtained target quantity was 11 645 (data not shown), equivalent to 85% of the 13 700 maize genome copies obtained with the standard primer and loaded as template input (**Figure 3**). The purity of the real-time PCR amplicons was analyzed by separating the reactions on a 2% agarose gel and ethidium bromide staining and visualization.

SYBR Green I Real-Time PCR. Per reaction, 37 ng of genomic DNA, 12.5 μL of SYBR green I PCR Master Mix (containing a uracil *N*-glycosylase; Applied BioSystems), and 300 nM of primers were added to each well in a total volume of 25 μL. Reaction thermal conditions were as follows: 50 °C for 2 min (UNG erase reaction) and 95 °C for 10 min, followed by 40 cycles at 95 °C for 15 s and 55 °C for 30 s and a final melt curve analysis (60−95 °C; ramp time = 19.59 min). Melting curve analysis was performed in order to verify the specificity of the reaction.

Data Analysis. *Analysis of the Amplification Efficiency.* The amplification efficiency of each reaction was determined using LinReg PCR 7.5 (*21*). Linear regression analysis of TaqMan dye fluorescent signal rate emission in the exponential phase of the PCR amplification was performed for each individual reaction well to determine the primer pair specific amplification efficiency (*E*). Further, the mean of the amplification efficiency values was calculated for each primer pair. The mean amplification efficiency per primer pair was calculated based on a variable number of data points depending on the number of runs performed and varies between 18 and 51 observations.

Analysis of C_T Values and Estimated DNA Quantities. To assess the effects of the mismatch type and/or position on the C_T values and the estimated DNA quantities generated by primer pairs 1−10, the data were analyzed as a two-way design (with runs set as blocking factor) using analysis of variance (ANOVA). Entries were calculated as an average of three or four technical replicates (runs) for 5% Mon810 and B73, respectively. To assess the effects of mismatch type on the C_T values and the estimated *SSIIb* DNA quantities generated by primer pairs 1, 4, 7, and 10 −16, the data were analyzed as a one-way ANOVA (with runs set as blocking factor). Entries were calculated as an average of three technical replicates for 5% Mon810 and B73. In both analyses, *F* statistics were calculated and significance was assigned to the main terms mismatch type and position effects and to their interaction, if relevant. The one-way and two-way ANOVA were performed separately for B73 and 5% Mon810. The analyses were performed using Genstat (*22*).

RESULTS

Mismatch near the 3′ End Can Lead to Low Amplification Efficiency. The LinReg analysis shows that most primer pairs have a high amplification efficiency. The values varied between 1.85 and 1.93 for primer pairs 1−14. Primer pair numbers 15 and 16 display the lowest amplification efficiencies: 1.58 and 1.76, respectively (**Figure 1**).

To check if the lower efficiency is due to low primer specificity, we performed dissociation curve analysis after SYBR Green I amplification. Each PCR product displayed a single melting curve peak at 87 °C, except for the melting curve obtained with primer pair 15, where a double peak curve was obtained with maxima at 87 and 77 °C (results not shown). The peak observed at 77 °C was higher and broader than the peak observed at 87 °C, indicating primer−dimer formation. Inefficient amplification using primer pair 15 was confirmed by analysis of TaqMan real-time PCR amplicons on agarose gel, where the amplicon with the expected length was present for all primer pairs except for primer pair 15 (results not shown).

Single Internal Mismatch along the Forward Primer Sequence Results in Small C_T Value Shifts. For all performed reactions, a single calibration curve was generated using plasmid DNA (Materials and Methods). The effect of primer/template mismatch was assessed by comparing the C_T values derived from the altered primers with those derived from the standard primer.

Primer pairs 2−10 (**Table 1**), harboring a single mismatch at different positions along the forward primer, show a shift in C_T values compared to the standard primer pair 1. Two-way ANOVA analysis shows a highly significant effect of the mismatch type on the C_T values in B73 ($p < 0.001$) and 5% Mon810 ($p = 0.001$). In contrast, the effect of the mismatch position was less significant (B73: $p < 0.05$) or not significant (5% Mon810: $p = 0.15$). The shift in the C_T varied between +0.25 and +1.25 for B73 and between −0.28 and +0.45 for 5% Mon810 (**Figure 2**). The largest shift was observed with primer 10, where T was replaced by a G at position 3, for both B73 and 5% Mon810 (**Figure 2**).

The shift of C_T values results in an incorrect estimation of the *SSIIb* quantity. The measured C_T values were converted to quantities to estimate the fold-change in under- and/or over-estimation as a result of a primer mismatch (see Materials and Methods). The absolute quantity in each reaction was calculated using the standard curve. This approach is analogous to what would occur during GMO routine analysis when an unknown sample carrying a polymorphism in the primer attachment site would be quantified using a standard curve derived from a reference material containing a perfect primer attachment site. Subsequently, the absolute quantities were converted to relative quantities by setting the standard primer to 100%. So, all estimated quantities obtained with the respective mismatch primer combinations are expressed relative to the standard primer.

Two-way ANOVA was used to assess the effect of the mismatch type and its position on estimated *SSIIb* quantities. The analysis showed a highly significant effect ($p < 0.001$) of the

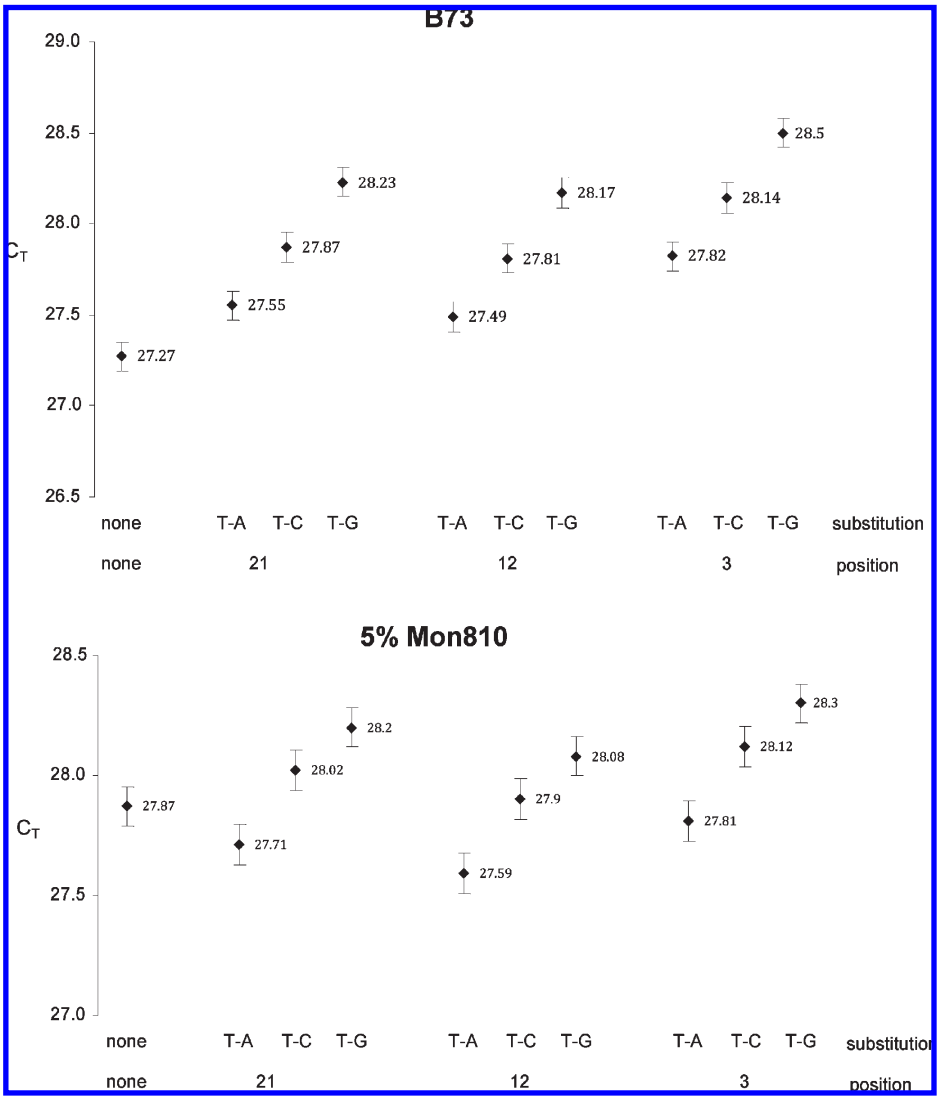

Figure 2. C_T means for substitution types at different positions, compared to the reference primer, in the B73 and 5% Mon810 matrices. Bars represent the standard error.

mismatch type on the estimated *SSIIb* quantities in both B73 and 5% Mon810. For B73, the primer 10 (T replaced by G; position 3) derived *SSIIb* quantity decreased to 45% in comparison with primer pair 1. The shift was less pronounced for 5% Mon810: it decreased to 78% of the *SSIIb* quantity obtained with primer pair 1. Overall, we observed that the substitution of a T with a G at positions 3, 12, or 21 resulted in a more pronounced decrease in the *SSIIb* quantities in comparison to the substitution of a T with a C or an A at the same positions (**Figure 3**).

Single Mismatch near the 3′ End Has Larger Effect on the Real-Time PCR Amplification and on *SSIIb* Quantification. As mismatches at position 3 show the largest shifts in C_T and *SSIIb* quantities, we were interested in studying the effect of mismatches when they occur at the 3′ penultimate and ultimate positions of the primer attachment site. We analyzed the data from the second set of experiments (using primers 1, 4, 7, and 10−16 in **Table 1**) by one-way ANOVA to assess the effect of the mismatch type and position. The mismatch type shows a significant effect ($p < 0.001$) on both C_T and *SSIIb* quantity in both B73 and 5% Mon810.

Primers 4, 7, and 10 were used in the second set of experiments to confirm the repeatability of the results obtained in the first set of experiments. A shift in the obtained C_T values in comparison with primer pair 1 was observed. The C_T shift varied between −0.17 and −0.92 for B73 and between −0.09 and −0.68 for 5% Mon810 (**Figure 4**). C_T values obtained in the first data set slightly differed from those observed in the second data set. However, the magnitude of C_T shifts was the same in both experimental data sets.

The C_T shifts caused by the primers harboring a mismatch at the penultimate position (numbers 11, 12, and 13) were between −0.74 and +0.76 for B73 and between −0.60 and +0.66 for 5% Mon810 (**Figure 4**). The derived *SSIIb* quantities represented 60−164% for B73 and 64−145% for 5% Mon810 of the *SSIIb* quantity obtained using the standard primer pair (**Figure 5**).

For primer 14, with a C by T substitution at the ultimate position, the C_T shift was relatively small (−0.14 for B73 and −0.28 for 5% Mon810). The shift in C_T values was more pronounced for other mismatch types at the same position. Indeed, when the 3′ ultimate C was replaced by a G (primer 16), a C_T value that is 7 C_T's higher compared to the standard primer pair was obtained for both matrices (**Figure 4**). A deviation in the estimated *SSIIb* quantity was also observed. The *SSIIb* quantity estimated with primer pair 14 was 8% (for B73) and 17% (for 5% Mon810) higher than the *SSIIb* quantity derived from the standard primer pair. Using primers 15 and 16 and for both B73 and 5% Mon810, the obtained *SSIIb* quantities represented a maximum of 1% of the standard primer pair derived *SSIIb* quantity (**Figure 5**). This large decrease is due to

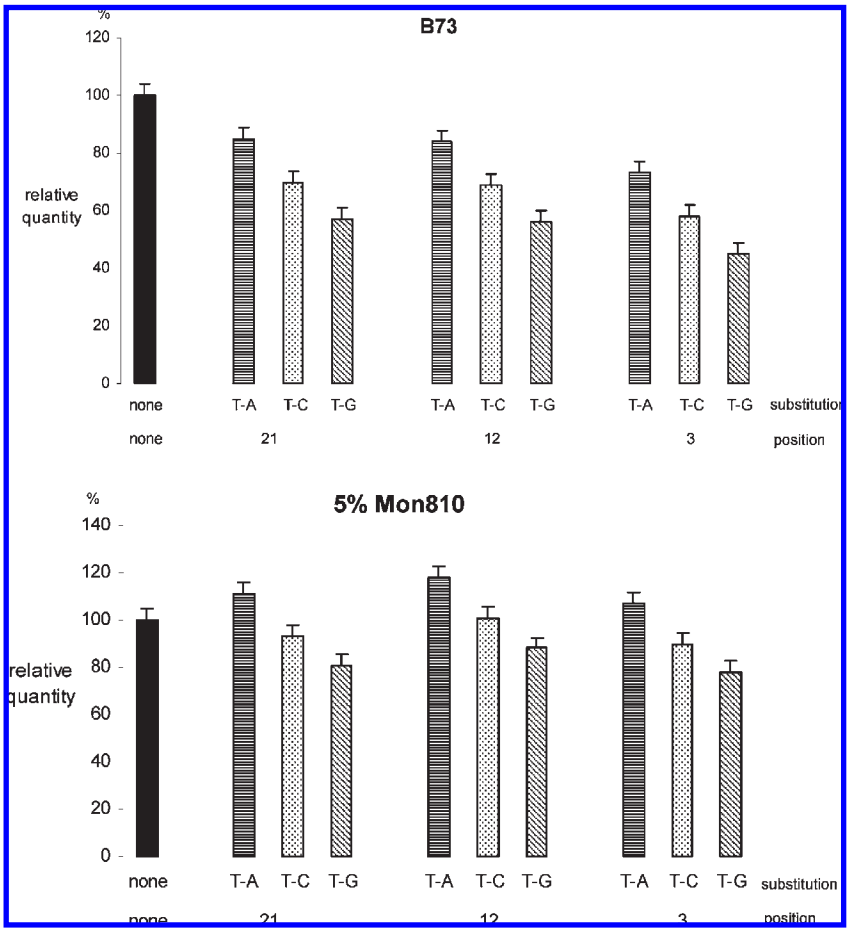

Figure 3. Relative *SSIIb* quantity means for substitution types at different positions, expressed as percentage of the quantity measured by means of the standard primer pair (indicated as none on the graph) for the B73 and 5% Mon810 matrix. Bars represent the standard error.

the low efficiency of primer pairs 15 and 16, 1.58 and 1.76, respectively (**Figure 1**).

DISCUSSION

In this study, we showed that a primer/template mismatch at different positions of the forward primer attachment site leads to a shift in the real-time PCR C_T values measured during maize *SSIIb* endogene quantification. Genomic DNA extracts from two matrices were used: B73 and the certified reference material 5% Mon810. Several mismatch types, located along the forward primer attachment site and at its 3′ end, were tested. The significance of the type and location of the single base pair substitution was assessed by evaluating the shift in measured C_T value as well as in the estimated *SSIIb* target quantities.

The type of the mismatch significantly influences the C_T values, leading to shifts ranging from +0.25 to +1.25 C_T for B73 and from −0.28 to +0.45 C_T for 5% Mon810. Due to the exponential nature of the real-time PCR, small fluctuations in C_T values translate into large variability in the measured DNA target quantity (*7*). In our study, the target DNA quantities calculated and derived from the C_T measurements with primers harboring a mismatch were 15−55% lower than the *SSIIb* quantities obtained in the reactions using the standard primer pair for B73. For 5% Mon810, the *SSIIb* quantity varied between −22 and +11% in comparison with the standard primer pair. Although the influence of the mismatch position was not statistically significant, the C_T shift was higher when the mismatch was located toward the 3′ end of the primer. Therefore, we additionally assessed the effect of mismatches located at the last two positions at the 3′ end of the primer sequence. Our findings reveal that, when the mismatch is located at the penultimate position, the C_T shifts were still relatively small, ranging from −0.74 to +0.76 C_T for B73 and from −0.60 and +0.66 C_T for 5% Mon810. The influence of a mismatch located at the ultimate 3′ end was dependent on the substitution type. Substitution of a C by a T at the ultimate position did not lower the amplification efficiency. The observed C_T shift was about −0.1 and −0.3 C_T for B73 and 5% Mon810, respectively. Substitution of a C by a G or an A at the same position yielded 1.76 and 1.58 amplification efficiencies, respectively. The C_T values obtained using these primers were very high compared to the standard primer pair (7−15 C_T higher), and the *SSIIb* quantities represented only 1% of that obtained with the standard primer pair. From these results, we can conclude that the position of the mismatch within the primer sequence is important for the stability of the primer annealing. In addition, our results show that mismatches located more toward the 3′ end of the primers are more critical and affect the PCR more dramatically than mismatches at other positions, which is consistent with the findings in several other studies (*14*, *15*, *18*, *23*).

Broothaerts et al. (*24*) reported a 2- to 4-fold decrease in the estimated *adh1* endogene quantity compared to the *hmg* endogene quantity in different non-GM and GM maize varieties. The decrease was due to the presence of a mismatch created by the substitution of a T by a C in the *adh1* genomic sequence in some maize varieties. The mismatch was located in the middle of the *adh1* reverse primer. In our study, using forward primers where the mismatch was located in the middle, the maximal obtained decrease in the *SSIIb* quantity was 55%. The difference between both studies

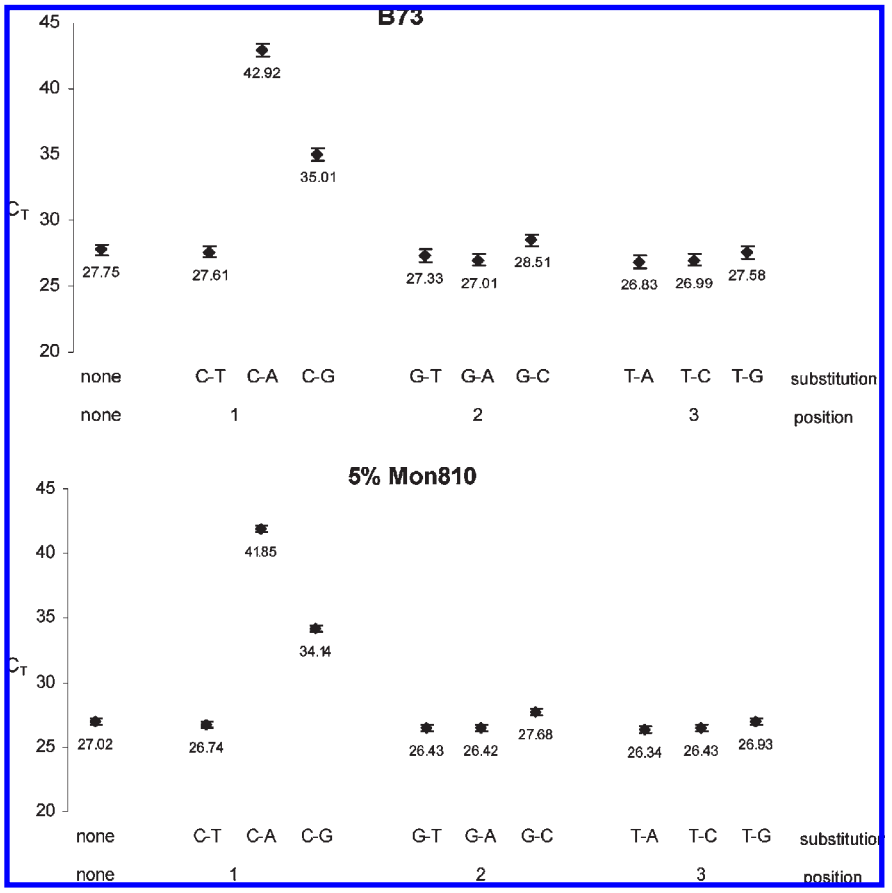

Figure 4. Mean C_T values for substitution type at different 3′ end positions for B73 and 5% Mon810 matrices. Bars represent the standard error.

regarding the extent to which a mismatch located in the middle of the primer is influencing the target DNA quantity estimation can be explained by several factors. Primer annealing temperature was set at 60 °C in our amplification reactions, while it was 56 °C in the study reported by Broothaerts et al. (*24*). Mismatch effects were described to be dependent on the annealing temperature (*15*, *25*). However, it was shown that the bias due to the presence of mismatches was reduced at lower annealing temperature (*25*). Primer length is also slightly different: 18 bp in Broothaerts et al. (*24*) study compared to 22 bp in our studies. The mismatch described in Broothaerts et al. (*24*) was located at position 10, while it was at position 12 in our primers.

GMO content is determined by the ratio between the GM event-specific DNA target quantity and the species- or taxon-specific DNA target quantity. We show that the presence of a primer/template mismatch during quantification of the endogenous reference gene will lead to a C_T and a DNA quantity deviation, hence leading to a deviation in the estimated GMO content. This deviation is dependent on the position of the mismatch within the primer attachment site. If the mismatch is located at the 3′ ultimate base, a high shift in the C_T and DNA quantities can be expected (except when a C was replaced by a T in the primer sequence). The high C_T shift will be immediately recognized and will indicate that the amplification reaction was severely hampered. If the primer/template mismatch is not located at the 3′ ultimate base of the primer, the deviation in the C_T values will be small (−1 to +1.25 C_T). Variations in measured C_T values within the range of one C_T are considered as normal variations within one run or between runs performed by the same operator, within a short time period, in the same laboratory (under so-called "repeatability conditions"). A difference of one C_T corresponds to a 2-fold difference in the estimated target quantity (+1 C_T equates to two times fewer targets while −1 C_T equates to two times more targets). Thus, the taxon-specific DNA target quantification will be affected, leading to an under- or overestimation of the GMO content. For maize, genes involved in metabolic pathways such as alcohol dehydrogenase (*adh*), chromatin-associated high mobility group (*hmg*), invertase (*ivr*), and *zein* genes are commonly used as taxon-specific reference genes for GMO quantification (*26*). Primers used for quantification are designed at coding sequences or intron/exon borders of these genes (*26*). SNP are single base pair positions in genomic DNA at which different alleles exist in individuals in a population, wherein the least frequency allele has an abundance of at least 1% (*27*). After examining 18 genes in 36 maize inbred lines representing the genetic diversity of the U.S. elite maize breeding pool, it has been shown that the frequency of SNP occurrence in maize coding sequences (on average one polymorphism per 124 bp) is lower than in noncoding regions (on average one polymorphism per 31 bp) (*13*). However, a SNP will only interfere with GMO quantification if it is located within the primer attachment sequence of the reference gene and/or transgene. The sequence length of the validated primers used for maize reference gene quantification ranges from 18 to 23 bp (*26*), which reduces the chance of a SNP occurrence in these regions of the DNA template sequence (36 to 46 bp including both primers attachment sites). Moreover, according to our results, a SNP that will severely hamper the amplification reaction and be noticed by the investigator must be located at the 3′ end of the primer attachment site, which is even more unlikely to occur.

Studying SNP frequency occurring in the reference gene sequences of commercially available maize varieties, in which the number is increasing and reached more than 1000 varieties registered in the EU (*28*), will be helpful to estimate the incidence

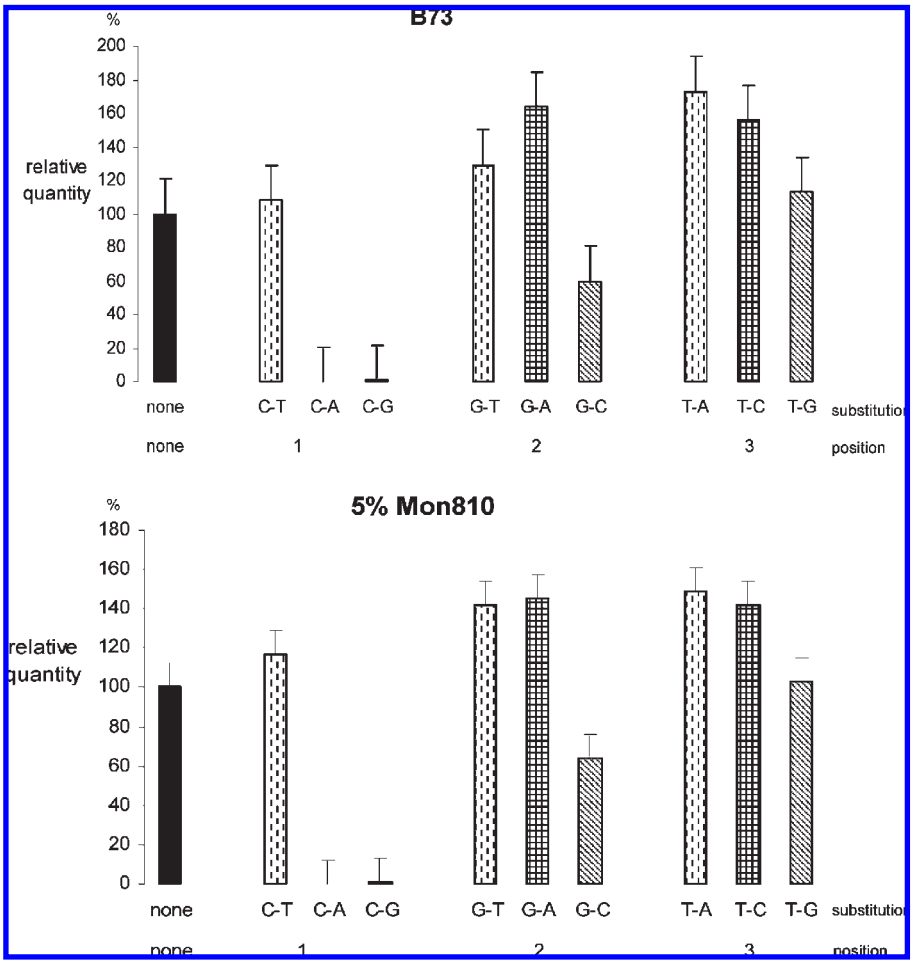

Figure 5. Relative *SSIIb* quantity means for substitution type at different 3′ end positions for B73 and 5% Mon810 matrices, expressed as percentage of the of the standard primer pair quantity (indicated as none on the graph). Bars represent the standard error.

of a SNP in a primer attachment site. Recently, in our laboratory, eight endogenous maize reference gene assays, amplifying sequences ranging in length from 70 up to 136 bp, were analyzed for their nucleotide sequence variation in a worldwide collection of different maize varieties and certified reference materials (*29*). One SNP in 11 out of more than 100 tested varieties was identified in the primer attachment site of the *Adh1* target sequence (136 bp). In another, *zein*-specific reference target (110 bp), a SNP was identified in 48 out of more than 150 tested varieties. However, this SNP was not located in the primer nor in the probe attachment sites of this reference assay (*29*). Hence, deviations in the calculated GMO content due to the presence of a base pair substitution in the genomic DNA are not likely to occur frequently. However, the impact of a primer/template mismatch might result in an inaccurate GMO content estimation. Thus, special attention should be given to the selection of species- or taxon-specific reference gene sequences for GMO quantification. In this selection, priority should be given to conserved (within a taxon) genomic regions with absence of SNPs and showing high nucleotide stability among the different cultivars.

For maize, reference assays for GMO quantification have been developed and validated based on genes encoding for high mobility group protein gene (*Hmg*), alcohol dehydrogenase 1 gene (*Adh1*), invertase (*Ivr*), *zein*, and the starch synthase type B (*SSIIb*) gene (*19*, *26*, *30*, *31*). Hence, it is possible to quantify multiple reference genes in parallel in order to overcome the risk of endogene quantification deviation due to the presence of a primer-template mismatch. A difference in one of the amplified gene quantity compared to the other(s) will be indicative of possible presence of a mismatch in one of its primer attachment sites. Amplifying two or more reference genes in parallel will, however, increase the workload and cost of the analysis. These can be reduced by using duplex and multiplex amplification methods (*32*−*36*), but multiplex reference assays have yet to be optimized.

ABBREVIATIONS

A, adenine; ANOVA, analysis of variance; C, cytosine; CRL, Community Reference Laboratory; CRM, certified reference material; C_T, threshold cycle; DNA, deoxyribonucleic acid; EC, European Commission; EU, European Union; G, guanine; GM, genetically modified; GMO, genetically modified organism; HGE, haploid genome equivalent; indels, insertions/deletions; PCR, polymerase chain reaction; SNP, single nucleotide polymorphism; *SSIIb*, maize starch synthase gene; T, thymine.

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