Chapter 16
Critical Evaluation of GM Cotton

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16.1 Introduction

Genetically modified (GM) crops mainly aimed to increase crop protection through the introduction of resistance against insects and diseases and tolerance to herbicides. Since their approval for commercial cultivation, they have been adopted in no less than 70 countries across the globe during the last 2 decades. During the span of 23 years (1996–2018), global area coverage of GM crops with now 2.5 billion hectares amounted to an upsurge of ~113-fold since 1996 (ISAAA 2018). Till 2018, GM cotton held a global area of 24.9 million hectares (Mha), making it the third most adopted GM crops, ranked right after soybean and maize (Burkitbayeva et al. 2016; ISAAA 2018). As per 2017 FAO global crop area coverage report, GM cotton shared 76% of the global cotton area in 2018 (ISAAA 2018). Notably, among all GM crops, Bt cotton has gained immense popularity and acceptability among smallholder farmers across the globe (Burkitbayeva et al. 2016). No wonder, Bt cotton has been the sole adopted and cultivated GM crops among several countries, particularly in developing nations like India (11.6 mha), Pakistan (2.8 mha), Myanmar (0.3 mha), Sudan (0.2 mha), Mexico (0.2 mha), and Eswatini (<0.1 mha) (ISAAA 2018). As a matter of concern, most of such developing nations are still lacking a sound regulatory milieu for strict monitoring of illegal and unauthorized dissemination of GM seeds or planting materials among the local cotton growers. As for instance, 6 out of 11 top GM crop-growing countries around the globe had recorded illegal cross-border permeation and released unauthorized GM seeds for sale preceding any approval from concerned regulatory authorities. Interestingly, all those

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six aforementioned countries happened to be developing nations, namely, China, India, Pakistan, Brazil, Bolivia, and Paraguay (Ramaswami et al. 2012; Sinebo and Maredia 2016). On the contrary, such unauthorized and reckless adoption of illegal GM seeds by allured farmers at times resulted in the cultivation of substandard GM crops with below par yield and quality traits. This often turns out to be disastrous, particularly for resource-poor farmers, who are easy targets by opportunistic local vendors who persuade and lure farmers to pay hefty amounts to purchase illegitimate spurious GM seeds. As a result, the poor farmers are often left marginalized and empty-handed with meager returns from their crops at the end. Moreover, this problem turns more troublesome if there is an accidental admixture of illegal GM seeds among non-GM or authorized GM seeds. GM crops containment is also necessary to restrict unwanted "transgene" (a gene which is artificially introduced into the other organism) gene flow across cross-compatible species, including wild or weed relatives (Ryffel 2014). Cotton being primarily a self-pollinated crop, most transgene flow in cotton is linked to uncontrolled GM seeds dissemination via livestocks and secondary cross-pollinations (Ryffel 2014), besides illicit man-made ventures. Hence, it is extremely crucial to trace illegitimate GM crops including largely popular Bt cotton, with robust precision to facilitate firm regulation over GM entities.

16.2 Development of Transgenic Plant

GM plants are also called transgenic plants whose DNA is modified with new useful traits using genetic engineering techniques which include resistance to insects, diseases, tolerance to adverse environmental conditions, high yield and nutritional quality, production of edible vaccine, etc. The process of transgenic development is to assemble a gene or combination of genes isolated from various sources to develop an improved crop plant. The very first transgenic plant was tobacco expressing antibiotic resistance gene in 1982 (Herrera-Estrella 1983). The first field trials of genetically engineered plants were for herbicide-resistant tobacco plants cultivated in France and the USA in 1986 (Clive 1996). The first genetically engineered insect-resistant plant was developed by incorporating genes from *Bacillus thuringiensis)* (Bt) that produced insecticidal proteins into tobacco (Vaeck et al. 1987). The first genetically modified food crop tomato (Flavr Savr™) (Kramer and Redenbaugh 1994) for consumption was developed for delayed ripening by Calgene's (USA) in 1994. Transgenic cotton resistant to lepidopteron insect with Bt gene (*cry1Ac)* is the first transgenic plants globally commercialized in 1995 (USA). In 1995, Bt cotton (Monsanto), bromoxynil-resistant cotton (Calgene), glyphosate-resistant soybeans (Monsanto), Bt maize (Ciba-Geigy), virus-resistant squash (Asgrow), and additional delayed ripening tomatoes (DNAP, Zeneca/Peto, and Monsanto) were developed. The principles involved in the generation of transgenic plant are discussed below. The process of genetic engineering and development of transgenic plants requires the following steps:

(a) Isolation of genes/gene constructs
(b) Genetic transformation methods
(c) Regeneration of plants through somatic embryogenesis

16.2.1 Isolation of Genes

Isolation of genes for agronomically important traits like higher yield, improved quality, pest and disease resistance, herbicide resistance, and tolerance to heat, cold, and drought is possible to produce millions of copies and determine their nucleotide sequence. Smith and Welcox (1970) discovered restriction enzymes which cut DNA at specific places, enabling to isolate genes from an organism (Roberts 2005). The identified gene of interest should be characterized for its regulation, effect on the plant, and interaction with other genes in the same biochemical pathway. Once a gene has been isolated, it is cloned in a bacterial vector with requisite modifications before transferring into a plant. Genetic engineering has broken down the species boundary, as the entire organisms have DNA as the basic material. The genes for transformation can be obtained from a wide range of sources, like the primitive organisms and virus to multicellular organism including human, and also can be synthesized de novo from the complete genome sequences of the organism. Much effort in recent years has been devoted to identifying potential target genes for use in genetic engineering for economically important traits especially biotic and abiotic stress resistance and improvement of quality traits. The process has been accelerated by reference to the rapidly expanding bioinformatics databases, by progress in elucidating the plant and bacterial genomes. There is no doubt that the use of insect resistant and herbicide tolerant (Howe and Jander 2008), singly and in combination, have been successful in practice, aside from social and environmental concerns. Gene pyramiding or stacking appears to confer relatively greater benefit as reported in case of increased expression of biotic stress regulatory gene (Claire 2005). The gene of interest should be free from technical complexity, issues of food/feed safety, and consumer health risk.

16.2.2 Gene Construct

Gene construct can be defined as engineered DNA fragment to be transferred, integrated, and expressed in the genome of the target plant. Apart from the gene of interest, promoter (“starter”) and a terminator (“stop signal”) are required for expression. In most cases, additional sequences are included, e.g., marker genes, which are essential for the selection of transformants and this gene also accompanied by a promoter and a terminator. In case of *Agrobacterium* gene construct, the left and right border sequences are essential units flanking the abovementioned

genes, and these are collectively called as gene construct. The promoter region is typically located at the 5′ upstream of a gene. Promoters are known for their function in governing gene expression, to an on/off switch. The promoters can be categorized into three main groups: constitutive promoters, tissue-specific promoters, and inducible promoters (Hernandez-Garcia and Finer 2014). The upstream of each gene contains regulatory information about how and when the gene is to be expressed. The area binds to proteins (RNA polymerase) that are needed for gene expression (transcription). All genes must have a promoter in order to be expressed. Genes transferred by genetic engineering must be accompanied by a promoter. Some promoters are active in all cells at all times called constitutive promoter (e.g., 35S CAMV promoter), while others are specific to different organisms or tissue types (e.g., seed specific). Others are sensitive to external signals such as temperature or the presence of a certain chemical. Such promoters can be used as controllable on/off switches for genes. The 35S CaMV promoter is very strong well-known constitutive promoter and widely for the plant transformation. It was discovered at the beginning of the 1980s, by Chua and collaborators at the Rockefeller University. The antibiotics kanamycin (*nptII*, encoding neomycin phosphotransferase) and hygromycin (*hptIV*, encoding hygromycin phosphotransferase, isolated from *E. coli*) are mostly used and in herbicides glyphosate (EPSPS, 5-enolpyruvate shikimate-3-phosphate synthase). PMI marker gene that allows metabolic selection for transgenic plants was used in golden rice (Hoa et al. 2003). Marker-free transgenics were developed with site-specific recombinases that cleave a marker gene within two specific sites (Hare and Chua 2002). The common reporter genes used to monitor plant transgene expression include *gus* (beta-glucuronidase), beta-galactosidase (*LacZ*), green fluorescent protein (GFP), luciferase (*Luc*), and *Chloramphenicol acetyltransferase* (CAT) (Jefferson et al. 1987), and reporter gene is most commonly used in the gene construct.

16.2.3 Genetic Transformation

16.2.3.1 Vector-Mediated Gene Transfer

Agrobacterium tumefaciens was discovered in the early 1970s, and it naturally transfers DNA (T-DNA) with "*onc* genes" in the Ti plasmid into the plant cell and produces crown gall (Christie and Gordon 2014). *A. tumefaciens* used for plant transformation are disarmed by removing the tumor-promoting and opine-synthesis genes and replaced with the desired foreign gene or selective markers, enabling the incorporation of foreign genes into plant's genome, transiently or stably (Van Montagu and Zambryski 2013). Vector is a DNA molecule which consists of insert (transgene), origin of replication, and a larger sequence that serves as the "backbone" of the vector. There are a number of vectors available; however, for genetic transformation in plant system, plasmid vectors are commonly used. Many plasmids are commercially available for such uses. The gene of interest is cloned along

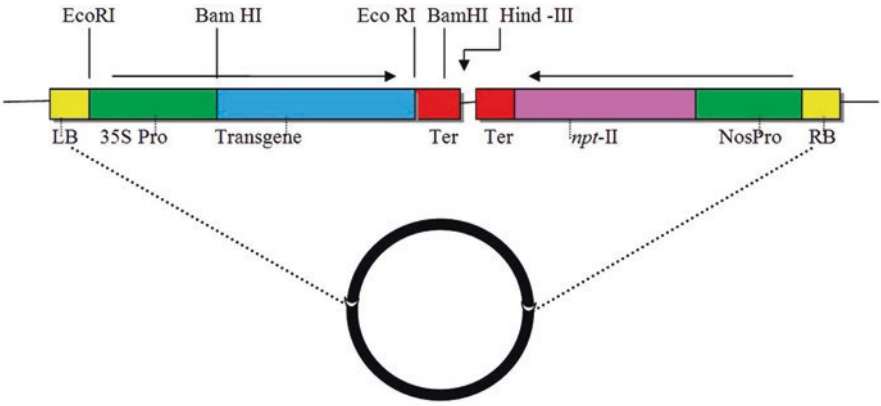

Fig. 16.1 A typical plant transformation binary vector map. *LB* left border of T-DNA, *35S Pro* CaMV promoter (constitutive), *Ter* terminator, *NosPro* nopaline promoter of *Agrobacterium*, *npt-II* neomycin phosphotransferase II gene, *RB* right border of T-DNA

with other selection marker genes (antibiotics) at multiple cloning sites (MCS, or polylinker), which has restriction site in a short region for the insertion of DNA fragments. T-DNA has left and right border repeat sequences which are essential genetic element (Fig. 16.1). T-DNA border repeat sequences (T-DNA borders) contain 25 bp that are highly conserved in all Ti and Ri plasmids (Barker et al. 1983). The binary vector system has cassettes with T-DNA (l0–15 kb) which are subsequently transferred into the plant genome with the *vir* genes residing on a separate helper plasmid that produces stable transgenics. A co-integration system developed through homologous recombination between disarmed Ti plasmid and an intermediate vector could be used to transfer many genes (about 150 kb) (De Framond et al. 1983). Among all the methods of transformation, *Agrobacterium* mediation is a good system to obtain transgenic plants with lower copy number, intact transgene, appropriate segregation, and transgene expression (Dai et al. 2001).

16.2.3.2 Direct Gene Transfer (Physical Methods): Particle Bombardment/Microprojectile

Particle bombardment was first described as a method for the production of transgenic plants (Sanford et al. 1987). In this method using a "gene gun" (Helios® Bio-Rad), the naked DNA/plasmid carrying the gene were coated with tungsten/gold particles and shot into the target tissue/plant cell under high pressure of helium gas. The fast-moving particles penetrate through the plant cell wall, directing the coated DNA into the nucleus. The efficiency of transformation is highest with gold particles in the range of 0.7–1.0 μm mean diameter (Southgate et al. 1995) especially for the recalcitrant cereals and major agronomic crops (McCabe and Christou 1993) as well as in the reduction of the amount of coated DNA on the microcarriers via

biolistic gun (Sivamani et al. 2009). The other direct methods such as electroporation (using electrical pulse with protoplasts, Hui 1995), microinjection (using micromanipulator with cells/protoplasts, Banks and Evans 1976), macroinjection (using immature embryo and pollen, Zhou et al. 1983), and pollen-tube pathway (PTP) utilize the normal fertilization cycle to eliminate the difficulty in regeneration. PTP-based transformation is an injection/delivery of naked DNA/drop of DNA solution to the stigma/top of the style into ovaries to produce transformed progeny (Touraev et al. 1997). This procedure was tried first in rice, wheat, and soybean. This method minimizes the time, expense, and recalcitrant plant cell culture and regeneration (Wang et al. 2013). Chemical methods such as polyethylene glycol (PEG) are used to disrupt cell membrane permeating the entry of foreign DNA (Lazzeri et al. 1991; Kofer et al. 1998). Silicon carbide fibers were used for wounding to improve frequency of *Agrobacterium*-based transformation in cotton (Arshad et al. 2013). However, *Agrobacterium tumefaciens*-mediated transformation is the best method over other transformation methods since, reduction in transgene copy number and intact gene sequence integration and segregation (Jones et al. 2005).

16.2.4 Regeneration of Plants Through Somatic Embryogenesis

The development of transgenic plants requires an efficient regeneration system. Regeneration through somatic embryogenesis is ideal over organogenesis because the entire plant is regenerated through a single somatic cell (Merkle et al. 1995). Shoemaker et al. (1986) induced somatic embryos in *G. hirsutum* cultivars Coker 201 and Coker 315 by manipulating culture media. The somatic embryos were derived from isodiametric, densely cytoplasmic cells and regenerated embryos from the hormone-free medium. Later a number of groups have regenerated Coker lines by somatic embryogenesis (Trolinder and Goodin 1987; Finer 1988; Firoozabady and DeBoer 1993) and other lines Sicala, Siokara (Cousins et al. 1991; Rangan and Rajasekaran 1996), Simian (Zhang et al. 2001), and Acala (Rangan 1993; Rangan and Rajasekaran 1996). Perlak et al. (1990) introduced *cry1Ab* and *cry1Ac* genes into cotton (*G. hirsutum*) plants, and transformed plants showed a high level of resistance to *Helicoverpa*. Bt gene inserted Coker-312 plants were used as mother plant to transfer Bt trait to other cotton cultivars by back crossing method.

The development of transgenic plants is severely constrained by the poor regenerative capacity of cotton plants (Zhang et al. 2011). Plant regeneration through somatic embryo is a long and complicated process. Initially, the transformed cells dedifferentiate into calli on the culture medium; then after several weeks, embryogenic callus undergoes into four stages like globular, heart-shaped, torpedo, and cotyledonary stages, which ultimately grows into a complete plant. The process of converting a non-embryogenic callus into embryogenic callus is the key bottleneck step (Zhang et al. 2011; Shang et al. 2009; Rajeswari et al. 2010). Somatic embryos

formation is a complex dynamic process that involves many intracellular metabolic changes and is influenced by various external environmental factors. The underlying temporal-specific expression of genes plays an important role in this process. Sun et al. (2018) studied the molecular mechanism of conversion from non-embryogenic callus into embryogenic callus in cotton which allows for the identification of novel genes involved. They compared transcriptome changes in the transformation from non-embryogenic callus into embryogenic callus and identified 46 transcripts that may contribute to initiating embryogenic shift. Analyzing the transcriptional activity of genes during the transition from non-embryogenic callus to embryogenic callus may help to reveal the molecular mechanisms involved in the acquirement of embryogenic potential, which, in turn, may provide ideas to facilitate the induction of embryogenic callus to further promote regeneration of a wider range of cotton cultivars for genetic modification.

16.2.5 Transgene Integration and Inheritance

The transgene integration into plant cells may be stable or transient. If the transgene integrates into the nucleus of the plant cell, then it is a stable transformation. The transgene gets integrated into the chromosome of the genome, and the copy number replicates along with the chromosome, and they are inherited in the next generation of the transgenic plant. In the transient expression, the transgene is not integrated into the nucleus, and they remain for a limited period and are not replicated and lost through cell division. Stable integration and expression depend on the gene construct and transformation method (Gelvin 2005). The transgene of a stable transgenic plant inherit in a Mendelian fashion. However, transgene inheritance depends on the location of integration and the copy number (Tizaoui and Kchouk 2012).

16.3 GM Cotton Detection Methods

Detection methods for genetically modified crops (GM crops) are primarily divided into gene-based and protein-based approaches (Holst-Jensen et al. 2012; Yu-jia et al. 2020). In addition, phenotype-mediated detection of GM crops is also considered as a cheap and effective tool (Holst-Jensen et al. 2012; Kamle et al. 2017) (Fig. 16.2). Traits like herbicide tolerance (HT) and insect resistance (IR) are often monitored through phenotyping via specific bioassays which are deemed to be accurate and user-friendly approach for quality assurance of GM crops (Kamle et al. 2017). For instance, phenotype-based herbicide bioassay for detection of glyphosate-tolerant Roundup Ready cotton is commercially available (Lübeck 2002). However, phenotype-based detection of GM crops has a narrow spectrum of application. As a matter of fact, for precise phenotype-mediated detection, the trait pertaining to the GM crops should ideally be phenotypically distinctive. Moreover,

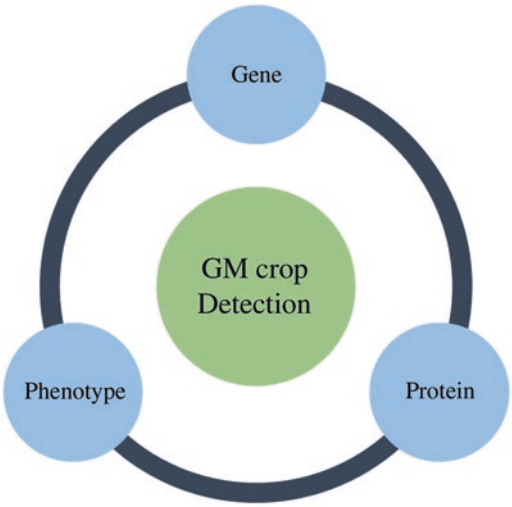

Fig. 16.2 The three basic biological raw materials for detection of GM crops

a GM crop must be grown for phenotype-based trait assessment which can be time-consuming as well. Hence, phenotype-based approaches for GM detection are not always apropos.

16.3.1 Gene-Based Approaches

Gene-/DNA-based approaches for GM detection encompass several biotechnological tools which include PCR-based tools, LAMP-PCR, microarray tools, etc. (Table 16.1). Moreover, the classical genomics tool, namely, southern blot analysis, can precisely reveal exact copy number of transgene integration in host crops (Kamle et al. 2017). Nevertheless, most of these gene-/DNA-based detection tools for GM crops are based upon four analytical modules, viz., species-/taxon-specific, element-specific, construct-specific, and event-specific (reviewed in detail by Holst-Jensen et al. 2012). Taxon-/species-specific modules are used to trace any specific DNA/gene sequence exclusive to a particular species. For instance, cotton-specific stearoyl-acyl carrier protein desaturase (*Sad1*) gene is often targeted as an endogenous reference for detection of GM cotton (Yang et al. 2005a, b). Element-specific modules can trace discrete inserted DNA motifs like promoter or terminator or intron/exon, etc., while construct-specific modules target the region bridging two distinct adjoining DNA elements, viz., promoter-transgene junction representing man-made transgene constructs (Randhawa et al. 2016). Event-specific modules target a DNA sequence motif spanning the region of junction between the transgene construct and the host's genomic DNA. Event-specific target sequence motif represents a unique signature tag pertaining to particular GM crops and, hence, provides explicit detection of a specific GM event with an irrefutable edge over the other

Table 16.1 Tools for gene-/DNA-based approach for GM detection, their applications, and key advantages

DNA-based GM crops detection tools	Application	Key advantages
Quantitative real-time PCR (Fraiture et al. 2015)	Can monitor PCR products with respect to time and quantify those amplicons by measuring a specific signal, often emitted through fluorescence signal	Sensitive, precise, and ease of quantification pertaining to GM crops detection
Multiplex PCR (Kamle et al. 2017)	Can concurrently target and amplify several DNA sequences in a single (PCR) reaction	Time-saving and cost-effective, yet sensitive, tool for GM detection
LAMP-PCR (Singh et al. 2020)	An isothermal nucleic acid amplification technique where target DNA sequence(s) can be amplified at constant temperature by the action of *Bst* polymerase (extracted from *Bacillus stearothermophilus*). The results can be obtained rapidly and visually observed by simple colorimetric approach	An excellent alternative to conventional PCR; does not require thermocycler and can be performed at a constant temperature. Simple, rapid, and practically adaptable tool, amenable for on-site application
Real-time LAMP-PCR (Randhawa et al. 2015)	An advanced LAMP-PCR where targets can be monitored with respect to time measured using a fluorescent dye-based detection of LAMP-PCR amplicons	Quick and sensitive, found to detect up to two target copies within 35 min, amenable for on-site application for detection of GM crops
Genome walking/ Long Template Rapid Amplification of gDNA Ends (LT-RADE) (Spalinskas et al. 2013)	Restriction-independent genome walking technique to detect unknown DNA regions adjoining a known DNA segment of transgene elements	User-friendly, can trace flanking regions of transgenic insert
Padlock probe ligation—microarray (Prins et al. 2008, 2010)	Padlock probes are detection probes which recognize and hybridize target sites within DNA sequence. Upon hybridization, the probe gets linearized and ligated to form a circular probe molecule, which can be amplified by using labeled primer, thus facilitating detection of target site. This can be combined with microarray detection module for high-throughput detection	Suitable for simultaneous detection of multiple unauthorized GM crops samples

(continued)

Table 16.1 (continued)

DNA-based GM crops detection tools	Application	Key advantages
Digital PCR (dPCR) (Vogelstein and Kinzler 1999; Yu-jia et al. 2020)	dPCR sample mix containing similar components as that in qPCR is distributed and partitioned into large number of individual wells before amplification, with an assumption that each well would contain either 1 or 0 targets following Poisson distribution. Following this, PCR amplification is carried out, to determine and quantify the number of positive vs negative reactions measured via fluorescence signal	Advanced form of qPCR, but independent of internal control and endogenous reference genes; absolute quantification of target gene(s) in GM crops can be calculated using Poisson's statistics
PCR capillary gel electrophoresis technology (Fraiture et al. 2015)	Fluorescently labeled primers are used for multiplexed PCR which facilitate distinguishing between varied amplicons of the same size	Resolution power is much higher as compared to conventional gel electrophoresis while detecting PCR products from multiplexed assay and, hence, suitable for GM crops detection

analysis modules (Holst-Jensen et al. 2012; Randhawa et al. 2016). Several reports for module-specific GM detection in cotton are cited in Table 16.2.

16.3.2 Protein-Based Approaches

Protein-based detection of GM crops is majorly restricted to fresh or frozen samples, as proteins are usually prone to denaturation due to heat or rigorous processing of plant samples. However, protein-based detection tools are remarkably reliable, as those can trace the actual product of the transgene from GM crops. Tools like enzyme-linked immunosorbent assay (ELISA), immunoassay strips, lateral flow strips, etc. are some of the popular and unswerving GM crops detection techniques that are often utilized as working principles behind several commercially available user-friendly kits. For instance, a series of ELISA and immunoassay-based detection kits, namely, "Bt-quant," "Bt-GUS," "Bt-Zygosity," and "Bt-Express," had been developed by ICAR-Central Institute of Cotton Research (India) for rapid detection and quantification of *cry* gene/*cry* toxin from Bt cotton transgenic plants (*http://www.cicr.org.in/tech_bank/Bt_kit.pdf; http://www.cicr.org.in/tech_bank/bt_express.pdf*). Especially, the "Bt-Express" kit became extremely popular and admired among cotton growers across India. It was largely because the kit was designed to be so simple and user-friendly that even illiterate farmers could detect presence or absence of Bt toxin from plant samples with ease, and hence, this detection kit deserves special mention. Apart from those, conventional proteomics techniques like western blot analysis (WB), mass spectrometry (MS), and two-

Table 16.2 Gene/DNA and protein-based tools for GM cotton detection

Detection approach	Basis of detection	GM detection tool	Detection modules	Application in GM cotton detection	References
Gene level (DNA)	PCR-based	qPCR	Event-specific	Cotton event DAS-81910-7	JCR GMOMETHODS, *http://gmo-crl.jrc.ec.europa.eu/gmomethods/*
		rtPCR	Element-specific	*cry2Ab2* detection	Dinon et al. (2011)
		qPCR	Taxon-specific	Fiber-specific acyl carrier protein	JCR GMOMETHODS, *http://gmo-crl.jrc.ec.europa.eu/gmomethods/*
		Multiplex real-time PCR	Taxon-specific	Stearoyl-acyl carrier protein desaturase gene	Cottenet et al. (2013)
		Multiplex PCR CGE	Taxon-specific	Acyl carrier protein 1 gene from cotton	Nadal et al. (2006)
		Multiplex PCR microarray	Taxon-specific	Stearoyl-acyl carrier protein desaturase gene	Prins et al. (2008)
		dPCR	Taxon-specific	*adhC* (alcohol dehydrogenase C gene from cotton)	Brod et al. (2014)
	DNA walking	LT-RADE (genome walking)	Construct-specific	LLCOTTON25 detection	Spalinskas et al. (2013)
	LAMP-PCR	LAMP-PCR	Construct-specific	*cry1Ac*, *cry2Ab2*, and *cp4-epsps* genes	Singh et al. (2020)
		Real-time LAMP	Event-specific	MON531 and MON15985	Randhawa et al. (2015)
	Microarray-based	PPLMD	Event-specific	*bar* MON1445	Prins et al. (2008)
	NGS		–	*cry1Ac*	Debode et al. (2019)

(continued)

Table 16.2 (continued)

Detection approach	Basis of detection	GM detection tool	Detection modules	Application in GM cotton detection	References
Protein level	ELISA	Monoclonal antibody-based sandwich ELISA	–	*vip3Aa*	Liu et al. (2020)
	Western blot	Western blot immunodetection	–	*cry*10Aa	Ribeiro et al. (2017)
	Immunostrips/ dipsticks	Immunostrip assay	–	*cry1Ac* and *cry2Ab*	Siddiqui et al. (2019)

NB: *PCR* polymeric chain reaction, *rtPCR* real-time PCR, *CGE* capillary gel electrophoresis, *qPCR* quantitative PCR, *dPCR* digital PCR, *LT-RADE* Long Template Rapid Amplification of gDNA Ends, *LAMP* loop-mediated isothermal amplification, *PPLMD* padlock probe ligation—microarray, *bar* phosphinothricin-*N*-acetyltransferases gene of *Streptomyces hygroscopicus*, *vip* vegetative insecticidal protein, *pat* phosphinothricin-*N*-acetyltransferase, *NGS* next-generation sequencing

dimensional SDS gel electrophoresis (2-DE) could be effectively employed to detect targeted proteins from GM crops (Ruebelt et al. 2006) (Table 16.2). In addition, using those techniques, the after-effects of transgene integration into GM crops with respect to their wild-type counterparts can be vividly studied. For instance, a comparative study encompassing Bt cotton and its non-transgenic counterpart was conducted to trace any transgene-induced unintended effects on Bt cotton. For this purpose, a combination of WB, 2-DE, and MS tools was used, which revealed that exogenous DNA could influence the growth and photosynthesis in GM cotton (Wang et al. 2015).

16.3.3 Advanced GM Detection Techniques

Apart from typical genomics and proteomics tools, several biophysical technique-based approaches are gaining popularity for their applications in detection of GM crops. Techniques like near-infrared (NIR) spectroscopy, surface plasmon resonance (SPR), surface-enhanced Raman scattering (SERS) spectroscopy, and biosensor-based detection are some of the biophysical tools which may be used in detection of GM crops (reviewed by Kamle et al. 2017), including GM cotton. In fact, SPR technology which is commonly used to study protein-ligand interactions could detect transgenic *cry1Ac* cotton with high accuracy, sensitivity, and rapidity (Zhao et al. 2013).

GM cotton is widely adopted and cultivated across the globe. The cutting-edge technology of BT cotton has been widely popular among cotton growers, including resource-poor farmers across the globe. Several countries, however, maintain strict biosafety regulatory monitoring following series of norms and standards before releasing and commercializing GM crops, including GM cotton. At times, to evade such strict biosafety regimes, local farmers and avaricious vendors often adopt illegitimate shortcuts, in a quest to maximize profits and, henceforward, promote spurious and illegal spread of unauthorized GM seeds and planting materials, overlooking the detrimental effects in the long run. Hence, to circumvent this, a set of robust yet simple and handy GM crops detection regimes is certainly the need of the hour. This will not only safeguard the authenticity of germplasm but also aid in delivering better quality of GM products, while serving the interest of farmers.

16.3.4 Characterization Under Contained Trials

After completion of molecular characterization, the transgenic plants and events should be subjected to contained (lab/growth chamber/greenhouse) trial for further study. Contained use is defined as any activity on GM crops should be quarantined/restricted for the safety of humans and the environment. During contained study, biosafety regulatory authority's clearance is required. Primarily the type of informa-

tion requested by the regulatory authority would be related to the details of plant species, biological document, category of genetic manipulation, detailed molecular characterization of the inserted DNA, as well as host genomic flanking sequences, e.g., the vector used and its resource, details of all functional nucleotides, primers used to amplify specific sequences of DNA, details of descriptions and functions, insertion site(s) and copy numbers of all inserted DNA (transgenes, regulatory sequences, vector backbone), etc.

16.3.5 Event-Specific Flanking Sequence Identification

The point of foreign gene/transgene integration and successful expression in a new genetic background made the transgene is a unique event. Event means the transgene integration at a particular host plant DNA of a single cell, which is transformed and regenerated into a complete transgenic plant. Each event will have different points of transgene integration and its positional effects in the host genome, and the introgressed position in the chromosome of host cell remains unchanged during segregation. Contained trials should be conducted to screen and identify the best event. A number of events are generated during transformation. Forwarding all the transgenic events is difficult and laborious and requires large investments. Thus, one should select the best event satisfying all the set parameter during contained event selection trial and forwarded to biosafety research trials.

The molecular details of transgene integration in the host plant and flanking sequences of the transgene are important for biosafety studies and tracing the transgenic event (Yang et al. 2013). Formerly, PCR-based methods like TAIL-PCR, genome walking, and DNA sequencing have been used to determine the point of transgene integration flanked with host DNA (Nakayama et al. 2001). However, these methods are time-consuming, complex, or cumbersome and may not work if the deletion, modification, or rearrangements occurred in transgene sequence during insertion (Wang et al. 2010). With the advent of high-throughput next-generation sequencing (NGS) technology, whole genome sequences can be obtained precisely within short period of time at low cost. NGS has been widely used in many crops to identify flanking sequences of transgene integration and its location in the chromosomes (Inagaki et al. 2015; Pauwels et al. 2015). Whole genome sequencing (WGS) with targeted bioinformatics analysis is a more sensitive and toil-effective method for characterization of GM plants. The WGS technology can divulge nucleotide sequence variations including single nucleotide polymorphism and InDels, which could detect even small sequence modifications (Pauwels et al. 2015). The WGS information could be used in evaluation of the potential toxicity, allergenicity of GM plants by verification of potential similarities in databases of toxins, targets of toxins, allergenic proteins, and anti-nutritional factors (Guo et al. 2016).

16.4 Confined Field Trials

The development of a genetically engineered (GE) crop plant follows a progression from experimentation in laboratory and other contained facilities to field studies and eventually to cultivation after pre-market environmental risk and food/feed safety assessments have been conducted by the appropriate regulatory authorities (Garcia-Alonso et al. 2014; Rüdelsheim 2015). The most early-phase activities of research and development are performed in laboratories, growth rooms, net house, and glasshouses known as contained trials. Under these conditions there is a physical barrier(s) that contains the material to avoid its direct contact with the environment. The Cartagena Protocol on Biosafety to the Convention on Biological Diversity defines "contained" use as "any operation, undertaken within a facility, installation or other physical structure, which involves living modified organisms that are controlled by specific measures that effectively limit their contact with, and their impact on, the external environment." The contained studies are followed by small-scale, proof-of-concept field trials and then by larger trials to further characterize and multiply material (principally seed) of the transformation events. These regulated field trials are known as confined field trials (CFTs) and are conducted with the permission from the appropriate competent authorities. Confined field trials (CFTs) are field experiments of growing a regulated, GE plant in the environment under specific terms and conditions that are intended to mitigate the establishment and spread of the plant. These are experimental activities conducted on a limited scale to collect data, including the data on potential biosafety impacts under the conditions of reproductive isolation known to mitigate dissemination of experimental plant, its persistence in the environment, and its introduction into food chain (GEAC 2015a, b). These represent greater environmental exposure than the contained studies and smaller degree of exposure than the commercial cultivation. These trials are meant to balance safety and exposure to environment and are considered as an essential component of GM research and development throughout the world.

The Cartagena Protocol on Biosafety (*http://bch.cbd.int/protocol/background/*) governs the movements of living modified organisms (LMOs) from one country to another and, therefore, does not govern the CFTs as CFTs are conducted basically to check the introduction of transgene or the transgenic plant into the environment. Therefore, many countries have developed or are developing regulatory frameworks for safe handling of GM crops. Though the guidelines and standard operating procedures for conduct and monitoring of the different types of CFTs vary with the country, the basic objectives of CFTs remain the same. CFTs are conducted to evaluate agronomic performance; to collect data on potential ecological and biosafety impacts; to understand the weedy characteristics of GE crop; to study the environmental fate of novel plant-expressed proteins; to understand the impact on beneficial, endangered, or other organisms; and to generate plant tissue for nutritional analyses, novel protein expression studies, feeding studies, and other studies (GEAC 2015b).

Conduct of CFTs involves the assignment of responsibilities and obtaining permits. Permitted CFTs are performed under a regime of management practices

designed to confine the trials so as to prevent the accidental release of plant material from the trial site, trait introgression into populations of sexually compatible species, or establishment of populations of the experimental GE plant in the environment (Garcia-Alonso et al. 2014). Since the CFTs are designed to understand the potential environmental impacts of GE crop, a regulatory oversight is required for conduct of CFTs, their monitoring, and risk management. It is the responsibility of Trail-in-charge to conduct the CFTs adhering to the norms and guidelines of the regulatory authorities. Safe and successful conduct of CFTs can only be accomplished through a combination of robust regulatory framework, science-based risk mitigation measures, trained personnel dedicated to abiding the terms and conditions of trial authorization, and a qualified monitoring staff (GEAC 2015b). Any weakness in any of these components puts the trust of public on regulatory system into a great risk. Public opinion and perception are considered crucial for acceptance and proliferation of GM technology. Potential risk mitigation procedures are to be in place that can prevent potential negative impacts of the possible known and unknown hazards.

The CFTs are conducted typically and in accordance with internationally accepted approaches to environmental risk assessment (ERA) of GE plants (OECD 1992; SCBD 2000), wherein a comparative assessment is followed where the GE plant is compared to its conventional counterpart, usually the isogenic or a near-isogenic line, which is included in the CFT as a control. Trial endpoints vary depending on the risk hypothesis being tested, but most CFTs aim at identifying any differences between the GE event and its non-GE comparator resulting from intended or unintended consequences of the genetic modification across a range of agroecosystems (OECD 1992; SCBD 2000). Design of CFTs is optimized to obtain data relevant to risk hypotheses while minimizing confounding factors that may interfere with the comparison (Garcia-Alonso et al. 2014).

16.4.1 Types of CFTs

The types of CFTs vary with the country as per the objective of study. Different types of CFTs permitted in India (GEAC 2015a, b) for evaluation of transgenic cotton are provided as follows:

1. *Event selection trials:* Many transgenic events with same gene(s) within the same crop are developed through the process of genetic engineering. These events have varying potential and utility owing to their site of integration in the plant genome, copy number, effect of background genome on its expression, nontargeted effects, and spatiotemporal stability of gene expression across generations. Therefore, it is desirable to evaluate these events under confined conditions to select the most promising event for further evaluation and commercialization. These trials are usually conducted in pots for preliminary evaluation of phenotypic expression.

2. *Biosafety Research Level-I (BRL-I) trials:* Review Committee on Genetic Manipulation (RCGM), Department of Biotechnology (DBT), Ministry of Science and Technology (MoS&T), Government of India, is the regulatory authority for BRL-I trials. These trials are limited in size to no more than 1 acre (0.4 ha) per trial site location and a maximum cumulative total of 20 acres (8.1 ha) for all locations for each plant species/construct combination, per applicant, per crop season.
3. *Biosafety Research Level-II (BRL-II) trials:* Genetic Engineering Appraisal Committee (GEAC) which functions in the Ministry of Environment Forest and Climate Change (MoEF&CC), Government of India, is the regulatory authority for BRL-II trials. These trials are limited in size to no more than 2.5 acre (1 ha) per trial site location, and a number of locations are decided on case-by-case basis for each plant species/construct combination, per applicant, per crop season.
4. *Experimental seed production:* With permission of RCGM and GEAC, the seeds of the selected events can be produced in confined conditions by the applicant for the next phase of trials.
5. *Production of planting material for food and feed studies:* Toxicity and feeding studies for assessment of food and feed safety demand plant material which can be generated under confined field conditions with prior permission of RCGM and GEAC.
6. *Other environmental safety studies:* In addition to BRL-I and BRL-II trials, developer may have to undertake some studies to generate specific information using specific experimental designs. With prior permission of RCGM and GEAC, such trials can be conducted as per guidelines and operating procedures of competent authorities.

16.4.2 Conduct and Monitoring of CFTs

The process of conduct of CFTs starts with the application by the developer which contains key information about description of genetically engineered plant, unmodified counterpart plant, site information with detailed map of the location, experimental protocols, trial management and risk mitigation procedures, and other details required by the concerned competent authority. Many countries have developed detailed guidelines and standard operating procedure for conduct and monitoring of CFTs (Adair and Irwin 2008; CFIA 2000, 2007; EU 2009; OECD 1986; OGTR 2001, 2013; EC 2001; Rüdelsheim 2015; USDA APHIS 2011; DBT 2008; GEAC 2015a, b). The transport, storage, evaluation, harvest, and postharvest operations were regulated through monitoring committees having experts and qualified staff. Lot of documentation and record keeping has to be attended by the concerned personnel at every stage (transport, storage, evaluation, harvest, and postharvest operations) which should be made available for review and scrutiny when demanded by the monitoring committee and/or associated competent authorities.

While evaluating the performance of regulated GE plant of upland cotton (*G. hirsutum*) in CFTs, it must be ensured that the pollen dispersal to other sexually compatible species like Egyptian cotton (*G. barbadense*) and other wild species is prevented. This can be achieved through following combination of different reproductive isolation methods like spatial isolation, temporal isolation, and early termination in line with the guidelines of the regulatory authorities. Spatial isolation is maintaining isolation distance between field trials of the regulated GE plant from the other plants of the same species or from sexually compatible relatives. The isolation distance of 50 m is to be maintained for cotton CFTs, and this isolation distance should be kept free from any other plants of the same or related species. Isolation areas surrounding the CFTs are to be regularly monitored, and prohibited plants found, if any, should be removed before flowering or seed set and should be rendered nonviable using appropriate methods at the trial site. Maintaining reproductive isolation through temporal isolation alone may not be suggestive as cotton is a long-duration crop with longer flowering window. Nevertheless, temporal isolation can be combined with spatial isolation for better risk mitigation in CFTs. Wherever it is compatible with the experimental objectives, early termination can be thought out wherein trial plants are destroyed before anthesis. Depending on the kind of material under trial and associated factors, devitalization of material under CFTs could be achieved through high temperature (autoclave sterilization), low temperature (freezing), chemical treatment (methyl bromide, chloropicrin, and chemosterilants), disinfectants, composting, and desiccation (Rüdelsheim 2015). Monitoring plays an important role in achieving the containment and safety. Designated teams or committees can undertake the monitoring of CFTs which include regular inspection visits to trial site before, during, and after conduct of trial; verification and inspection of different documents, reports, inventories, maps, etc.; inspection of storage facility; preparation of monitoring reports; and suggesting corrective action in case of noncompliance of guidelines and regulatory norms.

16.4.3 Global Status of CFTs: Crop and Trait-Wise Trends

CFTs were conducted for the first time in Canada and the USA in 1987. Since then thousands of CFTs are conducted and are regarded as an essential activity in the development of GM crops intended for commercial cultivation. Although information on the actual performance of CFTs is not systematically available, some information about regulatory applications for CFTs is publicly available in most countries with genetically modified organism (GMO) legislation. One can gain insights about traits and crops which are expected for commercial release in coming years through analysis of annual number of CFTs and the species and traits of the GM plants involved. Smets and Rüdelsheim (2018) conducted the comparative analysis CFTs during 2014–2017 using the information available in the public domain and after amicably addressing the associated challenges like inconsistencies in definition of GMO, basic unit of CFTs, form and format of available data, and data gaps across

countries. The comparative analysis revealed that the annual number of CFTs worldwide declined from 14,307 in 2014 to 6346 in 2017 although regional differences were observed. The highest number of CFTs was noted for maize (*Zea mays*; 21,846 CFTs) followed by soya bean (*Glycine max*; 10,896 CFTs), cotton (*Gossypium hirsutum*; 3045 CFTs), and oilseed rape (*Brassica napus*; 1425 CFTs). Among the traits, herbicide tolerance dominated the other traits followed by insect resistance and abiotic stress tolerance. Research institutes (not-for-profit research organizations) accounted for only 4.2% of all CFTs, while the rest 95.5% is accounted the industry. Industry comprised of both multinational players and smaller enterprises performed CFTs in one or more countries, in main crops, while public research institutes usually acted locally, focusing their efforts on lesser crops.

16.4.4 Transportability of CFTs Data: Need for Harmonization of Protocols Across Countries

In-country confined field trials are mandatory for unrestricted release of GM crops for commercial cultivation. It is important that the environment risk assessment for GM crops is done as efficiently and effectively as possible to avoid needless duplication of studies and to reduce unnecessary regulation in light of accumulated evidence and experience (Fedoroff et al. 2010; Raybould 2007). In a situation where GM crop is cultivated in a country where it is approved and crop produce or its products are targeted for import in other countries, transportability of confined field trial data of GM crops is advocated (Nakai et al. 2015) as it is considered particularly beneficial to public sector product developers and small enterprises who cannot afford to replicate redundant confined field trials (Garcia-Alonso et al. 2014). There is growing experimental evidence to consistently show that the differences between locations, years, genetic backgrounds, and agronomic practices contribute more to endpoint variation than the process of transgenesis (Harrigan et al. 2010; Ricroch 2012). The differences in endpoint measurements are often detected between different varieties of the same crop planted under very different conditions, but not between the GE and its non-GE counterpart grown under similar conditions (Garcia-Alonso et al. 2014). Conduct of CFTs at multiple sites and in multiple countries is both a logistical and financial challenge as they are highly regulated and resource intensive. It is more compounding to conduct the CFTs when there is a sufficient data already available from the earlier CFTs and peer-reviewed literature as well as practical experience crop breeding and cultivation to support the transportability of CFTs data for consideration by the competent authority to commercial release of GM crop or product in question. Since the CFTs are designed and conducted for comparative assessment of GM and its non-GM counterpart under controlled conditions, they are considered amenable to transportability. Efficient transportability of CFTs data could be achieved through harmonization of protocols at international level.

16.5 Food and Feed Safety Assessment

Successful applications of recombinant DNA technologies were reported in the early 1970s. Asilomar Conference 1975 by the scientific community was the first step toward fixing of guidelines for biotechnology and biosafety regulations. First formal guidelines for regulation of rDNA work in the USA were published in 1976 by the US National Institutes of Health (NIH). Gaining of momentum and rolling out of the biotech product in health and agriculture sector in the early 1980s resulted in the development of first international biosafety guidelines for use of GMOs in industry, agriculture, and environment based on the report on "Recombinant DNA safety Considerations 1986 from Organization of Economic Co-operation and Development (OECD)." Agreement on Cartagena Protocol on Biosafety resulted in the addition of more number of countries with biosafety regulatory framework. The Cartagena Protocol on Biosafety to the Convention on Biological Diversity was finalized and adopted on 29 January 2000 in Montreal, Canada, by more than 130 countries.

16.5.1 Food and Feed Safety: India

Food Safety and Standards Authority of India, Ministry of Health and Family Welfare (MoHFW), is a nodal organization for implementation of Food Standards and Safety Act, 2006, which includes genetically modified foods within the definition of food. Guidelines for the safety assessment of foods derived from genetically engineered (GE) plants, 2008, developed by Indian Council of Medical Research (ICMR), New Delhi, based on guidelines and principles of Codex Alimentarius Commission, 2003.

Framework safety assessment consists of well-structured questions that facilitate the realistic assessment of the safety of food and feed. The guidelines include dossier preparation checklists as an appendix seeking the following information from the applicant for the GE plants:

- Description of genetic-engineered plants includes pedigree, transformation events, and type and purpose of modification.
- Description of the non-transgenic host plant and its use as food includes botany, center of origin, and traits of plant harmful to human health, genotype and phenotype of host plant with relevance to safety, and history of safe use as food.
- Description of the donor organisms covering taxonomic classification, production of toxins, anti-allergens, anti-nutrients which concern to human health if any, pathogenicity nature of organisms, organisms presence in the food chain.
- Description of the genetic modification(s) comprising method of transformation of genetic material, description of all genetic material including their source and function, and description of modifications that affect expression of protein(s).

- Characterization of the genetic modification(s) covering sequence and structural details of target genetic materials inserted in the genome, copy number, gene rearrangement if any, event characterization data including flanking sequence of transgene cassette and details on generation of new fusion protein if any, additional information on the details of gene products of the inserted fragment, quantity of expression, tissue specificity, inheritance of gene, and position effect.
- Compositional analyses of key components: key nutrients, anti-nutrients, and major (fats, proteins, carbohydrates) and minor compounds (minerals, vitamins) of GE plants will be analyzed and compared with equivalent analysis with non-GM counterpart at the same point of time.
- Assessment of possible toxicity and assessment of possible allergenicity (proteins).

Protocols for food and feed safety assessment of GE crops in 2008 have been made available by the Department of Biotechnology (DBT), India, based on guidance and peer-reviewed publications available from the Codex Alimentarius Commission, FAO, WHO, OECD, and International Life Science Institute. It mainly contains acute oral safety limit study in rats and mice, sub-chronic feeding study in rodents, protein thermal stability, pepsin digestibility assay, and livestock feeding study (Codex Alimentarius 2003; DBT 1998; FAO/WHO 2001; OECD 1998, 2000a, b, 2002).

16.5.2 Acute Oral Safety Limit Study in Rats or Mice

Target gene products such as proteins are the test material for assessment of toxicity. Acute toxicity tests with proteins are preferred method for toxicity assessment worldwide. It has been observed that the proteins with toxic nature are effective even at low concentration and shorter time; hence acute oral safety studies are being suggested in protocol and guidelines to assess potential toxicity (Jones and Maryanski 1991; EPA 2000; NRC 2000). Acute oral toxicity test with purified protein and sub-chronic 90-day toxicity with whole plant material should be undertaken along with daily intake food/feed. Mortality, morbidity, or evident toxicity is considered for interpreting the toxicity potential of test substance.

16.5.3 Sub-chronic Feeding Study in Rodents

Ninety-day feeding studies are recommended with transgenic crops in rodents to evaluate the safety of edible parts of the plant as a food. The 90 days' study is advised to know the possible health hazards arise due to repeated exposure of the food containing the test substance.

16.5.4 Protein Thermal Stability

Thermal denaturation of protein molecule leads to loss of structure and function. It is necessary to study the possible allergenicity of newly expressed introduced protein. There is strong correlation between heat stability and allergenic potential. Hence, the protein thermal stability protocol is done to measure the thermolability of recombinant protein when exposed to heat. The purified protein is incubated at different temperature range from 25 to 95 °C for up to 30 min and cooled rapidly. Biological activity of the proteins samples will be used to assess the thermal stability. If the tested protein showed biological activity at higher temperature, it demands further tests to rule out likelihood of the test protein being allergenic.

16.5.5 Pepsin Digestibility Assay

Safe dietary proteins are characterized by their natural easy digestibility and act as dietary source of amino acids. For several food allergens, a correlation is observed between resistance to digestion by pepsin and their allergenic potential (Astwood et al. 1996). Pepsin digestibility is an assessment method to evaluate the potential digestibility of target test proteins in vitro using simulated gastric and intestinal fluids.

16.5.6 Livestock Feeding Study

The GM crop product should be mixed with animal food and feed to livestock such as milking cows or buffalos, goat, broiler chicken, fish, and rats and assess the safety level, and test data generated must be submitted to regulatory authority for approval.

16.6 Report on Environmental Assessment and Impact

Genetically modified (GM) crops have great potential to solve many of perceived problems in agriculture and feed the world. However, it attracts many safety issues, unknown fear, and strong opposition from few scientific and nonscientific groups. In the present contest, GM technology would be the best tool to improve the crop yield and reduce production challenges (Carzoli et al. 2018). Still many countries are hesitant to move forward with establishing biosafety laws and commercializing GM crops, primarily due to risk sensitivity and fear spread by anti-biotech groups. In fact, GM crops are well-studied and evaluated technology for safety to humans and the environment before its introduction into commercial cultivation.

It undergoes stringent biosafety tests formulated by international environment experts and qualifies for environment release. Most of the countries follow these standard operating protocols to assess the potential risk and safety of genetically modified organisms/crops to the environment and living organisms. These include unintentional effects such as impact on nontarget organisms, persistence of modified plants or its residue in the environment, possibility of invading into the new habitats, transfer of genes from GM to other species, etc. Insect resistance Bt cotton has been a rapidly adopted technology since its introduction in 1996. Farmers are using Bt cotton technology globally and benefited through increased productivity, reduced pesticide spray, and minimized environment contamination (Purcell and Perlak 2004). However, Bt cotton haggard on yield advantage, environment risk and resistance development by the insects.

16.6.1 Impact on Pesticide Usage

Cotton is one of the highest pesticide-consuming crops especially to control bollworms, which upset the environment through polluting land and water and poisoning humans and animals. Bt cotton was developed to control American bollworm *Helicoverpa armigera* (*Hubner*) families (Cunningham and Zalucki 2014). It was a minor pest of cotton, in India prior to 1980, but became a major pest due to indiscriminate use of synthetic pyrethroids and increased area under long-duration American cotton *Gossypium hirsutum* hybrid (Kranthi 2016). Bt cotton was introduced in India in 2002 mainly to control bollworm complexes. Evidently the Bt technology had significantly reduced chemical pesticide use and helped farmers not to depend on high-priced pesticides like Spinosad (broad-spectrum insecticide) and Indocarp (bollworm) which were used more frequently in conventional cotton (Veettil et al. 2014). Bt crops may favor biocontrol services and enhance economic benefit, and it was established with field studies indicating that Bt crops protected natural enemies in comparison with non-Bt crops which rely on conventional insecticide (Romeis et al. 2008). A number of studies have been reported that Bt cotton has led to a notable decline in acute pesticides poisoning cases among cotton growers in India and China (Pray et al. 2002; Huang et al. 2005; Kousar and Qaim 2011). Lu et al. (2012) reported a significant level of boost to predators such as ladybirds, lacewing, and spiders in China by adopting Bt cotton. In South Africa 90% of the smallholder Bt cotton producers achieved significant reduction in pesticide use (Ismael et al. 2001). In China, insecticide applications were reduced by an average of 67% and the kilogram of active ingredient by 80% (Huang et al. 2002a, b), and growers in the USA reduced insecticide use by 18.70 lakh pounds of active ingredient per year in 2001 (Gianessi et al. 2002). The reduction in Environmental Impact Quotient (EIQ) through Bt technology adoption has increased from 39% during 2002–2004 to 68% during 2006–2008. Bt adoption has contributed to higher environmental efficiency (Veettil et al. 2016). Krishna and Qaim (2012) analyzed the advantages of transgenic Bt cotton over time, using a

panel survey of farmers covering a decade. They claimed that Bt cotton pesticide applications increased yield gains in India. In the 2006–2008 periods, the Bt-induced net reductions in pesticide quantity were 52%. Bt cotton has also reduced pesticide application among the few remaining non-Bt farmers, because widespread adoption has contributed through area-wide suppression of bollworm populations. However, the secondary pests have increased. The recent report by Kranthi and Stone (2020) compared 20 years of Bt cotton data generated in India and showed that Bt adoption area was steadily increasing, but yield was not significant. However, a strong pesticide reduction was recorded initially. The transgenic cotton containing *cry1Ac* found still very efficient to control *H. armigera*, whereas the nontarget pest especially sucking pest, pink bollworm menace cotton cultivation in India and farmers are forced to spray more pesticides.

16.6.2 Pollen Flow

Outcrossing is the unintentional breeding of domestic crops with its related plants. Over the course of evolution, crop species like wheat, potatoes, corn, canola, and numerous others were modified from their original form because of hybridization with related species or weeds or cultivated strains growing nearby. Through this long-established mechanism for gene transfer, any gene in a cultivated crop can be transferred to its wild and semidomesticated relatives. There is potential risk that genes introduced in GM may "escape" (via pollen) to wild or weedy related species growing nearby which is often cited as one of the major risks (Daniell 2002). The degree of gene flow through pollen depends on a number of factors such as sexually compatible species, inheritance of the traits, size of pollen, viability of pollen over time, and distance. Transgene flow in the field between compatible plants can occur when they are close enough for pollen to reach a receptive stigma, the plants have synchronous flowering, and there are no reproductive barriers. Case-by-case study is required for a complete risk analysis. Combination of factors such as difference in experimental design, environmental conditions, and changing pollinator populations affects the results. Now more than two decades over, so far no outcrossed Bt cotton has emerged as a product of gene flow or Bt weeds or more sexually compatible Bt-wild species with transgenes. Experiments have confirmed that most cross-pollination in cotton occurs over distances of less than 50 m (Xanthopoulos and Kechagia 2000: Zhang et al. 2005). But honey bees can travel two miles or more which suggests that the 750 m radius at which pollen from Bt cotton is capable of outcrossing of non-Bt cotton resulted from movement of honey bees from Bt to non-Bt cotton fields (Beekman and Ratnieks 2000). The greatest risk is from herbicide-resistant crops to related weed species. Unwanted gene flow could be prevented or reduced using different barriers such as isolation distance, avoid synchronizing the flowering time, protective vegetation barriers, male sterility, etc. (Ellstrand 2003). Although gene flow has occurred, no examples have demonstrated an adverse environmental effect of gene flow from a transgenic crop to a wild, related plant species.

Heuberger et al. (2010) investigated seed-mediated and pollen-mediated gene flow in the seed production field. Seed-mediated gene flow yields adventitious Bt cotton plant from seed bags, and human error comprises over 15%; in contrast pollen-mediated gene flow affected less than 1% from field edges. Variation in outcrossing was better explained by the area of Bt cotton fields within 750 m of seed production fields than by area of Bt cotton within larger or smaller spatial scales. Variation in outcrossing was also positively associated with the abundance of honey bees (Heuberger et al. 2010).

16.6.3 Impact on Soil Microbial Activity

An imperative aspect of the biosafety assessment of genetically modified plants is to study its impact on soil ecosystem including changes in plant-associated microflora. Microorganisms present in the rhizosphere are affected by root exudates and play an important role in the growth and ecological fitness of their plant host. Rhizosphere microorganisms are considered to be an important component of soil ecological system (Li et al. 2018a, b). The report of the FAO on environmental effect of GM crops recommended that the environmental effect of Bt crops or any transgene protein should be assessed on a case-by-case basis including their potential impact on local soil microflora and biodiversity. Microbes are in close contact with all three soil phases (solid, water, and air), and they can sensitively and rapidly probe responses to soil perturbations. Bt cotton expressing *cry* proteins has no adverse effect on microbial population and enzymatic activity of rhizosphere soil (Zaman et al. 2015). Soil amended with purified *cry* protein and Bt-cotton tissue proteins decrease rapidly with a half-life of approximately 4 and 7 days, respectively (Palm et al. 1996). The difference in the bacterial population of Bt and non-Bt cotton soil might be attributed to variation in root exudates, quantity, composition, and root characteristics (Yasin et al. 2016). Microbes' dependent phosphatase activity significantly increased in the rhizosphere of Bt cotton as compared to non-Bt cotton fields. The higher soil enzyme activities might be due to more organic matter content, microbial activity, and available nutrients compared to non-Bt (Yasin et al. 2016; Singh et al. 2013). Bt cotton plant material had positive effect on acid and alkaline phosphatase activities, and alkaline activity was much higher than acid phosphatase activity (Sun et al. 2007), because alkaline phosphatase is associated with microorganisms, while the acid phosphatase is predominantly due to plants. Shen et al. (2006) recorded no significant differences in soil enzymes such as urease, phosphatase, DHA, PO, proteases, invertases, cellulases, and arylsulfatases in Bt and non-Bt cotton soil. More dehydrogenase activity was recorded in Bt cotton rhizosphere in contrast to non-Bt rhizosphere and could be due to presence of higher bacterial biomass. Dehydrogenase activity is also often used as an alternative to substrate-induced respiration and has been found to be correlated with microbial activity (Chaperon and Sauve 2007). In Bt rhizosphere the available phosphorus, Zn, and Fe contents were observed higher as compared to non-Bt field, and this

might be due to high root biomass-mediated exudates (Beura and Rakshit 2011). Cation exchange capacity, total nitrogen, extractable phosphorus, extractable potassium, active carbon, and Fe and Zn contents were higher in the rhizosphere of Bt cotton genotypes compared with non-Bt cotton genotypes (Yasin et al. 2016). Sarkar et al. (2009) demonstrated that the growth of Bt cotton had positive impact on most of the microbial and biochemical indicators, as microbial biomass carbon, microbial biomass nitrogen, microbial biomass phosphorus, and a range of soil enzyme activities and cultivation of Bt cotton appear to be no risk to soil ecosystem functions. Bt toxin released from root exudates and biomass of Bt corn has no apparent effect on earthworms, nematodes, protozoa, bacteria, and fungi in soil (Saxena and Stotzky 2001). Velmourougane and Sahu (2013) studied the effect of Bt protein on soil biological, microbiological, and diversity attributes at 0–30 cm soil depth and found no adverse effect of Bt cotton on soil microbial activity. Thus, the cultivation of Bt cotton expressing protein had apparently no negative effect on metabolic, microbiological activities and nutrient dynamics of soils.

16.6.4 Impact on Biodiversity

Human activities keep on dominating the planet causing rapid ecosystem changes and massive loss of biodiversity (biological diversity refers to the variety of life on earth) across the planet. Major direct threats to biodiversity include global industrialization, urbanization, and now development and use of genetically modified plants or organisms (Verma 2013). Still there is difference of opinion about the potential for "transgenes" to spread and establish in natural ecosystems. For example, transgenes are expected to have neutral or deleterious effects on antibiotic resistance, reproductive sterility, development of superweeds, etc. Further, people have been worried that the biodiversity will be threatened due to encouraging use of GM crops, which would favor monoculture and affect ecological stability (James et al. 1998; Shiva 1993; Sweet and Shepperson 1997). Although biotech new technology has many advantages to complement conservation of biodiversity, genetic pollution or gene contamination is among the environmental concerns by GM crops which need to be cleared or ascertained before releasing into the commercial use, because it can pollute the biodiversity and the natural or wild genetic pool which may cause irreversible damage (Yohannes and Woldesemayate 2017).

The rhizosphere microbial community structure is a rapid and sensitive indicator of anthropogenic (man-made) effects on soil ecology. Microorganisms present in the rhizosphere are affected by root exudates and play an important role in the growth and ecological fitness of their plant host. Kapur et al. (2010) assessed the culturable and non-culturable microbial diversities in Bt cotton and non-Bt cotton soil and found that cropping of Bt cotton did not affect adversely the diversity of the microbial communities. Li et al. (2018a, b) analyzed the diversity and dynamics of

rhizosphere fungal community on lateral and taproots of Bt cotton using qPCR and 18S rRNA gene sequencing and found no significant differences in population sizes of Bt and conventional cotton varieties root zones. Further, they suggested that the dominant and rare fungal taxa differentially contribute to community dynamics in different root microhabitats of both Bt and conventional cotton variety. Most studies have been carried out to assess the accumulation and persistence of Bt proteins in soil in which Bt crops have been continuously cultivated for several years (Head et al. 2002; Dubelman et al. 2005; Icoz et al. 2008; Zhang et al. 2019). The association between functional groups microorganisms involved in C, N, and P recycling and their influence on plant growth are potential indicators of the impacts of disturbance on the soil environment (Ferreira et al. 2003). Zhang et al. (2019) recorded significantly higher soil microbial communities in the transgenic Bt cotton field than the non-transgenic cotton field. Further, carbon sources including amino acids, amines, and carbohydrates were utilized significantly by the soil microbial communities. However, *cry1Ac* protein did not accumulate in the fields for the next crop season, but the functional diversity of soil microbial communities was affected continuously.

Herbicide-resistant crops are great concern for the loss of weed diversity, which would come out due to gene flow from herbicide-resistant crops to weeds. The currently available herbicide-resistant GM crops confer broad-spectrum herbicides like glufosinate and glyphosate. Since the introduction of glyphosate-resistant GM crops, about 38 weed species worldwide have been identified that have developed resistance to glyphosate, distributed across 37 countries and in 34 different crops and 6 non-crop situation (Heap and Duke 2018). Almost 50% surveyed farms are infested with glyphosate-resistant "superweeds," and these weeds are spreading very fast (www.ucsusa.org/superweeds). Thus, continuously spraying chemical toxic herbicide may alter the diversity of weeds field habitats. The diversity of weeds edible green leaf weeds get completely devastated in the agricultural landscape, which also affects reduction in the diversity of beneficial insects (Tappeser et al. 2014). Continuous use of herbicide has led to modification in the foraging behavior of insects. The best example is the reduced emigration and excessive feeding on crickets by wolf spiders in response to glyphosate application in the Western USA (Wrinn et al. 2012; Marchetti 2014). Another well-known example is the reduction of monarch butterfly populations in the USA and Mexico, which is due to nonavailability of milkweeds by continuous application of herbicide spray. Milkweeds are the main host plant for the monarch larvae (Brower et al. 2012). Increased application of glyphosate results in massive mortality of aquatic life on farmlands (Isenring 2010). Significant reduction in genetic diversity and variable population frequencies of many insects and weeds have been observed as a consequence of gene flow (NAS 2016). In the future glyphosate-resistant weeds are going to be a great threat to sustained weed control in major agronomic crops.

16.7 Insect Resistance to Bt Crops

After the first commercial release in 1995 and subsequent widespread adoption of insecticidal *cry* toxin (crystal protein from *Bacillus thuringiensis* bacterium)-expressing Bt crops, the evolution of resistance was anticipated in the target pest populations. However, despite the remarkable ability of pest populations to quickly adapt to a myriad of pest control strategies, the cases of field-evolved resistance in important target pests were not documented till 2003 (Tabashnik et al. 2003). Usually, the field populations of key target pests surviving on both Bt and nearby non-Bt host plants were continuously monitored for any sign of field-evolved resistance, which is defined as genetically based decrease in susceptibility of one or more field populations to a toxin in the field (Tabashnik and Carrière 2017). In 2008, based upon the extensive field monitoring datasets spanning before and after Bt commercialization (1992–2006), the first case of field-evolved resistance in some field populations *Helicoverpa zea* against *cry1Ac*-expressing Bt cotton was reported in the USA (Tabashnik et al. 2008). Thereafter, the total number of cases of field-evolved resistance with practical significance has gradually increased from 3 in 2005 to 22 cases in 2018 (Smith et al. 2019; Tabashnik et al. 2020), comprising of 10 insecticidal toxins (9 *cry* and 1 *vip3a* toxin) targeted against some 8 pest species (6 lepidopteron and 2 coleopteron) in 6 Bt crop-growing countries including 12 cases in the USA, 3 in Argentina, and 2 each in Brazil, India, Canada, and South Africa (Fig. 16.3).

In general, pest responses to Bt crops have been classified into three main categories, viz., (1) practical resistance, (2) early warning resistance, and (3) no decreases in susceptibility (Tabashnik et al. 2013; Tabashnik and Carrière 2019). Practical resistance and early warning resistance are field-evolved resistance characterized by a genetically based decrease in susceptibility in field-selected population upon exposure to a toxin in the field. In addition, the likelihood of evolution of cross-resistance against two or more toxins in field is also included. The practical resis-

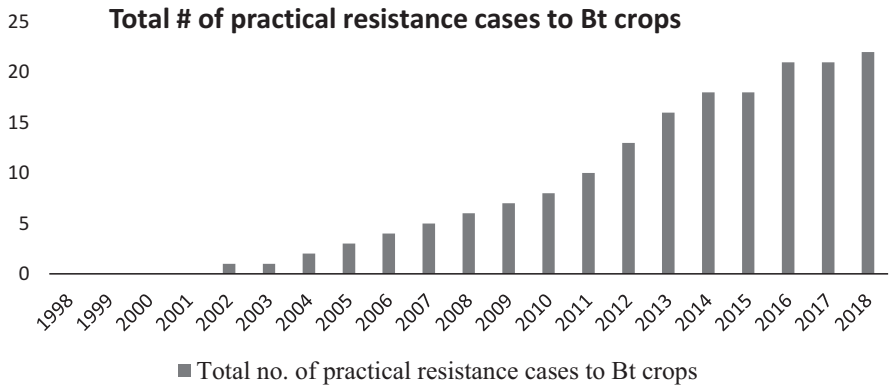

Fig. 16.3 Total number of practical resistance cases to Bt crops

tance can be defined as cases of a field-evolved resistance where more than 50% individuals have been found to be resistant to a toxin with practical field control problems evidenced with a significant reduction of Bt crop efficacy (Tabashnik et al. 2013; Tabashnik and Carrière 2017), while early warning resistance includes all cases of field-evolved resistance with a statistically significant decline in genetically based susceptibility without any evidence of reduced efficacy of Bt crop. The category 3 consists of all cases of pest responses to Bt crops where field resistance monitoring data suggests no sign of a statistically significant decrease in susceptibility in field pest population to the expressed toxin in Bt crops.

16.7.1 Insect Responses to Bt Cotton

A total of 19 pest responses to Bt cotton have been documented with 4 cases each in the category of practical resistance and early warning resistance, while 11 cases fit into the category 3 characterized with no significant decreases in susceptibility (Tables 16.3 and 16.4). In Bt cotton, cases of field-evolved resistance with practical implications are mainly associated with two major lepidopteron cotton pests, viz., *Helicoverpa zea* and *Pectinophora gossypiella* (pink bollworm) in the USA and India, respectively.

16.7.2 Reports of Insect Resistance to Bt Cotton in the USA

The analysis of field monitoring data (1992–2006) for some populations of *Helicoverpa zea* surviving on *cry1Ac*-expressing Bt cotton from southeastern states (Arkansas and Mississippi) of the USA reveals the first incidence of field-evolved resistance (Luttrell et al. 1999; Ali and Luttrell 2007; Ali et al. 2007; Tabashnik et al. 2008, 2009). In general, resistance ratio (RR) value greater than 10 often implies a significant decline in the heritable susceptibility in tested pest population (Tabashnik 1994). The laboratory bioassays with field-selected strains of *Helicoverpa zea* from Arkansas and Mississippi collected during 2003–2006 identified several strains with significantly increased resistance ratio from 1.2 to >(50–1000) compared to the population sampled during 1992–1993 (pre-Bt commercialization) (Ali et al. 2007; Tabashnik et al. 2008, 2009). The results of laboratory bioassays using diet-incorporated *cry1Ac* toxin and percent survival of field-resistant *Helicoverpa zea* larvae have suggested for a substantial decrease in the genetic-based larval susceptibility to *cry1Ac.* In December 2002, the second-generation pyramided Bt cotton called Bollgard II (*cry1Ac* + *cry2Ab*) has been commercially released and widely adopted. Field monitoring data from Bollgard II during 2002–2005 revealed a significant gain in the percentage (from 0 to 50%) of individuals of *Helicoverpa zea* populations with RR >10 and *cry1Ac* LC_{50} > 150 μg per mL of diet and indicated a positive correlation between percent survival

Table 16.3 Global status of responses of field populations of target pests against insecticidal toxins expressed in Bt cotton[a]

Category 1 (practical resistance: >50% resistant individuals with reduced field efficacy of Bt reported)							
Pest species	Country	Toxin	Year[b]	Years[c]	High toxin dose	Low initial resistance allele frequency	References
Helicoverpa zea	USA	*cry1Ac*	1996	6	No	No	Luttrell et al. (1999); Ali et al. (2007); Tabashnik et al. (2008)
	USA	*cry2Abcry2Ab*	2003	2	–	No	
Pectinophora gossypiella	India	*cry1Ac*	2002	6	No	–	Dhurua and Gujar (2011); Naik et al. (2018)
	India	*cry2Abcry2Ab*	2006	8	–	–	Naik et al. (2018)

– Data not available

[a]Data adopted from Tabashnik et al. (2013), Tabashnik and Carrière (2017, 2019)

[b]First year of Bt cotton introduction in the region surveyed for field pest population

[c]Total years from the initial year of Bt cotton introduction to the first evidence of field-evolved resistance in the region surveyed for pest resistance

and *cry2Ab* resistance (Ali and Luttrell 2007; Tabashnik et al. 2009). Similarly, LC_{50} values of both *cry1Ac* and *cry2Ab* in the 61 strains of *Helicoverpa zea* sampled during 2004–2006 were also found in positive correlation, $r = 0.32$. In comparison to susceptible strains, compelling data with five- to sevenfold high LC_{50} for *cry1Ac* and four- to sixfold increased survival on Bollgard II cotton leaves suggested that field-evolved resistance for *cry2Ab* in *Helicoverpa zea* was positively associated with *cry1Ac* resistance. The typically weak but statistically significant cross-resistance to *cry2Ab* in some field populations of *Helicoverpa zea* has unusually accelerated the *cry2Ab* resistance (Tabashnik et al. 2009; Tabashnik and Carrière 2013).

16.7.3 Reports of Pest Resistance to Bt in India

So far, Bt cotton is the only transgenic crop approved for commercial planting in India. The Bt cotton technology is known as Bollgard® (expressing single *cry1Ac* toxin) which was commercialized for the first time in India in 2002 and planted in 50,000 ha (Choudhary and Gaur 2010). Later on, in 2006, Bollgard II was also released for commercial planting with an idea of delaying the evolution of pest resistance and for continued benefits from Bt technology (Choudhary and Gaur 2010; Naik et al. 2018). The cotton cultivation in India is primarily dominated by Bt

Table 16.4 Global status of responses of field populations of target pests against insecticidal toxins expressed in Bt cotton[a]

Pest species	Country	Toxin	Year[b]	Years[c]	References
Category 2: early warning resistance cases (field-evolved resistance with statistically significant decline in genetically based susceptibility without any evidence of reduced efficacy of Bt crop)					
Helicoverpa zea	India	*cry1Ac*	2002	12	Kukanur et al. (2018)
	Pakistan	*cry1Ac*	2010	3	Rashid et al. (2008)[d]
	USA	vip3Aa	2010	8	Yang et al. (2019)
	China	*cry1Ac*	1997	20	Dandan et al. (2019)
Category 3: no significant reduction in genetically based susceptibility in field pest population					
Earias biplaga	South Africa	*cry 1Ac*	1998	15	Fourie et al. (2017)
Helicoverpa armigera	Australia	*cry 1Ac*	1996	16	Bird (2015)
	Australia	*cry2Abcry2Ab*	2004	11	
Helicoverpa punctigera	Australia	*cry 1Ac*	1996	19	Walsh et al. (2018)
	Australia	*cry2Abcry2Ab*	2004	11	Bird (2015)
Helicoverpa virescens	Mexico	*cry1Ac*	1996	11	Blanco et al. (2009)
	USA	*cry 1Ac*	1996	11	
	USA	*cry2Abcry2Ab*	2003	2	Ali et al. (2007)
Pectinophora gossypiella	China	*cry1Ac*	2000	15	Wan et al. (2017)
	USA	*cry 1Ac*	1996	12	Tabashnik and Carrière (2019)
	USA	*cry2Abcry2Ab*	2003	5	

[a]Data adopted from Tabashnik et al. (2013), Tabashnik and Carrière (2017, 2019)
[b]First year of Bt cotton introduction in the region surveyed for field pest population
[c]Total years from the initial year of Bt cotton introduction to the recent year of collection of field monitoring data
[d]Although illegal Bt cotton cultivation has been reported since 2004, commercial planting got official approval only in 2010 onward

cotton hybrids developed through the breeding of Bt variety expressing *cry* toxin(s) with non-Bt cultivar (Choudhary and Gaur 2010). In 2018, the total area under Bt cotton hybrids increased to 50,000 ha in 2002 to 11.6 million ha in 2018, benefiting more than 7.5 million farm families (ISAAA 2018). During the initial years of its introduction, the Bt cotton technology has been very effective in controlling major devastating pest of cotton including *Pectinophora gossypiella*, *Helicoverpa armigera*, *Earias vittella* (spotted bollworm), and *Earias insulana* (spiny bollworm) (Dhurua and Gujar 2011; Naik et al. 2018). In 2009, pink bollworm larvae surviving on *cry1Ac*-expressing Bt cotton plants from fields of four districts of Gujarat state

(India), viz., Rajkot, Amreli, Bhavnagar, and Junagarh, have confirmed the evolution of *cry1Ac* resistance in pink bollworm in laboratory bioassays (Monsanto Cotton India *http://www.monsantoindia.com/monsanto/layout/pressreleases/mmb*). However, the first case of field-evolved resistance with practical field control problem was reported from Amreli district of Gujarat state (Dhurua and Gujar 2011). In the 5-day-old offsprings of field-resistant *Pectinophora gossypiella* collected from Amreli district of Gujarat in 2008, a significantly higher resistance ratio (RR 44) was found to be associated with increased *cry1Ac* LC_{50} concentration (mean value of 1.64 μg per mL of diet). At the same time, the most susceptible population was recorded with a mean LC_{50} value of 0.050 μg per mL. In addition, a significant reduction in mortality (24–31 v/s 97%) in resistant insects was also recorded in bioassays using 1 μg per mL *cry1Ac.* In another study, the field-evolved resistance to *cry1Ac* in field-resistant pink bollworm progenies was also confirmed (Mohan et al. 2016). The study has found that offsprings of field-collected pink bollworm (Amreli, Gujarat) showed a sizable tolerance to *cry1Ac* concentrations of 1.0–10 μg per mL of diet. In a recent report, the pink bollworm response to Bollgard® (single *cry1Ac* toxin) and Bollgard II (*cry1Ac* + *cry2Ab*) was evaluated in the resistance monitoring data collected during 2010–2017 (Naik et al. 2018). A significantly higher resistance ratio to *cry1Ac* (26–262) and *cry2Ab* (1–108) with a substantially high percentage of average pest survival (28.85–72.49%) on Bt-II cotton (expressing *cry1Ac* + *cry2Ab*) was reported. The mean LC_{50} for *cry1Ac* has increased from 0.330 to 6.938 μg/mL from 2013 to 2017, while for *cry2Ab* increased from 0.014 to 12.51 μg/mL during 2013–2017. The study thus confirms the evidence of field-evolved resistance against *cry1Ac* + *cry2Ab* 2 expressing Bollgard II in India, particularly in central and south cotton-growing states.

However, with the introduction of Bt crops, refuge strategy (growing non-Bt hosts along with Bt crops) has also been extensively adopted to delay the evolution of resistance (Tabashnik 2008; Hutchison et al. 2010). The predictions from population genetic models suggested that the evolution of resistance can be postponed to more than 20 years with ≥ 5% refuge cover in a condition that the estimated initial resistance allele frequency should be 0.001 and the resistance preferably be governed by two alleles with single locus genetic architecture with completely recessive inheritance (h = 0) (Tabashnik et al. 2008). However, in most cases of field-evolved resistance reported globally, the non-recessive inheritance of resistance alleles along with low refuge abundance, not meeting high toxin dose standards, was considered among the major factors contributed to rapid evolution of field-evolved resistance (Tabashnik et al. 2008; Tabashnik and Carrière 2019).

16.8 Reports on Weed Resistance to Herbicides

Among the various tools, herbicides play essential roles in weed management of almost all the agricultural systems. However, frequent and indiscriminate use of few selected herbicides sharing a similar mode of action has resulted in the increasing

cases of the evolution of herbicide resistance in weeds (Beckie 2006). WSSA (the Weed Science Society of America) is a nonprofit professional society; aims to promote research, education, and awareness of weeds in managed and natural ecosystems; and defines herbicide resistance as "the inherited ability of a plant to survive and reproduce following exposure to a dose of herbicide normally lethal to the wild type" (http://wssa.net/). Globally, a total of 514 unique cases of herbicide resistance involving 262 weed species (152 dicots and 110 monocots) with confirmed herbicide resistance against 167 different herbicides targeting 23 of the 26 known herbicide sites of action have been documented in a regularly updating "International Herbicide-Resistant Weed Database" (http://www.weedscience.org/home.aspx) as on data updated in July 2020 (Table 16.5). In cotton, a total of 81 cases of herbicide resistance in 18 different weed species have been documented from 8 countries (Fig. 16.4). Palmer amaranth (*Amaranthus palmeri*) is a leading weed documented in 24 cases of multiple resistances with 5 sites of herbicide actions including EPSP synthase inhibitors (G/9), ALS inhibitors (B/2), PPO inhibitors (E/14), microtubule inhibitors (K1/3), and long-chain fatty acid inhibitors (K3/15).

16.9 GM Cotton Products Commercialized

Even though GM products have been developed for many economic traits, only GM products for insect and herbicide resistance have been commercialized successfully and occupied more than 90% of the total cultivated area. Globally GM cotton is available in the form of insect and herbicide tolerance either as single or stacked traits. In addition recently GM cotton for low gossypol developed by Texas A&M University was commercialized in the USA. Details of gene and transgenic events of insect and herbicide tolerance commercialized (Source: ISAAA's GM Approval Database. *http://www.isaaa.org/gmapprovaldatabase/*) are enlisted in Tables 16.6 and 16.7.

16.10 Global Status of Adoption of GM Cotton

GM cotton is one of the major crops that were first granted permission for commercial cultivation, and today it occupies 13% of the global area (Paul and Hennig 2019). Thirty-eight countries contribute to 98% of global cotton production. Genetically modified cotton is cultivated in 23 countries. Ninety percent of global cotton production comes from just ten countries—Australia, Brazil, Burkina Faso, China, India, Pakistan, Turkey, Turkmenistan, Uzbekistan, and the USA—and of these top cotton-producing countries, Turkey is the only exception as it does not cultivate GM cotton.

Turkey, Greece, and Spain serve a classic example for obtaining high yields without GM technologies. High yields are obtained in Turkey, without the adoption

Table 16.5 Global status of total number of confirmed cases of weed resistance to herbicides

Sr no	Weed species	Common name	Cases no.	Country	Herbicide site of action (WSSA code)
1	*Amaranthus palmeri*	*Palmer amaranth*	24	Brazil, Israel, Mexico, and USA	EPSP synthase inhibitors (G/9), ALS inhibitors (B/2), PPO inhibitors (E/14), microtubule inhibitors (K1/3), long-chain fatty acid inhibitors (K3/15)
2	*Conyza canadensis*	*Horseweed*	9	USA	EPSP synthase inhibitors (G/9), ALS inhibitors (B/2)
3	*Eleusine indica*	*Goosegrass*	9	Brazil and USA	ACCase inhibitors (A/1), EPSP synthase inhibitors (G/9), microtubule inhibitors (K1/3)
4	*Sorghum halepense*	*Johnsongrass*	7	Greece, Israel, and USA	ACCase inhibitors (A/1), ALS inhibitors (B/2), microtubule inhibitors (K1/3)
5	*Xanthium strumarium*	*Common cocklebur*	7	USA	Nucleic acid inhibitors (Z/17)
6	*Amaranthus tuberculatus (=A. rudis)*	*Tall waterhemp*	6	USA	EPSP synthase inhibitors (G/9), ALS inhibitors (B/2)
7	*Amaranthus retroflexus*	*Redroot pigweed*	3	Brazil	ALS inhibitors (B/2), photosystem II inhibitors (C1/5), PPO inhibitors (E/14)
8	*Lolium perenne* ssp. *multiflorum*	*Italian ryegrass*	3	USA	EPSP synthase inhibitors (G/9)
9	*Echinochloa colona*	*Junglerice*	2	Australia and USA	EPSP synthase inhibitors (G/9)
10	*Ambrosia artemisiifolia*	*Common ragweed*	2	USA	EPSP synthase inhibitors (G/9)
11	*Amaranthus spinosus*	*Spiny amaranth*	2	USA	EPSP synthase inhibitors (G/9), ALS inhibitors (B/2)
12	*Sonchus oleraceus*	*Annual sowthistle*	1	Australia	EPSP synthase inhibitors (G/9)
13	*Amaranthus viridis*	*Slender amaranth*	1	Brazil	ALS inhibitors (B/2), photosystem II inhibitors (C1/5)
14	*Ageratum conyzoides*	*Tropical whiteweed*	1	Brazil	ALS inhibitors (B/2)
15	*Digitaria sanguinalis*	*Large crabgrass*	1	China	ACCase inhibitors (A/1)
16	*Digitaria insularis*	*Sourgrass*	1	Paraguay	EPSP synthase inhibitors (G/9)
17	*Ambrosia trifida*	*Giant ragweed*	1	USA	EPSP synthase inhibitors (G/9)
18	*Kochia scoparia*	*Kochia*	1	USA	EPSP synthase inhibitors (G/9)

Source: INTERNATIONAL HERBICIDE-RESISTANT WEED DATABASE (*http://www.weed-science.org/Pages/crop.aspx*)

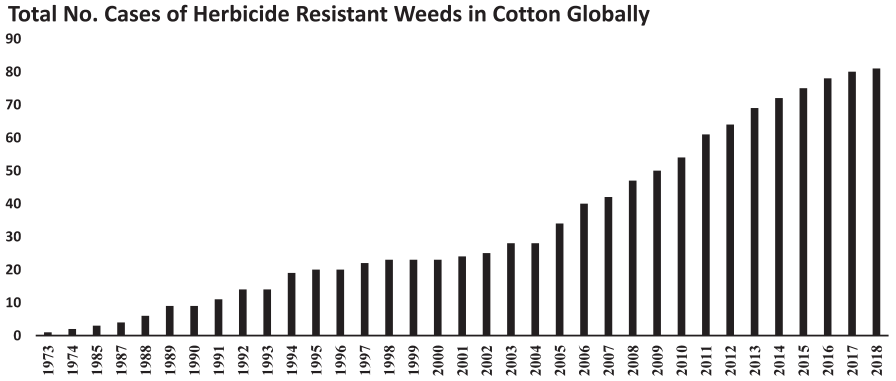

Fig. 16.4 Year-wise distribution of the total number of cases of herbicide-resistant weed species in cotton cropping system globally. Palmer amaranth (*Amaranthus palmeri*) is a leading weed documented in 24 cases of multiple resistances with 5 sites of herbicide actions including EPSP synthase inhibitors (G/9), ALS inhibitors (B/2), PPO inhibitors (E/14), microtubule inhibitors (K1/3), and long-chain fatty acid inhibitors (K3/15)

of Bt cotton. Turkey is an important producer and consumer of cotton. Turkey produces 10,000 tonnes of organic cotton which was expected to increase to 15,000 tonnes in the current 2020–2021 season. The private sector today provides almost all the hybrid cotton seeds in Turkey. Cotton is cultivated in three regions (GAP region, Cukurova region, and Aegean region), and the most dominant region is the GAP region that accounts for about 60.0% of the cotton acreage. For some time now, several steps were taken by the government to benefit cotton production in Turkey. In the GAP region, dams and irrigation channels were constructed that were expected to facilitate an irrigated area of 650,000 ha of land. Open canal system of irrigation was replaced with closed systems. Financial assistance and technical guidance for drip irrigation was provided by the government. Government incentivized cotton production by giving a bonus of 0.8 lira (US 12 cents) for every kilogram of cotton produced**.** Licensed storage facilities were set up in GAP and Izmir for 15,000 tonnes and 10,000 tonnes, respectively. Turkey spends US$ 77, US$ 400, US$ 546, and US$ 26 per hectare on seeds, fertilizers, pesticides, and manpower, respectively. The cost of cultivation is US$ 413 per hectare, and the production cost is US$ 1.55 per kg of lint (including seed value) and US$ 0.59 per kg of seed cotton (ICAC Cotton Data Book 2020).

Table 16.6 Details of genes for insect resistance and herbicide tolerance in commercialized GM cotton

Genes for insect resistance	Source of genes	Target trait
cry1Ac	*Bacillus thuringiensis* subsp. *kurstaki* strain HD73	*Resistance to lepidopteran insects*
cry1Ab	*B. thuringiensis* subsp. kurstaki	
cry1A	*B. thuringiensis*	
cry1Ab-Ac	*B. thuringiensis*	
cry1F	*Bacillus thuringiensis* var. *aizawai*	
cry2Ab2	*B. thuringiensis* subsp. *kumamotoensis*	
cry2Ae	*B. thuringiensis* subsp. dakota	
vip3A(a) codes for vegetative insecticidal protein	*Bacillus thuringiensis* strain AB88	
cpTI-trypsin inhibitor	*Vigna unguiculata*	*Broad spectrum*
mCry51Aa2	*B. thuringiensis*	*Hemipteran insects Lygus hesperus and L. lineolaris*
Herbicide tolerance in cotton		
cp4 epsps (aroA:CP4)	*Agrobacterium tumefaciens* strain CP4	*Glyphosate tolerance*
2mepsps	*Zea mays*	
pat gene coding for phosphinothricin-*N*-acetyltransferase (PAT)	*Streptomyces viridochromogenes*	*Glufosinate tolerance*
Bar phosphinothricin-*N*-acetyltransferase (PAT) enzyme	*Streptomyces hygroscopicus*	
Bxn produces nitrilase enzyme	*Klebsiella pneumoniae* subsp. *ozaenae*	*Bromoxynil tolerance*
S4-HrA herbicide-tolerant acetolactate synthase (ALS)	*Nicotiana tabacum* cv. Xanthi	Sulfonylurea tolerance
Dmo dicamba monooxygenase enzyme	*Stenotrophomonas maltophilia strain DI-6*	*Dicamba tolerance*
aad-12 aryloxyalkanoate di-oxygenase 12 (AAD-12) protein	*Delftia acidovorans*	*2,4,-D tolerance*
Modified p-hydroxyphenylpyruvate dioxygenase (hppd) enzyme (hppdPF W336)	*Pseudomonas fluorescens strain A32*	*Isoxaflutole tolerance*
Low gossypol seed		
dCS D-cadinene synthase gene	*Gossypium hirsutum*	*Low gossypol seed*

Source: ISAAA's GM Approval Database. *http://www.isaaa.org/gmapprovaldatabase/*

Table 16.7 Details of commercialized GM cotton transgenic events for insect resistance and herbicide tolerance

Event name	Gene	Trait	Country and year of approval	Trade name/ developer
GM cotton for insect resistance				
MON531	*cry1Ac*	Resistance to lepidopteran insects	USA 1995, Mexico 1996, South Africa 1997, Argentina 1998, India 2002, Australia 2003, Colombia 2003, Brazil 2005, Paraguay 2007, Costa Rica 2008, Sudan 2012	Bollgard™ cotton, Ingard™
MON757	*cry1Ac*	Resistance to lepidopteran insects	USA 1995, South Africa 1997	Bollgard™ cotton
MON1076	*cry1Ac*	Resistance to lepidopteran insects	USA 1995, South Africa 1997	Bollgard™ cotton
GK12	*cry1Ab-Ac*	Resistance to lepidopteran insects	China 1997	Chinese Academy of Agricultural Sciences
281-24-236	*cry1F*	Resistance to lepidopteran insects	Mexico 2004, USA 2004	Dow AgroSciences LLC
3006-210-23	*cry1Ac*	Resistance to lepidopteran insects	Mexico 2004, USA 2004	Dow AgroSciences LLC
COT102 (IR102)	*vip3A(a)*	Resistance to lepidopteran insects	USA 2005, Australia 2018, Costa Rica (seed production only) 2017	VIPCOT™ cotton
COT102 (IR102)	*vip3A(a)*	Resistance to lepidopteran insects	USA 2005, Australia 2018	VIPCOT™ cotton
Event 1	*cry1Ac*	Resistance to lepidopteran insects	India 2006, Eswatini 2018, Ethiopia 2018	JK 1
GFM *cry1A*	*cry1Ab-Ac*	Resistance to lepidopteran insects	India 2006	Nath Seeds/Global Transgenes Ltd (India)
MLS 9124	*cry1C*	Resistance to lepidopteran insects	India 2009	Metahelix Life Sciences Pvt. Ltd (India)
GHB119	*cry2Ae*	Resistance to lepidopteran insects	USA 2011	BASF

(continued)

Table 16.7 (continued)

Event name	Gene	Trait	Country and year of approval	Trade name/ developer
COT67B	*cry1Ab*	Resistance to lepidopteran insects	USA 2011, Costa Rica (seed production only) 2017	Syngenta
MON15985	*cry1Ac cry2Ab2*	Resistance to lepidopteran insects	USA 2002, Australia 2002, South Africa 2003, India 2006, Brazil, Burkina Faso, Costa Rica 2009	Bollgard II™ cotton
281-24-236 × 3006-210-23 (MXB-13)	*cry1Ac cry1F*	Resistance to lepidopteran insects	Mexico 2004, Australia 2009, Brazil 2009, Costa Rica 2009	WideStrike™ cotton
COT102 × COT67B	*vip3A*(a) *cry1Ab*	Resistance to lepidopteran insects	Costa Rica 2009	VIPCOT™ cotton
COT102 × MON15985	*vip3A(a) cry1Ac, cry2Ab2*	Resistance to lepidopteran insects	Australia 2014	Bollgard® III
281-24-236 × 3006-210-23 × COT102	*cry1Ac cry1F, vip3A(a)*	Resistance to lepidopteran insects	Brazil 2018	Dow AgroSciences LLC
MON88702	*mcry51Aa2*	Resistance to hemipteran insects	Australia, Canada, New Zealand (food), Japan, USA 2018 (food and feed purpose)	Monsanto Company (including fully and partly owned companies)
GM cotton for herbicide tolerance				
BXN10211/ BXN10215 (10215)/ BXN10222 (10222)/ BXN10224 (10224)	*bxn*	*Bromoxynil tolerance*	USA 1994	BXN™ Plus Bollgard™ cotton
MON1445	*cp4 epsps (aroA:CP4)*	*Glyphosate tolerance*	USA 1995, Mexico 2000, South Africa 2000, Argentina 2001, Australia 2003, Japan 2004, Columbia 2004, Brazil 2008, Costa Rica 2008, Paraguay 2013	Roundup Ready™ cotton
MON1698	*cp4 epsps (aroA:CP4)*	*Glyphosate tolerance*	USA 1995, Mexico 2000, South Africa 2000	Roundup Ready™ cotton
19-51a	*S4-HrA*	Sulfonylurea tolerance	USA 1996	DuPont (Pioneer Hi-Bred Int. Inc.)

(continued)

Table 16.7 (continued)

Event name	Gene	Trait	Country and year of approval	Trade name/ developer
LLCotton25	*bar*	*Glufosinate tolerance*	USA 2003, Brazil 2008, Costa Rica 2009, Colombia 2010	Fibermax™ LibertyLink™
MON88913	*cp4 epsps (aroA:CP4)*	*Glyphosate tolerance*	USA 2004, Mexico 2006, Australia 2006, Japan 2006, South Africa 2007, Costa Rica 2009, Brazil 2011	Roundup Ready™ Flex™ cotton
GHB614	*2mepsps*	*Glyphosate tolerance*	USA 2009, Costa Rica 2009, Brazil 2010, Argentina 2012, Australia 2016	GlyTol™
GHB614 × LLCotton25	*2mepsps, bar*	*Glyphosate tolerance Glufosinate tolerance*	Brazil 2012, Colombia 2013, Argentina 2015	GlyTol™ LibertyLink™
MON88701	*Dmo bar*	*Dicamba tolerance Glufosinate tolerance*	USA 2014, Brazil 2017, Costa Rica (seed production only) 2016	Monsanto Company (including fully and partly owned companies)
81910	*pat*	*Glufosinate tolerance*	USA 2015, Brazil 2018, Costa Rica (seed production only) 2016	Dow AgroSciences LLC
MON88701 × MON88913	*dmo, bar*, *cp4 epsps (aroA:CP4)*	*Dicamba tolerance Glufosinate tolerance Glyphosate tolerance*	Australia 2016 Brazil 2018	Monsanto Company (including fully and partly owned companies)
GHB811	*hppdPF W336, 2mepsps*	*Isoxaflutole tolerance Glyphosate tolerance*	USA 2018, Brazil 2019	BASF
Insect resistance and herbicide tolerance				
31807	*Bxn cry1Ac*	Oxynil tolerance Resistance to lepidopteran insects	USA (1997)	BXN™ Plus Bollgard™ cotton
31808	*Bxn cry1Ac*	Oxynil tolerance Resistance to lepidopteran insects	USA (1997)	BXN™ Plus Bollgard™ cotton

(continued)

Table 16.7 (continued)

Event name	Gene	Trait	Country and year of approval	Trade name/ developer
MON531 × ON1445	*cry1Ac cp4 epsps (aroA:CP4)*	Resistance to lepidopteran insects Glyphosate tolerance	Mexico 2002, Australia 2003, Japan 2004, South Africa 2005, Columbia 2007, Argentina 2009, Brazil 2009, Costa Rica 2009, Paraguay 2013	Roundup Ready™ Bollgard™ cotton
GHB119	*cry2Ae bar*	Resistance to lepidopteran insects Glufosinate tolerance	USA 2011	Bayer Crop Science
T303-3	*cry1Ab bar*	Resistance to lepidopteran insects Glufosinate tolerance	USA 2012	Bayer Crop Science (including fully and partly owned companies)
T304-40	*cry1Ab bar*	Resistance to lepidopteran insects Glufosinate tolerance	USA 2011	Bayer Crop Science (including fully and partly owned companies)
MON15985 × MON1445	*cry1Ac cry2Ab2 cp4 epsps*	Resistance to lepidopteran insects Glyphosate tolerance	Australia 2002, Costa Rica 2009	Roundup Ready™ Bollgard II™ cotton
COT102 × COT67B × MON88913	*cry1Ab vip3A(a) cp4 epsps*	Resistance to lepidopteran insects Glyphosate tolerance	Costa Rica 2009	VIPCOT™ Roundup Ready Flex™ cotton
MON-88913-8 × MON-15985-7	*cry1Ac cry2Ab2 cp4 epsps*	Confers resistance to lepidopteran insects Glyphosate tolerance	Mexico 2006, Australia 2006, Japan 2006, South Africa 2007, Columbia 2007, Costa Rica 2009, Brazil 2012, Paraguay 2017	Roundup Ready™ Flex™ Bollgard II™ cotton
3006-210-23 × 281-24-236 × MON1445	*cp4 epsps cry1F cry1Ac, bar*	Glyphosate tolerance Resistance to lepidopteran insects Glufosinate tolerance	Japan (2006) Mexico (2005) Costa Rica (2009)	WideStrike™ Roundup Ready™ cotton Monsanto Company and Dow AgroSciences LLC

(continued)

Table 16.7 (continued)

Event name	Gene	Trait	Country and year of approval	Trade name/ developer
3006-210-23 × 281-24-236 × MON88913	*cp4 epsps cry1F, cry1Ac bar*	Glyphosate tolerance Resistance to lepidopteran insects Glufosinate tolerance	Japan (2006), Costa Rica (2009)	WideStrike™ Roundup Ready Flex™ cotton
LLCotton25 × MON15985	*cry1Ac cry2Ab2 bar*	Confers resistance to lepidopteran insects Glufosinate tolerance	Australia 2006	Fibermax™ LibertyLink™ Bollgard II™
COT102 × COT67B × MON88913	*cry1Ab vip3A(a) cp4 epsps*	Resistance to lepidopteran insects Glyphosate tolerance	Costa Rica (2009)	VIPCOT™ Roundup Ready Flex™ cotton Syngenta and Monsanto Co.
T304-40 × GHB119	*cry2Ae cry1Ab Bar*	Resistance to lepidopteran insects Glufosinate tolerance	Brazil 2011 USA 2011 Argentina 2014	TwinLink™
GHB614 × T304-40 × GHB119	*2mepsps Bar, cry2Ae cry1Ab*	Glyphosate and glufosinate tolerance Resistance to lepidopteran insects	Brazil 2012	GlyTol™ × TwinLink™
GHB614 × LLCotton25 × MON15985	*cry1Ac cry*2Ab2 *Bar 2mepsps*	Resistance to lepidopteran insects Glufosinate tolerance Glyphosate tolerance	Japan 2011	Bayer Crop Science
COT102 × MON15985 × MON88913	*cry1Ac, cry2Ab2 vip3A(a) cp4 epsps*	Resistance to lepidopteran insects Glyphosate tolerance	Australia 2014 Brazil 2016	Bollgard® III × Roundup Ready™ Flex™

(continued)

Table 16.7 (continued)

Event name	Gene	Trait	Country and year of approval	Trade name/ developer
MON88701 × MON88913 × MON15985	*cry1Ac, cry2Ab2 cp4 epsps bar, dmo*	Resistance to lepidopteran insects, Glyphosate tolerance Glufosinate tolerance Dicamba tolerance	Japan 2015 (for type I use only)	Monsanto Company (including fully and partly owned companies)
DAS-24236-5 × DAS-21Ø23-5 × SYN-IR1Ø2-7 × DAS-81910-7	*cry1F, cry1Ac vip3A(a) pat*	Resistance to lepidopteran insects Glufosinate tolerance	Brazil 2019, Japan 2016 (food and feed)	Dow AgroSciences LLC
DAS-21Ø23-5 × DAS-24236-5 × MON-88913-8 × SYN-IR1Ø2-7 × DAS-81910-7	*cry1F, cry1Ac vip3A(a) cp4 epsps pat*	Resistance to lepidopteran insects Glyphosate tolerance Glufosinate tolerance	Japan 2015, Mexico 2016, South Korea 2016–2017 (food and feed)	Dow AgroSciences LLC
COT102 × MON15985 × MON88913 × MON88701	*cry1Ac, cry2Ab2, vip3A(a) cp4 epsps Bar, dmo*	Resistance to lepidopteran insects Glyphosate tolerance Glufosinate tolerance Dicamba tolerance	Australia 2016 Brazil 2018	Monsanto Company (including fully and partly owned companies)
GHB614 × T304-40 × GHB119 × COT102	*2mepsps Bar, cry2Ae cry1Ab vip3A(a)*	Glyphosate and glufosinate tolerance Resistance to lepidopteran insects	Australia 2016 Brazil 2017	GlyTol™ × TwinLink™ × VIPCOT™ cotton
GHB614 × T304-40 × GHB119	*2mepsps Bar, cry2Ae cry1Ab vip3A(a)*	Glyphosate and glufosinate tolerance Resistance to lepidopteran insects	Approved for food (South Korea) and feed (Taiwan 2016) Feed (South Korea 2015)	GlyTol™ × TwinLink™ × VIPCOT™ cotton

(continued)

Table 16.7 (continued)

Event name	Gene	Trait	Country and year of approval	Trade name/ developer
3006-210-23 × 281-24-236 × MON88913 × COT102	*cry1F, cry1Ac vip3A(a) cp4 epsps pat*	Resistance to lepidopteran insects Glyphosate tolerance Glufosinate tolerance	Japan 2013, South Korea 2014–2015 (food and feed), Mexico 2014 (food)	WideStrike™ × Roundup Ready Flex™ × VIPCOT™ cotton
GHB614 × LLCotton25 × MON15985	*2mepsps Bar, cry1Ac, cry2Ab2*	Resistance to lepidopteran insects Glyphosate tolerance Glufosinate tolerance	Japan 2010, South Korea 2013 and 2011 (food and feed), Mexico (food) 2010, Taiwan (food) 2015	Bayer Crop Science (including fully and partly owned companies)
T304-40 × GHB119 × COT102	*cry2Ae cry1Ab, vip3A(a), bar*	Resistance to lepidopteran insects Glufosinate tolerance	Brazil 2018	Bayer Crop Science (including fully and partly owned companies)
GHB811 × T304-40 × GHB119 × COT102	*cry2Ae cry1Ab, vip3A(a), Bar, hppdPF W336*	Resistance to lepidopteran insects Glufosinate tolerance Isoxaflutole tolerance	Brazil 2019	BASF and Bayer Crop Science (including fully and partly owned companies)
Low seed gossypol				
TAM66274		Low seed gossypol	USA 2018	Texas A&M AgriLife Research University

Source: ISAAA's GM Approval Database. *http://www.isaaa.org/gmapprovaldatabase/*

16.10.1 Brazil

Brazil cultivates cotton over six provinces of which Motto Grasso cultivates the largest area under cotton. With an area of 1.6 Mha and a production of 2.77 million tonnes (ICAC Cotton Data book, 2019), Brazil in 2018 grew genetically modified cotton over 94% of the area. BRS-430 B2RF, BRS-432 B2RF, and BRS-433 B2RF, all with Bollgard II Roundup Ready Flex technology (Monsanto), were released by Embrapa/Bahia foundation, in addition to the foreign cultivars.

GM cotton events commercialized in Brazil:

Name	Event	Year of release	Target pest/herbicide
Lepidopteran pest tolerance	MON 531	2005	Bollworm tolerance
Roundup-resistant cotton	MON 1445	2008	Roundup Ready
LibertyLink cotton	LL cotton 25	2008	Glufosinate
Bollgard II cotton	MON 15985	2009	Enhanced spectrum of lepidopteran pest control
Bollgard RRF	MON 531 × MON 1445	2009	Lepidopteran and Roundup Ready resistant
GlyTol	GHB614	2010	Tolerant to glyphosate
TwinLink	T304-40 × GHB119	2011	Resistant to lepidopteran and tolerant to glufosinate
Glyphosate tolerance	MON 88913	2011	Roundup resistant
GlyTol × TwinLink	GHB614 × T304-40 × GHB119	2012	Tolerance to glyphosate and glufosinate ammonium, insect resistance
GlyTol × LibertyLink	GHB614 × LLCotton25	2012	Tolerance to glufosinate
Two-gene Bt cotton and glyphosate	MON15985 × MON88913	2012	Resistance to some lepidopteran and glyphosate tolerance

16.10.2 India

Six events have been approved for commercial cultivation in India, since the first approval of a GM event in the country in 2002. These events are Mon531, Mon15985, JK event 1, BNLA-601, *cry1C*Metahelix event, and the Nath event of which MON15985 is most widely cultivated. While almost all countries cultivate GM cotton varieties, Bt cotton was introduced as hybrids in India of which the Monsanto's events have been consistently the most popular. MON531 was soon replaced by hybrids containing MON15985. More than 95% of the area under cotton is cultivated with Bt cotton hybrids with MON15985 event. Unauthorized cultivation of GM cotton is often seen—illegal MON531 was being cultivated between 2002 and 2007, and currently unapproved cultivation of Roundup Ready Flex cotton is reported. Despite resistance to *cry* toxins detected in the pink bollworm since 2008 to single-gene Bt cotton and to the dual genes since 2010 and increase in the use of insecticides against sucking pests, the area under GM cotton has not substantially declined. It must be mentioned here that the single- and dual-gene BT cotton hybrids are still effective against *Helicoverpa*. An attempt is being made to cultivate short-duration, single-gene Bt varieties, by the public sector, and compact Bollgard II hybrids (by the private sector) under high-density planting, as an approach to overcome yield stagnation.

16.10.3 Pakistan

Cotton contributes 7.8% toward value addition in agriculture and fulfils 55% of country's domestic cooking oil requirements (Nazli et al. 2010). Only six GM events, two in cotton and four in maize, are approved in Pakistan. In 2005, Pakistan Atomic Energy Commission (PAEC) commercialized four varieties of Bt cotton exhibiting insect resistance (IR), i.e., IR-CIM-443, IR-CIM-448 (approved as IR-NIBGE-3701), IR-NIBGE-2 (approved as IR-NIBGE-1524), and IR-NIBGE-901 (Arshad et al. 2018). Punjab Seeds Council (PSC) approved 40 varieties, and by 2016, an additional 50 Bt cotton varieties were commercialized. At present, 96% of the total cotton production in Pakistan is Bt cotton which is planted on a total area of three million hectares. These varieties are backcross of Mon531 event which carries *cry1Ac* gene from Monsanto. At present more than 80 Bt cotton varieties having the single gene, i.e., *cry1Ac* (MON531 event), are available to farmers, and a couple of varieties having double genes, i.e., *cry1Ac* + *cry2Abcry2Ab* (CEMB-2 event), are in the pipeline.

Factors limiting seed cotton production in Pakistan are poor germination and limited availability of certified seed, reliance on single-gene event that has decreased efficacy against bollworms, increasing incidence and damage due to emerging pests, and narrow genetic base of the cotton germplasm from which varieties have been bred. The government, this year, has procured enough quantity of certified bioengineered seed of the latest cotton varieties that will increase farmers' choice to plant improved cultivars with the availability of free certified seed for 100,000 acres, through balloting. Arrangements for timely supply of fertilizer and pesticides were made. Regional trials with Bt varieties are recommended to identify the best performing ones with enhanced pest tolerance, leaf curl virus disease tolerance, and yield, for site-specific or region-specific recommendations (Karar et al. 2020).

16.10.4 Australia

Cotton is cultivated over 52,000 Ha in New South Wales and the rest of the 82,000 Ha is found in Queensland. Cotton production in Australia stands at 114,000 tonnes. All the 113 cotton varieties released for commercial cultivation in Australia are from CSIRO. Australia cultivates GM cotton with insecticide-resistant and herbicide-tolerant traits. GM cotton was first cultivated in 1996—Ingard 1 with *cry1Ac* was followed by Bollgard II and WideStrike. The herbicide-tolerant traits are Roundup Ready and Roundup Ready Flex, tolerant to glyphosate, and LibertyLink cotton, tolerant to glufosinate ammonium. With all their cultivars being GM, Australian cotton growers have put in place integrated insect pest management and integrated resistance management of weeds. Fall armyworm is being monitored having recently entered Australia. It is likely to remain within tropical areas away from most of the Australian cotton-growing regions, thereby having minimal impact

on cotton production. However, pest management programs have been developed for its management should it infest biotech cotton (Biki and Flake 2020). Climate change is being experienced severely in Australia and with increasing degree days; cotton crop is seen to display rank vegetative growth that may necessitate changes in the doses of growth regulators used in canopy management. The stewardship provided by Australian cotton is facilitated across the cotton-growing regions through different committees, such as the insecticidal transgenic technical panel, insecticide technical panel, herbicide-tolerant crops technical panel, and in-season troubleshooting technical committee, with members whose responsibilities are assigned for a given region.

16.10.5 China

China is the largest cotton producer in the world and is also one of the largest pesticide users, overtaking the USA (FAOSTAT 2017). Almost 30–40% of pesticides, of which 40% are belonging to the extremely or highly hazardous, as categorized by the WHO are used on cotton (FAOSTAT 2013).

Introduction of genetically modified crops in China began with insect-resistant cotton, expressing *cry1Ac*, in 1997. From an area of 5.7%, the total planting area reached more than 3.7 mHa with 96% adoption in 10 years. The area planted with cotton decreased to 2.9 MHa in 2016–2017 due to structural changes in policies governing the sector. And*cry1Ac*, *cry1Ab1Ac*, and *cry1A + CpTI* events are cultivated in China (Li et al. 2017).

The practice of applying excessive amounts of highly toxic pesticides has continued even after the adoption of Bt cotton (Qiao et al. 2017; Pemsl et al. 2005; Yang et al. 2005a, b). Risk averseness and poor knowledge on IPM by cotton farmers, upsurge of secondary pests, and poor Bt seed quality are the suggested causes for increasing pesticide use on GM cotton in China.

New approaches to GM cotton involve pyramiding of RNA interference along with *cry* toxins as a resistant management tool (Li et al. 2020). A National Scientific and Technological Innovation Plan issued by the government proposed to strengthen research on GM crops and promote the industrialization of new varieties of Bt cotton, Bt corn, and herbicide-tolerant (HT) soybeans over the next 5 years (Deng et al. 2019).

16.10.6 Burkina Faso

South Africa introduced Bt cotton in 1997. While it performed well in the initial years of its commercialization, the technology was soon found unsustainable. Introduction of GM cotton in Makhathini (South Africa) disrupted, indirectly, easy access to institutional credit and a guaranteed market. Long-term impacts and benefits of the technology were not fully appreciated, and the data generated covered

certain categories of farmers who benefit from these technologies over others, and these technologies disrupted farming systems. Burkina Faso followed South Africa to emerge as one of the leading adopters of agricultural biotechnology in sub-Saharan Africa (Vitale and Greenplate 2014). Genetically modified (GM) cotton, Monsanto's Bollgard© II, was legalized by the government of Burkina Faso in 2007 and was commercially introduced in 2009 (Vitale and Greenplate 2014). The success of Bt cotton in terms of yield gain and insecticide use reduction over conventional cotton was expected to impact the adoption of GM technology in Western and sub-Saharan Africa. Bt cotton outperformed conventional cotton by up to 2.2% in terms of yield. However, fiber quality was reduced in the upper half mean length and fiber strength by −1.45 to −2.09 mm and −19.7 to −40.57 kNm/Kg, respectively, while micronaire, maturity, short fiber index, reflectance, and yellowness were similar between Bt and conventional cotton. The reputation of cotton fiber from Burkina Faso was affected in the international market, and a penalty was promulgated on all export sales of cotton produced from 2010 (Fok 2016). In Burkina Faso, the decision to phase out Bt cotton was made by the cotton companies and not cotton farmers. "The higher yield of Bt cotton meant more income for farmers while the lower ginning ratio and shorter staple length meant less fiber, and of a lower quality, for cotton companies to sell. The case of Bt cotton in Burkina Faso exposed the conflicting interests within the cotton value chain, underlining how GM crops can produce different outcomes for different stakeholders" (Dowd-Uribe and Schnurr 2016). With this experience, steps such as introgression of traits into local varieties with backcrossing being made at the site, increasing the number of backcrosses, are now being put in place to secure the future road map for GM cotton in Burkina Faso.

Three regions cultivate cotton in Burkina Faso—Sofitex, Faso Cotton, and Socoma. Of the 615,000 ha, Sofitex cultivates 520,000 ha and cotton production in Burkina Faso is 170,000 tonnes. Three varieties are popularly cultivated in Burkina Faso that were released 6–24 years ago of which FK37, a 20-year-old variety, occupies 94% of the area. Fiber length of these varieties ranged from 28.1 to 30 mm, and fiber strength from 29.1 to 30.7 g/tex with a micronaire of 4–4.2.

16.10.7 USA

Thirty-two events have been reported in the USA, with traits responsible for insect resistance, herbicide tolerance, or both insecticide resistance and herbicide tolerance, according to ISAAA. Bollgard II (Mon15985), Bollgard, and VIPCOT regulate resistance in cotton plants to a wide range of lepidopteran pests. Of the herbicide-tolerant toxins, BXN (tolerance to oxynil herbicides), GlyTol (resistance to glyphosate), LibertyLink cotton (tolerance to glufosinate), and Roundup Ready and Roundup Ready Flex cotton (tolerance to glyphosate) have been listed under the ISAAA website. Two insecticide-resistant and herbicide-tolerant traits are registered—TwinLink cotton (glufosinate+ lepidopteran pest tolerance) and BXN plus Bollgard cotton (oxynil herbicide and lepidopteran pest tolerance) to combat weed and insect problems, simultaneously.

16.11 Economic Benefits of BT Cotton

There is a big debate still going on regarding the economic benefits derived due to the adoption of Bt cotton across the globe. Early studies proved that the major benefits from Bt cotton include effective control of bollworms leading to significant yield increase, drastic reduction in chemical sprays, and substantial increase in net profit to farmers (Manjunath 2007; Sadashivappa 2008; Subramanian and Qaim 2009; Rahman et al. 2015). The numbers of pesticide sprays and expenditure on insecticides decreased substantially, and higher yields were realized by the Bt cotton adopters (Dev and Rao 2007). The farmers in major cotton-growing states in India benefitted significantly from adopting Bt technology through higher profitability mainly due to reduced pest control costs and higher yields, though there was considerable variation in key variables like yield, cost, pesticide use, etc. (Ashok et al. 2012). In India the Bt cotton adoption rate has increased tangible socioeconomic benefits for small farm holders. Further, living standard of poor and small farm holders has increased by 18% by adopting Bt cotton; thus this technology contributes to positive socioeconomic development (Kathage and Qaim 2012). During the period 2002–2015, the total benefit gained due to the adoption of Bt cotton has been estimated to be of 220 billion with 85% share accruing to producers and 15% to the private seed companies/marketing firms (Ramasundaram et al. 2014). Brookes and Barfoot (2020) reviewed the global, socioeconomic, and environmental impacts of GM crops since its introduction for commercial cultivation from 1996 to 2018. Farmers around the world have adopted GM crops and continued to use the technology for their production system. The direct global farm income through GM in 2018 was $18.95 billion. Cotton sector significantly gained higher yields and lowers the production cost since 1996. Farm income level in 2018 was increased by $4.57 billion. Cumulatively global GM cotton farmers have benefitted as additionally of 65.8 billion since 1996 (Brookes and Barfoot 2020). The huge income gain for developing countries farmers has been through insect-resistant GM cotton and herbicide-tolerant soybean (Clive 2014). But some of the researchers attribute the increase in the yield during the Bt era to increased fertilizer use and the introduction of new insecticides.

16.12 Future Outlook

Technologies evolve concurrently with scientific advances. No technology is perfect. Every technology presents advantages and disadvantages. Because genetic changes are inheritable, GM technologies have the potential of offering unique advantages that many other nongenetic technologies do not. However, these heritable genetic changes may have the propensity to influence biodiversity in a significant manner. Therefore, GM technologies have attracted more attention of environmental activists, and the need for biosafety has been emphasized more with

GM products than with any other technologies. GM crops should not be seen as silver bullets. They are generally developed for one or two traits that can indeed have a strong impact on crop production and crop protection. Impact of any technology depends on stewardship and its methods of deployment. The 25 years of global GM cotton experience taught several lessons. GM technology has been readily accepted by majority of farmers because of the ease in adoption; GM technology is not a silver bullet; GM technology is not invincible, because insects display strong potential to develop resistance to Bt toxins and weeds develop resistance to herbicides, because of which the technology is rendered unsustainable. Experience also highlights the importance of preemptive strategies that must be developed and complied with, so as to delay resistance in insects and weeds and to conserve efficacy of the GM traits for the longest possible time. Experience in India shows that restricting the Bt technology to hybrid cotton varieties did not benefit rainfed regions, because of the suboptimal performance of input-intensive hybrids in rainfed conditions, thereby resulting in poor yields, for example, as in predominantly rainfed states such as Maharashtra.

Across the globe, except China and Uzbekistan, all other countries have adopted GM cotton products that were developed by multinational or transnational companies. The two main GM technologies, namely, herbicide-tolerant (HT) cotton and insect-resistant (IR) cotton, have been adopted in more than 75% of the global cotton acreage. A global analysis shows that *Bt* cotton technology has been effective so far in protecting cotton against the *Heliothine* species, except a few isolated cases of field resistance in the USA, China, and Pakistan. However, pink bollworm resistance to Bt cotton highlights the need for stringent compliance with insect resistance management (IRM) strategies. More and more genes are being continuously added in pyramids over the recent years to enhance the efficacy, increase the spectrum of efficacy, and decrease resistance risk. However upgrading a technology with new genes requires investment, which is recovered back eventually by the technology developers from farmers in the form of trait fee or technology fee or royalties. With continuous pyramiding of genes, GM products are progressively becoming expensive. With progressive exposure of the pyramided GM products to the ecology and environment, their efficacy is declining over time, which further necessitates gene pyramiding. It remains to be seen as to how long this cyclic pattern would continue to be technically feasible and economically acceptable especially by smallholder farmers who comprise more than 95% of the global cotton farmers.

Thus far Asia and African countries have been cultivating Bt cotton with either a single gene or with two genes. The two technologies that are being used in other countries and which India, Pakistan, and Africa (except South Africa) do not have are the “three-gene-based Bt cotton” and “herbicide-tolerant (HT) cotton.” The three-gene-based *Bt* cotton neither kills the “Bt-resistant pink bollworms of India” nor any other insects that are not killed by the two-gene Bt cotton. Herbicide-tolerant cotton only facilitates weed control with herbicide spray; there is no evidence anywhere to show that the introduction of these technologies can help India to increase yields or reduce pesticide usage (Kranthi 2020).

What is the future outlook? What kind of GM products will be available to cause a breakthrough in Asia and Africa to increase yields? Will smallholder farmers be able to afford the more expensive multigene GM pyramid products? Have smallholder farmers in Asia been enticed into a technology trap that forces them progressively to adopt newer and more expensive technologies without allowing them any alternative options to move out? Smallholder farms obtain low yields in India, Pakistan, and Africa. While crop protection is just one facet of crop production, the main question remains: are there any GM traits available anywhere in the world that can increase yields or help to increase cotton yields?

A large section of the farming community in developing and developed countries has experienced the advantages and disadvantages of GM cotton over the past 25 years. With the existing technologies, a breakthrough in yields hasn't been apparent, at least until date. With new options of gene editing technologies such as CRISPR/Cas or RNA interference (RNAi) on the anvil, there is new hope that there could be newer technologies with better selectivity, with positive impacts on yields, and with least negative impacts on biosafety and biodiversity.

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