

# Chapter 2

## Genetically Modified Cotton in India and Detection Strategies

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### Abstract

India is one of the largest cotton-growing countries. Cotton is a fiber crop with varied applications from making tiny threads to fashionable clothing in the textile sector. In the near future, cotton crop will gain popularity as a multipurpose crop in India. The commercialization of *Bt* cotton in 2002 and consequently the fast adoption of *Bt* cotton hybrids by cotton farmers have enhanced the cotton production in India. Presently, genetically modified (GM) cotton has occupied 21.0 million hectares (mha) that comprise 14% of the global area under GM cultivation. In the coming years, improved cotton hybrids, with stacked and multiple gene events for improved fiber quality, insect resistance, drought tolerance, and herbicide tolerance, would further significantly improve the cotton production in India. With the dramatic increase in commercialization of GM crops, there is an urgent need to develop cost-effective and robust GM detection methods for effective risk assessment and management, post release monitoring, and to solve the legal disputes. DNA-based GM diagnostics are most robust assays due to their high sensitivity, specificity, and stability of DNA molecule.

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### 1. Introduction

Cotton (*Gossypium hirsutum* L.), a fiber crop, is being cultivated in an area of 11.0 mha in India (1), the largest cotton-growing country in the world. Though cotton is a fiber crop, it is regarded as a multipurpose crop in India because of its usage in the form of both cotton lint and cottonseeds. Cotton is used as (1) an edible oil for human consumption, (2) de-oiled cake as an animal feed, and (3) kapas for fiber (2). Keeping in view the economic importance of cotton and major biotic threat to cotton production, due to insect pests, GM cotton for insect resistance was developed. *Bt* cotton expresses insect resistance transgene from *Bacillus thuringiensis*, conferring resistance to bollworm, a lepidopteron insect

pest of cotton. In India *Bt* cotton was commercialized for the first time in 2002. Presently, Bt cotton is being cultivated in an area of more than 10.6 mha in India, which is 86% of the total cotton-growing area (1).

### 1.1. Commercialization of GM Crops in India

So far, cotton is the only GM crop which has been commercialized in India, occupying 15.4% of the global area under GM cultivation. The first *Bt* cotton event, i.e., MON531 (Bollgard® I), was commercialized in India way back in 2002. In 2011, 883 hybrids and 1 variety of six events, i.e., MON531 with *cryIAc* gene, MON15985 (Bollgard® II) with *cryIAc* and *cry2Ab* genes, Event1 with *cryIAc* gene, GFM-*cryIA* with fused *cryIAb* and *cryIAc* genes, Dharwad Event (Bt Bikaneri Nerma) in a variety with truncated *cryIAc* gene, and 9124 Bt cotton with synthetic *cryIC* gene in hybrids, have been commercially released (1) (Fig. 1). Out of these six commercialized events, three events, MON531, MON15985 (Maharashtra Hybrid Seeds Co. Ltd.), and GFM-*cryIA* (Nath Seeds Ltd.), have been imported in the years 1995, 2000, and 2002, respectively, whereas the other three events are indigenously developed, i.e., Event 1 developed in the year 2002 at IIT, Kharagpur, using indigenous *cryIAc* gene (3) and commercialized by J.K. Agrigenetics Ltd. while BN-Bt (4) was developed and commercialized by Central Institute of Cotton Research (CICR), Nagpur, in 2008 and event 9124 was developed and commercialized by Metahelix Life Sciences, Bangalore, in 2009 (1) (Table 1).

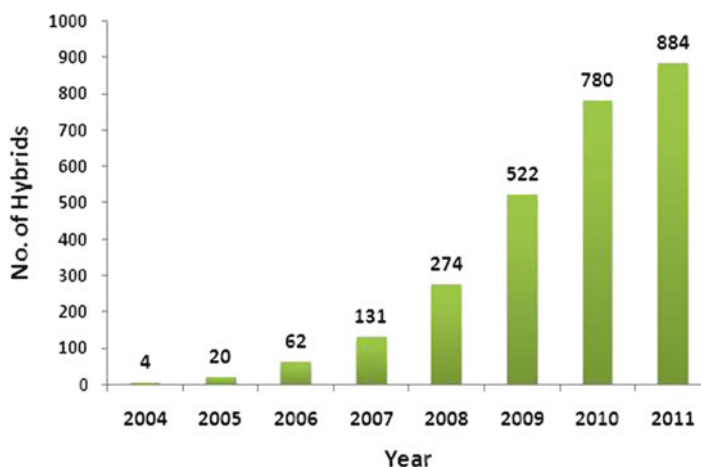


Fig. 1. Year-wise commercialization of Bt cotton hybrids (ISAAA, 2011).

**Table 1**  
**Commercially released hybrids/variety of six Bt cotton events in India**

S. no	Event	No. of hybrids/variety	Developer
1.	MON-531	215	Mahyco/Monsanto
2.	MON-15985	528	Mahyco/Monsanto
3.	Event-1	41	JK Agri-Genetics
4.	GFM event	96	Nath Seeds
5.	BNLA-601	2 <sup>a</sup>	CICR(ICAR) & UAS Dharwad
6.	MLS-9124	2	Metahelix Life Sciences

Source: ISAAA, 2011

<sup>a</sup>Bt Cotton variety

## 1.2. GM Cotton Events Commercialized, in Field Trials, and Imported Events in India

### 1.2.1. Commercialized GM Cotton Events

Till date, 883 hybrids and one variety of six events of Bt cotton have been commercialized in India. Out of these hybrids and variety, 59.7% hybrids are of stacked Bt cotton event (Bollgard® II) (Fig. 3).

1. Single gene GM cotton events: Five insect resistance commercialized Bt cotton events are single gene events, viz., (1) MON531 expressing *cryIAC* gene, (2) Event 1 expressing synthetic *cryIAC* gene, (3) GFM-cry1A expressing fused *cryIAC-IAb* gene, (4) BN-Bt expressing truncated *cryIAC* gene, and (5) MLS-9124 expressing *cryIC* gene. First three events occupy 24.3%, 4.63%, and 10.85% of total GM cotton area, respectively, whereas cotton hybrids of BN-Bt event and MLS-9124 event collectively cover 0.4% (Fig. 2).
2. Stacked GM cotton events: MON15985 (Bollgard® II), expressing insect resistance *cryIAC* and *cry2Ab* genes, is the only stacked event amongst the six commercialized events, covering 59.7% of total Bt cotton hybrids (Fig. 3). MON15985 has a trait for enhanced protection against a range of insects, viz., Spodoptera (a leaf-eating tobacco caterpillar), American bollworm, Pink bollworm, and Spotted bollworm. Due to better performance of stacked event of *Bt* cotton, higher profits are being earned due to cost savings associated (1) with lesser sprays for Spodoptera control and (2) increasing yield by 8–10% over single gene *Bt* cotton hybrids.

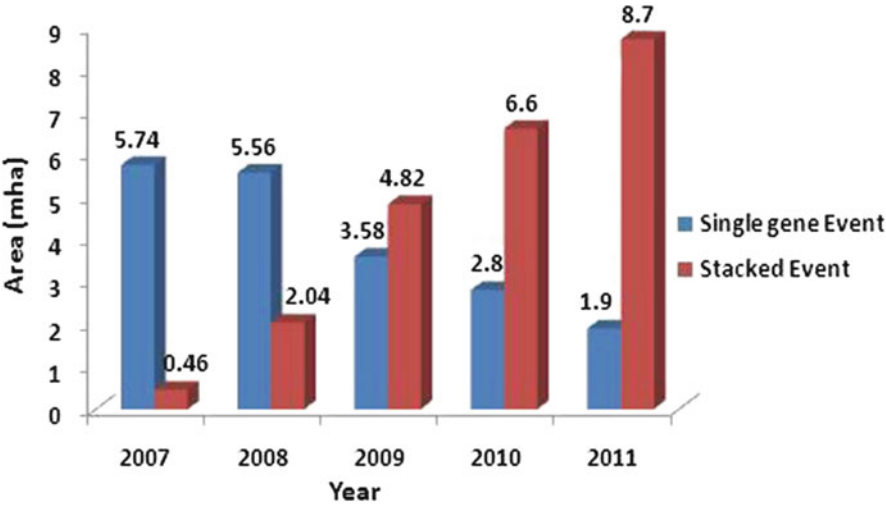


Fig. 2. Area under single gene and stacked gene Bt cotton hybrids (2007–2010). Source: ISAAA, 2011.

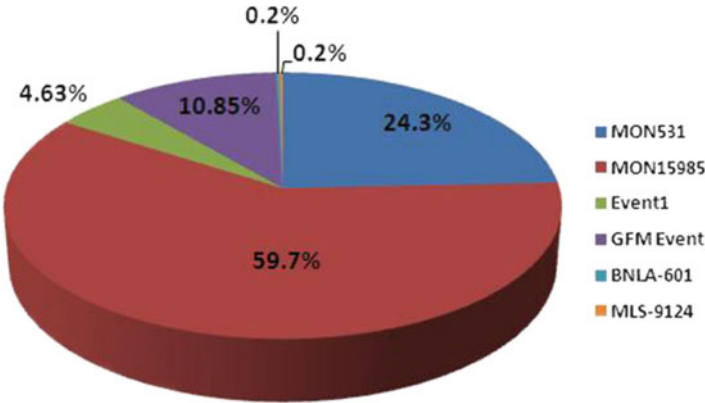


Fig. 3. Commercialized Bt cotton hybrids of six events. Source: ISAAA, 2011.

1.2.2. GM Cotton Events in Field Trials

Amongst the GM cotton events under field trials in India from 2009 to 2011, 54.6% are single gene events and 44.4% of the events are stacked (Table 2).

1. Single gene GM cotton events: Single gene events under field trials express *cryIAc*, *cryIEc*, *cryIF*, *cry2Ac*, and *cryIAb* for insect resistance and *epsps*, *2mepsps*, and *pat* for herbicide tolerance.
2. Stacked GM cotton events: Stacked GM events under field trials include:
  - (a) MON15985 x MON88913 (Bollgard®II-Roundup Ready Flex (BGIIRF®)) of Mahyco, expressing *cryIAc*, *cry2Ab*, and *epsps* genes for both insect and herbicide resistance.
  - (b) Widestrike of Dow Agrosiences, expressing *cryIF* and *cryIAc* genes for insect resistance.

**Table 2**  
**GM cotton in field trials in India (2009–2011)**

Event	Gene	Trait	Institute/company
2009			
Event-1	<i>cry1Ac</i>	Insect resistance	JK Agrigenetics Ltd, Hyderabad Dow Agrosciences India Pvt. Ltd., Mumbai
Event-24	<i>cry1EC</i>		
Widestrike	<i>cry1 Ac</i> and <i>cry1F</i>		
Event 3006-210-23 x Event 281-24-236			
2010			
WideStrike	<i>cry1Ac</i> and <i>cry1F</i>	Insect resistance	Dow AgroSciences India Pvt. Ltd., Mumbai
Event 3006-210-23 x Event 281-24-236			
Event-1	<i>cry1Ac</i>		JK Agri Genetics Ltd., Hyderabad Central Institute for Cotton Research, Nagpur
Event-24	<i>cry1EC</i>		
ILK-Bt 77-1 to ILK-Bt 77-7	<i>cry1Ac</i>		
Anjali-AcBt-1, Anjali- AcBt-2, Anjali-AcBt-3	<i>cry1Ac</i>		
Anjali-FBt-1, Anjali-FBt-2			
G-822-Bt (Desi Bt G 822-1 to Desi Bt G-822-4), PA255-Bt (CICR Bt Desi-1 to CICR Desi Bt-6)	<i>cry1F</i> <i>cry1Ac</i>		
Events MIR-cotton (1–131)	<i>cry1Ac</i> and <i>cry1EC</i>	Insect resistance	Krishidhan Seeds Ltd., Jalna
SP499 G, SP503 G, SP7017 G, SP7140 G, SP7139 G, SP7152 G, SP7230 G	<i>2mEPSPS</i>	Herbicide tolerant	Bayer Bioscience Pvt. Ltd, Hyderabad
2011			
GHB 119 T304-40	<i>cry2Ae/ PAT</i> <i>cry1Ab/PAT</i>	Insect resistance and herbicide tolerant	Bayer Bioscience Pvt Ltd., Hyderabad
MLS9124 or GFM CryIA event		Insect resistance	Metahelix Life Sciences Pvt. Ltd., Bangalore
MON 88913	<i>cp4epsps</i>	Herbicide tolerant	Maharashtra Hybrid Seeds Company Ltd., Jalna

Source: <http://www.igmoris.nic.in>

(c) Event-1 x Event-24 of J.K. Agrigenetics, expressing *cryIAc* and *cryIEc* genes for insect resistance.

### 1.2.3. Imported GM Cotton Events

Twenty six (26) GM cotton planting material have been imported for research purposes by various public and private research institutions from the USA, China, and Israel, through National Bureau of Plant Genetic Resources (NBPGR), the nodal organization under Indian Council of Agricultural Research (ICAR) for

**Table 3**  
**Details of imported GM cotton planting material for research purposes**

S. no	Trait (no. of imports)	Transgenes	Importing institute/company
1.	Insect resistance (14)	<i>cry1Ac</i> , <i>cry2Ab</i> , <i>cry1Ab</i> , <i>cry2Ae</i> , <i>cry1F</i> , <i>GFM cry1A</i>	<ul style="list-style-type: none"> <li>• Syngenta India Pvt. Ltd., Pune</li> <li>• Nath Seeds Ltd., Aurangabad</li> <li>• De-nocil Crop Protection Pvt. Ltd., Mumbai</li> <li>• Proagro, PGS Ltd., Gurgaon</li> <li>• Vikki's Agrotech Pvt. Ltd., Hyderabad</li> <li>• Bayer Bioscience Pvt. Ltd., New Delhi</li> </ul>
2.	Herbicide tolerance (5)	<i>epsps</i> , <i>2mepsps</i> , <i>bar</i>	<ul style="list-style-type: none"> <li>• Maharashtra Hybrid Seeds Co. Ltd., New Delhi</li> <li>• Maharashtra Hybrid Seeds Co. Ltd., Mumbai</li> <li>• Bayer Bioscience Pvt. Ltd., New Delhi</li> </ul>
3.	Insect resistance and herbicide tolerance (4) (Stacked Events)	<i>cry1Ac</i> , <i>cry2Ab</i> , <i>cry1F</i> , <i>epsps</i>	<ul style="list-style-type: none"> <li>• Emergent Genetics India Pvt. Ltd., Hyderabad</li> <li>• Monsanto Genetics India Pvt. Ltd., Mumbai</li> </ul>
4.	Abiotic stress tolerance (3) Drought tolerance Drought and salinity tolerance	<i>35 S rol A, B, and C</i> ; <i>Mannosyl transferase</i> <i>At A-20</i> , <i>At SOS1</i> , <i>At SOS2</i> , <i>At-ANP1</i> , <i>At-CBF3</i>	<ul style="list-style-type: none"> <li>• Nath Seeds Ltd., Aurangabad</li> <li>• Ankur Seeds Pvt. Ltd., Nagpur</li> </ul>

import and quarantine processing of transgenic planting material (Table 3).

1. Single gene GM cotton events: More than 50% of these imports are for insect resistance, and other GM events are for herbicide tolerance, for both insect and herbicide resistance, and for abiotic stress tolerance (Fig. 4a, b).

These imports constitute a range of GM traits with an array of transgenes:

- (a) Insect resistance: *cry1Ac*, *vip3A*, *cry1Ab-cy1Ac*, *cry1F*, *cry2Ab*, *cry1Ab*, and *cry2Ae*.
- (b) Herbicide tolerance: *epsps*, *2mepsps*, and *bar*.
- (c) Insect Resistance and Herbicide Tolerance (Stacked Events): *cry1Ac*, *cry2Ab*, *cry1F*, and *epsps*.
- (d) Abiotic stress tolerance: *35 S-rolA*, *B*, and *C*, *Mannosyl transferase*, *At A-20*, *At SOS1*, *At SOS2*, *At-ANP1*, and *At-CBF-3*.

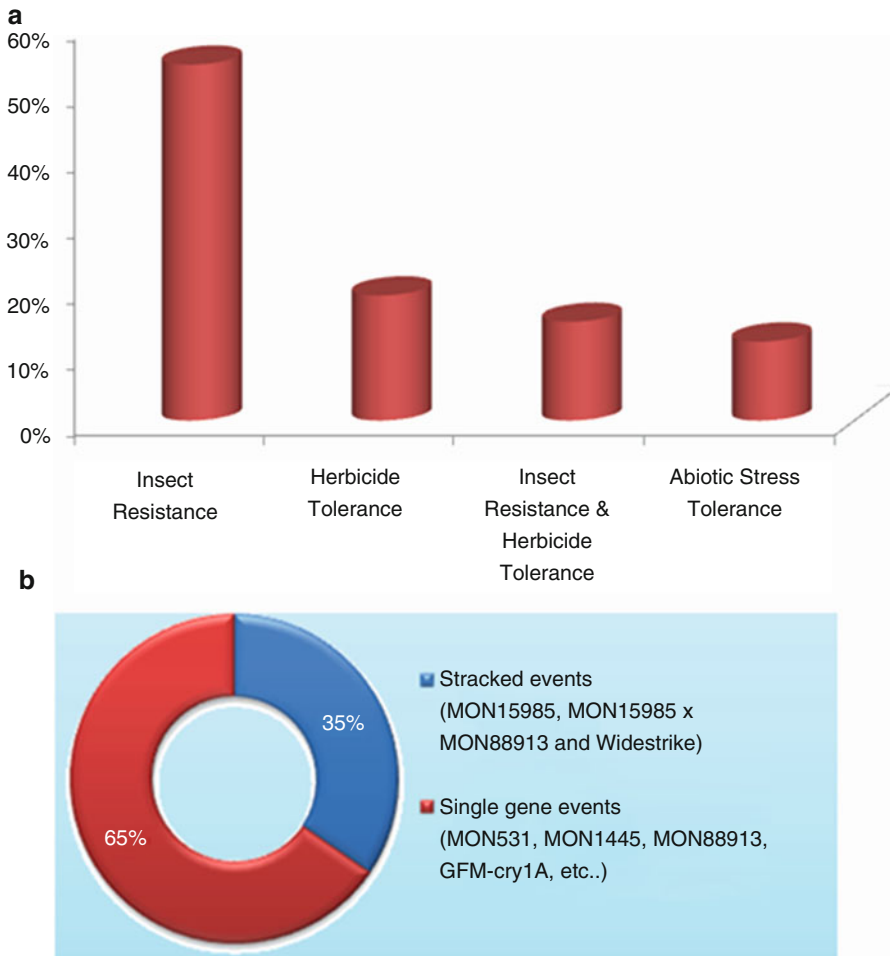


Fig. 4. (a) Trait-wise analysis of imported GM cotton planting material in India; (b) stacked and single gene events in imported GM cotton in India.

2. Stacked GM cotton events: Out of the 26 imports of GM cotton planting material, 34% of imports are of stacked GM events including Bollgard®II, BGIIRRF® and Widestrike.

The trend of GM cotton commercialized, imported, and under field trials, in India, clearly indicates that the stacked events are going to be much more in demand in the near future.

## 2. Detection Methods Employed for GM Cotton

The development, commercialization, and deployment of GM crops, both in terms of acreage of cultivated land as well as event/trait diversification, are increasing dramatically. The availability of reliable and robust assays, reference materials, and analytical methods that

allow identification and accurate determination of GM content/trait in crops is an important key element to meet the regulatory obligations and legislative requirements as well as to effectively address the biosafety issues pertaining to GM crops.

Among conventional PCR technologies, multiplex PCR is time efficient and cost-effective, which can detect multiple target sequences of inserted gene construct in a single reaction. The multiplex PCR assays for screening of different *Bt* crops either commercialized or under field trials in India have been developed (5). Several multiplex PCR methods have been developed and validated for precise and accurate monitoring, tracing, and regulation of GM cotton (6–9). A decaplex PCR and real-time PCR for identification and differentiation of MON531 and MON15985, two major commercialized events of *Bt* cotton in India, have also been reported (6) (Table 4).

To increase the accuracy, sensitivity, and reproducibility for detection of GM crops and for automatic and high throughput, multiplex PCRs have been coupled with other methods. In 2009, Nadal et al. developed a multiplex PCR assay coupled to capillary gel electrophoresis for amplicon identification by size and color (multiplex PCR-CGE-SC) for simultaneous detection of cotton species and five events of GM cotton, viz., Bollgard®I, Bollgard®II, Roundup Ready, 3006-210-23, and 281-24-236 (10). Real-time quantitative PCR method for detection of Widestrike GM cotton event 281-24-236/3006-210-23 was developed based on detection of DNA sequences in the junction between the transgene insert and cotton genome (11). Lee et al. (2007) reported the qualitative and quantitative detection of GM cotton events MON15985 and MON88913 using two kinds of specific primer pairs, probes, and one standard plasmid, and confirmed the applicability for practical use by in-house validation experiment (12). Real-time PCR assays have also been developed by our laboratory for quantification of *cry1Ac* and *cry2Ab* genes in two *Bt* cotton events, viz., MON531 and MON15985 of (6).

For rapid screening of GM cotton expressing *chitinase* (*chi*) gene and *Bt* cotton containing the *cry1A(b)* gene, a visual and rapid loop-mediated isothermal amplification (LAMP) assay was developed by Rostamkhani et al. in 2011 (13). This method can amplify nucleic acids with high specificity, sensitivity, and speed under isothermal conditions (14).

Tohidfar et al. have reported PCR and southern blot analysis to confirm the integration of *cry1Ab* and *nptII* transgenes into the GM cotton genome. Western immunoblot analysis of proteins extracted from leaves of GM cotton revealed the presence of an immunoreactive band with a molecular weight (MW) of approximately 67 kDa in transgenic cotton lines using the anti-Cry1Ab polyclonal antiserum (15).



**Table 4**  
**Summary of DNA-based detection systems being employed for GM cotton**

GM event/crop	PCR system	Target gene	References
MON531 and MON15985	Decaplex PCR	<i>cry1Ac</i> and <i>cry2Ab</i> transgenes; <i>nptII</i> , <i>aadA</i> , and <i>uidA</i> marker genes; <i>CaMV</i> 35S promoter and <i>nos</i> terminator; two construct-specific sequences, i.e., <i>cry1Ac</i> transgene construct and <i>cry2Ab</i> transgene construct; and endogenous <i>Sad1</i> gene	(6)
	Real-time PCR	Quantification of <i>cry1Ac</i> and <i>cry2Ab</i> genes	
GM cotton (VipCot14, VipCot29)	Multiplex; construct-specific PCR	<i>vip-s</i> gene and <i>vip3A</i> -like genes, <i>CaMV</i> 35 S promoter, <i>nos</i> terminator, and <i>npt II</i> marker gene	(9)
MON15985	Multiplex PCR	<i>Cry2Ab</i> , promoter, terminator, and <i>nptII</i> genes	(7)
Widestrike cotton (Event 281-24-236x 3006-210-23)	Quantitative real-time PCR	A cotton-specific endogenous reference gene <i>SAH7</i> and events 281-24-236 and 3006-210-23	(11)
Mon531, GK19, SGK321	Conventional as well as quantitative	Cowpea trypsin inhibitor ( <i>CpTI</i> ) gene of SGK321 cotton and the specific junction DNA sequences containing partial <i>CryIA(c)</i> gene and <i>NOS</i> terminator of Mon531, GK19, and SGK321 cotton varieties	(8)
Bollgard I, Bollgard II, Roundup Ready, 3006-210-23, and 281-24-236	Multiplex PCR-CGE-SC	Bollgard I, Bollgard II, Roundup Ready, 3006-210-23, and 281-24-236	(10)
MON15985, MON88913	Event-specific qualitative PCR and quantitative real-time PCR	MON15985, MON88913	(12)
GM Cotton	LAMP	<i>Chitinase</i>	(13)
Bt Cotton	PCR, Southern Blot and Western Blot	<i>CryIAb</i> , <i>nptII</i>	(15)

### 3. Detection Methods for GM Cotton in India

CICR, Nagpur, has developed three Bt cotton testing kits, namely, Cry1Ac Bt-Quant, an ELISA kit; Cry 1Ac Bt-detect, a dot-blot assay kit; and Cry1Ac Bt express, a dip-stick format, for the detection of Bt toxin. These kits have been effectively deployed to verify

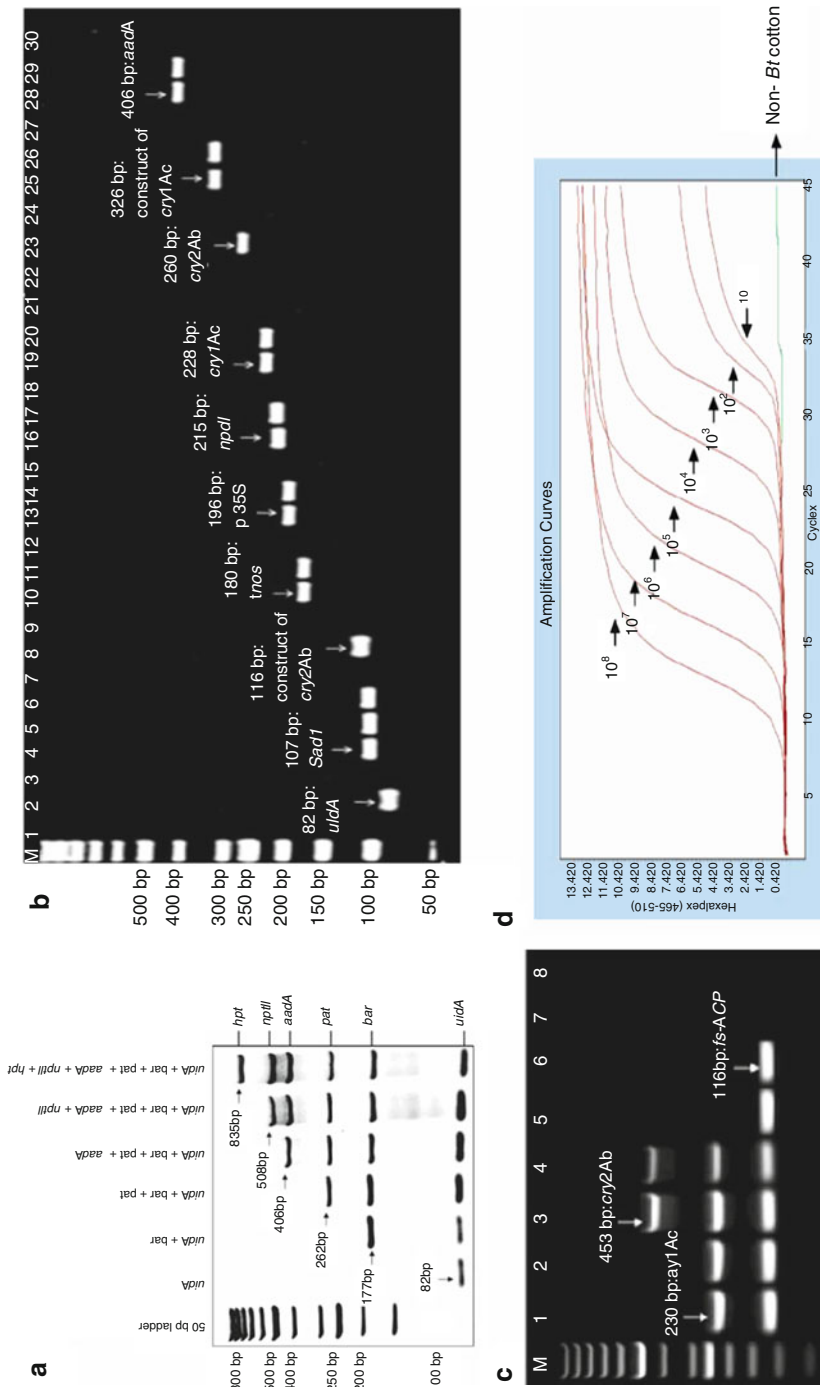


Fig. 5. (a) Hexaplex PCR for simultaneous amplification of six commonly used marker genes, i.e., *uidA*, *bar*, *pat*, *aadA*, *nptII*, and *hpt*; (b) simplex PCR for amplification of inserted genes, construct-specific sequences, and endogenous gene in two Bt cotton events, i.e., MON531 and MON15985, using primer pairs for *cry1Ac* and *cry2Ab* transgenes, *nptII*, *aadA*, and *uidA* (continued) marker genes, *CaM/35S* promoter, *nos* terminator, endogenous *Sad1* gene, and specific gene constructs in MON531/MON15985 and MON15985; (lane M) 50 bp ladder; (lanes 1, 4, 7, 10, 13, 16, 19, 22, 25, 28) samples of MON531 cotton; (lanes 2, 5, 8, 11, 14, 17, 20, 23, 24, 29) samples of MON15985 cotton; (lanes 3, 6, 9, 12, 15, 18, 21, 24, 27, 30) samples of non-GM cotton; (c) triplex PCR to differentiate MON531 and MON15985 Bt cotton events, Lane M: 50 bp ladder, Lanes 1–2: MON531, Lanes 3–4: MON15985, Lanes 5–6: Non-GM Cotton, Lane 7: Water control; (d) amplification curves generated for eight serial dilutions of standard plasmid with 10 to 10<sup>8</sup> copies of *cry2Ab* gene.

the purity of Bt seed and ensure the supply of quality Bt hybrid seed to the farming community.

NBPGR, New Delhi, has also developed robust DNA-based GM diagnostics for initial screening and for identification and quantification of GM content in more than ten GM crops. Some of these GM diagnostics, with special reference to GM cotton, are the following:

1. For initial screening of GM crops for checking the GM status of a sample irrespective of crop and trait, PCR assays have been developed targeting commonly used markers, promoter and terminator genes in simplex and multiplex formats.
2. Decaplex and triplex PCR assays have been developed to differentiate between two major commercialized Bt cotton events (covering more than 80% of the total cultivated area for GM cotton) in India, viz., MON531 and MON15985 along with simplex PCRs for each transgene element present in these two Bt cotton events (5, 6) (Fig. 5b).
3. Real-time PCR-based quantitative analysis of *cry1Ac* gene in Bt cotton events, MON531 and MON15985; *cry2Ab* gene in MON15985 has also been developed (6) (Fig. 5c).
4. Rapid and cost-effective diagnostic kits for GM cotton events, viz., Bollgard®I (MON531) and Bollgard®II (MON15985), have also been developed.
  - (a) Hexaplex PCR assay has been developed for simultaneous amplification of commonly used six marker genes, i.e., *aadA*, *bar*, *hpt*, *nptII*, *pat*, and *uidA* (16) (Fig. 5a).
  - (b) Heptaplex PCR assay simultaneously amplifying a combination of marker genes, *nptII*, *aadA*, *pat*, and *uidA* and regulatory elements, viz., *CaMV* 35 S, *nos* promoters, and *nos* terminator, has also been developed.

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