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DNA Content in Embryo and Endosperm of Maize Kernel (*Zea mays* L.): Impact on GMO Quantification

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PCR-based techniques are the most widely used methods for the quantification of genetically modified organisms (GMOs) through the determination of the ratio of transgenic DNA to total DNA. It is shown that the DNA content per mass unit is significantly different among 10 maize cultivars. The DNA contents of endosperms, embryos, and teguments of individual kernels from 10 maize cultivars were determined. According to our results, the tegument's DNA ratio reaches at maximum 3.5% of the total kernel's DNA, whereas the endosperm's and the embryo's DNA ratios are nearly equal to 50%. The embryo cells are diploid and made of one paternal and one maternal haploid genome, whereas the endosperm is constituted of triploid cells made of two maternal haploid genomes and one paternal haploid genome. Therefore, it is shown, in this study, that the accuracy of the GMO quantification depends on the reference material used as well as on the category of the transgenic kernels present in the mixture.

KEYWORDS: DNA; genetically modified organisms; certified reference materials; maize; endosperm; embryos; tegument

INTRODUCTION

Maize (*Zea mays* L.) is among the major sources of food and feed in the world. In the past two decades, tremendous advances have been achieved in genetic modification of many crop species including maize. This has led to the release of many transgenic corns that are increasingly grown in a couple of countries including mainly the United States and Canada. The main introduced traits are, to date, insect resistance and herbicide tolerance (*1*). The marketing of genetically modified organisms (GMOs) has raised ideological and ethical concerns during recent years. The debate has led the European Commission (EC) to approve the production and commercialization of food and feed of many genetically modified crops including transgenic corn and to regulate, through guidelines and directives, the intentional release, commercialization, and labeling of GMOs. This includes the Novel Food Directive 258/97/EEC (*2*), which regulated the marketing of GMOs for food use, and the Council's directives 1139/98/EEC (*3*), 49/2000/EEC (*4*), and 50/2000/EEC (*5*) that established the requirement of labeling of food and products containing GMOs. According to these directives, products containing >1% per ingredient of transgenic material must be labeled as containing GMOs. Other countries have also regulated the labeling of transgenic food using different GM thresholds.

Many quantitative methods for GMO analysis have been developed (*6*). Undoubtedly, the most used is based on the Polymerase Chain Reaction (PCR) and more particularly the quantitative real time PCR (*7*). This technique has been shown to provide the most accurate determination of GMO content in foodstuff as reported in many ring test studies (*8*). This technique is based on the specific amplification of DNA fragments within the transgenic event. Reference materials containing known ratios of GMOs are used as standards for quantification. The reference material used is often a set of commercial standards produced at the Institute for Reference Materials and Measurements (IRMM) (Geel, Belgium). This consists of a dried powder of a particular transgenic event. The powder is obtained by mixing powder from ground transgenic maize kernels with powder from nontransgenic kernels at the corresponding ratio. For maize, the transgenic kernels used are hemizygous (obtained through the crossing of a homozygous transgenic parent with a nontransgenic one).

Maize kernels are made, mainly, of a tegument, an embryo, and an endosperm. Endosperm accounts for 80−90% of total kernel's weight. Endosperm development begins with intensive mitosis followed by an endoreduplication of the DNA. This leads to large cells containing 3C (C being the haploid DNA content per nucleus) to up to 690C (*9*, *10*). The embryo is made of small and dense diploid cells (*11*). The DNA origins of the embryo and endosperm tissues are different. Whereas endosperms are triploid, resulting from the fusion of two maternal polar nuclei with one sperm nucleus, embryos are diploid, resulting from the fusion of one haploid maternal nucleus and

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Table 1. Genome Composition of Hemizygous Transgenic Kernel Tissues with Regard to the Transgenic Parent

	male transgenic	female transgenic
teguments	2C[a]	2C*[b]
embryo	1C*/1C	1C/1C*
endosperm	1C*/2C	1C/2C*

[a] C indicates a haploid genome. [b] C* indicates a haploid genome containing the transgene locus.

one haploid male nucleus. Finally, teguments are diploid and wholly of maternal origin. Thus, a hemizygous transgenic kernel might have different transgene copies depending on the transgenic parent and the pollination event as summarized in **Table 1**.

In GMO quantification using PCR-based methods, GMO proportion is given by calculating the transgenic genome copy number in the total maize genome copy number. Thus, it is obvious that the GMO quantification would be dependent on the transgenic parent and the DNA content of the teguments, embryos, and endosperms. Microscopy studies have shown that embryos are made of small and dense cells, whereas most of the endosperm cells are larger and vacuolated (*11*, *12*). This has led to the assumption that the kernel's total DNA is mainly made of the embryo's DNA. Using this hypothesis, GMO quantification is no longer dependent on the transgenic parent. However, given the fact that endosperm cells can contain as much as 690C, this might not be true. To our knowledge, no research group has yet determined the total DNA content in the different tissues of the maize kernels or addressed the impact on the accuracy of the GMO quantification.

Moreover, in real time PCR quantification of GMO, corn kernels, or any other starting materials, are ground and DNA is extracted from the powder. Two PCR reactions, one amplifying the transgene and the other an endogenous gene in maize, are used to determine the number of copies of transgene and the total number of genome copies of maize in the sample. Results are expressed in percentage by dividing the first by the second and assumed to be the weight-to-weight ratio in the initial maize mixture. This would be right only if all of the maize kernel cultivars, including transgenic ones, contain similar amounts of DNA per mass unit. This was always implicitly assumed but, to our knowledge, never checked.

The goal of this paper is to test the truthfulness of these two widely used hypotheses. The DNA content in teguments, embryos, and endosperms of individual kernels and the total DNA content of maize kernels from 10 different cultivars were measured. The impact of the results here found on the quantitative detection of GMOs by PCR-related techniques is discussed.

MATERIALS AND METHODS

Materials. Ten different maize cultivars randomly chosen among different maize classes were used in this study: Anjou 285, Cergi, F1444, DK512, Naudi, Anthares, DK585, Monumental, Chambord, and Prinz. These cultivars represent different types of grains (from dent to flint) and precocity (from early to late). All are hybrids except F1444.

Kernel Dissection and DNA Isolation. Kernels from each cultivar were incubated in *N*-cetyl-*N*,*N*,*N*-trimethylammonium (CTAB) extraction buffer [20 g/L CTAB; 1.4 M NaCl; 0.1 M tris[hydroxymethyl]-aminomethane (TRIS); 20 mM ethylenediaminetetraacetic acid (EDTA), pH 8] for 48 h. Teguments, embryos, and endosperms were separated from individual kernels. Tissues were lightly ground with a 7 mm solid steel bead in a paint shaker SO-10m Fluid Management (Sassenheim, The Netherlands) for 2 min. Tissues were lyophilized for 24 h, weighed, and stored until use. The entire tegument powder, 20 mg of endosperm, and 6 mg of embryo were further ground with metal beads for 2 min as previously described. The obtained powders were incubated for 30 min at 65 °C, with occasional shaking, in 1.2 mL of CTAB extraction buffer containing α-amylase (Sigma) (10 μL of a 10 mg/mL solution) and RNase A (Sigma) (10 μL of a 20 mg/mL solution). Twenty microliters of a 40 mg/mL solution of proteinase K (Sigma) was added and the mixture further incubated for 30 min at 65 °C. Proteins were extracted with chloroform treatment, and the DNA was precipitated for 1 h on ice with a 0.25 volume of 10 M of ammonium acetate and a 0.6 volume of 2-propanol. The pellet was recovered by centrifugation for 30 min at 15000*g* at 4 °C. The pellet was washed with 500 μL of 70% ethanol, recovered by centrifugation, and dried. The DNA was dissolved for 12 h in 0.1× TE buffer (1 mM TRIS, 0.1 mM EDTA, pH 8).

DNA Quantification. DNA quantity was determined using the picogreen ds DNA quantification kit (Molecular Probes, Leiden, The Netherlands). Fluorescence was detected using the FLx800 microplate fluorescence reader (Bio-Tek Instruments) and analyzed by KC4 software (2000). DNA was quantified relative to four standards (0.2, 2, 20, and 200 ng).

Table 2. DNA Quantity Extracted per 0.1 g of Maize Powder from 10 Cultivars

cultivar	DNA quantity (μg)	CV
Anjou 285	22.9	0.056
Cergi	23.0	0.090
DK512	19.3	0.100
F1444	23.5	0.103
Monumental	22.8	0.072
Anthares	24.5	0.039
Chambord	23.8	0.120
Prinz	20.8	0.122
Naudi	24.5	0.072
DK585	21.5	0.057

RESULTS AND DISCUSSION

Total DNA Content in Maize Kernels. Four random kernels of each of the selected cultivars were individually ground, and 25 mg was subjected to DNA extraction. The average DNA quantity was obtained as a mean value of three independent DNA quantifications using a fluorometer. The average value of the four average DNA quantities for each cultivar and the coefficient of variation are shown in **Table 2**.

The results show that by using our experimental procedure, the DNA quantity that can be extracted from 0.1 g of maize powder ranges from 19.3 to 24.5 μg for the 10 cultivars. Considering the extreme values of DNA content in 0.1 g of maize powder obtained, 1% (w/w) of DK512 powder in Naudi powder will correspond to 0.79% of DK512's DNA to total DNA extracted from the mixture, whereas 1% of Naudi in DK512 corresponds to 1.26% of Naudi's DNA to total DNA.

The maize cultivars used in this study are nontransgenic. However, it can be assumed that any transgenic maize would have comparable DNA contents per mass unit. Indeed, transgenic events are generated in maize lines selected for high transformation rates and other interesting traits for laboratory use and manipulation. Once a transformed plant is selected, the transgenic locus is transferred through back-crosses to different existing cultivars. Thus, one transformation event can be introduced into many cultivars, giving an isogenic transgenic cultivar. For example, the Mon810 event has been introduced into the DK512 cultivar, giving the DK513 transgenic cultivar. It is assumed that the only difference between the original cultivar and the transgenically derived cultivar is the transgene product. Theoretically, no significant difference would be

Table 3. DNA Quantity Extracted per 0.1 g of Maize Endosperm or Embryo Powder from the Cultivar Cergi

	starting material (mg)	DNA (μg)	CV
endosperm	10	4.588	1.16
	20	13.725	0.21
	40	13.089	0.08
embryo	1.5	60.135	0.09
	3	70.992	0.02
	6	111.588	0.25

observed in total DNA content per mass unit of these transgenic cultivars compared to the nontransgenic ones.

Taken together, in the total DNA extracted from a maize mixture, the DNA ratio of any specific cultivar, whether it is transgenic or not, cannot be automatically assumed to be proportional to the weight ratio of that cultivar in the maize mixture. Caution should be taken before PCR-based quantifications of GMOs, which are given as DNA ratios, are converted into weight-to-weight ratios.

Optimization of Extraction Procedure for Teguments, Embryos, and Endosperms. To compare the DNA contents in the different tissues of the individual kernels, the DNA extraction procedure should be finely optimized for each of these tissues. DNA extraction efficiency is known to be dependent on the particle size (*13*, *14*). We checked first that our grinding procedure gives powders made of <100 μM particles for all tissues (data not shown). In the second round, we determined the optimal quantity of the endosperm and of the embryo powder to be extracted. Using the same experimental procedure, we extracted the DNA from increasing amounts of starting endosperm and embryo powder. The total DNA quantity that can be extracted from 0.1 g of endosperm or embryo powder was calculated, and the results are shown in **Table 3**.

Higher starting quantities of endosperm and embryo powders were tested and gave lower yields (data not shown). The results, shown in **Table 3**, indicate that, using our experimental extraction procedure, the highest yield would be achieved using around 20 mg of endosperm powder and 6 mg of embryo powder as starting material. Actually, smaller quantities might have led to lower yields because of a weak carrier effect during the precipitation, whereas all of the DNA could not be extracted efficiently using 1.2 mL of extraction buffer and higher starting amounts of powder. We also have found that all of the material that was obtained by grinding a kernel's tegument should be treated at once (data not shown).

Kernel Tissue DNA Content and Impact on GMO Quantification. For each cultivar, the DNA of the tegument, endosperm, and embryo of four different kernels was extracted according to the procedure described. Three independent quantifications were realized for each DNA solution. The total DNA content of each of the tissues was calculated according to the starting material weight used in the extraction and the total tissue weight. Average values of a particular tissue's DNA to total DNA and standard deviations are given in **Table 4**.

The average tegument DNA ratio to total DNA ranges from 0.64 to 3.51%. The endosperm DNA ratio ranges from 36.27% for cv. DK512 to 59.41% for cv. Chambord. Finally, the embryo ratio varies from 38.56% (Chambord) to 59.55% (cv. Monumental).

These results were obtained using a CTAB-based buffer for the extraction procedure from the different tissues. CTAB-based extraction buffers are the most widely used for GMO quantifications (*6*). The ratio of a particular tissue's DNA to total DNA

Table 4. Relative DNA Content Ratio of Teguments, Endosperm, and Embryo to Total Genomic DNA for 10 Cultivars from the Average Value of Four Independent Kernels

	tegument		endosperm		embryo	
	av (%)	SD	av (%)	SD	av (%)	SD
Anjou 285	1.86	0.76	51.83	3.42	46.31	3.77
Cergi	1.56	0.47	54.63	3.22	43.80	3.47
DK512	1.49	0.43	36.27	2.37	62.24	2.78
F1444	0.64	0.14	52.64	1.05	46.72	0.94
Monumental	2.99	0.88	37.46	2.69	59.55	2.52
Anthares	2.95	0.87	48.86	5.39	48.19	5.09
Chambord	2.03	0.52	59.41	0.75	38.56	0.48
Prinz	1.99	0.32	40.71	21.58	57.29	21.50
Naudi	3.51	0.78	52.57	4.28	43.92	4.36
DK585	2.37	0.62	42.15	1.94	55.48	1.48

Table 5. Calculation of Total Haploid Genome Copies Harboring the Transgene Locus in 100 ng of Total Genomic DNA of the IRMM's Mon810-Certified Materials

GMO (%)	Ct	EmCt	EmCt*	EnCt	EnCt*	Ct*
0.1	36.7	19.25	9.625	19.25	6.41	16.04
0.5	192.5	96.25	48.125	96.25	32.08	80.21
1	385	192.5	96.25	192.5	64.17	160.41
2	770	385	192.5	385	128.33	320.83
5	1925	962.5	481.25	962.5	320.83	802.08

can be inaccurate if this buffer extracts the DNA more efficiently from one tissue powder than from the others. To test this hypothesis, we used an SDS-based extraction buffer (100 mM TRIS; 50 mM EDTA; 500 mM NaCl; 0.5% SDS; 3.8 g/L sodium bisulfite) and the same downstream experimental procedure. We have found comparable ratios using this buffer (data not shown), eliminating the possibility that the CTAB-based buffer would extract DNA from one tissue powder more efficiently than from the others.

Thus, these results indicate that about half of the total DNA extracted from the maize kernels originates from the endosperm tissue and the other half from the embryo tissue. The endosperm's DNA and the embryo's DNA are of different natures as the first is made of two maternal haploid genomes and one paternal haploid genome, whereas the second is made of one maternal and one paternal haploid genome. Tegument DNA, which is wholly maternal, can be neglected as it reaches at maximum 3.5% of total DNA. These findings have a strong implication over GMO quantification. Indeed, certified reference materials (CRMs) produced by the IRMM and used for maize GMO quantification are made of mixtures of non-GMO powder and a powder obtained by grinding transgenic hemizygous kernels. Using PCR-based quantification would allow, theoretically, an accurate quantification of only the hemizygous transgenic maize obtained by a comparable pollination event.

For Mon810 IRMM's certified material, for example, the transgenic hemizygous kernels were obtained by crossing a transgenic male with a nontransgenic female. In PCR-based quantification, 100 ng of total DNA is usually used for the PCR reaction, which corresponds to 38500 haploid genomes (*14*). Mon810 IRMM's certified material is available as a set of five powders containing 0.1, 0.5, 1, 2, and 5% of transgenic material. In 100 ng of DNA extracted from these powders, the total haploid genome copies harboring the transgene locus (Ct*) can be calculated for any of these reference powders as shown in **Table 5** and using the following formulas:

$$Ct = 38500 \times GMO\%$$

Table 6. Simulation of GM Content on Different Contamination Situations in Maize Using Mon810-like CRMs

situation	1% of the GM contamination by	Ct	EmCt	EmCt*	EnCt	EnCt*	Ct*	estimated GMO %
1	hemizygous GM locus from male parent transgenic	385	192.5	96.25	192.5	64.17	160.42	1
2	hemizygous GM locus from female parent transgenic	385	192.5	96.25	192.5	128.33	224.58	1.4
3	homozygous GM locus	385	192.5	192.5	192.5	192.5	385	2.4

where Ct is the total genome copies originating from the transgenic kernels;

$$EmCt = Ct/2$$

where EmCt is the total genome copies issuing from the embryos of the transgenic kernel as approximately 50% of the total DNA extracted from a kernel originates from the embryo tissue;

$$EnCt = Ct/2$$

where EnCt is the total genome copies issuing from the endosperm of the transgenic kernel as ~50% of the total DNA extracted from a kernel originates from the embryo tissue;

$$EmCt^* = EmCt/2$$

where EmCt* is the total genome copies harboring the transgene and deriving from the embryo's DNA of the hemizygous transgenic kernels;

$$EnCt^* = EnCt \times 1/3$$

where EnCt* is the total genome copies harboring the transgene and deriving from the endosperm's DNA of the hemizygous transgenic kernels (Indeed, in Mon810 IRMM's certified material, the transgenic kernels are hemizygous and were obtained using a transgenic male parent that brings only one haploid transgenic genome to the triploid endosperm tissue.); and

$$Ct^* = EmCt^* + EnCt^*$$

where Ct* is the total genome copies harboring the transgene.

According to this, the total copies genome number containing the transgene can be linearly linked to the GMO% in this specific IRMM's certified material with the following equation: $Ct^* = 160.41 \times (GMO\%)$. In the quantification of the Mon810 event GMO content in a given sample, at least three theoretical situations of contaminations can be possible: (1) the sample contains hemizygous GM locus kernels arising from the pollination of a nontransgenic female with transgenic pollen; (2) the sample contains hemizygous GM locus kernels arising from the pollination of a transgenic female with nontransgenic pollen; and (3) the sample contains homozygous GM locus kernels. Considering the case of a 1% (w/w) GMO contamination, and using the above given formulas, the theoretical haploid genome containing the GM locus in 100 ng of DNA extracted from the mixture is 160, 224.6, and 385 for situations 1, 2, and 3, respectively, as shown in **Table 6**. Using the IRMM's Mon810 reference material and the equation $Ct^* = 160.41 \times (GMO\%)$, the 1% (w/w) GMO containing samples 1, 2, and 3 would be assessed at 1, 1.4, and 2.4, respectively. Most cases of GMO contamination in food, feed, and seeds would correspond to situation 1. These contaminations can be quantified accurately. However, using Mon810-like CRMs, GMO quantification will be overestimated in situations 2 and 3. These situations may be very few in the real contaminations of food and feed but can be more frequent in maize seed production. The overestimation of GMO content in samples might increase the risks of sellers, whereas the buyers' risks are limited.

Table 7. Calculation of Haploid Genome Copies Harboring GM Locus in 100 ng of Total Genomic DNA of 1% Mon810-Certified Reference Material (IRMM413-3) According to the Percent of DNA Extracted from the Endosperm

endosperm DNA ratio (%)	Ct	EmCt	EmCt*	EnCt	EnCt*	Ct*	estimated GMO %
36	385	264.4	123.2	138.6	46.2	169.4	1.06
50	385	192.5	96.25	192.5	64.17	160.4	1
60	385	154	77	231	77	154	0.96

These results were obtained using the assumption that half of the total DNA arises from endosperm cell DNA. However, in the 10 cultivars studied, we have shown that the endosperm's DNA to total DNA ratio can range from 36.27 to 59.41%. Still, **Table 7** shows that the variation of the percentage of DNA extracted from endosperm found in the present study has only an insignificant influence on the total genome copies harboring the GM locus and therefore on GMO quantification.

Taken together, the results found in this study indicate that GMO quantification depends on the nature of the reference material and the nature of the transgenic material present in the analyzed sample. We have shown that, theoretically, an accurate determination is possible only if these two materials are of the same nature.

ABBREVIATIONS USED

GMO, genetically modified organisms; CRM, certified reference materials; PCR, Polymerase Chain Reaction; CTAB; *N*-cetyl-*N*,*N*,*N*-trimethylammonium bromide; TRIS, tris[hydroxymethyl]aminomethane; EDTA, ethylenediaminetetraacetic acid; SDS, sodium dodecyl sulfate.

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