Chapter 7
Detection of Genetically Modified Plants in Seeds, Food and Feed

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7.1 Introduction

In the context of the development and approval of a growing number of genetically modified (GM) plants which are field-tested in the environment or cultivated as crop plants, the methodology for their detection and identification has become an important issue. Detection methods and techniques used by researchers and in development laboratories for the characterisation of transformants are generally different to those applied by official testing laboratories and public analysts. Enforcement laboratories apply specific methods and analytical strategies for the detection of GM plants used in the foods, feed or seeds sectors, having in mind that the commercialisation of transgenic crop plants is regulated in different ways depending on national legal frameworks. In the European Union (EU) for example a validated transformation event-specific detection method, including sampling, extraction, identification and quantification, has to be provided by the applicant if authorisation of a certain GM event as food and feed is intended (EU 2003a). In contrast, for example in the United States, GM plants become deregulated for use as food, feed or for cultivation when they have been reviewed by the competent regulatory agencies. Moreover, according to international agreements laid down in the Cartagena Protocol on Biosafety (UN 2000), the trade and transfer of living GM organisms (e.g. seeds and propagable grains) across national borders may require information for the specific detection and identification of that GM organism.

Under certain circumstances the GM crop content needs not only to be detected and identified but also to be quantified in terms of certain thresholds for labelling the foods and feeds which contain or are produced from GM plants. Threshold levels also depend on national legislations and, for example in the EU, labelling is

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not required if the proportion of GM material is not higher than 0.9% of the food ingredient, provided that the presence of this material is adventitious or technically unavoidable, whereas for example in Japan the labelling threshold is 5%. For GM plants not authorised according to EU regulations a zero tolerance is applied, making the sensitive detection of such GM materials an emerging challenge for the official testing laboratories.

This review describes the current techniques used for detection of transgenic plant materials and the different analytical strategies applied by the official control laboratories responsible for enforcement from an European perspective. In addition, the limitations of current methodologies and finally the recent developments in GMO detection area applying advanced or alternative amplification techniques are reviewed.

7.2 Techniques Used to Detect a Transgenic Plant

To detect genetic modifications in plants in general (for the methods of genetic modification, see Chaps. 1, 2) two different techniques could be applied (Anklam et al. 2002; Holst-Jensen 2007). One is based on the detection of genetic material (DNA), for example by polymerase chain reaction (PCR). This technique is most versatile for the detection of GM plants and therefore preferably used and chosen for many applications (Lipp et al. 2005). The alternative approach is detecting the newly expressed protein(s) which most GM plants contain as a result of the insertion of the new gene(s). Here specific antibodies are applied and used in lateral flow strip tests or complex ELISA assays (Grothaus et al. 2007). As compared to PCR, protein techniques are more restricted in their applicability but can be very useful for certain raw commodities. DNA is relatively stable and is often still present in many products, even after processing of the plant material. Therefore genetic modifications in plants are more easily and reliably detected at the DNA level. However, this does not apply to highly processed GM materials or ingredients, such as oil, sugars or starch, which may no longer contain any DNA. Here, the EU regulations for example demand the traceability of the product through every phase of marketing, i.e. over the entire production and processing chain (EU 2003b).

7.2.1 DNA-Based Detection

DNA-based detection of transgenic plants targets the novel DNA sequences introduced into the crop genome. These methods show the absence or presence of GM plant material in a sample and can also measure the relative quantity (percentage) in a tested sample.

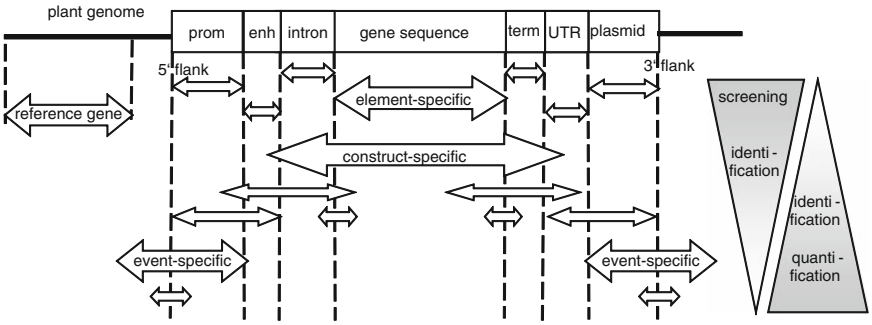

Fig. 7.1 Analytical strategy and targeted sequences. Detection of genetically modified plants in food, feed and seed samples is generally conducted by consecutive PCR tests targeting the genetic elements (element-specific) and constructs (construct-specific). For event-specific identification and quantification of GM plants the 5′ or 3′ junction regions around the integration sites are targeted. A plant taxon-specific reference gene is targeted for relative quantification of the GM content. Element- and construct-specific methods are applicable mainly for screening purposes, event-specific methods are required for identification and quantification

7.2.1.1 Polymerase Chain Reaction

DNA-based testing for GM plants is commonly performed using PCR, amplifying specifically a short segment of the targeted DNA (Fig. 7.1). The design of specific primers depends on a knowledge of the precise and comprehensive DNA sequence information of the actually integrated DNA. If the method is to detect specifically a certain transformation event, information about the inserted DNA sequence and the 3' and 5' flanking plant genome sequences is required. For element-specific PCR-based screening and construct-specific detection the DNA sequences of the inserted elements and gene constructs are targeted, respectively.

PCR-based detection and particularly the quantitative measurement of the GM content in a sample actually involves the use of two PCR systems, one for determination of the inserted GM-derived DNA sequence and another system specific for an endogenous, plant-taxon specific reference gene sequence (Fig. 7.1). The latter is also thought to serve as a control for the quality and quantity of the extracted DNA.

7.2.1.2 Conventional Qualitative PCR

Conventional PCR methods are mainly used for qualitative testing to obtain yes/no answers concerning the presence of GM plant material. PCR products are analysed by agarose or polyacrylamide gel electrophoresis (Sambrook and Russel 2001) and visualised using UV fluorescence with ethidium bromide as fluorophor or by other means. It may be necessary to confirm GM-positive test results by further analyses, either by restriction analyses, Southern hybridisation or DNA sequencing (ISO 2005a).

Before the PCR method is applied the primer combination has to be optimised and validated for their performance requirements. The important performance criteria for qualitative PCR methods are the sensitivity in detecting the transgenic DNA sequences and the specificity for the targeted DNA segment. At optimal reaction conditions a limit of detection (LOD) of 1–10 copies of the target sequence can be achieved in less than 40 PCR cycles (Hübner et al. 2001). Practically the LOD of the PCR method should allow that the presence of the target sequence is detected in at least 95% of the time, with $\leq 5\%$ false negative results (ENGL 2008). The length of the amplified product influences the PCR performance and should therefore selected in a way that it matches to the size range of DNA fragments which can be extracted from the sample matrix. For raw materials like seeds or leaves containing less fragmented DNA a broader range of PCR product size up to maximally 250 bp is applicable, whereas for processed food or feed with higher DNA fragmentation the PCR product should be ideally 80–150 bp. The specificity of the method should be tested theoretically by sequence similarity search with the primer sequences against nucleic acid sequence databases (e.g. Blast search in EMBL, GenBank, etc.) and empirically by testing the GM target event(s), very similar non-target GM events and different non-GM plants in order to confirm that the primers can discriminate between the target and closely related non-target sequences. For the reference gene-specific PCR methods different varieties should be tested to demonstrate that the target sequence is conserved between different plant lines (Hernandez et al. 2004, 2005; Broothaerts et al. 2008).

7.2.1.3 Quantitative Real-Time PCR

The most preferred technique to quantify GM material in a sample is real-time PCR. It allows the detection and measurement of increasing fluorescence proportional to the amount of amplification products generated during the PCR process. Of the various chemistries TaqMan fluorogenic probes (Holland et al. 1991) are most commonly applied in real-time PCR-based detection and quantification of GM plant materials. Real-time PCR is mainly used for quantification purposes, but it is increasingly utilised also for qualitative testing to screen or to identify the GM event (Zeitler et al. 2002; Rho et al. 2004; Reiting et al. 2007; Waiblinger et al. 2007).

The limit of quantitation (LOQ) of a real-time PCR method depends on the optimisation of the PCR detection method and on the accepted standard deviation of the measurement. The LOQ is experimentally determined during method validation and should reach 30–50 target molecules, which is close to the theoretical prediction (Hübner et al. 2001). As shown in Table 7.1, the LOD/LOQ values depend primarily on the characteristic plant genome size (C value) and range from 0.004%/0.02% for papaya to 0.16%/0.7% for wheat. The obvious effect here is that PCR is inhibited when the amount of input DNA is exceeding approx. 8 ng/µl of reaction volume. For example for maize, according to its genome size a 200-ng DNA sample contains approximately 39 000 genome copies and thus a given sample with a GM plant content of 0.1% corresponds to 39 copies for a single-copy

Table 7.1 Plant genome size and theoretical LOD/LOQ in real-time PCR assays

Common name	Scientific name	Nuclear DNA content[a]		Genome copies	LOD[c]	LOQ[d]
		Mbp/1C	pg/2C[b]	(in 200 ng)	(%)	(%)
Alfalfa/ lucerne	*Medicago sativa (2n=4X)*	1.510	3.09	64 768	0.02	0.06
Barley	*Hordeum vulgare*	4.873	9.97	20 070	0.05	0.2
Cotton	*Gossypium hirsutum*	2.246	4.59	43 544	0.02	0.1
Maize	*Zea mays*	2.504	5.12	39 058	0.03	0.1
Oilseed rape	*Brassica napus*	1.182	2.42	82 741	0.01	0.05
Papaya	*Carica papaya*	0.372	0.76	262 903	0.004	0.02
Pea	*Opisum sativum*	4.172	8.53	23 442	0.04	0.2
Peanut	*Arachis hypogaea(2n=4X)*	2.813	5.75	34 767	0.03	0.1
Potato	*Solanum tuberosum (2n=4X)*	1.730	3.54	56 548	0.02	0.07
Soybean	*Glycin max*	1.115	2.28	87 713	0.01	0.05
Sugarbeet	*Beta vulgaris ssp. Saccharifera*	0.758	1.55	129 024	0.01	0.03
Sunflower	*Helianthus annuus*	3.030	6.20	32 277	0.03	0.1
Tobacco	*Nicotiana tabacum (2n=4X)*	4.434	9.07	22 059	0.05	0.2
Tomato	*Lycopersicon esculentum*	0.954	1.95	102 569	0.01	0.04
Rice	*Oryza sativa*	0.441	0.90	221 769	0.005	0.02
Wheat	*Triticum aestivum (2n=6X)*	15.966	32.65	6 126	0.16	0.7

[a]Nuclear DNA content values were taken from Arumuganathan and Earle (1991)
[b]1 picogram (pg) = 978×106 base pairs (Dolezel et al. 2003)
[c]Relative limit of detection (LOD) based on an LOD (CI=95%) of 8–12 copies of the GM target sequence (Burns and Valdivia 2008)
[d]relative limition of quantification (LOQ) based on an LOQ of 40 copies for the GM target sequence (Hübner et al. 2001)

transgene. A quantitative real-time PCR assay should be carefully optimised for the specific LOD/LOQ needed for GM content detection and quantification. The precision of the quantitative real-time PCR methods is commonly expressed as relative standard deviation (RSD) which can vary over 10–30% with respect to intra-laboratory repeatability and over 15–50% for inter-laboratory reproducibility, depending on the range of target copies analysed.

7.2.1.4 Alternative DNA-Based Techniques

To solve the challenge that the increasing number of GM plant events is covered by appropriate analytical methodologies it is expected that multi-target analyses are necessary. The DNA microarray technology could be an option to parallelise the multi-analyte detection of several PCR products in a single run. Arrays that have been developed consist of various oligonucleotide probes that are immobilised on a glass support and used for screening of genetic elements, for constructs and events including detection of plant taxon-specific reference genes (Hamels et al. 2007; Xu et al. 2007; Leimanis et al. 2008). However, this approach is based on the use of multiplex PCR before hybridisation of the PCR products to the microarray and, as has been shown elsewhere, PCR is limited in its multiplexing capacity within one

reaction due to the reduced sensitivities of the individual PCR systems. Therefore, alternative amplification methods are currently investigated for their potential use for GMO detection in the future, particularly to cover the increasing number of GM host plants and diversity in genetic elements and constructs. Several alternatives are being tested for improvements in GMO detection, e.g. loop-mediated isothermal amplification (LAMP; Fukuta et al. 2004), ligation-depended probe amplification (LPA; Moreano et al. 2006), SNPlex technology (Chaouachi et al. 2008), padlock probe ligation in combination with microarray detection (Prins et al. 2008) and nucleic acid sequence based amplification using transcription techniques (NASBA) in combination with microarray detection (Morisset et al. 2008). In addition, to circumvent the limitations concerning the availability or reference materials (e.g. for unauthorised GM events), the use of multiple displacement amplification (MDA) for whole-genome amplification has been described to generate reference material for GMO detection (Roth et al. 2008).

7.2.2 Protein-Based Detection

Detection of the novel proteins expressed by GM crops is based almost exclusively on the application of immunoassay technology. Several immunoassays are available for different traits present in diverse GM plant crops and are used in a variety of applications, including testing for unauthorised events and determining the relative GM content (Grothaus et al. 2007). Immunoassays are based on the reaction of an antigen (e.g. the GM-derived protein) with a specific antibody to give a antigen-antibody complex that can be indirectly measured. The immunoassay formats commonly used for GM-protein detection are the enzyme-linked immunosorbent assay (ELISA) and the lateral flow device (LFD).

7.2.2.1 Lateral Flow Strip

Lateral flow strip devices (LFD) are used for qualitative or semi-quantitative detection of antigens and, in the case of novel GM proteins, antibodies are used in the same sandwich immunoassay format as in ELISA, except that the secondary antibody is labelled with a coloured particle such as colloidal gold rather than an enzyme as a means of generating a visible signal. A typical LFD has linked simultaneously a second antibody on the strip to provide visual control that the test has worked correctly. LFDs are available for several traits, require low instrumentation and allow rapid testing also in the field. They are show to be sufficiently specific, but concerning sensitivity only up to the 0.1% range is achievable. LFD represent a useful tool to detect GM proteins in raw materials such as seeds and leaves, however in food and feed products their applicability is restricted to samples containing sufficient GM plant material where the GM protein is expressed. The more drastic limitation for the application of LFDs for food and feed testing is

obviously the physico-chemical instability of proteins when products are processed and heat-treated. The CP4-EPSPS protein is considered as a useful GM protein marker in food/feed products and the Cry1Ab protein to a lesser extent (van den Bulcke et al. 2007).

7.2.2.2 Enzyme-Linked Immunosorbent Assay

Enzyme-linked immunosorbent assays (ELISAs) are commonly 96-well microplates with removable strips of 8–12 wells coated with a primary antibody to capture a target antigen in the sample. A secondary antibody, conjugated to an enzyme such as horseradish peroxidase, is used to detect the presence of the bound antigen, which results in a sandwich of the analyte between the primary and secondary antibodies.

In general ELISAs are quantitative and provide high-throughput capability to the laboratory analysis, considering that the protein is not denatured. Detection limits for Cry1Ab protein is reported to be below 0.1% for dried maize flour (Ermolli et al. 2006). To determine the concentration of the targeted protein in a sample, standards correlating to known concentrations of the antigen are used to produce a calibration curve to determine the unknown concentration of the antigen in the sample. Either recombinant proteins, which contain a similar or identical amino acid sequence and immunoreactivity as the plant-expressed protein, or uniform preparations of actual samples with known concentrations of GM proteins (such as maize or soybean flours available as certified reference materials) may also be used as calibration standards. Since processing affects the detectability of proteins, ELISA is not applicable to most processed food or feed matrices. Furthermore, ELISA does not allow event-specific identification and may fail to detect novel GM proteins.

7.2.3 Method Validation and Standardisation

Validation of detection methods is an essential component to assess the reliability of test methods. By using validated and standardised methods, control laboratories assure that the analytical procedures applied are harmonised at the national or even international level. The process of validation establishes numerical values for the different performance criteria (specificity, sensitivity, applicability, robustness, etc.) and consists at the beginning of an in-house validation in the developers' laboratory followed by a collaborative trial to determine the method's repeatability and reproducibility in order to estimate the transferability of a method between laboratories (Codex 2009). If a collaborative trial-validated method is to be implemented in a laboratory, it is of course also necessary to confirm that the method performs as well under the local conditions as it did in the inter-laboratory method validation study.

To harmonise the procedures applied for the detection of GM plants in foodstuffs and derived products, the International Standardization Organisation (ISO) has

published a series of internationally agreed standards for nucleic acid extraction (ISO 2005c), for qualitative nucleic acid analysis (ISO 2005a), for quantitative nucleic acid analysis (ISO 2005b) and for protein-based methods (ISO 2004). Furthermore, general requirements and definitions involving these different working steps are described in a generic standard document (ISO 2006). These ISO standards prescribe what method performance and validation studies have to be conducted to establish data and the performance characteristics for the specific method application. At the European level a guidance document of the European Network of GMO Laboratories (ENGL) provides practical recommendations how event-specific PCR methods shall be evaluated in the context of the approval of a GM food or feed according to EU Regulation 1829/2003 and defines minimum performance requirements for acceptance of these methods (ENGL 2008).

7.3 Detection Strategies

Detection of the presence of GM plants is an analytical process involving several working steps. It includes: (i) the sampling step, (ii) the extraction step for isolating DNA or protein fractions from the ground material and (iii) the final analysis for identification and/or quantification of GM material. The detection of GM plant DNA can be used for qualitative and for quantitative testing. In quantitative PCR assays, the amount of the specific target DNA present in the sample is estimated, whereas in qualitative PCR tests the presence or absence of a specific GM target sequence is determined.

A commonly applied strategy for testing the presence of GM plants in food, feed and seeds is to first perform screening tests with qualitative methods (Fig. 7.2). This is typically done with DNA-based PCR tests targeting the genetic elements that are most frequently present in GM plants. In the next working step the identification of the GM event is performed by construct-specific or event-specific PCR methods, followed by real-time PCR-based quantification of the relative proportion of transgene DNA copy number versus the plant taxon-specific DNA copies present in the analysed DNA sample (Holst-Jensen et al. 2003). Target sequences to be detected by analytical PCR methods include sequences integrated in the GM event (screening, construct-specific, event-specific), sequences for plant taxa-specific reference genes and occasionally sequences from the donor organisms in order to exclude false-positive results, e.g. possible plant infections with cauliflower mosaic virus (Cankar et al. 2005).

7.3.1 Screening

For the expression of newly integrated genes, GM plant developers use a limited number of regulatory elements (promoters and terminators). Since these elements

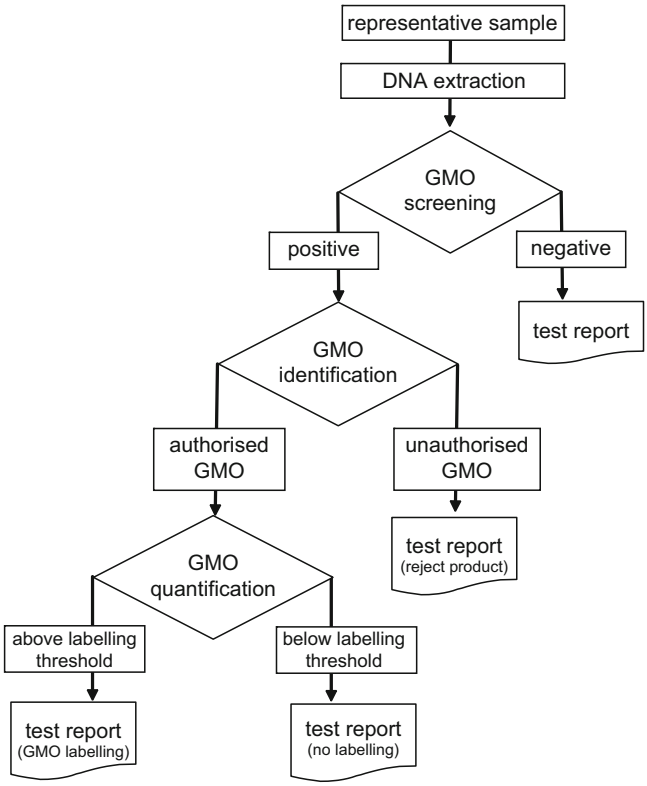

Fig. 7.2 Procedure for GMO testing of food, feed and seeds from a European perspective. A stepwise approach consisting of GMO screening, identification and quantification is commonly applied for testing food, feed and seed products for compliance with European authorisation and labelling regulations

have been frequently used they are ideal candidates for the screening of a large number of samples and are useful to assess whether or not a sample under investigation is likely to contain GM-derived material (Fig. 7.2). To identify these elements Bruderer and Leitner (2003) systematically surveyed which genetic components have been introduced into GM crops at the worldwide level. Correspondingly, the widely applied screening methods target the constitutive 35S promoter (P-35S) sequence from cauliflower mosaic virus (CaMV) or derivatives of this promoter and the terminator sequence isolated from the nopaline synthase (*nos*) gene of *Agrobacterium tumefaciens* which are found in 43 events (P-35S) and in 37 events (T-nos), respectively (Bruderer and Leitner 2003). The survey identified also a few genes with significant numbers of application in GM plants (see Table 7.2). Herbicide-tolerance genes like the *cp4epsps* gene derived from *A. tumefaciens* sp. strain CP4, the phosphinothricin acetyltransferase *(bar)* gene from *Streptomyces hygroscopicus* or from *S. viridochromogenes* (*pat*) have been identified to be reasonable targets for screening (Zeitler et al 2002; Waiblinger et al. 2005).

Table 7.2 Genetic elements and constructs present in selected GM crops (Bruderer and Leitner 2003; Waiblinger et al. 2008)

Genetic element/ construct	GM crop (event name)						
	Canola	Cotton	Maize	Potato	Rice	Soybean	Sugar beet
P-35S	23-198 23-18-27 (Laurical) GS40/90 Topas19/2 OXY235 Liberator L62 T45	MON1445 MON1698 MON15985 31807 31808 MON531 757 1076 BXN	676, 678, 680 DAS-59122-7 B16 (DLL26) Bt11 Bt10 Bt176 CBH-351 DBT418 LY038 MON80100 MON809 MON810 MON832 MON863 MON88017 MON89034 MS3, MS6 NK603 T14, T25 TC1507	ATBT04-X (NewLeaf) BT X (NewLeaf) SPBT02-5 SPT02-7	LL62 LL06 LL601	356043 A2704-12 A2704-21 A5547-127 A5547-35 G94-1 G94-19 G-168 GTS40-3-2 GU262 W62 W98	GTSB77 T120-7
T-nos	MS1 RF1 RF2 MS1xRF1 MS1xRF2 MS8 RF3 MS8xRF3 OXY235	MON1445 MON1698 MON15985 MON531 757 1076	3272 Bt11 CBH-351 GA21 LY038 MIR604 MON80100 MON802 MON809	ATBT04-X (NewLeaf) BT X (NewLeaf) RBMT15-X (NewLeaf) RBMT21-X (NewLeaf) RBMT22-X (NewLeaf) SPBT02-5, SPT02-7	Bt63	G94-1 G94-19 G-168 GTS40-3-2 W62 W98	

			MON832 MON863 MON88017 MON89034 MS3, MS6 NK603				
ctp2-cp4epsps	GT200 GT73	MON1445 MON1698	MON80100 MON802 MON809 MON832 MON88017 NK603	RBMT22-X (NewLeaf)		MON89788 MON87754-1	GTSB77 H7-1
bar	MS1 RF1 RF2 MS1xRF1 MS1xRF2 MS8 RF3 MS8xRF3 PHY23 PHY36		B16 (DLL26) Bt176 CBH-351 DAS-06275-8 DBT418 MS3, MS6		LL62 LL06 LL601	W62 W98	
P35S-pat	GS40/90 Topas 19/2 Liberator L62 T45		676,678,680 DAS-59122 DAS-59132 Bt11 T14, T25 TC1507			A2704-12 A2704-21 A5547-35 A5547-127 GU262	T120-7 T252

The δ endotoxin (*cry*) genes from *Bacillus thuringiensis* (see Chap. 10) belong to the most frequently used genes in transgenic crops. However, screening methods targeting the different *cry* genes have not been established, because theses genes and gene variants are target-organism specific, often synthetic or modified and in some cases truncated or fused, thus making this gene group less suitable for screening purposes.

If for example canola seed samples (canola see Chap. 21) have to be screened for the presence of GM events it is not advisable to use the P-35S specific method, since many GM canola events remain undetected (Table 7.2) and CaMV can infect rapeseed, thus increasing the chances of false-positive results. A screening concept for canola seeds proposed by the German official control laboratories therefore applies a combination of four different construct-specific PCR tests, allowing the detection of 13 known GM canola events (LAG 2006). As described in this concept, the combination of four PCR tests (*P35S-pat*, *pFMV-epsps, pSSUAra-bar*, *P35S-nptII*) covers 13 events and, if one test is positive, further analyses for identification of the GM event have to be performed.

Recently, also real-time PCR arrays based on multi-target analytical systems were developed to serve as less laborious analytical tools for the screening of unauthorised GM crops in the EU and Japan (Querci et al. 2008; Mano et al. 2009). The formats are 96-well or 384-well PCR plates prepared with primers and probes specific for the simultaneous detection of as many GM elements, constructs and events as possible.

7.3.2 Identification

The next step in the work flow of analysing samples which reacted positive in screening tests is the identification of the plant species and the GM events which may be present (Fig. 7.2). If the results of the screening tests indicate the presence of several different GM events, they must of course be first carefully analysed as to which specific tests have to be performed next to identify the GM plant with the most effective strategy. Depending on the sample it may thus be useful to verify first the plant taxa before numerous identification tests are performed. For example, if only DNA from one plant taxon is present, the testing scheme for GM event identification could be much less complex. Another alternative could be to first perform a sub-screening with construct-specific PCR methods targeting transgenic events containing identical gene constructs which have been used to generate several transformation events or to introduce the specific trait in different crop plants (see Fig. 7.1). If for example a construct-specific *ctp2-cp4epsps s*creening is performed (Waiblinger et al. 2005, 2008), solely the different events tolerant to glyphosate (Roundup Ready, see Chap. 9) will be detected, such as canola GT73, maize MON88017 and NK603, soya MON89788 and sugar beet H7-1.

7.3.3 Quantification

For quantification of the GM plant material present in a sample, real-time PCR assays are commonly employed to determine the amount of sequence copies of the GM target versus the reference gene target, which obviously is not generating a direct weight-to-weight measurement (ENGL 2007). These assays use standard curves generated with a serial dilution of DNA of known GM content and target sequence concentration. In this way two calibration curves are constructed, one for the targeted GM sequence and one for the plant taxon-specific reference gene. The calibration DNA can be the DNA extracted from certified reference materials or plasmids (Block and Schwarz 2003), or hybrid amplicons carrying both target sequences can be used (Pardigol et al. 2003). The standard curves and the sample DNA are analysed in the same PCR run and, by extrapolating the Ct obtained, quantitative information for the targets is obtained. The copy numbers are calculated for the GM target sequence and the reference gene and used to estimate the relative amount and percentage of the GM plant event present in a given sample. Target DNA copy numbers of standards and quantitative positive controls must be precisely quantified before use, for example by fluorometric techniques (Ahn et al. 1996) or by spectrophotometric analysis (ISO 2005c). The DNA concentration measured is converted to copy number equivalents by using conversion factors, as reported by Arumuganathan and Earle (1991), or by referring to the plant DNA C-value database (Bennett and Leitch 2005). If certified reference materials of a certain percent GM content are used, the percentage of the material must be considered when calculating GM copy number equivalents for these materials. However, it should be also noted that quantitative PCR methods often measure the GM content in relation to specific reference materials, thus the genetic situation (zygosity, degree of ploidy, copy number per genome, etc.) is not considered, which could be an important issue particularly for maize (Papazova et al. 2005a, b; ENGL 2007).

Because of the relatively high measurement uncertainty (MU) accompanied with DNA-based quantitative analysis of the GM plant content in a given sample, it is important that testing laboratories apply procedures to calculate the combined standard deviation accumulating during the whole analytical process. Such a practical approach was recently described for the calculation of the overall MU for decision-making concerning the European 0.9% labelling threshold (Zel et al. 2007). These authors report that, for event GTS-40-3-2 (Roundup Ready soybean, see Chaps. 9, 24), the expanded uncertainty was 23.2%.

7.3.4 Detection of Stacked Events

A growing number of GM plant events containing stacked traits are approved and already cultivated in some countries (Tavaniers et al. 2008). Of the different approaches for the production of gene stacks, crossing GM events which express

different traits (e.g. by combining the Bt trait for insect resistance with a trait for herbicide tolerance) is preferably applied to rapidly obtain stacked events for commercialisation. This type of stacked event is indeed widely accepted by breeders and forms also the basis for the OECD definition of a unique identifier for gene stacks (OECD 2006).

In general, for the purpose of qualitative testing it is not necessary to discriminate between stacked and non-stacked events, since event-specific methods are already available for most of the commercialised parental GM lines and may be used to identify and, if necessary, to quantify the single events present in the stack. However, if a sample is positive for two or more single events which have been used for the production of a stacked event, it is hardly possible to discriminate between a mix of the single events (parents of the stack) and the hybrid (stacked) GM plant. The only currently available way to circumvent this analytical problem is to analyse single plants or seed kernels for example by using multiplex event-specific real-time PCR assays (Akiyama et al. 2005) or protein flow strips (Ma et al. 2005). However, these single kernel-based analyses are laborious and cost-intensive; sophisticated technical simplifications will be required for any routine application.

7.3.5 Detection of Unauthorised/Unknown GMOs

For GM plants not authorised for marketing as products, EU regulations stipulate a zero tolerance (Fig. 7.2). Examples of unauthorised GM products that have been identified at the European market are GM papaya (‘SunUp’ events 55-1, 63-1), several maize events (‘StarLink’ CBH-351, Bt10, ‘event 32’ DAS-59132-8, MIR604) and rice (LL601, LL62, ‘Bt63’). One of the reasons for these incidences most likely was that protein-based ELISA and LFD tests were used by seed producers to test for the adventitious presence of GM events during scale-up and production. These tests cannot distinguish between different events, which had already potentially caused these problems with the basic seed material by contamination with unauthorised events carrying the same trait. For example, in 2005 the authorised event Bt11 maize was found to be mixed with event Bt10 which was not intended for further propagation and commercialisation and therefore not approved in the United States or in any other country at that time. A recent case (unapproved maize event DAS-59132-8 in DAS-59122-7) shows that protein-based seed quality testing is still causing problems when commercialising GM plants.

The detection for unapproved events is of course an extreme analytical challenge, since in most cases only limited information on such events is available or only partial characterisation has been reported. In these cases specific detection methods have to be developed (Mäde et al. 2006; Cankar et al. 2008), or have to be provided by the concerned seed companies and official authorities, e.g. the USDA. However any PCR-based detection strategy depends on the detailed knowledge of the genetic modification and of the DNA sequence of the insert in order to select

appropriate oligonucleotide primers. For a GM plant which is unknown to the control laboratories this approach is not applicable due to the lack of information on the genetic elements and DNA sequences. Other analytical strategies than PCR-based methods have to be applied to detect this category of GM events, e.g. fingerprinting and fragment profiling techniques (AFLP, RAPD; Theuns et al. 2002), whole genome amplification (Roth et al. 2008) and extensive DNA sequencing. Recently, a pilot study with high-density microarrays showed it was applicable for the screening or profiling of discrete transgene elements present in unknown GMOs (Tengs et al. 2007). However, this method needs pure and relatively high sample DNA concentrations because no PCR amplification of target DNA is performed before the hybridisation step and these microarrays are very cost-intensive. Further optimisation of this approach will clarify whether such an array-based method could be a helpful tool not only for research on plants, but also for detection of unknown GM events in general.

7.3.6 Method Databases

Reports and public databases provide information about the genetic elements contained in GM plants (Bruderer and Leitner 2003; AGBIOS 2008). At the European level detailed information is provided on GM plants for which an application for authorisation has been submitted or which are authorised in the EU. There are also lists of methods and databases available which are valuable sources to find information on validated protein and DNA-based methods used for the identification of GM plants (Bonfini et al. 2007; CRL-GMFF 2008; Dong et al. 2008; JRC 2008).

7.3.7 Sampling Issues

The sampling procedure includes different steps and consists of: (i) taking a composite of increments from a lot to form a bulk sample, (ii) reducing the bulk sample to the laboratory sample and (iii) after grinding/homogenisation again, taking a portion for the actual analysis (test portion). An optimal sampling plan is adapted to the lot size to yield a representative laboratory sample and is of course always a compromise between costs and accepted sampling error. Guidance for the sampling of food and feed products can be found in general standards published by ISO (1999, 2002). At the European level specific documents and recommendations have been established, particularly for GMO sampling of food (CEN/TS15568; EU 2004). Sampling of seeds should follow internationally agreed practices according to the appropriate regulations of the International Seed Testing Association (ISTA). On that basis it is generally agreed that a test sample taken for the GMO analysis should contain at least 2995 seeds to detect a GM seed content of 0.1% with a

confidence level of 95%. It is noted that information on the sampling procedure is of course essential for the correct interpretation of an analytical report.

7.4 Conclusions

The application of appropriate methods and strategies applied for sensitive and specific GM plant detection in seeds, in food and in feed products has become a challenging issue because the global cultivation rates and species of GM crop plants, as well as the diversity of inserted genes and regulatory elements, are constantly increasing. This is currently reflected by accelerated efforts to study and develop new methods and tools with the aims of solving the technical problems, achieving scientific advancement and harmonising GMO detection approaches and testing regimes. It has to be awaited whether technical solutions can be provided for pending problems, for example like the correct distinction and correct quantification of (multiple) stacked events. Concerning the detection of unauthorised GM events it is noted that research institutions and biotechnology companies should contribute as much as possible to minimise the risk that GM plants developed and studied for research purposes are not dispersed accidentally into the environment or marketed through impurities in non-GM seed lots. As demanded for the analytical GM testing process, strict and reliable quality management systems may contribute to the positive public perception concerning the use of GM plants.

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