DNA-based GMO Screening Strategies:

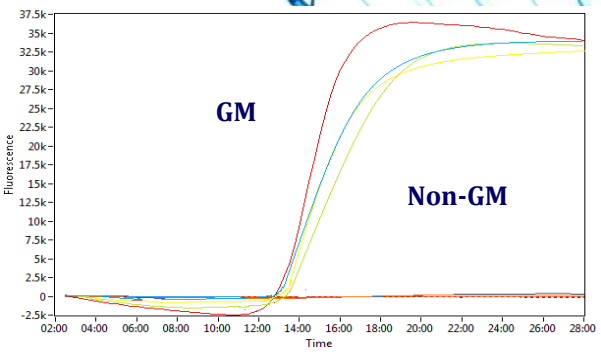
Rapid Monitoring the GM Status of Products in Food and Supply Chain

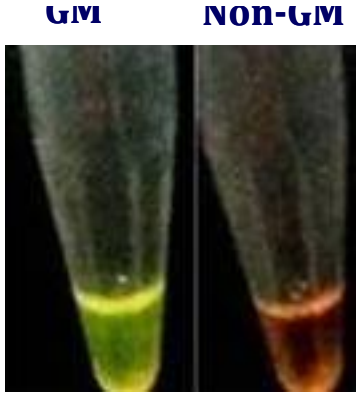




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GM Crops

WHAT?

GM crops with desired traits developed by introducing **"gene or genetic construct of interest"** employing recombinant DNA techniques.

GM crops carries "transgene(s)" conferring either

(i) A **new trait** to the plant, which was not earlier present in native form (e.g., insect resistance, as in Bt cotton) or

(ii) Enhance an already existing trait (e.g., quality enhancement).



WHY?

- ✓ Food Security
- ✓ Increased Crop Productivity
- ✓ Mitigating Climate Change

RISKS?

Biosafety issues need to be properly addressed

- ✓ Food Safety
- ✓ Environmental Safety

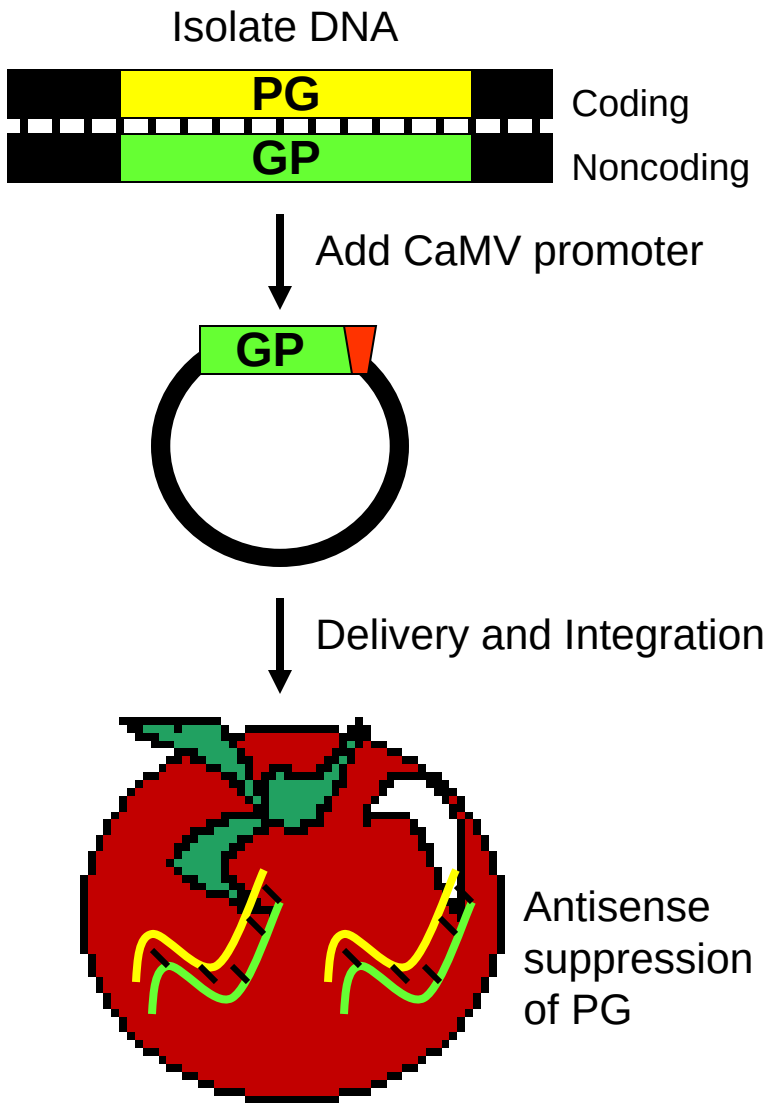
First Approved GM Food Crop…..Flavr-Savr Tomatoes: Antisense Technology

Polygalacturonase (PG): an enzyme expressed during ripening causing depolymerization of the pectin fraction of the cell wall, which **results in softening of ripe tomatoes.**

The PG gene was identified in the tomato and inserted into a plasmid in such a way that **a PG antisense transcript is produced**

The antisense transcript binds the PG sense mRNA blocking access of the translational machinery eliminating 99% of the PG product

Increased Shelf Life…..

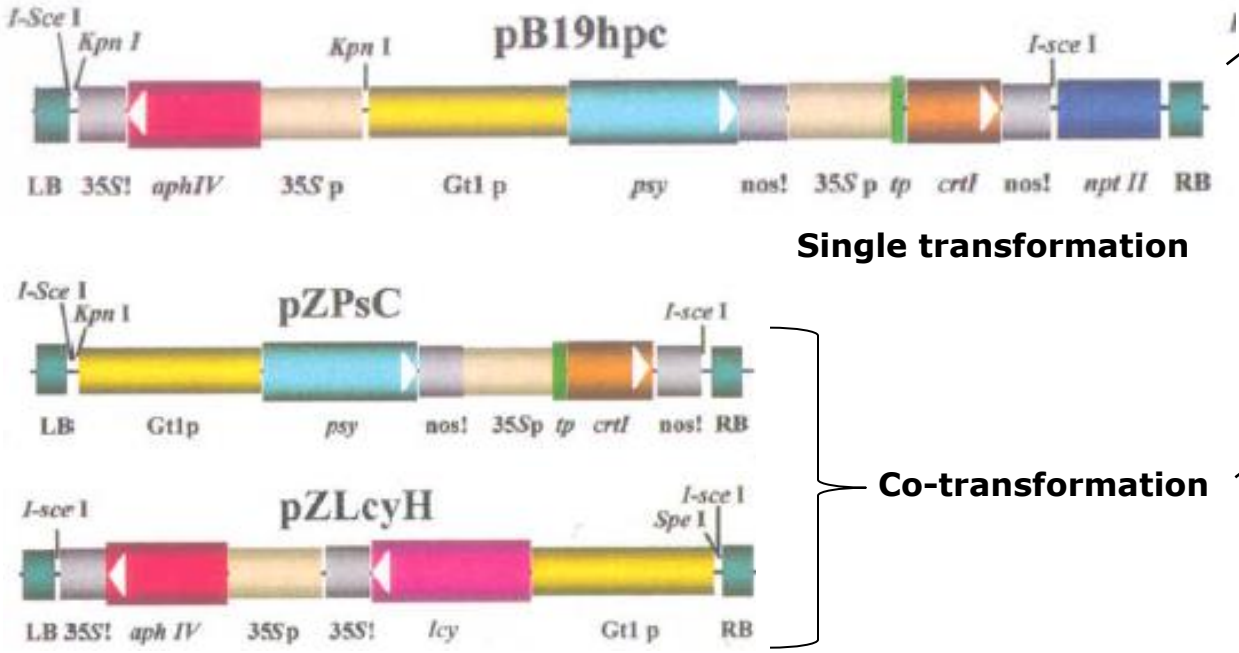


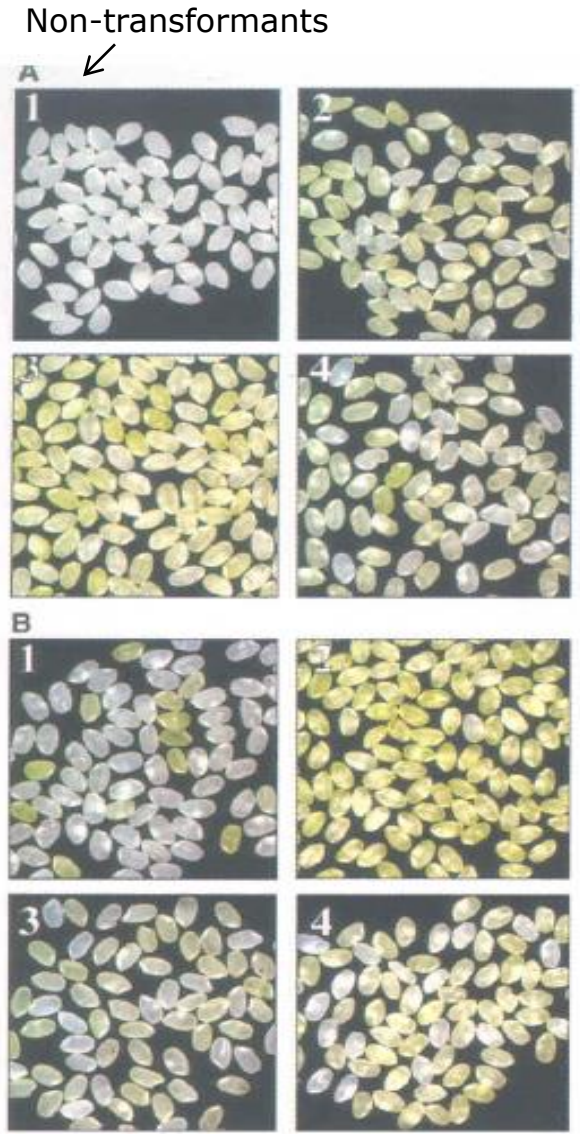
Golden Rice: *Classical Example of GM Food Crop*

Provitamin A (β-carotene) biosynthetic pathway in rice endosperm

✓**phytoene synthase (*psy*) and lycopene β-cyclase (β-*lcy*) both from *Narcissus pseudonarcissus***; under control of **endosperm-specific glutelin promoter**

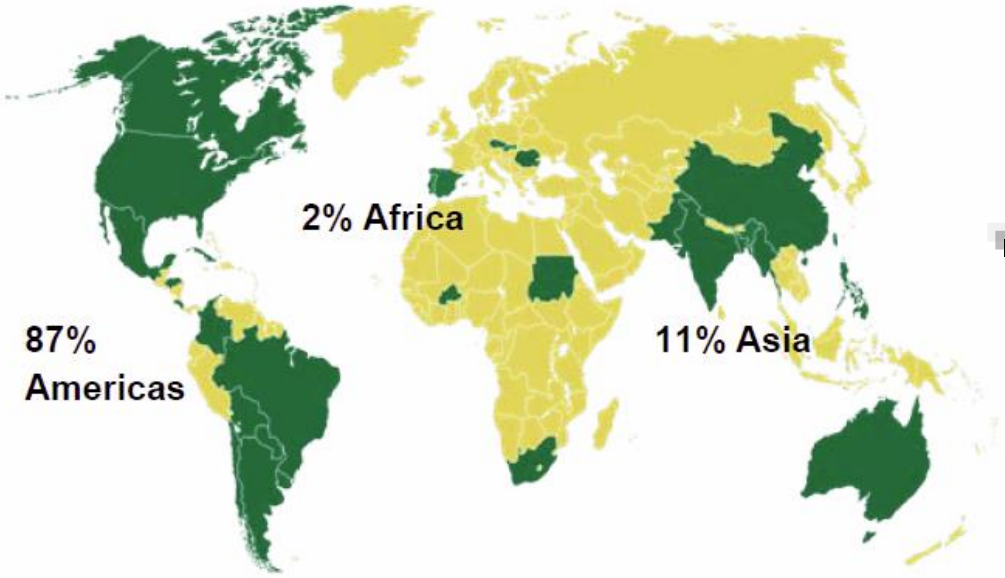
✓bacterial **phytoene desaturase (*crt*I, from *Erwinia uredovora*** under **constitutive 35S promoter** control).





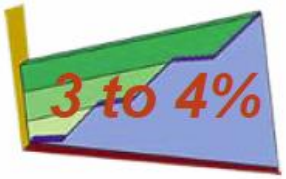
Source: Ye et al. (2000) Engineering the provitamin A (beta-carotene) biosynthetic pathway into (carotenoid-free) rice endosperm. Science, 287(5451):303-5.

Global Status of GM Crops



28 countries which have adopted biotech crops

Increase over 2013



In 2014, global area of biotech crops was 181.5 million hectares, representing an increase of 3 to 4% over 2013, equivalent to 6.3 million hectares.

Source: Clive James, 2014.

Biotech Mega Countries

50,000 hectares (125,000 acres), or more

		Million Hectares
1.	USA	73.1
2.	*Brazil**	42.2
3.	*Argentina**	24.3
4.	*India**	11.6
5.	Canada	11.6
6.	*China**	3.9
7.	*Paraguay**	3.9
8.	*Pakistan**	2.9
9.	*South Africa**	2.7
10.	*Uruguay**	1.6
11.	*Bolivia**	1.0
12.	*Philippines**	0.8
13.	Australia	0.5
14.	*Burkina Faso**	0.5
15.	*Myanmar**	0.3
16.	*Mexico**	0.2
17.	Spain	0.1
18.	*Colombia**	0.1
19.	*Sudan**	0.1

Less than 50,000 hectares

*Honduras** | Romania
*Chile** | Slovakia
Portugal | *Costa Rica**
*Cuba** | *Bangladesh**
Czech Republic

** Developing countries*

>20 GM food crops comprising 75% of globally commercialized GM events approved for use as food (direct or in processed form) or for cultivation.

GM Papaya Resistant to Papaya Ring Spot Virus (PRSV): A GMO Success Story

1986: Efforts initiated to develop virus resistant GM papaya by transforming Hawaiian papaya with CP gene of PRSV by Gonsalves & coworkers.

1991: GM line (**55-1**) resistant to PRSV identified.

1992: Two PRSV resistant transgenic cultivars '**SunUp**' and '**Rainbow**' developed.

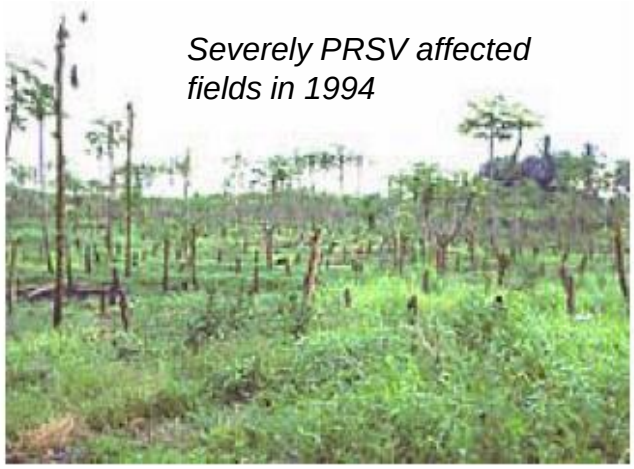
1998: License to commercialize papaya in Hawaii.

Approved in: USA, Canada & Japan *(As Food for direct use or in processed form)*



Two varieties of papaya resistant to papaya ringspot virus have been developed using biotechnology: SunUp, *left*, and Rainbow, *right*. They have performed well for Hawaiian growers, even under prolonged and heavy disease pressure.

Source: Gonsalves (2004) California Agriculture, 58 (2): 92-93



Severely PRSV affected fields in 1994



Green healthy transgenic Rainbow papaya in 1999

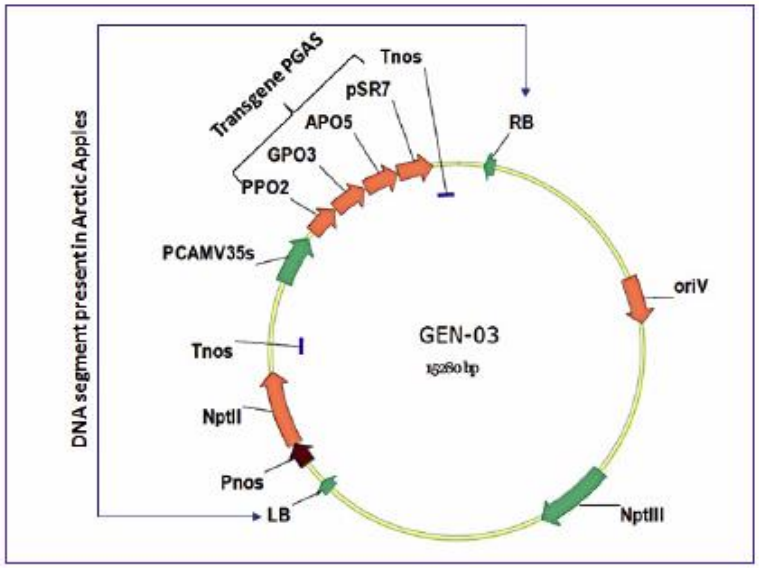
Source: Gonsalves et al. (2004) APSNet (Feature Story)

Recently Approved GM Apple with Non-Browning Phenotype

- ✓ **Two GM Events "Arctic Granny Smith Apple" and "Arctic Golden Delicious Apple", developed by Okanagan Specialty Fruits (a Canadian firm)**
- ✓ **Contains *PGAS PPO* suppression gene** (PGAS: comprises 4 groups - namely PPO2, GPO3, APO5 and pSR7 of 10 genes; PPO: **polyphenyl oxidase**)

"Arctic Granny Smith Apple" and "Arctic Golden Delicious Apple", with slices.

Approved in 2015: USA & Canada *(As Food for direct use or in processed form)*





Source: Xu K (2013) New York Fruit Quarterly, 21 (3): 8-10

Status of GM Crops in India

- Commercialized

GM (*Bt*) Cotton

Area under *Bt* cotton: 11.6 mha (>95% of total area under cotton cultivation)



Event	Developer	Year of Approval
MON531	Mahyco/ Monsanto	2002
MON15985	Mahyco/ Monsanto	2006
Event 1	JK Agri-Genetics	2006
GFM	Nath Seeds	2006

- Imported for Research Purposes (>195 imports of 15 crops)

- Field trials during 2006-2013: GM events of 18 crops*

*brinjal, cotton, corn (maize), cabbage, castor, cauliflower, chickpea, groundnut, mustard, okra, papaya, potato, rice, rubber, sorghum, sugarcane, tomato, watermelon

- Research and development by public and private sector

Status of GMO Labelling in Food Products



Consumers could make "informed choice" on whether they want to buy packaged food products that are "GM"

The Ministry of Consumer Affairs, in an extraordinary gazette notification, has made an amendment to make labelling of every package* containing GM food mandatory from January 1, 2013.

*19 commodities to be covered: include baby food, biscuits, breads, edible oils, milk powder, cereals, pulses

Source: The Hindu, June 21, 2012

DNA-based GMO Testing: A Stepwise Approach

Step 1 — PCR with endogenous reference gene

-ve → DNA is contaminated with PCR inhibitors

+ve



Step 2 — Initial screening targets commonly used elements

-ve → Sample is non-GM

+ve → Sample is GM



Cuts down the cost of GMO testing *(by reducing the number of test samples & eliminating the need for testing for each and every GM event)*

Step 3 — Identification of specific transgene

Step 4 — Construct-/event-specific PCR for more specificity of the particular event

Step 5 — Quantification using Real-time PCR to check the % GM content and copy number of the transgene and to test the contamination

GMO Screening Matrix: A Decision Support System

- GMO screening matrix, **for 141 GM events of 21 crops** with the information on **106 genetic element targets for detection**, was developed;

-The matrix includes commercially cultivated *Bt* cotton events and other GM events, under field trials during the past six years (imported/indigenously developed);

- Ten most frequently present targets, *viz*., ***P-35S, T-nos, Os-Msca1, cry1Ab, cry1Ac, cry1C, cry2Ab, GA20 oxidase1, nptII, bar*** were identified to screen these events

Success story on developed GMO matrix

- Published in SciDev Net on 22 April, 2014
- Telecasted on Rajya Sabha TV (RSTV) in - Gyan Vigyan (31 May, 2014);
- Science Monitor (1 June, 2014)

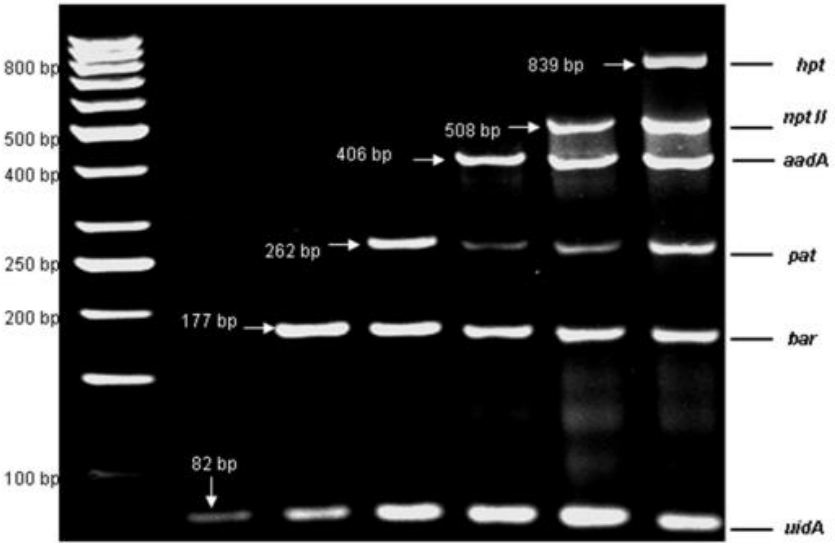


Event	Number of known genetic elements	P-35s	TMV enhancer	P-rd29A gene	P-FMV	P-NOS	P-nos	P-OCS	PR2 (Wound inducible promoter	chimeric promoter	P-T-A29	P-(PHA)-L(dlec2) (seed specific)	Endosp-erm specific glutelin promoter	maize ubiquitin1 promoter	P-ubi (a th)	Fruit specific promoter LeAC54	Arabidopsis SSU promoter	PubiZm	P-HaT4D/A	Pa7a7	T-35S	T-E9	T-nos	T-ocs	T-histone/A	T-hsp 17	β- conglycinin	aadA	OS ARGoS like leucine zipper gene	AT - CDKB12	Alternate Oxidase	Arg	Cry1Ab-Ac fusion gene	cry6C	avp1	glutelin	mepsps	bean alpha amylase inhibitor	Has	ScFv	AFP-AG
Bt Brinjal containing *cry1Ac* gene (Event EE1)	3	1																								1														
Bt Brinjal containg *cry1Aa* and *cry1Aabc*	2																																							
Event 142	1																																							
Cabbage containing *cry* 9C gene	1																																1							
Castor containing *cry1Aa* and *cry1Ec* genes (AMT-1,	2																																							
CF-3, CF-4, CF-5, CA-1, CA-2, CA-6	1																																							
Bt Cauliflower containing *cry1Ac*, *cry1Ba* and *cry1*	3																																							
Bt Cauliflower containing *cry1Ba*, *cry1Ca* and *bar*	3																																							
Chickpea containing Bean alpha-amylase inhibitor	4	1										1																										1			
Chickpea containing cry2Aa (BS5A.1 (T2), (BS5A.1 (T2)18-1 PI), BS5A.1 (T2)18-2 PI), BS5A.2(T2) (BS5A.2(T2) 19-1 P2,	4																1						1																	
Chickpea containing DREB1A gene	5				1		1																1																	
Bt11	4	1																					1																	
Bt11 x GA21	3	1																					1																	
Bt176	4	1																			1																			
GA21	3	1																					1																	
MON 810	4	1																			1																			
MON 89034	8	1			1																		1			1													
MON 89034 x NK603	9	1			1																		1																	
MON89034 x NK603 x TC1507	6	1			1																1		1			1													

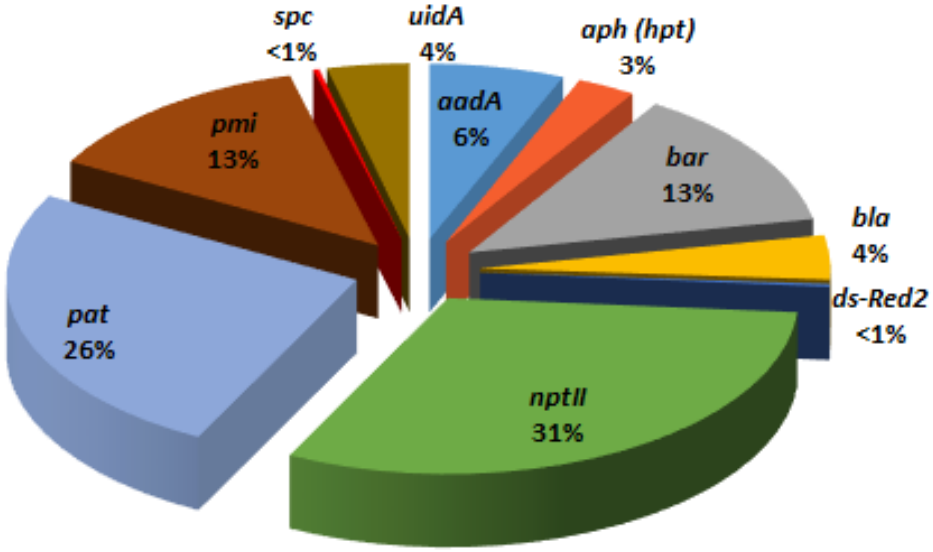
Source: Randhawa GJ, Morisset D, Singh M & Žel J (2014) GMO matrix: A cost-effective approach for screening unauthorized GM events in India. Food Control, 38: 124-129

Multiplex (6-plex) PCR-based GMO Screening:
Checking the GM status irrespective of crop & GM trait

Targets: Six marker genes - *nptII, aadA, bar, pat, hpt* and *uidA*



Gel profile of hexaplex PCR



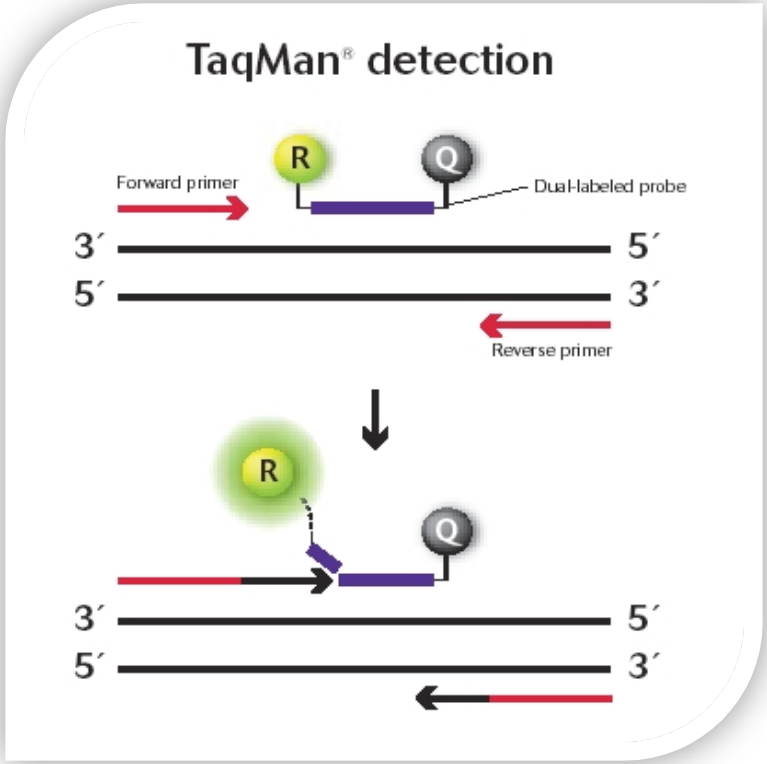
*Screening for presence of **over 80% of globally commercialized GM events** and all the GM cotton events commercialized in the country*

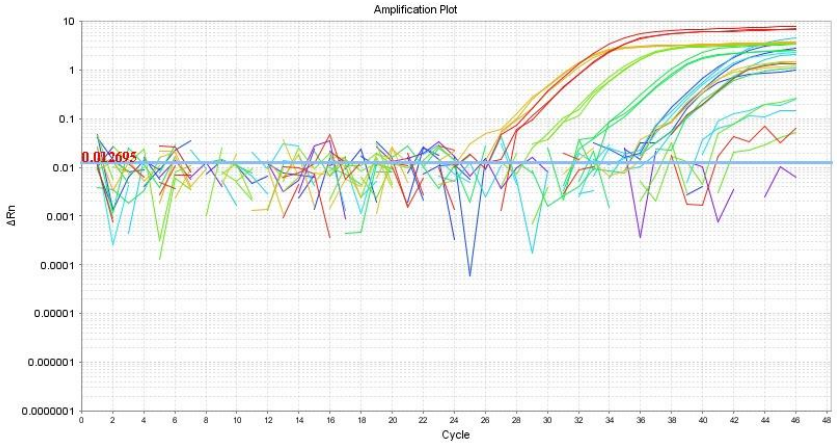
Randhawa G.J. et.al (2009) Multiplex PCR-based simultaneous amplification of selectable marker and reporter genes for screening of GM crops. J. Agri. Food Chem. 57 (12): 5167-5172.

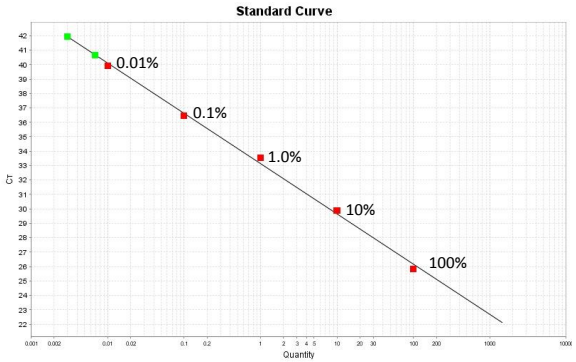
Duplex Real-time PCR targeting *P-35S* and *T-nos*

Screening for presence of **over 80% of globally commercialized GM events**; and all the GM cotton events commercialized in the country

- **Limit of detection up to 0.01% GM content**
- Employed in proficiency testings and validation programs.
- ***Could be efficiently utilized by GMO testing laboratories to check the GM status***







Amplification profile of duplex real-time PCR targeting *P-35S* and *T-nos*; Standard curve for *T-nos*

Real-time PCR based Multi-target System for GMO Screening

Primers & probes lyophilized on Pre-spotted Plate

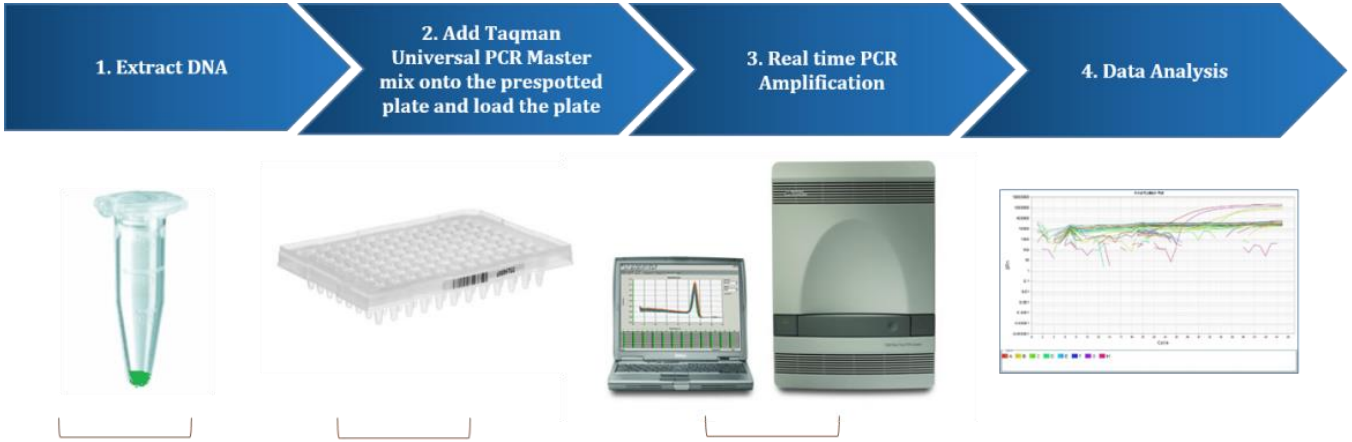
47 Targets: Events (21): Maize (12), Cotton (6), Rice (1), Brinjal (1), Soybean (1)
Endogenous genes (6): Maize, Cotton, Rice, Brinjal, Soybean, Potato
Construct-specific (5), Transgenes (11), Control elements (4)

	1	2	3	4	5	6	7	8	9	10	11	12
A	*Adh1*	GA21	3272	LL Rice	*cry1Ac*	*P-35S*	*Adh1*	GA21	3272	LL Rice	*cry1Ac*	*P-35S*
B	*Sad1*	NK603	59122	EE1	*cry1Ab*	*P-nos*	*Sad1*	NK603	59122	EE1	*cry1Ab*	*P-nos*
C	*sps*	MIR162	MON531	GTS 40-3-2	*cry1Ab/Ac*	*T-nos*	*sps*	MIR162	MON531	GTS 40-3-2	*cry1Ab/Ac*	*T-nos*
D	*β-fructosidase*	MIR604	MON15985	*P35S - cry1Ac*	*cry2Ab*	*T-35S*	*β-fructosidase*	MIR604	MON15985	*P35S - cry1Ac*	*cry2Ab*	*T-35S*
E	*Lectin*	MON810	MON1445	*P35S-cry1C*	*cry3A*	*nptII*	*Lectin*	MON810	MON1445	*P35S-cry1C*	*cry3A*	*nptII*
F	*UGPase*	MON863	MON88913	*P35S-uidA*	*cry1C*	*pat*	*UGPase*	MON863	MON88913	*P35S-uidA*	*cry1C*	*pat*
G	Bt11	MON89034	Event281	*ctp2-cry2Ab*	*epsps*	*bar*	Bt11	MON89034	Event281	*ctp2-cry2Ab*	*epsps*	*bar*
H	Bt176	TC1507	Event3006	*ctp2-cp4epsps*	*AmA1*	control	Bt176	TC1507	Event3006	*ctp2-cp4epsps*	*AmA1*	control



Limit of Detection : Up to 0.1-0.01% for different targets

Work flow and approximate timing for GMO analysis using the multi-target system



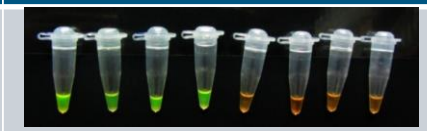
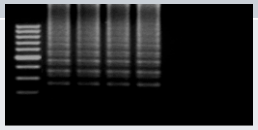
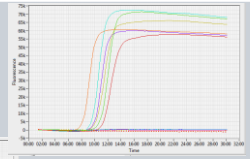
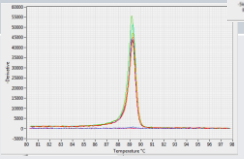
Source: Randhawa GJ, Singh M, Sood P & Bhoge RK (2014) Multi-target real-time PCR-based system: Monitoring for unauthorised GM events in India. J. Agric. Food Chem. 62 (29), 7118 -7130.

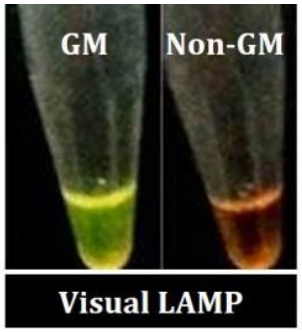
Loop-mediated Isothermal Amplification (LAMP): A New Generation GM Diagnostics

- An **isothermal nucleic acid amplification technique:** amplification is done at constant temperature
- *Bst* DNA polymerase large fragment (a *Bacillus stearothermophilus* DNA Polymerase protein with 5′ → 3′ polymerase activity, but lacks 5′ →3′ exonuclease activity
- Less sensitive to inhibitors
- Can be subjected to Crude samples
- Does not require sophisticated equipment, and has the potential to be deployed on-site
- Six primers: F3/B3, FIP/BIP, Loop-F/R
- High sensitivity and specificity,

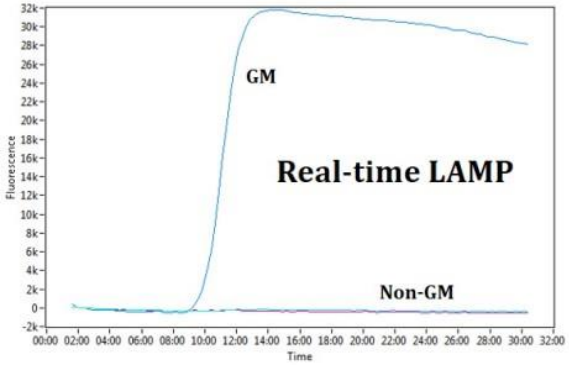
Visual and Real-time Loop-mediated Isothermal Amplification (LAMP): Rapid/On-site GMO Screening

TARGETS:
Promoters: *P-35S, P-FMV*
Marker genes: *aadA, nptII, uidA, pat, pmi*
Transgenes: *cry1Ac, cry2Ab2, cp4-epsps*

System	Chemistry	Detection Method	LOD	Completion Time
Heating Block	Bst DNA Pol.		40 copies	75 min
Thermal cycler	Bst DNA Pol.	End Point 	40 copies	75 min
Light Cycler®480 Real-time System	OptiGene Isothermal Master Mix	Real-time 	10 copies	45 min
Isothermal Real-time System (Genie II)	OptiGene Isothermal Master Mix		4 copies	35 min



LAMP assays for LMO detection





LAMP assays when combined with a fast DNA extraction method faciliate rapid, on-site GM detection at port of entry and farmer fields

Source: Singh M, Randhawa GJ, Sood P & Bhoge RK (2015) Food Cont. 51: 283-292.
Randhawa GJ, Singh M, Morisset D, Sood P and Žel J (2013) J. Agric. Food Chem. , 61(47): 11338-11346.

Conclusion

- ***…With the dramatic increase in number and diversification of GM events, appropriate GMO screening assays cut down the cost of GMO testing by eliminating need of specific assays for each and every GMO***

The developed DNA-based GMO screening assays would assist in rapid/cost-efficient GMO testing to check the GM status of a product, which would be utilized by GMO testing laboratories, regulatory and enforcement bodies & key stakeholders

The technology transfer and knowledge sharing is a step towards national capacity building and make in India effort…..availability of GMO testing kits in the country at lower costs

Acknowledgments:

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National Agricultural Innovation Project
Department of Biotechnology
Department of Science & Technology

