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Strategies to monitor the adventitious presence of transgenes in *ex situ* collections

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ABSTRACT

With the advent of genetic engineering technologies enabling transfer of foreign genes in plants, immense benefits have been drawn in terms of increased yield, better nutritional quality and resistance to biotic and abiotic factors. With such immense benefits, there is also the need to undertake risk assessment studies systematically to allay the fears about the impact of GM crops on the genetic diversity. National Bureau of Plant Genetic Resources (NBPGR) is the nodal institute for management of indigenous and exotic plant genetic resources for food and agriculture and to carry out related research. National Genebank at NBPGR conserves more than 377 000 germplasm accessions of field and horticultural crops and their wild relatives. As the main purpose of Genebanks is to collect, conserve and make genetic resources available to the breeders, so the maintenance of the purity of genetic identity of the accessions is of critical importance. All possible efforts need to be made to prevent the adventitious presence of transgenes into the samples conserved in the Genebanks. To achieve this, most appropriate and effective strategies to monitor the unintentional presence of transgenes in *ex situ* collections are required to be put in place.

Key words: Adventitious presence, Biodiversity, *Ex situ* collections, Genebank, PCR-based detection

Revolutionary advances in plant biotechnology over the past decade have enabled the plant breeders to overcome several barriers associated with conventional hybridization making the entire global gene pool of plants, animals and microorganisms accessible for crop improvement. Biotechnology holds immense potential for agriculture in terms of increased yield, better nutritional quality and resistance to biotic and abiotic stresses and reduced post-harvest losses. The first genetically modified (GM) crop released in 1994 for commercial production was the *FlavrSavr* tomato in the United States. Thereafter, the global area under GM crops has continued to grow steadily over the last decade touching 134 million ha in 2009, from merely 1.7 million ha in 1996. Forty six per cent of this area, i e 61.5 million ha is being grown in the 16 developing countries (Clives, 2009), showing that the immense potential of this technology for enhancing crop productivity as well as quality has been accepted worldwide. In India, *Bt* cotton was the first GM crop commercialized in 2002. By 2009, 618 hybrids of 6 events, i e MON 531, MON 15985, GFM-cry1A, Event-1, 9124 event and 1 variety of Dharwad Event (*Bt Bikaneri Nerma*) of *Bt* cotton were commercially released. Out of the 6 events commercialized, 4 events MON 531, MON 15985 and GFM-cry1A and 9124 event were imported, whereas the other 2 events were indigenously developed, i e Event-1 developed at Indian Institute of Technology, Kharagpur using indigenous *cry1Ac* gene and commercialized by J K Agrigenetics Ltd, Dharwad Event developed by UAS Dharwad (Karihaloo and Kumar 2009 and Randhawa and Chhabra 2009a). GM crops with more than 2 dozen events, are also being developed and are at different stages of field testing in India (Table 1) (IGMORIS 2008).

India has rich genetic diversity in cultivated crops and their wild relatives, and is one of the 12 Mega-diversity centres and one of the Vavilovian Centres of Origin of crop plants. About 29% of flowering plants occurring in India are endemic and 2 of the 25 hot spots of biodiversity exist here. With immense potential benefits of GM crops, the impact of introduction of GM crops on the genetic diversity of crop landraces and wild relatives in areas of crop origin and diversification may be of concern, if systematic risk assessment and management studies are not undertaken.

NATIONAL REGULATORY MECHANISM

In India, the regulatory mechanism for the development, evaluation and release of GM crops has evolved over the

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Table 1 Genetically modified crops under field trials in India, 2008

Crop	Developer organization	Transgene/event
Brinjal	Indian Agricultural Research Institute, New Delhi	*cry1Aabc*
	Sungro Seeds Ltd., New Delhi	*cry1Ac*
	Maharashtra Hybrid Seeds Company, Jalna	*cry1Ac*
	Tamilnadu Agricultural University, Coimbatore	*cry1Ac*
	University of Agricultural Sciences, Dharwad	*cry1Ac*
	Bejo Sheetal, Jalna	*cry1Fa1*
Cabbage	Nunhems, Gurgaon	*cry1Ba and cry1Ca*
	Sungro Seeds Ltd., New Delhi	*cry1Ac*
Castor	Directorate of Oilseeds Research, Hyderabad	*cry1Aa and cry1Ec*
Cauliflower	Sungro Seeds Ltd., New Delhi	*cry1Ac*
	Nunhems, Gurgaon	*cry1Ac, cry1Ba and cry1Ca*
Corn	Monsanto, Mumbai	MON89034 and NK603
Groundnut	International Crops Research Institute of Semi Arid Tropics, Hyderabad	Rice *chit and DREB*
Okra	Maharashtra Hybrid Seeds Company, Mumbai	*cry1Ac*
	Sungro Seeds Ltd., New Delhi	*cry1Ac*
	Bejo Sheetal, Jalna	*cry1Ac*
	Arya Seeds, Gurgaon	*CP-AVP1*
Potato	Central Potato Research Institute, Shimla	*RB*
	National Institute of Plant Genome Research, New Delhi	*AmA1*
Rice	Indian Agricultural Research Institute, New Delhi	*cry1Aabc, DREB, GR-1 & GR-2* (Golden Rice)
	Tamil Nadu Agricultural University, Coimbatore	*chi11*
	MS Swaminathan Research Foundation, Chennai	*MnSOD*
	Directorate of Rice Research, Hyderabad	*cry1Ac*
	Maharashtra Hybrid Seeds Company, Mumbai	*cry1Ac, cry2Ab*
	Bayer Crop Sciences, Hyderabad	*cry1Ca, cry1Ab, bar*
	Avesthagen, Bengaluru	*NAD9*
Tomato	Indian Agricultural Research Institute, New Delhi	Antisense *replicase, osmotin, DREB*
	Maharashtra Hybrid Seeds Company, Mumbai	*cry1Ac*
	Avesthagen, Bengaluru	*NAD9*

Source: Indian GMO Research Information System (IGMORIS), 2008

last decade at a fairly fast pace. The Ministry of Environment and Forests enacted the Environment Protection Act (EPA), 1986 for regulation of matters related to protection and improvement of environment. The rules under the Act cover all areas of research including the release of genetically modified organisms (GMOs) and related products. A coordination mechanism based on interaction between various committees and different departments of Government of India has been established for commercialization of indigenously developed GM crops (Fig 1). Some of the important committees vested with powers and responsibilities to regulate different aspects are: The Recombinant DNA Advisory Committee (RDAC); Institutional Biosafety Committee (IBSC); Review Committee on Genetic Manipulation (RCGM); Genetic Engineering Approval Committee (GEAC); State Biotechnology Coordination Committee (SBCC) and District Level Committees (DLC). GEAC is the apex committee for authorizing release of GMOs and products thereof into the environment, while RCGM regulates the research work at laboratory level and also at small-scale field trials of transgenic crops and directs generation of data on biosafety. IBSC is responsible for compliance of guidelines at the Institute level (GOI 1990). Revised guidelines for research in transgenic plants and guidelines for testing toxicity and allergenicity of transgenic seeds, plants and plant parts (GOI 1998) issued by the Department of Biotechnology (DBT) are already in force. The Government of India has also amended relevant Acts and Rules such as the Protection of Plant Varieties and Farmer's Rights (2001) and the National Seed Policy (2002) to incorporate and provide for the required regulatory provisions relating to transgenics. The Environment Protection Act (EPA) (GOI 1989) has vested upon NBPGR, the authorization for issue of permits (on the technical clearance of RCGM, DBT) for import of transgenic planting material for research purposes.

ROLE OF NATIONAL BUREAU OF PLANT GENETIC RESOURCES

The National Bureau of Plant Genetic Resources (NBPGR), under the aegis of Indian Council of Agricultural Research (ICAR), is the nodal institute at the national level

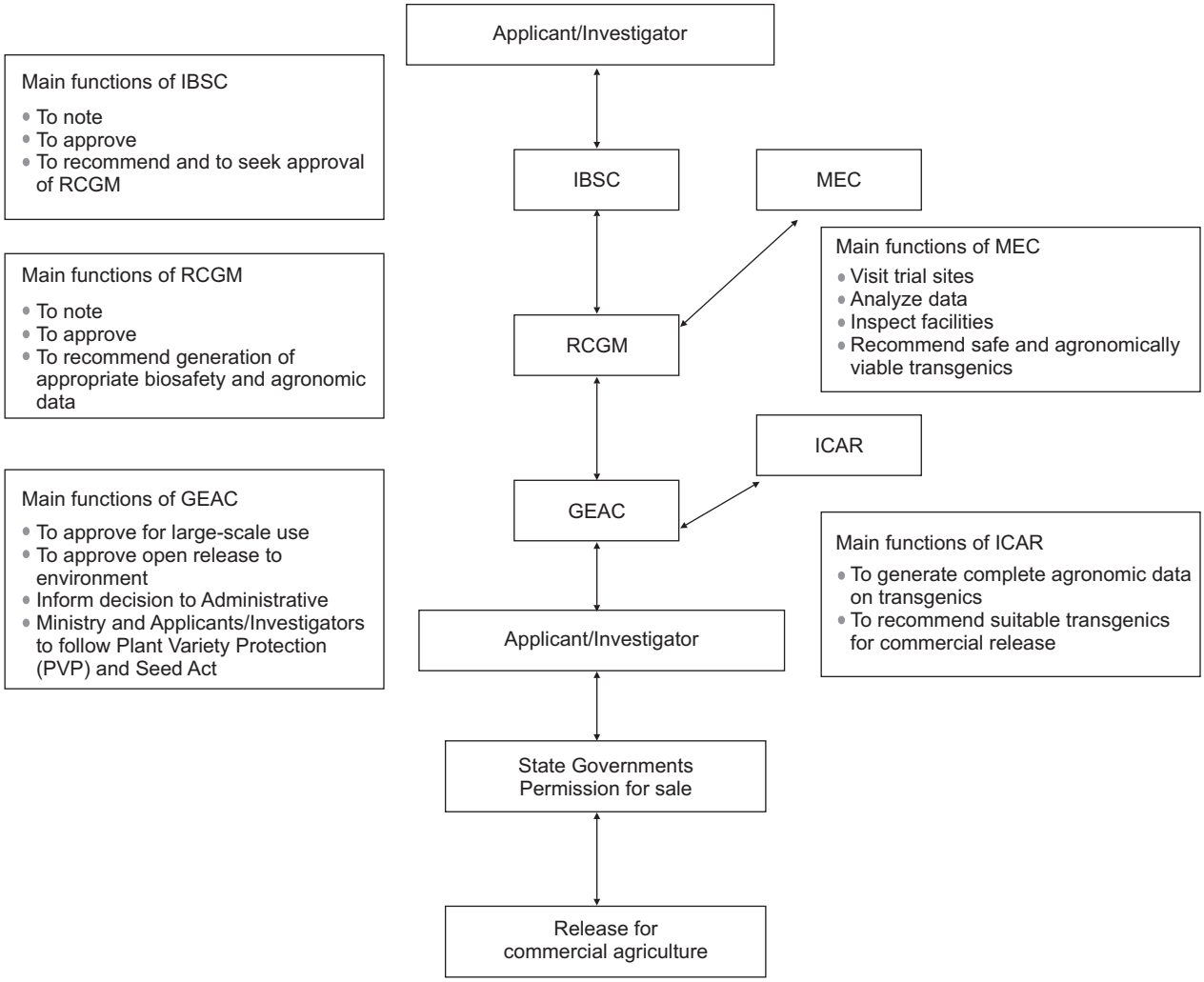

Fig 1 Procedures involved in commercialization of indigenously developed transgenic crops
Source: Sharma *et al.* 2003

for management of indigenous and exotic plant genetic resources for food and agriculture and to carry out related research and human resources development for sustainable growth of agriculture. National Genebank at NBPGR holds more than 377 000 germplasm accessions of field and horticultural crops and their wild relatives, which are conserved, under seed bank as base collection. In addition, cryobank is conserving more than 9 200 accessions and *in vitro* bank also conserves more than 2 000 accessions. The crop germplasm management system at the national level in India has NBPGR at its apex with National Active Germplasm Sites (NAGS) across the country, which comprises ICAR Institutes, Project Directorates, National Research Centres, All India Coordinated Research Projects and State Agricultural Universities.

NBPGR and the Department of Biotechnology, Government of India have jointly established the National Containment-cum-Quarantine Facility of CL 4, the highest level for testing transgenic planting material. A GM detection laboratory has also been established, to ensure the absence of terminator technology (embryogenesis deactivator gene) in the imported transgenic planting materials, which is mandatory as per the legislation; the seeds of all the imported transgenic lines are being tested using polymerase chain reaction (PCR) analysis with the primers for *cre-lox* system and plasmid cloned with *cre* sequence used as positive control. The imported transgenic lines are regularly tested for the specific transgenes/promoters/marker genes. So far, 85 consignments of transgenic planting material comprising 12 crops with an array of transgenes have been imported from different countries through NBPGR for various public and private research institutions engaged in transgenic research (Randhawa and Chhabra 2009a). NBPGR has also been designated as a Referral Centre for Molecular Diagnosis of Transgenic Planting Materials by the Department of Biotechnology, Government of India.

GLOBAL INITIATIVES

International Maize and Wheat Improvement Centre (CIMMYT) has shared its expertise and advice with the Mexican institutions to help in identifying the type and source of the introduced genes. CIMMYT has formulated a project on studying gene flow in maize, which was implemented at the Oaxaca and Chipas regions of Mexico. The guidelines for maize conservation and related aspects were also prepared. The best practices to maintain genetic integrity of wheat and maize in genebanks are being followed. In this context, a workshop on 'Development of Consultative Group of International Agricultural Research (CGIAR) policies to address the possibility of adventitious presence of transgenes in CGIAR *ex situ* collections' was also organized at Rome in 2004 by the International Plant Genetic Resources Institute (IPGRI) to discuss various strategies for handling the unintentional presence of transgenes in germplasm collections. The workshop mainly aimed to evolve the technical consensus for developing an appropriate approach to handle the probability of unintentional introgression of transgenes into the *ex situ* collections in the genebanks. And also to collect and share technical and economic information associated with institutional strategies and practices for collecting, managing and distributing materials to reduce this risk, including screening for GM elements and associated costs so as to formulate an action plan to minimize the adventitious presence of transgenes.

It was emphasized that the effective germplasm management should be based on 5 major principles, i e ethics, transparency, accountability, risk analysis and quality control. As the main purpose of genebanks is to collect, conserve and make genetic resources available to the breeders, the maintenance of the purity of genetic identity of the accessions is of critical importance. Therefore, all possible efforts need to be made to prevent the unintentional introgression of transgenes, into the samples conserved in the genebanks.

Systematic germplasm management procedures and practices vary from crop to crop, in terms of its mode of pollination, breeding system, and whether the crop is annual or perennial. The available techniques and procedures do not enable the complete exclusion of unintentional presence of transgenes, in genebank accessions and therefore do not provide an absolute guarantee, without testing every single seed or plant that any given accession is free from transgenes. Only the best practices and management strategies will achieve a 'required' degree of statistical probability to the effect that an accession in the genebank does not include unintentional presence of transgenes.

Tina *et al*. (2007) conducted a study to check the adventitious presence of other varieties in oilseed rape (*Brassica napus*) from seed banks and certified seeds to obtain information on possible sources of contamination of the seed harvest of oilseed rape (*Brassica napus* L., spp *napus*) by other varieties (adventitious presence) and to investigate the purity of certified seed lots, the abundance and origin of volunteers, and longevity and origin of seeds in the soil seed bank using Inter Simple Sequence Repeat (ISSR) markers of volunteers collected in the field and seedlings derived from the soil seed bank. DNA profiles of the volunteers and seedlings were obtained, and these profiles were compared with ISSR profiles from an assortment of 14 of the most commonly cultivated oilseed rape varieties from 1985 to 2004. Results of ISSR analysis of the 14 reference varieties showed that 3 of the certified seed lots contained other varieties above the permitted threshold.

As a risk management strategy for the commercialized GM crops, it is better to use the separate combines, lorries, storage rooms, etc. for GM, conventional and organic production and careful cleaning needs to be performed.

As the area under cultivation of GM is increasing globally and in India also more than a dozen GM crops are in the pipeline for commercialization, the issue of adventitious presence has become very important to be addressed in proper perspective by having crop-wise risk assessment plan based on their biological behaviour (self-pollinated or cross pollinated if cross pollinated by undertaking gene flow studies that how far the pollen can travel).

FUTURE STRATEGIES AND THRUST AREAS

The genebanks need to take proactive steps to determine the risk of unintentional presence of transgenes, in *ex situ* collections. Information can be compiled addressing the crop-specific guidelines for best genebank management practices, covering the crop-specific risk analysis procedures (i e risk assessment, management, and communication) addressing critical specific control points.

The main genebank operations that need to be critically evaluated are collection, acquisition, regeneration, characterization, delivery, conservation, testing health and viability, evaluation and documentation. The major area of unintentional introduction of transgenes is the collection and acquisition stage since the genetic resources may have been exposed to gene flow outside the control of the genebank. The strategies therefore need to minimize the gene flow of the transgenes at these stages. As part of their risk analysis, when collecting or acquiring new accessions by other means, the genebanks should ensure the following aspects before testing:

1. Whether transgenic events (commercial or research) in the relevant taxa are likely to be present in the area of exploration/collection;
2. The distance between the collecting site and areas where transgenic events (commercial or research) is being cultivated;
3. Whether germplasm providers can give adequate documentation of their germplasm management practices with respect to the material in question.

With respect to existing accessions, genebank testing procedures need to be guided by the following criteria:

1. No testing would be required when:
   - There are no transgenic events (commercial or research) in the relevant taxa
   - There were no transgenic events (commercial or research) in the relevant taxa at the time of acquisition (e g *Bt* cotton prior to March 2002 in India)
   - There are transgenic events (commercial or research) present, however, proper management practices have been followed and documented in the management of the accession
2. Once an accession has been determined to be either not requiring testing or has tested negative, the genebank needs to follow best practices on regeneration and maintenance procedures to maintain the genetic integrity, as for all accessions.

A database on the global and national status of GM research and development for the crops needs to be maintained by the genebanks for facilitation of effective risk analysis.

ISSUES AND GAPS IN GM DETECTION

There are certain issues pertaining to the development of methods for transgene testing which need to be addressed on priority. These are as follows:

1. Self certification/disclosure from source countries or personnel
2. Obtaining gene sequences for synthesizing probes and primers from the developer of transgenic crop in case of indigenously developed transgenic crop and from the importer in case of imported transgenic crop
3. Classifying areas and crops as per the possibility of contamination: Certain hot spots, where the possibility of accidental contamination by transgenic seeds is possible have to be identified
4. Any suspected material should be subjected to molecular and grow-out tests and the whole lot discarded, if found contaminated.
5. The likelihood of contamination can be avoided by clearly defining the appropriate thresholds of introgression of transgenes for different GM crops.
6. Cost of detection: Less for protein detection in the field, though developmental cost for the kits is high; moderate, if it is for one gene and one sample in case the primer or probes of the transgenes are already available and high if several samples are to be analyzed and it is not known as to which transgene should be analyzed and fresh primers/probes need to be designed and synthesized.

PRECAUTIONS TO BE TAKEN DURING FIELD TESTING OF GM CROPS

1. The following procedures should be followed in the field in order to prevent mixture of seeds
   (i) A perimeter fallow zone depending on the pollination pattern of the crop needs to be established.
   (ii) The planting of food and feed crops in the same location in the following season should be avoided if there is a chance for volunteer seedlings to be inadvertently harvested with the following crop.
   (iii) Dedicated planting and harvesting should be arranged and storage facilities be provided.
   (iv) Personnel training programme should be organized to address these critical issues
2. Awareness at grass root level should be created with the help of already established extension network.
3. Multiple field inspections should be conducted and proper records need to be maintained.
4. There is need to formulate a set of guidelines for explorers, especially if the explorations are carried out where transgenic crops are being grown in the vicinity.
5. Capacity building for transgene detection and monitoring needs to be strengthened.
6. Stringent action should be taken against the defaulter companies/research institutes.

The role of national genebank is therefore of prime importance. Although it is difficult to make an absolute guarantee that the collection is free from adventitious presence of transgenes, but all efforts discussed above can be made to minimize the introgression of transgenes to achieve an optimal purity level.

INITIATIVES AT NATIONAL LEVEL

PCR-based methods for GM detection are imperative to ensure seed quality and to monitor for adventitious presence of transgenes in *ex situ* collections of genebanks. The expertise and capacity for PCR-based GM detection has been strengthened and upgraded significantly at NBPGR. Multiplex PCR assays simultaneously amplifying the most commonly used marker genes, i e *nptII*, *aadA, bar, pat, hpt* and *uidA* have been developed, which will be useful for initial screening of GM planting materials, irrespective of the crop and GM trait (Randhawa *et al.* 2009b). Multiplex PCR assays have been developed for simultaneous detection of c*ry1Ac* gene, *CaMV* 35S promoter and endogenous *SRK* gene in *Bt* cauliflower (Randhawa *et al.* 2008), *cry1Ab* gene for insect resistance*, CaMV* 35S promoter, *nptII* marker gene and endogenous *UGPase* gene in GM potato (Randhawa *et al.* 2009c); o*smotin* gene, *CaMV* 35S promoter and endogenous *LAT52* gene in GM tomato (Randhawa *et al.* 2009d); *AmA1* gene and *cry1Ab* gene along with validated potato-specific endogenous *ST-LS1* gene in GM potato lines with *AmA1* and *cry1Ab* genes (Randhawa *et al.* 2009e); *AmA1* gene for better

protein quality with *nptII* marker gene, 35S promoter, *nos* terminator and *UGPase* gene in GM potato (Randhawa *et al.* 2009f). Qualitative and quantitative molecular testing methodologies for *Bt* cotton, *Bt* brinjal, *Bt* rice, *Bt* cauliflower, *Bt* potato and *Bt* okra have also been developed (Randhawa *et al*. 2010). PCR-based diagnostic kits for GM crops under different stages of limited or large-scale field trials have also been developed. These diagnostic kits are reliable, sensitive, economical and efficient, as more than one target sequences can be detected in a single assay (Randhawa 2008). Since *Bt* cotton is the only commercialized GM crop in India, the screening for adventitious presence of transgenes using PCR/ real-time PCR assays in *ex situ* collections of cotton collected from the different cotton growing regions of India, conserved in National Gene Bank, NBPGR, New Delhi, is already underway.

Meticulously planned introduction and monitoring of GM crops after planning and executing all risk assessment and management stratgeties and conserving *ex situ* collections which is clear of unintentional presence of transgenes in the National genebanks would be needed to fully harness the benefits of GM crops without affecting the biodiversity.

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