

Challenges in detecting GM crops

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Abstract

GM crops represent the state of art technologies in modern Agriculture. The benefits of GM crops have been harvested all over the world to increase productivity while reducing dependence on chemical-intensive farming methods. However, since the technology has enormous impact on agriculture, concerns have been raised from time to time on the need for a careful assessment of biosafety, profitability and sustainability of the eco-systems in which the technology forms an important component. Detection of GM crops is one of the most important prerequisites to address biosafety concerns. However, considering the fact that new genes are being discovered and deployed in GM crops all the time in almost all over the world, development of GM crop diagnostic kits has emerged as a challenging task. It is extremely important to detect GM-crop material that would have been introduced intentionally or inadvertently into the country apart from monitoring the spread of transgenes in the environment and ensuring quality control and genetic purity of legal transgenic seed.

Introduction

The ability to introduce desirable genes into plants has enabled the genetic manipulation of crop plants to modify them to our advantage. Genetic transformation has now become a routine technique for several plant species. While several economically important traits are being conferred to crop plants through genetic engineering, resistance to insects, viruses and drought have been the most widely considered traits. The recent advances made in transgenic research over the past decade and the advent of insect resistant transgenic (genetically modified) crops, have opened up exciting possibilities, new areas of research and new avenues especially in eco-sustainable pest management.

Over the past decade GM crops have changed the pest management scenario world over. Out of 306 transgenic events developed so far in 45 crops, 105 events have been released for commercial cultivation globally until April 2006. Biosafety of all GM crops is now a universal concern. Post-release detection and monitoring of the spread of transgenes in the environment is a significant challenge. The deliberate or inadvertent mixing of GM products with non-GM products carries the risk of adversely affecting international seed trade and is incompatible with biosafety norms. Detection of unapproved transgenic events and unapproved transgenic varieties in the environment is important from biosafety perspective. Additionally, quality control and genetic purity of legal transgenic seed is an important concern. Reliable, efficient and cost-effective techniques for detection, identification and quantification of GMOs are essential to ensure that bio-safety concerns are efficiently addressed. Currently, there is no single, rapid and inexpensive test available

to verify whether a crop or crop sample is free of transgenic traits. Technology for reliable detection of unapproved events and unapproved varieties is not as yet available anywhere in the world. Therefore specific initiatives are imperative to address the challenge of developing appropriate diagnostic tools to ensure biosafety.

How many transgenes?

Thus far nearly 105 events of 20 GM crops have been released for commercial cultivation in 21 countries. Additionally, about 201 events in 45 crops have been reported from public funded institutions of 15 countries, and are in various stages of testing (Cohen, 2005). Of the 152 events examined, 133 events were related only to insect pest (51) and disease (82) management. However, amongst the commercially released GM crops, cotton, soybean, maize and canola were main crops to occupy nearly 99% of the total GM area. In 2004, 56% of the 86 M ha of soybean; 28% of the 32 m ha cotton; 14% of the 140 m ha maize and 19% of the 23 m ha canola were genetically modified. In 2005, the global area under biotech crops reached 90 m hectares. Nearly 70% of the total global transgenic crop area was herbicide tolerant. Rest was insect resistant, mainly Bt. The global area under Bt cotton alone was 8.5 m hectares in 2005. At least nine Bt-Cry-toxins have been released globally till date. Thus, clearly Bt Cry toxins were the main source of insect resistance for the development of insect resistant transgenic crops. Genes from the soil bacterium *Bacillus thuringiensis* that encode insecticidal crystal proteins and genes that code for inhibitors of insect gut proteases have been used so far for the generation of cotton transgenics. Over 406 genes of the 179 holotypes that encode the Cry toxins have now been sequenced and enable the toxins to be assigned to more than 50 groups on the basis of sequence similarities. Currently, about 21 insecticidal crystal (Cry) proteins are being actively pursued for their potential in plant protection.

Three groups of genes appear to be of sufficiently robust utility and suitability for wide use. They are the insect specific Cry genes from *Bacillus thuringiensis*, coat protein of plant viruses and genes conferring herbicide tolerance. Fifty-one events for insect resistance have been reported of which 35 events represent Bt, 5 represent the snowdrop lectin, 4 the pin gene, 2 events for trypsin inhibitors, and 2 events for Bt and trypsin inhibitor together. Apart from these we have 1 event each for the alpha amylase inhibitor and one for gall midge resistance. The top ten crops used for GM are rice, potato, maize, papaya, tomato, cotton, soybeans, wheat and alfalfa. Thus we have unique crop and trait combination available today. Eight phenotypic categories are being used to improve agronomic properties, bacterial, insect, fungal, viral resistance, herbicide tolerance and product quality. Of the seven Asian countries involved, India is privileged to be working with all the eight traits in 11 crops. China on the other hand is concentrating on only 4 traits namely, agronomic properties, fungal, insect and viral resistance in 10 crops. Of the percentage of different phenotypic groups among the 201 transformation events, over half involve single genes that confer biotic resistance either to viral or insect stresses to the host plant. Most countries are focusing on genes already available and have already been characterized, but a few are also investing in their own gene discovery such as South Africa, Malaysia, Brazil, India and China.

In India, eleven crops (cotton, corn, brinjal, cabbage, cauliflower, ground nut, mustard, okra, pigeonpea, rice and tomato) have been genetically transformed for enhanced resistance to insects and viruses and are in various stages of testing. Six Cry (crystal) genes (*cryIAa*, *cryIAb*, *cryIAc*, *cryIF*, *cryIB*, *cry2Ab*) and *vip-3A* gene from *Bacillus thuringiensis* were used for insect resistance in nine crops. The Government of India approved Bt-cotton, in 2002 as the first transgenic crop for commercial cultivation. Since then, the total area under Bt-cotton increased from an initial 32,000 hectares in 2002 to 38 lakh hectares in 2006. Recently two more Cry1Ac events, developed in India and China, were released.

Current issues

The key issues confronting farmers, regulators and all stakeholders are as follows:

1. Clandestine import and sale of unapproved Bt-cotton and other transgenic crops. Moreover, counterfeit, duplicate and fake Bt-cotton products are also available in the market.
2. Post-release detection and monitoring of the spread of transgenes in the environment is a significant challenge. Moreover it is important to ensure that the transgenic product available in the market is the same transgenic-event that was tested for biosafety approval; contains the same genes as those tested for biosafety approval and is genetically the same seed/plant variety that was for biosafety approval
3. Quality control and genetic purity of legal transgenic seed is an important concern.
4. Researchers need simple and rapid techniques to detect transgene integration into the host genome. Such tools are not available for many genes and gene products.
5. Detection of unapproved transgenic events and unapproved transgenic varieties in the environment is important from biosafety perspective. Technology for reliable detection of unapproved events and unapproved varieties is not as yet available in the country. How do we assess for the presence of unreported trans-genes in consumable products imported intentionally or introduced unintentionally? Methods to detect and identify GMOs that were released inadvertently into the environment or imported unintentionally are not available
6. The deliberate or inadvertent mixing of GM seed with non-GM seed lots carries the risk of adversely affecting international seed trade and is incompatible with biosafety norms. Reliable, efficient and cost-effective techniques for detection, identification and quantification of GMOs in non-GM seed lots are not available as yet. Currently, there is no single, rapid and inexpensive test available to verify whether a crop or crop sample is free of transgenic traits.
7. Permissible thresholds of GMOs in non-GM seed lots are not defined as yet.

8. Quarantine personnel need to establish whether an incoming/ outgoing grain lot has any transgenic trait or not.

There is a need to develop simple cost effective methods to assist farmers in the detection of transgenic purity of the product before they use the seed for sowing. Apart from assisting farmers, the GMO detection kits will help regulators and quarantine personnel to detect and track down the spread of approved, unapproved and unintentionally released GMOs in the environment.

Global status of research on diagnostics

Detection of transgenic products has been carried so far using techniques such as PCR (Polymerase chain reaction), ELISA (Enzyme linked immunosorbent assay) and immuno lateral flow assays, which emerged as the methods of choice all over the world. Other rapidly emerging methods include mass spectrophotometry, NIR, microfabricated devices, Real Time PCR, DNA chip technology and nanoscale GMO analysis.

For routine PCR based screening purposes 'generic' markers representing genetic control elements such as cauliflower mosaic virus 35S promoter (P-35-S), neomycin phospho transferase-II (*npt-II*) and the *Agrobacterium tumefaciens* nos terminator (nos3') are used as these are present in majority of GMOs currently being cultivated commercially. Degenerate primers (Hemmer, 1997) have also been designed to detect the 8 variants of P-35-S and are being used routinely to detect GMOs. However, these tests identify GMOs but not the specific trait.

Immunological techniques such as ELISA and immunochromatography (dip-sticks) have now become indispensable tools for the detection of GMOs. Dumbroff *et al.*, (1993), Bindler *et al.*, (1999) and Brett *et al.*, (1999) described immunological methods to detect transgene expression in GMOs. Several immunoassay-based methods, mainly ELISA, have so far been developed that are specific for gene products widely expressed in transgenic plants such as the *npt-II* gene product, the enzyme 5-pyruvylshikimate-3 phosphate synthase (EPSPS), the *Bacillus thuringiensis* (Bt) insecticides Cry1Ab, Cry1Ac, Cry1C, Cry3A, Cry2A, Cry9C, CP4-EPSPS and herbicide-tolerance phosphinothrin acetyl transferase (PAT) protein (Rogan *et al.*, 1999, Wood *et al.*, 1995, McKenzie *et al.*, 2000). ELISA methods offer a high degree of automation and sample throughput. Field variants of this technique, such as the lateral flow strips or dip-stick kits, have been recently developed and offer a semi quantitative test of considerable practical value for field testing with simple laboratory set up (Stave 1999, Anklam *et al.*, 2002). Some of these kits are also available commercially in the developed countries and can be imported through Envirologix Inc., & Strategic Diagnostic USA. However, they are expensive and specific for specific transgenic traits.

Status of Indian research on diagnostics

Research on development of farmer usable transgenic diagnostic kits is just beginning in India. Several testing methods (Bt-Quant ELISA kit, Bt-PCR-zygosity test, Bt-Express

Lateral flow strips kit and Bt-detect dot-blot kit) were developed at the Central Institute for Cotton Research (CICR), Nagpur to assist regulators, farmers, seed industry and researchers in identifying Bt-cotton. The institute has an Indian patent on the technology and is credited with being the first from within the ICAR system to file an international patent in seven countries. The Cry1Ac detection kits were commercialized in 2002 and have become very popular with all the stakeholders of the technology and thus far more than 10,00,000 strips have been used by various stakeholders for quality control to ensure transgene seed purity. Amongst these, farmer usable diagnostic kits were developed at the institute and have been regarded widely as the most significant contributions to the regulated use of transgenic crops in India. Kranthi *et al.*, (2005) used the ELISA kits to quantify Cry1Ac in Indian varieties of Bt-cotton.

The Central Institute for Cotton Research was recognized as the 'Bt-referral laboratory' for purposes of testing. Amongst the (more than 1000) unapproved brands of seed samples tested until December 2006, only 30-32% were found to be genuine F-1 seed packets, whereas the rest were either non-Bt or F-2 seeds or mixtures of variable proportions of Bt seeds in non-Bt packets. From the results it was estimated that at least 55-60% of the unapproved seeds did not contain any Cry toxin in them.

Diagnostic tools: research gaps in India

- a. There is an imminent need for indigenous diagnostic methods/kits for a range of transgenic crops based on proteins such as Cry1Ab, Cry1Ac, Cry1Aa, Cry1F, Cry1B, Cry2Ab and Vip-3A, that will be released shortly for commercial cultivation in India. Such kits are unavailable in the country. Only a few kits that were developed for specific transgene products are available in the USA. Cry2Ab was released recently and thus far there appear to be no efforts anywhere in the country to develop detection kits and the relevant guidelines for quality regulation.
- b. There is a need to develop GMO detection kits based on generic markers and specific transgene inserts to ensure an adequate level of protection in the field of safe transfer, handling and use of LMOs/GMOs taking also into account risks to human health, and specifically focusing on trans-boundary movement. India is a party to the Cartagena Protocol on Biosafety under the Convention on Biological Diversity (CBD), which is an attempt to establish a globally harmonized regime for biosafety especially enabling countries to regulate trans-boundary movement of transgenes. Currently, GMO detection kits to enable regulate trans-boundary movement are unavailable in the country. Technology for reliable detection of unapproved events and unapproved varieties is not as yet available in the country.
- c. Empowering farmers with 'on-the-spot' test kits to detect transgenic seed purity will enable improvement in the overall quality seed availability in India. Quality control of inputs, especially seeds, is a serious issue in India. Farmer usable diagnostic kits are not available in India, except the ones developed by CICR, Nagpur for Cry1Ac detection. Reliable and economic kits are essential to ensure a quality check at all levels by all the stakeholders, including farmers.

- d. Seed testing laboratories require simple, but robust test methods, which can be used as quality checks to test transgenic seed purity for all the events approved by the GEAC.

Diagnostic kits: approach to fill research gaps

World over, GMOs are detected reliably either by detecting DNA segments or specific proteins that are unique to the transgenic crop. DNA detection is primarily through PCR-based methods and protein detection is mainly through immunoassays such as enzyme-linked immunosorbent assay (ELISA) and lateral flow strip methods. PCR is a powerful method, but requires considerable technical skills and expertise, apart from being expensive for conventional seed testing. ELISA is suited for qualitative as well as quantitative detection proteins and can be used as a high-through-put test for simultaneous handling of a large number of samples in routine testing as ELISA. The lateral flow strip (dipstick format) is based on immuno-chromatography. It is simple and reliable. The test can be conducted on-the-spot by even illiterate farmers by crushing seeds or plant tissues with the extraction solution in plastic vials provided with the kit and by dipping the lateral flow strips into the sample thus prepared. Results are obtained within 5-10 minutes and provide sensitive and accurate qualitative (presence or absence) results. Ideally, a single detection test is needed to verify the presence/ absence of GMOs in a sample irrespective of the crop and transgenic trait. Majority of the commercially available GMOs contain following marker genes: 1. npt-II (Neomycin phosphotransferase); 2. hpt (Hygromycin phosphotransferase); 3. pat (Phosphinothricin-N-acetyl transferase); 4. bar (Bialophos resistance); 5. uidA (B-D-Glucuronidase)-reporter and 6. ipt (Isopentenyl transferase). Methods can be developed to detect each of these independently and also to detect all these in a single test using ELISA/ dip-stick method/ PCR. Dip-stick method, detecting all these markers using a single strip, would be very helpful to regulatory authorities and ELISA would be highly useful for quarantine personnel to test the material being imported and exported. Immunoassays can be used for screening and PCR for confirmation.

Research approaches must include identification of important transgene elements that will be used as candidate molecules for the development, validation, commercialisation and dissemination of PCR based DNA kits, and antiserum based ELISA and lateral flow assay kits.

1. Database must be developed to enlist all genes, markers, promoters, traits and crops that have been released for commercial cultivation in India and elsewhere in the world. The database must include genes, markers, promoters, traits and crops that are under active consideration in transgenic research and are likely to be released soon for commercial cultivation.
2. Generic markers and the most commonly used trait conferring genes and promoters must be short-listed to be used for detection methods.
3. Locus specific tests must be designed for all the approved transgenic events based on sequences flanking transgene inserts.

4. Degenerate primers must be designed to develop PCR methods to detect GMOs. The primers should be tested on positive and negative DNA samples isolated from the available transgenic crops/putative samples to establish their reliability and limits of detection.
5. New methods must be developed to design lateral flow strips that can detect DNA from plant samples 'on-the-spot' without having to isolate DNA or carry out PCR and electrophoresis.
6. A universal lateral flow strip must be designed to enable the detection of any of the most commonly cultivated GMOs, to be used at port of entry for quarantine purposes.
7. The methods and kits must be validated through many test centers, to validate accuracy, reliability and robustness. Standard quantifiable benchmark parameters for specificity, sensitivity, repeatability, reproducibility, limits-of-detection, overall accuracy and robustness (includes product stability and user friendliness) of transgenic detection kits for validated regulatory testing purposes should be developed through repeated testing at all participating centers.

Diagnostic kits to detect insect resistance to the transgenes

Nearly 30% of the area under GM crops is covered by insect resistant transgenics. Toxins from *Bacillus thuringiensis* form the main source of transgenes in almost all the insect resistant transgenic crops that are under commercial cultivation. The cotton bollworm *Helicoverpa armigera* (Hübner) is one of the main target pests of the Bt technology in India. It feeds on at least 181 plant species and is a major pest of cotton, redgram, chickpea, tomato, cabbage, cauliflower etc. all of which are being transformed with Bt-toxin genes. The bollworm has a history of developing resistance to almost all the insecticides used for its control. Cry1Ac is the most toxic of the *Bacillus thuringiensis* insecticidal proteins to *H. armigera*. Laboratory experiments to select for resistance in India, Australia and China have shown that *H. armigera* is capable of developing resistance to Cry1Ac. Bt cotton expresses the Cry1Ac toxin in all parts of the plant throughout the growth period. The pest would thus be exposed to a continuous selection pressure, thereby causing resistance in field populations. Resistance represents an ecological hazard. It is important to ensure that the technology, which represents the state of art of pest management, remains effective in controlling target pests for the longest possible time. It is possible to develop simple diagnostic kits to detect resistant individuals in field populations. Such research should be encouraged so as to facilitate diagnose the problem at its initial stage and initiate appropriate measures to delay resistance.

Immunodiagnostic kits

Based on the existing literature the following farmer usable lateral flow immunoassay 'on-the-spot' test kits (Dip-sticks) and ELISA quantification kits must be developed on priority before the transgenic crops incorporating these genes are released for commercial cultivation.

Immunological tests (ELISA and Dip-stick kits) must be developed to detect and quantify npt-II (neomycin phospho transferase II), GUS (glucuronidase), EPSPS (5-enoylpyruvyl shikimate-3-phosphate synthase), PAT (neomycin phospho transferase II), ALS (acetolactate synthase), HPT (hygromycin phospho transferase), IPT (isopentenyl transferase), AAD (Aminoglycoside adenyl transferase), *Bacillus thuringiensis* toxins such as Cry1Aa, Cry1Ab, Cry1B, Cry1C, Cry1F, Cry2A, Cry3A, Cry3B, Cry34Ab, Cry35Av, Cry9C, Vip3A, protease inhibitor, viral CP (coat protein), chitinases and glucanases. Additionally it would be very useful to develop multiplex universal dip-stick/ELISA to detect GMO.

Molecular diagnostics

DNA based PCR methods can help in detecting transgenes that may or may not express in the crop and do not constitute the economically important trait. Several promoters, enhancers, reporters and marker genes fall in this category. It is important to devise reliable PCR methods to detect such genes along with the main transgenes that confer the quality trait.

PCR based kits must be developed to detect the presence of *P-35S* (Cauliflower mosaic virus 35S promoter), *E9 3'* (3' sequence of small subunit of rbcS E9 pea gene), *NOS 3'* (NOS terminator sequence from *A. tumefaciens*) *P-NOS* (Nopaline synthase) *npt-II* (neomycin phospho transferase II) *als* (acetolactate synthase gene) *aad A* (Aminoglycoside adenyl transferase) *pat/bar* (phosphinothricin acetyltransferase) *bxn* (bromoxynil nitrilase) *uid A* *gus* (glucuronidase) *ipt* (isopentenyl transferase) *hpt* (hygromycin phosphotransferase) *epsps* (5-enoylpyruvyl shikimate-3-phosphate synthase) *Bacillus thuringiensis* toxin genes such as *cry1Aa*, *cry1Ab*, *cry1B*, *cry1C*, *cry1F*, *cry2A*, *cry3A*, *cry3B*, *cry34Ab*, *cry35Av*, *cry9C*, *vip3A*, protease inhibitor, viral *CP* (coat protein), *chitinases* and *glucanases*. Additionally it would be very useful to develop multiplex universal PCR test to detect any GMO.

Human Capacity building efforts

GM detection kits would be extremely valuable for farmers, regulating agencies, seed industry, researchers, administrators and all other stakeholders of the technology. The technologies will help the country build up its capacity to be a strong partner in the Cartagena Protocol Convention. Apart from strengthening the regulatory systems, the kits will assist in tracing labeled and unlabelled transgenic products introduced unintentionally or otherwise into the environment in the country.

It is important to form a strong human resource that can assist in safe guarding public safety with reference to GM crops while ensuring that misapprehensions if any, are eliminated. Seed testing inspectors, referral lab personnel, quarantine officers, researchers Seed industry personnel and regulatory authorities must be trained in the use of diagnostic kits and their applications.

It must be remembered that GM crops may not pose any immediate threat to the environment and unintended non-target organisms, but can emerge as strong technologies that can have a significant impact on ecology and eco-systems that include human beings and the non-target organisms. Therefore it is important to be able to stringently assess the GM products for their short term and long term potential impact on biosafety and the environment before they are introduced, and to evaluate their direct impact on the environment after they are introduced for general public use. Diagnostic methods, especially consumer usable simple diagnostic kits will be immensely valuable in fulfilling such objectives.

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