Beiträge zur Tabakforschung International · *Contributions to Tobacco Research* Volume 19 · No. 2 · July 2000

DOI: 10.2478/cttr-2013-0698

Current PCR Methods for the Detection, Identification and Quantification of Genetically Modified Organisms (GMOs): a Brief Review*

by

Ferruccio Gadani, Gregor Bindler, Hans Pijnenburg, Luca Rossi, Jacques Zuber

Philip Morris Europe
Research and Development
CH-2003 Neuchâtel
Switzerland

SUMMARY

Analytical methods based on the polymerase chain reaction (PCR) technology are increasingly used for the detection of deoxyribonucleic acid (DNA) sequences associated with genetically modified organisms (GMOs). In the European Union and Switzerland, mandatory labeling of novel foods and food ingredients consisting of, or containing GMOs is required according to food regulations and is triggered by the presence of newly introduced foreign DNA sequences, or newly expressed proteins.

In order to meet regulatory and consumer demand, numerous PCR-based methods have been developed which can detect, identify and quantify GMOs in agricultural crops, food and feed. Moreover, the determination of genetic identity allows for segregation and traceability (identity preservation) throughout the supply chain of GM crops that have been enhanced with value-added quality traits.

Prerequisites for GMO detection include a minimum amount of the target gene and prior knowledge of the type of genetic modification, such as virus or insect resistance traits, including controlling elements (promoters and terminators). Moreover, DNA extraction and purification is a critical step for the preparation of PCR-quality samples, particularly for processed agricultural crops such as tobacco. This paper reviews the state-of-the-art of PCR-based method development for the qualitative and quantitative determination and identification of GMOs, and includes a short summary of official and validated GMO detection methods.[a] [Beitr. Tabakforsch. Int. 19 (2000) 85–96]

ZUSAMMENFASSUNG

Analytische Methoden, die auf der Technologie der Polymerase-Kettenreaktion (PCR) beruhen, werden in zunehmendem Maße zum Nachweis von Desoxyribonukleinsäuresequenzen (DNA) bei genetisch veränderten Organismen (GVO) verwendet. In der EU und der Schweiz besteht eine durch lebensmittelrechtliche Verordnungen festgelegte Kennzeichnungspflicht für Novel Food und Lebensmittelzutaten, die GVOs darstellen oder solche enthalten, wenn diese durch Hinzufügen von DNA-Sequenzen aus Fremdorganismen oder durch Neuexpressionen von Proteinen erzeugt wurden.

Um den gesetzgeberischen Vorgaben und den Forderun-

* Received: 20th February 2000 – accepted: June 2nd 2000

Presented, in part, at the Joint Meetings of the CORESTA Smoke and Technology Study Groups (Innsbruck, Austria, September 1999) and Agronomy and Phytopathology Study Groups (Suzhou, China, October 1999).

[a] *Note*: The use of trade names and commercial sources throughout this paper is for information purposes only and does not imply endorsement by the authors.

gen der Konsumenten Folge zu leisten, wurde eine Vielzahl von PCR-basierten Methoden entwickelt, mit denen genetisch veränderte Organismen in landwirtschaftlichen Produkten, in Nahrungs- und Futtermitteln nachgewiesen, identifiziert und quantifiziert werden können. Darüber hinaus ermöglicht die genetische Identifizierung die Abgrenzung und den Nachweis (Erhaltung der Identität) genetisch veränderter Kulturpflanzen, die mit qualitätssteigernden Eigenschaften optimiert wurden, durch den ganzen Versorgungskreislauf.
Voraussetzung für den Nachweis von GVOs ist das zur Verfügung stehen einer kleinen Menge an Zielgenen und vorhandenes Wissen über die Art der genetischen Veränderung, wie zum Beispiel Resistenzmerkmale gegenüber Viren oder Insekten, einschließlich der Kontrollelemente (Promotoren und Terminatoren). Außerdem ist die Extraktion und Reinigung von DNA zur Herstellung von qualitativ hochwertigen Proben für die PCR-Analyse, insbesondere bei verarbeiteten landwirtschaftlichen Produkten wie Tabak, ein schwieriger Schritt.
Diese Arbeit berichtet über den aktuellen Stand der PCR-basierten Methodenentwicklung zur qualitativen und quantitativen Bestimmung und Identifizierung von GVOs und beinhaltet einen kurzen Überblick über die offiziellen und anerkannten Nachweismethoden für GVOs. [Beitr. Tabakforsch. Int. 19 (2000) 85–96]

RESUME

Les méthodes analytiques basées sur la technologie PCR sont utilisées de façon croissante pour la détection de séquences d'acide désoxyribonucléique (ADN) associées aux organismes génétiquement modifiés (OGM). L'étiquetage des nouveaux aliments et ingrédients alimentaires contenant ou consistant en organismes génétiquement modifiés qui sont déclenchés par la présence de séquences d'ADN étrangères nouvellement introduites ou par l'expression de protéines nouvelles est préscrit par la législation dans l'Union Européenne et en Suisse.
Pour répondre aux réglementations et aux demandes des consommateurs, de multiples méthodes PCR ont été développées avec lesquelles ont peut détecter, identifier et quantifier les OGM dans la production agricole et dans l'alimentation humaine ou animale. En outre, la détermination de l'identité génétique permet de séparer et de suivre à la trace les OGM (préservation de l'identité) à travers le transport des aliments, dont les propriétés ont été améliorées.
Les conditions préalables à la détection d'OGM sont une quantité minimum du gène cible et la connaissance préalable du type de modification génétique, tels que les caractères de résistance à des virus ou à des insectes, comprenant des éléments de contrôle (promoteurs et terminateurs). De plus, l'extraction et la purification d'ADN constituent une démarche difficile pour la préparation d'échantillons PCR de qualité, en particulier pour les productions agricoles comme le tabac.
Cette communication fait l'état actuel des connaissances des méthodes PCR appliquées à la détermination qualitative, quantitative et l'identification des OGM et comprend un bref résumé des méthodes officielles et validées des OGM. [Beitr. Tabakforsch. Int. 19 (2000) 85–96]

INTRODUCTION

Plant biotechnology, along with modern genetics and plant breeding, provides tools for crop improvement and generates greater crop management options. The recombinant deoxyribonucleic acid (DNA) techniques used to obtain the desired genetic modifications involve the isolation and subsequent introduction of discrete segments of heterologous DNA containing the gene of interest into the recipient plant. Genes isolated from a variety of organisms can now be used to improve specific crop traits such as protection from diseases and pests, yield increase, and quality improvement. As modern crop biotechnology is coming of age, the commercial cultivation of plant varieties with genetically modified (GM) agronomic (or input) traits has grown to 40 million hectares in at least twelve countries (23), which represents 4% of the total world acreage. In the USA, more than 35% of all corn, 55% of all soybeans, and almost half of all cotton have been reported to be genetically engineered in 1999. However, the presence of genetically modified organisms (GMOs) in food products is becoming increasingly controversial in several countries because of public concern with regard to the potential impact of GMOs on the health and the environment (12). Moreover, recent EU and Swiss regulations have introduced GMO labeling provisions that require verification of the GM or non-GM status by the use of methods for the analysis of DNA and proteins (54). This has led most official and several industry laboratories to develop DNA analytical methods that can assist in the enforcement of the labeling provisions.
More recently, genetic modification has been successful in enhancing quality (or output) traits of crop plants, e.g. modified fatty acid or amino acid profiles in vegetable oils and cereals, respectively. DNA analysis techniques are therefore needed for tracing these value-added GM agricultural materials/traits throughout the entire supply chain, from seed to shelf, i.e. for the implementation of identity preservation (IP) programs.
Tobacco (*Nicotiana tabacum* L.) is among the most widely used model systems for investigations in the areas of plant molecular biology and genetic engineering (5, 10, 27, 49), and was the first plant to be genetically modified in 1983. Potential commercial applications of biotechnology to tobacco as a crop have been limited to field trials conducted in several countries, although there are examples where new GM tobacco varieties have been developed and are awaiting approval before commercialization. The recently developed methods for GMO analysis are critical

tools in the research, development and monitoring of GM tobacco varieties. PCR-based molecular marker technologies for variety identification, such as amplified fragment length polymorphism (AFLP) (38, 39) add to the tools at disposal of analytical laboratories to assist in the implementation of tobacco IP programs.

Since the discovery of DNA in 1953 (55), different methods for the detection of this molecule have been developed. One method which has revolutionized and expanded all fields of life sciences is the polymerase chain reaction (PCR)[b], which was described for the first time in 1985 (40). In 1993, Kary B. MULLIS received the Nobel Prize in chemistry for the invention of this technique. Because of its extraordinarily high sensitivity, PCR is now the most popular DNA detection method, used in a vast array of different applications, including the detection of GM crops (9, 11, 19).

This paper reviews the current approaches of PCR-based GMO analysis and includes information on official and validated GMO detection methods.

DNA EXTRACTION AND PURIFICATION

Obtaining a suitable quality and quantity of genomic DNA is the first and most critical step for a successful GMO analysis. In processed agricultural raw materials, such as cured tobacco leaf, DNA is often degraded and contaminated by compounds (e.g., polyphenols, polysaccharides, lipids) and particulate matter which can interfere with the PCR reaction. It has been recently reported that the quality of DNA extracted from dry tobacco leaves depends on the variety and the type of curing and processing, with DNA of dark fire-cured tobacco being the least suitable template for PCR (11a). HEMMER (19) has reported the average DNA fragment length in processed plant foodstuffs to be in the range of 100 base pairs (bp) (e.g., soy protein) to 300–400 bp (e.g., bread, tomato paste). The result of DNA degradation can be the production of false negatives, while contaminants can alter the specificity with which primers interact with the DNA template and lead to artifactual PCR products, e.g. false positives (46). Consequently the analytical reliability and sensitivity are greatly influenced by the choice of the appropriate DNA extraction and purification methods. Moreover, strict laboratory procedures and physical containment precautions (such as separation of pre- and post-PCR working areas) need to be put in place in order to reduce the rate of false positives from carry-over DNA contaminants.

Numerous methods for plant DNA preparation have been described in the literature (42, 53, 8) including a number of modifications for very complex matrices such as woody species (7), cotton, coffee, rubber tree (52), dry seeds (17), and market samples of dry tea (44). Typically, plant tissue is disrupted by freezing and grinding, and genomic DNA is extracted and purified using organic solvents and alcohol/salt precipitation. In addition, several companies are selling kits for DNA isolation from plant materials, many of which are based on the property of DNA to adsorb to silica matrices in the presence of chaotropic salts.

Rossi *et al.* (38, 39) have recently reported on the successful use of two DNA extraction procedures on the three main types of cured tobacco leaf (flue-cured, Burley and Oriental): a slightly modified Nucleon PhytoPure™ Plant DNA extraction (Amersham Pharmacia Biotech AB, Uppsala, Sweden) and the CTAB (cethyl-trimethyl-ammonium-bromide) method. Genomic DNA suitable for PCR analysis has also been obtained from these three types of cured tobacco leaf by PIJNENBURG *et al.* (34) by the use of the Qiagen DNeasy™ Plant Mini Kit (Qiagen GmbH, Hilden, Germany).

Examples of DNA extraction methods published in the German and Swiss official methods, respectively, are the CTAB method (29,30) and the Wizard extraction method (2, 45).

CONVENTIONAL PCR METHODS FOR GMO SCREENING: END-POINT ANALYSIS

The polymerase chain reaction (40) is a method for the *in vitro* enzymatic amplification of nucleic acids carried out by repeating heating and cooling cycles in the presence of template-specific oligonucleotide primers and other reagents. In particular, the enzyme Taq DNA polymerase (which can operate at temperatures over 70 °C), in the presence of deoxynucleoside triphosphates (dNTP), synthesizes a new complementary strand of DNA starting from the primers that flank a target sequence. The PCR reaction results in the exponential accumulation of a discrete DNA fragment (called *amplicon*) to millions of copies starting from picogram amounts of genomic DNA, i.e. within a very large background of irrelevant sequences. In the case of tobacco, for example, a 100 bp target DNA sequence represents approximately less than one millionth of its total genomic DNA (3.8×10^9 bp, distributed on 48 chromosomes). The presence and the identity of the amplicon can be confirmed by analytical gel electrophoresis. In this procedure the sample is applied to the agarose gel, separated via electrophoresis, stained with ethidium bromide and visualized by exposure to UV light.

Oligonucleotide primer design is of crucial importance to the specificity, accuracy and reliability of the GMO analysis test. Optimal primers are usually determined empirically, although a set of parameters should be considered when selecting primer sequence: e.g., primer

[b] The PCR process is covered by U.S. Patents 4,683,195 and 4,683,202 and foreign equivalents owned by Hoffmann-LaRoche AG.

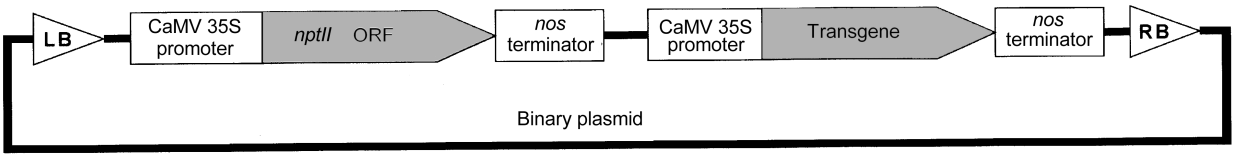

Figure 1.
Example of construct for *Agrobacterium*-mediated plant genetic modification with the different domains for GMO analysis (LB, left border; RB, right border; CaMV, cauliflower mosaic virus; *nos*, nopaline synthase; *nptII*, neomycin phosphotransferase II; ORF, open reading frame).

length, melting temperature (Tm^c), self-complementarity, % of guanine + cytosine (G+C), etc. Careful primer design should also take into account the size of the target amplicon, as the genomic DNA extracted from processed materials may be degraded to fragments smaller than the target gene.

A major drawback of the conventional PCR process described above is that accurate quantification is not possible because measurements are performed during late PCR cycles, i.e. at the end of the log-linear phase, when the PCR efficiency drops dramatically ("plateau effect").

ANALYTICAL OPTIONS FOR GMO ANALYSIS

Genetic modification of plants is commonly achieved by the use of *Agrobacterium tumefaciens*-mediated transformation (58, 36). In nature *A. tumefaciens* can transfer a fragment of its own DNA, the transfer DNA or T-DNA, to cells of most dicotyledonous plants. This mobile DNA fragment is then transported into the nucleus and integrated in the chromosomal DNA of the plant. The T-DNA is flanked by two 25 bp direct repeats called right (RB) and left border (LB) that are recognized by the bacterial transport machinery. The genes located between the border sequences are not necessary for the plant transformation process and can thus be substituted with genes that scientists want to introduce in plants. This transformation method is frequently used since it allows integration of intact single copy genetic modification constructs into plant genomes (36, 37).

A typical plant genetic modification construct contains two genes, each being composed of three different domains (Figure 1) that can be used for diagnostic purposes: a promoter, which drives the expression of the protein-coding region of the gene, the coding region that encodes for the desired trait or for the selectable marker used for the transformation, and a terminator, the sequence that marks the end of the gene. The cauliflower mosaic virus (CaMV) 35S-promoter and the *A. tumefaciens* nopaline synthase (*nos*) terminator are the two most widely used ancillary DNA sequences in plant transformation. In 1997, HEMMER (19) published a survey of promoters and terminators used in GM crops, showing that 35S is found in nearly all of the approved GM plant varieties whereas *nos* was used in at least 16 of the 28 products considered. Therefore, screening of the vast majority of genetically modified plant varieties can be currently achieved by the detection of these two markers. However, the detection of the generic markers 35S and/or *nos* is only an indication that the analyzed sample may contain DNA from a genetically engineered plant and does not provide information on the specific genetic modification. In case of positive results, further investigation has to be performed in order to detect the introduced gene with more selective tests or methods, and to exclude any false positive result due to possible contamination with laboratory carry-over products or naturally occurring CaMV and *A. tumefaciens*. Appropriate standards and endogenous controls must be used to detect accidental contamination of samples and reagents.

Negative results in the screening assay do not exclude the presence of a GMO in the samples; they only exclude the presence of a 35S-promoter and *nos*-terminator. It should also be stressed that a poor knowledge of the target sequences often leads to inappropriate choice of oligonucleotide primers, which in turn can produce a false negative. This and other critical points for quality assurance in the GMO analysis laboratory have been recently reviewed by HÜBNER *et al.* (21).

To address the issue of lack of prior knowledge of the specific genetic modifications introduced in transgenic organisms, MCCORMICK and colleagues (31) have proposed to "tag" approved GMOs with a unique synthetic DNA sequence marker that would facilitate post-release monitoring and discrimination from non-approved GMOs.

An additional method that is often used for screening in GMO analysis is the detection of genes coding for selectable markers employed in plant genetic transformation, such as the gene coding for kanamycin resistance neomycin phosphotransferase II (*nptII*).

Multiplex PCR, a technique that uses more than one pair of primers in the same assay, allows the simultaneous detection and identification of different recombinant genes expressed in a transgenic plant. It is an effective technique for the identification of plants carrying multiple GM traits.

[c] The temperature at which the transition from double-stranded nucleic acid to single-stranded nucleic acid is 50% complete.

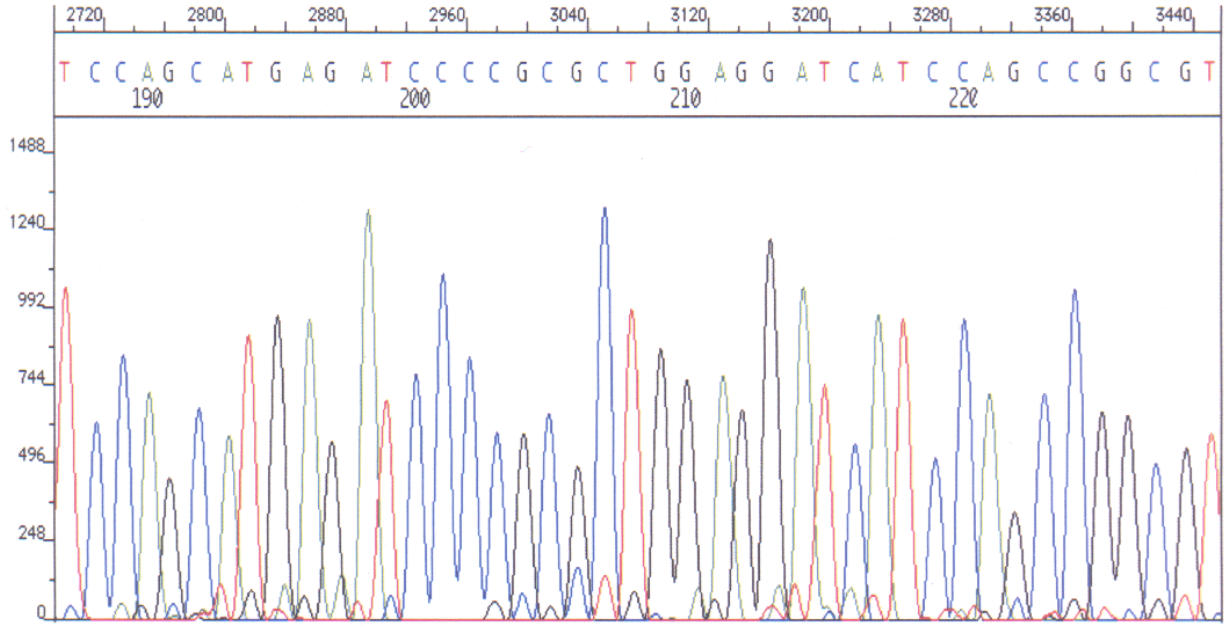

Figure 2.
Example of electropherogram featuring automated nucleotide cycle sequencing and fluorescent detection of the *nptII* gene, as generated by the PE Applied Biosystems ABI PRISM® 310 Genetic Analyzer (PE Applied Biosystems, Foster City, CA) and using the ABI PRISM® BigDye™ Terminator Cycle Sequencing Ready Reaction Kit with AmpliTaq® DNA Polymerase (H. PIJNENBURG and G. BINDLER, unpublished). The four colors correspond to four dyes that identify the A, C, G and T extension reactions.

GMO IDENTIFICATION

Samples of agricultural raw materials that have been detected as GMO by the use of the qualitative screening methods should be submitted to gene-specific PCR procedures that can discover the specific GM trait of the modified crop, e.g. tolerance to RoundUp® herbicide (*A. tumefaciens* EPSPS gene) or resistance to insects (*Bacillus thuringiensis*-Bt *cryIA* genes). This allows the discrimination of the GM plant varieties that have been approved by the regulatory authorities from the non-approved materials that may have inadvertently entered the commercial supply chain.

The specificity of the test can be increased by selecting target sequences that overlap the promoter- or the terminator-transgene region (Figure 1) (e.g., 35S–*cryIA* or *cryIA*–*nos*). Moreover, the complete determination of the nucleotide sequence of the junction region and/or of the transgene can confirm the identity and eventually the origin of the genetic modification at biological variant level (e.g., a gene coding for resistance to a particular strain of tobacco mosaic virus [TMV]). Figure 2 illustrates the PCR-based cycle sequencing of DNA, a simple method that uses a succession of denaturation, primer annealing and extension in a thermal cycler, coupled with a fluorescent labeling of either the primers or the dideoxynucleotide (ddNTPs) terminators. The dideoxy sequencing method was originally developed by SANGER (41).

A variety of confirmatory methods is used in many laboratories to add specificity and increase sensitivity in GMO analysis. The most commonly used methods are: the nested or semi-nested PCR, which consists in reducing the threshold target copy number for detection by performing a second round of PCR with a set of primers that are internal to the first pair (57); the restriction enzyme digestion of the PCR amplicon, which confirms the gene identity by detection of a specific internal sequence (restriction endonuclease site); and probe hybridization (Southern blot), an assay which can provide information on chromosomal integration of the transgene. Southern analysis is, however, a labourious procedure that requires substantial amounts of highly pure DNA.

PCR METHODS FOR QUANTITATIVE GMO DETECTION

The above-described standard formats of PCR are qualitative detection methods that can give only limited quantitative information (26, 35). As mentioned earlier, towards the end of the PCR reaction, products are formed with an unknown reaction rate and in a non-logarithmic fashion. However, in order to have a maximum sensitivity, product formation is often measured at this stage, i.e. when the correlation between the product concentration and the concentration of the initial target molecules is

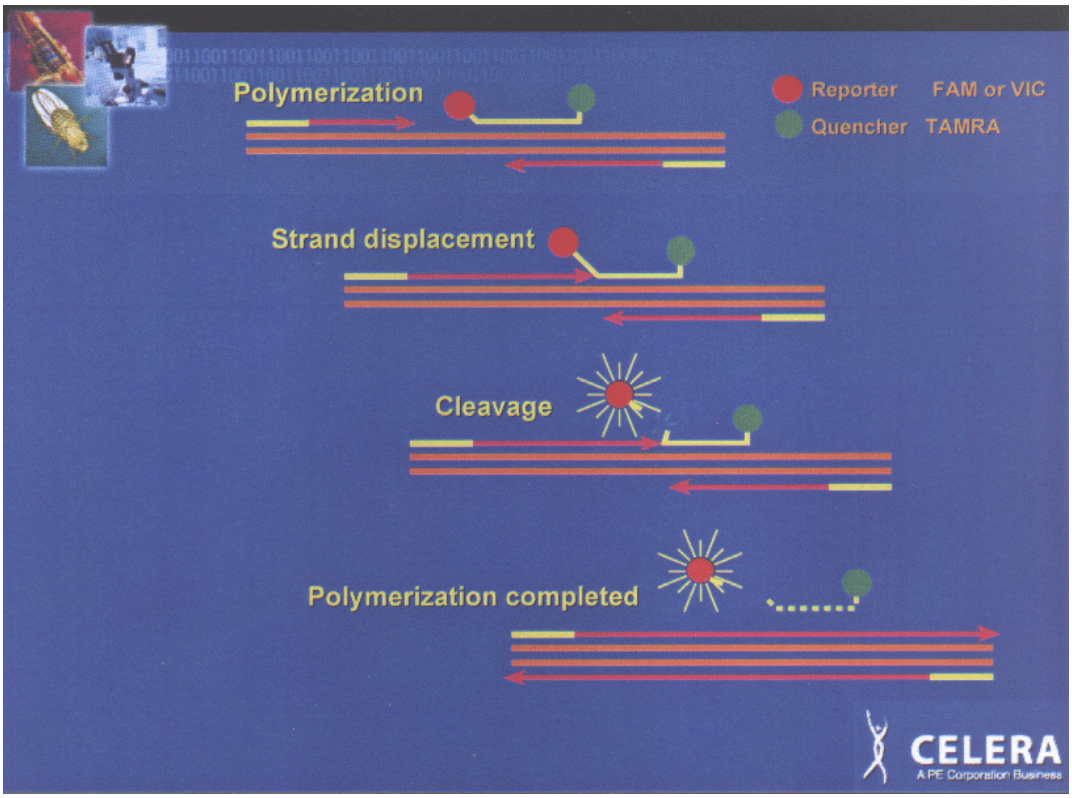

Figure 3.
Diagram showing the main steps of the real-time quantitative PCR (TaqMan® assay) which exploits the 5'-nuclease activity of the Taq polymerase. Reactions are performed with all the normal components for PCR, with the addition of the target-specific TaqMan® probe. TaqMan® fluorogenic oligonucleotide probes consist of an oligonucleotide with a 5'-reporter dye (FAM or VIC) and a 3'-quencher dye (TAMRA). The 3'-terminus also contains a blocking group to prevent probe extension. Fluorescence of the reporter group is inhibited by the quencher located at the 3'-end. If hybridization of the TaqMan® probe to a target sequence occurs, probe cleavage by the Taq polymerase takes place during polymerization of the targeted amplicon, resulting in separation of the reporter and quencher, and causing the reporter dye fluorescence to increase. Conversely, when the probe is intact, the proximity of the reporter dye to the quencher dye results in suppression of the reporter fluorescence.
(*The diagram was kindly provided by Dr. Marc Malandro of PE Celera AgGen.*)

very poor. One option for a better quantitative reaction is to stop the reaction in an earlier (logarithmic) stage and use a more sensitive product detection method.

Since the early 90's, PCR-based techniques have been developed to overcome some of the problems associated with quantification of nucleic acids by PCR, such as quantitative competitive PCR (QC-PCR) and real-time PCR. These methods are currently widely used for GMO quantitation in agricultural crop raw and processed materials (15, 50).

In QC-PCR, the specific DNA sequence and an internal standard are simultaneously amplified with appropriate primers in a PCR reaction (6, 13, 16, 43, 51). The PCR products are then separated by electrophoresis and assessed for the expected products by comparison to length standards. The PCR amplification products and the internal standard can be distinguished by a small difference in size (20–80 bp). Multiple PCR reactions are needed as each sample is amplified with increasing amounts of internal standard, while keeping the sample volume/concentration constant. Quantification is achieved by comparing the equivalence point at which the amplicon from the internal standard gives the same signal intensity as the target DNA.

The QC-PCR method described by STUDER *et al.* (47), and more recently by HARDEGGER *et al.* (14), was validated and successfully tested in an inter-laboratory test in Switzerland and a similar ring test has been launched for the European Union. The method may be a recommended method for labeling compliance according to revised Food Ordinance in Switzerland (3).

Real-time PCR (18) was originally developed in 1992 by HIGUCHI *et al.* (20) and is rapidly gaining popularity due to the introduction of several complete real-time PCR apparatuses that perform closed-tube assays, and the integration of the specific 5'-3'-exonuclease activity of the Taq DNA polymerase. The amplification of the DNA target sequence is followed during the whole reaction by

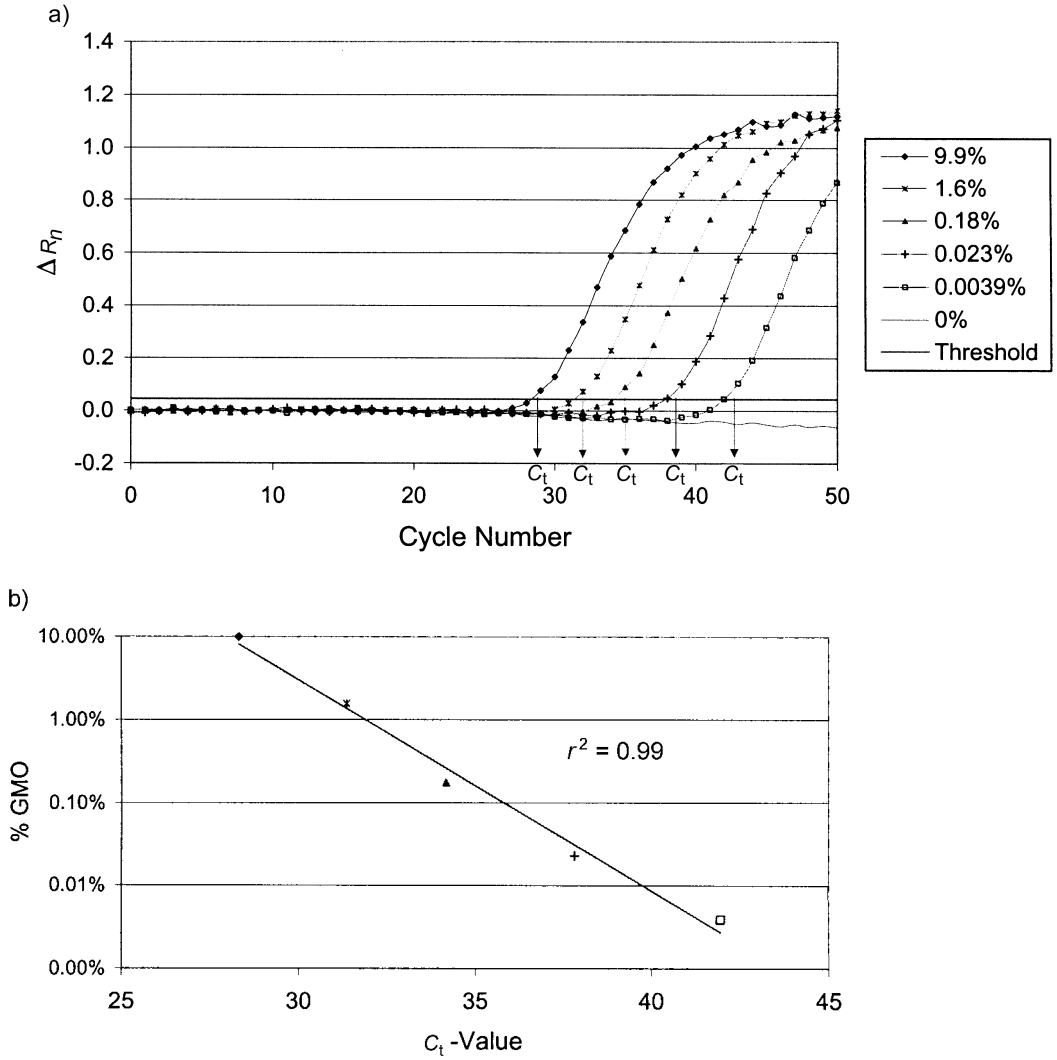

Figure 4.
a) Real-time PCR diagram showing the accumulation of the target analyte 35S-promoter at six different ratios of GMO/non-GMO material (% w/w). PCR product formation is visualized in real-time by taking fluorescence measurements (ΔR_n) at each cycle. The initial template concentration is determined based on the threshold cycle (C_t), i.e. the PCR cycle at which fluorescence is first detected statistically significant above background. C_t is inversely proportional to the logarithm of the number of target copies present in the sample.
b) Linear regression diagram showing the logarithmic relation between the GMO/non-GMO ratios and the C_t-values.

monitoring fluorescence formation in the PCR reaction. In particular, a fluorogenic probe consisting of an oligonucleotide with both a reporter and a quencher dye attached (TaqMan® probe), anneals specifically to the amplification product (target DNA) between the forward and reverse primers (Figure 3). When the probe is intact, the proximity of the reporter dye to the quencher dye results in quenching of the reporter fluorescence. If hybridization occurs, the probe is cleaved by the 5'-3'-exonuclease activity of the Taq DNA polymerase, and the reporter dye is separated from the quencher dye thus generating a sequence-specific signal. This process occurs in every cycle, with the increase of fluorescence intensity being monitored in real-time during the PCR.
The number of PCR cycles necessary to generate a signal statistically significant above the noise is taken as a quantitative measure and is called cycle threshold (C_t), which is inversely proportional to the logarithm of the initial amount of target molecules (Figure 4). When compared to QC-PCR, real-time PCR has higher specificity and is considerably less labor-intensive (e.g., multiple PCRs and gels are not needed), and is therefore suitable for high throughput routine analysis.
The relative quantification of a target gene is possible by preparing a standard curve of an additional endogenous gene (33, 34), such as the tobacco nitrate reductase gene (*nia*), and extrapolating from the linear regression. The PE ABI PRISM 7700 Sequence Detection System is able to carry out the latter reaction in the same tube by a multiplex TaqMan® PCR.

Other real-time techniques have been recently described which make use of hairpin-shaped amplicon-specific probes: the Molecular Beacons (48) and the Scorpion method (56).
Real-time PCR can be considered the most advantageous method for quantitative PCR available at the moment, although the high investment costs for the purchase of instruments (e.g., PE ABI PRISM 7700 or ABI PRISM 5700 Sequence Detection Systems; Boehringer-Mannheim Lightcycler) might still be an obstacle for many laboratories to start with this technique.

OVERVIEW OF OFFICIAL AND VALIDATED GMO ANALYSIS METHODS

The adoption of official or validated methods as a tool for detecting GMOs in raw agricultural materials and finished products is in its initial stage. Germany and Switzerland are currently the only countries having official methods whereas the European Union (EU) has recently validated a screening method. The screening methods published or validated in the EU, Germany and Switzerland are all based on the publication of PIETSCH *et al.* (32), and the procedures set forth in each method are based on the detection of the CaMV 35S-promoter and *A. tumefaciens nos*-terminator, the most widely used promoter and terminator sequences used in plant genetic modification.

European Union validated methods

According to the Novel Food Regulation EC 258/98 and Council Regulation EC 1139/98 of the European Union (EU), GMOs have to be labeled if modified DNA or newly expressed proteins can be detected and their content exceeds a threshold amount. EC 1139/98 was recently amended with the Commission Regulation EC 49/2000 of January 10, 2000, to introduce a 1% threshold (expressed as proportion of GMO-derived material(s) present in the food ingredients as individually considered, or in a food comprising a single ingredient). It is important to stress that the labeling threshold can only apply when one can demonstrate by documentary evidence that the presence of the GMO material is adventitious.
Although the EC 1139 regulation does not specify test methods to be used or criteria for selecting a method for different applications, the European Union Commission Joint Research Centre (JRC) has recently validated a "Screening method for the identification of genetically modified organisms (GMO) in food" (1, 25), and other methods are in the stage of evaluation and validation. Reportedly 26 out of the 28 GM crops already approved or being considered for approval in the EU can be detected using this qualitative method. The EU method was originally introduced by Swiss and German scientists (32) and was developed using soybean and corn, the two most common crop plants used in the food industry. It is a screening method based on the detection of the DNA controlling elements 35S-promoter and *nos*-terminator.
The EU method was validated through a European ring test with twenty-five EN 45000 accredited laboratories from 13 countries (16 governmental organizations, seven private and two university laboratories). The JRC provided the European laboratories with the appropriate reference materials containing Bt-176 corn and RoundupReady® soya and produced at the Institute for Reference Material and Measurements of the JRC (Geel, Belgium).
The results of the validation study demonstrated that the EU screening method is suitable for the detection of GMO in raw material derived from soybean and corn having a GM concentration of 0.5% or higher. Samples containing 2% of GMO (both soybean and corn) were always correctly identified by all the laboratories, thus demonstrating the validity of the method for screening purposes (see 6 October 1998 press release of the EC Joint Research Center in http://www.jrc.org/jrc/index.asp).
It should be noted that this method has been validated for the qualitative detection of GM raw agricultural products and that quantitative PCR methods will be needed to determine whether GMO levels are above or below the threshold established in the EU (1).
This screening method has already become an official Swiss method (2) and the same principle is also the basis for the official German method (29, 24).

Official methods – Germany

In Germany GM crops are regulated under the Gentechnikgesetz (GenTG) and EU Novel Food Regulation. The working group of the German Federal Institute for Health Protection of Consumers and Veterinary Medicine (Bundesinstitut für gesundheitlichen Verbraucherschutz und Veterinärmedizin [BgVV], Berlin) coordinates the development of the official methods, which are included after validation in the official method collection according to article 35 of the German Food Act (Lebensmittel- und Bedarfsgegenständegesetz [LMBG]).
The two methods published in the LMBG are crop-specific and include a description of procedures for DNA extraction, PCR analysis and a confirmatory test (Southern blot).
In the method "Detection of genetically modified soybeans" (29, 24), the genetic modification is detected as specific DNA sequences encoding the CaMV 35S-promoter sequence linked to the *Petunia hybrida* chloroplast transit-signal sequence. Confirmation is performed by hybridizing a labeled CaMV 35S-promoter sequence to the amplified product. The amplification capacity is checked with primers specific to the lectin gene.
The method "Detection of genetically modified potato" (30) detects specific DNA sequences encoding the hygromycin phosphotransferase gene, the sequence of which is

used in a confirmatory hybridization assay. The DNA suitability for PCR is checked using primers specific to a chloroplast tRNA gene.
Quantitative methodologies are also being investigated. JANKIEWICZ *et al.* of BgVV (24) have recently published a semi-quantitative study of theoretical and practical detection limits in GM crops, which were reported to be 0.005% and 0.1% GMO/non-GMO (w/w), respectively.

Official methods – Switzerland

According to the Swiss Ordinance on Food Stuffs of 1995 and its 1999 amendments (4, 3), all foodstuffs, food additives and processing aids consisting of/or derived from GMOs must have premarket approval and have to be labeled.
The Swiss Federal Office of Public Health has published an official method for the screening of GMOs in food in the Swiss Food Compendium 52B: "Methods in Molecular Biology" (2). The PCR method is derived from PIETSCH *et al.* (32), whereby the detection of the 35S-promoter and/or *nos*-terminator indicates the presence of a genetically modified plant and triggers GMO labeling. Positive samples have to be confirmed with specific methods according to the example described in Method 52B/4: "Detection procedure for genetically modified Roundup Ready™ soybeans (RRS) in foods".
An evaluation of quantitative competitive PCR techniques is being performed in order to provide a tool for labeling compliance testing after the recent introduction of a GMO threshold value of 1% for the labeling of foods and foodstuffs (3).

Standardization of GMO analysis methods and availability of GMO reference materials

While an increasing number of food control laboratories are adopting PCR as the technology of choice for GMO detection, international standardization and validation of GMO analysis methods by harmonized and accepted protocols is still in its early phases and is currently limited to the EU and Switzerland.
Standardization bodies such as the European Committee for Standardization (CEN, Brussels, Belgium) and the French Standardization Association AFNOR (Paris, France) have recently acknowledged the need for harmonized protocols to validate GMO analysis methods.
In particular, CEN has started a harmonization effort in the area of GMO detection methods through its technical committee TC 275 WG11 "Methods for GMO detection". AFNOR has recently created a standardization committee named "Detection of GMO and derived products" (*see* press release in http://www.afnor.fr), whose activities are linked to CEN. The work program concerns food crops and is carried out in cooperation with service laboratories and agro-food companies under the coordination of the National Institute for Agronomic Research (INRA). It includes the preparation of guidelines on nucleic acid extraction, the definition of qualitative and quantitative methods, and sampling procedures. AFNOR expects to publish experimental norms or documentation on these subjects in 2000.
Moreover, discussions are being held at various standardization associations to consider the proposal of involving the International Standards Organization (ISO, Geneva, Switzerland) in the standardization of GMO analysis methods.
Another international body that recently got involved in the standardization and validation of GMO analysis methods is the International Union of Pure and Applied Chemistry (IUPAC, Research Triangle Park, NC, USA; http://www.iupac.org/). The IUPAC Division of Chemistry and the Environment, Commission on Food Chemistry (VI.5) has worked in cooperation with the EU Commission JRC for the validation of the EU screening method (28).
In the United States, the United States Department of Agriculture (USDA) has recently announced that it will establish a reference laboratory to evaluate and verify the validity of GMO analysis methods as applied to grains and oilseeds. The new laboratory will be based at the Grain Inspection, Packers and Stockyards Administration's (GIPSA) Technical Center in Kansas City, MO, and should open in time for monitoring the year 2000 crops.
The development, validation and use of GMO analysis methods ideally demand the availability of certified reference materials. The EU commission Institute for Reference Materials and Measurements (IRMM) has recently produced reference materials for the detection of GM soybean and corn in the form of stable pure powders of guaranteed GMO-free flour and containing mass fractions of 0.1%, 0.5% and 2.0% of GM flour. The reference materials are commercially available from Fluka Chemie AG (Buchs, Switzerland).
Moreover, it has been proposed that a central repository, perhaps a European Union facility, be created for storage of information (e.g., sequence data, primers, novel proteins) on, and samples (e.g., DNA, relevant protein products) of traditional and GMO reference materials (*see* press release, International Life Sciences Institute-ILSI: http://www.ilsi.org/). This should help ensure the quality and stability of reference standards and facilitate the standardization and distribution of materials and information needed for assay development.

CONCLUSION

PCR is among the most powerful technologies ever used by analytical scientists and is increasingly applied worldwide in food control laboratories to determine compliance of agricultural raw materials and ingredients with GMO regulations and to address consumer demand for

non-GMO product segregation. The use of PCR for the detection of genetic modification markers and transgenes is also necessary for the development, quality assurance and post-release monitoring of genetically modified crops, including tobacco.

Reliable quantitative detection methods are being developed to address the introduction of GMO ceilings, which take into account the possibility of inadvertent commingling of GM with conventional materials during agricultural production, shipping or processing. Threshold values that trigger product labeling have been recently established in Switzerland and the EU.

There appears to be an urgent need of increased cooperation among all the stakeholders in GM crop development, from seed developers to processors and manufacturers, in order to verify PCR methods performance and achieve standardization of GMO detection procedures and methods. The efficient utilization of modern PCR techniques for GMO detection will largely depend on the availability of accurate information on target gene sequences and certified reference materials.

New and more sophisticated PCR-based or non-PCR technologies are emerging which promise to increase the accuracy, precision and sample throughput in detecting and measuring GM markers in plant products. While most technical issues are today resolved for conventional PCR, sensitivity, detection limits and other performance parameters of each new method are to be investigated, thoroughly evaluated and defined in the GMO analysis laboratory.

Acknowledgements. The authors wish to thank the members of the CORESTA Task Force Genetically Modified Tobacco – Detection Methods for contributing and discussing some of the information reviewed in this paper. The authors are very grateful to Dr. Serge Kocher of Biolytix AG for performing real-time PCR experiments and for critical reading of the manuscript, and to Dr. Marc Malandro of PE Celera AgGen for providing the diagram representing the TaqMan® assay.

REFERENCES

1. Anklam, E. (1999) The validation of methods based on polymerase chain reaction for the detection of genetically modified organisms in food; Anal. Chim. Acta 393 (1999) 177–179.
2. Anonymous: Schweizerisches Lebensmittelbuch (Swiss Food Manual); *in*: Molekularbiologische Methoden, edited by Bundesamt für Gesundheit, Chapt. 52B, Eidgenössische Drucksachen und Materialzentrale, Bern, Switzerland, 1998.
3. Anonymous: Lebensmittelverordnung (LMV, Swiss Food Ordinance) sur les denrées alimentaires (ODAI) – Modification du 14 juin 1999; Eidgenössische Drucksachen und Materialzentrale, Bern, Switzerland, 1999.
4. Anonymous: Lebensmittelverordnung (LMV, Swiss Food Ordinance), Vol. SR 817.02, Art.15, 22, 23, and Lebensmittelgesetz Art. 9; Eidgenössische Drucksachen und Materialzentrale, Bern, Switzerland, 1995.
5. Brandle, J.D., D. Bai: Biotechnology: uses and applications in tobacco improvement; *in*: Tobacco: production, chemistry and technology, edited by D.L. Davis and M.T. Nielsen, Blackwell Science Ltd., Oxford, UK, 1999, pp. 49–65.
6. Borson, N.D., M.A. Strausbauch, P.J. Wettstein, R.P. Oda, S.L. Johnston and J.P. Landers: Direct quantitation of RNA transcripts by competitive single tube RT–PCR and capillary electrophoresis; Biotechniques 25 (1998) 130–137.
7. Cheng, F.S., S.K. Brown, N.F. Weeden: A DNA extraction protocol from various tissues in woody species; Hortscience 32 (1997) 921–922.
8. Csaikl, U.M., H. Bastian, R. Brettschneider, S. Gauch, A. Meir, M. Schauerte, F. Scholz, C. Sperisen, B. Vornam, B. Ziegenhagen: Comparative analysis of different DNA extraction protocols: a fast, universal maxi-preparation of high quality plant DNA for genetic evaluation and phylogenetic studies; Plant Mol. Biol. Reptr. 16 (1998) 69–86.
9. Gachet, E., G.G. Martin, F. Vigneau, G. Meyer: Detection of genetically modified organisms (GMOs) by PCR: a brief review of methodologies available; Trends Food Sci. Technol. 9 (1999) 380–388.
10. Gadani, F., D. Ayers, W. Hempfling: Tobacco: a tool for plant genetic engineering research and molecular farming, Part I; Agro Food Ind. Hi-Tech 6 (1995) 19–24.
11. Gadani, F., G. Bindler, H. Pijnenburg, L. Rossi, and J. Zuber: The application of polymerase chain reaction (PCR) technology to the detection, identification and quantification of genetically modified organisms (GMOs): current approaches; Joint Meeting of the Agronomy and Phytopathology Study Groups, Suzhou, China, 10–14 October 1999, Cooperation Centre for Scientific Research Relative to Tobacco (CORESTA), Conference Proceedings, Abstract no. AP44, p. 56.

11a. Gadani, F., M. Maunders, M. Ward (eds.): Report of the CORESTA Task Force Genetically Modified Tobacco: Detection Methods; CORESTA Bull. 4: Cooperation Centre for Scientific Research Relative to Tobacco (CORESTA), Paris, France, 1999, 77–148.

12. Gaskell, G., W.M. Bauer, J. Durant, N.C. Allum: Worlds apart? The reception of genetically modified foods in Europe and the U.S.; Science 285 (1999) 384–387.
13. Gilliland, G., S. Perrin, K. Blanchard, and H.F. Bunn: Analysis of cytokine mRNA and DNA: Detection and quantitation by competitive DNA

polymerase chain reaction; Proc. Natl. Acad. Sci. USA. 87 (1990) 2725–2729.
14. Hardegger, M., P. Brodmann, A. Hermann: Quantitative detection of the 35S promoter and the NOS terminator using quantitative competitive PCR; Eur. Food Res. Technol. 20 (1999) 83–87.
15. Härri, A. and S. Kocher:TaqMan® real-time quantitative PCR: a new method to quantify genetically modified organisms (GMOs) and pathogens; Poster 02, Infotag "Molekulare Analytik von Lebensmitteln", 11 November 1998, DECHEMA-Haus, Frankfurt am Main, 1998.
16. Hayward-Lester, A., P.J. Oefner and P.A. Doris: Rapid quantification of gene expression by competitive RT–PCR and ion-pair reversed-phase HPLC; Biotechniques 20 (1996) 250–257.
17. Hee Wan Kang, Y., U.H.Y. Gu Cho, E. Moo Young: A rapid DNA extraction method for RFLP and PCR analysis from a single dry seed; Plant Mol. Biol. Reptr. 16 (1998) 1–9.
18. Heid, C.A., J. Stevens, K.J. Livak and P.M. Williams: Real-time quantitative PCR; Genome Res. 6 (1996) 986–994.
19. Hemmer, W.: Foods derived from genetically modified organisms and detection methods; Report 2-97 of the Biosafety Research and Assessment of Technology Impacts of the Swiss Priority Program Biotechnology of the Swiss National Science Foundation (BATS), Basel, Switzerland, 1997.
20. Higuchi, R., G. Dollinger, P. Sean Walsh, and R. Griffith: Simultaneous amplification and detection of specific DNA sequences; Bio/Technology 10 (1992) 413–417.
21. Hübner, P., E. Studer, D. Häfliger, M. Stadler, C. Wolf, and C. Looser: Detection of genetically modified organisms in food: critical points for quality assurance; Accred. Qual. Assur. 4 (1999) 292–298.
22. Hübner, P., E. Studer, and J. Lüthy: Quantitation of genetically modified organisms in food; Nature Biotechnol. 17 (1999) 1137–1138.
23. James, C.: Global review of commercialized transgenic crops: 1999; ISAAA Brief No. 12, The International Service for the Acquisition of Agri-biotech Applications, Ithaca, NY, 1999.
24. Jankiewicz, A., H. Broll, J. Zagon: The official method for the detection of genetically modified soybeans (German Food Act LMBG & Sect. 35): a semi-quantitative study of sensitivity limits with glyphosate-tolerant soybeans (RoundupReady®) and insect-resistant Bt maize (Maximizer); Eur. Food Res. Technol. 209 (1999) 77–82.
25. Joint Research Center of the European Commission, Environmental Institute, Consumer Protection and Food Unit: Screening method for the identification of genetically modified organisms (GMO) in food. Detection of the CaMV 35S promoter and NOS terminator by means of polymerase chain reaction (PCR); JRC, Ispra, Italy, 1998.
26. Köhler, T.: General aspects and chances of nucleic acid quantitation by PCR; *in*: Quantitation of mRNA by DNA polymerase chain reaction, nonradioactive PCR methods, edited by T. Köhler, D. Labner, A.K. Rost, B. Thamm, B. Pustowoit, and H. Remke, Springer-Verlag, Heidelberg, 1996, p.166.
27. Koller, D.: Genetics and the future of tobacco: issues and research opportunities; *in*: Recent Advances in Tobacco Science, Proceedings of the 53rd Tobacco Science Research Conference (TSRC), 12–15 September 1999, Montreal, Canada., 1999, pp. 37–60.
28. Lipp, M., P. Brodmann, K. Pietsch, J. Pauwels, and E. Anklam: IUPAC collaborative trial study of a method to detect genetically modified soy beans and maize in dried powder; J. AOAC Int. 82 (1999) 923–928.
29. LMBG Lebensmittel- und Bedarfsgegenständegesetz: Detection of genetically engineered soy beans by amplification of the altered DNA sequence using the PCR (polymerase chain reaction) and hybridization of the PCR product with a DNA probe; LMBG 23.01.22, Beuth Verlag GmbH, Berlin, Köln, March 1998.
30. LMBG Lebensmittel- und Bedarfsgegenständegesetz: Detection of genetically engineered potato by amplification of the altered DNA sequence using the PCR (polymerase chain reaction) and hybridization of the PCR product with a DNA probe; LMBG 24.01, Beuth Verlag GmbH, Berlin, Köln, 1996 and January 1997.
31. McCormick, C.A., H.G. Griffin, H.M Underwood and M.J. Gasson: Common DNA sequences with potential for detection of genetically manipulated organisms in food; J. Appl. Microbiol. 84 (1998) 969–980.
32. Pietsch, K., H.U. Waiblinger, P. Brodmann, and A. Wurz: Screeningverfahren zur Identifizierung "gentechnisch veränderter" pflanzlicher Lebensmittel; Dtsch. Lebensmitt. Rundsch. 93 (1997) 35–38.
33. Pijnenburg, H.: Relative quantification calculation methods; First Swiss Sequence Detection Systems User Meeting, December 3–4, Wengen, 1998, Proceedings, p. 12.
34. Pijnenburg, H., G. Bindler, S. Kocher, A. Härri, L. Rossi, F. Gadani, and J. Zuber: Personal communication.
35. Raeymaekers, L.: Quantitative PCR: theoretical considerations with practical implications; Analyt. Biochem. 214 (1993) 582–585.
36. Rossi, L., B. Hohn, B. Tinland: Integration of complete transferred DNA units is dependent on the activity of virulence E2 protein of *Agrobacterium tumefaciens*; Proc. Natl. Acad. Sci. USA 93 (1996) 126–130.

37. Rossi, L., B. Tinland, B. Hohn: Role of virulence proteins of *Agrobacterium* in the plant; *in*: The rhizobiaceae: molecular biology of model plant associated bacteria, edited by H.P. Spaink, A. Kondorosi, and P.J.J. Hooykaas, Kluwer Academic Publishers, Dordrecht, Netherlands, 1998, pp. 303–320.
38. Rossi, L., G. Bindler, H. Pijnenburg, and F. Gadani: Potential of molecular marker analysis for assessment of genetic diversity in tobacco varieties; Joint Meeting of the Agronomy and Phytopathology Study Groups, Suzhou, China, 10–14 October 1999, Cooperation Centre for Scientific Research Relative to Tobacco (CORESTA), Conference Proceedings, Abstract no. AP45, p. 57.
39. Rossi, L., G. Bindler, H. Pijnenburg, F. Gadani: Evaluation of molecular marker technologies for tobacco variety identification; Plant & Animal Genome VIII Conference, San Diego, CA, January 9–12, 2000, Conference Proceedings, Scherago International, Inc., New York, USA, Abstract no. P473, p. 169.
40. Saiki, R.K., S. Scharf, F. Faloona, K.B. Mullis, G.T. Horn, H.A. Erlich, and M. Arnheim: Enzymatic amplification of γ-globin genomic sequences and restriction site analysis for diagnosis of sickle cell anemia; Science 230 (1985) 1350–1354.
41. Sanger, F., S. Nicklen, and A.R. Coulson: DNA sequencing with chain-terminating inhibitors; Proc. Natl. Acad. Sci. (USA) 74 (1977) 5463–5467.
42. Scott, O.R., A.J. Bendich: Extraction of DNA from plant tissues; Plant Mol. Biol. Manual A6, 1988, pp 1–10.
43. Siebert, P.D., and J.W. Larrick: Competitive PCR; Nature 359 (1992) 557–558.
44. Singh, M., Bandana and P.S. Ahuja: Isolation and amplification of genomic DNA from market samples of dry tea; Plant Mol. Biol. Reptr. 17 (1999) 171–178.
45. Spoth, B., and E. Strauss: Screening for genetically modified organisms in food using Promega's Wizard resin; Promega Notes Magazine 73 (1999) 23–25.
46. Straub, J.A., C. Hertel, and W.P. Hammes: Limits of a PCR-based detection method for genetically modified soya beans in wheat bread production; Z. Lebensm. Unters. Forsch. A 208 (1999) 77–82.
47. Studer, E., C. Rhyner, J. Lüthy, and P. Hübner: Quantitative competitive PCR for the detection of genetically modified soybean and maize; Z. Lebensm. Unters. Forsch. 207 (1998) 207–213.
48. Tyagi, S. and F.R. Kramer: Molecular beacons—probes that fluoresce upon hybridization; Nature Biotechnol. 14 (1996) 303–308.
49. Tso, T.C.: Production, physiology and biochemistry of tobacco plant; Ideals, Inc., Beltsville, MD, 1990.
50. Vaïtiligom, M., H. Pijnenburg, F. Gendre, and P. Brignon: Quantitative detection of genetically modified organisms in food; J. Agric. Food Chem. 47 (1999) 5261–5266.
51. Verhaegen, M. and T.K. Christopoulos: Quantitative polymerase chain reaction based on a dual-analyte chemiluminescence hybridization assay for target DNA and internal standard; Anal. Chem. 70 (1998) 4120–4125.
52. Vroh Bi, I., L. Harvengt, A. Chandelier, G. Mergeai, and P. Du-Jardin: Improved RAPD amplification of recalcitrant plant DNA by the use of activated charcoal during DNA extraction; Plant Breeding 115 (1996) 205–206.
53. Wang, H., M. Qi, A.J. Cutler: A simple method of preparing plant samples for PCR; Nucleic Acids Res. 21 (1993) 4153–4154.
54. Ward, M.R.: A review of the regulations governing the production and use of genetically modified crops; *in*: Recent Advances in Tobacco Science, Proceedings of the 53rd Tobacco Science Research Conference (TSRC), 12–15 September 1999, Montreal, Canada, pp. 81–91.
55. Watson, J.D., and F.H.C. Crick: A structure for deoxyribose nucleic acid; Nature 171 (1953) 737.
56. Whitcombe, D., J. Theaker, S.P. Guy, T. Brown, and S. Little: Detection of PCR products using self-probing amplicons and fluorescence; Nature Biotechnol. 17 (1999) 804–807.
57. Zimmerman, A., W. Hemmer, M. Liniger, J. Luthy, and U. Pauli: A sensititve detection method for genetically modified MaisGard™ corn using a nested PCR-system; Lebensm.-Wiss. Technol. 31 (1998) 664–667.
58. Zupan, J.R., and P. Zambryski: The *Agrobacterium* DNA transfer complex; Crit. Rev. Plant Sci. 16 (1997) 279–295.

Address for correspondence:

F. Gadani
Philip Morris Europe
Research and Development
CH-2003 Neuchâtel
Fax: (+41) 32- 888 68 08
e-mail: gadani.ferruccio@pmintl.ch