Chapter

Molecular Identification of Genetically Modified Crops for Biosafety and Legitimacy of Transgenes

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Abstract

Crops undergo artificially DNA modifications for improvements are considered as genetically modified (GM) crops. These modifications could be in indigenous DNA or by introduction of foreign DNA as transgenes. There are 29 different crops and fruit trees in 42 countries, which have been successfully modified for various traits like herbicide tolerance, insect/pest resistance, disease resistance and quality improvement. GM crops are grown worldwide and its area is significantly increasing every year. Many countries have very strict rules and regulations for GM crops and are also a trade barrier in some situations. Hence, identification and testing of crops for GM contents is important for identity and legitimacy of transgene to simplify the international trade. Normally, molecular identification is performed at three different levels, i.e., DNA, RNA and protein, and each level has its own importance in testing about the nature and type of GM crops. In this chapter, current scenario of GM crops and different molecular testing tools are described in brief.

Keywords: biotechnology, genetic engineering, transgenic plants, molecular testing, polymerase chain reaction, enzymes linked Immuno-sorbent assay

1. Introduction

Biotechnology is a set of scientific tools in which living organisms are used for the welfare of mankind. This technique is efficiently used to modify and improve plants, animals and other microorganisms to increase their value. Biotechnology has a very wide range of applications and almost every field of daily science get benefit from this technology. Application of biotechnology in the field of agriculture has been practiced for a long time as people have wanted to improve agriculturally important crops by selection and breeding. In 1970s with the advancements in molecular biology, researchers were able to modify DNA which is a chemical building block and specify the features of living organisms at molecular level. This modification in genetic material or DNA is called as recombinant DNA technology or genetic engineering [1]. With the involvement of genetic engineering in agriculture, one can transfer useful hereditary/genetic information from distant sources into targeted crop which was not possible through traditional breeding methods. This genetic information is coded in the form of DNA or genes. Genes from any living organisms (human, animal, plant and microorganism) could be easily manipulated

and transferred into other organisms to enhance their value. Organisms artificially modified at genome level using genetic engineering tools are termed as genetically modified organisms (GMOs). Microorganisms, i.e., bacteria and viruses have been genetically modified for the production of different kinds of medicines, pharmaceuticals and food ingredients [2]. Genetic engineering also has a great role in the field of agriculture by developing the transgenic crops for various traits. For example, a useful gene from bacteria, fungi and animals etc. could be isolated, cloned and integrated into desired crop to develop resistance against diseases and pests, drought and salinity tolerance or to improve the quality related traits etc. and are known GM crops [3]. After transformation, the transgenes replicate with indigenous plant genes and produce specific protein [4]. Biotechnology supports in practical exploitation of genetic material for the betterment of mankind. By using latest trends in genetic engineering one can create the new face of existing cultivars with improved and desirable characteristics. In addition to the improvement of agronomic traits, scientists are also looking in the production and expression of commercially valuable protein in plants like spider silk protein and polymers used in surgery [5]. A huge number of human vaccines, antigens and other pharmaceutical products are very efficiently expressing in transgenic plants. GMO offer many benefits to humans, but at the same time people also worry about the possible threats of using GMOs. These risks include the possible introduction of allergens in GM foods and transfer of selection marker genes which are normally antibiotic resistant genes to gut flora [6–8].

With the introduction of foreign genes, there are also some biosafety issues linked with GM crops. Such crops are often unintentionally or intentionally used for food and feed production. In some conditions, GM crops spread globally by trading, transportation and storage either intentionally or unintentionally and contaminate GM free items. Many countries have very strict rules and regulations for the development, cultivation, commercialization and labeling of GM crops and is also a trade barrier in some situations [9]. For example, USA has an optional labeling of GM in food items, whereas European Union has very strict rules for approval, cultivation and use of GM crops, including a compulsory labeling system [10]. They require very comprehensive information about such crops like type of targeted crop and transgene, safety for humans, environment, animals and effects on other related non-modified crops [11–13]. The increase in GM crop production has been coupled with an intricate and asynchronous international regulatory approval system, requiring identification and testing of food and agricultural products for the presence of GM content to simplify international trade. Molecular identification of GM crops confirms the identity and type of modified product at each stage and assures compliance with import for GM food and feed [14]. The testing of GM crops could be performed in open field or under controlled laboratory conditions that depends upon type of samples and sensitivity of test performed. Normally, molecular identification and testing of GM crops is performed at three different stages, i.e., DNA, RNA and protein. Each testing level has its own importance in testing the nature and type of GM crops. Generalized GM development methodology, global status, testing methods, possible biosafety issues and other benefits etc. are discussed in brief.

2. Global scenario of GM crops

The rapid acceptance of GM crops shows the significant benefits realized by large and small growers in both developed and under-developed countries growing GM crops commercially. Around 99% of global GM crops area is occupied by four major crops, i.e., soybean, maize, cotton and canola. USA is leading in the area under GM crops with 75.0 million hectares followed by Brazil and Argentina with 50.2 and 23.6 million hectares, respectively. In 2017, 24 countries planted 189.8

million hectares with an increase of 3.0% than 2016 [15]. Despite the possible health risks, cultivation area of GM crops is regularly increasing and introduction of new GM crops is continued. There are 29 different crops and fruit trees in 42, which countries have been successfully modified for various traits. A brief detail of GM crops, targeted/GM traits, number of GM events with responsible transgenes has been given in **Table 1**. Among GM trait distribution, herbicide

Sr. #	GM crops	GM events	GM traits	Transgenes
1	Alfalfa	05	Herbicide tolerant, Modified Product Quality	*cp4 epsps (aroA:CP4), ccomt (inverted repeat)*
2	Apple	03	Modified Product Quality	*PGAS PPO suppression gene*
3	Argentine Canola (*Brassica napus*)	41	Modified Product Quality, herbicide tolerant, Pollination control system	*te, gat4621, pat, cp4 epsps (aroA:CP4), goxv247, bar, barnase, barstar, phyA, bxn, Lackl-delta12D, Picpa-omega-3D, Micpu-delta-6D, Pyrco-delta-6E, Pavsa-delta-5D, Pyrco-delta-5E, Pavsa-delta-4D,*
4	Bean	01	Disease resistance	*ac1 (sense and antisense)*
5	Carnation	19	Herbicide tolerance , Modified flower color, Delayed ripening/senescence	*dfr, hfl (f3′5′h), surB, bp40 (f3′5′h), dfr-diaca, cytb5, acc (truncated)*
6	Flax	01	Herbicide tolerance	*als*
7	Maize	231	Male sterility , Fertility restoration , Modified alpha amylase , Herbicide tolerance, Insect resistance, Phytase production, Modified amino acid, Increased Ear Biomass, Drought stress tolerance,	*ms45, zm-aa1, amy797E, cry1Ab, pat, mepsps, mcry3A, ecry3.1Ab, cry1Fa2, cry1F, cry34Ab1, cry35Ab1, vip3Aa20, cp4 epsps (aroA:CP4), cry3Bb1, aad-1 , dam, zm-hra, gat4621 , vip3A(a), cry2Ab2, cry1A.105 , phyA2 , bar , cry9C, pinII, cry1Ac, 2mepsps , cordapA, goxv247,athb17 , dvsnf7, dmo, cspB, barnase, mocry1F epsps grg23ace5*
8	Cotton	60	Herbicide tolerance, insect resistance	*S4-HrA, pat, cry1Ac, cry1F, vip3A(a), aad-12, cp4 epsps (aroA:CP4), bar, bxn, cry2Ab2, cry2Ae, 2mepsps, cry1Ab-Ac cry1C, dmo, CpTI,*
9	Potato	48	insect resistance , Modified starch/carbohydrate, Reduced Acrylamide Potential , Reduced Black Spot, Viral disease resistance, Fungal Disease Resistance, Herbicide Tolerance	*cry3A, gbss (antisense fragment), asn1, ppo5, pvy_cp, plrv_orf1, plrv_orf2, cp4 epsps (aroA:CP4), Rpi-vnt1*
10	Linseed	01	herbicide tolerance	*als*
11	Eucalyptus	01	Volumetric Wood Increase	*cel1*
12	Eggplant	01	insect resistance	*Cry1Ac*
13	Creeping Bentgrass	01	Herbicide tolerance	*cp4 epsps (aroA:CP4)*
14	Chicory	03	Herbicide Tolerance, Male sterility	*bar, barnase*
15	Soybean	40	Modified Product Quality, Herbicide Tolerance, Insect resistance,	*gm-fad2-1 (silencing locus), pat, csr1-2, 2mepsps, aad-12, cp4 epsps (aroA:CP4), cry1Ac, cry1F, gm-fad2-1 (partial sequence), gat4601, gm-hra, hppdPF W336, Hahb-4, fatb1-A (sense and antisense segments), fad2-1A (sense and antisense), dmo, bbx32, cry1A.105, cry2Ab2, Pj.D6D, Nc.Fad3, avhppd-03, bar*
16	Poplar	02	Insect Resistance	*cry1Ac, API*
17	Plum	01	Viral disease resistance	*ppv_cp*
18	Petunia	01	Modified Product Quality	*chsA*
19	Papaya	02	Viral disease resistance	*prsv_rep, prsv_cp*
20	Melon	02	Delayed ripening/senescence	*sam-k*
21	Rose	02	Modified Product Quality	*5AT, bp40(f3′5′h)*
22	Rice	08	Modified Product Quality, Insect Resistance, Herbicide Tolerance	*crt1, psy1, 7crp,cry1Ab, cry1Ac, bar, cry1Ab (truncated)*
23	Squash	02	Viral disease resistance	*cmv_cp, zymv_cp, wmv_cp*
24	Sugar beet	03	Herbicide tolerance,	*cp4 epsps (aroA:CP4), goxv247, pat*
25	Sugarcane	04	Insect resistance, Drought stress tolerance	*cry1Ab, EcBetA, RmBetA*
26	Sweet pepper	01	Viral disease resistance	*cmv_cp*
27	Tobacco	01	Nicotine reduction	*NtQPT1 (antisense)*
28	Tomato	11	Delayed ripening/senescence , Insect resistance, Delayed fruit softening, Modified Product Quality, Viral disease resistance	*acc (truncated), sam-k, cry1Ac, accd, pg (sense or antisense), anti-efe, cmv_cp*
29	Wheat	01	Herbicide tolerant	*cp4 epsps (aroA:CP4)*

Table 1.
Summary of GM crops with modified traits and introduced transgenes.

tolerance (HT) enjoys the top position with 47% of the GM crops area. Stacked traits and insect resistance (IR) occupy 41 and 12% of the cultivated area of GM crops in 2017, respectively. The cultivation area under stacked traits, i.e., HT/IR is increasing very fast and various stacked gene products were got approved for food/feed and general commercialization. Soybean, maize and cotton are major crops developed with stacked traits [16]. Countries approving GM crops for food, feed and general cultivation are also increasing every year. In year 2017, 18 countries issued 176 approvals regarding GM crops cultivation, commercialization and use as food/feed [15].

3. GM crop's development methodology

Plants, in which one or more foreign genes are introduced artificially instead of plant getting them under natural conditions of cross-breeding or normal recombination, are known as GM plants. The introduced gene, known as transgene, could be from identical species or from different species within the same kingdom or other kingdom [17]. The process of introducing the transgene is called as genetic transformation that has become an important tool for crop improvement. Different steps are involved in the genetic transformation work like selection and identification of gene of interest (transgene), isolation from source organisms, cloning into suitable plasmid vector. Followed by development of expression vector containing all regulatory elements, i.e., promoters and terminators for regulation of transgene expression in targeted plants [18]. In addition, another gene cassette of selection is also the part of expression vector which serves as the primary selection of putative transgenic cells on artificial plant media. Normally two types of selection markers are used, antibiotic and visual selection markers, which depend upon the type of work. Final expression cassette is multiplied in suitable bacterial media and verified using various molecular biology techniques before transformation [19]. Integration of final expression cassette into plant can normally be achieved by two methods: (i) direct DNA delivery system, i.e., using biolistic gene gun by coating DNA on gold or tungsten particles and shooting on plant tissue with a specific pressure of helium gas (ii) introduction of gene by using biological vectors like disarmed Ti-plasmid of *Agrobacterium tumefaciens*. Both methods have successfully been used for the introduction of transgenes in plants [20]. Following genetic transformation, the transformed tissues are initially screened for transgene integration using selective plant tissue culture media. The regenerated plantlets on selective media supposed to have the transgenes and called as putative transgenics. Because there are three possibilities that the developed plantlets may be (i)

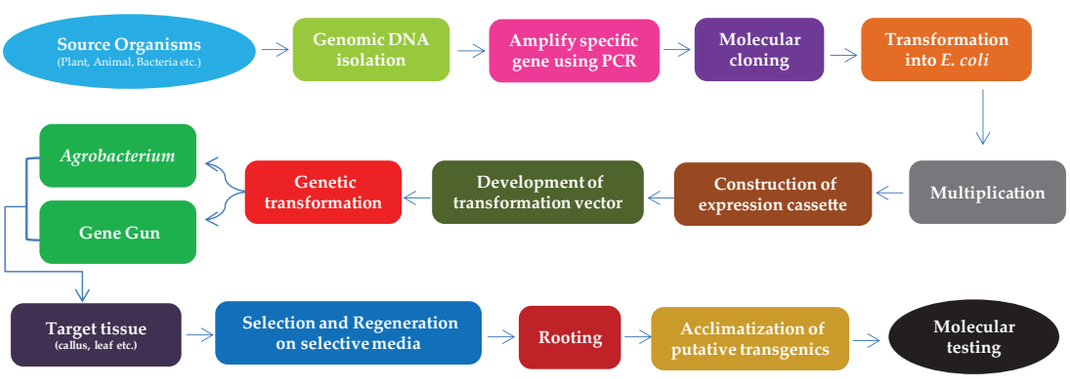

Figure 1.
General methodology for the development of genetically modified (GM) plants.

true transgenics (ii) escapees (iii) mutants. Hence, various molecular biological techniques like PCR, blotting, *E*nzyme-*l*inked *I*mmuno*S*orbent *A*ssay (ELISA) etc. are used to confirm transgene integration and true transgenics. The overall methodology for gene isolation, cloning, transformation and selection of putative transgenics has been shown in **Figure 1**.

4. Molecular test methods for the identification of GM crops

Introduction of GM crops and their products in markets required to be monitored and need to know the presence and type of GM elements. Labeling rules and trade requirements vary from country to country which necessitates for the development of reliable methods for the detection, identification and quantification of GM crop varieties and their products. GM crops can be tested by identifying either transgenes at DNA level, at transcriptional level by mRNA of transgene or using resulting transprotein. There are many other methods like chromatography and mass spectrometry etc. which have their own importance in GMO testing. An overview of test methods used for detection and identification for GM crops has been given in **Figure 2**. Every test method has its own significance and value towards the final conclusion of GM crops. A brief summary of these methods has been shown in **Table 2**.

4.1 DNA based test methods

There are three main types of DNA based GMO testing methods.

4.1.1 Qualitative PCR

Polymerase Chain Reaction (PCR) is a primary method for screening of GM crops at DNA level. Qualitative analysis comprises of specific detection of target DNA sequence in test samples. Qualitative results clearly validate the presence or absence of GM elements under study, comparative to suitable controls and within the detection limits of analytical technique used, and test portion analyzed [21, 22]. This method has found very broad and wide applications in GMO detection as commonly accepted tool for regulatory purposes. In this method target gene/GM element multiplied to millions or billions by using gene specific primers. PCR process is basically comprised on three main steps, i.e., denaturation, annealing and extension in one cycle. In first step the double stranded DNA is separated into two single strands, primers then identify their homologous sequence and are annealed to each strand in second step. Third and final step involves making two identical copies of original DNA strand by adding exact nucleotides with the help of DNA polymerase at an appropriate temperature. These cycles repeated normally 40–50 times which results in an exponential amplification of target DNA/gene. Amplification of target gene occur in-vitro through a reaction catalyzed by a DNA polymerase in the presence of oligonucleotide primers and deoxyribonucleoside triphosphates in a defined reaction buffer [23, 24]. This amplified DNA can be visualized by using gel electrophoresis techniques. The results of this method will be either positive or negative for specific GM elements.

There are four testing methods which includes (i) Target-taxon specific (ii) Screening (iii) Construct-specific and (iv) Event-specific, these methods are generally used for the detection and identification of GM crops using PCR. Selection of specific and suitable primers is the most critical step in GMO detection which depends upon the testing method used. Brief detail of qualitative PCR based testing methods is given below:

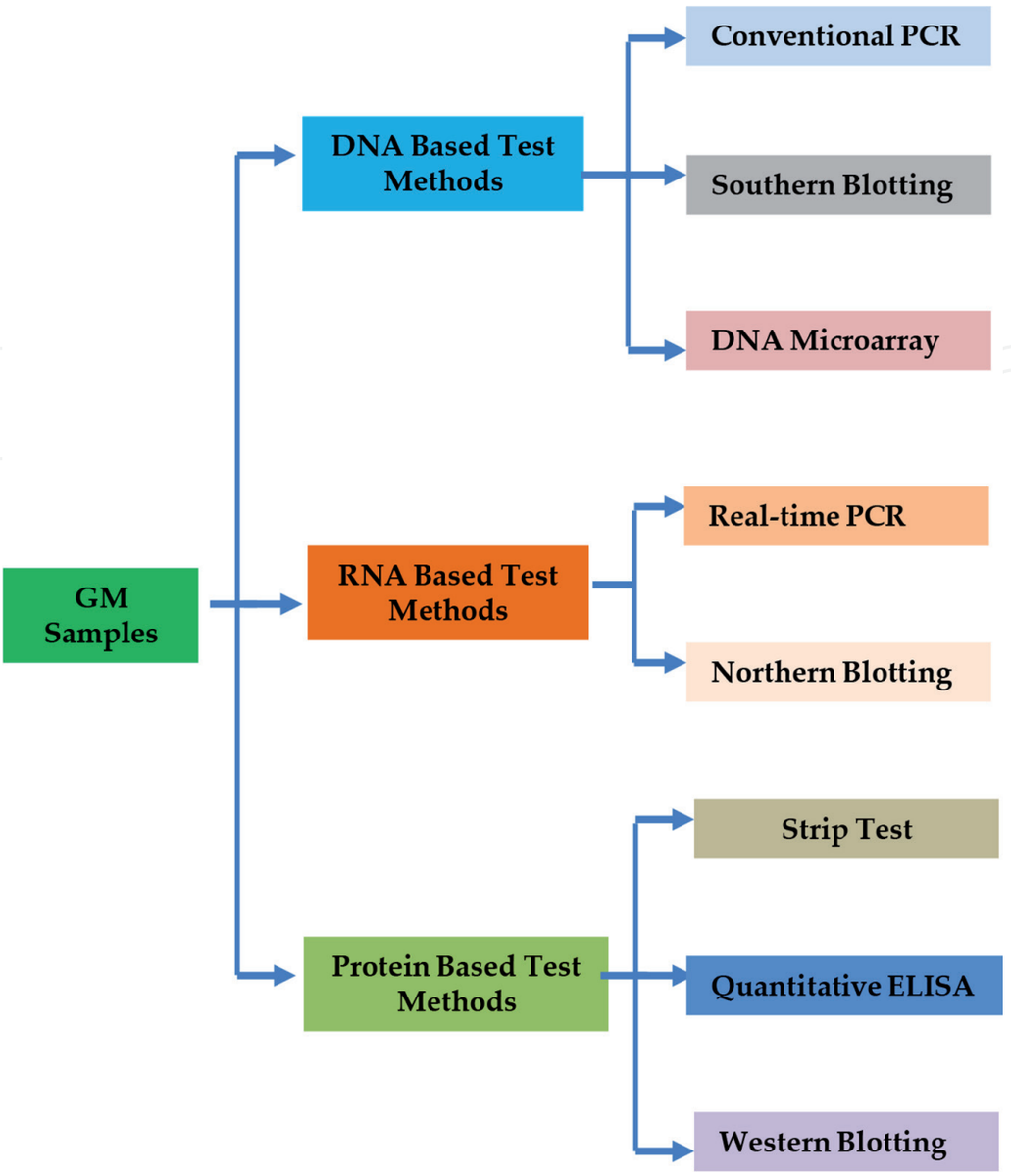

Figure 2.
Diagrammatic presentation of molecular test methods of GM crops.

4.1.1.1 Target-taxon specific method

PCR with various barcoding methods normally used for plant identification from mixed food samples particularly prepared from different plants. DNA barcode is broadly used technique for the detection and identification plants, animals or fungi texa by sequencing an optimized short DNA fragment. PCR and barcoding approaches identify specific texa very intelligently within samples of different origins [25–27]. This approach also plays very important role in the detection of mislabeled species and accidental or intentional species exchanges in food samples [28, 29]. The success of this method for identification and detection of species depends on the selected loci, because DNA barcode constitute a small portion of genome coupled with other PCR limiting factors, no single locus has been selected as universal DNA barcode region for all plant identification. For example lectin gene Le1 for soybean [30], chloroplast trnL intron for the identification of multicopy

Test methods	Test Name	Testing sample	Sensitivity /limit of detection	Conditions required	Advantages/ disadvantages
DNA based	Conventional PCR	DNA from Leaf/seed/processed food/feed items etc.	Highly sensitive/ 0.01%	Laboratory	Easy, broad range, used worldwide for GMO analysis
	Southern blotting		Highly sensitive		Require more time, sophisticated equipment/expertise,
	DNA Microarray		Highly sensitive		expensive, not used frequently
RNA based	Quantitative Real-time PCR	RNA converted in to cDNA before used in PCR	Highly sensitive	Laboratory	Broad range, allow actual quantification, require sophisticated equipment/expertise, expensive
	Northern blotting	RNA	Highly sensitive		Require more time, sophisticated equipment/expertise, expensive, not used frequently
Protein based	Strip test	Protein	Low sensitive/ 0.1-1.0%	May be perform in field	Easy, quick, cheap, not suitable for processed food
	Qualitative ELISA		Highly sensitive/ 0.01-0.1%	Laboratory	Not suitable for processed food, require expertise, expensive, results may vary from person-to-person and lab –to-lab of same sample
	Western blotting		Highly sensitive		Require more time, sophisticated equipment/expertise, expensive, not used frequently

Table 2.
Brief summary of GMO test methods.

DNA sequences in plants [31], polygalacturonase gene (PG gene) codes for a PG-enzyme that is linked with ripening in GM Zeneca tomato etc. [32].

4.1.1.2 Screening method

This is a most generalized method and widely used for the screening of GM crops from non-GM materials. This is not crop specific and can detect the GM elements even in raw and processed matrices like food and feed products developed from GM crops. In this method promoter, terminator and selection marker genes are the target elements in PCR. These are the bacterial gene sequences used to regulate the transgenes and selection of transgenic cells on artificial plant media [33, 34]. These genetic elements include cauliflower mosaic virus CaMV 35S promoter, *Agrobacterium tumifaciens* nopaline syhnthase NOS terminator and neomycin phosphotransferase NPTII etc. present in most commercialized GM crops in market. Hence, one can easily detect and identify the presence of GM crop by using specific primers of these genetic elements in PCR [35, 36].

4.1.1.3 Construct-specific method

In this method specific primer pairs normally got designed from the transformed gene construct. These construct could be transformed more than one crop for genetic improvement. The construct-specific detection method involves targeting the junction between two elements, and it is not able to distinguish two different events transformed with the same plasmid [37]. These methods either DNA or protein based. For practical reasons, several DNA sequences are shared by many GM crops and protein based methods detect the product of inserted DNA like Bt toxin in GM crops. Since different GM crops may produce the identical protein, this test method can detect a sample for several GMOs in one step. For examples GTS-40-3-2 GM construct for the development of roundup ready soybean, Zeneca F282 GM tomato, Bt11, Bt176 and T25 for GM maize etc.

4.1.1.4 Event-specific method

The junction sequences in the transgene integration points in the plant genome can be used to identify and detect the specific transformation event. The transgene integration site usually unique and specific for each transformation event due to lack of homologous recombination. Hence, different GM crops could be produced with similar gene construct and this event-specific detection method will be the only approach to differentiate between GM crops having similar transgenic cassette. This method can distinguish legitimate transgenic events from related unauthorized genotypes/varieties having identical transgene construct, thus this approach frequently used to assess the legality of GM crops [24]. Examples are Mon-531 event for Bollgard cotton, Mon-1445 event for Roundup Ready cotton, Mon-89,034 event for YieldGard VT Pro maize etc.

4.1.2 Southern blotting

Another DNA based GM crops identification techniques is southern blotting which was described by Southern in 1975 [38]. This test method is frequently used for the identification of specific DNA fragments transformed into the genome of transgenic plants or its products. This method could also be used in gene discovery and mapping, evolution and developmental studies, diagnostics and forensics etc. This test method involved five steps (i) DNA isolation and enzyme restriction (ii) electrophoresis for DNA separation (iii) shifting and fixing of separated DNA on suitable membrane (iv) hybridization with labeled probe and (v) detection by chemiluminescence or radioactive methods. This is very reliable method that provides the molecular evidence of the transgene integration and also estimates the copy number of introduced gene into the GMO genome. In comparison with PCR, this method associated with some limitations like it requires large amount of DNA, expensive, requires more time, proper infrastructure and trained manpower etc.

4.1.3 DNA microarray

A microarray is a laboratory method used to identify the expression of more than one gene in a single test. It is DNA based and new in comparison to previous protocols. This test method has been included in GMO screening as a method for simultaneous detection of more than 250,000 targets in single assay/chip [39–41]. This method consists of pre-amplification step of the desired targets, followed by hybridization on a chip having specific probes, and then detection step [42, 43]. So far, it is used for qualitative information of GMO, sometime semi-quantitative.

Use of microarray technology for the GMO detection is restrained as it require very special and costly equipment for scanning microarrays, chances of cross contamination and laborious in comparison with other techniques.

4.2 RNA based test methods

Transgenic DNA must be translated into protein to be an effective and have effects in an organism. This translation process occurs when DNA transcribed into messenger RNA (mRNA), and is considered as the intermediate step transferring information contained in DNA to protein. The presence of mRNA is directly associated with gene expression. Different molecular biology techniques used to monitor and study the gene expression in GMOs include real-time PCR, northern etc. These methods could be used to identify the transgene expression in various plant tissues and at different developmental phases in GMOs.

4.2.1 Quantitative real-time PCR

Gene expression normally verified in RT-PCR using isolated mRNA from GMOs. This test method is based on reverse transcription of mRNA and synthesis of complementary DNA (cDNA) which is then used as template in PCR amplification of target gene. The amplified fragment electrophoresed and visualized using agarose gel under UV. Intensity of amplified band in agarose gel give some indications of target mRNA in tested sample [44]. Quantitative RT-PCR is an up-to-date method, principally based on RT-PCR and is generally known as qRT-PCR. It is more robust, specific and sensitive, provides good quantitative results. The process of amplification is presented in real-time by capturing a fluorescent signal in more sophisticated way. In real-time assay of transgene in GMOs, the amplification and detection occur simultaneously [45].

4.2.2 Northern blotting

Similar to RT-PCR, northern blotting also requires mRNA as tested material from GMOs. This is a standard method for the analysis of size and level of target RNA in a complex GMO samples. Likewise southern blotting, it also composed on five steps, only difference is that the starting material is mRNA instead DNA and the labeled probe is complementary DNA (cDNA), which hybridizes the RNA. It gives comparative amount of gene expression at the RNA level. This is comparatively simple to perform, cheap and not overwhelmed by artifacts [46]. Recent advancements of hybridization membranes and buffers have resulted in increased sensitivity, closing the gap to the more laborious nuclease protection experiments. It is considered that this test method can study gene expression for a limited number of genes per analysis. This can be very useful to monitor the up- or down regulation of transgene for specific problem, but is not useful in monitoring the up- or down regulated genes are unknown.

4.3 Protein based test methods

Immunoassay protocols for the detection of GMOs by antibodies are the impressive for the detection of various types of proteins either qualitatively or quantitatively [47]. Two types of antibodies, i.e., monoclonal and polyclonal could be used depending on the need and specificity of detection method. Normally, *E*nzyme-*l*inked *I*mmuno*S*orbent *A*ssay (ELISA) and western blot methods have been used for the protein analysis in GMOs.

4.3.1 Qualitative strip test

Most common antibody based test for GMO screening is strip test method also known as lateral flow or dipstick test. It is qualitative in nature and gives the information about the presence or absence of specific proteins in tested samples. In this method, thin strip made-up of nitrocellulose membrane used which protected by a sample pad on one end and a wicking pad on other end. Test samples normally homogenized in suitable buffer solutions and membrane on strip wicks up the solution and it will move upward via capillary movement and protein will bind to its specific antibody. The results shown in the form of visible lines on the strip depicting that the specific protein is present in test sample. There are normally two lines appears on the strip, one for tested protein and second of control line showing the authenticity of all test procedure and strip used. The appearance of only control line on the strip, shows that sample is negative for transgenic protein, but the test was performed accurately [48]. This is very quick method to test GMOs which normally take 5–15 minutes to gives results [49]. In addition, it is cheap, easy to perform and not require specific equipment and special trained manpower. It can be performed in open field as well. Currently, strips are available to detect multiple proteins in single assay [50].

4.3.2 Quantitative ELISA test

Another more sensitive antibody-based protein identification method is *E*nzyme-*l*inked *I*mmuno*S*orbent *A*ssay (ELISA) also called a plate test or quantitative ELISA. It gives information about the quantity of protein in tested samples. In this assay protein specific antibody coated multi-well plate is used to identify and quantify the specific protein. Specific protein present will bind to antibody, following washing, another antibody specific for protein of interest and tagged with an enzyme is added to well [51]. The enzyme linked identification antibody will bind with specific protein and unbound antibody removed by washing. The color of the solution will change from blue to yellow by the addition of substrate for enzyme. Intensity of yellow color is directly proportional to amount of protein present in well. This GMO test method is more sensitive in comparison with strip test and can detect target protein even in very low concentrations. However, it requires more time, trained manpower and good laboratory facilities in contrast to strip test.

4.3.3 Western blotting

This is very specific method and provides the qualitative results of the target protein in GM crop sample. This method is very useful to analyze the insoluble proteins [47, 50]. Like other blotting techniques samples are solubilized with detergents and reducing agents and separated by electrophoresis and shifted to membrane. Binding immunoglobulin sites on membrane are blocked by dried nonfat milk and specific sites are probed with antibodies. Detection carried out using different staining agents silver nitrate of Coomassie, alkaline phosphatase etc. [18]. Its detection limit varies with test ample like 0.25% for seeds and 1.0% for toasted meal [52]. In comparison with other protein based assays, it is difficult method, and is capable of studying only a few samples at a time. Therefore, it is not frequently used in GMO testing activities but it is more used in research purpose to verify initial results generated by other testing method.

5. Certified/standard reference materials for GMO testing

Validity and authenticity of GMO testing results is doubtful until the use of positive and negative controls at each testing step. Use of certified reference material (CRM) or standard reference material (SRM) during testing produce not only validate the testing results but at the same time, assess the performance of test method, equipment, personnel and other environmental conditions in which testing being performed [52]. CRM must contain the certificate of analysis, should be prepared by following ISO-Guide 34, have information about which GM events or elements present and what is its concentration, storage requirements, preparation and expiry date etc. While SRM have all the similar information but lacks the certificate of analysis and was not prepared by a certified company. Bothe the CRM and SRM could be used to validate the testing results but CRM is more reliable and globally acceptable. Each GMO needs specific CRM which is used in testing and conclusion about the presence of specific GM event/element in testing samples. Normally seeds of GM and Non-GM crops are mixed at specific percentage and homogenized to make powder before analysis [51]. The availability of CRM is presently restricted due to some concerns of IPR and expenses [53]. The Institute of Reference Materials and Measurements at the Joint Research Center (JRC) in Geel, Belgium, FAPAS Fera Science Ltd., Sand Hutton UK, American Oil Chemists Society (AOCS), Urbana, Illinois, USA etc. are authorized companies to paper and sale of CRMs for GMO testing.

6. Pros and cons of GM crops

The most common improvement by the introduction of GM crops is the increase in yield and quality. There are many yield limiting factors like insect/pest, diseases, drought, heat, salinity, rapidly changing climatic conditions etc. Conventional approaches like irrigations, sprays and use of fertilizers etc. done a great job but the problem was increasing day-by-day. By the introduction of recombinant DNA technology in agricultural sector, scientists successfully develop the new face of existing cultivars with improved and desirable traits. The GM technologies increase the opportunities for plant breeders to develop crops that are protected from climatic stresses and attacks of insects and diseases [54, 55]. The crops have been successfully modified for herbicide tolerant, insect/pest resistance, disease resistance, abiotic stress tolerance, micronutrient enrichment etc. Furthermore, this technology helping us to improve the nutritional quality, longer shelf life, foods that are more appealing to eat and easier to transport. Development of various biopharmaceuticals and expression of human therapeutic proteins in plants also a great contribution of GM technology to improve the human life [56].

On the other hand there are also some biosafety issues linked with the use of GM crops. Biosafety means the need to protect human and animal health from possible adverse effects of GM technology. There are some reports about the potential threats linked with the use of GMOs like risks of allergineicity, development of herbicide tolerant weeds and resistant insects, harms to non-target organisms, selection marker gene could induce antibiotic resistant and reduce the effectiveness of antibiotics to cure disease etc. [7, 57–58]. Turning on of certain genes due to the use of strong promoters and might be harmful in humans, effects on the nutritional profiling, transgene may flow from non-target crops/weeds etc. Biosafety is an essential to modern biotechnology and the adoption of biotech products requires to be balanced with acceptable biosafety safeguards. Participation of different

stakeholders and dissemination of information and knowledge in public about GM products is much important to safe use of this technology.

7. National scenario of GM crops, biosafety, labeling and trade aspects

Agriculture sector of Pakistan plays a dominant role in the economy with 18.9% contribution in Gross Domestic Product (GDP) and engages 42.3% labour force. It is also a chief source of foreign exchange earnings and provide raw material for progress of other sectors [59]. Pakistan stands at seventh position among 26 countries growing GM crops, and insect resistant GM cotton of Mon-531 event is the only crop grown in the country with adoption reaching 2.9 million hectares of total 3.0 million hectares cotton crop area. Mon-531 is the only approved commercialized GM event in the country having insecticidal *Cry1Ac* gene of *Bacillus thurengiiensis* to control the lepidopteron insects. In 2015, US$398 million economic gain was estimated with the adoption of GM cotton [60]. Moreover, field trials of GM maize hybrids have successfully been conducted for single and stacked insect resistant (IR) and herbicide tolerances (HT) traits. For single HT trait, Monsanto event NK-603 was tested, while for stacked traits, i.e., IR/HT, Mon-89,034 x NK-603, TC-1507 x Mon-810 x NK-603 and TC-1507 x NK-603 were studied. These GM traits were officially approved for commercial cultivation by National Biosafety Committee in 2016. Field performance trials were completed as the part of regulatory requirements and varietal registration by Federal Seed Certification and Registration Committee of National Food Security and Research ministry [15]. In near future, GM maize having IR and HT traits will be grown by farmers, and it will be the second approved GM crop in the country.

Pakistan is signatory to World Trade Organization (WTO) and has sanctioned Convention on Biological Diversity (CBD) in 1994 and Cartagena Protocols in 2009. Different legislations under the Agreement of Trade Related Aspects of Intellectual Property Rights have been disseminated in the country. In addition, Pakistan Biosafety rules were designed in 2005, which are responsible for safe use of GM technology, manufacturing, import and storage of GMOs. Following these, National Biosafety Guidelines were developed in which the procedures to undertake all linked activities to GMO work were highlighted. These guidelines were framed in accordance with the recommendations of Food and Agriculture Organization (FAO), World Health Organization (WHO), United Nations Industrial Development Organization (UNIDO) and United Nations Environment Programme (UNEP). As per these guidelines the biosafety aspects of GMO work are monitored at three different levels, i.e., Institutional Biosafety Committee (IBC), Technical Advisory Committee (TAC) and National Biosafety Committee (NBC). The applications related to GMO work are submitted to IBC, and after thorough evaluation, the case is submitted to TAC for assessment and recommendations, while NBC is the final body to take further action regarding its approval or rejection. NBC is responsible to looks after the laboratory research, field studies, commercial release, imports, exports and sale/purchase of GMOs and their products [61].

Pakistan exports rice, cotton, fruits (oranges and mangoes), vegetables and fish to its neighboring states, Middle East and Central Asian countries. IR cotton of Mon-531 event is the only one GM crop officially approved for general cultivation in the country. Very comprehensive testing procedures are adopted to test and verify the status of approved events in the candidate cotton varieties. Around 49 universities and 07 research institutes are actively involved in the teaching and research related to the development and testing of GM crops in the country [62]. In Pakistan, Agricultural Biotechnology Research Institute (ABRI) at Ayub Agricultural Research Institute (AARI), Faisalabad and SGS Laboratories Karachi have GMO testing labs, which have been accredited by Pakistan National Accreditation Council

(PNAC) for ISO-17025. These labs are efficiently working on the testing and identification of GM crops and are equipped with state-of-the-art facilities needed for the detection, identification and quantification of GMOs. All crop seeds being imported from other countries are first tested for the presence and type of GM elements from these laboratories and then allowed for cultivation in the country. All the import and export activities are strictly monitored with reference to GMOs.

8. Conclusion

Testing of GM crops is important issue for the legitimacy, biosafety and regulatory purposes. The area under GM crops is increasing very rapidly and many new genes are being introduced in major crops. For the safety of humans, environment, animals and other related micro-flora, a comprehensive molecular testing of newly developed GMO is very important before commercial release. Regulatory processes for GM crops approval need comprehensive risk analysis for each case separately. The detection and identification of GMOs is also of great value in identifying the purity of sample, labeling food and trade reasons. Therefore, combined use of more than one testing methods would be advantageous for complete analysis, authenticity and biosafety assessment of GM samples.

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