Laboratory networking for safety management and knowledge sharing with special reference to Bt Cotton and Bt Brinjal

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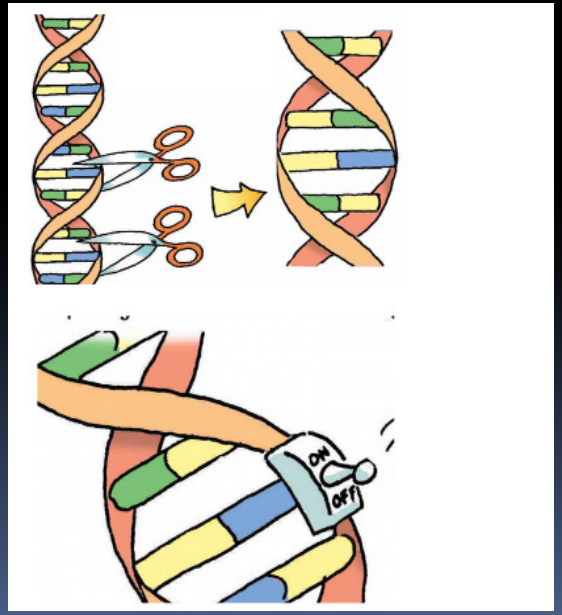
What is a Genetically modified organisms (GMO) ?

Combining genes from different organisms is known as recombinant DNA technology, and the resulting organism is said to be genetically modified , whose genetic material has been altered/modified in a manner that does not occur through natural recombination.

How?

- **Inserting a specific gene**
- **Switching a gene on or off**
- **Removing a specific gene**



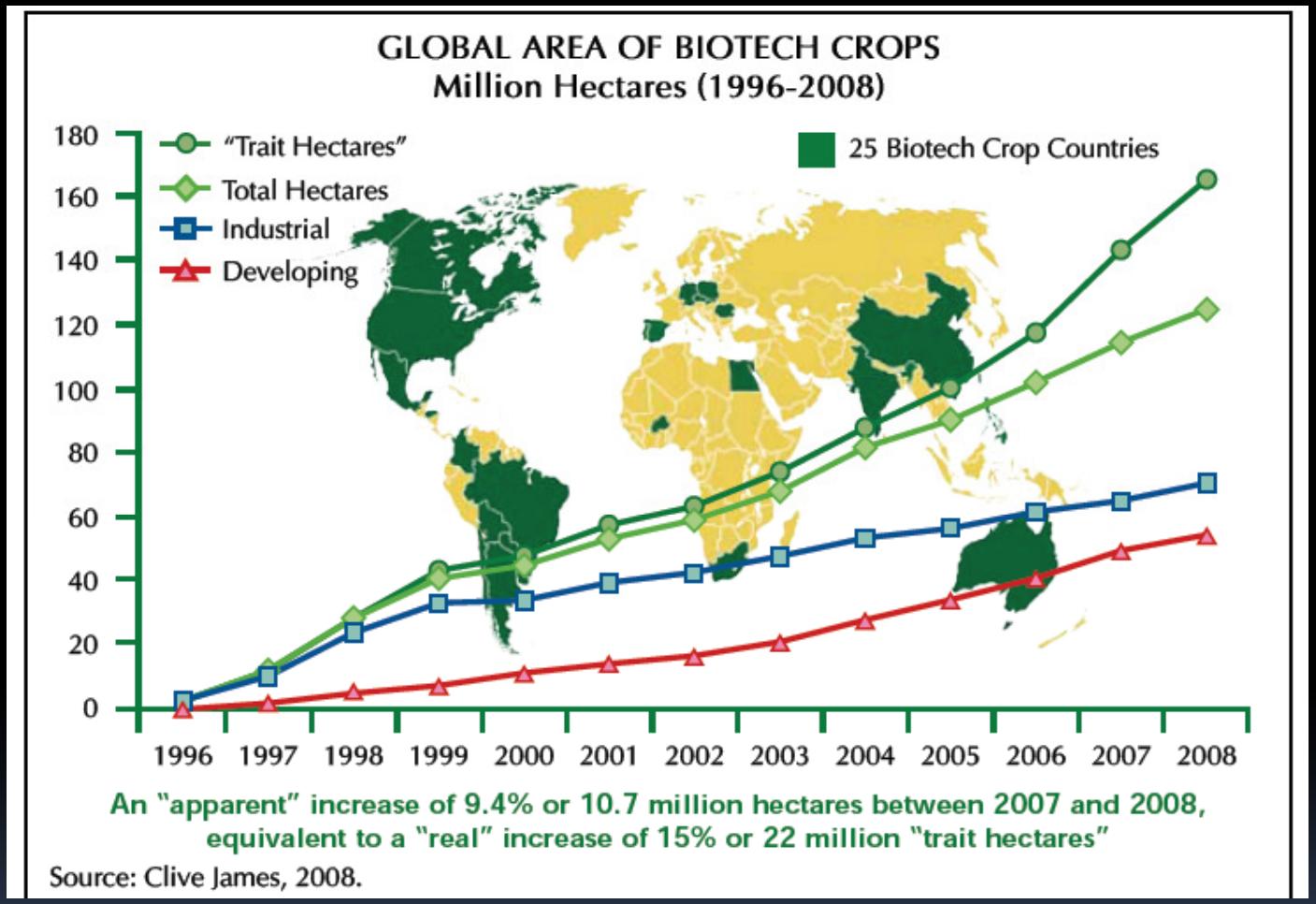
Why GMO?

- Increased world population
- Increasing global food demand
- Decreased land for cultivation
- conventional cultivation could not reach the global demand.

What is the aim of Genetic engineering technology?

- Improving productivity
- Decreasing cost of cultivation
- Increasing nutritive values of food
- Increasing shelf life

GLOBAL PERSPECTIVE



The number of countries adopting GM crops have increased steadily from 6 in 1999, to 25 in 2008.

The global planted area under GM crops has increased dramatically from 1.66 mha in 1996 to 125 mha in 2008.

Global status of GM crops

Rank	Country	Area (million hectares)	Biotech Crops
1*	USA*	62.5	Soybean, maize, cotton, canola, squash, papaya, alfalfa, sugarbeet
2*	Argentina*	21.0	Soybean, maize, cotton
3*	Brazil*	15.8	Soybean, maize, cotton
4*	India*	7.6	Cotton
5*	Canada*	7.6	Canola, maize, soybean, sugarbeet
6*	China*	3.8	Cotton, tomato, poplar, petunia, papaya, sweet pepper
7*	Paraguay*	2.7	Soybean
8*	South Africa*	1.8	Maize, soybean, cotton
9*	Uruguay*	0.7	Soybean, maize
10*	Bolivia*	0.6	Soybean
11*	Philippines*	0.4	Maize
12*	Australia*	0.2	Cotton, canola, carnation
13*	Mexico *	0.1	Cotton, soybean
14*	Spain *	0.1	Maize
15	Chile	<0.1	Maize, soybean, canola
16	Colombia	<0.1	Cotton, carnation
17	Honduras	<0.1	Maize
18	Burkina Faso	<0.1	Cotton
19	Czech Republic	<0.1	Maize
20	Romania	<0.1	Maize
21	Portugal	<0.1	Maize
22	Germany	<0.1	Maize
23	Poland	<0.1	Maize
24	Slovakia	<0.1	Maize
25	Egypt	<0.1	Maize

* 14 biotech mega-countries growing 50,000 hectares, or more, of biotech crops

Source: Clive James, 2008.

INDIAN PERSPECTIVE

List of GM crops under development and field trials in India

Institute	Crop	Transgene inserted	Aim
Central Tobacco Research Institute	Tobacco	*Bt* toxin genes	Resistance to Tobacco Caterpillar *(Spodoptera* completed; *litura)*
Central Potato Research Institute, Shimla	Potato	*Bt* toxin gene	Resistance to Potato Tuber Moth *(Phthorimaea opurculella)*
Indian Agricultural Research Institute (IARI), New Delhi	Brinjal	*Bt* toxin gene	Resistance to Shoot and Fruit Borer *(Leucinodes arbonalis)*
IARI, New Delhi	Rice	*Bt* toxin gene	Resistance to Yellow Stem Borer *(Scirpophaga incertulas)*
IARI, New Delhi	Tomato	*Bt* toxin gene	Resistance to Fruit Borer *(Helicoverpa* further *armigera)*
IARI, New Delhi	Cabbage	*Bt* toxin gene	Resistance to DiamondBack Moth *(Plutella xylostella)*
IARI, New Delhi	*Tomato*	*ACC synthase gene*	*Delayed fruit ripening*

List of GM crops under development and field trials in India

Institute	Crop	Transgene inserted	Aim
IARI, New Delhi	Brassica Juncea	*Annexin gene* from *Arabidopsis*	Tolerance to moisture stress
IARI, New Delhi	Potato	Osmotin	Tolerance to moisture stress
Directorate of Rice Research (DRR), Hyderabad	Rice	*Bt* toxin gene	Resistance to Yellow Stem Borer
Directorate of Rice Research (DRR), Hyderabad	Rice	*Chitinase* gene	Resistance to sheath blight disease
Bose Institute, Calcutta	Rice	*Bt* toxin gene	Resistance to Yellow Stem Borer
Delhi University, South Campus, Delhi	Mustard/ rapeseed	Barnase and Barstar	Pollination control for hybrid development
Jawahar Lal Nehru University, New Delhi	Potato	*Ama-1* gene from *Amaranthus*	To improve nutritional quality
Central Institute of Cotton Research, Nagpur	Cotton	*Bt* toxin gene	Resistance to lepidopteron insect pest

Need for Biosafety regulations

Consumers are concerned about the safety of GM foods

Animal and human health issues :-

- ***Allergens***
- ***Transfer of antibiotic resistance***
- ***Toxicity***
- ***Digestibility***

Environmental issues :-

- ***Unintended transfer of transgenes through cross-pollination***
- ***Emergence of new Weeds***
- ***Effect on subsoil and other 'non-targeted' organisms***
- ***Gene variability / Instability***
- ***New pests and diseases.***

Ethical issues :-

- ***Violation of natural organisms intrinsic values***
- ***Tampering with nature by mixing genes among species***
- ***Objections to consuming animal genes in plants and vice versa***

What is Biosafety?

Genetically modified organisms (GMOs)

Risks

Biosafety:
Measures to avoid risks to human, animal health and environment resulting from GMOs

National regulatory systems in India

Two nodal agencies, Ministry of Environment and forests (MoEF) and Department of Biotechnology (DBT), Ministry of science and technology are responsible for implementation of the regulations in India.

The MoEF notified the rules and procedures for the handling of genetically modified organisms (GMO) under the environment protection act 1986(EPA).

There are six competent authorities to handle various issues viz.,

1) Recombinant DNA Advisory Committee (RDAC)
2) Institutional Biosafety Committee (IBSC)
3) Review Committee on Genetic Manipulation (RCGM)
4) Genetic Engineering Approval Committee (GEAC)
5) State Biotechnology Coordination Committee (SBCC)
6) District Level Committee (DLC)

Competent authorities for GM regulations in India

COMPETENT AUTORITY	FUNCTIONS
1) **Recombinant DNA Advisory Committee (RDAC) :**	To review biotechnology developments at national and international levels; to implementation safety regulations in research and applications of GMOs and products thereof.
2) **Institutional Biosafety Committee (IBSC):**	It has mandate to approve low –risk (category I & II) experiments and to ensure adherence to r-DNA safety guidelines; It recommends category III or above experiments to Review Committee on Genetic Manipulations (RCGM).
3) **Review Committee on Genetic Manipulation (RCGM) :**	To review all ongoing projects involving high-risk (category III) and controls field experiments. It approves applications for generating research information on transgenic plants.
4) **Genetic Engineering Approval Committee (GEAC) :**	To authorize commercial use of GMOs or their products. It can authorize approval and prohibition of any GMO for import, export, transport, manufacture, processing use or sale.
5) **State Biotechnology Coordination Committee (SBCC) :**	To inspect, investigate and take punitive actions in case of violations of the statutory provisions. This committee also nominates state government representatives in the committee constituted for field inspection of GM crops.
6) **District Level Committee (DLC) :**	To monitor the safety regulations in installations engaged in the use of GMOs in research and applications. To act as nodal agency at district level to assess damage, if any, from release of GMOs and take on site control measures.

Cartagena Protocol on Biosafety

India ratified the Cartagena Protocol on Biosafety (CPB) on 23rd January 2003 and it came in to force in September 2003.

What is the Cartagena Protocol?

- A binding international agreement
- Linked to the international Convention on Biodiversity
- Covers the safe transfer, handling and use of GMOs & LMOs
- Specific focus on trans-boundary movement

Capacity Building for GMO research in India

Institutions with responsibility of developing and validating detection methods of GMO

DNA based testing:-

- **CDFD (Centre for DNA Fingerprinting and Diagnostics), Hyderabad, Andhra Pradesh**
- **CFTRI (Central Food Technological Research Institute), Mysore**
- **NBPGR (National Bureau of Plant Genetic Resources), New Delhi**

Protein based testing:-

- **ITRC (Industrial Toxicology Research Centre), Lucknow, Uttar Pradesh**
- **NIN (National Institute of Nutrition), Hyderabad, Andhra Pradesh**

Institutions with responsibility of developing and validating Safety Evaluation Methods

- **ITRC (Industrial Toxicology Research Centre), Lucknow, Uttar Pradesh**
- **IGIB (Institute of Genetics and Integrative Biology), Delhi**

Current developing & validating detection methods for GM crops at CDFD

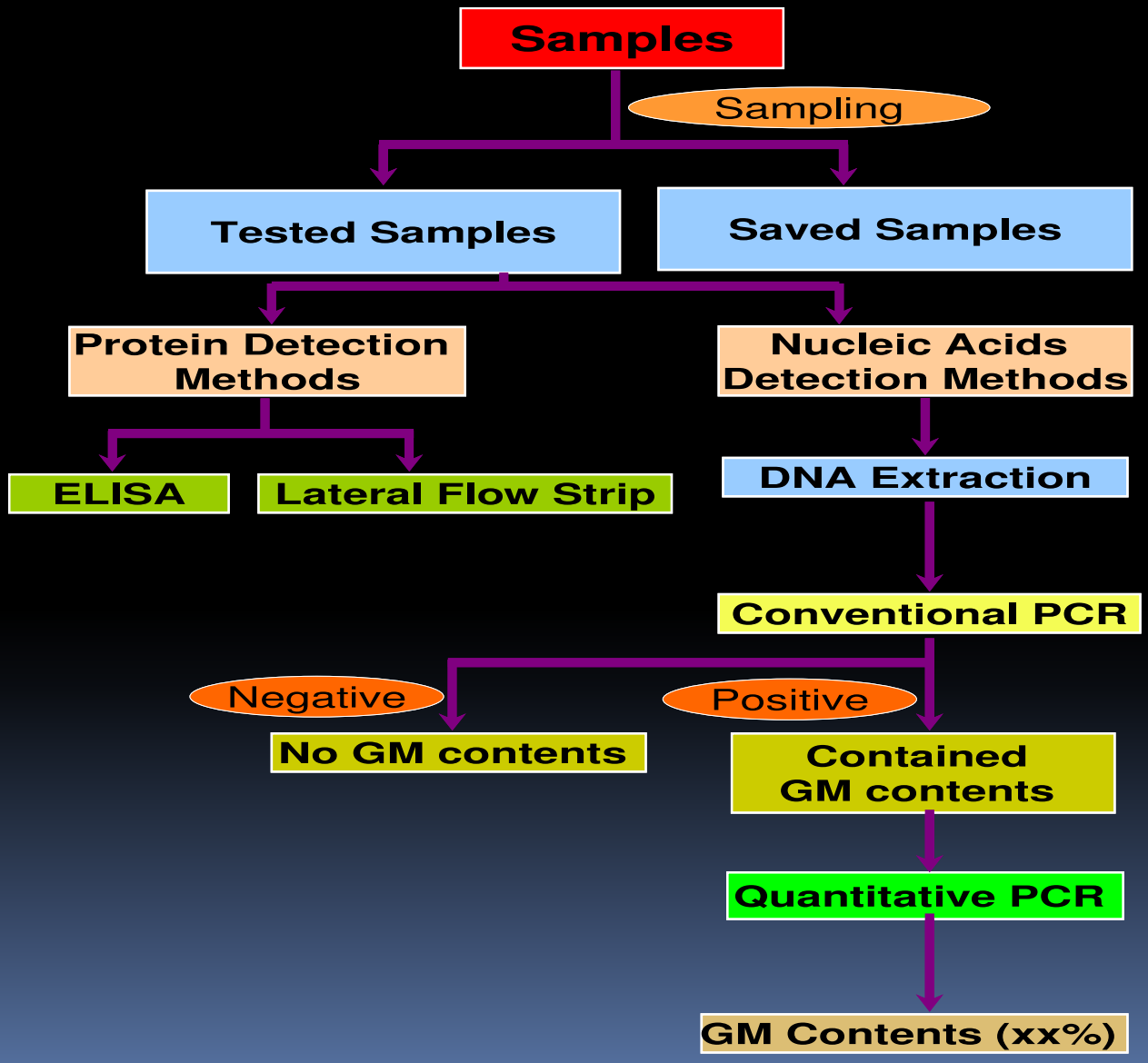
Bt cotton is the first GM crop commercialized in India. It contains *Cry* genes , which confers resistance to lepidopteron insects , including American bollworm (*Helicoverpa armigera*), the spotted bollworm (*Earias vittella*), and the pink bollworm (*pectinophora gossypiella*).

i. MON 531 contains *Cry1AC*
ii. MON 15985 contains stacked *cry1Ac* and *cry2Ab2* genes.

Bt brinjal is likely to be the first biotech food crop commercialized in India. It contains *Cry* gene , which confers resistance to most important insect pest of brinjal, the fruit and shoot borer (FSB) *Leucinodes orbonalis.*

- Event -142 expressing *CryFa1* gene

GM-crops and derived foods detection procedure



Development of GMOs analysis methods

PCR based methods (qualitative and quantitative)

- **Endogenous reference genes**

  fsACP (*fiber specific acyl carrier protein*) for cotton , ***smcp*** (*Solanum melongena cysteine proteinase*) for Brinjal

- **Screening PCR method :**

  CaMV 35S promoter; NOS terminator; Npt II marker gene .

- **Gene-specific PCR method:**

  Cry1Ac,Cry2AB2 & Cry 1Fa

- **Construct-specific PCR method:**

  MON 15985

- **Event-specific PCR method:**

  ✓ *ESP MON 531, ESP MON 15985 & ESP BR-142 event.*

- **R&D Reference molecules**

  Tandem markers plasmid (plasmid with multiple targets) .

Endogenous reference genes

The importance of a reference gene

Real Time quantitation of GM-event is based upon the notion of species-specific reference gene

An optimal reference gene should be:

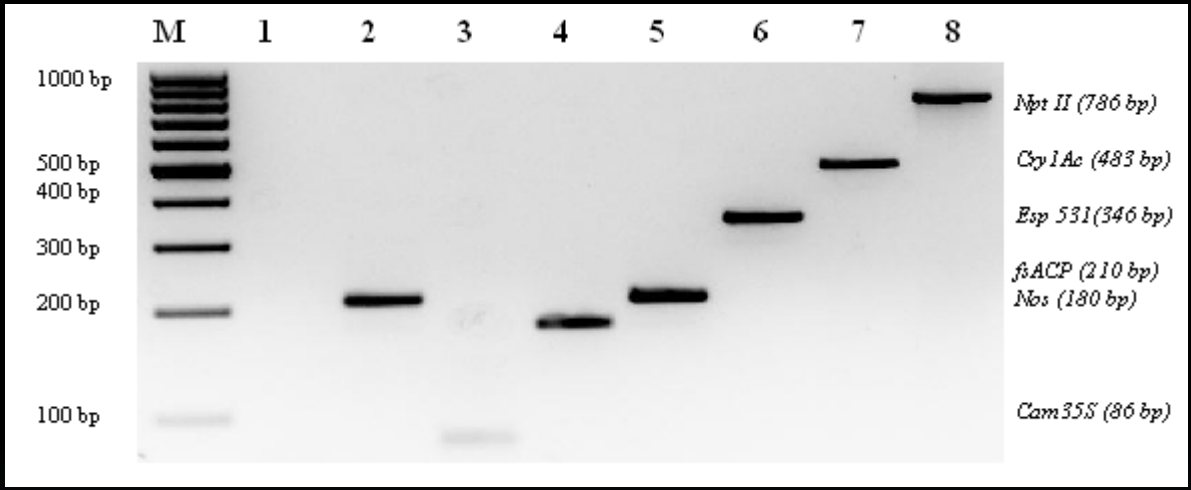
1. **single (or low and known) copy number**
2. **low allelic variation in cultivars**
3. **species specificity**

Ideally, reference gene should be proposed by the scientific community as golden standard for a given taxon

List of primers used for the development of multiplex PCR for the detection of two cotton events MON 531 and MON 15985

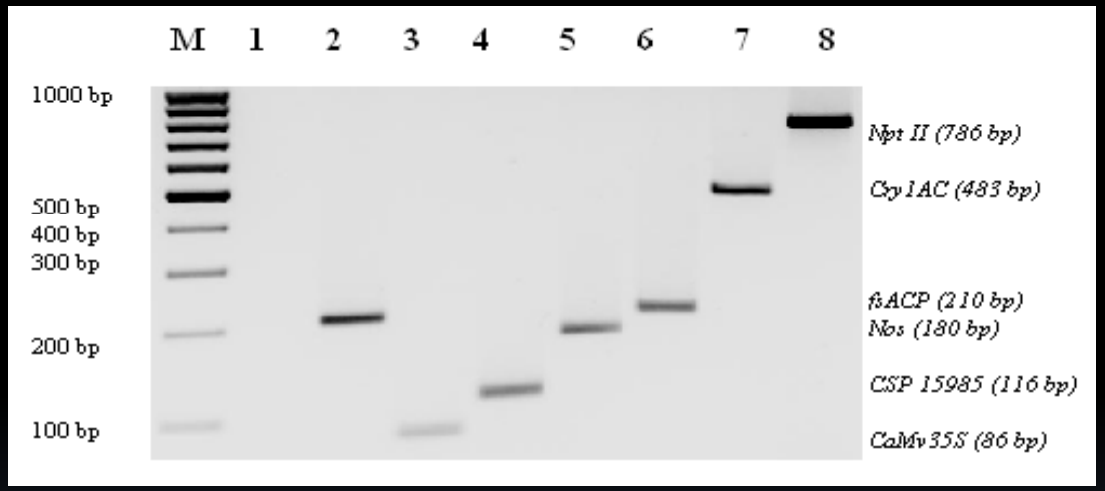
Gene	primer	Primer sequence	Expected size (bp)
CaMv35s promoter	*CaMv35S*	F TCCACTGACGTAAGGGATGAC R TCTCCAAATGAAATGAACTTCC	86
Nos terminator	Nos	F GAATCCTGTTGCCGGTCTTG R TTATCCTAGTTTGCGCGCTA	180
fsACP	fsACP	F TTGTGTTGGGACTTGAGGAA R GTTCACACATGATTTCCCCC	210
Event specific Mon531	Esp531	F AAGAGAAACCCCAATCATAAAA R GAGAATGCGGTAAAGATACGTC	346
Cry1AC	Cry1Ac	F GGGAGGAGATGCGTATTCAA R CTATACCCTGGGCAGAACCA	483
Npt II	Npt II	F GAACAAGATGGATTGCACGC R GAAGAACTCGTCAAGAAGGC	786
Construct specific Mon 15985	CSP15985	F ATTGAAGAAGAGTGGGATGACGTTA R GACCAGAGTTCAGGACGGAGTT	116

Singlet PCR for MON 531 event



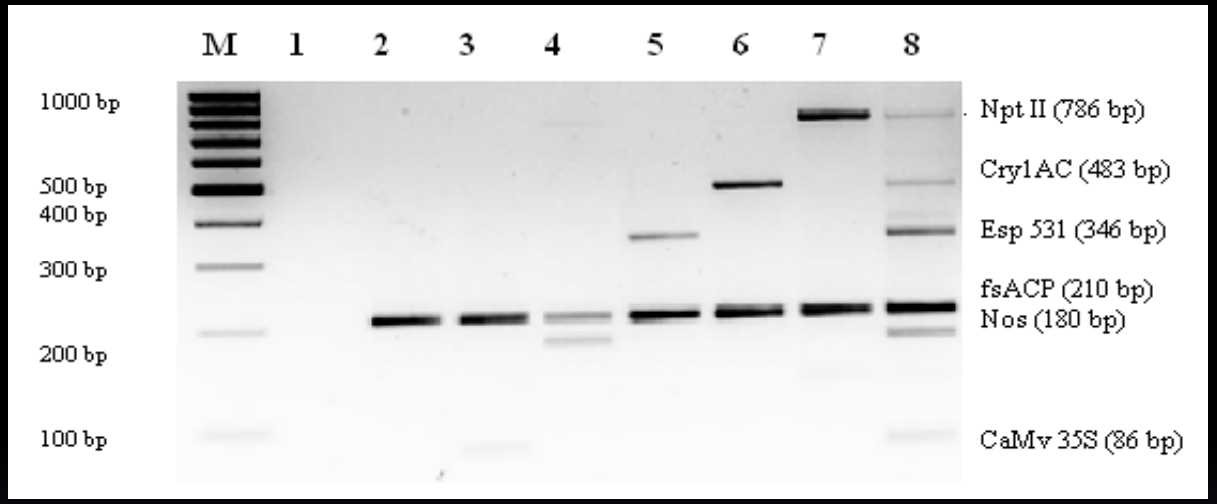
Singlet PCR assay for testing of primer pairs specificity for Mon 531 event. Lane M,100 bp ladder; lane 1, negative control; lane 2, non-GM cotton with *fsACP* (210 bp); lane 3 to 8, amplified DNA fragments of 86 bp, 180 bp, 210 bp, 346 bp, 483 bp, and 786 bp correspond to the *CaMv35S* promoter, *Nos* terminator, *fsACP*, *ESP 531*, *Cry1Ac* and *Npt II* genes

Singlet PCR for MON 15985 event



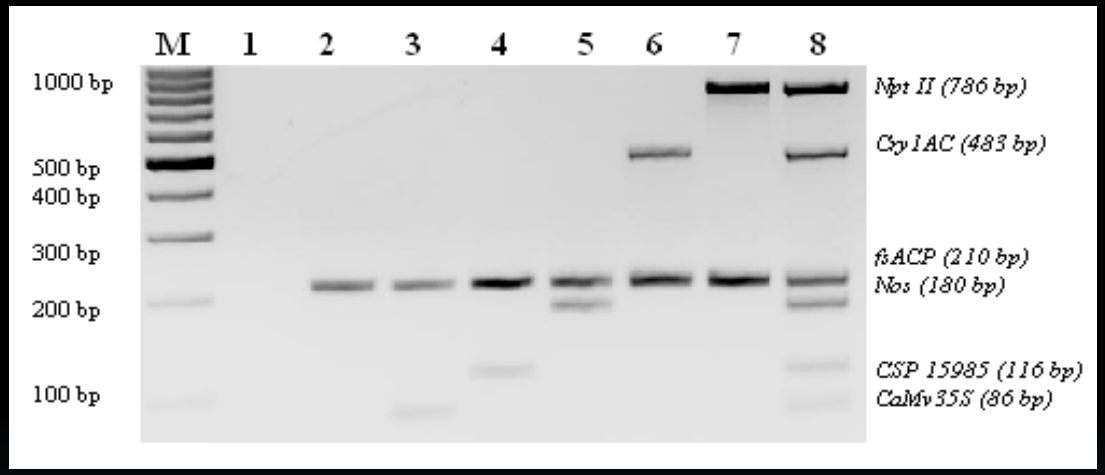
Singlet PCR assay for testing of primer pairs specificity for Mon 15985 event. Lane M,100 bp ladder; lane 1, negative control; lane 2, non-GM cotton with *fsACP* (210 bp); lane 3 to 8, amplified DNA fragments of 86 bp, 116 bp, 180 bp, 210 bp, 483 bp, and 786 bp correspond to the *CaMv35S* promoter, *CSP 15985*, *Nos* terminator, *fsACP*, *Cry1Ac* and *Npt II* genes.

Duplex and multiplex PCR methods for the detection of MON 531 event



Detection of Mon 531 event by duplex and multiplex PCR methods. Lane M, 100 bp ladder; lane 1, negative control; lane 2, non-GM with *fsACP* (210 bp); Lane 3, *CaMv35S* and *fsACP* (86 bp & 210 bp); lane 4, *Nos* and *fsACP* (180 bp and 210 bp); lane 5, *ESP 531* and *fsACP* (346 bp & 210 bp); lane 6, *Cry1AC* and *fsACP* (483 bp & 210 bp); lane 7, *Npt II* and *fsACP* (786 bp & 210 bp). Lane 8, multiplex PCR,amplified DNA fragments of Mon 531 event with 86 bp, 180 bp, 210 bp, 346 bp, 483 bp, and 786 bp correspond to the *CaMv35S*, *Nos*, *fsACP*, *ESP 531*, *Cry1Ac* and *Npt II* genes.

Duplex and multiplex PCR methods for the detection of MON 15985 event



Detection of Mon 15985 event by duplex and multiplex PCR methods. Lane M, 100 bp ladder; lane 1, negative control; lane 2, non-GM with *fsACP* (210 bp); Lane 3, *CaMv35S* and *fsACP* (86 bp & 210 bp); lane 4, *CSP 15985* and *fsACP* (116 bp & 210 bp); lane 5, *Nos* and *fsACP* (180 bp and 210 bp); lane 6, *Cry1AC* and *fsACP* (483 bp & 210 bp); lane 7, *Npt II* and *fsACP* (786 bp & 210 bp). Lane 8, multiplex PCR,amplified DNA fragments of Mon 15985 event with 86 bp, 116 bp, 180 bp, 210 bp, 483 bp, and 786 bp correspond to the *CaMv35S , CSP 15985*, *Nos*, *fsACP*, *Cry1Ac* and *Npt II* genes.

Sensitivity assay to determine the limit of Detection (LOD)

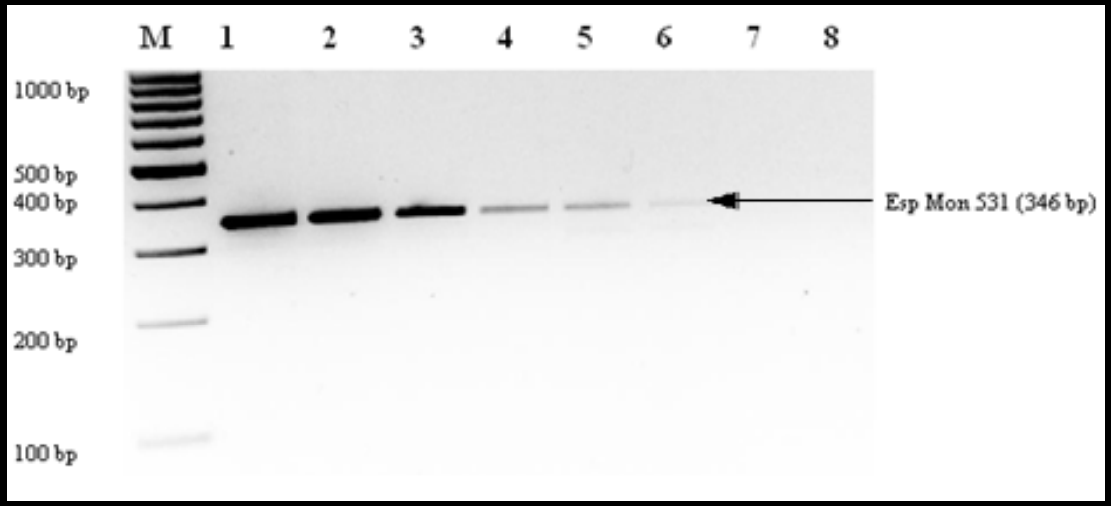
➢DNA mixture was prepared with each of two cotton events and non-GM DNA using serial dilution of 100 ng/µl DNA sample of GM cotton (100%) with 100 ng /µl of non-GM DNA (100%).

➢ the copy number in the sample is determined by dividing the sample DNA weight (in picograms) by the published average 1C value for cotton genome 2.33 picograms. Ref Armuganathan, K. *et. al* **1999.**

Calculation of genetically modified organisms (GMO) genome copies in GM cotton

S.No	GM DNA	Non GM DNA	GM %	GMO genome copies
1	100	0	100	42918
2	10	90	10	4291
3	1	99	1	429
4	0.1	99.9	0.1	42
5	0.05	99.95	0.05	21
6	0.03	99.97	0.03	12
7	0.01	99.99	0.01	4
8	0	100	0	0

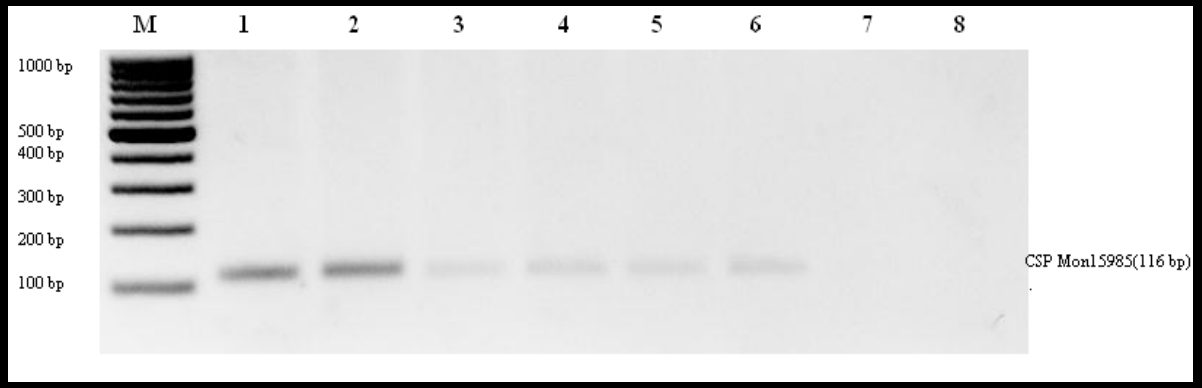
Sensitivity assay of MON 531 event



✓PCR was performed with ESP 531 primers. The LOD of MON 531 is **0.03%**

Sensitivity assay of MON 531 event. Lane M, 100 bp ladder; lane 1-8: serially diluted DNA of MON 531 event containing 100%, 10%, 1%, 0.1%, 0.05%, 0.03%, 0.01% and 0%.

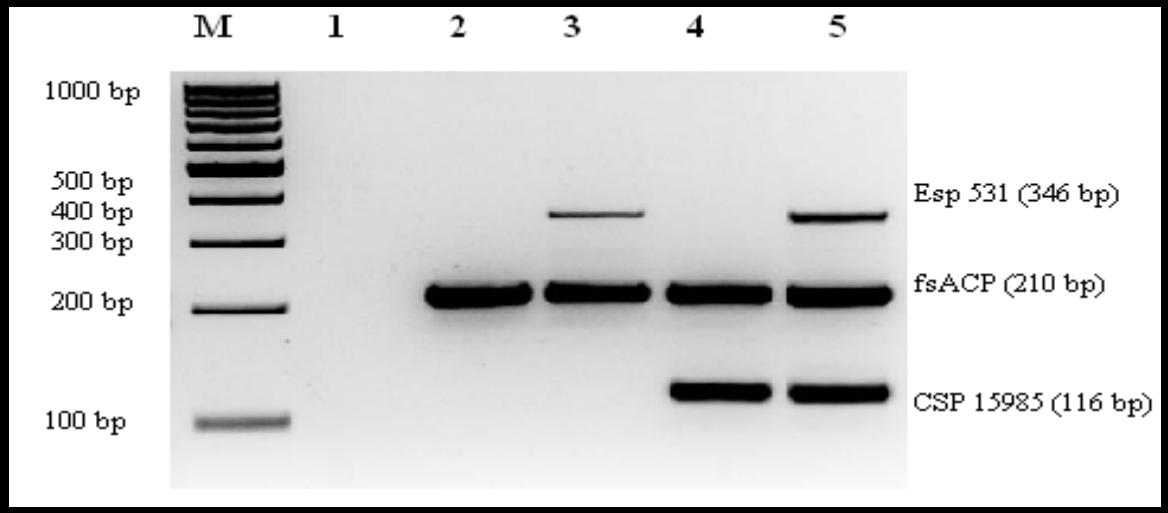
Sensitivity assay of MON 15985 event



✓PCR was performed with CSP 15985 primers. The LOD of MON 15985 event is **0.03%**

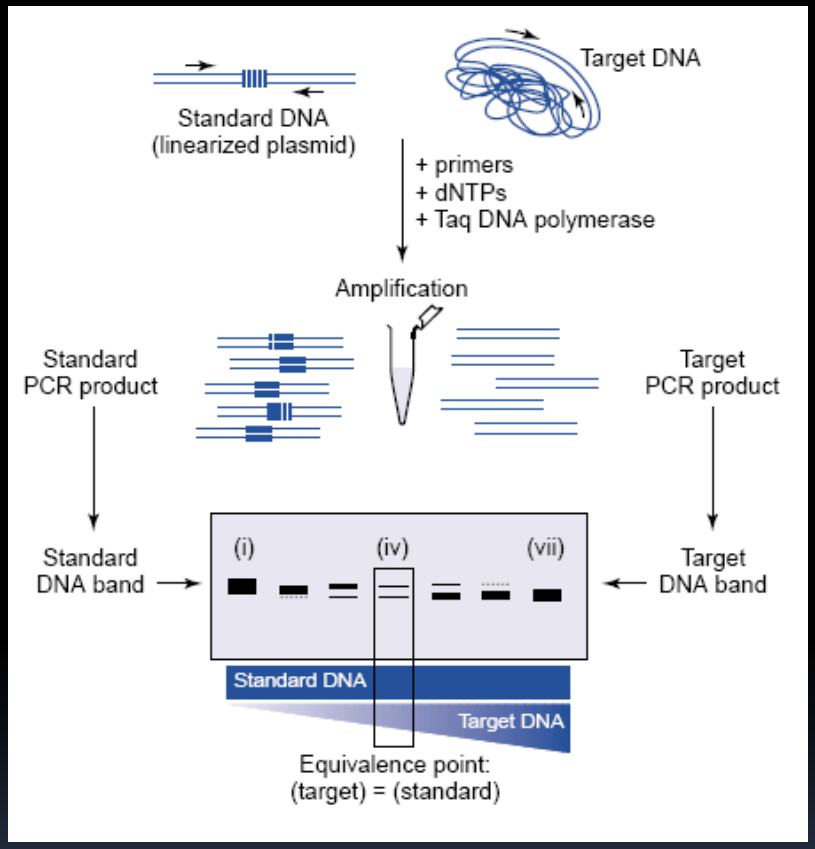
Sensitivity assay of MON 15985 event. Lane M, 100 bp ladder; lane 1-8: serially diluted DNA of MON 15985 event containing 100%, 10%, 1%, 0.1%, 0.05%, 0.03%, 0.01% and 0%.

PCR for the detection of artificial event mixing of MON 531 and MON 15985 events



PCR for the detection of artificial mixing of MON 531 & MON 15985 events. Lane M, 100-bp ladder; lane 1, negative control; lane 2, non-GM with *fsACP* (210 bp); lane 3, Mon 531 with *fsACP* & *Esp 531*(210bp & 346 bp); lane 4, Mon15985 with *fsACP* & *CSP15985* (210 bp & 116 bp); lane 5, mix of two events with CSP 15985, *fsACP* & *Esp531* (116 bp, 210 bp & 346 bp).

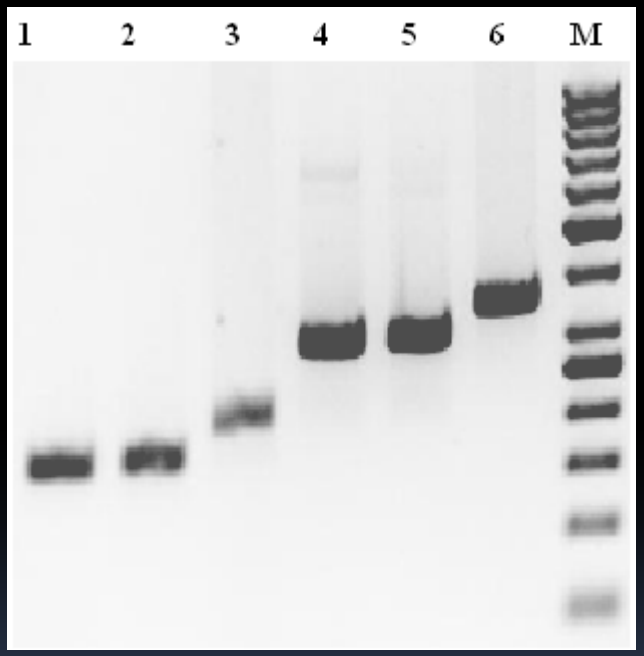
Quantitative competitive PCR



Reference: Farid E. Ahmed. Detection of genetically modified organisms in foods. *Trends in Biotechnology* Vol.20 No.5 May **2002.**

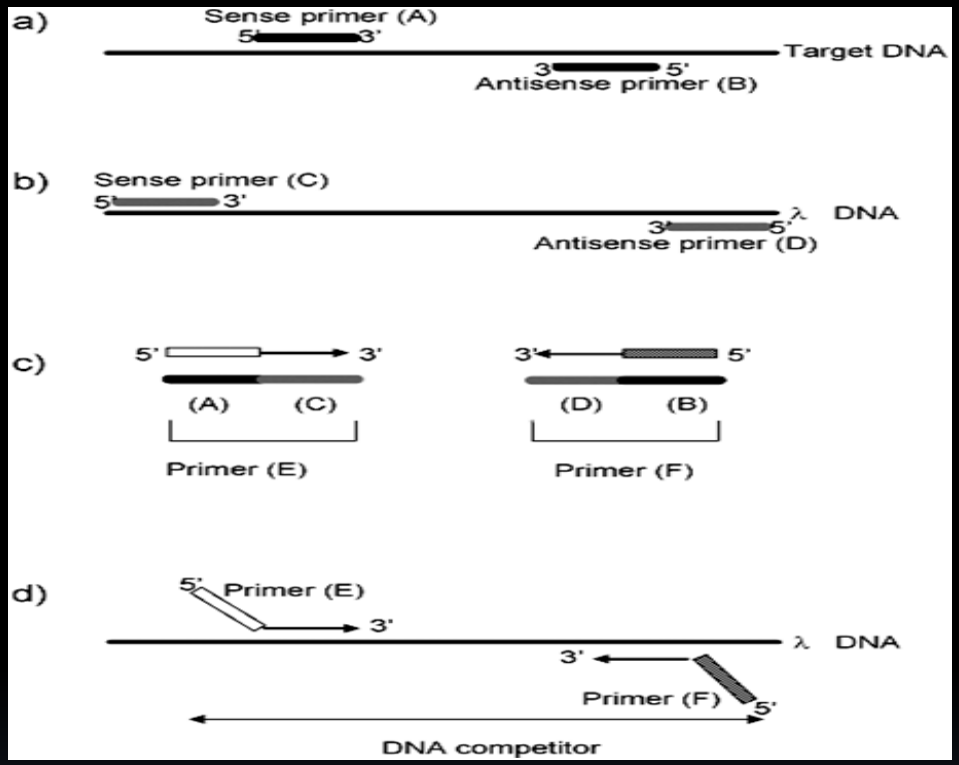
1) Construction of competitors by deleting few base pairs by using restriction enzymes

competitors were constructed by deleting 22 bp from the amplified endogenous gene (*fsACP*), by using restriction enzymes (SfaN I & Alu I) and Event specific competitor (Mon 531) was constructed by deleting 35 bp from the amplified event specific gene by using restriction enzymes (SfaNI & Cac8I).



Agarose gel showing PCR product of (*fs ACP* and *ESP531)* and respective competitors. Lane 1- 2, competitor of fsACP (188 bp): lane 3, PCR product of *fsACP* (210 bp); lane 4-5 competitor of ESP Mon 531 (311 bp); lane 6, PCR product of ESP Mon 531 (346 bp): lane M, 50 bp ladder.

2) Construction of competitors by using special primers



Primer sequences:

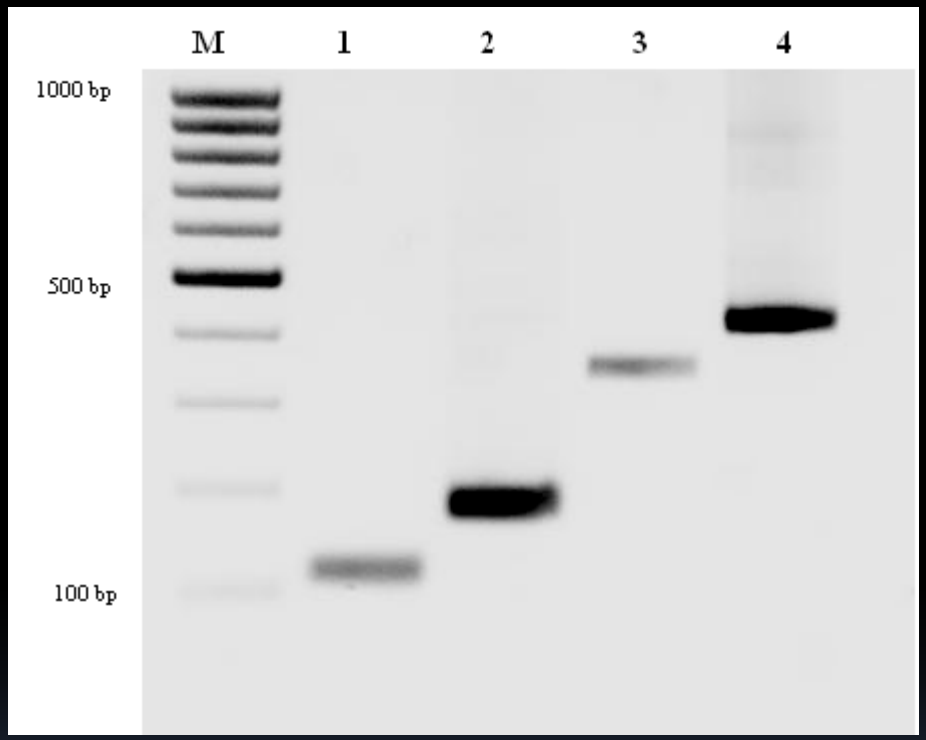
fs ACPco F 5' CAAACAAGAGACCGTGGATAAGGTATGTACCGGCTGTCTGGTATG 3'

fs ACPco R 5' CAAGAGAATCAGCTCCAAGATCAAGGTTTCAGGGCAAAACTCAGC 3'

ESP531co F 5' AAGAGAAACCCCAATCATAAAAAACCTTCGTAAACACCACGC 3'

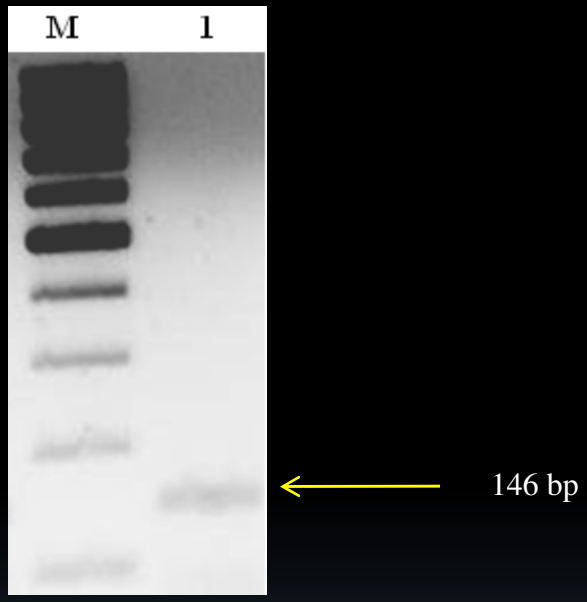
ESP531co R 5' GAGAATGCGGTAAAGATACGTCGCGTTCATACACAATGGTCG 3'

Construction of competitors by using special primers



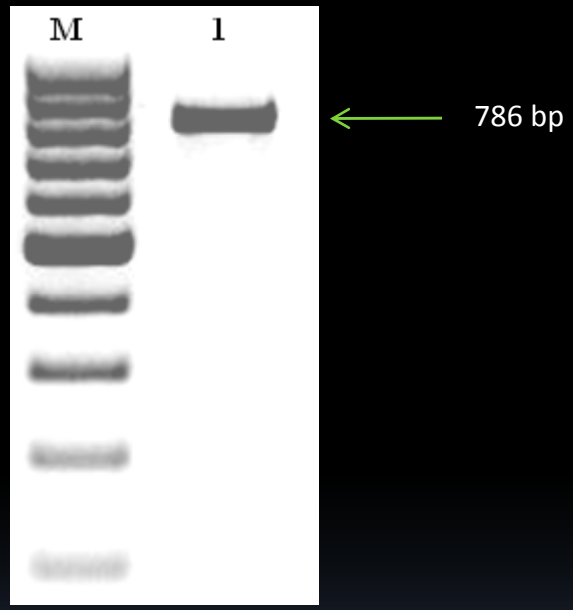
Agarose gel showing competitors of *fs ACP* and *ESP531*. Lane M, 100 bp marker; lane 1, *fsACP(116 bp) ;* lane 2, fsACP competitor (178 bp); lane 3, ESP 531 (346 bp); lane 4, ESP531 competitor (414 bp).

Singleplex PCR for Brinjal endogenous gene (smcp)



Agarose gel showing amplification of *smcp* gene (146 bp) in Brinjal. Lane M, 100 bp ladder, lane 1, amplified product of *smcp* (solanum melongena cysteine proteinase) gene.

Singelplex PCR for the detection of Npt II gene of Bt Brinjal



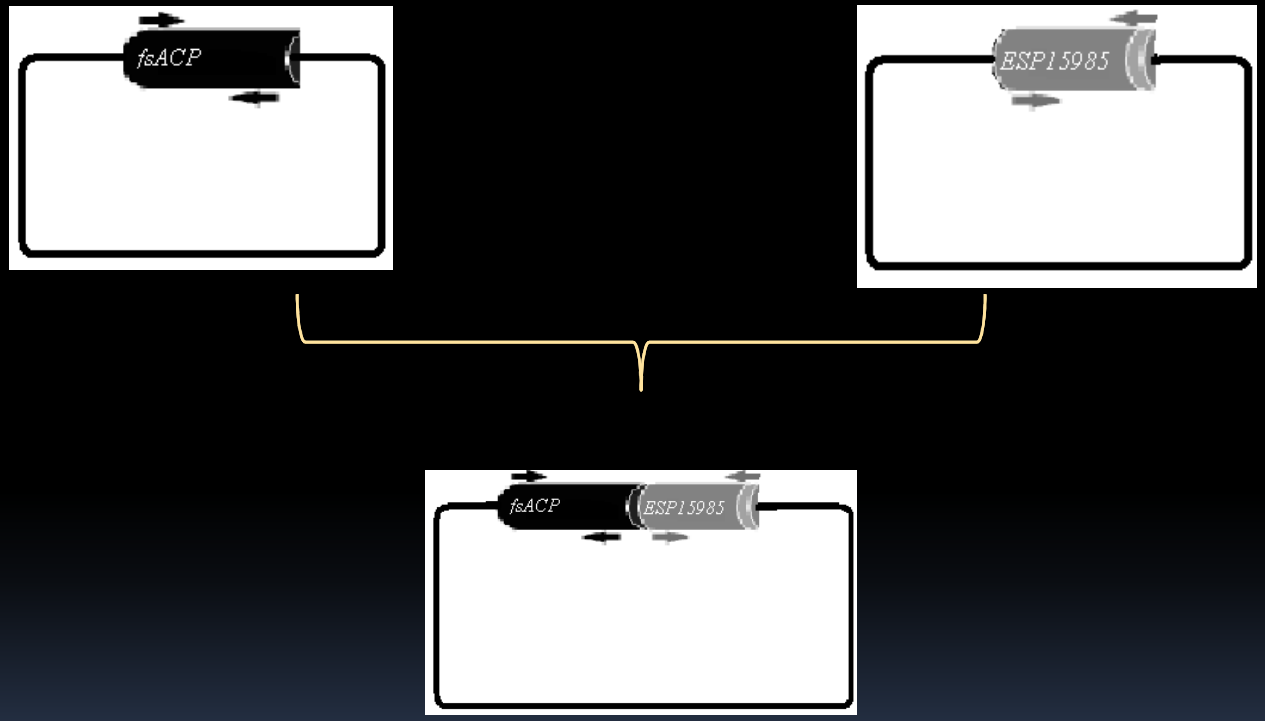
Agarose gel showing amplification of *Npt II* gene (786 bp) in Bt Brinjal event-142. Lane M, 100 bp ladder, lane 1, amplified product of *Npt II* gene.

R&D Reference molecules

➢**The certified reference materials for GMOs analysis is not easily obtained. Several CRMs have been R&D by Institute for Reference Materials and Measurements (IRMM), and series of CRMs might be purchased from Fluka. However, the price is very expensive.**

✓**Reference molecule can be used in GMOs analysis instead of CRMs**

Development of reference plasmid containing multiple targets



Plasmid containing the event-specific junction sequences for transgenic cotton events and cotton specific endogenous reference gene *fsACP* respectively. These indigenously generated plasmid reference samples are used for quantification of GM cotton using real-time PCR.

What are the factors discouraging Biotech R&D in developing countries?

- High research costs
- Insufficient regulatory capacity
- Lack of scientific resources and skilled personnel
- Unfavorable intellectual property arrangements

What are the steps to be taken to exploit the benefits of modern biotechnology ?

- Allocation of additional public resources to agricultural research
- Improvement in the seed distribution and extension systems
- Capacity building of the public sector in biotech R&D
- Public education
- Policies and regulatory framework on biosafety, food safety and intellectual property rights, (IPRs)
- Strong public-private sector links for both international and local collaborative undertakings.

New facilities to be created for GMO information exchange

- GMO biosafety database
- GMO detection method database
- GMO detection, new method validation, and training service
- GMO public education

Thank you!