21

Biosafety and Detection of Genetically Modified Organisms

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1. Introduction

Biosafety is a set of actions focused on preventing, minimizing and eliminating risks associated with research, production, teaching, use, technology development and services related to genetically modified organisms (GMOs) with the aims of protecting human and animal health and environmental preservation.

Transgenic organisms, or GMOs, are organisms in which genetic material has been altered by recombinant DNA technology. Biotechnology allows the insertion of one or more genes into the genome of an organism from a different organism or species (e.g., animals, plants, viruses, bacteria); the expression of the introduced gene results in a new feature in the phenotype of the modified organism. A shortened definition of genes is that they are DNA sequences that contain the necessary information to affect phenotypic expression in an organism, such as the shape of a seed or resistance to a specific pest. The information encoded by the gene is expressed through two principal steps: transcription, in which the coding region of the DNA is copied into single-stranded RNA; and translation, in which the amino acid sequence encoded by mRNA is assembled and translated into protein. Thus, for the creation of a GMO, it is necessary to introduce the gene responsible for a particular trait into the genome of the target organism through recombinant DNA techniques.

Several products derived from recombinant DNA technology are commercially available worldwide. GMO products already on the market include human insulin, somatropin and transgenic varieties of crops, such as maize, soybeans, cotton and common beans. The United States, Brazil and Argentina are among the principal countries engaged in the commercial production and marketing of GMOs (James, 2010).

The emergence of genetic engineering in the early 1970s in California, USA, with the isolation, introduction, and expression of the insulin gene in *Escherichia coli* provoked a strong reaction from the scientific community all over the world, which led to the Asilomar Conference in 1974. At that time, the scientific community proposed a moratorium on genetic engineering. They argued that rules and safeguards should be established to ensure

the use of genetic engineering techniques without risking human life and the environment. In a relatively short period of time, biosafety regulations were developed for the appropriate use of these technologies in the laboratory. After over 35 years of research on and commercial use of biotechnology, there have yet to be any reports about the adverse effects of the use of genetic engineering on human and animal health or the environment. Therefore, to ensure the appropriate generation and utilization of this technology, biosafety regulations and monitoring mechanisms have been developed in different countries around the world. Several field tests with transgenic varieties have been performed in the USA, Argentina, Bolivia and Chile since 1991. However, in Brazil, these tests only began in 1997.

2. Transgenic plants and their advantages

In the future, there will be difficulties in meeting the food demand in developing countries due the increasing trends of food prices and population growth. Therefore, it is necessary to employ new technologies, such as the use of transgenic varieties, to increase the productivity per unit area, especially in the developing nations. Qaim and Zilberman (2003) reported that in developing nations (i.e., China, India and Sub-Saharan Africa) farmers can achieve a greater than 60% grain yield advantage by using transgenic plants modified with the *Bt* gene instead of conventional varieties. In their work, these authors showed that the yield advantage comes solely from the impact of the *Bt* gene on the control of insect pests.

Biotechnology research currently plays a key role in food production because it helps to increase productivity, improve the nutritional quality of agricultural products and reduce production costs. Qaim and Zilberman (2003) reported that using the *Bt* gene for the control of insect pests gave a US $ 30 per ha advantage over conventional cotton. It is also advantageous because it allows reduction of the use of highly hazardous chemicals, such as organophosphates, carbamates, and synthetic pyrethroids, which belong to international toxicity classes I and II.

The commercialization of transgenic plants in Brazil has also strongly affected the agrochemical sector, which has annual profits of approximately 20 billion US dollars. Of this, approximately 8 billion US dollars per year corresponds to pesticides used for the control of diseases, insects and weeds. In some cases, the cost of pesticides in relation to the total cost of production reaches approximately 40%, as in cotton. However, varieties developed by genetic engineering that are tolerant to herbicides and resistant to insects, fungi, bacteria and viruses have led to reductions in the cost of agricultural production and, consequently, reduction of the impact of agrochemical wastes that have an adverse effect on the environment and human health.

The findings described above, obtained in developed and developing nations, demonstrate the contribution of transgenic plants to increasing the productivity per unit area to fulfill the increasing demand for agricultural products to feed the growing population of the world.

GMO technology is currently widely employed throughout the world. The global area of biotechnology crop coverage in 2010 reached approximately 148 million ha in 29 countries on five continents. The major biotechnology crops cultivated worldwide are maize, soybean, canola, cotton, sugar beet, alfalfa and papaya (James, 2010). The geographic distribution of biotech crops throughout the world is presented in Table 1.

3. Food biosafety

The food safety of transgenic plants is assessed in accordance with risk analysis. This methodology was initially developed with the aim of assessing deleterious effects on human health arising from potentially toxic chemicals present in food, pesticide residues, contaminants and food additives and was subsequently applied in assessing the food safety of GM plants.

One of the main foundations of risk analysis methodology is that transgenic plants are not inherently more dangerous than conventional crops; i.e., the potential health risks that may be associated with a transgenic variety are not because it is GM but rather are related to the possible chemical changes that may result from genetic modification (Konig et al., 2004). For example, a genetically modified common bean expressing an allergenic protein from the allergen Brazil Nut (*Bertholletia excelsa*) was not prohibited from being produced because it was obtained by genetic engineering, but because the genetic modification was incorporated in a gene that promotes the synthesis of an allergenic protein in this variety.

Order	Country	Area (millions of hectares)
1st	USA	66.8
2nd	Brazil	25.4
3rd	Argentina	22.9
4th	India	9.4
5th	Canada	8.8
6th	China	3.5
7th	Paraguay	2.6
8th	Pakistan	2.4
9th	South Africa	2.2
10th	Uruguay	1.1

Table 1. The ten major producers of transgenic crops in the world (adopted from James, 2010)

In general, most transgenic plants are modified to synthesize proteins that are absent in conventional varieties. These proteins are encoded by a transgene and introduced precisely for the purpose of conferring the desired trait. However, beyond this difference, other biochemical changes may result from the introduction of a transgene, and all of this is investigated during risk analysis.

In the case of transgenic plants, risk analysis is performed by comparing them with their non-GM counterparts, which are considered to be safe on the basis of their usage records. In risk analysis, instead of attempting to identify every hazard associated with the GM variety, one can seek to identify only new hazards that are not present in the traditional variety.

This type of comparative study is referred to as substantial equivalence analysis and is based on comparison of the biochemical profile of the transgenic variety with the conventional variety. The GM variety can be classified as substantially equivalent or substantially non-equivalent.

At this point, it should be noted that food security assessment of a GM plant is not restricted to applying the concept of substantial equivalence. This constitutes only the starting point for this assessment, and it aims to identify differences that will be analyzed later. Further analyses include allergenicity and toxicity tests performed *in silico*, *in vitro* and *in vivo* in animal models (i.e., rodents, birds, fish, and other species) to assess toxicity levels. In these tests, the LD_{50} (lethal dose in 50% of cases) is generally determined as an indicator of acute (i.e., short-term) toxicity.

The risk assessments are performed in three steps (Borém and Gomes, 2009):

Step 1. Risk assessment: This step can be defined as the evaluation of the probability of adverse health effects arising from human or animal exposure to a hazard. Risk assessment consists of four segments:

i. Hazard identification, which entails the identification of biological, chemical and physical hazards found in food that may cause adverse health effects;
ii. Hazard characterization, which entails an evaluation of an identified hazard in qualitative and quantitative terms and often involves the establishment of a dose-response relationship due to the magnitude of exposure (dose) to a physical, chemical, or biological hazard and the severity of adverse health effects;
iii. Exposure assessment, which entails a quantitative and qualitative assessment of the likelihood of ingestion of physical, chemical and biological agents through food;
iv. Risk characterization, which entails a qualitative and quantitative estimation of the likelihood and severity of an adverse effect on health based on identification and hazard characterization and on exposure assessment.

Step 2. Risk management: Risk management is measured from the results of risk assessment and other legitimate factors to reduce risks to the health of consumers. Measure of risk management may include labeling, imposition of conditions for marketing approval and post-trade monitoring.

Step 3. Risk communication: Risk communication includes the information exchange that must occur between all stakeholders, including the government, industry, the scientific community, media and consumers. It should occur throughout the assessment and risk management processes and should include an explanation to the public of the decisions made, ensuring access to documents obtained from the risk assessment and, at the same time, respecting the right to safeguard the confidentiality of industrial information.

Food biosafety analyses performed by different national and international organizations, such as the World Health Organization, the International Council for Science, the United Nations Food and Agriculture Organization, the Royal Society of London and the National Academies of Sciences from Brazil, Mexico, India, the United States, Australia, and Italy have demonstrated that transgenic varieties can be considered safe for human consumption.

4. Environmental biosafety

Similar to food risk assessment, environmental risk assessment considers three important points: the possibility, probability and consequences of a hazard, which should always be assessed on a case-by-case basis. This means that, following the identification of a possible

danger, you should consider whether that danger is possible, if it is likely and, if it were to occur, what the result would be (Conner et al., 2003).

In the specific case of risk assessment for GM plants, a fourth point should also be considered: the risks of non-adoption of this technology.

An essential element in any risk assessment is the establishment of correct benchmarks. As described for the assessment of food security, a GM crop plant is compared with its non-GM counterpart. Similarly, the environmental impact of transgenic plants should be evaluated in relation to the impact caused by conventional varieties.

These principles are essential for providing guidance regarding which tests should be conducted and what questions should be answered to generate information that will assist in making the decision to use or not use a specific transgenic variety. Failure to follow these principles can result in unnecessary and unhelpful evaluations in risk assessments.

For example, the cultivation of insect-resistant transgenic cotton in Brazil has raised concerns about gene escape, i.e., the possibility of the transgenic variety crossing with wild species of the genus *Gossypium* that are native in Brazil and thus sexually compatible with cultivated cotton (Freire and Brandão, 2006). The main issue is the possibility of the pollen of transgenic cotton plants fertilizing wild cotton. The offspring of such crosses could have consequences for the maintenance of genetic diversity, although this point remains very controversial, as several research groups do not believe that the introduced gene would produce any adaptive advantage when exposed to the natural environment.

Gene escape from transgenic plants can occur in three main ways:

i. When the transgenic plant becomes a weed or an invasive species (e.g., for crops with weed-like characteristics, such as sunflower, canola, and rice), the transgenic gene found in the transgenic plant may allow the crop to become weedier and more invasive;
ii. Intraspecific and interspecific hybridization, such as when transgenic DNA is transferred by crossing to other varieties of cultivated species and wild species, respectively;
iii. When transgenic DNA is asexually transmitted to other species and organisms.

For a gene to escape and be transferred to different species, certain conditions are necessary:

i. The two parental individuals must be sexually compatible;
ii. They must be located in neighboring areas and with flowering overlap between the two parental types;
iii. A sufficient amount of viable pollen must be present and transferred between individuals;
iv. The resulting progeny should be fertile and ecologically adapted to environmental conditions where the parents are located.

To avert gene escape from transgenic varieties to conventional varieties, isolation distance should be maintained. For example, maize is a wind-pollinated species, and the distances that pollen can travel depend on the wind pattern, humidity and temperature. In general, fields with transgenic varieties should be isolated from other conventional varieties with a distance of at least 200 m (Weeks et al., 2007). The risk of gene escape from soybeans and maize to wild relatives in Brazil is considered by most scientists to be small or nonexistent.

The risk of gene escape associated with transgenic soybeans in China and maize in Mexico to their wild relatives are different because China and Mexico are the centers of diversity of the respective species.

Additionally, transgenic crops may have an effect on a non-target organism. Evidence has shown that the lethal dose (LD_{50}) of *Bt* varieties for beneficial insects, such as bees and ladybugs, is far higher when such insects are exposed to fields of these transgenic varieties. Some studies have also reported the safety levels of *Bt* varieties for the monarch butterfly (Tabashnik, 1994; Tang, 1996).

A study under the auspices of the European Union addressing the environmental impacts caused by cultivation of GM crops was conducted for 15 years (1985-2000) involving 400 public research institutions and reached the following conclusion: "Our research shows that, according to standard risk assessments, GM organisms and their products do not present risks to human health or the environment. In fact, the use of more precise technology and conducting the most accurate analysis possible during the regulatory process associated with these varieties make these products even more secure than their conventional forms (European Union, 1999 – available at
http://bio4eu.jrc.ec.europa.eu/documents/FINALGMcropsintheEUBIO4EU.pdf).

5. Biosafety regulations

The need for official regulation of genetically modified organisms became more evident in the mid-1980s, when biotechnology companies sought permission to perform research on genetically modified organisms.

Currently, implementation of biosafety rules is determined on a case-by-case basis around the world based on technical and scientific data, with transparent decision-making and consistency, building public confidence. There is no international standard established, and each country is responsible for creating its own regulations for research, trade, production, transport, storage and disposal.

5.1 Regulatory agencies

Field testing of the first transgenic plants began in the early 1980s. To date, there have been more than 25,000 field tests performed worldwide, half of which have been in the United States and Canada. In South America, the greatest number of releases occurred in Argentina. The commercialization of GM crops began in 1994, with tomatoes genetically engineered by Calgene. Transgenic varieties of soybean, maize, cotton, canola and papaya, among other crops, already represent a significant share of agriculture in the United States, Brazil, Canada, and Argentina. These varieties have been modified for resistance to insects and viruses and tolerance to herbicides. Field testing and laboratory evaluation of GMOs have been performed by regulatory agencies in each country to evaluate the risks of the GMOs to human and animal health and the environment.

In the United States, the agencies that examine the safety of genetically modified varieties include the Environmental Protection Agency (EPA), the Food and Drug Administration (FDA), and the Animal and Plant Health Inspection Service (APHIS) of the United States Department of Agriculture (USDA).

The USDA-APHIS regulates field tests of both plants and genetically modified microorganisms. This is the agency that reviews the licensing procedures for field testing by industry, universities and nongovernmental organizations (NGOs). The processes related to the agricultural and environmental safety of herbicide organisms, such as Roundup Ready™ (RR) soybeans, are also reviewed by USDA-APHIS.

The FDA evaluates the safety and nutritional aspects of genetically modified varieties that are used for human food and feed for animals. The FDA guidelines are based on the fact that food derived from GMOs must meet the same rigorous safety standards required for conventional foods.

The EPA is responsible for ensuring the safety of GMOs and varieties that produce pesticide elements and chemical and biological substances for distribution, consumption and trade. Under U.S. law, the jurisdiction of the EPA is limited to pesticides. For example, a plant that has been genetically modified to resist insects falls within its jurisdiction, but not a plant modified to resist drought. Plant resistance to a pest is under the authority of the EPA because the plant produces a substance that acts as a pesticide. In contrast, drought resistance may be due to factors such as deeper roots, and this transgenic plant would be subject to regulation by the USDA-APHIS.

With respect to pest-resistant varieties, the EPA has four categories of analysis: product characterization, toxicology, effects on non-target organisms and disposal in the environment. The characterization of a product includes a review of its origin and how the transgene is expressed in living organisms, the nature of the pesticide, the modifications introduced to the trait (compared with what is found in nature) and the biology of the receiving plant. To analyze the toxicology, the level of acute oral toxicity of the pesticide substance is evaluated in rats. For proteins toxic to insects, the EPA also requires a digestibility test, which evaluates the time required for the protein to be digested by gastric and intestinal juices. The EPA also analyzes the allergenicity of the protein. With regard to environmental impacts, the agency examines the exposure and toxicity of the transgenic plant to non-target insects and beneficial insects.

The regulation of biosafety is governed through local agencies in each country, such as Health Canada and the Canadian Food Inspection Agency in Canada; the Ministry of Agriculture, Forestry and Fisheries and the Ministry of Health and Labour in Japan; and Conabia in Argentina.

Several countries in Latin America, including Brazil, Argentina, Chile, Mexico and Venezuela, have established biosafety rules through specific legislation to regulate the use of genetic engineering and the release of the products of this technology into the environment. In Brazil, these rules are guided by Federal Law 11,105, enacted on March 24, 2005. This law also created the National Board of GM Biosafety (CTNBio), the National Biosafety Council (CNS), also known as the Council of Ministers, and the Biosafety Information Service (SIB). Fontes (2003) discussed the regulatory and legal concerns in Brazil in detail.

CTNBio is composed of 27 members, and their backups appointed by the scientific community, have deep scientific knowledge in the areas of biotechnology associated with humans, animals, plants and the environment, along with representatives of the several Ministries. CTNBio has developed a set of Normative Resolutions that now regulate most

aspects of modern biotechnology in the country. To date, the Board has authorized the commercial release of 31 GM events for use in agriculture and vaccines for husbandry in Brazilian territory.

CTNBio analyzes the requests that are forwarded to it by issuing opinions that are specific to each transgenic target of evaluation. Before any GMO product is released for planting, trade or use, it must have been subjected to analysis of possible risks to humans, animals and the environment. The results of these tests are evaluated by CTNBio, which then makes a recommendation for release of GMOs that do not pose a risk to human or animal health or the environment. Genetically modified products suspected to have any adverse effect on human or animal health or the environment are banned from commercial use by CTNBio.

5.2 Other recently developed techniques to avoid environmental and human health concerns

5.2.1 Chloroplast engineering

The chloroplast is the principal organelle of plant cells and eukaryotic algae where photosynthesis is carried out. This is the site where food production starts. Therefore, incorporation of genes in the chloroplast genome and their expression provide a relative advantage compared with expression in the nucleus (Wang et al. 2009). The existence of many copies of chloroplast plastids, in some cases up to 10 thousand per cell, mean that chloroplast DNA comprises approximately 10-20% of cellular DNA. The other advantage of chloroplast engineering is that it is associated with greater visibility of the effects of the transgene on the production of proteins and carbohydrates. In addition, a chloroplast transgene has an advantage over a nuclear DNA transgene because it reduces the impact of contamination of the transgene via pollen to wild relatives and other similar species. This reduces the risk of developing weeds resistant to toxins and herbicides. Furthermore, the expression of a transgene in chloroplast-transgenic plants is more stable than a nuclear transformant because transgenes integrate into the chloroplast genome by homologous recombination (Elizabeth, 2005). The other advantage of chloroplast transformation is the possibility of transforming several transgenes under the control of one promoter. Chloroplast transformation has been used by different researchers to develop cultivars resistant to diseases and pests, weeds, and abiotic stresses (Wang et al., 2009, Wani et al., 2010).

Chloroplast engineering has several applications in the fields of medicine, biology and agriculture. This technique is used for improvement of plant traits, such as resistance to biotic and abiotic stress (Rhodes and Hanson, 1993), introduction of insect-resistant transgenes into crop plants (Dufourmantel et al., 2005), biopharmaceutical production (Daniell et al., 2001), metabolic pathway engineering (Lossl et al., 2005), and research on RNA editing (Hayes et al., 2006). A detailed review on chloroplast engineering was presented by Wang et al. (2009).

5.2.2 Producing marker-free transgenic plants

The objective of developing marker-free transgenic plants is of great urgency, as most of the transgenic plants developed contain the marker gene used during the selection phase. Selection genes include resistance to ampicillin (or other antimicrobials) and herbicides. The existence of this type of gene in the environment has raised a great deal of concern from

environmentalists and consumer protection groups, as this might have unpredictable consequences for human and animal health. In specific cases, an herbicide-resistant gene used in the selection process could pass to weeds, resulting in the development of weeds that are resistant to herbicides. With respect to consumers, if a gene resistant to antibiotics is present in food products, it could spread to the human population, though there is no evidence for this at the moment. Therefore, developing appropriate methods of selection for transgenes without transferring the selectable marker to the environment will increase the commercialization of transgenic plants in the world by reducing the cost of developing and commercializing genetically modified crops and will make consumers more comfortable.

The currently available transformation techniques are not efficient in transforming a number of genes responsible for quantitative traits and other important agronomic traits in a single transformation. This makes joint gene transformation in a target organism impossible and increases the cost of the transformation and development of transgenic organisms. According to Puchta (2003), there are four possible ways to avoid marker genes from genetically modified crops: avoiding the use of selectable marker genes; employing marker genes with no harmful effects; joint transformation of the target trait gene and marker gene, followed by their segregation; and removing the selectable marker gene from the gene of interest (the transgene) through successful site-specific recombination or homologous recombination. Among the above four techniques used to eliminate marker genes from transgenic plants, the fourth one has recently received more attention because of its efficiency and acceptance.

The specific-site recombination system involves the Cre protein and two lox sites within the transgene construct. This system is used in a number of different types of genome manipulations. Two lox sites in direct orientation are required for the excision of the marker gene (Russell et al., 1992), and excision is performed with the expression of Cre. Elimination of the marker gene from the target gene in the transgenic plant occurs through site-specific recombination, in which the two lox sites are required for removing the marker gene from the plant genome by the use of the Cre recombinase. This technique has two principal advantages: it requires only a single round of genome manipulation; and it reduces the time required to obtain a marker-free transgenic plant (Thomason et al., 2001).

6. Detection of genetically modified organisms

There are several reasons to support detailed research on transgenic organisms, especially to allow their easy and quick identification among conventional organisms. The ability to identify a GMO is strongly related to animal, human and environmental biosafety, and it is within the rights of the consumer to know what he/she is eating. In the case of Brazil, which is the second largest transgenic producer, local legislation specifies that all food containing more than 1% material derived from GMOs should have a label indicating the presence of a GMO in its composition. The presence of genetically modified seeds in conventional seed samples has become a growing problem for international trade and may result in severe consequences for food exporters, such as Brazil. For compliance with laws and GMO regulatory measures to be effective, it is necessary to apply techniques that enable the sensitive, reliable detection and quantification of GMOs. The following section will describe the techniques used for the detection of GMOs.

6.1 Techniques for GMO detection

The genetic modifications introduced into an organism should be well known to better understand what techniques can be employed in the detection of GMOs. The basic structure of an exogenous DNA sequence inserted into a GMO is composed of three main elements, as described by Conceição and co-workers (2004): the promoter region, which is responsible for gene transcription; the gene itself, which defines the desired characteristic; and the terminator region, which is responsible for transcription termination. All detection systems are based on the elements present in the DNA sequence inserted into the GMO, either through direct detection of an exogenous DNA molecule inserted in the genome or indirectly through the protein product and by-products resulting from expression of the DNA insert.

6.1.1 Direct assays: Detection of the presence of exogenous DNA

6.1.1.1 PCR

The polymerase chain reaction (PCR) was developed by Mullis and Faloona in 1987, and it is the main technique used in molecular biology laboratories to detect GMOs. The technique is based on the replication of specific sequences of exogenous DNA. For this purpose, small pieces of DNA known as primers bind to exogenous DNA present in the GMO, and during the PCR amplification process, which is catalyzed by the DNA polymerase enzyme, thousands copies of the specific sequence are produced. Copies of DNA produced by PCR are easily visualized by electrophoresis in agarose gels. A DNA intercalating agent (e.g., ethidium bromide) is used for visualization of DNA bands present in the gel. If the same primers are used in a non-transgenic organism, the band will not displayed in an agarose gel, as there will be no detection of copies of exogenous DNA because it is not present in the wild or conventional organism.

The PCR technique is very specific, sensitive and safe and is able to detect both events of genetic modification (Bertheau et al., 2002; Giovannini and Concillo, 2002) and distinguish events associated with different gene constructs expressing the same protein (Yamaguchi et al., 2003). However, this technique also presents some limitations, such as: 1) the difficulty involved in designing primers, as it is necessary to know the genetic sequence of the DNA introduced into the GMO, and this information is usually confidential (Holst-Jensen et al., 2003); 2) the need for appropriate equipment and trained personnel; 3) the relatively high cost because the test is specific for each genetic alteration introduced; and 4) the special care required to avoid sample contamination (Miraglia et al., 2004, Yamaguchi et al., 2003).

The quality of DNA extracted is crucial to the success of the PCR method. There are several procedures described in the literature for DNA extraction from leaves, seeds, and even processed foods. The CTAB (cetyltrimethylammonium bromide) method is widely used in molecular biology laboratories. There are also several commercial kits that employ silica resin with high affinity for DNA molecules. Poor quality, degraded or low purity DNA can negatively influence the success of PCR, preventing the identification of foreign DNA in the sample. This low purity DNA can occur from the presence of inhibitors in DNA extracts (e.g., proteins, polysaccharides, and polyphenols) that hinder the annealing of primers to target DNA and/or inhibit the activity of the DNA polymerase enzyme (Ahmed, 2002). Consequently, the reaction will occur with a low efficiency or may not occur at all. For quality control in PCR, it is always necessary to use a standard reaction (control) that

evaluates the quality of the extracted DNA and avoids false-negative results. The default reaction may be performed using specific primers for any known endogenous gene.

The limit of detection for PCR is between 20 picograms and 10 nanograms of exogenous DNA. Thus, it is possible to detect a single genetically modified seed among 1,000 to 10,000 conventional seeds (Luthy, 1999).

6.1.1.2 Real-time PCR

Various PCR techniques are used in molecular analysis of transgenic events for different purposes. The real-time qPCR (quantitative PCR) technique was developed and has been used in the identification of GMO events and products. Real-time qPCR is used not only for the detection of specific DNA but also for the quantification of copy number of a particular target DNA sequence inserted in a GMO. The difference between conventional PCR and qPCR is the specificity and sensitivity of the latter method. In real-time qPCR, equipment capable of detecting the fluorescence emitted by the reaction during each cycle of amplification of the target DNA molecule is used whereas in conventional PCR, the result is visualized in an agarose gel after 30-45 cycles of amplification. Real-time qPCR is monitored from the first cycle of amplification until the last one by detecting the fluorescence emitted. Then, it is possible to recognize the exact time (i.e., cycle) at which the amplification of the target molecule can be detected (Figure 1). These data allow inference of the number of copies of the transgene present in the GMO based on an endogenous control reaction. Basically, when the number of amplification cycles required to detect the emitted fluorescence is reduced, the copy number of the transgene inserted in the GMO is higher (an inverse correlation). The sensitivity of the method is based not only on the uptake of the fluorescent signal but also on how fluorescence is emitted during the reaction. Most real-time PCR applications require only one fluorescent agent for double-stranded DNA. However, some applications require greater specificity, such as the TaqMan® system.

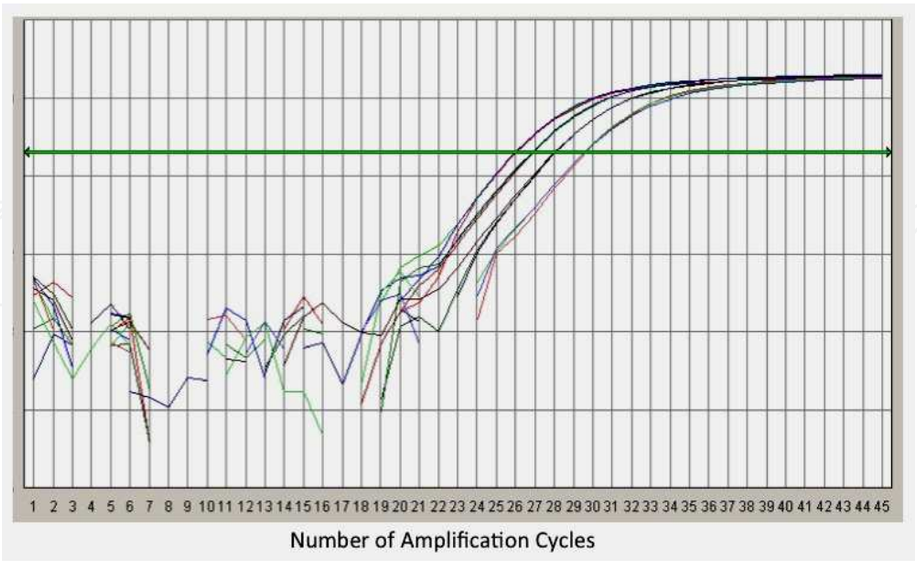

Fig. 1. Real-time qPCR. The curves in the graph show the progress of target molecule amplification. Capture of the fluorescent signal occurs throughout all PCR cycles. Source: Camargo (2009).

Real-time qPCR using the TaqMan® detection system employs a pair of primers and a probe labeled with a fluorophore (a marker that emits fluorescence when stimulated). These three oligonucleotides (two primers and a probe) are specific to the target sequence, thereby contributing to the greater specificity of the technique. In the process of primer amplification; the probe binds to the target DNA molecule, and a DNA polymerase enzyme initiates polymerization of the new molecule. When the enzyme meets the probe linked to the target DNA, it severs the probe and allows fluorescence to be emitted and then detected by the equipment. Therefore, the process of fluorescence emission is dependent on the joint action of four elements: two primers, one probe and the DNA polymerase enzyme.

There are factors related to amplification conditions that may adversely affect the reliability of the results obtained, such as the use of inappropriate temperatures for primer and probe annealing; low specificity of the primers and probe with respect to annealing to the DNA template; and inappropriate conditions for the activity of the polymerase enzyme (e.g., unadjusted salt concentration or pH of the buffer).

6.1.1.3 Southern blotting

The Southern blot technique, described by Southern in 1975, is also frequently used in laboratories to detect specific fragments of exogenous DNA integrated into the genomic DNA of a transgenic organism and its products. This technique essentially consists of five steps: 1) extraction and digestion of genomic DNA with one or more restriction enzymes; 2) separation of DNA fragments by electrophoresis in an agarose gel; 3) transfer and fixation of DNA present in the gel to a nitrocellulose or nylon membrane; 4) hybridization of DNA present in the membrane against a DNA probe that has sequence homology to the target DNA; and 5) visualization by autoradiography or colorimetry.

Southern blot analysis is very reliable and is considered molecular evidence of the integration of exogenous elements in a GMO genome. In addition, it is also possible to estimate the number of copies that were introduced into the genome of the recipient organism with this method (Figure 2).

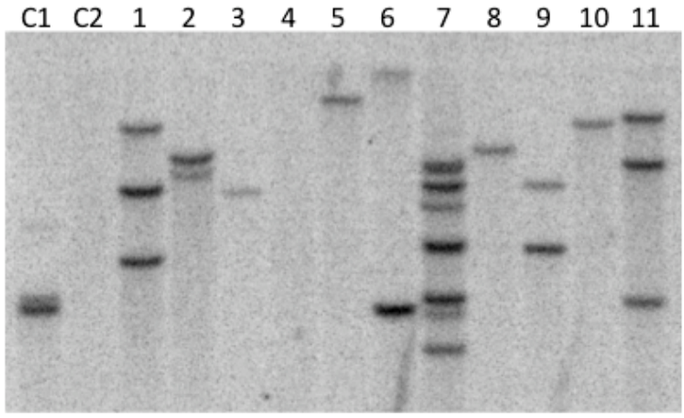

Fig. 2. Southern blot analysis. Analysis of transgenic events using the Southern blot technique. C1) Positive control (transgenic event); C2) negative control (wild plant); 1 to 11) transgenic events in the analysis. In this case, the number of bands refers to the copy number of target DNA sequences present in the genome of each event analyzed. Source: Camargo (2009).

However, this technique is associated with the limitation of requiring a large amount of genomic DNA (between 20 to 40 micrograms), which is sometimes difficult to obtain depending on the type of sample used. Other limitations to this technique are its high cost, operational complexity, long period required to perform the experiment and obtain results, the use of radioactive probes, and that it requires an appropriate infrastructure and adequate training for the handling and storage of radioactive products and waste disposal.

6.1.2 Indirect assays: Detection based on the presence of RNA

The genetic information in DNA must be translated into protein to be effective and have effects in an organism. The translation of information from DNA to protein occurs only because of the previous transcription of DNA into molecules of messenger RNA (mRNA). mRNA synthesis can be considered as the intermediate stage of the process of transferring the information contained in DNA and reflects the level of transcription activity, as the presence of mRNA is directly related to gene expression.

There are different molecular techniques that can be used in studies of gene expression. These include the northern blot and RT-PCR (reverse transcription - polymerase chain reaction) techniques. These techniques can be employed to determine the gene expression in different tissues and/or different stages of development of the organism under study and to monitor the gene expression of exogenous DNA in GMOs.

6.1.2.1 Northern blotting

The northern blot technique, also referred to as an RNA blot, was developed to study gene expression through the detection of RNA molecules present in a sample (Alwine et al., 1977). The execution of northern blotting is very similar to the Southern blot technique and basically consists of 5 steps: 1) total RNA extraction, 2) separation of RNA fragments by electrophoresis in an agarose gel, 3) transfer and fixation of the RNA present in the gel to a nitrocellulose or nylon membrane, 4) hybridization of the RNA present in the membrane against a DNA or RNA probe with homology to the target RNA sequence, and 5) revelation by autoradiography.

One of the most important steps in this technique is the extraction of total RNA from the sample because to obtain reliable results, it is necessary to obtain intact and pure RNA. Exercising care during the technique is much more critical than in Southern blotting because RNA degrades easily. To prevent its degradation, it is necessary to treat all objects with specific solutions to eliminate or minimize the presence of RNases, which are enzymes that are very effective in degrading RNA molecules.

6.1.2.2 RT-PCR

RT-PCR is widely used to verify gene expression by detecting mRNA molecules. This technique is based on reverse transcription of mRNA followed by PCR amplification. The reverse transcription reaction is based on the synthesis of complementary DNA (cDNA) from an mRNA molecule template by the reverse transcriptase enzyme. The product of this amplification is visualized in an agarose gel. The intensity of the bands visualized in the gel provides some indication of the amount of target mRNA present in the sample.

Quantitative RT-PCR (qRT-PCR) is a modern method based on the principles of RT-PCR (i.e., cDNA production followed by PCR). Therefore, qRT-PCR is more robust, specific and

sensitive; consequently, it provides better quantitative results. The amplification progress of the target molecule is displayed in real time by capturing a fluorescent signal in more sophisticated thermal cyclers, such as those described for the qPCR technique.

6.1.3 Indirect assays: Detection based on the presence of protein

6.1.3.1 Bioassays

In most of the GM varieties commercialized to date, genes have been introduced conferring tolerance to herbicides and/or resistance to viruses, fungi or insects (Borém and Almeida, 2011). The bioassay technique for the detection of GMOs in these cases is relatively simple, inexpensive and easy to establish in both laboratories and greenhouses, but a relatively long time is required to obtain results (the results are usually obtained after a week).

Bioassays for herbicide tolerance can be conducted using a plant or even seeds. In case of plants, a dose of the herbicide is sprayed on the leaves. Then, the plants are monitored daily to verify the presence or absence of any phenotype (i.e., symptoms) resulting from the application of the herbicide (Figure 3). The leaves of plants with no tolerance to the herbicide initially become yellow and then dry (i.e., the tissue undergoes necrosis). The leaves of herbicide-tolerant plants exhibit no or few symptoms of necrosis, and the plants continue to look as healthy as before the herbicide application. In the case of bioassays performed with seeds, the seeds are germinated in a medium containing a diluted solution of the herbicide. If the seeds are tolerant to the herbicide, germination will occur, and the plant will develop normally, as is seen for the seeds of transgenic Liberty Link™ corn and Roundup Ready™ soybeans, which are tolerant to glyphosate. If the seeds are sensitive to the herbicide, no germination will be observed. Currently, bioassays for herbicide tolerance are commonly used by companies that export seeds and grains to prove the authenticity and quality of their products (Torres et al., 2003).

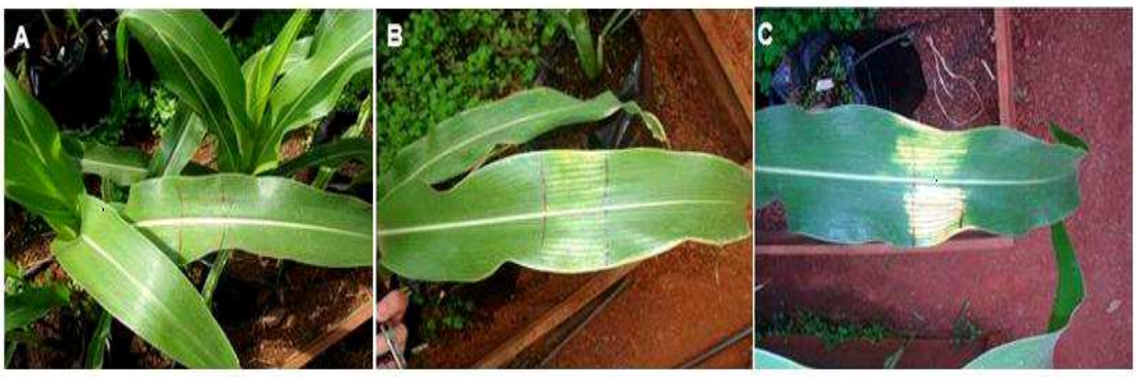

Fig. 3. Bioassay for herbicide tolerance in genetically modified maize plants overexpressing the *bar* gene. This gene encodes the enzyme phosphinothricin-N-acetyltransferase (PAT), which confers tolerance to the herbicide ammonium gluphosinate (PPT). a) Leaf of a genetically modified maize plant showing PPT tolerance after 10 days of herbicide application; b) leaf of a genetically modified maize plant showing mild PPT susceptibility after 10 days of application; c) leaf of an unmodified maize plant (negative control) showing an intense PPT susceptibility after 10 days of application. Source: Camargo (2009).

Insect-resistant plants can also be analyzed by bioassays. In this case, the bioassay can be performed by placing insects or their larval form, according to the cycle of the insect that attacks the plant, on the plants to be analyzed or even on leaf discs from the plants. Two types of information can be obtained from these experiments: 1) the mortality rate of the pest; and 2) the damage caused by the pests to the analyzed tissue. Analysis of these data will indicate whether or not the plant is resistant to the insect under investigation.

Such bioassays are based on the gene expression and phenotype of interest presented by the GMO. However, a disadvantage of this method is that the results of bioassays are generally not sufficiently definitive proof of the integration of exogenous DNA into the genome of a transgenic organism, and other evidence (i.e., results from other techniques) is necessary for its verification.

6.1.3.2 Immunoassays

Immunoassays are ideal methods for the qualitative and/or quantitative detection of specific proteins produced from an exogenous DNA sequence introduced into a GMO. The main immunoassays used for the detection and quantification of target proteins present in GMOs are the enzyme-linked immunosorbent assay (ELISA), western blotting and the lateral flow immunoassay (LFI).

6.1.3.2.1 ELISA

An ELISA identifies a target protein present in a protein extract containing a population of other proteins by using specific antibodies that bind to the target protein. The antigen-antibody reaction identifies the presence of exogenous protein in a qualitative and even quantitative form.

There are several types of ELISA, including direct ELISA, indirect ELISA, sandwich ELISA and competitive ELISA. The sandwich ELISA method, which uses two specific antibodies (Abs) for a target protein, is the most sensitive of these techniques and is used for the detection of GMOs (Yates, 1999). In this type of immunoassay, two types of antibodies specific for a target protein are used: a primary (i.e., capture) Ab, which is used to sensitize a plate to capture the target protein (i.e., the antigen, Ag) present in the sample; and a secondary (i.e., combined) Ab, usually in conjunction with an enzyme (e.g., peroxidase or alkaline phosphatase) that acts on a given substrate to produce color (in a colorimetric assay) or fluorescence (in a fluorimetric assay). The intensity of the color produced is directly related to the amount of antigen present in the sample, as the color will only occur when the target protein binds to the capture antibody on the plate, followed by binding of the enzyme-conjugated antibody to the immobilized target protein (Figure 4). Free antibodies and protein that did not form Ab-Ag-Ab complexes are discarded during microplate washing steps. Thus, there is little possibility for false-positive results to occur. For quantitative assays, a standard curve of protein at known concentrations is used.

Direct and indirect ELISA assays are similar to the sandwich ELISA, but with some modified steps. A direct ELISA assay uses only a specific antibody conjugated to an enzyme (i.e., the "secondary Ab"). Protein extract is added to a plate followed by the conjugated antibody. In the next step, the appropriate substrate is added; the enzyme then acts on it, and the reaction is revealed. An indirect ELISA assay uses a primary Ab specific for a target protein and an enzyme-conjugated Ab that is specific to the primary Ab (anti-IgG of the

organism in which the primary Ab was produced, usually rabbit or mouse). In this case, protein extract is added to a plate, followed by the primary Ab. The secondary Ab is added to the reaction, recognizing the primary Ab, and then binding to the Ab-Ag complex. The development stage occurs in the same way as in the direct ELISA. In many cases, the sandwich ELISA assay is two to five times more sensitive than the direct or indirect ELISA.

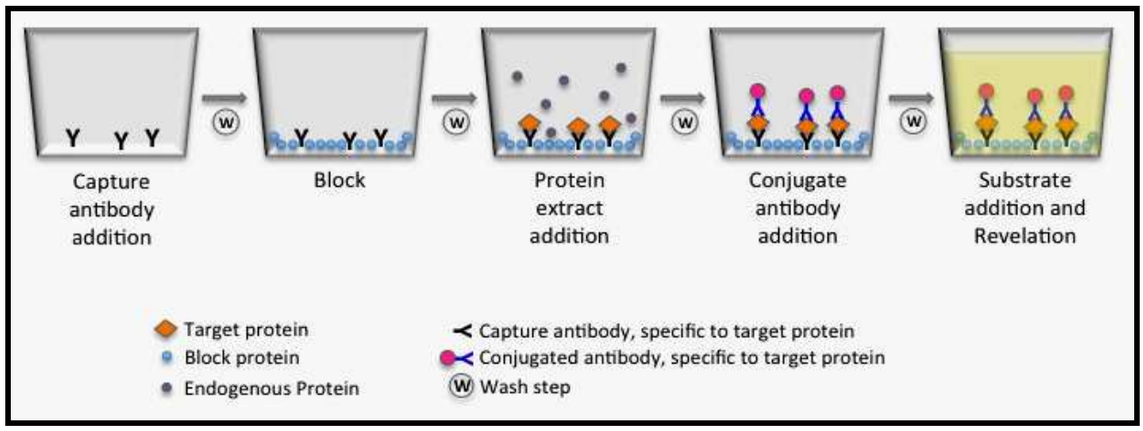

Fig. 4. Steps of the sandwich ELISA assay. The assay starts with sensitization of the plate with the capture antibody. After washing, the plate is blocked. Then, the protein extract is added. The plate is washed again, and conjugated antibody is added. After incubation, the plate is washed and then the protein present is revealed by adding the substrate. Source: Camargo (2009).

Another kind of ELISA is the competitive assay, in which the target protein in the sample and a standard protein conjugated to an enzyme compete for the binding of a capture antibody. In this type of test, the amount of target protein is inversely proportional to the colorimetric intensity produced. The more standard protein binds to the antibody, the more intense the color produced by the reaction. Some drawbacks of competitive ELISA are its restriction for use with only one specific antibody each time it is used and that it is less sensitive than the sandwich method.

The type of antibody used in an immunoassay can also contribute to the greater sensitivity and specificity of the assay. Monoclonal antibodies contribute to increasing the specificity of this type of technique while polyclonal antibodies increase sensitivity because they can recognize different epitopes of a target protein (Ahmed, 2002).

ELISA is a very sensitive, specific, robust, safe and rapid technique for the detection of GMOs in the laboratory. Moreover, it is the ideal technique for simultaneous analysis of a large number of samples under routine diagnosis.

6.1.3.2.2 Western blotting

The western blot technique is based on the separation of proteins present in the protein extract from a sample in non-denaturing polyacrylamide gels and subsequent transfer to nitrocellulose or nylon membranes. Detection of the target protein is performed by means of specific antibodies that recognize epitopes of the protein of interest. Visualization of the assay occurs through a colorimetric reaction or radiographic detection. Thus, western blot

analysis combines the resolution of electrophoresis with the specificity of immunological detection (Brasileiro and Carneiro, 1998).

The electrophoretic separation of proteins in the sample usually occurs under denaturing conditions. Thus, problems of solubility, aggregation and co-precipitation of the target protein with other proteins present in the sample are eliminated (Sambrook and Russel, 2001). However, antibodies against conformational epitopes of the target protein may not recognize these epitopes when denatured.

The western blot technique is semiquantitative, specific and sufficiently sensitive to detect proteins (Brett et al., 1999). The limit of detection for the target protein depends on a number of factors, including the type of membrane and the detection system used. In most cases, this limit corresponds to approximately 20 femtomoles (10-15 moles). Thus, it is possible to detect approximately 1 nanogram of a protein with a molecular weight of 50 kDa (Brasileiro and Carneiro, 1998). In seed analysis, the minimum limit of detection is 0.25% (Yates, 1999). Western blot analysis is a laborious technique, and it is capable of analyzing only a few samples simultaneously. Therefore, western blotting is rarely used in routine analysis of GMOs. This technique is usually used to confirm preliminary results generated by other detection techniques.

6.1.3.2.3 Lateral flow immunoassay

The lateral flow immunoassay (LFI) is widely used for analysis of material still in field trials and product testing because it is practical, inexpensive and fast. Its results are obtained within 5 to 15 minutes. Other advantages of this method are that it does not require special equipment and trained personnel. However, this technique is not sufficiently robust for quantification of GM material present in a sample, but it is a very sensitive technique for the qualitative detection of GMOs (Urbanek et al., 2001).

The principle of the lateral flow immunoassay is similar to the sandwich ELISA. The detection antibody is located at the end of a strip that is inserted into a sample solution. The target protein present in the sample binds to antibody present in the strip, and the Ag-Ab complex then migrates by capillary action to the other end of the strip, where there are two capture zones. In these areas, there are specific antibodies to capture the target protein or detection antibody. When the Ag-Ab complex passes through the capture zones, there is a colorimetric reaction (Figure 5). The presence of two colored bands on the strip indicates that the test is positive (i.e., the transgenic protein is present in the sample). The presence of only one band indicates that the sample is negative (i.e., that it contains no traces of the transgenic protein, but the test was performed correctly) (Conceição et al., 2004).

IFL strips are produced commercially for the detection of a wide range of proteins used in the production of GMOs. Soybeans, maize, canola, cotton and sugar beets genetically modified to contain the endotoxin Cry (Ab) from *Bacillus thuringiensis* or the CP4-EPSPS protein from *Agrobacterium tumefaciens* can be easily analyzed using this technique (Lipton et al., 2000).

In addition to their usefulness in the detection of GMOs, immunoassays are powerful tools for assessing the expression of a transgene. It is possible to identify the location of transgene expression in a plant, in which tissues the protein is present (e.g., roots, leaves, seeds) and the ratio of expression among different tissues (i.e., tissues that show more or less expression of the transgene).

6.1.4 Alternative techniques for the detection of GMOs

Because of the increasing number of GMOs and the complexity of the genetic changes that are emerging, new techniques are being developed or improved with the goals of increasing sensitivity and reliability, lowering costs and allowing simultaneous analysis. DNA microarrays, chromatography and mass spectrometry are examples of other techniques for detecting GMOs.

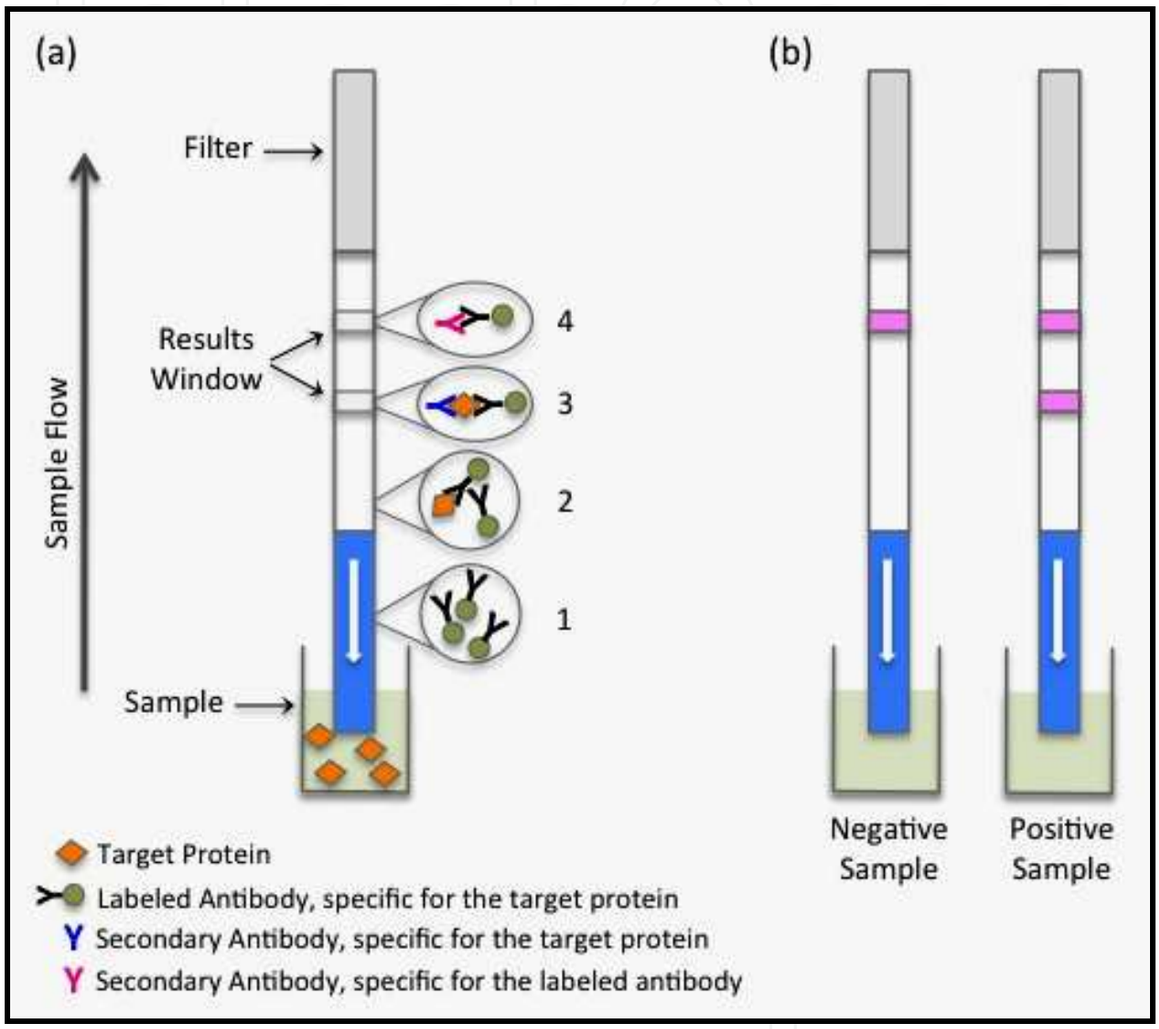

Fig. 5. Diagram of a lateral flow immunoassay (IFL). a) One end of the IFL strip is inserted into the sample. By capillarity, the sample travels up the strip toward the other end. During this run, the sample passes through a region where there are capture antibodies (1), and the target protein binds. Capture antibodies that are either complexed with the target protein or free (2) migrate and bind to specific antibodies for the target protein (3) or specific antibodies to the capture antibodies (4), respectively. b) A single band revealed in the results window indicates that the sample is negative (i.e., absence of the target protein), and two bands indicate that the sample is positive (i.e., presence of the target protein). Source: Camargo (2009).

7. Conclusions

Regulatory procedures for GM crops require extensive risk analysis on a case-by-case basis for modified organisms. Information regarding the number of copies of foreign DNA inserted into the genome, the expression level of the protein of interest, the parts of the plant in which the protein is present, the toxicity and allergenicity of the protein and its possible adverse effects for non-target organisms and for the environment is required. Thus, numerous techniques have been used to evaluate the biosafety of GMOs. The detection and identification of these organisms are also of great interest for identifying the purity of seed samples, labeling food, and trade reasons.

Knowledge regarding the genetic constitution of a GMO and the main features of each technique is essential for the implementation of these tests and to obtain accurate and reliable results with respect to detecting and evaluating a transgenic organism. In some cases, the simple integration of exogenous DNA into the host genome does not mean that the gene is being expressed and that the target protein is being produced. Thus, the combined use of more than one detection technique may be necessary for the complete assessment of GMOs.

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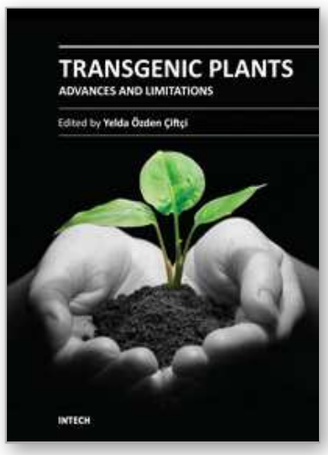

Transgenic Plants - Advances and Limitations

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Development of efficient transformation protocols is becoming a complementary strategy to conventional breeding techniques for the improvement of crops. Thus, Transgenic Plants - Advances and Limitations covers the recent advances carried on improvement of transformation methods together with assessment of the impact of genetically transformed crops on biosafety. Each chapter has been written by one or more experienced researchers in the field and then carefully edited to ensure throughness and consistency.

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