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Review

Techniques for detecting genetically modified crops and products

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The cultivation of genetically modified crops is becoming increasingly important; more traits are emerging and more acres than ever before are being planted with GM varieties. The release of GM crops and products in the markets worldwide has increased the regulatory need to monitor and verify the presence and the amount of GM varieties in crops and products. Labeling legislation and trade requirements differ from one country to another, leading to the necessity for the development of reliable and sensitive analytical methods for detection, identification and quantification of GM varieties in crops and their products. GM crops and their products can be identified by detecting either the inserted genetic material at DNA level, the resulting protein or phenotype. Several analytical methods such as methods based on the polymerase chain reaction (PCR) for detecting the inserted DNA, immunological assays for detecting the resulting protein, or using bioassays to detect the resultant phenotype have been developed. So far only PCR has found broad application in GMO detection as a generally accepted method for regulatory purposes. Presently, real-time PCR can be considered as the most powerful tool for the detection and quantification of GM crops and products.

Key words: Genetically modified crop, genetic transformation, detection.

INTRODUCTION

A genetically modified (GM) crop is a plant into which one or more genes have been artificially inserted instead of the plant acquiring them under natural conditions of cross-breeding or natural recombination. The inserted gene sequence, known as the transgene, may be from same species, a different species with in the same kingdom or even from a different kingdom (e.g. genetically modified Bt corn, which produces the natural insecticide, contains a gene from a bacterium). The world of biotechnology is moving very fast, more traits are emerging and more acres than ever before are being planted with genetically modified varieties of an ever-expanding number of crops. The biotechnology sector is investing billions of dollars in consolidations to ensure access to these rapidly growing markets, while investing billions more in research and development. The public debate about the future of agricultural biotechnology is more mature as the public becomes better informed and sees more clearly the benefits associated with biotechnology.

The regulatory need to monitor and verify the presence and the amount of GM varieties in crops and products has increased with the expansion of the cultivation of the GM crops. Labeling legislation and trade requirements differ from one country to another, leading to the necessity for the development of reliable and sensitive methods for detection of GM varieties. However, GM samples vary from raw commodities to highly processed foods and testing requirements extend from a general GM screen to a method capable of identifying and quantifying a specific GM crop. This review summarizes the technologies for production of GM crops, their global status, potential benefits; and description of technologies capable of detecting, identifying and quantifying either the DNA introduced or the protein(s) expressed in GM crops and products.

GENETIC TRANSFORMATION FOR PRODUCTION OF GM CROPS

Genetic transformation has become an important tool for crop improvement. The successful genetic transformation in plants requires the production of normal, fertile plants expressing the newly inserted gene(s). The process of genetic transformation involves several distinct steps, namely identification of useful gene, the cloning of the

gene into a suitable plasmid vector, delivery of the vector into plant cell (insertion and integration) followed by expression and inheritance of the foreign DNA encoding a polypeptide. A gene construct consists typically of three elements: 1) The promoter functions as an on/off switch for when and where the inserted/modified gene is active in the recipient plant; 2) The transgene encodes a specifically selected trait, 3) The terminator functions as a stop signal for transcribing the inserted/altered gene. In addition marker genes for distinguishing GM from non-GM varieties during crop development may be present.

Methods of gene insertion in plants can be achieved by direct gene transfer like microprojectile bombardment or through biological vectors like a disarmed Ti (tumour inducing)-plasmid of *A. tumefaciens*.

Direct gene transfer to plant cells

Methods of direct gene transfer are used especially for the transformation of plant species, which are recalcitrant and not susceptible to agroinfection. The methods of DNA delivery into plant cells are fundamentally different from agroinfection since the foreign DNA is introduced through physical means and no biological carriers are involved. Therefore, these techniques are not limited to the constraints characteristic of *Agrobacterium*-mediated transformation. The direct gene transfer methods include microprojectile bombardment, liposome fusion, microinjection, PEG-mediated DNA uptake and electroporation. Microprojectile bombardment is a process by which transforming DNA is coated onto metal microcarriers of tungsten or gold that is accelerated to high velocity either by a gunpowder device or through compressed gases. DNA carried on the microprojectiles remains biologically active inside the cell and can be expressed transiently or by integration into the chromosomal DNA of the host resulting in stable transformation.

Microprojectile bombardment has become one of the major techniques for the transformation of plant cells where the cell wall need not be considered as an obstacle (Hamilton et al., 1992). It has become the most convenient means of introducing DNA for stable transformation in number of agricultural and horticultural crop plants including rice, wheat, soybean, maize, papaya, banana and sugarcane (Becker et al., 2000; Bower and Birch, 1992; Christou et al., 1989; Fitch et al., 1990; Gordon-Kamm et al., 1990; Vasil et al., 1992; Wang et al., 1988). This method is not limited by the species or the type of tissues bombarded and frequently used for transformation of monocotyledonous species.

Agrobacterium-mediated transformation

Plant transformation mediated by the soil plant pathogen *Agrobacterium tumefaciens* has become the most commonly used method for plant transformation. *A. tumefaciens,* a gram-negative phytopathogen, naturally infects the wounded sites in dicotyledonous plant causing the formation of the crown gall tumours.

The basis of the crown gall formation is a transfer of a segment of bacterial tumour inducing plasmid (Ti) DNA, the T-DNA, into the nuclear genome of the infected plant cells. The T-DNA contains two types of genes: the oncogenic genes, encoding for enzymes involved in the synthesis of auxins and cytokinins and responsible for tumour formation; and the genes encoding for the synthesis of opines. Outside the T-DNA are located the genes for the opine catabolism, the genes involved in the process of T-DNA transfer from the bacterium to the plant cell and for the bacterium-bacterium plasmid conjugative transfer genes (Hooykaas and Shilperoort, 1992; Zupan and Zambryski, 1995). The T-DNA fragment is flanked by 25-bp direct repeats, which act as *cis* element signals for the T-DNA transfer. *A. tumefaciens* infects only wounded, actively dividing plant cells, which excrete phenolic compounds, such as acetosyringone and hydroxy-acetosyringone. These phenolics act both as chemo-attractants for *Agrobacterium* and inducers of the virulence genes (Stachel et al., 1985).

For the development of plant transformation systems using *A. tumefaciens*, T-DNA genes can be replaced by other defined gene(s) of interest, which can be transferred to the plant genome. As a consequence of the removal of the plant hormone biosynthetic T-DNA genes, the transformed plant cells do not proliferate into tumorous tissues, but can regenerate into normal plants.

Protocols have been developed for efficient *Agrobacterium*-mediated transformation in both dicotyledonous and monocotyledonous plants, including a large number of crop species. Compared to direct gene transfer methodologies, *Agrobacterium*-mediated transformation offers several advantages such as the possibility to transfer only one or few copies of DNA fragments carrying the genes of interest at higher efficiencies with lower cost and the transfer of very large DNA fragments with minimal rearrangement (Gheysen et al., 1998; Shibata and Liu, 2000). The most important advantage however is the possibility of producing GM plants, which is free of marker genes (Mathews et al., 2001). This will continue to have enormous implications with regards to approval by regulatory agencies, public acceptance and market availability of GM crops.

GLOBAL STATUS OF GENETICALLY MODIFIED PLANTS

The global area of GM crops increased 47 fold, from 1.7 million hectares in 1996 to 81 million hectares in 2004, with an increasing proportion grown by developing countries (James, 2004). Almost one-third (30%) of the global transgenic crop area, was grown in developing

countries where growth continued to be strong. The main GM crops which are being commercialized include soybean (60%), corn (23%), cotton (12%), canola (5%) and potato (~1%). The traits for which GM varieties have been produced are herbicide tolerance (71%), insect resistance (28%) and quality traits (1%). However, research efforts are being made to genetically modify most plants with a high economic value such as cereals, fruits, vegetables, floriculture and horticulture species.

Recently, it has been reported that there are fourteen countries growing about 50,000 hectares or more of GM crops (James, 2004). These includes 9 developing countries and 5 developed countries; they are, in order of hectarage, USA, Argentina, Canada, Brazil, China, Paraguay, India, South Africa, Uruguay, Australia, Romania, Mexico, Spain and the Philippines. Thus, despite the continuing controversy about GM crops, the hectarage and number of farmers growing GM crops have continued to grow at a double digit rate or more, every year since their introduction in 1996. More than 8 million farmers are benefiting from this technology (James, 2004). About 90% of the beneficiary farmers are resource-poor farmers from developing countries, whose increased incomes from biotech crops contributed to the alleviation of poverty.

THE POTENTIAL CONTRIBUTION OF GM CROPS

World population is growing very fast. Estimates of population growth suggest that food requirements are likely to rise substantially in the next 20 years. More than 800 million people in developing countries, including one third of the population of sub-Saharan Africa, are undernourished. More than 90 percent of these are suffering long-term malnourishment and micronutrient deficiency. More than one billion people in the world live on less than one dollar a day. Genetic engineering has tremendous potential to solve these problems. Researchers from the Swiss Federal Institute of Technology's Institute for Plant Sciences inserted genes from a daffodil and a bacterium into rice plants to produce "golden rice," which has sufficient beta-carotene to meet total vitamin A requirements in developing countries with rice-based diets. Vitamin A deficiency leads to blindness in millions of children every year in the developing countries. This crop has potential to significantly improve vitamin uptake in poverty-stricken areas where vitamin supplements are costly and difficult to distribute. The GM crops can offer a range of benefits by contributing to:

1. Increasing crop productivity by production of GM crop resistant to biotic (disease and pest) and abiotic (like drought, frost, acid or salty soil) stresses, and thus contribute to global food security.
2. Conserving biodiversity, as a land-saving technology for higher productivity.
3. Improving the nutritional quality of foods through GM crop varieties containing additional nutrients that are lacking from the diets of many people in develop developing countries, thus contributing to human health.
4. More sustainable agriculture and environment, reduction in use of pesticides and other chemicals; and
5. Improvement of economy and poverty alleviation in developing countries through increasing income of farmers.

DETECTION OF GM CROPS AND PRODUCTS

GM crops and their products can be identified by detecting either the inserted genetic material at DNA level, the mRNA transcribed from the newly introduced gene, the resulting protein, metabolite or phenotype. The analytical tests are generally carried out with the polymerase chain reaction (PCR method) detecting the inserted DNA, immunological assays detecting the resulting protein, or using bioassays to detect the resultant phenotype. Although much progress has been achieved in the development of genetic analysis methods, such as those based on the use of PCR, several other analytical technologies that can provide solutions to current technical issues in GM sample analysis are emerging. These methods include mass spectrometry, chromatography, near infrared spectroscopy, micro fabricated devices and, in particular, DNA chip technology (microarrays). So far only PCR has found broad application in GMO detection as a generally accepted method for regulatory purposes.

In general the procedure consists of three distinct steps:

1) Detection: The objective is to determine whether a product is GM or not. For this purpose, a general screening method can be used. The result is a positive/negative statement. The screening methods are usually based on the PCR, immunoassays or bioassays. Analytical methods for detection must be sensitive and reliable enough to obtain accurate and precise results.

2) Identification: The purpose of identification is to find out which GM crop or product are present and whether they are authorized or not in the country.

3) Quantification: If a crop or its product has been shown to contain GM varieties, then it become necessary to assess compliance with the threshold regulation by the determination of the amount of each of the GM variety present. Normally, quantification is performed using Real-time PCR.

METHODS FOR DETECTING GM CROPS AND PRODUCTS

The analytical methods differ in many levels. The methods are DNA-based, protein-based or trait-based.

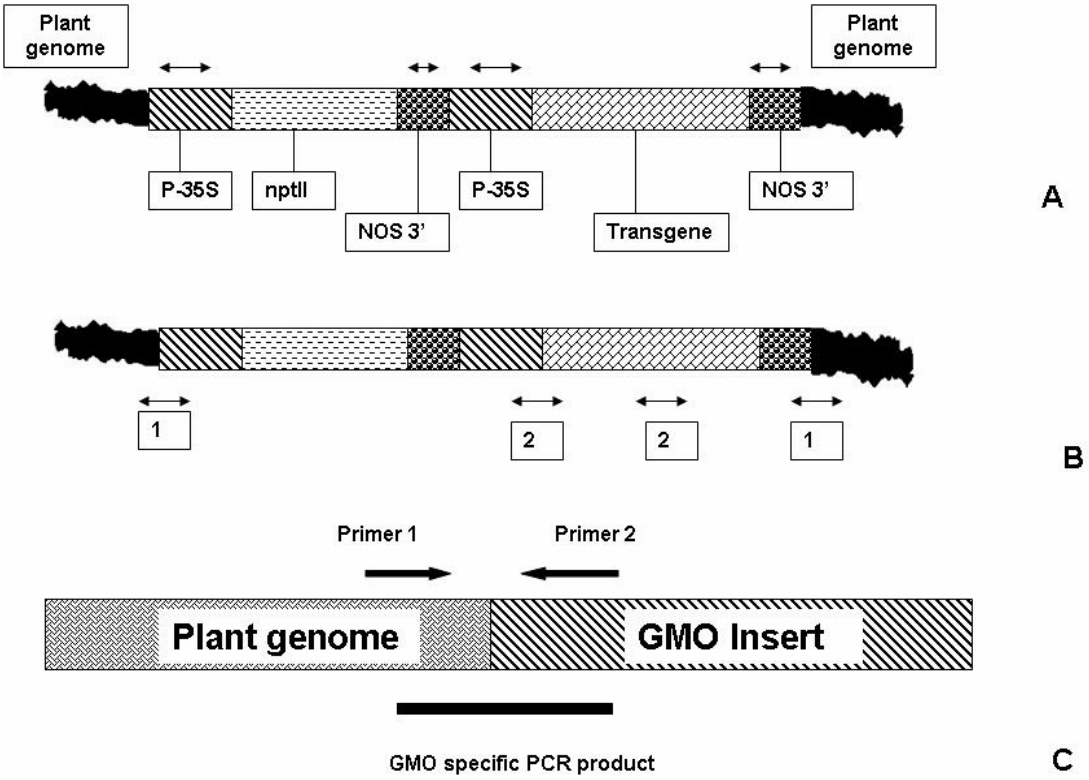

Figure 1. Primer selection for detection of GM crop by PCR analysis: **A.** primer selection for general screening purposes; **B.** primer selection for identification of GM crop, 1-event specific, 2-construct specific; and **C.** primer selection for detecting a specific transformation event.

DNA-based methods

DNA based methods are based on detection of the specific genes, or DNA genetically engineered into the crop. Although, there are several DNA based methodologies, the most commercial testing is conducted using PCR technology. The PCR technique is based on multiplying a specific target DNA allowing the million or billion fold amplification by two synthetic oligonucleotide primers. In PCR, the first step in a cycle involves separation of the two strands of the original DNA molecule. The second step involves binding of the two primers to their oligonucleotide primers. The third step involves making two perfect copies of the original double stranded DNA molecule by adding the right nucleotides to the end of each primer, using the strands as templates. Once the cycle is completed, it can be repeated, and for each cycle the number of copies is doubled, resulting in an exponential amplification. The amplified fragment can be detected by gel electrophoresis or hybridization techniques.

The process consists of extraction and purification of DNA, amplification of the inserted DNA by PCR and confirmation of the amplified PCR product. In principle, PCR can detect a single target molecule in a complex DNA mixture.

I. Qualitative PCR analysis

The most critical parameter for successful PCR is the design of primers. A poorly designed primer can result in little or no product due to non-specific amplification and/or primer-dimer formation, which can become competitive enough to suppress product formation. It is essential that care should be taken in the design of primers for PCR. Several parameters including the length of the primer, %GC content and the 3' sequence need to be optimized for successful PCR. Certain of these parameters can be manually optimized while others are best done with computer programs. The selection of primers is the most important component for detection of GM crop by PCR and it depends upon the choice of target gene. The strategies for choosing an appropriate target are as follows:

The detection of GM crops: For general screening purposes the focus should be on target sequences that are characteristic for the group to be screened (Figure 1A). Genetic control elements such as the cauliflower mosaic virus 35S promoter (P-35S) and the *Agrobacterium tumefaciens* nos terminator (nos3') are present in many GM crops currently on the market. The general screening PCR detects the presence of GMO, which then need to be identified.

The identification of GM crop: Primer selection has to be based on target sequences that are characteristic for the individual GM variety. The junction sequences between two adjoining DNA segments can be the target for a specific detection of the genetic construct like the cross-border regions between integration site and transformed genetic element of a specific GM variety, or specific sequence alterations (Figure 1B). Only a continuous survey of all data available on GM crops - especially the introduced genetic elements and their integration sites, can be a guaranteed comprehensive detection of GM crops.

To detect a particular event: The junction sequences in the integration site (plant-construct junction fragment) can be used to detect a specific transformation event (Figure 1C). When the GM crop is the result of a non-homologous recombination, the integration site is unique. When the same gene construct is used to produce different GM crops, this will be the only strategy to distinguish between GM crops containing the same gene construct.

The sequential test scheme for GM crop detection is to initially screen samples for species-specific DNA, known as housekeeping genes as *e.g.* lectin gene (soybean samples) or invertase gene (maize samples) to determine whether DNA from that species can be detected. If DNA is detectable, samples are then screened using the general genetic elements for the detection of GM varieties. Positive results from this initial screening are further confirmed using tests, which screen for the specific genes or constructs used in the most common GM crops. Then identification tests used depends on the DNA sample (e.g. Cry genes, EPSPS gene, Pat gene), or, more ideally, for the plant-construct junction fragments.

II. Multiplex PCR-based detection methods

With multiplex PCR-based methods, several target DNA sequences can be screened and detected in a single reaction. The advantage of multiplex methods is evidently that fewer reactions are needed to test a sample for potential presence of GMO-derived DNA. Development of multiplex assays requires careful testing and validation. After the PCR the resulting pool of amplified fragments needs to be further analysed to distinguish between the various amplicons. Several research groups are currently developing a number of multiplex assays, but only one paper has been published presenting a multiplex assay for detection of five GM-maize (Bt11, Bt176, Mon810, T25 and GA21; Matsuoka et al., 2001).

III. Quantitative PCR

In principle, PCR based quantitation can be performed either after completion of the PCR (end-point analysis), or during the PCR (real-time analysis).

Quantification using conventional PCR: Conventional PCR measures the products of the PCR reaction at the end point in the reaction profile. End-point analyses are based on comparison of the final amount of amplified DNA of two DNA targets, the one to be quantified and a competitor (an artificially constructed DNA that is added in a small and known quantity prior to the PCR amplification and that is co-amplified with the target, which is to be quantified). The competitor has the same binding sites for the same primer pair but is different in size. This is called competitive quantitative PCR, and the two DNA targets are amplified with equal efficiency. A dilution series of the DNA to be analysed is prepared, and a constant amount of the competitor is added. After completion of the PCR the resulting amplification products are visualized through gel electrophoresis and when both DNA targets yield the same amount of product it is assumed that the starting amount was also the same. By setting up two competitive PCRs, one for the GM crop (e.g. Bt Corn) and one for the species of interest (e.g. maize), and including competitors in both, the quantity of GM crop relative to the species can be estimated by extrapolation from the degree of dilution and concentration of the competitors. The competitive PCR methods are semi-quantitative.

Quantification using Real-time PCR: Real-time PCR is a system based on the continuous monitoring of PCR products. This is done via fluorometric measurement of an internal probe during the reaction. In real-time analyses the amount of product synthesized during PCR is estimated directly by measurement of fluorescence in the PCR reaction. Several types of hybridisation probes are available that will emit fluorescent light corresponding to the amount of synthesized DNA. However, the amount of synthesised product can also be estimated with fluorescent dyes, e.g. SYBR Green I that intercalates double-stranded DNA. With the latter, it is not possible to distinguish between the specific product and non-specific products, and consequently the use of specific hybridisation probes is normally preferred. The quantitative estimate is based on extrapolation by comparison of the GM crop sequence relative to the reference of interest (e.g. gene sequence from Roundup Ready soybean and lectin gene from soybean). The idea is that with the use of fluorescence it becomes possible to measure exactly the number of cycles that are needed to produce a certain amount of PCR product. This amount corresponds to the amount producing a fluorescence signal clearly distinguishable from the background signal and measured well before the plateau effect becomes a problem. The number is called the Ct-value. Then by comparison of Ct-values for the GM crop target sequence, e.g. Roundup Ready soybean 3' integration junction, and the reference gene, e.g. soybean lectin, it becomes possible to estimate the ratio of the GM target sequence to the reference sequence in terms of difference in number of cycles needed to produce the

same quantity of product. Since one cycle corresponds to a doubling of the amount of product, a simple formula can be presented to estimate the ratio in percent. While real-time PCR requires more sophisticated and expensive equipment than competitive PCR, it is faster, automated and more specific. Presently, real-time PCR can be considered as the most powerful tool for the detection and quantification of GM crops and products.

Protein based methods

Immunoassay is the current method for detection and quantification of new (foreign) proteins introduced through genetic transformation of plants. Immunoassay is based on the specific binding between an antigen and an antibody. Thus, the availability of antibodies with the desired affinity and specificity is the most important factor for setting up immunoassay systems. Immunoassays can be highly specific and samples often need only a simple preparation before being analysed. Moreover, immunoassays can be used qualitatively or quantitatively over a wide range of concentrations. Western blot, ELISA (Enzyme-Linked Immunosorbent Assay) and lateral flow sticks are typical protein-based test methods.

The antibodies can be polyclonal, raised in animals, or monoclonal, produced by cell cultures. Commercially available polyclonal antiserum is often produced in rabbits, goats or sheep. Monoclonal antibodies offer some advantages over polyclonal antibodies because they express uniform affinity and specificity against a single epitope or antigenic determinant and can be produced in vast quantities. Both polyclonal and monoclonal antibodies may require further purification steps to enhance the sensitivity and reduce backgrounds in assays. The specificity of the antibodies must be checked carefully to elucidate any cross-reactivity with similar substances, which might cause false positive results.

I. ELISA (Enzyme Linked Immunosorbent Assay)

In ELISA the antigen-antibody reaction takes place on a solid phase (microtiter plates). Antigen and antibody react and produce a stable complex, which can be visualised by addition of a second antibody linked to an enzyme. Addition of a substrate for that enzyme results in a colour formation, which can be measured photometrically or recognised by naked eye.

ELISA test kits provide the quantitative results in hours with detection limits less than 0.1%. However, some companies operate with slightly higher quantification levels as *e.g*. 0.3%. ELISAs have been designed to detect a novel GM protein or trait. There are the reports that some ELISA detected the novel protein such as the CP4 EPSPS protein from *A. tumefaciens* expressed in Roundup Ready Soybeans.

II. Lateral flow sticks

The lateral flow test (dipstick format) uses a membrane-based detection system. The membrane contains two capture zones, one captures the bound GM protein, the other captures color reagent. Paper strips or plastic paddles are used as support for the capture antibody that is immobilized onto a test strip in specific zone. Most tests are provided usually in kit form. The lateral flow test strip is dipped into the prepared sample in extraction solution and the sample migrates up the strip by capillary action.

As the sample flows through the detection antibody strip and the capture antibody strip, the protein of interest will accumulate and thus give a high intensity band, but the volume is not as well controlled. These tests generally provide qualitative or semi-quantitative results using antibodies and color reagents incorporated into a lateral flow strip.

Lateral flow techniques are qualitative or semi-quantitative. By following appropriate sampling procedures, it is possible to obtain a 99% confidence level of less than 0.15% GMO for a given lot.

Phenotypic characterisation (herbicide bioassays)

Phenotypic characterisation allows detection of the presence or absence of a specific trait. So far only tests for traits as herbicides tolerance are available. Such methods can be used to test for presence or absence of herbicide resistant GM varieties and is termed herbicide bioassays. They consist of conducting germination tests on solid germination media in the presence of a specific herbicide, where non-GM and GM seeds show distinct characteristics. The detection level is dependent on germination of the seed and the germination methods should ensure that all viable seeds of the tested sample germinate. Seeds tested positive should be exposed to subsequent tests for confirmation.

The herbicide bioassay tests are claimed to be accurate, inexpensive, and useful as a preventative test primarily for seed companies. Companies are using the herbicide bioassays to check individual shipments as a quality assurance program. Negative trait and positive trait seeds should be included as controls with every sample testing. At the moment herbicide bioassays are available for Roundup Ready soybean, maize, cotton and oilseed rape, and Liberty Link maize. In the future bioassays for insect-resistant or other GM varieties may be developed.

Comparison of the different methods

The comparison of various detection methods is summarized in Table 1. At present, only PCR offers a

Table 1. Comparison of the different methods.

Methods	Suitability	Duration	Advantage	Disadvantage
PCR	Detection, identification, quantification	5-7 days	Very sensitive and most precise in terms of detection limits, qualitative and quantitative measurement of levels of target DNA sequence	Expensive and require specialized equipment and trained personnel
ELISA	Trait identification	2-4 days	Faster, less expensive, quantitative measurement of protein levels	Cannot detect denatured protein, require some specialized equipment and trained personnel, cannot identify a GM sample where several varieties may have the same trait incorporated
Lateral flow stick	Trait identification	10-20 min	Quick, Qualitative measurement of presence or absence of target protein	No quantification of protein, cannot identify a GM sample where several varieties may have the same trait incorporated
Herbicide bioassays	Trait identification	7-10 days	Inexpensive, very accurate, identifying GM crops with the particular trait in samples of viable seed/grain	Only viable seeds can be tested, no processed products can be tested, bioassays require separate tests for each trait in question and at present the tests will detect only herbicide tolerance traits

way for performing a general screening for GM varieties and detection of particular "events". Phenotypic characterisation and immunoassays detect particular traits that may be present in several GM crops (*e.g.* the Cry1a protein and genes, conferring insecticide resistance, are present in a range of different GM Maize: MON80100, MON801, MON802, MON809, 176, BT11).

One of the major considerations in analytical testing of almost any GM crop or its product is the sampling procedure. The sample analysed must be representative of the material from which it is taken otherwise the testing regime is flawed. Sample preparation for both DNA-based and protein-based methods is critical for detection and/or quantification. It is important to know the limitations of each procedure as well as the purpose of detection. Both the sample size and sampling procedures dramatically impact the conclusions that may be drawn from any of these testing methods.

CONCLUSIONS

The release of GM crop and products in environment and markets worldwide has resulted in public debate in many part of the world. Despite the continuing controversy about GM crops, the hectarage and number of farmers growing GM crops have continued to grow at a double digit rate or more, every year. Currently, there are fourteen countries, 9 developing countries and 5 developed countries, growing GM crops. The need for identification and detection of GM crops and products has increased with the rapid expansion in the cultivation of GM crops. Labeling and traceability of GM material is way forward to address the concerns of consumers and regulators. The establishment of relevant, reliable and economical methodology for detection, identification and quantification of GM crops continues to be a challenge at international level. A great number of different strategies and methods are available for testing of GM material and the quality of these results depends not only on the methodology and the equipment but also the sampling, the theoretical expertise and the practical skills of the regulatory officers handling the testing of the sample. Therefore, it is important to understand the methods and their applications for detection of GM crops and their products. This paper describes the technologies capable of detecting, identifying and quantifying either the DNA

introduced or the protein(s) expressed in GM crops and products. Currently, available methods for detecting GM crops and products are almost exclusively based on PCR, because of their high sensitivity, specificity and need for only a small amount of DNA. Especially, real-time PCR has been regarded as the most powerful tool for the detection and quantification of GMO despite its high expense.

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