JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

Coherence between Legal Requirements and Approaches for Detection of Genetically Modified Organisms (GMOs) and Their Derived Products

ARNE HOLST-JENSEN,*,† MARC DE LOOSE,‡ AND GUY VAN DEN EEDE§

National Veterinary Institute, Ullevaalsveien 68, P.O. Box 8156 Dep., 0033 Oslo, Norway; Department for Plant Genetics and Breeding, Centre for Agricultural Research, Caritasstraat 21, B-9090 Melle, Belgium; and Food Products and Consumer Goods Unit, Institute for Health and Consumer Protection, European Commission, Joint Research Centre, I-21020 Ispra (VA), Italy

Analytical methods for the qualitative and quantitative detection of genetically modified (GM) products may serve multiple purposes. Legal requirements differ among jurisdictions, ranging from no requirements to mandatory use of event-specific quantitation and implementation of production chain traceability. Although efforts have been taken to harmonize the analytical methodology at national, regional, and international levels, no normative international standards have yet been established. Lack of coherence between analytical methodologies and their applicabilities, on the one hand, and legislation, on the other hand, is a major problem. Here, key points where coherence is lacking are discussed. These include the definition of units of measurements, expression of GM material quantities, terminology, and inconsistent legal status of products derived from related but slightly different transformation routes. Finally, recommendations to improve the coherence are brought forward, including guidance to stakeholders for prediction of product-specific GM material quantities from gene ratios in the originating seed.

KEYWORDS: Feed; food; gene stacking; genetically modified organisms; GMO; method harmonization; reliability; seed; terminology; unit of measurement

INTRODUCTION

Since the first appearance of genetically modified (GM) plants in the early 1990s a wide range of detection methods have been developed for a multitude of purposes, see, for exampel, refs *1* and *2*. In the late 1990s the lack of coherence between different testing approaches led to the establishment of working groups with the purpose of establishing standards at national (e.g., AFNOR in France, DIN in Germany, CFQLCS in Japan), regional (CEN in Europe), and international (ISO and CC-MAS) levels. However, the different purposes for which methods were developed and applied, as well as regional preferences and legislation, have elucidated another related problem, that is, the lack of regulatory and analytical coherence.

The European Network of GMO Laboratories (ENGL, http://engl.jrc.it), which was established in 2002 under the auspices of the European Union (EU), has met regularly to discuss all kinds of genetically modified organism (GMO) detection related problems. Early on, the ENGL recognized the lack of methodological coherence as a major problem and pointed out that there was a need for harmonization between legislative requirements and detection methodologies. The work of ENGL and individual laboratories has influenced the present EU legislation in the GMO area on a scientific basis, for example, the Qpcrgmofood project (http://www.vetinst.no/Qpcrgmofood/Qpcrgmofood.htm) (see also refs *3* and *4*). Yet, there are a number of problems remaining before the legislation is truly coherent with detection methodology and vice versa.

GMO LEGISLATION

The current EU-based GM legislation is complex, but core elements include preauthorization safety assessments (*5*) by the European Food Safety Authority (EFSA) (*6*), availability of validated detection methods, reference materials, and thresholds for labeling (*7*, *8*), postmarket monitoring, and postmarketing traceability requirements (*9*, *10*).

In all countries where there is GMO legislation in place, the basic requirement is that any GMO shall go through an authorization procedure before it can be grown or used. This procedure is primarily in place to ensure that only safe products are placed on the market and usually applies the principle of substantial equivalence (reviewed in ref *11*). The authorization procedure may differ among different jurisdictions, and although different terms are applied that cover slightly different product categories, the most widely used term to refer to a specific GMO is probably "transformation event". This term, however, is not

* Author to whom correspondence should be addressed (telephone +47-23216243; fax +47-23216202; e-mail arne.holst-jensen@vetinst.no).
† National Veterinary Institute.
‡ Centre for Agricultural Research.
§ European Commission.

10.1021/jf052849a CCC: $33.50 © 2006 American Chemical Society
Published on Web 03/22/2006

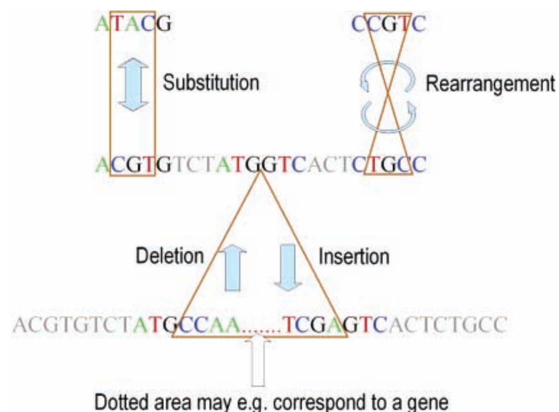

Figure 1. Genetic modification always includes the insertion of exogenous DNA (middle to bottom); in addition, the modification may involve substitution (middle left to top left), rearrangement (middle right to top right), or deletion (bottom to middle) of a sequence motif.

defined as consistently as desirable. The relevance of the definition of this term in the legal context is discussed in ref *4*.

DNA SEQUENCE MODIFICATIONS

In genetic engineering, transformation means the modification of a cell by the uptake and incorporation of exogenous (foreign) DNA. Transformation can be achieved by use of gene technology, and the result of the process is a "modified sequence" (ModSeq); that is, genetic information is inserted into the genome of the cell (organism) being modified (**Figure 1**). Notably, our definition of the ModSeq here is limited to the single functional genetic construct, although in principle a single inserted sequence contig may carry two or more functional genetic constructs. Here the definition of genetic modification also covers the possible deletion, rearrangement, or substitution of a part of the genome of the cell (**Figure 1**), but not in the absence of uptake and incorporation of exogenous DNA. Related terms such as "transgene", "expression cassette", and "inserted DNA fragments" have slightly different meanings. The former term is sometimes also used to refer to a GMO and may therefore be confusing. The term "expression cassette" implies that the sequence concerned is expressed, which is not necessarily true for a ModSeq. The term "inserted DNA fragment" may also be used to refer to the exogenous DNA inserted in the plant genome, without taking into consideration other changes to the sequence resulting from transformation as explained in **Figure 1**. For practical reasons the terms "transformation" and "genetic modification" may be used synonymously throughout the following text.

The basic perception of the transformation event is derived from the classical description of transformation of GM plants and from the classification of current DNA-based analytical methods, reviewed in ref *12* (cf. **Figure 2**).

ANALYTICAL TARGET RELIABILITY

The subject to genetic modification is DNA, whereas RNA and proteins are gene products produced through transcription and translation of DNA and mRNA sequences (see **Figure 3**). The transcription of the DNA sequence is regulated by a set of transcription factors, the concentration of gene products, and the physicochemical conditions that the cell is exposed to. RNA transcripts from a DNA sequence may therefore be produced in very different quantities under different conditions. The translation of mRNA sequences to protein depends on translation

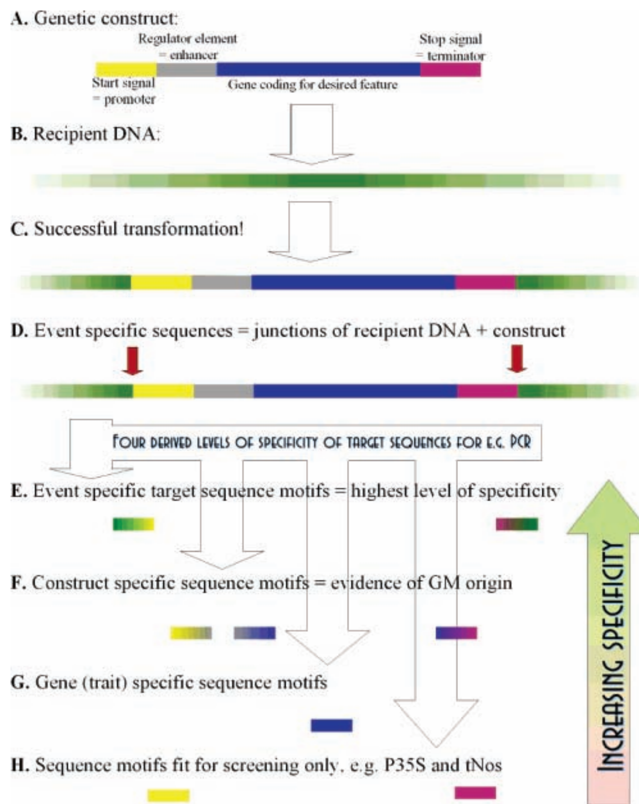

Figure 2. Relationships between genetic elements included in gene constructs, transformation, and specificity of alternative derived target sequences for detection of GM-derived materials. Adapted from ref *12*.

factors, the specific mRNA sequence, and the availability of corresponding tRNA molecules and amino acids in the cell. The translation of mRNA to protein is therefore highly variable depending on the conditions and needs of the cell. Consequently, in the single plant a direct quantitative relationship exists between the number of cells and the number of copies of a particular nuclear DNA sequence. Endo-reduplication is a common phenomenon in plants that represents an exception to this rule (*13*). Notably, however, endo-reduplication amplifies the entire genome and therefore does not alter the ratio of the copy numbers, a fact of great importance in the context of GMO detection and quantitation. In contrast, there is no linear relationship between the quantities of mRNAs and/or proteins derived from the genetic modification. Furthermore, a particular gene or RNA sequence may translate into different proteins as a result of RNA editing (*14*), although this phenomenon does not appear to be common for plant nuclear genes.

It is sometimes argued that construct-specific and event-specific methods are equally reliable with respect to identifying and quantifying GM-derived material, provided that the construct in question has been used only in one (authorized) event. Recently it was discovered that a globally unauthorized and publicly unknown GM maize Bt10 had been illegally grown in the United States and sold on the world market for several years (*15*). Bt10 was transformed using the same transforming plasmid, namely, pZO1502 (EMBL/GenBank accession no. AR110602), that was used to transform the widely authorized Bt11 maize, and the differences at sequence level between the inserted genetic constructs of the two events are limited to three single nucleotide substitutions and the additional insertion of the *bla* gene in Bt10 (letter from Syngenta to the European Commission's Joint Research Centre, March 31, 2005). Thus, the two events cannot be distinguished by application of the

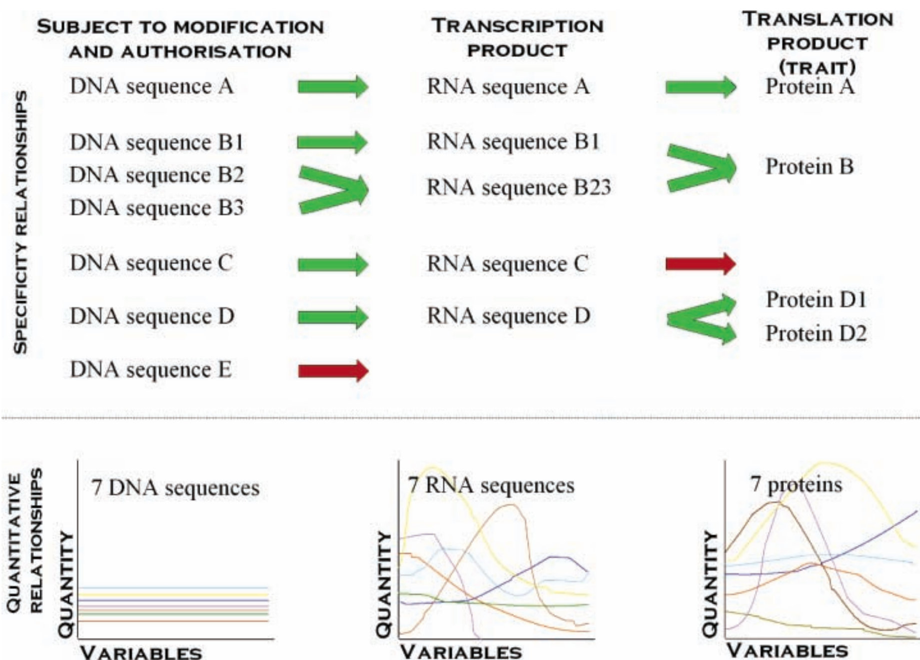

Figure 3. Specificity and quantitative relationships between candidate analytes for GMO testing. (Top) DNA sequences are subject to genetic modifications and authorization. Although there may be cases when one gene yields one mRNA sequence yields one protein (A), it is also possible that more than one gene sequence can give rise to the same mRNA sequence and that more than one mRNA sequence can give rise to the same protein (B). Sometimes RNA is degraded before it is translated to protein (C), and sometimes DNA is not transcribed (E). RNA editing may result in the potential synthesis of more than one protein (D). (Bottom) Although there is a linear relationship between the copy number of a given DNA sequence per cell (exemplified with seven hypothetical DNA sequences), independent of variables such as time and cellular conditions (tissue, physiological parameters, developmental stage), the same is not true for RNA (seven hypothetical RNA sequences) and proteins (seven hypothetical proteins).

construct-specific methods presently included in the European and international standards (ISO 21569 [annex C3] and ISO 21570 [Annex C7]; *16*, *17*). Bt10 is an "unauthorized GMO" for which the current EU legislation explicitly has zero tolerance (*7*, *8*). This clearly demonstrates that construct- and event-specific methods are not equally reliable. Although efforts have been initiated to cope with the technical side of this challenge (see http://www.coextra.org/WP/WP6.html), it is not clear how coherence between legislation and analytical realities can be ensured.

Although there are presently several GMOs on the world market that carry unique traits or unique genetic constructs, it is possible that the gene encoding the trait or the genetic construct present in such GMOs is also introduced to other new GMOs, as described above for Bt10 and Bt11. This is the basis for the current requirement for event-specific detection methods in the EU legislation (*7*, *8*, *18*). For insertions it is straightforward to identify event-specific sequence motifs (**Figure 2**). For deletions and substitutions, the situation is more uncertain. The substituted sequence motif or the fusion of the two sequence motifs flanking the deleted sequence would, in principle, create a unique sequence motif. However, in contrast to insertions, it may be that the deletion or substitution can be perfectly repeated, in which case the resulting sequence motif is not event-specific. Notably, however, the definitions of transformation and genetic modification require that exogenous DNA is inserted, in which case there is always an event-specific sequence motif available.

TERMINOLOGY

The product resulting from the transformation is often narrowly referred to as an elite event or, more widely, as a transformation event. However, it is commonly the case that two or more ModSeqs are created during the transformation process, for example, as a result of two physically separated insertions in the genome of the transformed cell, and therefore we need a more detailed terminology (see **Figure 4**).

A transformant resulting from a single-copy single ModSeq may be termed a "unique event" or "single event" (UniEv). A transformant resulting from multiple-copy and/or multiple ModSeqs may be termed a "multiple event" (MulEv). Notably, the MulEv results from a single transformation, but the ModSeqs may be located at the same locus or at separate loci. Both UniEvs and MulEvs are the primary transformants and may therefore be jointly termed "transformation events" (TraEvs). Because exogenous DNA is inserted into single chromosomes, the resulting ModSeq is not present on both homologous chromosomes, and consequently TraEvs are heterozygous for the ModSeq. Crossing of two TraEvs produces a "stacked event" (StaEv), and TraEvs and StaEvs together may be termed "elite events" (EliEvs). All EliEv production is taking place prior to authorization and marketing. The authorization and marketing concern the use of the EliEv to produce seed and products derived from planting of the seed. Seed is produced by crossing an EliEv with a conventional seed, typically adapted to local conditions where the seed will be planted. The resulting GM seed may be termed a "commerce seed" (ComSe). This more specific and detailed terminology should facilitate further the establishment of a more coherent traceability system for GM products. Hereafter this distinction will be used throughout the paper. Although the following description is somewhat simplified, the production of a ComSe essentially follows one of two routes. In some plant species the ComSe is heterozygous, whereas in other species the ComSe is produced by backcrossing of the F_1 generation to produce a homozygous F_2, which is the ComSe. The above description is slightly simplified, and it may therefore be that there is also a need for a more detailed terminology for the production and propagation of ComSes.

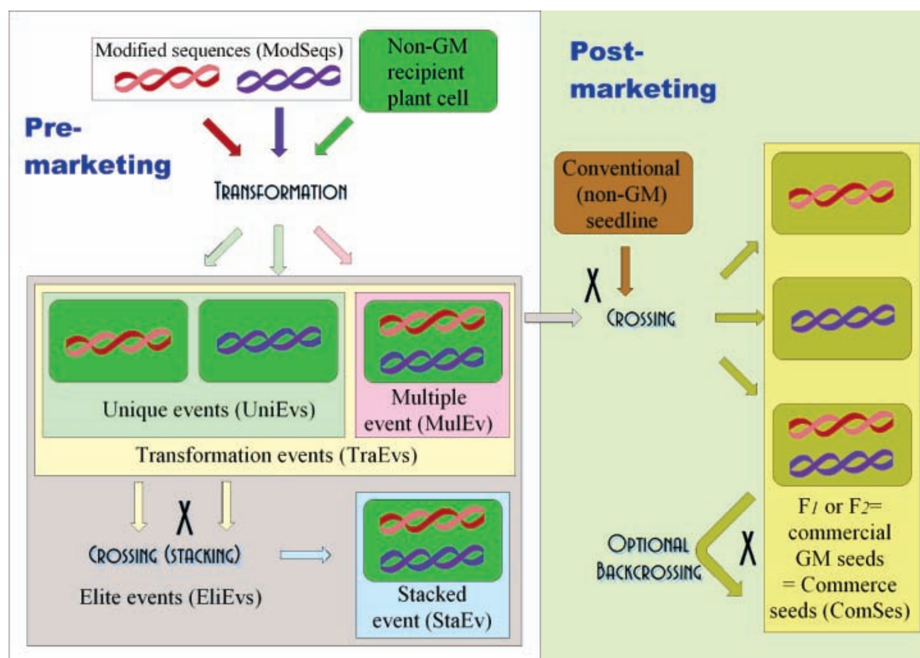

Figure 4. Distinction between development stages and corresponding genetically modified (GM) cell types and proposed terminology for reference to the different cell types. The modified sequences (ModSeqs; top left) may be identical or different. The ModSeqs are shown as blue or red double-stranded DNA (dsDNA) molecules. This is a simplified illustration; in reality, the cells contain several chromosomes, each present in two copies, and each ModSeq is a short fragment on a single chromosome. From the analytical point of view there may be no difference between a multiple event (MulEv) and a stacked event (StaEv) containing the same ModSeqs and no possibility to distinguish products derived from the simultaneous presence of individual single event (UniEv) and StaEv, except when a single individual plant or fruit (seed, kernel, etc.) is being tested. Postmarketing involves the production of commercial seeds from crossing of the elite event (EliEv) with conventional (non-GM) seed, and the resulting seed is further bred including optional backcrossing to produce a homogeneous commercial seed (ComSe) combining the characteristics of the GM EliEv with those of the conventional seed. Notably, the EliEvs are usually backcrossed to become homozygous for the ModSeq prior to the postmarketing phase. The ComSe is heterozygous for the ModSeq unless it is produced from backcrossing, which is often used to produce homozygous ComSes for certain crops, for example, soybean.

Gene stacking leads to the creation of an offspring containing the inserted genetic constructs of both parental TraEvs. The derived plant consequently contains two or more physically unlinked inserted genetic constructs. From a detection point of view this is no different from any TraEv containing two or more physically unlinked genetic constructs, for example, TraEvs produced by successful transformation with two or more transformation vectors (**Figure 4**). A StaEv may be considered a serially transformed MulEv. It may therefore be justifiable to ask whether a quantitative analysis of a MulEv should be treated differently from a quantitative analysis of a stack between two UniEvs, for example, if the ModSeqs found in the two UniEvs are exactly the same as those found in the MulEv.

Terminology is not only a problem in relation to defining the "event" but also in relation to the terms "genome size" and "haploid genome". This is extensively discussed in ref *19*, where the term "monoploid chromosome complement" (with chromosome number x) distinct from the "holoploid chromosome complement" (whole chromosome complement with chromosome number n irrespective of the degree of generative polyploidy) is used. The terms "holoploid genome", "monoploid genome", and the abbreviated term "C-value", referring to the "holoploid genome size", are derived from this. The term "haploid genome" may refer to both the monoploid and holoploid genomes. Note that both "monoploid" and "holoploid" refer to the haploid condition. Commission Recommendation 787/2004 (*20*) states that "*Seed or other plant propagating material lot quality level and its associated statistical uncertainty are defined in relation to thresholds for GMOs and relate to the percentage of GM-DNA copy numbers in relation to target taxon specific DNA copy numbers calculated in terms of haploid genomes*" (from recommendation IV, 1, last paragraph) and "*The results of quantitative analysis should be expressed as the percentage of GM-DNA copy numbers in relation to target taxon specific DNA copy numbers calculated in terms of haploid genomes*" (from recommendation V, 4, fifth paragraph). In light of the terminology proposed in ref *19*, this leaves too much room for interpretation because it is not clear if the reference to haploid genome shall be understood as the monoploid or holoploid genome. The question is not just of academic interest because most food crops are polyploid derivatives of ancestral wild plant species. For example, the holoploid genome of wheat ($n = 3x = 21$) is composed of three monoploid genomes ($n = x = 7$). Consequently, the interpretation of the term "haploid genome" will have a highly significant impact on how GM is quantified.

INHERITANCE OF GENES

DNA is inherited in a highly predictable manner. In eukaryotes, the nuclear DNA is transferred to successive generations in a Mendelian manner, whereas extranuclear DNA found in organelles (chloroplasts and mitochondria) is normally inherited uniparentally, most frequently maternally. Organelles may exist in highly variable numbers in each cell, and within each organelle the number of copies of the chromosome also varies extensively (*21*). It may be very difficult to introduce a ModSeq to, for example, all chloroplasts in a cell, and RNA editing is a very common phenomenon in plant organelles (*14*). Currently, all authorized genetically modified plants

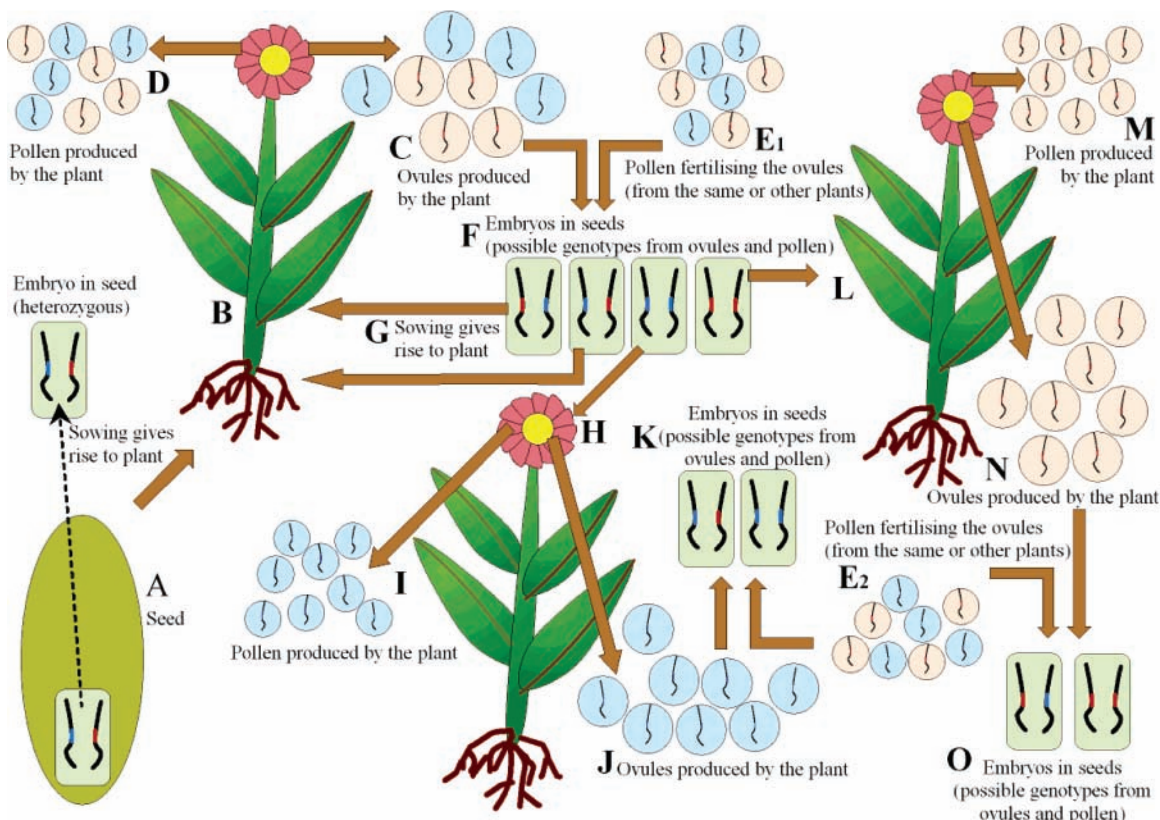

Figure 5. Genetic inheritance in plants: (**A**) The seed embryo contains two sets of genetic information (holoploid genomes), one maternally derived and one paternally derived (shown here as blue or red, i.e., the plant is heterozygous). (**B**) The plant contains the markers of the seed. (**C**) Ovules (female germcells) will be produced from both sets of holoploid genomes. Each individual ovule will contain only one of the markers, whereas on the plant the ovule pool has equal frequency of both markers. (**D**) Pollen (male germcells) will also contain only one of the markers, with equal frequency of both markers in the pollen pool on the plant. (**E**) Pollen is dispersed, resulting in a global pool of pollen for the mating population (here all pollen from D, I, and M). This pollen may stem from the same plant, from neighboring plants, or from plants more or less distantly located. (**F**) Pollen from the global pollen pool pollinates the ovules, resulting in the production of new seeds/embryos. The genetic constitution of each new embryo depends on the specific combination of ovule and pollen that gave rise to the new embryo. The resulting frequency of heterozygous (red + blue or blue + red) and homozyogous (blue + blue or red + red) depends on the frequency of occurrence of either of the markers in the two germline pools. (**G**) Heterozygous seed will give rise to heterozygous plants. (**H**) Homozygous seed (blue + blue) will give rise to homozygous plants (all cells have only one marker). (**I**) Homozygous plants give rise only to one type of pollen. (**J**) Homozyogous plants give rise only to one type of ovule. (**K**) Seed produced on the homozygous plant will always contain the marker from the parent plant, and in the embryo this will be combined with the markers present in the pollen population with a probability corresponding to the marker frequencies of the global pollen pool (E_2). (**L**) As for **H**, but with red and blue substituted. (**M**) As for **I**, but with red and blue substituted. (**N**) As for **J**, but with red and blue substituted. (**O**) As for **K**, but with red and blue substituted.

carry the genetic modification in their nuclear DNA. Genetic modification of extranuclear DNA may potentially introduce a high level of uncertainty in relation to stability and expression level. However, it also has some potential advantages, such as uniparental inheritance that may be exploited to minimize the risk of unintended dispersal of the genetic material. Nuclear genetically modified DNA is inherited according to Mendelian principles (see **Figure 5**), but extranuclear DNA will not follow these principles. From the analytical perspective, one of the advantages of Mendelian inheritance is that we may predict with a defined uncertainty the probable distribution and frequency of the genetically modified DNA from one generation to the successive generations.

After several years of scientific debate it was decided by the European Commision in the autumn 2004 that the concentration of genetically modified material shall be expressed as the ratio of an event-specific target sequence to a species-specific reference gene in terms of haploid genomes (*20*). This ratio must be established for each species and gene (see, e.g., refs *22*−*24*).

GMO MARKER FREQUENCIES, AN EXAMPLE CASE AND GUIDANCE TO DECISION SUPPORT

Trifa and Zhang (*25*) reported on the contribution of DNA from the seedcoat, the endosperm, and the embryo in maize seeds. Papazova et al. reported on the tissue-specific DNA extractability of maize (*26*) and assessed the influence of maize kernel genetic structures on DNA-based (real-time PCR) quantitation (*27*). These data can be extrapolated to produce an estimate of the genome equivalent-based concentration of GM-derived DNA in maize seeds (cf. **Figure 6**).

Each holoploid genome (n) may contain (n_+) or not (n_-) the ModSeq of interest, and the relationship between the associated probabilities $P(n_+)$ and $P(n_-)$ can be described as

$$P(n_+) + P(n_-) = 100\% \qquad \text{(I)}$$

Within a normal diploid cell, the frequency of the ModSeq (${}^cF_{n+}$) can be expressed relative to the holoploid genome content. The frequency of absence of the ModSeq in a normal diploid cell (${}^cF_{n-}$) can be expressed similarly. If, in a normal diploid

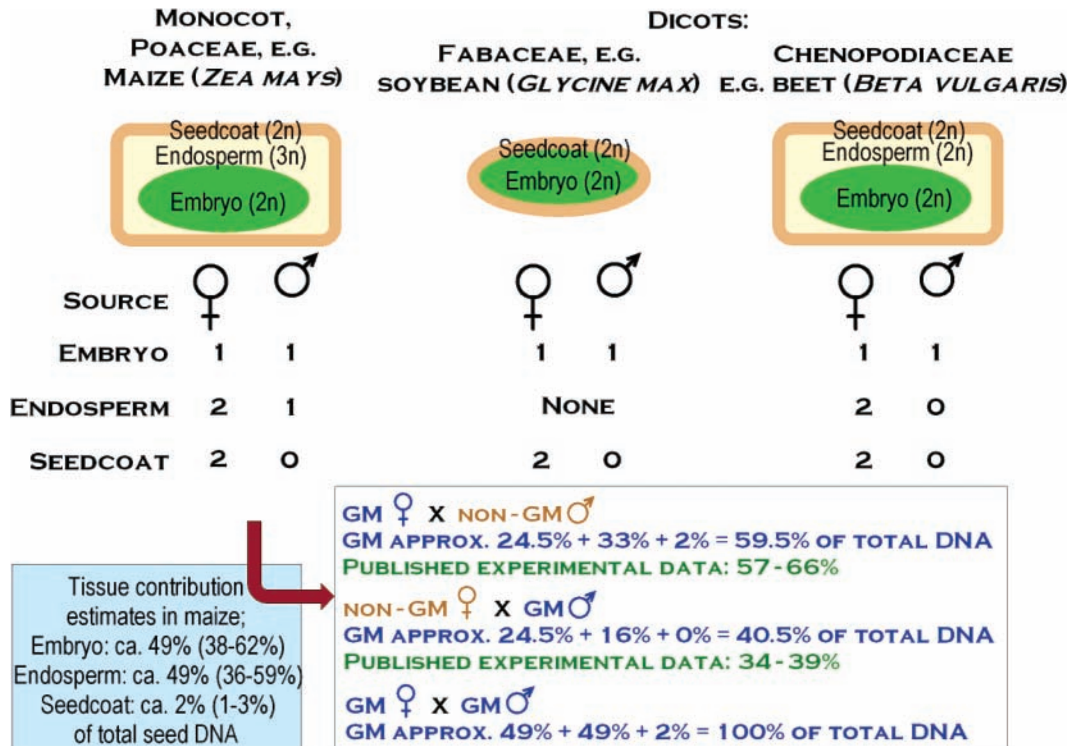

Figure 6. Contribution of haploid genomes from the parental gametes in plant seeds varies among taxa, as well as among tissues of the seeds. The influence of parents on the DNA-based GMO content is notable, as exemplified with maize on the basis of extrapolation from data published in refs *25–27.* The beet seed does not have an endosperm, but instead has a strictly maternal pericarp and perisperm with endosperm function.

cell, neither of the two holoploid genomes contains the ModSeq, then ${}^{c}F_{n+} = 0$ and ${}^{c}F_{n-} = 100\%$. If only one of the two holoploid genomes contains the ModSeq, then ${}^{c}F_{n+} = 50\% = {}^{c}F_{n-}$. Finally, if both holoploid genomes contain the ModSeq, then ${}^{c}F_{n+} = 100\%$ and ${}^{c}F_{n-} = 0\%$. Each cell must express one of these three ratios. In a population of normal diploid cells, it may be appropriate to distinguish between a population derived from a single plant and a population derived from more than one plant. On the single plant, the situation may be compared to the single cell. However, in a population derived from more than one plant, the frequency may take any value from 0 to 100%.

An embryo will receive one holoploid genome from each of its parents. The holoploid genome may be n_+ or n_-. The embryo may consequently contain either of four combinations of holoploid genomes: $(2n_+)$, $(n_+\ n_-)$, $(n_-\ n_+)$, or $(2n_-)$. The frequency of each of these combinations is established by the frequency of n_+ in the maternal plant $({}^{m}F_{n+})$ and in the paternal plant $({}^{p}F_{n+})$, respectively, in their diploid stage:

$$F(2n_+) = {}^{m}Fn_+ \times {}^{p}Fn_+ \qquad \text{(II)}$$

$$F(n_+ + n_-) = {}^{m}Fn_+ \times {}^{p}Fn_- \qquad \text{(III)}$$

$$F(n_- + n_+) = {}^{m}Fn_- \times {}^{p}Fn_+ \qquad \text{(IV)}$$

and

$$F(2n_-) = {}^{m}Fn_- \times {}^{p}Fn_- \qquad \text{(V)}$$

From eqs I−V, the probability for any given embryo to contain the ModSeq of interest $[{}^{e}P(n_+)]$ can be established

$${}^{e}P(n_+) = ({}^{m}Fn_+ \times {}^{p}Fn_+) + ({}^{m}Fn_+ \times {}^{p}Fn_-) + ({}^{m}Fn_- \times {}^{p}Fn_+) \qquad \text{(VI)}$$

and

$${}^{e}P(n_-) = ({}^{m}Fn_- \times {}^{p}Fn_-) \qquad \text{(VII)}$$

This may be of particular importance in relation to seed production, especially if GM seed ratios are defined as the ratio of seeds containing the ModSeq to seeds not containing the ModSeq of interest. However, in relation to product testing, it is more important to establish the frequency distribution of n_+ in the product, which is usually produced from a very large number of individual plants.

The relative frequency of a ModSeq relative to the holoploid genomes in a given tissue $({}^{t}F_{n+})$, expressed in percent, is a function of the number of maternal holoploid genomes (X) and the number of paternal holoploid genomes (Y) in the nucleus of the normal tissue cells and the relative frequency of the ModSeq in each of the two parental holoploid genome populations, $({}^{m}F_{n+})$ and $({}^{p}F_{n+})$, respectively. This frequency can be expressed according to the equation

$${}^{t}F_{n+} = \frac{X \times {}^{m}F_{n+} + Y \times {}^{p}F_{n+}}{X + Y} \qquad \text{(VIII)}$$

Estimates of ${}^{m}F_{n+}$ may be obtained by analyzing a representative leaf sample of the maternal plant population, whereas it may be more difficult to obtain good estimates of ${}^{p}F_{n+}$ because pollen is not only produced within the maternal plant population (where ${}^{p}F_{n+} = {}^{m}F_{n+}$) but may also be produced in neighboring fields. Pollen migration routes may be predicted from crop-by-crop-

Table 1. Examples[a] of the Relationship between Relative Frequency of a Modified Sequence (ModSeq) in the Maternal (${}^{m}F_{n+}$) and Paternal (${}^{p}F_{n+}$) Populations of Holoploid Genomes and the Resulting Predictive Relative Frequency of the ModSeq in Tissues (${}^{t}F_{n+}$) Produced from the Parental Holoploid Genomes

ploidy status of tissue = ♀ + ♂ = $n + n$[b]			ploidy status of tissue = 2♀ + ♂ = $2n + n$[c]			ploidy status of tissue = 2♀ + 0♂ = $2n + 0$[d]		
ModSeq frequencies in parent holoploid genome populations (%)		expected frequency in tissue (%)	ModSeq frequencies in parent holoploid genome populations (%)		expected frequency in tissue (%)	ModSeq frequencies in parent holoploid genome populations (%)		expected frequency in tissue (%)
♀ = ${}^{m}F_{n+}$	♂ = ${}^{p}F_{n+}$	${}^{t}F_{n+}$	♀ = ${}^{m}F_{n+}$	♂ = ${}^{p}F_{n+}$	${}^{t}F_{n+}$	♀ = ${}^{m}F_{n+}$	♂ = ${}^{p}F_{n+}$	${}^{t}F_{n+}$
0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1
	0.5	0.3		0.5	0.233		0.5	0.1
	1	0.55		1	0.4		1	0.1
	1.5	0.8		1.5	0.567		1.5	0.1
	1.8	**0.95**		1.8	**0.667**		1.8	**0.1**
0.5	0.1	0.3	0.5	0.1	0.367	0.5	0.1	0.5
	0.5	0.5		0.5	0.5		0.5	0.5
	1	0.75		1	0.667		1	0.5
	1.5	**1**		1.5	**0.833**		1.5	**0.5**
1	0.1	**0.55**	1	0.1	**0.7**	1	0.1	**1**
	0.5	**0.75**		0.5	**0.833**		0.5	**1**
	0.7	**0.85**		0.9	**0.967**		0.9	**1**
1.5	0.1	**0.8**	1.5	0.1	**1.033**	1.5	0.1	**1.5**
	0.5	1		0.5	1.167		0.5	1.5

[a] Bold numbers are cases where different tissues respectively comply and not comply with the current EU labeling threshold (0.9%) (*7*). [b] Representative tissues are, e.g., embryo, leaves, etc. in most plants. [c] Representative tissue is endosperm of monocot seeds and grains. [d] Representative tissue is seedcoat of all plants and endosperm of some dicot seeds and grains.

based studies, taking into consideration, for example, climatic conditions. Results from ongoing, for example, SIGMEA (http://sigmea.dyndns.org/) and Co-Extra (WP1; http://www.coextra.org) and future research projects, may provide a better basis for estimation of ${}^{p}F_{n+}$. At the same time it would facilitate establishment of a link between the way GM concentrations are determined in seed, feed, and food and in the environment, that is, paving the way for true coexistence between GM and non-GM supply chains and harmonization of monitoring approaches. Focus may logically be on ModSeq (allele) frequencies in populations.

In normal diploid tissues, for example, in embryos and leaves, $X = Y = 1$, and consequently from eq VIII, ${}^{t}F_{n+} = ({}^{m}F_{n+} + {}^{p}F_{n+})/2$. In particular, in diploid tissues, such as the seedcoat and endosperm in seeds of some species, and in polyploid tissues, such as the endosperm in seeds of some other species, $X \neq Y$. Consequently, for these tissues the frequency is more difficult to determine. On the basis of eq VIII, however, it is possible to tabulate the expected frequencies from predefined values of X, Y, ${}^{m}F_{n+}$, and ${}^{p}F_{n+}$ (**Table 1**).

Notably, depending on the relative frequency of the ModSeq in each of the two parental holoploid genome populations, (${}^{m}F_{n+}$) and (${}^{p}F_{n+}$), respectively, two different tissues on the same plant derived from the same parental holoploid genomes may contain the ModSeq at a frequency lower than and higher than a specified threshold (e.g., 0.9%), respectively. Two examples may demonstrate this (see **Table 1**): (1) If ${}^{m}F_{n+} = 0.1\%$ and ${}^{p}F_{n+} = 1.8\%$, then ${}^{t}F_{n+} = 0.95\%$ in derived embryos or leaves, whereas ${}^{t}F_{n+} = 0.667\%$ in derived endosperm if the plant is, for example, maize. (2) If, however, ${}^{m}F_{n+} = 1.5\%$ and ${}^{p}F_{n+} = 0.1\%$, then ${}^{t}F_{n+} = 0.8\%$ in derived embryos or leaves, whereas ${}^{t}F_{n+} = 1.033\%$ in derived endosperm if the plant is, for example, maize.

Table 1 and eq VIII may therefore be used to predict the overall ModSeq frequency in harvests from a population of seeds with a known embryonic frequency of the ModSeq. The ModSeq concentration in the harvest (product) can be estimated by application of the equation

$$ {}^{T}F_{n+} = \frac{(A \times {}^{a}F_{n+}) + (B \times {}^{b}F_{n+}) + ... + (K \times {}^{k}F_{n+})}{(A + B + ... + K)} \quad \text{(IX)} $$

where ${}^{a}F_{n+}$ is the expected frequency of the ModSeq in a particular tissue (see **Table 1** and eq VIII) contributing A% of the total number of holoploid genomes in the product, ${}^{b}F_{n+}$ is the expected frequency of the ModSeq in a particular tissue (see **Table 1** and eq VIII) contributing B% of the total number of holoploid genomes in the product, etc., and ${}^{T}F_{n+}$ is the expected overall frequency (concentration) of the ModSeq in the product taking all tissues in the product into consideration. Notably, $A + B + ... + K = 100\%$. If the product is maize seed, then ${}^{a}F_{n+}$ can be the ModSeq frequency in the embryo, ${}^{b}F_{n+}$ can be the ModSeq frequency in the endosperm, and ${}^{k}F_{n+}$ can be the ModSeq frequency in the seedcoat; A can be the relative contribution of holoploid genomes from the embryonic tissues, B can be the relative contribution of holoploid genomes from the endosperm tissues, and K can be the relative contribution of holoploid genomes from the seedcoat. For this example, estimates of A, B, and K can be extrapolated from refs *25* and *26*. Further experimental data may also be found in ref *27*.

The actual values for the tissue-specific variables X and Y in eq VIII and A, B, ..., K in eq IX need to be established on a crop-by-crop or product-by-product basis, and the resulting values may be tabulated, including confidence ranges, to be used as decission support tools for stakeholders.

Estimates of holoploid genome-equivalent-based concentrations of GM-derived DNA may be used to establish biological uncertainty ranges around predicted distributions of GM and non-GM DNA in harvests produced from planted seeds. From **Figure 6**, taking GM maize seeds as an example, it is clear that ${}^{t}F_{n+} = 100\%$ in the homozygous GM and ${}^{t}F_{n+} = 0\%$ in the homozygous non-GM seed, respectively, whereas ${}^{t}F_{n+} \approx 50 \pm 16\%$ in hemizygous GM maize seed. Measuring the holoploid GM DNA ratio yields a measurement that we may refer to as N%. With respect to the uncertainty, it is clear from

Table 2. Expected DNA-Based GM Quantity in Flour and Total Kernels,[a] with Kernel Number Based GM Quantity = 1%

no. of ModSeqs in the kernel[b]	parental origin of GM DNA	DNA ratio embryo:endosperm in kernels			
		50%: 50% (%)[c]	varA 62.2%: 36.3% (%)[d]	varB 38.6%: 59.4% (%)[e]	flour
unique event (UniEv)	♂	0.42	0.43	0.39	0.33
	♀	0.58	0.55	0.59	0.67
	♂+♀	1	1	1	1
two traits	♂	0.83	0.86	0.78	0.67
	♀	1.17	1.1	1.18	1.33
	♂+♀	2	2	2	2
three traits	♂	1.25	1.29	1.17	1
	♀	1.75	1.66	1.77	2
	♂+♀	3	3	3	3

[a] Kernels and seeds would yield the same DNA-based GM quantities. [b] Result is the same whether the number of ModSeqs in the kernel stems from a multiple event (MulEv) or a stacked event (StaEv). [c] Ratio to be assumed if there is no prior knowledge about the true DNA content ratio in the kernel. [d] Ratio reported in ref *25* for the maize variety DK512. [e] Ratio reported in ref *25* for the maize variety Chambord.

the above that the biological uncertainty exclusively stems from hemizygous seeds.

Let us for simplicity at this point consider a situation in which we have no analytical uncertainty, that is, no uncertainty associated with the analytical method itself. In this case the scenario with the highest degree of biological uncertainty is when all ModSeqs are derived from hemizygous GM seeds. The biological uncertainty of the measurement concerning the true GM content of the embryos is approximately $\pm N/3$ of the measurement in hemizygous maize seeds (cf. **Figure 6**).

This allows us to provide very accurate and narrow estimates of the lowest quality level (LQL) and acceptable quality level (AQL) associated with selling and buying the seed. In comparison, if seed was the unit, the LQL and AQL would have to describe the expected frequency of GM in the harvest on the basis that all GM seeds were homozygous.

IMPACT OF THE UNIT OF MEASUREMENT ON GMO QUANTITY ESTIMATES

Until recently, the prevailing regulatory line of thinking was that GMOs should be quantified on the basis of weight units. Consequently, there would be no de facto quantitative difference between any lot derived from a mix of 1 kg of GM and 99 kg of non-GM grain, independent of whether the GM grain is produced from an EliEv of the UniEv, MulEv, or StaEv type. Genetically, however, there is a considerable difference with respect to the GM content of these grain lots (see **Figure 6**).

Trifa and Zhang (*25*) provided data on the relative DNA content ratio of the endosperm (tegument), endosperm, and embryo of four different kernels for each of 10 different maize cultivars. The two most extreme ratios were found in the variety DK512 (varA in the following) and the variety Chambord (varB in the following), respectively. Products derived from maize kernels may include only particular tissues. For example, maize flour for human consumption is produced only from the endosperm. The embryo is removed from the kernels before processing to flour because the embryo contains high levels of oil that would result in reduced shelf life of the flour (*28*). **Table 2** explains how variations in relative DNA content in maize kernels, parental origin of the GM ModSeq, and degree of gene stacking may affect DNA-based GM quantitation in the total kernel and in derived flour.

Figure 7 illustrates how the determined GM quantity is affected by the relative DNA content of the kernel in different maize varieties and by parental origin of the GM trait in the kernel, in total kernels, and in kernel-derived flour.

Figure 8 gives another example of a potential problem created by lack of commutability between estimates of GM concentration on the basis of weight or particle ratios. This example consider beet fruits (seeds), derived from crossing a non-GM maternal plant with GM pollen (paternal plant). The resulting seed, cf. **Figure 8**, would be heterozygous, and only the embryo would carry the ModSeq. In this case, each seed would be 100% GM as determined by the particle- or weight-based approach, but significantly less when the DNA-based haploid genome equivalent approach was applied. Imagine further that in a pool of seeds, 75% of these carry the ModSeq of interest (**Figure 8**), and the seeds are processed by grinding. Only particles that are derived from the embryo of GM seeds would carry the ModSeq. The effect may be that a population of predominantly GM seeds is converted into a population of predominantly non-GM particles derived from the endosperm and the seedcoat with a smaller number of GM particles derived from the GM embryos. In other words, a 75% GM material is processed into a material with a detectable GM content of much lower than 75%. In contrast, having applied a DNA-based quantitation approach, the quantity of GM in the processed seed would have been invariant from that of the seeds prior to the processing.

GENE-STACKED AND MULTIPLE-EVENT GMOS

The genetic modification is per definition a modification of DNA, and consequently it may seem logical to define the unit of measurements in GM testing with respect to DNA. The modification itself is defined by a ModSeq and its locus, and each ModSeq locus may therefore be considered as a TraEv, cf. **Figure 4**. If the same ModSeq or different ModSeqs have been inserted into separate loci, the resulting GMO may be considered as a StaEv, as discussed above. Because it is not possible to define a unique molecular marker for a StaEv that will be vertically transmitted along with the cluster of associated TraEv-specific ModSeqs, it is logical to define that the detection method for the StaEv is the set of detection methods for each of the associated TraEvs. For the purpose of establishing the ModSeq concentration in the product, each UniEv must be detected and quantified. This can be done only if at least one unique detection method is available per UniEv. From a StaEv the UniEv-specific sequences may become segregated in the succeeding generation. This may have a significant impact on legal requirements, because authorization of a StaEv may require the availability of validated UniEv-specific detection methods for each of the involved UniEvs. Legally, the current EU legislation requires that a StaEv must go through separate

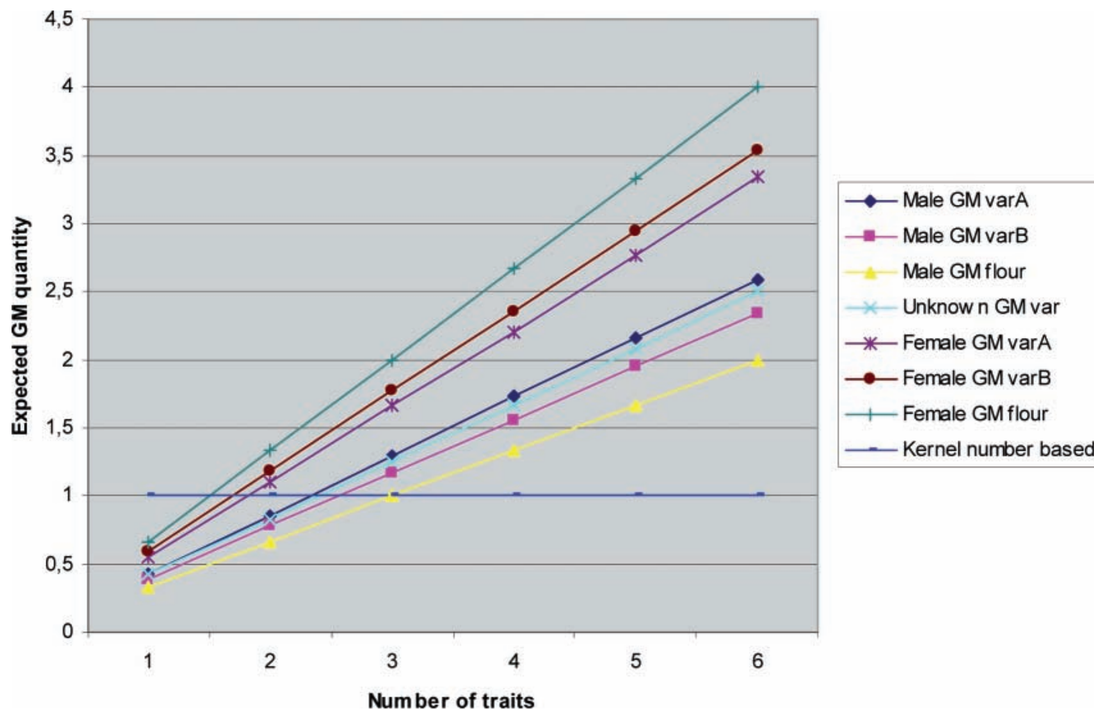

Figure 7. Expected DNA-based GM quantity in flour and total kernels, with fixed kernel number based GM quantity of 1% and heterozygous kernels. Flour here refers to flour for human consumption, not for animal feeds (*28*). Notably, in the example, for single traits, labeling with the present EU labeling threshold (*7*, *8*) will be required only for the kernels. For double traits, labeling in the EU will be required if the GM trait originates from the maternal parent, but not if it originates from the paternal parent. For triple and multiple traits, labeling in the EU will always be required in the example.

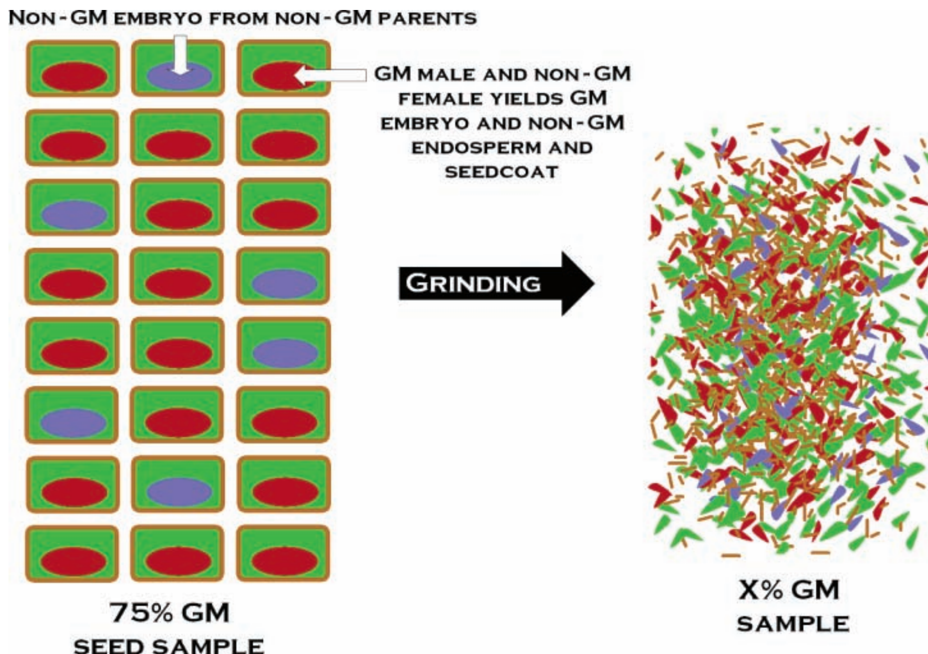

Figure 8. Processing, for example, grinding, may have significant effect on weight- or particle-based quantitation. A hypothetical example is shown where beet fruits (seeds) have been produced by crossing a GM male and a non-GM female, yielding heterozygous GM seeds, where only the embryo contains the GM target sequence. Red, GM-embryo-derived material; green and brown, nonembryonic endosperm and seedcoat material; blue, non-GM embryonic material. Applying a weight- or particle-based approach, each seed is defined to be GM or non-GM. However, once the material is processed, there is no clear link between the number of individual particles or mass units and the original sample material that is GM. The actual percentage of GM particles or mass of GM material (X) will depend on the analytical methodology applied and the relative size of the embryo as well as the particle sizes of the various tissue-derived materials after grinding. In contrast, processing will have no effect if determination of GM concentration is DNA based.

authorization procedures, independently of whether each of the involved parental TraEvs has been authorized. However, whereas the term "gene stacking" usually refers to intended crosses between TraEvs, gene stacking may also result from unintended crosses between two or more TraEvs, for example, when GM pollen from one TraEv is pollinating a GM ovule from another TraEv in a commercial field. Consequently, from an analytical perspective it would make no sense to authorize

a StaEv without simultaneously or previously authorizing each of the parental TraEvs.

Recently, a method for DNA-based single-seed testing for the presence of StaEvs was published (*29*). This method could be used to test seed lots, but it would require individual testing of a large number of seeds per lot. Furthermore, it would not provide accurate information about the StaEv content of the resulting harvest expressed in mass or particles (e.g., kernels), only about the probable UniEv frequency. Consequently, application of the method could comply with the requirements of the International Seed Testing Association (ISTA), but it would not provide the information necessary to reassure the farmers that their successive harvests would comply with, for example, labeling regulations.

Yet another uncertainty in current legislation was elucidated above: Shall the quantity of StaEv-derived material be expressed with reference to DNA or to other units? It may be argued that compared to a weight- or particle- (e.g., seed/kernel) based approach, a holoploid genome based approach may result in overestimates of the GMO quantity, because the presence of, for example, two TraEv-specific ModSeqs in a single holoploid genome would yield an estimated GMO concentration of 200%. At this point, it is reasonable to recall that the hemizygous single UniEv-derived GM maize seed is deemed only 40−60% GM (cf. **Figures 4** and **7** and **Table 2**). In contrast, if seed is the prevailing unit, the same seed is deemed 100% GM. Thus, there will be cases when the measured GMO content is lower and cases when it is higher depending on the applied approach. The main difference is whether the approach applied is consistent and coherent. As explained above, it may be justifiable to claim that the haploid genome based approach is much more consistent and coherent than any of the alternatives. A consistent application of this approach would appear (1) to unambiguously refer to either mono- or holoploid genome ratios to express the concentration of GM material, (2) to quantify GM materials on the basis of every motif corresponding to a UniEv in the product, and (3) to treat MulEvs and StaEvs similarly, with respect to identification and quantitation of derived material.

Genomes are plastic, and both MulEvs and StaEvs may be subject to mutational forces. Infrequent losses of ModSeqs in MulEvs and StaEvs may consequently render them nondetectable by particular detection methods. By defining the StaEv and MulEv associated detection methods as described above, it may as a matter of fact become very easy to handle a situation in which eventually a line derived from a GM has gone through unintended genetic changes, that is, single nucleotide substitutions and loss of particular targets for detection methods. If these changes occur on the single plant, they are likely to go undetected, and in a population of plants the effect on ${}^{T}F_{n+}$ will be ignorable on any derived product. However, on a seed production plant such unintended changes should be detected prior to commercializing the seed, simply by applying quantitative detection methods for each UniEv motif to a representative sample of the seed batch. The recent Bt10 incident may suggest that the seed producers may need an incitement to ensure that the seeds they commercialize are pure and of the correct genetic constitution. Legal requirements may function as such an incitement.

BOTANICAL IMPURITIES

A final example of the lack of coherence is with respect to taxonomic and other impurities (see also ref *30*). These are, for example, the presence of soybeans or soybean-derived materials (dust, fragments, etc.) in a maize grain lot or maize-based ingredient stemming from surviving (weedy) seeds from crops grown earlier in the same or preceding seasons or contamination transferred to the product from, for example, machines during processing. These impurities, if not taken into consideration in the establishment of the GM concentration in a product, may lead to serious problems for the analysts. If, for example, a maize ingredient with 1% botanical impurity stemming from 100% GM soybean is mixed with a perfectly non-GM soybean ingredient, then the mixed product may contain GM soybean derived DNA exceeding a defined threshold. The presence of botanical impurity in composite products may effectively preclude any reliable GMO determination in the absence of prior assessment of the ingredient-based impurity level. One solution to this problem could be to require that botanical impurities must be considered part of the ingredient (i.e., they would themselves become ingredients), although this is likely to have wide ranging negative implications for key stakeholders. Another solution could be to require ingredient-based impurity determination prior to mixing.

CONCLUDING REMARKS

Here we have listed and discussed what we believe have emerged as some of the most important challenges to coherence between GMO legislation and the analytical world. Particularly difficult technical challenges, for example, associated with cost-efficient screening and identification, however, have not been the subjects of this paper.

Undoubtedly, the decision by the European Commission to recommend the use of DNA ratios to express GMO quantity was a major step toward true coherence, although it is yet to be fully implemented even within the European Union. For reasons highlighted here we strongly recommend its immediate implementation in all GMO-related areas, including determination of seed impurity and gene stacking levels. From the ModSeq frequency of the seeds sown by the farmer, the GM content in the resulting harvest and derived products may be predicted more reliably than from seed-based numbers. The main uncertainty associated with the precision of the DNA-based predictions stems from uncertainty in the determination of the ModSeq frequency in the pollen pool of the mating population (including influx from neighboring fields). We also point out the need for a more refined terminology and propose several new terms. We hope that this paper will form the basis for further discussions, and we remind all stakeholders of the urgency of true coherence in this field.

ACKNOWLEDGMENT

We express our sincere gratitude to numerous colleagues in the European Network of GMO Laboratories (ENGL) and CEN/TC 275/WG 11 for long and fruitful discussions on issues related to the subject of this paper.

LITERATURE CITED

(1) Miraglia, M.; Berdal, K. G.; Brera, C.; Corbisier, P.; Holst-Jensen, A.; Kok, E.; Marvin, H. J. P.; Schimmel, H.; Rentsch, J.; van Rie, J. P.; Zagon, J. Detection and traceability of genetically modified organisms in the food production chain. *Food Chem. Toxicol.* **2004**, *42*, 1157−1180.

(2) Hernandez, M.; Rodríguez-Lázaro, D.; Ferrando, A. Current methodology for detection, identification and quantification of genetically modified organisms. *Curr. Anal. Chem.* **2005**, *1*, 203−221.

(3) Holst-Jensen, A.; Berdal, K. G. The modular analytical procedure and validation approach, and the units of measurement for genetically modified materials in foods and feeds. *J. AOAC Int*. **2004**, *87*, 927−936.

(4) Lezaun, J. Creating a new object of government: making genetically modified organisms traceable. *Social Stud. Sci.* **2006**, in press.

(5) European Commission. Directive 2001/18/EC of the European Parliament and the Council of 12 March 2001 on the deliberate release into the environment of genetically modified organisms and repealing Council Directive 90/220/EEC. *Off. J. Eur. Communities* **2001**, *L 106*, 1−38.

(6) European Commission. Regulation (EC) 178/2002 of the European Parliament and of the Council of 28 January 2002 laying down the general principles and requirements of food law, establishing the European Food Safety Authority and laying down procedures in matters of food safety. *Off. J. Eur. Communities* **2002**, *L 31*, 1−24.

(7) European Commission. Regulation (EC) No. 1829/2003 of the European Parliament and of the Council of 22 September 2003 on genetically modified food and feed. *Off. J. Eur. Union* **2003**, *L 268*, 1−23.

(8) European Commission. Commission Regulation (EC) No. 641/2004 of 6 April 2004 on detailed rules for the implementation of Regulation (EC) No. 1829/2003 of the European Parliament and of the Council as regards the application for the authorisation of new genetically modified food and feed, the notification of existing products and adventitious or technically unavoidable presence of genetically modified material which has benefited from a favourable risk evaluation. *Off. J. Eur. Union* **2004**, *L 102*, 14−25.

(9) European Commission. Regulation (EC) No. 1830/2003 of the European Parliament and of the Council of 22 September 2003 concerning the traceability and labelling of genetically modified organisms and the traceability of food and feed products produced from genetically modified organisms and amending Directive 2001/18/EC. *Off. J. Eur. Union* **2003**, *L 268*, 24−28.

(10) European Commission. Commission Regulation (EC) No. 65/2004 of 14 January 2004 establishing a system for the development and assignment of unique identifiers for genetically modified organisms. *Off. J. Eur. Union* **2004**, *L 10*, 5−10.

(11) Schauzu, M. The concept of substantial equivalence in safety assessment of foods derived from genetically modified organisms. *AgBiotechNet 2* **2000**, *ABN 044*, 1−4.

(12) Holst-Jensen, A.; Rønning, S. B.; Løvseth, A.; Berdal, K. G. PCR technology for screening and quantification of genetically modified organisms (GMOs). *Anal. Bioanal. Chem*. **2003**, *375*, 985−993.

(13) Larkins, B. A.; Dilkes, B. P.; Dante, R. A.; Coelho, C. M.; Woo, Y.-m.; Liu, Y. Investigating the hows and whys of DNA endoreduplication. *J. Exp. Bot.* **2001**, *52*, 183−192.

(14) Brennicke, A.; Marchfelder, A.; Binder, S. RNA editing. *FEMS Microbiol. Rev*. **1999**, *23*, 297−316.

(15) Macilwain, C. US launches probe into sales of unapproved transgenic corn. *Nature* **2005**, *434*, 423.

(16) ISO. *ISO 21569*; foodstuffs−methods of analysis for the detection of genetically modified organisms and derived products−qualitative nucleic acid based methods; International Standardization Organization: Geneva, Switzerland, 2005.

(17) ISO. *ISO 21570*; foodstuffs−methods of analysis for the detection of genetically modified organisms and derived products−quantitative nucleic acid based methods; International Standardization Organization: Geneva, Switzerland, 2005.

(18) European Commission. Guidance documents on the website of the Community Reference Laboratory; accessed Sept 9, 2005; http://gmo-crl.jrc.it/guidancedocs.htm.

(19) Greilhuber, J.; Doležel, J.; Lysák, M. A.; Bennett, M. D. The origin, evolution and proposed stabilization of the terms ‘genome size’ and ‘C-value’ to describe nuclear DNA contents. *Ann. Bot.* **2005**, *95*, 255−260.

(20) European Commission. Commission recommendation 2004/787/EC of 4 October 2004 on technical guidance for sampling and detection of genetically modified organisms and material produced from genetically modified organisms as or in products in the context of Regulation (EC) No. 1830/2003. *Off. J. Eur. Union* **2004**, *L 348*, 18−26.

(21) Griffiths, A. J. F.; Gelbart, W. M.; Miller, J. H.; Lewontin, R. C. *Modern Genetic Analysis*; W. H. Freeman: New York, 1999.

(22) Hernandez, M.; Rio, A.; Esteve, T.; Prat, S.; Pla, M. A rapeseed-specific gene, acetyl-CoA carboxylase, can be used as a reference for qualitative and real-time quantitative PCR detection of transgenes from mixed food samples. *J. Agric. Food Chem*. **2001**, *49*, 3622−3627.

(23) Hernandez, M.; Duplan, M.-N.; Berthier, G.; Vaïtilingom, M.; Hauser, W.; Freyer, R.; Pla, M.; Bertheau, Y. Development and comparison of four real-time polymerase chain reaction systems for specific detection and quantification of *Zea mays* L. *J. Agric. Food Chem*. **2004**, *52*, 4632−4637.

(24) Qpcrgmofood. Reliable, standardised, specific, quantitative detection of genetically modified food; accessed Nov 14, 2005; final report−workpackage 2; http://www.vetinst.no/Qpcrgmofood/Deliverable2.pdf.

(25) Trifa, Y.; Zhang, D. DNA content in embryo and endosperm of maize kernel (*Zea mays* L.): impact on GMO quantification. *J. Agric. Food Chem*. **2004**, *52*, 1044−1048.

(26) Papazova, N.; Malef, A.; Degrieck, I.; Van Bockstaele, E.; De Loose, M. DNA extractability from the maize embryo and endosperm−relevance to GMO assessment in seed samples. *Seed Sci. Technol*. **2005**, *33*, 533−542.

(27) Papazova, N.; Taverniers, I.; Degrieck, I.; Van Bockstaele, E.; Joost, H.; De Loose, M. Real time polymerase chain reaction (PCR) quantification of T25 maize seeds−influence of the genetic structures in the maize kernel on the quantitative analysis. *Seed Sci. Technol*. **2006**, in press.

(28) Butzen, S.; Hobbs, T. Corn processing. *Crop Insights* **2002**, *12* (10), 1−6.

(29) Akiyama, H.; Watanabe, T.; Wakabayashi, K.; Nakade, S.; Yasui, S.; Sakata, K.; Chiba, R.; Spiegelhalter, F.; Hino, A.; Maitani, T. Quantitative detection system for maize sample containing combined-trait genetically modified maize. *Anal. Chem.* **2005**, *77*, 7421−7428.

(30) Taverniers, I. Development and implementation of strategies for GMO quantification in an evolving European context. Ph.D. Thesis, University of Gent, Gent, Belgium, 2005; ISBN 90-5989-049-3.

Received for review November 16, 2005. Revised manuscript received February 19, 2006. Accepted February 27, 2006. This paper could not have been prepared without the financial support of the European Commission and the Research Council of Norway.

JF052849A