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This book is dedicated to Dr. Brent Eugene Zehr, starting with maize breeding he grew more and more interested in cotton and the impact of biotechnology in cotton in India, dedicating the last 10 years of his life to improving cotton.

Preface

The food, feed, fiber, and fuel needs of the changing world pose the challenge of doubling or tripling of world food, feed, and fiber production by the year 2050 to meet the needs of a 11 billion global population. In addition, the dramatic changes in food prices in the recent years further warrant that production and productivity need to be enhanced to ensure adequate supplies. Biotechnology can make a significant contribution to this effort as demonstrated by cotton and other crops; the new advances in biotechnology have made it possible to develop plants that contain genes that were not possible to be developed by sexual means. Cotton has been a leader in the use of biotechnology. With the introduction of Bt cotton, followed by stacked cotton products (insect and herbicide tolerance) and extensive use of molecular breeding tools, cotton cultivation has been much improved.

The contributions in this book illustrate the scientific advances that are going on in cotton and the impact they continue to deliver for all cotton growers. Twelve percent of the global cotton area is now under biotech products at 15.5 million ha. The primary benefits from using genetically engineered cotton include reduced insecticide use, lower production costs, improved yields, lower farming risks, and increased opportunities to grow cotton in areas of severe pest infestation. Secondary benefits include higher populations of beneficial insects in cotton fields, reduced pesticide runoff and air pollution, less exposure to the farm worker, reductions in labor and fuel use, and improved soil and water quality.

Cotton crop is used for multiple purposes, fiber being the key component. However, cotton oil, cotton meal, and plant residues are also utilized by various industries. The impacts of GE cotton on human health and the environment have been studied, showing a positive impact.

Countries where Bt cotton or other genetically engineered cotton has been cultivated have in place stringent regulatory mechanisms usually monitored by several ministries. Under these guidelines, studies have concluded that genetically engineered cotton does not pose any different risks to human or animal health than non-GE cotton and environmentally, it has a positive impact, including on biodiversity.

Studies in India and China show that Bt cotton has impacted the farm, the farmer, and the consumers by increasing the yields and reducing insecticide use in both countries.

Also, the benefits of the technology have been seen at all farm size levels, whether it is with the small holder farmers or with the large farms of North America.

The new research highlighted in this book also shows how molecular genetics is leading the way to improved efficiency by understanding the cotton genome and applying this information to the identification of markers, genes, or metabolic processes to enhance breeding efficiencies, as well as new product development.

The future generation of biotech cotton products are beginning to address more complex traits such as drought, salinity, or fertilizer use. A cotton revolution has already taken place, and these new technologies will continue to improve cotton production around the world. They will have a positive impact on the livelihoods of millions of cotton farmers and will supply the growing needs of the consumer for this most important natural fiber.

November 2009 Usha Zehr

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Chapter 1
Cotton: An Introduction

B.M. Khadi, V. Santhy, and M.S. Yadav

1.1 Introduction

Cotton is currently the leading plant fibre crop worldwide and is grown commercially in the temperate and tropical regions of more than 50 countries (Smith 1999), with a total coverage of 34 million ha. The cotton seed coat extends into tubular fibre and is spun into yarn. Specific areas of production include countries such as USA, India, China, the Middle East and Australia, where climatic conditions suit the natural growth requirements of cotton, including periods of hot and dry weather, and where adequate moisture is available, often obtained through irrigation. Among the five major cotton growing countries, China holds the highest productivity level (1,265 kg/ha), followed by USA (985 kg/ha), Uzbekistan (831 kg/ha), Pakistan (599 kg/ha) and India (560 kg/ha) (Table 1.1). India ranks first in terms of cultivated area, occupying over a quarter of the world cotton area, followed by China, USA, and Pakistan. About 26.247 million metric tons of cotton are produced globally, and the major countries contributing the most are China, India, USA and Pakistan followed by Uzbekistan, Turkey, Australia, Greece, Brazil and Egypt.

The cotton species recognized in the world are about 50, of which 4 are cultivated. Two of these (*Gossypium arboreum and G. herbaceum*) are diploids, and two (*G. hirsutum* and *G. barbadense*) are tetraploids. More than 80% of the world's cotton area is covered by tetraploids. However, diploid cottons are cultivated in Asia and the Middle East. India is the only country where all the cultivated species and some of their hybrid combinations are commercially grown.

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Table 1.1 Major cotton growing countries of the world (2007–2008)

Sl. No.	Country	Area (000 ha)	Production (000 metric tonnes)	Productivity (kg/ha)
1	China (M)	6,385	8,078	1,265
2	USA	4,245	4,182	985
3	India	9,555	5,355	560
4	Pakistan	3,082	1,845	599
5	Uzbekistan	1,450	1,206	831
6	Turkey	520	675	1,298
7	Australia	63	126	2,000
8	Brazil	1,077	1,603	1,487
9	Egypt	246	224	909
10	Greece	300	285	950
11	Argentina	311	152	489
12	Others	6,129	2,516	
	World average	33,363	26,247	787

Source: Cotton: World Statistics, November 2008

The diversity of cotton cultivars and cotton agro climatic zones in India is larger when compared to other major cotton growing countries in the world.

1.2 History and Taxonomy

The first reference to cotton is found in a *Rig-Veda* hymn, which was written about the fifteenth century BC. The use of cotton in about 800 BC is recorded in Manu's "Dharmashastra". The Sanskrit word *karpasa,I,* which is connected to kapas of modern Hindustani, was used in ancient literature. The technological and agricultural term in English, Cotton, which describes cultivated species of *Gossypium,* comes from the Arabic word *qutum* or *kutum* (Brown and Ware 1958). Systematic taxonomic study of cotton started with the description of *Gossypium* by Linnaeus in 1953.

The work of Sir George Watt entitled "The wild and cultivated cotton plants of the world" provided a new dimension to the taxonomic studies. The cytological studies of Zaitzev (1928) cited in the paper "A contribution to the classification of genus *Gossypium*" was a landmark in cotton classification. Kohel (1973) has addressed the description of genetic mutants based on the rules of the International Committee on Genetic Symbols and Nomenclature.

Among the 50 species recognized in the dicotyledonous genus *Gossypium*, belonging to family Malvaceae about 45 are diploids divided into three geographical groups and corresponding subgenera viz. *Sturtia, Houzingenia* and *Gossypium*, five species are tetraploids included in one subgenus viz. *Karpas* (Fryxell 1984; Wendel and Cronn 2003; Cronn and Wendel 2004) (Table 1.2).

The diploid species with 26 chromosomes are placed in eight cytogenetic genome groups designated A–G and K and tetraploids with 52 chromosomes in

Table 1.2 Classification of Genus *Gossypium*

Primary distribution	No. of species	Subgenus	Section	Subsections	Examples of species
Africa (Africa and Arabian peninsula)	14	*Gossypium*	*Gossypium*	*Gossypium*	Asiatic diploids
			Pseudopambak	*Anamola*	*G. anomalum*
				Pseudopambak	*G. stocksii*
				Longibola	*G. longicalyx*
Australia (NW Kimberly region)	17 (16 taxonomically described)	*Sturtia*	*Sturtia*		*G. sturtianum*
			Grandi calys		*G. costulatum*
			Hibiscoidea		*G. australe*
America (West Mexico Galapagos islands and Peru)	14 (13 taxonomically described)	*Houzingenia*	*Houzingenia*	*Houzingenia*	*G. thurberi*
				Integrifolia	*G. davidsonii*
				Caducibractealaa	*G. harknessii*
			Erioxylum	*Erioxylum*	*G. aridum*
				Selera	*G. gossypioides*
				Astromericana	*G. raimondii*
American Pacific	5	*Karpas*	–	–	All tetraploid species including New World cultivar

one group designated AD (Endrizzi et al. 1985; Fryxell 1992; Stewart 1995; Wendel and Cronn 2003) according to the genome affinities. The five allotetraploid species are the united version of Old World A and New World D genome in A genome cytoplasm (Skovsted 1937; Brubaker et al. 1999a) (Table 1.3).

Table 1.3 *Gossypium* species grouped according to germplasm pool

Pool	Species	Genome	Seed	Notes
Primary	*G. hirsutum*	AD_1	+	Current and obsolete cultivars, breeding stocks, land races, referral and wild accessions
	G. barbadense	AD_2	+	Current and obsolete cultivars, breeding stocks, land races, referral and wild accessions
	G. tomentosum	AD_3	+	Hawaiian Islands
	G. mustelinum	AD_4	+	NE Brazil
	G. darwinii	AD_5	+	Galapagos Islands
Secondary	*G. herbaceum*	A_1		Cultivars, landraces of Africa and Asia minor, one wild from Southern Africa
	G. arboreum	A_2	+	Cultivars, landraces from Asia minor, SE Asia and China; some African
	G. anomalum	B_1	+	Two subspecies, Sahel and SW Africa
	G. triphyllum	B_2	+	SW Africa
	G. capitis-viridis	B_3	+	Cape Verde Islands
	G. trifurcatum	$B_?$	–	NE Somalia
	G. longicalyx	F_1	+	Trailing shrub, Sudan, Uganda, Tanzania
	G. thurberi	D_1	+	Sonora Desert, North America
	G. armourianum	$D_{2\text{-}1}$	+	Baja California (San Marcos Island)
	G. harkenssii	$D_{2\text{-}2}$	+	Central Baja California
	G. davidsonii	$D_{3\text{-}d}$	+	Southern Baja California
	G. klotzchianum	$D_{3\text{-}k}$	+	Galapagos Islands
	G. aridum	D_4	+	Arborescent, Pacific slopes of Mexico
	G. raimondii	D_5	+	Pacific slopes valleys of Peru
	G. gossypioides	D_6	+	Central Oaxaca, Mexico
	G. lobatum	D_7	+	Arborescent, Central Michoacan, Mexico, West Central Mexico
	G. tumerui	D_{10}	+	NW Mexico, coastal
	G. schwendimanii	D_{11}	+	Arborescent, El Infiernillo Valley, SW Mexico
Tertiary	*G. sturtianum*	C_1	+	Ornamental, Trans central Australia arid zone
	G. robinsonii	C_2	+	Western Australia
	G. bickii	G_1	+	Central Australia arid zone
	G. australe	G	+	Trans Australia, North arid zone
	G. nelsonii	G	+	Central Australia
	G. costulatum	K	+	North Kimberley (wet–dry tropical Western Australia)
	G. cunninghamii	K	+	Northern NT, Australia
	G. enthyle	K	+	North Kimberley, WA
	G. exgiuum	K	+	Prostrate, North Kimberley, WA
	G. nobile	K	+	North Kimberley WA
	G. pilosum	K	+	Terailing, Nort Kimberley, WA
	G. populifolium	K	+	North Kimberley, WA

(*continued*)

Table 1.3 (continued)

Pool	Species	Genome	Seed	Notes
	G. pulchellum	K	+	North Kimberley, WA
	G. rotundifolium	K	+	Prostrate, North Kimberley, WA
	G. sp. nov.	K	+	North Kimberley, WA
	G. stocksii	E_1	+	Arabian Peninsula and Horn of Africa
	G. somalense	E_2	+	Horn of Africa to Chad
	G. areysianum	E_3	+	Yemen
	G. incanum	E_4	+	Yemen
	G. benadirense	E	–	Ethiopia, Somalia, Kenya
	G. bricchettii	E	–	Somalia
	G. vollesenii	E	–	Somalia

1.3 Origin and Distribution

DNA-sequence phylogenetic data suggest that 6–7 million years ago, following a trans-oceanic dispersal event, a D genome diverged from the African lineage that eventually gave rise to the A genome, and became a separate lineage in the Americas (primarily Mexico) (Senchina et al. 2003; Wendel and Cronn 2003; Cronn and Wendel 2004). From another long-distance dispersal event 1–2 million years ago, a tetraploid originated through hybridization of an African plant of the A-genome group, perhaps most closely related to the present-day species *G. herbaceum*, with a resident plant of the D-genome group, most closely related to the present-day species *G. raimondii* (Wendel et al. 1992; Senchina et al. 2003; Wendel and Cronn 2003; Kebede et al. 2007). The nascent disomic AD allotetraploid from that single polyploidisation event evolved into the five present-day tetraploid species (Endrizzi 1962). Comparative RFLP mapping was used to construct genetic maps for the allotetraploids (AD genome $n = 26$) and diploids (A & D genome $n = 13$) (Brubaker et al. 1999b). The study showed that allotetraploid A and D genomes and A & D diploid genomes are recombinationally equivalent despite nearly two fold difference in physical size. Polypoidisation in *Gossypium* is associated with enhanced recombination as genetic lengths for allotetraploid genomes are over 50% greater than those of their diploid counterparts. The concept of organismal and genome relationships of diploid and allopolyploid taxa in the genus *Gossypium* have been given in Fig. 1.1.

Gossypium raimondii, a rare species of northwestern Peru, is considered to be the diploid with the genome that has retained the most similarity to this ancestral D-genome species (Guo et al. 2007). It is one of the more recently evolved of the DD species, having diverged in isolation as a result of a long-distance dispersal event from Mexico (Wendel and Cronn 2003; Alvarez et al. 2005).

Soon after separation of the D-genome lineage, African *Gossypium* further diverged with a long distance dispersal event resulting in establishment of an Australian lineage (which evolved into the three genome groups C, G and K). The lineage in Africa evolved further into four genome groups, first with divergence of

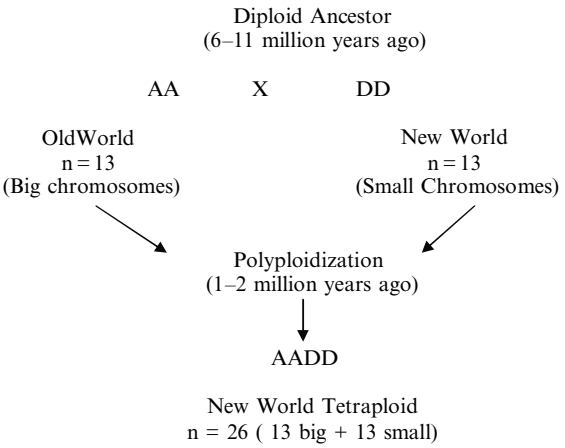

Fig. 1.1 Evolution and phylogenetic relationships among *Gossypium* genus (Endrizzi et al. 1985; Wendel 1989)

the E-genome lineage, subsequently the B-genome lineage, and most recently the F- and A-genome lineages (Cronn et al. 2002; Cronn and Wendel 2004).

The means and route of the relatively recent long-distance dispersal of the A-genome fruit/seed(s) and place of origin of the progenitor allotetraploid continue to be researched (Wendel and Cronn 2003). The A and D genomes of the South American tetraploid *Gossypium mustelinum* (northeastern Brazil) are genetically most similar to the ancestral type that differentiated into the five present-day widely dispersed tetraploids (Wendel et al. 1994). The disseminule of an AA species may have travelled via sea currents from Africa to the Americas (Renner 2004). Then, pollen from an American diploid (DD) species fertilized the immigrant, and chromosome doubling produced the original AADD tetraploid.

1.3.1 Origin, Domestication and Distributioin of Diploids

Wild (non-feral) *Gossypium herbaceum* subsp. *Africanum* occurs naturally in the savanna biome across southern Africa (Vollesen 1987; Wendel et al. 1989), whereas the domesticated plant *G. herbaceum* subsp. *herbaceum* is found disjunctly farther to the northeast, being grown mainly from Ethiopia to Central Asia, northwestern China and India (Wendel et al. 1989; Guo et al. 2006). The original ranges or centres of domestication of *G. herbaceum* subsp. *herbaceum* are unclear. *G. herbaceum* consists of five races: (1) *Africanum*, (2) *Acerifolium*, (3) *Persicum*, (4) *Kuljianum* and (5) *Wightianum*. Circumstantially, *G. herbaceum* subsp. *herbaceum* might be from Southwest Asia and *G. arboreum* possibly from India (Santhanam and Hutchinson 1974).

Gossypium arboreum is grown primarily across Asia farther to the east, from India to Korea (Wendel et al. 1989; Basu 1996; Guo et al. 2006). The genome of

G. arboreum is derived from that of *G. herbaceum* and these two species are set apart by a reciprocal translocation (Gerstel 1953). Genetically both species are closely related and are good functional diploids. *G. arboreum* arose as an incipient species with the origin and fixation of the translocation. The accumulation of genes due to consistent isolation and selection supported by hybridization resulted into eventual reemergence of *G. arboreum* as a full fledged species (Fryxel 1984). The races evolved from *G. arboreum* are (1) *Indicum*, (2) *Burmanicum*, (3) *Sinense*, (4) *Sudanense* and (5) *Cernum*.

The cultivated AA diploids of the Old World are typically short-staple cottons, with a fibre length of less than 23 mm. These cottons can be important regionally, and still may be preferred especially in harsh or dry growing conditions (Basu 1996; Rajendran et al. 2005).

The wild diploid species belonging to B, E and F genome have distribution in Africa and Arabia and the entire D genome has distribution in South and Central America (Fryxell 1992). Kimberly cottons belonging to *Grandicalyx* (K) and other diploids belonging to *Sturtia* (C) and *Hibiscoidea* (G) have been found distributed in Australian continent (Craven et al. 1995).

1.3.2 Origin, Domestication and Distribution of Allotetraploid Cottons

The two other cultivated species (*G. hirsutum* and *G. barbadense)* are in the AD allotetraploid genomic group and contain one genome similar to those of the A-genome diploids, and one similar to those of the D-genome diploids (Endrizzi et al. 1985; Wendel et al. 1989).

Originally wild (i.e. non-feral) *Gossypium barbadense* is considered to occur naturally in the dry coastal region of northern Peru and southern Ecuador (Perey and Wendel 1990; Westengen et al. 2005). Cultivated *G. barbadense* is considered to be native to South America and was adapted to the coasts of the Islands of Carolinas and Georgia, hence called "Sea Island cotton." The introductions of *G. barbadense* in Egypt during early nineteenth century brought the name "Egyptian cotton". Subsequent reintroduction of the Egyptian cotton into the Southwest USA added another synonym "Pima cotton". The two races developed in *G. barbadense* are *brasilliense* and *darwinii*.

G. hirsutum is considered native to Mexico and Central America. Since these were grown in upland sites they are called "Upland cotton". Crosses between many varieties of introduced cottons have caused the worldwide expansion of upland cotton (Iqbal et al. 2001). The upland type of *Gossypium hirsutum* and derived varieties are the mainstay of the worldwide industry (May and Lege 1999). The seven races evolved in *G. hirsutum* are *Palmeri*, *Morilli*, *Richmondii*, *Yucatenanse*, *Punctatum*, *Marie galante* and *Latifolium*. The race *Latifolium* is widely cultivated in the world.

Way back in 1937, Skovsted based on his cytogenetic studies hypothesized that the chromosome complement of allo-tetraploid was made up of one set of 13 smaller homologous chromosomes of New World diploid species. Later Beasley (1940) and Hutchinson (1940) independently confirmed Skovsted's hypothesis. Gerstel (1963) exhibited the contribution of *G. herbaceum* as A genome and *G. raimondii* as D genome in the development of allotetraploid based on the univalent frequencies in hexaploids of crosses between tetraploid, *G. hirsutum* with different diploids. Study of multivalent frequencies and genetic segregation ratios showed that *G. raimondii* (D_5) closely approached allotetraploid behaviour and must be related to the D sub genome contributor of the AD allotetraploid (Gerstel 1963; Phillips 1963). Further, the data on DNA content of A and D genomes and their near additivity in tetraploids also confirmed the polyploidisation between A and D genomes (Gomez et al. 1993). The diploid like behaviour of allotetraploids was confirmed by Endrizzi and Phillips (1960) and Endrizzi (1962). The restriction endonuclease studies on cpDNA (chloroplast DNA) indicated the presence of A genome cytoplasm in tetraploids (A_1) (Wendel et al. 1989).

For all practical purposes, the progenitors of the New World allotetraploid cottons have now been fixed as A_1 (*G. herbaceum var. africanum*) and D_5 (*G. raimondii*). However, in the studies on the spread of dispersed repetitive DNA into slot-blot hybridization, about 400,000 copies of A genome specific dispersed repeats were found widely distributed throughout the genome of *G. gossypioides* (Zhao et al. 1998). This discovery adds support to a suggestion previously made, based on evidence from only a single genetic locus (Wendel et al. 1995) that this species may be either the closest living descendent of the New World (D genome) cotton ancestor or an adulterated relic of polyploid formation. The present understanding of Cladogenetic relationships in *Gossypium* genus is presented in Fig. 1.2.

The large body of cytological data indicating the similarity in the chromosome end arrangement in all five tetraploid New World cottons and the perfect meiotic

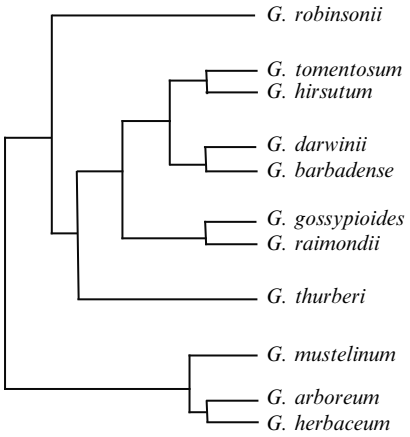

Fig. 1.2 Present understanding of Cladogenetic relationships in *Gossypium* genus

pairing, suggested monophyletic origin of allotetraploids. However, the existence of several diploid species in the New World coupled with their geographical distribution suggested a polyphyletic origin. This concept of polyphyletic origin was supported by chromatographic studies of Parks et al. (1975), studies on electrophoretic banding pattern of seed protein by Johnson (1975) and investigation of RFLP data by Reinisch et al. (1994).

After polyploidation, the AD genome group diverged into three distinct lineages (Brubaker and Wendel 1994; Small et al. 1998) presently represented by five species, including economically important cottons of *G. hirsutum* and *G. barbadense* (Fig. 1.3).

Through intensive study of germplasm collections from the widespread complex in the region, Hutchinson (1959) distinguished six domesticated races (not botanical varieties) and one wild race based mainly on their habit and morphology, and found that these races had generally distinct geographic distributions, with the most differentiation of the domesticated types in southern Mexico:

- Morrilli – inland montane, southern Mexican plateau and northward
- Palmeri – pacific slope, southern Mexico west of Isthmus of Tehuantepec
- Richmondi – pacific slope in Gulf of Tehuantepec region

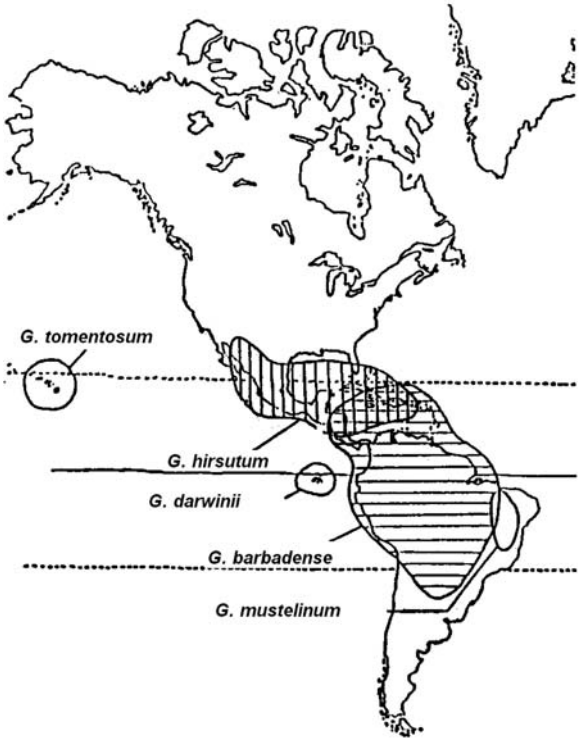

Fig. 1.3 Distribution of allotetraploid species of *Gossypium* genus

- Punctatum – Yucatam Peninsula, and northward on Atlantic slope, to Florida (USA) and Bahamas
- Yucatanense – wild, northwestern coast of Yucatan Peninsula
- Latifolium – Guatemala (both slopes) and southernmost Mexico (Chiapas), nearby areas
- Marie-galante – northern Central America (Guatemala) southward to Colombia on both coasts, Caribbean region (Antilles) and northeastern Brazil

1.4 Germplasm Resources in Cotton

Large collections of *Gossypium* germplasm at several centres around the world, including the United States Department of Agriculture (USDA) collection of Primitive types of *G. barbadense* and *G. darwinii* at Phoenix, Arizona, obsolete cultivars of *G. hirsutum* at Stoneville, Mississippi, primitive types and genetic markers of *G. hirsutum* and all other species at College Station, Texas are being maintained. The National Seed Storage Laboratory at Fort Collins, Colorado has genetic stocks of all the above mentioned. The Central Institute for Cotton Research (CICR) in India maintains large collections (~10,000 accessions) of all four cultivated species and 26 wild species. The cotton germplasm collected from Central and South American area by Mauer and Bukasor are maintained in Uzbekistan. In addition over 2,300 accessions of various species introduced from 18 Asian, 17 African, 9 latin American and 2 North American countries are maintained in China.

Stewart (1995), based on the model concept of germplasm pools described by Harlan and DeWet (1971), classified cotton gene pool as primary, secondary and tertiary according to ease with which genes could be transferred from source to the tetraploid cultivated cotton.

Based on the ability to generate fertile hybrids, cotton is grouped into three gene pools as detailed below.

1.4.1 Primary Gene Pool

The primary pool of cotton germplasm consists of all natural G*ossypium* allotetraploids (2AD) which cross with *G. hirsutum* lines resulting in direct genetic recombination between the parental genomes. Production of sexual hybrids requires no special technique in this pool other than synchronized flowering. The gene pool comprises the subgenus *Karpas:* the three wild tetraploid species (*G. mustelinum*, *G. darwinii*, *G. tomentosum*) and the wild, commensal, landrace, cultigens and feral *G. barbadense* and *G. hirsutum*. Favourable traits have been incorporated from this gene pool (particularly from *G. hirsutum* and *G. barbadense*) into the modern crops (Endrizzi et al. 1985; Meredith 1991; Stewart 1995).

1.4.2 Secondary Gene Pools

All species in these gene pools are diploids. In addition to cytological barriers to hybridization, varied physiological barriers exist between the diploids and *G. hirsutum,* the usual focus for improvement. In vitro culture of ovules partly solves the problem (Stewart and Hsu 1977). Three main breeding strategies have been devised to overcome sterility barriers and can lead to successful introgression of desirable traits (Endrizzi et al. 1985; Meredith 1991; Stewart 1995). In two schemes, crossing a diploid with *G. hirsutum* results in sterile triploids (3×), with few rare exceptions (Meyer 1973). Hexaploids (6×) are then made (using colchicine) by chromosome doubling of the triploid genome. The hexaploid can then be crossed with a different diploid and result in a tri-species tetraploid hybrid. Or, *G. hirsutum* can be crossed with the hexaploid; the resultant pentaploids (5×) can be self-crossed, or crossed again with *G. hirsutum,* resulting in a tetraploid.

The secondary gene pool includes the evolutionarily closer diploids, thus comprising the D-genome species (subgenus *Houzingenia*) and the A-genome species, as well as the African B- and F-genome species (Phillips 1963; Phillips and Strickland 1966; Wendel and Cronn 2003).

Bridge-crosses between two diploid species, induced genome doubling, and then crossing with *G. hirsutum* are other useful strategies for gene transfer. Such an approach using the A-genome and D-genome species produces synthetic AD tetraploids, which may be readily crossed with *G. hirsutum*. Genes from the A or D Genome may thus be transferred to the upland cotton crop (Saravanan et al. 2007). For example, the ATH tri-species hybrid (*G. arboreum* × *G. thurberi*) × *G. hirsutum* has been used to introduce fibre strength.

1.4.3 Tertiary Gene Pool

This gene pool includes the evolutionarily distant diploids, thus comprising the African-Arabian E-genome species, and the Australian C-, G- and K-genome species (Wendel and Cronn 2003). It has not been possible to obtain hybrids of *G. hirsutum* with G-genome species, where as hybrids can be obtained with C-genome species and variable results with K-genome species (Brown and Brubaker 2000). The diploid species possess traits of agronomic value. The sterility behaviour of their hybrids with allotetraploid can be overcome using synthetic polyploids as introgression bridges (Mergeai et al. 1999; Lopez and Brubaker 2007). Desirable traits include gossypol-free seeds, which occur in both the C- and G-genome species. The first gene from the tertiary gene pool introgressed into *G. hirsutum* came from the C genome's *G. sturtianum* (Benbouza et al. 2008). The gossypol formation is reduced through control of terpenoid aldehyde methylation (Brubaker et al. 1999c). Although *G. sturtianum* is the species in subgenus *Sturtia* that crosses most readily with *G. hirsutum*, the F_1 is completely infertile.

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Chapter 2
Cotton in India

B.M. Khadi, V. Santhy, and M.S. Yadav

2.1 Introduction

India is the second largest cotton producer in the world (31.5 million bales), with the largest area (9.56 million ha) under cultivation. The productivity in India is poor (560 kg lint/ha) despite being the only country to have successfully adopted hybrid cotton on significant area. The Indian cotton scenario has shown a positive trend with consistent increase in area, production, and productivity during 2001–2002 to 2008–2009 (Fig. 2.1). On the basis of agro-climatic situations, cotton growing areas have been classified into four zones (Table 2.1). As described in Chap. 1, India grows all four cultivated species besides hybrids of cotton with majority of the area being used for the tetraploid cottons. *Gossypium hirsutum* represents 90% of the hybrid cotton in India, and the current Bt hybrids are of *G. hirsutum*. Cotton is the major cash crop of India and accounts for 65% of the fiber used in the textile industry. Cotton impacts the lives of an estimated 60 million people in India. By way of exports, foreign exchange earnings of cotton amount to about INR760 billion or US$19 billion, which is one-third of the total foreign exchange earning of our country.

Cotton is cultivated in four distinct agro-ecological regions (north, central, south, and east) of the country. The northern zone is almost totally irrigated, while the percentage of irrigated area is much lower in the central (23%) and southern zone (40%). The largest area under cotton cultivation is in the central zone (66.66%) of India.

The north zone (Punjab, Haryana, and Rajasthan) occupies only 15.61% of the total cultivated area but contributes more than 14.92% of the production and

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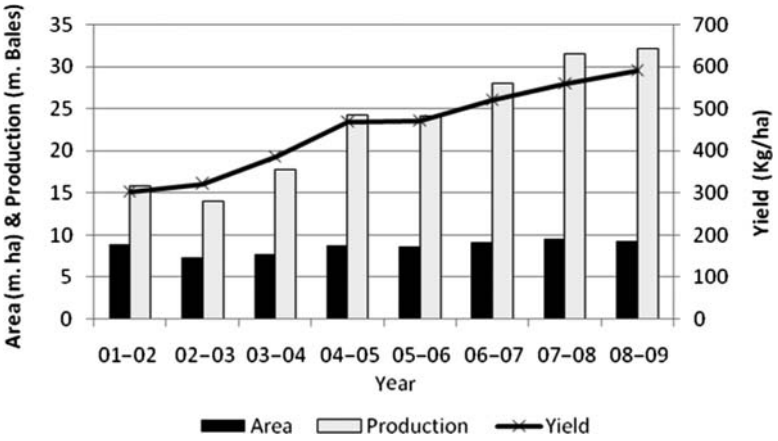

Fig. 2.1 India's cotton area, production and yield

Table 2.1 Cotton growing zones in India

Zone	Species grown	% of cotton area	% of cotton production
Northern zone	*G. hirsutum, G. arboreum,* Inra-*hirsutum*, intra *arboreum* hybrids	15.61	14.92
Central zone	*G. hirsutum, G. arboreum* G. *herbaceum* and Inra-*hirsutum,* Inter-specific tetraploid, diploid hybrids and intra-*arboreum*	66.66	61.90
Southern zone	*G. hirsutum, G. herbaceum, G. arboreum, G. baradense,* Intra-*hirsutum* and Inter-specific tetraploid hybrids and intra-*arboreum*	16.89	18.73
Eastern zone	*G. arboreum*		

varieties/hybrids (including Bt hybrids) limited to only *G. hirsutum* and *G. arboreum*. The central zone (Maharastra, Madhya Pradesh, and Gujarat), occupying more than 66.66% of the total area, contributes less than 61.90% to the total production and is characterized by rampant proliferation of hybrids. Bt technology has been extensively adopted in this region. The south zone (Karnataka, Andhra Pradesh, and Tamil Nadu) is typical of all types of cotton, hybrids (inter and intra-specific, diploid, and tetraploid), and varieties (diploid and tetraploid). The south zone is occupying 16.89% of area and contributing nearly 18.73% in national production. The all India cotton productivity trends during 2001–2002 to 2008–2009 are depicted in Fig. 2.2.

Among nine major cotton growing states, Maharashtra (3.2 million ha) has the largest area followed by Gujarat (2.5 million ha) and Andhra Pradesh (1.0 million ha). The three states contribute approximately 50% of lint to the Indian cotton basket. High productivity has been obtained in Gujarat (757 kg/ha), Andhra Pradesh

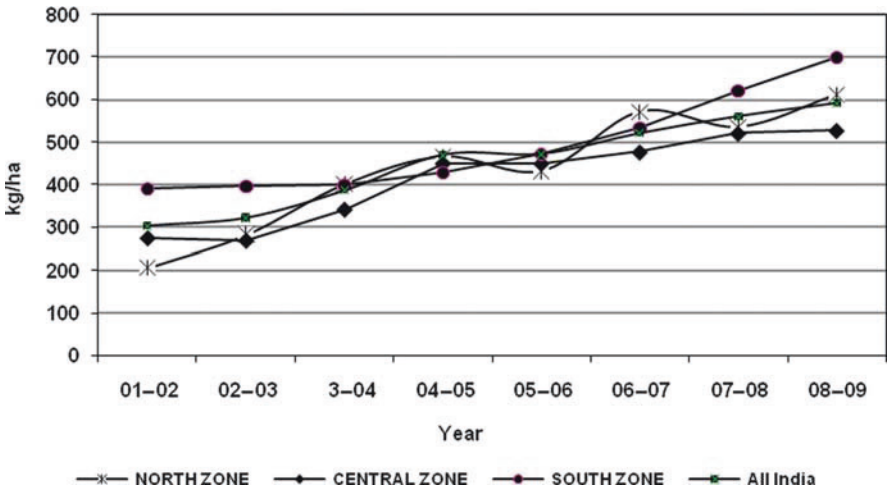

Fig. 2.2 All India cotton productivity trends during 2001–2002 to 2008–2009

Table 2.2 Species composition of Indian cotton

Species	Area (million ha)					
	1947–1948	%	1955–1956	%	2007–2008	%
G. hirsutum	0.14	3	3.21	41	1.80	19
G. arboreum	2.79	65	2.84	36	0.90	10
G. herbaceum	1.39	32	1.78	23	0.40	5
Hybrids	–	–	–	–	6.4	66
Total	4.32	–	7.83	–	9.5	–

(714 kg/ha), Tamil Nadu (654 kg/ha), and Punjab (583 kg/ha). These states cultivate hybrid cotton in approximately 50% of their cotton area.

Species composition of cultivated cotton in India has also changed over the years (Table 2.2). In 1947, 97% of the area was used for *G. arboreum* and *G. herbaceum,* while only 3% was occupied by *G. hirsutum*. Now, 19% of area is occupied by *G. hirsutum* varieties, and 66% of area is occupied by the intra-*hirsutum* and *hirsutum-barbadense* hybrids. About 0.01% of area is still occupied by *G. barbadense* (Gururajan 2008).

The per se contribution of *G. barbadense* is negligible, but as male parent of inter specific hybrids, it is the chief source of extra long staple cotton in India. The present species composition with about 15% of area (1.3 million ha) under the cultivation of *G. arboreum* and *herbaceum* varieties should be maintained, so that the genetic base for tolerance to abiotic and biotic stresses such as moisture stress, resistance to leaf curl virus disease, and jassids is not depleted.

Staple-wise production of cotton in India has also witnessed a significant change. During 1960–1961, all the cotton produced in India had fiber length below 24 mm.

At present, approximately 48% of cotton produced in India fall under the medium to superior medium category (upto 24 mm length) and 40% under long staple (more than 24.5 mm staple length). The reason for increase in diversity of staple classes and net staple length of Indian cotton is attributed to the introduction of tetraploids from New world. The variability in spinning potential in India has also increased and ranges between 6s and 120s, which is largely due to hybrid cotton (Narayanan et al. 1990).

In India, cotton research activities in the public sector are being carried out under the umbrella of All India Co-ordinated Cotton Improvement Project (AICCIP) operating in 11 main centers and 16 sub centers located in different cotton growing states with headquarters at Coimbatore. There are two central institutes such as Central Institute for Cotton Research (CICR), Nagpur and Central Institute of Research on Cotton Technology (CIRCOT), Mumbai that play a pivotal role in cotton research. Research efforts are also on in private sector for development of hybrids and GM products.

2.2 Cotton Improvement in India

Breeding objectives for cotton improvement are formulated by taking into consideration the species in which improvement is sought. However, in India, all four species have equal importance with regard to judging their genetic improvement programs. By and large, breeding objectives in India vary according to the cotton growing zones and region specific requirements.

The breeding objectives in cotton include higher yield, earliness, fiber quality, resistance to biotic and abiotic stresses, quality of oil, wide adaptability, synchronous maturity, and to some extent on colored cotton.

Cotton is predominantly a self pollinated crop though varying degrees of cross pollination have been reported. In USA, 30–50% cross pollination has been reported, and some researchers (Richmond 1950; Simpson 1948) have reported as high as 81% (California) and 50% (Tennessee) cross pollination. In India, natural out-crossing has been observed only up to 2%. Hence, breeding methods employed for cotton involve procedures of both self and cross pollinated crops.

Breeding and selection approaches utilized in cotton can be classified into the following:

1. *General selection procedure*: This includes introductions, followed by mass selection and pure line selection. Methods used include Pedigree method, Texas method of bulked progeny selection, type selection, line test, Sudan technique, and honey comb method of selection or a combination.
2. *Methods to exploit heterosis or recombination*: India grows majority of its acreage under hybrids and combining ability or heterosis is critical in any breeding program. For development of hybrids, synthetics, and multilines, this method is used.

3. *Population improvement approaches*: Disruptive mating, biparental mating, and recurrent selection.
4. *Breeding methods for specific purpose*: Multi adversity resistance (MAR) and multi environment stress resistance (MESR) through back cross method. Mutation breeding and utilization of wild species have been achieved with special efforts for incorporating specific traits. A number of varieties have been developed for commercial cultivation directly or as parental lines of popular hybrids in cotton employing the above methods.

2.2.1 General Selection Procedures

The new varieties were used as sources of many characters for cotton improvement and provided a dramatic impact on cotton scenario of India. Sea Island Andrews, a variety of Egyptian cotton introduced from West Indies, was directly released for commercial cultivation in Tamil Nadu. *G. hirsutum* germplasm introduced from American continent has changed species composition of cultivated cottons in India. Introduction of American Nectariless has lead to the development of first commercial intra-*hirsutum* hybrid, H4. Introduction of Russian *barbadense* SB 289E resulted in production of inter specific hybrids in tetraploid cotton.

Mass selection is usually practiced in indigenous or introduced genetic material in the initial phase of any breeding program. It is also exercised in deriving composite population for isolation of superior genotypes. Varieties developed using this method include Bikaneri Nerma, H777, etc., in *G. hirsutum* and G27, HD11, LD 133, etc., in *G. arboreum*.

Pure line selection has been widely used for genetic improvement of cotton and begins with individual plant selections culminating into a uniform variety. Examples of varieties developed using the method include MCU 5 VT in *G. hirsutum*, Lohit in *G. arboreum*, and Sujata in *G. barbadense*.

In cotton, majority of the varieties were developed by pedigree method of breeding. Segregating populations of single, double, three way, and multiple crosses have been handled for isolating varieties. Some notable varieties have been developed by pedigree method of breeding, such as Surabhi, LRA 5166, LRK 516, Rajat, DHY 286, HS 45, etc. in *G. hirsutum* and AKA 5, AKA 7, AKA 8401, etc. in *G. arboreum*.

2.2.2 Methods to Exploit Heterosis and/or Recombination

2.2.2.1 Breeding Cotton Hybrids

The basic principles, genetic basis, mechanisms, and other details are described later under the heading of "heterosis breeding."

2.2.2.2 Synthetic Varieties

Synthetic varieties offer an opportunity to utilize a degree of heterosis in cross pollinated or often cross pollinated crop plants. Douglas and Weaver (1963) suggested that the components of synthetic varieties should have large general combining ability. Kohel and Richmond (1969) compared synthetic varieties produced under high (42%) and low (12%) levels of natural crossing. In each location, a few selected flowers were selfed and others were left for open pollination. In the first year, selfed and open pollinated seeds were harvested on individual plant basis. For subsequent generations, seeds were taken from plants that had both selfed and open pollinated bolls to avoid possible divergence between the two. The population was grown to develop synthetics by harvesting in bulk and taking random sample of seeds. The assumption was that the heterosis present in the population would help in increasing genetic plasticity and seed cotton yield. Varieties produced at high levels of natural out crossing areas were superior to those produced at lower levels of natural crossing.

2.2.2.3 Multilines

Mechanical mixtures of cotton seeds from two or more selected lines are made to constitute a population intended for wide spread distribution.

Walker (1963) experimented on a multiline BP 52 in Uganda. When component lines of BP 52 were crossed in diallel fashion, substantial amount of heterozygosity was detected which conferred advantages because of heterosis and homeostasis. Significant interaction component was found among the components of BP 52 but the size of interaction was too small to explain the difference between the yield levels of multilines and weighted mean of its components. After successive growing for a few generations, the differences became more pronounced and multiline yield exceeded the yield of maximum yielding component. High degree of natural crossing is necessary for multilines to function properly.

Mechanical mixtures of different lines of cotton were also released for commercial cultivation in Tanzania during 1946–1958. Such mixtures were termed as multiline issues (Peat and Brown 1961). The approach has rarely been used for cultivar development in cotton.

2.2.3 Population Improvement Approaches

In cotton, population improvement procedures like recurrent selections, disruptive selection and bi parental mating systems have been used to a limited extent.

2.2.3.1 Recurrent Selection

Various traits where recurrent selection was practiced to improve cotton have been summarized: fiber strength (Shephered 1965), lint percentage, earliness, seed per locule, and seed cotton yield (Miller and rawlings 1967).

1. *Disruptive selection*: Selection of the two extreme types of a character in segregated generations followed by inter mating is called disruptive selection. Three cycles of disruptive selections was found to release the genetic variability and increase the transgressive segregants for traits such as yield per plant, bolls per plant, boll weight, halo length, and ginning percentage (Narayanan et al. 1987a, b, 1988a, b).
2. *Bi parental mating*: Singh et al. (1989) crossed randomly selected plants in F_2 and found it to be effective in breaking negative association of boll number and boll weight and increasing the concentration of favorable genes in the populations. It has been rarely used in cotton.

2.2.4 Breeding Methods for Specific Purposes

These are methods used in cotton for transferring specific traits such as male sterility restorer genes, disease resistance, morphological characters, getting new phenotypes through mutations, getting introgression of selected traits from wild species, and genetic transformation.

2.2.4.1 Back Cross Method

In cotton, this method is very effective for transfer of resistance, male sterility, inter specific gene transfer, and development of multiline varieties. V 797 and Digvijaya have been improved by backcross method. Some specific characters transferred through back cross method are frego bract (Jones and Andries 1969), nectariless (Meyer and Meyer 1961), okra leaf (Andries et al. 1969), storm proof (Niles and Richmond 1962), and male sterility (Meyer 1975).

2.2.4.2 Mutation Breeding

Induced and spontaneous mutations have played significant role in the genetic improvement of cotton. Singh et al. (1994) have reviewed the role of induced mutations in cotton improvement. Besides development of breeding material, mutations could also provide some varieties and parental lines of cotton. Earliness, dwarfness and compactness, as well as improvement in boll weight, seed oil,

induction of male sterility, and glandless characters are some of the traits achieved by mutation breeding.

Apart from these traits, improvement in fiber properties was also noticed by many workers (Raafat 1998; Badigannavar et al. 2002). Out of 16 improved varieties developed, six have been released for commercial cultivation in India. These varieties are Indore-2, MCU 7, Rashmi, DB 3-12 (spontaneous mutant), MCU 10, and DS_1. One mutant, namely SB (YF) 425, has served as a parent of the inter specific hybrid DCH-32.

2.2.4.3 Utilization of Wild Species

More than 50 species of *Gossypium* have many special characters which have been transferred to either tetraploid or diploid cultigens. Some characters that were successfully introgressed into cultivated species (Gotmare and Singh 2004) are mentioned in the Table 2.3.

Derivatives of *G. hirsutum* × *G. tomentosum* have been utilized for development of varieties such as B-1007, Khandawa 2, etc., in India. A *hirsutum* variety, Arogya was developed through *G. hirsutum* × *G. anomalum* hybridization program, and released for rainfed cultivation in the Central Zone. Immunity to bacterial blight and tolerance to sucking pests were introgressed into this variety from *G. anomalum*. A new genetic stock of *G. arboreum* with five loculed bolls has been registered which was developed by tri-species cross involving *G. anomalum*.

Stewart (1995) has discussed the details of different introgression schemes on the basis of which genes could be transferred from the donor source to the crop species.

The extent of success in production of a hybrid between two *taxa* is species specific. Hybridization among tetraploid species has no genetic barriers. Similarly, species such as *G. harknessii* and those from the Kimberly region of Australia hybridize readily when *G. hirsutum* is used as the maternal parent. A few diploid species, namely *G. davidsonii*, *G. klotzschianum*, and *G. gossypioides*, carry complementary lethal genes that cause necrosis of hybrid embryos or seedlings. Further,

Table 2.3 Character transferred to introgression hybridization using wild species

Character	Species transferred from	Species transferred to
Jassid resistance	*G. tomentosum*	*G. hirsutum*
Smooth for boll weevil resistance	*G. armourianum*	*G. hirsutum*
Rust resistance	*G. raimondii*	*G. hirsutum*
Fiber length	*G. thurberi, G. raimondii*	*G. hirsutum*
Firbre strength	*G. thurberi*	*G. hirsutum*
Cytoplasmic male sterility	*G. anomalum, G. harknessii, G. aridum*	*G. arboreum, G. hirsutum, G. hirsutum*
Fertility restorer	*G. harknessii*	*G. hirsutum*
Hairiness	*G. tomentosum*	*G. barbadense*
Caducous bract	*G. armourianum, G. harknessii*	*G. hirsutum, G. hirsutum*

species such as *G. thurberi*, *G. bickii*, and "A"-genome species are very difficult to hybridize with tetraploid cottons because of endosperm abortions. In such cases ovule culture (Stewart and Hsu 1977, 1978; Gill and Bajaj 1987; Altman 1988) allowed those hybrids to be obtained that were unattained by traditional crossing. Colchicine is used in most cases for chromosome doubling.

Inter specific hybridization has been utilized in India to generate diverse genetic stocks for commercial hybrids (Gotmare and Singh 2004).

Cotton breeding efforts in India have catered to the needs of location specific culivars. For this reason, India has about 45 major ruling varieties in cultivation. Cultivar development and seed problems in India have been critically analyzed indicating essentiality of SWOT analysis (strength, weaknesses, opportunities and threats) of each cotton breeding program in terms of genetic stocks, germplasm enhancement procedures adopted, and prioritization of objectives. The necessity of maintenance breeding has been emphasized to prevent the genetic mix-up of the released varieties.

2.2.5 Composite Crossing

High level of resistance to insects, plant pathogens, and abiotic stresses are important for stabilized production. To achieve this, two approaches were almost simultaneously started during 1970s in USA and India:

1. Multi Adversity Resistance Genetic improvement (MAR) (Bird 1982)
2. Multi Environment Stress Resistance (MESR)

Both required development of gene pools through composite crossing and achieving progressive improvement in the targeted characters. These gene pools were exposed to many biotic and abiotic stresses and careful selections were made for relevant morphological, physiological, and biochemical characters for maintaining a strong agronomic base in the populations. At present, Tamcot varieties produced from MAR system and CPD and JK series of lines from MESR system are cultivated in large areas. Still these gene pools are a source of variability for many characters.

2.3 Heterosis Breeding in Cotton

The success of heterosis breeding in maize provided an impetus to work on different species combinations of cotton for evaluating F_1 superiority over better parent (Heterobeltiosis) and commercial check variety (standard heterosis).

The hybrid vigor or heterosis in cotton has been critically reviewed by Davis (1978), Singh et al. (1980), Bhale (1987), Narayanan et al. (1989) and ICAC (1997). India has done pioneering work in the development and commercial cultivation of

hybrids and has the distinction of spreading hybrid technology (mostly intra specific) to 66% of its cotton area, which was in North, South and Central India. Inter-specific hybridization between *hirsutum* and *barbadense* has also been successful in developing Varalaxmi– the world's first inter-specific hybrid. Today, the hybrids cover the entire country.

2.3.1 General Features of Hybrid Vigor in Cotton

1. Heterosis is observed for many characters in different combinations of *Gossypium*.
2. F_1 hybrids produce exceptionally high and uniform quality of fiber.
3. The parental lines G67, Laxmi, SB289E, Khandwa 2, G.cot 10, AK 32, Bikaneri Nerma, LRA-5166, etc, have contributed to the production of high heterotic sustainable hybrids.
4. Very high range of fiber category requirements has been met by hybrids.
5. The degree of inbreeding depression is low and implies the potential of exploitation of F_2 heterosis also.
6. The extent of heterosis observed in inter specific hybrid was 10% to 138% and in intra-*hirsutum* hybrid 7% to 50%. In both cases, under highly favorable environments, 80% to 187% heterosis has been observed in India.

Though, Mell (1884) and Balls (1908) had reported heterosis in tetraploid cotton, it was Patel (1971) who demonstrated heterosis at commercial level. The first hybrid cotton H4 (Shankar 4) developed by him was an intra-*hirsutum* (G-67 × American Nectariless). In the same year Katarki (1971) showed heterosis in interspecific tetraploid hybrid, through the release of Varalaxmi (Laxmi × SB 289E). The two events transformed the entire cotton scenario of India. C.T. Patel is called Father of Hybrid Cotton Technology.

2.3.2 Hybrid Seed Production Techniques in Cotton

Methods of production of hybrid cotton seed can be classified as per the following scheme.

2.3.3 Identification of Good Combiners

Good general combining ability is mandatory for parents, while specific combining ability is the final measure in choice of parents. Apart from this, the female parent

should have good boll weight, good seed index, more seeds per boll, ease of emasculation, and availability of genetic marker for easy grow out test.

2.3.4 Hand Emasculation and Pollination

Doak's method (Doak 1934) of hand emasculation, with some modifications, and pollination is still being used in conventional hybrid seed production in India. Patel (1955), Srinivasan and Gururajan (1983), Mehta and Patel (1983), and Mehta et al. (1983) have reviewed the literature and suggested modifications to basic Doak's method. Flower buds that are likely to open the next day are emasculated during afternoon or evening hours by removing anther column. The emasculated flowers are tagged with red tissue paper bag or twine thread for identification of the bud for pollination the next day and to identify crossed boll at the time of harvest, respectively. The stigma is receptive between 8 a.m. and 12 a.m. Pollen from one male flower is used to pollinate four to five emasculated female flowers. To maintain high genetic purity, uncrossed flowers are removed every day and uncrossed bolls (if any) are removed at the time of harvest. Similarly, male plants are removed after crossing period is over. During peak flowering period 25–30 persons are required for effective crossing operation. Though commonly adopted, this method is not suitable for developing diploid hybrids as the flower buds of these are small and the style is short and brittle.

2.4 Use of Male Sterility

Hand emasculation and pollination is laborious and results in high cost of hybrid seed. Use of male sterility can make hybrid seed much cheaper, and its utilization for commercial hybrid seed production is a better approach for teraploid and diploid hybrids.

2.4.1 Male Sterility in Tetraploid

2.4.1.1 Genetic Male Sterility

A total of 11 loci have been identified controlling genetic male sterility (GMS) and 10 of them are in *G. hirsutum* and one in *G. barbadense*. Four genes, namely Ms_4, Ms_7, Ms_{10}, and Ms_{11} are dominant and produce complete sterility while the remaining seven are recessive. Among the recessive genes, ms_1, ms_2, and ms_3 cause male sterility when present singly while ms_5, ms_6, ms_8, and ms_9 behave as duplicate recessive genes. GMS system involving ms_5ms_5 ms_6ms_6 found in Greg MS, is the

only stable source, utilized in India, Pakistan, and USA. All *G. hirsutum* genotypes which carry Ms_5 or Ms_6 (or both genes) are restorers. Any *G. hirsutum* line can be converted into GMS system by repeated back crossing with alternate selfing and selection. Maintenance of GMS lines involves sib mating between male sterile (ms_5ms_5 ms_6ms_6) and fertile (ms_5ms_5 MS_6 ms_6 *or* Ms_5ms_5 ms_6ms_6) plants. The seed production plots of GMS female contain 50% fertile plants which need to be rouged out during flowering but before pollination. For this reason GMS was considered non profitable mechanism for hybrid seed production.

2.4.1.2 Cytoplasmic Genetic Male Sterility

Interaction between nucleus of *G. hirsutum* and cytoplasm of *G. arboreum*, *G. anomalum*, *G. harknessii*, *G. longicalyx*, *G. aridum*, and *G. trilobum* produced male sterility (Meyer 1973, 1975; Meshram et al. 1992). However, *G. harknessii* (D_2 CMS), *G. trilobum* (D_8 CMS), and *G. aridum* (D_4 CMS) are sources of cytoplasms which induce stable male sterility for practical hybrid seed production. Most of the *G. hirsutum* lines are maintainers (B lines) for these CMS lines (A lines). Any B line can be converted into MS line by repeated backcrossing and selection. Restorer line can also be converted through backcross method by alternate selfing and selection with the background of sterile cytoplasm.

The restoration in D_2 CMS involves monogenic dominant action with one enhancer gene (Sheetz and Weaver 1980). Similarly in D_8 CMS, restoration is monogenic dominant.

It has been found that with the same set of parents, the performance of hybrids is superior, when F_1 seed is produced by emasculation and pollination as compared to seed produced by employing cytoplasmic genetic male sterility system.

2.4.2 Male Sterility in Diploids

Asiatic hybrids between cultivated diploid species have produced very high level of exploitable heterosis (up to 200%) when crossed through conventional technique. The hybrids DDH 2, G. Cot Dh 7, and G. Cot DH9 give high yields but have seed production problems. There are at present two sources of GMS.

1. *Hissar source*: The recessive GMS in *G. arboreum* cotton variety, DS 5 (GMS-1) has white small flowers with petal spot. The GMS-1 was isolated as a spontaneous mutation. Semi-closed corolla is drawn back in GMS-1 and it has been over come in GMS-2.
2. *Akola Source*: The GMS line GAK-423A is developed by transferring the genome of *G. arboreum* (AKH 4) into *G. anomalum* cytoplasm (Meshram and Wadodkar 1992). This source has yellow, larger flowers than DS 5 GMS and possesses dark petal spot. Most of *G. herbaceum* and *G. arboreum* lines restore

fertility when used as males. GMS has enabled the exploitation of heterosis in diploids and easy hybrid seed production.

Up to 185% of useful heterosis has been reported by Rajput et al. (1998) in GMS-based diploid hybrids. Superior performance of diploid hybrids over tetraploid by 10% to 102% was observed in the preliminary study conducted by Narayanan et al. (1989). Sufficient seed set has been obtained by using GMS, and seed production can be made economical. India is the first country to release a GMS-based hybrid (AAH-1) in diploids.

2.4.3 Thermo Sensitive Genetic Male Sterility

The discovery of environment sensitive genetic male sterility system in rice laid the foundation for replacing the three line hybrid production system using A, B, and R line by two line system (Lopez and Virmani 2000). The work on TGMS in rice has been extensively done in India (Reddy et al. 2000). In cotton a spontaneous mutant of *G. arboreum* GMS line was observed showing sensitivity to temperature regimes (Khadi et al. 2001). Gradual alteration from sterility to fertility occured when temperature was reduced to less than 18°C. These lines have been stabilized and are helpful to overcome the problem of crossing for maintenance of male sterile line and hence no fertile segregants need to be rogued out. Prevention of linkage drag along with male sterile cytoplasm during transfer and overcoming the problem of sterility restoration are some of the other advantages of this male sterility system. Histological studies in Petunia showed that post meiotic male sterility is caused as a result of non-release of microspores after the tetrad stage. This in turn is due to non-degradation of callose wall surrounding the tetrad (Izhar and Frankel 1971). In cotton TGMS, it is assumed that Callase enzyme may become active only at lower temperatures enabling the degradation of Callose wall making lines to be fertile by releasing the pollen grains. Work is under progress towards exploitation of the system for commercial hybrid seed production.

2.5 Bee Pollination

Under any of the hybrid seed production systems, cotton pollen needs to be transported. Seed set is a limitation when male sterility system is employed because other, more attractive, options distract bees from acting as pollinators. Studies on honey bee pollination showed less uniform transfer of pollen (Bhale and Bhat 1989) resulting in lower boll setting compared to hand emasculation and pollination.

2.6 Factors Affecting Yield and Quality of Hybrid Seed

Factors affecting yield and quality of hybrid seed are the planting ratio of male and female parents, number of male flowers used in pollinating female flowers, staggering of parental lines (Khadi et al. 1995), period of crossing, etc. (Doddagondar et al. 2006). Using one male flower to pollinate more than three female flowers is found to decrease the hybrid seed yield and quality. If staggered sowing of parental lines is not followed, the required seed yield will decrease because of failure in synchronization. Seeds produced after crossing during the second fortnight (15 days) of flower initiation have better germination, vigor of female parental seed, pollen production ability of male parent, and position of boll set on the plant when compared with the later produced hybrid seeds. For example, early crossed bolls generally develop at lower portion, remain for longer period, absorb more nutrients, and develop seeds with high seed index.

2.7 Apomixis

Apomixis is a phenomenon observed in many crop plants especially gramineae (Hanna and Bashaw 1987) which can overcome all the demerits mentioned above for hybrid seed production. It enables production of seed without meiosis and fertilization. Apomixis helps to fix the hybrid vigor once a desirable cross combination has been obtained resulting in hybrids which can be grown like varieties. Progenies derived from a tri-species cross of cotton involving *G. arboreum*, *G. hirsutum*, and *G. barbadense* showed abnormal chromosome number and exhibited apomictic characters (Bhatade et al. 2004). Efforts have already been initiated on genetics and breeding strategies of apomixis in various crop species combined with molecular methods to analyze the apomictic and sexual mode of reproduction (Savidan 2001). Already work has been initiated for utilization of apomictic lines in heterosis fixation of Bt cotton hybrids.

A comparison of conventional and non-conventional method with respect to the need for emasculation and/or pollination has been depicted in Table 2.4.

Table 2.4 Comparison of need for emasculation and/or pollination in conventional and non-conventional breeding methods

Emasculation	Pollination
A. Hand emasculation	Hand pollination
B. Use of male sterility	Hand pollination
• Genetic male sterility	Bee pollination
• Cytoplasmic genetic male sterility	• Employ bees, through reared colonies • Pollination through natural populations of wild bees
C. Use of chemical induced male sterility	Hand pollination, bee pollination
D. Thermo- sensitive male sterility	Hand pollination, bee pollination
E. Apomixis	No need of emasculation and pollination

2.8 Breeding for Insect Resistance

Pest infestation is a major destabilizer of cotton production. The significance of pest control can be gauged by the fact that cotton accounts for 22.5% of all root insecticide sales worldwide because insect fauna inhabitating cotton has been estimated to number over 1,326.

Cotton insects are classified into following two groups on the basis of feeding behavior.

1. *Sucking pests*: This group includes jassids (*Amrasca bigutulla bigutulla*), whitefly (*Bemisia tabaci*), aphids (*Aphis gossypii*), thrips (*Thrips tabaci*), and mites (*Tetranynchus* sp.).
2. *Tissue feeders*: This group includes bollworms and weevils including American bollworms (*Helicoverpa armigera* and *H. virescens*), pink bollworm (*Pectinophora gossypiella*), spotted bollworms (*Earias vitella* and *E. inbsulana*), tobacco cut worm (*Spodoptera litura*), bollweevil (*Anthonomous grandis*), red bollworm (*Diparopsis castanea*), and shoot weevil (*Alcidodes affaber*). Among these jassids, aphids, whitefly, and bollworms (*H. armigera* and *P. gossypiella* and *Earias* sp.) are serious pests in India. In general losses due to sucking pests (5%–10%) are much less than from bollworms (25%–50%). During reproductive period, bollworms not only cause reduction in the yield but also affect fiber properties. Sucking pests, active during reproductive period, are vectors for many pathogen and viruses; the best example is whitefly, the vector for cotton leaf curl virus.

There are two approaches for developing genotypes with pest tolerance:

1. Conventional breeding approach
2. Biotechnological approach

2.8.1 Conventional Breeding Approach

It involves manipulation of morphological traits and selection of levels of plant biochemical products that act as feeding deterrent. The concept of conventional breeding program is based on three mechanisms of resistance:

1. Antixenosis (non-preference)
2. Antibiosis
3. Avoidance

Host plant resistance studies have been reviewed by many workers (Maxwell et al. 1972; Thompson and Lee 1980; Jenkins 1989; Khadi 1996).

2.8.1.1 Antixenosis (Non-Preference)

It is mediated through morphological traits that are unacceptable to the insects and inhibit their feeding and reproduction. In this mechanism, morphological and anatomical characters indicate tolerance to various pests (Table 2.5).

The anatomical characters contributing to resistance are as follows:

1. Dense cortical cells per unit area of petiole stem tip and midrib, compact mesophyll cells of leaves, and thick coating of leaf – resistance to sucking pests
2. More collenchymas (mechanical tissue), more compact epidermal cells, and less cell inter space – resistance to sucking pests
3. More collenchymas and compact cell arrangement with less cell inter space of boll rind – tolerance to bollworms
4. Long and compact palisade layer, more distance to the phloem, and the presence of adaxial layer of palisade and epidermis in *G. arboreum* -resistance to sucking pests

2.8.1.2 Antibiosis

This mechanism involves production of variable quantity of feeding deterrents or allelochemicals by host plants. The allelochemicals act as repellent, insect growth retardant, or factor responsible for reduction in insect survivability. Allelochemicals such as terpenoid aldehyde (esp. gossypol), phenols (esp. tannins), proteins, and sugars are involved in pest tolerance. Bollworm tolerance is conferred by production of high levels of gossypol and tannin, and low levels of proteins and sugars.

2.8.1.3 Avoidance or Escapism

This mechanism of insect resistance involves preponement of maturity or earliness so as to escape from insect damage.

In short duration cotton, the period available for insect development is reduced and so are the levels of overwintering populations. Such compact flowering period varieties are found to escape boll weevil and pink bollworm damage (Liu et al. 1990; Wallhood et al. 1981). Escape from boll worms due to reduced boll period was indicated by Rao et al. (1996).

The conventional breeding approach in cotton for resistance to insect pests has yielded variety of genetic stocks which are either tolerant to boll feeders or resistant to sucking pests.

In general, diploid cultivated species are comparatively more tolerant to many pests as compared to the tetraploid species.

Table 2.5 Reaction of insects towards morphological features of cotton

Characters	Sucking pests	White flies	Bollworms	Boll weevils
Hairiness	R	R	S	R
Glabrous	S	R	T	N
Glaborous upper surface hairy lower surface	R	S	T	-
Red pigmentation	N	R	T	R
Nectariless	N	N	T	N
Frego bract	N	N	T	R
Thick boll rind	N	N	T	R
Hardness of the boll	N	N	T	R
Okra leaf/small leaf	N	R	T	R
Thin lamina	R	R	N	N
Pedicel length	N	N	T	R
Less bracteole teeth	N	N	T	R
Gossypol glands	R	R	T	R

S susceptible; T tolerant; R resistant; N neutral; – and/or not affected

2.8.2 Biotechnological Approach

2.8.2.1 Tissue Culture

Embryo rescue is employed to obtain plants from inter specific hybridizations that will abort (Mehetre and Aher 2004). A research focus in the 1960s and 1970s was development of new cell culture methods. Callus cultures were the starting point to isolate protoplasts, with a view to making wide crosses via protoplast fusion with sexually incompatible germplasm (Carlson et al. 1972). Price et al. (1977) first defined the conditions for establishment of callus cultures, from six cotton species. The first report of a cell culture system to obtain somatic embryos from cotton callus cultures was by Price and Smith (1979), and improvements have continued (Kumar and Tuli 2004; Sun et al. 2006).

2.8.2.2 Genetic Engineering

Recent developments in plant molecular biology permit the introduction and expression of alien genes in cotton to impart resistance against herbicides and some of the major insect pests. The alien gene is called a transgene and the cotton in which such a gene has been introduced and expressed is called transgenic cotton. Some of the other traits of continuing interest to incorporate into cultivated cottons include drought and salt tolerances (Basu 1996; Paterson and Smith 1999; Wilkins et al. 2000; Hake 2004). The first agronomically important gene inserted into cotton was for insect resistance, cry 1Ab from *Bacillus thuringiensis* (Bt) (Perlak et al. 1990). Among 51 transgenic events for insect resistance 35 events

represent Bt, where insect resistance gene from *B. thuringiensis* is introduced. The Bt gene directs the synthesis of a crystal protein in the plant tissues, and the insects that feed on these tissues are killed. An insecticidal gene AaHIT from scorpion is also being explored in transgenic cotton against some lepidopteron. Among the 11 crops genetically modified in India (cotton, corn, brinjal/eggplant, cabbage, groundnut, mustard, okra, pigeon pea, rice, and tomato), Bt cotton is the first transgenic approved by Government of India for commercial cultivation.

2.9 Bt Cotton

More details are available in Chapters 10 and 12 of this book. Bt cotton, which confers resistance to Lepidopteron pests of cotton, was first adopted in India as a hybrid in 2002 after stringent assessment for bio-safety and profitability. In India, after extensive testing of Bt cotton hybrids (with *cry*1 Ac gene) in All India Coordinated Cotton Improvement Project (AICCIP) and farmers' fields, Government of India has approved commercial cultivation of Bt cotton hybrid with effect from 2002 crop season.

The transgenic hybrids released in the country can be categorized in different ways on the basis of transgene involved. They can be categorized into two groups viz., (1) Bollgard (single gene) (2) Bollgard II (double gene) and on the basis of species involved, they can again be classified into two distinct types: (1) Intra-*hirsutum* and (2) Inter-specific hybrids (*hirsutum* × *barbadense*).

The large scale cultivation of Bt cotton is likely to usher in an era of eco-friendly cotton cultivation with reduction in the number of insecticidal applications(40%–60% less) which in turn will enable better sustenance of parasites and predators in cotton crop creating ideal condition for wide spread adoption of IPM. The relatively short duration of Bt cotton is likely to bring about reduced water requirement by the crop and in a situation as it prevails in north zone will enable timely sowing of wheat. Spread of Bt cotton area in India from 2002–2003 to 2008–2009 is given in Fig. 2.3.

The textile policy of 2000 aims at achieving the target of textile and apparel exports of US$50 billion by 2010 of which the share of garments will be US $25 billion. Consequently, the requirement of cotton is likely to increase perceptibly in the coming years in respect of internal consumption as well as to meet the targeted export demand, necessitating the need for concerted research, development, and extension efforts to make Indian cotton compete effectively in terms of quantum, quality, and competitiveness in the globalized trade scenario. The requirement of cotton by the textile industry by 2010 is estimated to be 35 million bales. Bt cotton hybrids as well as varieties along with related production and protection technologies are likely to play a pivotal role in realizing the targets set for cotton production (Table 2.6).

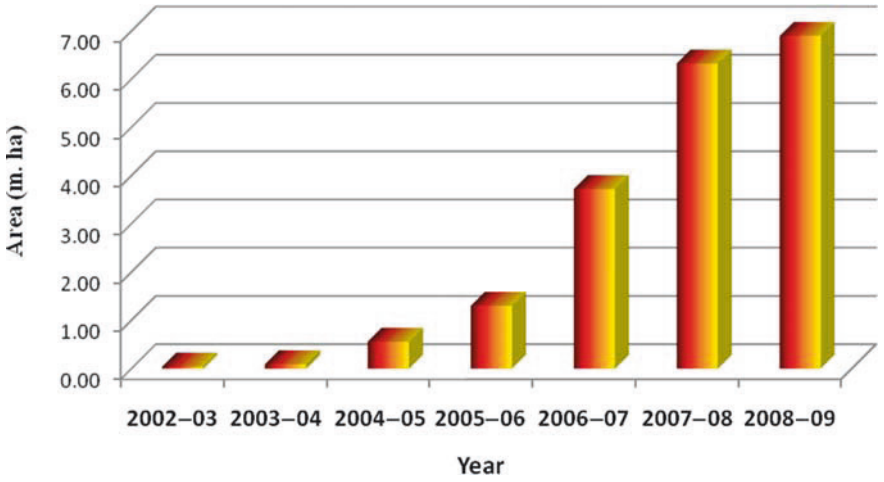

Fig. 2.3 Spread of Bt cotton area in India

Table 2.6 Statewise Bt cotton area in India (in million ha)

State	Year						
	2002	2003	2004	2005	2006	2007	2008
Punjab	0.000	0.000	0.000	0.0704	0.2810	0.5750	0.4760
Haryana	0.000	0.000	0.000	0.0107	0.0420	0.2790	0.3800
Rajasthan	0.000	0.000	0.000	0.0023	0.0050	0.0380	0.1210
NORTH ZONE	0.000	0.000	0.000	0.0834	0.3280	0.8920	0.9770
Gujarat	0.0091	0.0417	0.1259	0.1493	0.4070	1.3000	1.4500
Maharashtra	0.0120	0.0218	0.1615	0.5088	1.6550	2.5620	2.5720
Madhya pradesh	0.0014	0.0133	0.0861	0.1362	0.3020	0.4710	0.5140
CENTRAL ZONE	0.0225	0.0768	0.3735	0.7943	2.3640	4.3330	4.5360
Andhra pradesh	0.0038	0.0054	0.0712	0.0904	0.6570	1.0000	1.1430
Karnataka	0.0021	0.0030	0.0343	0.0293	0.0800	0.1460	0.1720
Tamil nadu	0.0003	0.0076	0.0120	0.0170	0.0320	0.0600	0.0720
SOUTH ZONE	0.0062	0.0160	0.1175	0.1367	0.7690	1.2060	1.3870
Total	0.0287	0.0930	0.4980	1.0150	3.4610	6.3340	6.8900
Total cotton area	7.3900	7.8350	8.9700	9.1580	9.1580	9.5550	9.3730
% of total cotton area	0.388	1.187	5.552	11.083	37.792	66.290	73.616

Source: Directorate of Economics & Statistics as on April 1, 2009

One clear impact of Bt cotton on Indian agriculture appears to be the replacement of large tracts of varietal areas of north India, with Bt hybrids, as the technology is available in India only in the form of hybrids. Bt-cotton seems to have reduced the overall quantity of insecticide substantially, only in some parts of the country, coupled with spectacular yield increases reported from Gujarat, while rest of the states have been showing mixed results despite increase in the area under Bt-cotton.

Five events have been approved by the Genetic Engineering Approval Committee (GEAC) for commercial release in India. They are *cry*1 Ac (Mon 531) developed by Mahyco Monsanto Biotech Limited and sourced from Monsanto, *cry*1 Ac and *cry*2 Ab (Mon 15985), stacked gene event developed by Mahyco Monsanto Biotech Ltd. and sourced from Monsanto, *cry*1 Ac (Event 1) developed by JK Agri Genetics Seeds Ltd and sourced from IIT, Kharagpur, and *cry*1 Ab and *cry*1 Ac (GFM event) developed by Nath Seeds and Sourced from China featuring fused genes, *cry*1 Ac (Dharwad event) developed under ICAR (CICR Nagpur, UAS Dharwad, NRCPB New Delhi) system.

Currently 284 Bt hybrids covering the four events and developed by 31 seed companies have been approved for commercial cultivation. Out of this majority of the hybrids belong to intra *hirsutum* category and the rest to the inter specific (*G. hirsutum* × *G. barbadense*) category. The first Bt cotton variety Bikaneri Nerma Bt developed by the Public sector has been released by CICR, Nagpur.

Cultivation of Bt cotton hybrids under diverse agro climatic conditions during the last 7 years has amply proved the following points:

- The high yield potential of the Bt hybrids due to effective pest control
- Reduced quantity of pesticides used to control lepidopteron insects
- Conservation of beneficial insects, parasites, and predators
- Reduction in environmental pollution
- Reduction in cost of cultivation

However, large scale and continued use of Bt cotton genotypes may bring into focus the problem of insect resistance. Hence adequate precautions are necessary to delay the development of resistance to Bt toxin. They are as follows:

- Strict adherence to the planting of Refugia
- Encouraging the cultivation of other alternate host crops
- Gene stacking
- Use of alternate genes that do not share common resistance mechanism as that of *cry*1 Ac
- Reduced Bt cotton surviving population of *H. armigera* by conventional methods of insect control

Resurgence of secondary insect pests such as mirid bugs (*Creontides biseratence*) and mealy bugs as major pests and issues related to Bt seed purity have become priority in the wake of increased Bt hybrid cotton cultivation in India. The tobacco caterpillar, *S. litura*, was also found to stage a comeback as an economic pest of Bt cotton. Data showed that thus far there are no symptoms of resistance to *cry*1 Ac in any of the bollworm field populations tested. With cultivation of Bt cotton, the overall pesticide use has dramatically gone down. Many pesticides used are broad spectrum pesticides which also provide control against the secondary pests. In instances where no control measures have been taken for these pests, the resurgence is seen.

Resistance to *cry*1 Ac has not been recorded from any of the Indian populations of *H. armigera* or *Earias* sp. thus far until April 2009, despite cultivation of Bt cotton for 7 years.

2.9.1 Development of Bt Kits

Adoption of Bt cotton has also brought along the need for diagnostic tools to ensure quality norms. Several players in the country are providing such services which can determine both qualitative and quantitative levels of the protein.

The Central Institute for Cotton Research, Nagpur, India has also developed several Bt detection kits including the "Bt Express," which became extremely popular with farmers and seed testing officers all over the country and has empowered farmers to test seeds and plants to ensure quality. Six kits (Bt express, Bt quant, Bt Detect, Bt Zygosity, Bt Express-II, and Bt-ELISA II) that include four scar markers, three ELISA kits, two dot-blot, and two immunochromatographic dip sticks have been developed.

In a landmark achievement the CICR, in collaboration with UAS, Dharwad and IARI, New Delhi, developed a Bt transgenic *G. hirsutum* variety "Bikaneri Nerma," which was released in 2008 for commercial cultivation. Elite Indian Cotton *G. hirsutum* CV viz., LRA 5166, Anjali (LRK 516), and *G. arboreum* CV viz., RG-8, PA255, PA402 have been transformed with Bt *cry*1 Ac and *cry*1 Aa3 genes. The transformed plants have been analyzed for the Bt genes integration by PCR and Southern blot techniques and the expression was assayed by ELISA test.

2.9.2 Disease Resistance

Bacterial blight caused by *Xanthomonas* spp. has been well studied globally. In *Xanthomonas compestris pv malvacearum* (*Xcm*) a total 32 races have been reported (Verma and Singh 1974) and at least 22 major genes (B) for resistance have been identified (El-zik and Thaxton 1995). Majority of these genes are completely or partially dominant for resistance. Although a single B gene confers resistance to a few races of *Xcm* (vertical resistance), vulnerability to other races of pathogen remains. Combination B_2B_3 Bs_m conferred immunity to all races found in USA and resistance has been stable for 22 years. An African strain, S 295 bred through recurrent selection in Chad is known to be resistant to all known races and isolates of *Xcm* including the highly virulent race HV1 (Girardot et al. 1986; Wallace and El-zik 1989). Comprehensive reviews on this disease have been published by Verma (1986) and Hillocks (1992). Cultivars like Stoneville-20, Albar, MCU 10, RKR 4145, and Supriya are resistant sources for blight.

Vegetative compatibility (V-C) tests and isozyme analysis of wilt caused by *Verticillum dahliae* have shown that there are four genetically isolated populations (V-C groups) within the species and that each V-C group has at least two subgroups (Strausbaugh et al. 1992). Major terpenoid phytoalexins formed in xylem of upland cotton were found to be involved in active defense mechanism and Russian varieties like Tashkent 1, 2 and Tashkent 3 are widely used resistance sources in the world against *V. dahliae*. Wilt is more pronounced in winter cotton area of Tamil Nadu where a resistant cultivar MCU 5VT has been recommended.

Fusarium wilt (*Fusarium oxyporium* f.sp. *vasinfectum*) is most prevalent in parts of USA, India, Tanzania, and Sudan. Armstrong and Armstrong (1980) have reported six races across cultivars of different cotton species and other crops. In India, all the currently released diploid and most of the tetraploid varieties are resistant to *Fusarium* wilt. Detailed account of resistance sources for different fungal and bacterial diseases available in the germplasm of all four cultivated species in India is given by Sheo (1992).

Some wild species reported to be resistant to bacterial blight, *Fusarium,* and *Verticillium* wilt are listed in Table 2.7.

Among the viral diseases, cotton leaf curl virus (CLCuV) transmitted by adult whitefly (*B. tabaci*) was devastating in North India and Pakistan during the 1990s. During 1993, CLCuV disease affected almost the entire 221,600 ha cotton area in Pakistan (Rahaman 1997) but in India the disease incidence was only in patches (Narula et al. 1999). However, during 1997 the entire North Indian cotton (218,610 ha) was found affected by CLCuV (Narula et al. 1999).

Genetics of resistance in the resistant *G. barbadense* strains was worked out and found monogenic dominant inheritance with modifiers. In India, the short compact *G. hirsutum* variety RS875 was found resistant to CLCuV, and has been recommended for double cropping systems of North India. LHH 144, an intra hirsutum resistant hybrid has been recommended for cultivation in North India. LRA-5166 has also been found to be resistant to CLCuV. In Pakistan, LabAcala (69) 11, C 1743, FH 643, C1M 1100, and CIM 448 have been found resistant to CLCuV. All diploid cottons belonging to *G. herbaceum* and *G. arboreum* are immune to the CLCuV. Transgenic approach is being adapted in developing ClCuV resistant cotton using RNA interference technique.

Another viral disease, called blue disease, transmitted by *A. gossypii* prevails in Vietnam. When Indian *G. arboreum* and *G. hirsutum* cultures were screened against the disease, *G. arboreum* cottons were found to be more resistant than *G. hirsutum*;

Table 2.7 Resistance sources in wild species

Bacterial blight	Wilts	
	Fusarium	Verticillium
G. anamolum	*G. sturtianum*	*G. thurberi*
G. armourianum	*G. harknesii*	*G. bickii*
G. davidsonii	*G. thurberi*	*G. sturtianum*
		G. raimondii
		G. harknesii

10 to 13 lines of *G. arboreum* exhibited immunity. Some intra *hirsutum* hybrids such as Bio seed 7 and 2 were resistant to blue disease (Singh and Singh 1999).

2.9.3 Fiber Quality and Its Improvement

Quality of cotton refers to suitability of the fiber to produce better quality yarn and fabric. Improved fiber quality traits include length, strength, fineness, maturity, and uniformity.

Tetraploid cultivated species possess longer and finer fibers. The diploid Asiatic species generally have shorter and coarse fiber. *G. barbadense* cultivars exhibit longest fibers of high fineness and are used to produce very fine fabrics. *G. herbaceum* and *G. arboreums* are used for coarse fabric manufacture. Fiber quality improvement in tetraploid cotton has reached high standard with release of MCU-5 in *G. hirsutum,* Suvin in *G. barbadense*, and Varalaxmi, DCH-32, HB-224, DHB105, TCH 213 as inter specific hybrids. Increase in length in *G. hirsutum* has also been due to introgression of genes from *G. barbadense* and found suitable for maximum 80s (as is the case in extra long staple cotton) counts spinning instead of 20s spinning (Kairon et al. 1998)

The diploid species, especially *G. arboreum*, have also been improved for their fiber traits through introgression of genes from *G. hirsutum* and the resultant lines had 30 mm staple length with 3.1 to 5.0 micronaire. In view of decreasing area of diploids, intensive program on promotion of high yielding, superior fiber quality diplod cottons, which will be useful for low and marginal farmers of rainfed areas, are being undertaken in India.

2.10 Value Addition for Cotton

Fiber is the primary product of cotton and oil is considered to be secondary. Cotton has distinct opportunity to become dual purpose crop for both fiber and oil as there is no major negative association reported between oil content, seed size, lint yield, and quality. Cotton seed meal or flour is also sometimes used for human consumption when derived from gossypol-free varieties, or if the gossypol has been extracted or is present in the food at low levels.

Cotton seed is valuable food stuff for cattle, combining high energy, high fiber, and high protein (Ensminger et al. 1990), and is used as whole seed, hulls flour, and cake. The whole seed of *G. hirsutum* also includes linter fibers (~10% of seed weight), which are nearly pure cellulose and highly digestible. The seed oil gives it high energy value (Coppock 1987). Cattle and sheep are fed cotton seed hulls as an important source of roughage. Gin trash is also fed to ruminants, and has 90% of the food value of the hulls (Ensminger et al. 1990).

Though cotton seed is an inexpensive and rich source of edible oil and protein, breeding efforts in this direction are limited (Dani 1990). Considerable genetic variability was found to exist for oil content in cotton (Dani 1984; Kohel 1980). Tetraploid cotton contains more oil than diploids (Murthy and Appu Rao 1963).

Seed and embryo size in cotton are determined predominantly by maternal parent. Maternal effects as a source of variation in cotton seed oil were reported by Kohel (1980) and Dani (1992). Glandless genotypes possess marginally higher seed oil content than glanded genotypes (Kohel 1980; Harry and Richard 1986). Glands on cotton seed which are called pigment glands are the main source of gossypol that imparts color to the oil. Gossypol is toxic to the nonruminant animals (Murthy and Achaya 1975).

Cyclopropenoide fatty acids (CPFAs) present in the cotton seeds, and tannins in the leaves and flower buds act as deterrents to insects. The level of CPFAs is generally higher in *G. hirsutum* than *G. barbadense* (Frank 1987). CPFAs are anti-nutritional compounds, which interfere with the metabolism of saturated fats.

Processed cotton fiber contains over 99% cellulose (Wakelyn et al. 2007), and is used widely in pharmaceutical and medical applications because of its low capacity to cause irritation. Inhalation of cotton dust by mill workers can cause an asthma-like condition called byssinosis (Samantha et al. 2004), which may be complicated by fungal contamination of the cotton dust.

Lint is the main component of cotton crop. Cotton seed is next in importance due to its oil and protein content. Plant biomass, linter, cotton seed hull, and ginning wasters are other important byproducts from which value added products can be obtained. Another major byproduct is cotton stalks, which can be effectively utilized for the production of particle boards, corrugated boxes, paper, and other products for which the technology is already available with CIRCOT, Mumbai.

The consumption of cotton in the country has been rising year after year and there is a boost in cotton consumption subsequent to the abolition of Quota Regime from 1 January 2005. The VISION STATEMENT prepared by the confederation of Indian Textile Industry places the requirement of cotton by the textile industry by 2010 at 35.0 million bales (this figure is likely to go up depending upon the export scenario). The rising trend in cotton consumption during the last decade would be evident. During the year, consumption of mills, including the small scale units, is likely to be 21.5 million bales.

2.11 Export

India is gradually emerging as a major player in the World Cotton Trade. Export from the country boomed to 4.7 million bales during 2005–2006. The uptrend continued during 2007–2008 when export rose to a new record of 8.5 million bales and is likely to be around 7.5 million bales only during the current year as a result of economic recession leading to lower international demand.

2.12 Naturally Colored Cotton

Some species of cotton produce naturally colored cotton. The most common colors observed are different shades of brown and green. Varieties with brown and green are presently being cultivated in restricted areas of USA (Fox 1987a, b). Different aspects of colored cotton have been reviewed by Khadi and Kulkarni (1996) and Waghmare and Koranne (1998). Sunlight has profound influence on development of colour in the fiber (Khadi et al. 1996).

Considering its ecofriendly nature, breeding efforts to produce colored cottons are under way in some of the Cotton Research Institutes in India. Presently the colored genotypes in India have inferior yielding ability and poor fiber properties. Sufficient heterosis has been observed in white × colour linted hybrids (Amudha and Raveendran 1997; Singh et al. 1996). Contamination of white cotton throughout crossing and mechanical mixing during ginning are potential threat to the development of naturally colored cottons.

2.13 Molecular Marker Techniques Used in Cotton Improvement

Although morphological features are indicative of the phenotype, they are affected by environmental factors and growth practices. Therefore, more reliable markers such as DNA markers that are larger in number and have less pleotropic effect are being used. Applications of molecular markers in cotton in areas such as phylogenetic mapping and genetic diversity, construction of linkage maps and marker assisted selection for acceleration of introgression of genes from wild species to cultivated donor lines, for pyramiding of genes, and QTL mapping have been studied. (Chapter 3 deals with this subject in greater detail later in this book.)

2.14 Future Strategies

Cotton remains the chief raw material for textile industry. The demand for cotton textile products is increasing every day because of increasing global population. To meet the challenges ahead, viable strategies need to be deployed. Increase in net productivity, quality improvement, resistance to abiotic stresses, resistance to biotic stresses, identification of stable QTLs, and development of reliable transgenic testing methods are needed as we look to the future.

Only thoroughly planned scientific research can ensure that the emerging challenges facing cotton farming are addressed from time to time, while harnessing the full potential of our natural resources, manpower, and technologies so that cotton farming becomes a sign of prosperity. Some of the emerging challenges are related to transgenic cotton and harnessing the full potential of the technology. Thus far

280 Bt cotton hybrids have been approved by the Genetic Engineering approval Committee (GEAC) until August 2008 and 6.89 million ha were under Bt cotton cultivation in 2008 consisting 73.3% of the total area. It is anticipated that by 2010, 90% of the area would be covered by Bt cotton. The future strategies to combat various emerging issues are the following:

- Superior varieties and hybrids developed by the public research system will not be adopted easily, as more than 75% of the area would be under commercial Bt cotton hybrids developed by the private seed companies. Exploiting apomixis in Bt hybrids will be the priority area of research.
- Harmonizing package of crop production and protection practices for the innumerable Bt cotton hybrids is not possible and famers will find it very difficult to make a proper choice and harness the full potential of the hybrids thus resulting in confusion, unwarranted experimentation, and relatively low yields. Efforts for identification and cultivation of only promising zone specific Bt hybrids are needed.
- High susceptibility of hybrids to minor pests such as mealy bugs, mirid bugs and several diseases, thereby facilitating proliferation of the pests and diseases. Characterization of emerging new pests and their management strategies including bollworm resistance management is already underway.
- Spurious product and seed quality becomes more concerning in a scenario which has a large number of genetically modified events. Development of highly sensitive detection methods and strict regulatory seed laws are needed.
- Breeding for improved fiber qualities as per emerging needs will receive due attention.
- Extension of cotton to non-traditional areas in rice fallows in South and East India is needed to promote cotton cultivation.
- Establishing better linkage between Research & extension officials for successful adoption of new technologies.
- Organizing more number of Front Line Demonstrations (FLDs) of newer technologies including newly released Bt cotton hybrids.
- Mass production and supply of quality biocontrol agents and remunerative price to the farmer are to be effectively followed for achieving more production with profitability to all concerned.

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Chapter 3
Cotton Genomics

A.H. Paterson

3.1 The Need for Cotton Biotechnology

The National Research Council reports that a competitively priced bio-based products industry will eventually replace much of the petrochemical industry (Council 2000). Cotton, one of the world's most important crops, enjoys a host of opportunities to participate in a bio-based products revolution. The value of cotton fiber and byproducts grown in the USA is typically about $6–7 billion/year. More than 440,000 domestic jobs are related to cotton processing, with an aggregate influence of ~$120 billion/year on the US gross domestic product and ~$500 billion/year worldwide. Increased durability and strength of cotton fiber offers the opportunity to replace synthetic fibers that require ~230 million barrels of petroleum per year to produce in the USA alone. Its seed oil and also byproducts of cotton processing are raw materials for biofuel production (Holt et al. 2003). The unique structure of the cotton fiber makes it useful in bioremediation, and accelerated cotton improvement also promises to reduce pesticide and water use.

The over-exploitation of a few genetic backgrounds has dangerously eroded the cotton genetic base, slowing breeding progress and increasing vulnerability of the entire cotton industry to crop failure (Helms 2000). Cotton has a very narrow gene pool resulting from its evolutionary history, domestication, and modern crop improvement practices. The elite Upland cotton gene pool derives from only a small subset of progenitors from the Mexican-Guatemalan border that was brought into the USA and also dispersed into China, India, Australia, and other countries (Hutchinson et al. 1947). The lack of genetic diversity in modern cotton germplasm has recently been further exacerbated by breeding programs repeatedly crossing only a few closely-related genotypes to one another to generate new cultivars

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(May et al. 1995). Study of more than 320 cultivars/lines (from the US National Plant Germplasm System) with 250 DNA markers shows that cotton has a lower level of DNA variation than most of the major crops (Chee et al. 2004; Lubbers et al. 2004).

Sewall Wright, among the leading population geneticists of all time, envisioned that selection would drive a gene pool to *scale an adaptive peak* of a height determined by the initial genetic diversity available in the gene pool (Wright 1968). Having reached its peak, the gene pool could only be shifted to a new, higher peak either by a change in environment or by the infusion of new genetic diversity. Gains in cotton productivity have been realized by changing the environment with addition of fertilizers, pesticides, irrigation, and other factors – indeed, cotton protection alone typically accounts for 10% of the total US agri-chemical market, and is also among the largest consumers of fresh water. However, the high economic and environmental cost of such changes creates *an urgent need to reinvigorate the infusion of genetic diversity into the elite Upland cotton gene pool that can provide environmentally benign solutions to the needs of cotton producers, processors, and consumers*.

With about 50 species in the *Gossypium* genus, cotton enjoys large primary, secondary, and tertiary gene pools from which valuable new alleles might be drawn, and these have been underutilized. Moreover, rapid progress in functional genomics of angiosperms offers still additional alleles, some naturally occurring and others modified (for example, by over expression) that can be utilized in cotton improvement by extra-genetic approaches that have long been established for cotton. In this chapter, I review advances in key resources in the toolbox of the cotton biotechnologist.

3.2 Genetic Mapping and DNA Marker-Assisted Breeding

3.2.1 Genetic Mapping and DNA Markers

There exist at least a dozen published genetic maps for various cotton crosses, most made to map specific traits (QTLs), collectively including about 5,000 public DNA markers (~3,300 RFLP, 700 AFLP, 1,000 SSR, and 100 SNP). By far, the most detailed sequence tagged site (STS)-based map and a source of probes for many of the other maps are genetic maps for diploid (D) and tetraploid (AtDt[1]) *Gossypium* genomes have been employed in a range of structural, functional, and evolutionary genomic studies (detailed in project description). The reference maps include respectively, 2,584 loci at 1.72 cM (~600 kb) intervals based on 2,007 probes (AtDt); and 1,014 loci at 1.42 cM (~600 kb) intervals detected by 809 probes (D)

[1]Dt refers to the D-subgenome found in tetraploid cottons (to distinguish it from the genome of D-diploid cottons). Likewise, at refers to the A-subgenome of tetraploid cottons.

(Rong et al. 2004c, 2005b). There is a very high degree of colinearity among the respective genome types (Rong et al. 2004b) – to take advantage of this, we inferred the gene order of a hypothetical common ancestor of the At, Dt, and D genomes. This map included 3,016 loci identified by 2,337 probes, spanning 2,324.7 cM.

Additional maps that are particularly marker-rich and/or have been widely used as reference maps for QTL studies have been developed from three additional interspecific crosses (Lacape et al. 2003, 2007; Guo et al. 2007; Yu et al. 2007).

Other mapping resources include aneuploid substitution stocks that were derived from tetraploid TM-1 (*G. hirsutum*) × 3–79 (*G. barbadense*) (Endrizzi and Ramsay 1979) and TM-1 × *G. tomentosum* (Saha et al. 2006b). Together, monosomics and telosomics identify 23 of the 26 cotton chromosomes and three remain unidentified. Using these resources, many SSR and RFLP markers have been assigned to chromosomes.

3.2.2 Trait and QTL Mapping

Cotton molecular maps have been employed in identification of diagnostic DNA markers for a wide range of traits. Genetic analysis of characteristics related to fiber yield and quality has, naturally, been a high priority (Jiang et al. 1998; Kohel et al. 2001; Ren et al. 2002; Guo et al. 2003; Paterson et al. 2003; Zhang et al. 2003; Mei et al. 2004; Chee et al. 2005c,d; Draye et al. 2005b; He et al. 2005, 2007, 2008; Shen et al. 2005, 2006b, 2007; Ulloa et al. 2005; Wang et al. 2006a, 2007a; Abdurakhmonov et al. 2007, 2008; Wan et al. 2007; Wu et al. 2007; Asif et al. 2008; Guo et al. 2008; Mir et al. 2008; Qin et al. 2008; Saha et al. 2008; Zhao et al. 2008). The heavy water use of cotton has drawn attention to molecular dissection of drought tolerance (Saranga et al. 2001b, 2004; Zhao et al. 2008). Disease resistance has also been a major focus, with published information for *Verticillium* (Bolek et al. 2005; Wang et al. 2008; Yang et al. 2008), *Xanthomonas* (Wright et al. 1998; Rungis et al. 2002), *Thielaviopsis* (Niu et al. 2008), and root knot nematode (Shen et al. 2006a; Wang et al. 2006b; Wang and Roberts 2006; Ynturi et al. 2006; Niu et al. 2007). Interest in hybrid cottons in some countries has drawn attention to a nuclear restorer of a cytoplasmic male sterility system (Guo et al. 1998; Lan et al. 1999; Zhang and Stewart 2004; Feng et al. 2005; Wang et al. 2007b). Morphological features such as the pubescence that is characteristic of *G. hirsutum* (Wright et al. 1999; Lacape and Nguyen 2005; Desai et al. 2008), as well as leaf morphology (Jiang et al. 2000b; Song et al. 2005; Waghmare et al. 2005b; Hao et al. 2008), and unique features such as nectarilessness (Mei et al. 2004; Sajid Ur et al. 2008) have also received attention. The value of cotton seed has led to interest in mapping variation in seed physical characteristics and nutritional value (Song and Zhang 2007). It is probable that this list is incomplete (and the author apologizes for any inadvertent omissions).

Comparison of multiple QTL mapping experiments by alignment to a common reference map offers a more complete picture of the genetic control of a trait than

can be obtained in any one study, and reveals the genomic organization of trait variation. A total of 432 QTLs have been aligned to a high-density reference genetic map which consists of 3,475 loci in total, including QTLs for cotton fiber quality (Jiang et al. 1998b; Paterson et al. 2003; Chee et al. 2005a, b; Draye et al. 2005a), yield (Jiang et al. 1998b; Saranga et al. 2001a, 2004; Rong et al. 2005c), leaf morphology (Jiang et al. 2000b; Waghmare et al. 2005b), flower morphology, resistance to bacteria (Wright et al. 1998), trichome distribution and density (Wright et al. 1999), and other traits that were mapped in a total of 11 populations. All QTLs were also projected onto a consensus map which was inferred to resemble the DNA marker arrangement of the hypothetical ancestor of the two "sub-genomes" of tetraploid cotton (Rong et al. 2005a). The consensus map has improved our ability to deduce cotton-*Arabidopsis* synteny relationships, and thus fosters study of correspondence between the cotton QTLs and fiber or trichome-related *Arabidopsis* genes. To encourage further utilization and online community access to these data, a CMap resource was developed and can be accessed at http://chibba.agtec.uga.edu/cgi-bin/cmap/viewer.

3.2.3 Genome-Wide Introgression

The most immediate and promising opportunities to enhance the genetic diversity of Upland cotton derive from crossing elite cultivars with the four divergent allotetraploid relatives in the secondary gene pool, which include the cultivated species *G. barbadense* and three nondomesticated species *G. mustelinum*, *G. darwinii*, and *G. tomentosum*. Each of these species is cross compatible with Upland cotton and may contain novel alleles with potential crop improvement value. In particular, the allotetraploid *G. barbadense* is a valuable fiber crop in its own right that has been described as Pima, Egyptian, or Sea Island cotton. In our genetic diversity survey of 320 *G. hirsutum* cultivars/lines, *G. barbadense* alleles were restricted to a small group of germplasm lines that according to historical accounts were clearly developed through interspecific hybridization (Chee et al. 2004). However, none of these introgressive alleles were found in modern cotton cultivars, suggesting that the potential benefits of introgression from *G. barbadense* into upland cotton are still largely unrealized.

These opportunities have not gone unnoticed. A host of scientists have made interspecific crosses, in particular between *G. hirsutum* and *G. barbadense* (Davis 1974, 1979; Palomo and Davis 1983); however, the lack of tools such as DNA markers that facilitate separation of rare desirable alleles from many nearby alleles associated with interspecific compatibilities often led to their abandonment. Early DNA marker studies confirmed that there had been an important role played by introgression from *G. hirsutum* chromatin into five specific genomic regions of cultivated *G. barbadense* (Wang et al. 1995), however strong segregation distortions in interspecific populations, especially in advanced generations (Jiang et al.

2000a), cast some doubt on whether introgressed chromatin would actually persist in such populations.

The employment of backcross-inbred approaches, testing the effects of chromosomal segments from exotic donors in largely-elite cotton backgrounds, offers promise of making better use of exotic cotton germplasm (and has also been very successful in other taxa such as tomato and rice (Tanksley et al. 1996; Xiao et al. 1996)). In one series of studies in cotton, workers developed *G. hirsutum* genotypes containing small introgressed chromosomal segments from each of two divergent cotton species. The multigeneration backcrossing procedure used (Chee et al. 2005a, b; Draye et al. 2005a, b), provided several segregating generations in which to expose interspecific gene combinations that led to sterility, prohibitively late flowering, or other phenotypes that dramatically reduced fitness. Accordingly, these procedures may have filtered out the portions of the exotic genomes that would not be useful in breeding programs. While the filtered portions of the exotic genomes may include some desirable alleles, this is more than compensated for by the advantage of concentrating effort on those regions of the genome that are most likely to be immediately useful. Similar studies of segmental introgressions from *G. tomentosum* and *G. mustelinum* are in progress (A.H. Patterson, O.L. May, and P. Chee, pers. comm.).

3.3 The Cotton Genomes and Progress Towards Their Sequencing

3.3.1 The Cotton Transcriptome and Patterns of Gene Expression

A first logical step toward the sequencing of a genome is to use well-established techniques to isolate mRNA and sequence the corresponding DNA to gain insight into the most frequently and abundantly expressed genes. The *Gossypium* transcriptome refers to all transcribed sequences in any of approximately 45 diploid and 5 tetraploid species in the genus. A recent review (Udall 2009) describes in detail the extent of knowledge of the cotton transcriptome, which is presently heavily biased toward genes expressed in fibers. Four large EST (Arpat et al. 2004; Udall et al. 2006) contributions comprise 72% of the ESTs in Genbank and were funded by either the National Science Foundation Plant Genome Research program or the USDA-ARS. Smaller, but significant, contributions have been made from numerous other cotton genome research projects and represent transcriptional profiles from either specialized tissues or specific experimental treatments (Udall 2009). *Gossypium* ESTs have been derived from three different species of cotton: *Gossypium arboreum* (A_1-genome), *G. hirsutum* (AD_1-genome), and *G. raimondii* (D_5-genome).

Toward the deduction of complete sequences of the expressed portions of cotton genes, EST assemblies have been both created and viewed by two separate programs: the Program for Annotating and Viewing ESTs (PAVE) developed at the

University of Arizona (http://www.agcol.arizona.edu/), and the Cotton Gene Index at the Computational Biology and Functional Genomics Laboratory located at the Dana-Farber Cancer Institute and the Harvard School of Public Health (CBFGL; formerly part of The Institute of Genomic Research). Comparisons of the distinct unigene sets suggest that the similarity between the two assembly programs is high, but some homologous contig alignments generated by these programs may be different. Both of these programs are important to the *Gossypium* transcriptome because their respective EST assemblies can be browsed or searched by the cotton community. A final methodology was used to create the most comprehensive EST assembly by ESTInformatics (http://www.estinformatics.org). Currently, this platform does not have web-based browsing, viewing, or EST search capability. A recently-inferred total number of unigenes (54,085: Udall 2009) agrees closely with recent estimates of diploid cotton gene number of 53,550 (Rabinowicz et al. 2003) based on methyl-filtration estimates and 50,128 $\pm$ 7,489 based on sequencing whole genome shotgun libraries.

Large-scale experiments for investigating cotton gene expression patterns are presently based largely on cDNA- and oligonucleotide-based microarrays, with the latter including both long-oligo arrays and short-oligo arrays that distinguish between homoeologs. (Arpat et al. 2004; Udall et al. 2006, 2007). A host of studies to date have concentrated on detailed analysis of cotton fiber development (reviewed in Udall 2009), but these same genomic tools are also beginning to be applied to other aspects of cotton biology and improvement.

A particularly striking finding is the dramatic changes in gene expression that have occurred in tetraploid cottons relative to their diploid progenitors (Adams and Wendel 2005a, b). Corresponding genes from the two diploid progenitors, when combined in a common nucleus, appear in many cases to have subfunctionalized (subdivided their functions), with only one member of homoeologous gene pairs being expressed in some tissues and the alternative member being expressed in others. While the mechanism by which this subfunctionalization occurs remains unclear, the process appears to occur very rapidly, being found in "synthetic polyploids" made experimentally by humans. A tantalizing hypothesis warranting further investigation is that this complementarity of gene function may be related to novel features of polyploid cotton such as its genetic potential for higher fiber yield and quality than its diploid progenitors (Jiang et al. 1998a, b).

3.3.2 *Beyond the Transcriptome – Sequencing of Entire Cotton Genomes*

The genomes of most major crops are likely to be sequenced in the next 10 years (Paterson 2006), and cotton genome sequencing is likewise proceeding. Cotton is unusual, although not truly unique, in that we will need to sequence not only cultivated (tetraploid) genotypes but also their diploid progenitors, to understand

how tetraploid cottons have come to "transgress" the productivity and quality of their progenitors. The cottons that dominate commerce are allotetraploid, originating in the New World from interspecific hybridization between an A-genome African diploid species resembling *G. herbaceum,* and a D-genome American diploid species (Skovsted 1934; Beasley 1940) resembling *G. raimondii* or *G. gossypioides* (Gerstel 1958; Phillips 1963). The A- and D-genome groups are estimated to have diverged from a common ancestor 5–10 MYA, then been reunited via polyploidization in an A-genome cytoplasm (Wendel 1989; Small and Wendel 1999) about 1–2 MYA (Wendel and Cronn 2003) following trans-oceanic dispersal to the New World of an A-genome propagule closely resembling the extant species *G. herbaceum*. Domesticated AD-tetraploid cottons appeared in the New World by 3500–2300 BC. (Hutchinson et al. 1947; Jiang et al. 1998a). Domesticated A-genome diploids existed in the Old World by 2700 BC (Chowdhury and Burth 1971), and remain intensively bred and cultivated in India, China, and Pakistan. The D-genome cotton species do not produce spinnable fiber, but have had a significant impact on fiber traits in the allotetraploids as evidenced by marker-assisted QTL localization (Jiang et al. 1998a) and chromosome substitution line performance (Saha et al. 2006a).

Sequencing of representatives from each diploid clade, and preferably each genome, will be important to molecular dissection of numerous evolutionary patterns and biological phenomena, including the genomic and morphological diversity that has permitted species within the genus to adapt to a wide range of ecosystems in warmer, arid regions of the world. Given the expected continuing progress in improving sequencing throughput and reducing cost (Paterson 2006), a strong case can be made for complete sequencing of one or more representatives of each *Gossypium* genome type, including a tetraploid. Efficient approaches to capturing the unique information available from the genus will need to consider several constraints, as follows, that may require the use of different sequencing strategies for different taxa.

1. A high degree of colinearity and synteny among the A, D, and tetraploid genomes (Reinisch et al. 1994; Brubaker et al. 1999; Rong et al. 2004a; Desai et al. 2006) suggests that complete sequencing of a small number of genotypes together with reduced-representation sequencing of representatives of additional nodes might be cost-effective. There has been some additional rearrangement of tetraploid chromosome structure relative to their diploid progenitors (Brubaker et al. 1999; Rong et al. 2004a; Desai et al. 2006), including some evidence of cryptic rearrangements based on genetic maps that may not be obvious (Waghmare et al. 2005a). In partial summary, the high degree of transferability of information about gene content and order among the respective genome types suggests that whole-genome efforts in favorable taxa will provide strong guidance for future efforts in the most difficult taxa.
2. Efficient strategies for capturing the sequence diversity represented within the *Gossypium* genus will be greatly influenced by large differences in genome size and organization across the genus. The diploid genomes vary about threefold in

DNA content, but have the same chromosome number and similar gene content. The smallest haploid genome size is estimated to be ~880-Mb for *G. raimondii* Ulbrich, with a size of ~1.75-Gb for *G. arboreum* L., and ~2.5 Gb for tetraploid *G. hirsutum* L. (Hendrix and Stewart 2005). DNA content of the allopolyploids is approximately the sum of those of the A and D-genome progenitors, and nearly all of >22,000 AFLP fragments surveyed are additive in the allopolyploids (Liu et al. 2001).

3. Much of the size variation among the diploid genome types is due to dispersed repetitive DNA (Zhao et al. 1998), which appears to be largely retrotransposon-like elements (Hawkins et al. 2006). There appears to have been large expansions of repetitive DNA content in the A/B/E/F and C/G/K genome clades in the 5–10 million years since the divergence of the diploid clades; therefore, many of these element families may include large numbers of relatively recently-derived members that are problematic for whole-genome shotgun approaches. By contrast, the D genome clade appears to have only a minimum of such recently-amplified repetitive DNA, and may be more amenable to whole-genome shotgun approaches. A survey of about 100 of the most abundant families in the tetraploid genome showed only four to be abundant in the D genome but found them to be rare or absent in the A genome (Zhao et al. 1998). Thus, most high-copy repetitive DNA families in the D genome are older than the A–D divergence (5–10 million years old), an age that renders them likely to be amenable to assembly by a whole-genome shotgun approach. By contrast, the alternative A genome progenitor contains about 50 repetitive element families that are rare or absent from the D genome, suggesting that these families amplified in this same 5–10 million year period. Most of these A-genome repetitive element families contain thousands of members, and have continued to amplify and transpose since polyploid formation about 1–2 million years ago (Zhao et al. 1998), rendering the A and tetraploid "AD" genomes potentially less amenable to whole-genome shotgun approaches.
4. The tetraploid clades combine the properties of the A and D genome diploids with modification by intergenomic concerted evolution. Concerted evolution of the repetitive DNA fraction (Wendel et al. 1995a, b; Cronn et al. 1996; Zhao et al. 1998) has been clearly shown. The possibility of intergenomic exchange of low-copy DNA remains somewhat unclear, with evidence for (Reinisch et al. 1994) and against it (Cronn et al. 1999), but growing data from other taxa strongly suggest that it may be an important dimension of polyploid evolution (Hughes and Hughes 1993; Moore and Purugganan 2003; Gao and Innan 2004; Chapman et al. 2006; Wang et al. 2007c). The possibility of intergenomic concerted evolution, much like the presence of recently-amplified repetitive DNA families, would tend to support the need for a BAC-based rather than a whole-genome shotgun approach in the affected genome(s).

Given these four considerations, one logical conclusion is that the whole-genome shotgun sequence of the smallest *Gossypium* genome would be likely to expediently provide fundamental information about gene content and organization across the genus. *G. raimondii* has the smallest genome size in the genus (~880 Mb)

and lowest amount of repetitive DNA sequences, with most of its repetitive DNA relatively old and therefore likely to be comprised of well-differentiated family members. A fully sequenced *G. raimondii* genome would establish an initial "template/backbone" toward the long term goal of characterizing the spectrum of diversity among the eight *Gossypium* genome types and three polyploid clades. Whole-genome shotgun based characterization of this smallest genome is in theory the most cost effective and easiest of the whole genome approaches at present.

For these and additional reasons, the US Department of Energy Joint Genome Institute (JGI) recently completed a "pilot project" of ~0.5 genome equivalents of *G. raimondii*, toward formulating an efficient strategy for its sequencing. On the basis of the results of the pilot study, which confirmed that *G. raimondii* was amenable to assembly of a good-quality whole-genome shotgun sequence, JGI committed to sequence an additional 1.2-genome equivalents (1 Gb), comprised largely of paired-end Sanger sequences from fosmids, for release in 2009. Pilot studies are in progress for the species *G. arboreum* and *G. hirsutum*, the next priorities after *G. raimondii* in the international strategy for characterizing the spectrum of *Gossypium* (cotton) diversity (Chen et al. 2007).

The economic importance of cotton fibers and scientific interests in polyploidy suggest an ultimate goal of sequencing a *G. hirsutum* tetraploid. The possibility of intergenomic concerted evolution, much like the presence of recently-amplified repetitive DNA families, may tend to support the need for a BAC-based rather than a whole-genome shotgun approach. Concerted evolution of the repetitive DNA fraction (Wendel et al. 1995a, b; Cronn et al. 1996; Zhao et al. 1998) has been clearly shown. The possibility of intergenomic exchange of low-copy DNA remains somewhat unclear, with evidence for (Reinisch et al. 1994) and against it (Cronn et al. 1999), but growing data from other taxa strongly suggest that it may be an important dimension of polyploid evolution (Hughes and Hughes 1993; Moore and Purugganan 2003; Gao and Innan 2004; Chapman et al. 2006; Wang et al. 2007c).

The possibility of intergenomic concerted evolution, much like the presence of recently-amplified repetitive DNA families, would tend to support the need for a BAC-based rather than a whole-genome shotgun approach in the affected genome(s). Using a finished diploid genome as a template and guide, a BAC-based sequence of a tetraploid will elucidate the types and frequencies of changes that have distinguished polyploid from diploid cottons. A reasonable approach is to establish a minimum tiling path of finger-printed BAC contigs (FPC) using genetically-anchored DNA markers and BAC-end sequences largely as described for other taxa (Chen et al. 2002; Bowers et al. 2005). While hybridization probes may anchor multiple homoeologous loci, 5–10 million years of divergence will provide for adequate differentiation of BAC contigs in most instances. Any exceptions can be resolved using routine techniques (Lin et al. 2000). Contig assemblies might be further validated, and any rogue contigs lacking genetic markers anchored to their chromosomal locations, using BAC fluorescence in situ hybridization (FISH) (Hanson et al. 1995; Stelly et al. 1995; Zwick et al. 1998; Kim et al. 2005a, b; Wang et al. 2006c).

3.4 After the Sequence – Analysis of Cotton Gene Functions

To understand and manipulate the features that make cotton unique will require a host of enabling tools, technologies, and resources; in particular targeting portions of the sequence that are substantially different from those of other organisms. As the basic gene set for angiosperms has been revealed by sequencing of several botanical models, a natural priority in sequencing cotton will be to reveal that genes are related to their unique features. There are few, if any, other examples of seedborne epidermal plant cells that reach 1–2" or more in length and are nearly pure cellulose. How will we recognize the genes that confer these features, and how will we determine how they work?

3.4.1 Deductions from Correspondence of Cotton Genes to Those of Other Organisms

The relatively close relationship of cotton and *Arabidopsis*, the first fully-sequenced angiosperm and the crown botanical model for genomics, is of especially high value. At least 59% of the cotton map and 53% of the *Arabidopsis* transcriptome show correlated gene arrangements (Rong et al. 2005b). Ongoing progress in completing the cotton sequence, together with new methods (Tang et al. 2008a, b) to deal with lineage-specific genome duplications in both cotton and *Arabidopsis* since their divergence (Rong et al. 2005b), will permit scientists to deduce the relationships of cotton chromosomes to those of other sequenced angiosperms. This will enable researchers focusing on particular cotton genes to quickly deduce the corresponding gene(s) in *Arabidopsis*. This approach is expected to quickly suggest the functions of many cotton genes based on their known functions in *Arabidopsis* – in some instances, this may lead to the identification of economically-important genes directly (for example, disease resistance). In other cases, this may reveal genes that have relatively conserved, "housekeeping" functions that are not likely to contribute to novel (economically-important) features of cotton, thus redirecting attention to more promising targets.

3.4.2 Mutagenesis of Cotton Genes to Determine the Phenotypic Effects of Their Loss

To determine the functions of cotton genes that lack clear homologs or for which homologs in other taxa are of unknown function, de novo analysis will be necessary. Comprehensive mutant populations, using chemical mutagenesis in combination with high throughput screens for novel alleles in specific target genes (McCallum et al. 2000; Till et al. 2003; Slade et al. 2005; Comai and Henikoff 2006) provide

a means by which functional analysis of genes can be carefully-targeted to complement and supplement more extensive resources for *Arabidopsis* and other botanical models. These techniques are likely to become much faster and less costly using new resequencing technologies. Several populations of mutants have been produced in cotton, but their use is complicated by (a) the practice of single-boll, rather than single-seed descent, which has the consequence that some mutants in the resulting population(s) may be identical by descent, and (b) the cost and difficulty associated with selfing of many thousands of cotton plants at each generation, to avoid the possibility of outcrossing.

An attractive complement to chemical mutagenesis is a transposon mutagenesis strategy based on the *Dslox* system, which combines the advantages of transposon-tagging using maize *Ac*/*Ds* elements for producing insertion mutants (Liu et al. 2000), and *Cre-lox* site-specific recombination for inducing gross chromosomal rearrangements (Medberry et al. 1995). Approximately 200 Dslox transgenic cotton lines have been produced, a sufficient number that one or more Dslox elements is expected to be located on each of the 26 tetraploid chromosomes. Ac-mediated transposition of *Dslox* has been demonstrated in transient assays (Trolinder 2009), establishing that the *Ac*/*Ds* transposon-tagging system functions in cotton as in a number of other species. These lines form the foundation of genetic stocks suitable for "saturation mutagenesis" of the cotton genome, permitting production of large deletions, inversions, or translocations that aid in the functional analysis and can be targeted to any region (or gene) in the genome by virtue of the locations of the *Dslox* sites. *Dslox* insertion sites are by far most effective for mutating nearby genes – accordingly, to permit researchers to select among the existing genetic stocks for those that are most effective for analysis of their target genes, the cotton genomic regions flanking each *Dslox* insertion site need to be sequenced and used to map each insertion site within the cotton genome.

Recently, progress was reported toward an efficient but transient viral-induced gene silencing system, another attractive complement for rapid analysis of specific genes of interest (Tuttle et al. 2009).

These approaches will provide for both the study of genes/gene families that are less tractable in other plants, and also for targeting functional analyses to specific genes implicated in key cotton traits by association genetics or other approaches. Such resources are ideally needed for each of the two cultivated tetraploids (to permit study of duplicated gene fates during all-important adaptation to the polyploid state) and each of the diploid genome types, with priority placed on the progenitor A and D genomes that contributed to the tetraploid.

3.4.3 Transformation

Cotton was the first crop for which genetically-engineered genotypes were commercialized in the USA, enjoying the advantage that it was not a food crop and that its wild relatives were largely not found in the USA (save for one diploid relative

and some localized feral populations). The success of transgenic cotton for reducing pesticide use and facilitating water- and energy-efficient minimum-tillage strategies has motivated much ongoing investment in cotton transformation. An extensive literature on this subject has recently been reviewed by one of its pioneering practitioners (Trolinder 2009).

A persistent and continuing problem has been the genotype-specificity of cotton somatic embryogenesis. Cultural procedures could not be uniformly applied to all cultivated cottons, indicating a recalcitrance of certain genotypes to regeneration by somatic embryogenesis (Trolinder and Chen 1989). Highly embryogenic lines, particularly Coker 312 and Coker 5110, were identified. These lines were the products of a cross between Coker 100 W and Delta Pine 15. Reconstitution of this cross identified 100 W as the progenitor of the embryogenic trait in these lines. Extensive screening has identified many embryogenic cultivars, including, but not limited to GC510, Pee Dee germplasm, Mar(Multi-Adversity Resistance) germplasm, Sure Grow 501, DP90, Texas Racestock T25, Chinese variety Lu 1, and Acala lines – however, only a limited number yield the frequencies desired for efficient transformation (Trolinder 2009).

The genotype-specificity of cotton somatic embryogenesis has been only a relatively minor stumbling-block in the development of commercial products, where only one or two transformation events producing a specific product can afford to be taken through the regulatory approvals necessary for commercialization. Therefore, this procedure has substantially broadened the gene pool that is accessible for cotton improvement. However, to utilize transformation as a tool for cotton genomics, it would be desirable to be able to transfer genes readily in any background.

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Chapter 4
Cotton Transformation

D.R. Duncan

4.1 Introduction

The genetic transformation of cotton provides the cotton breeder with the opportunity to develop plant traits that are very difficult or impossible to develop through conventional breeding. For instance, genetic transformation can or has yielded traits, such as resistance to the herbicides 2,4-D (Bayley et al. 1992), and glyphosate (Zhao et al. 2006), resistance to aphids (Wu et al. 2006) and bollworm (Perlak et al. 1990; Rashid et al. 2008), as well as *Aspergillus flavus* (Chlan et al. 2003), and the reduction of gossypol in cotton seed (Sunilkumar et al. 2006). Transformation of any species requires a means to deliver DNA into a plant's cells and subsequently, create an entire plant from that cell. This process has traditionally required tissue culture, and cotton has not been an exception to this paradigm. Cotton has been cultured in various forms since the early 1970s (Schenk and Hildebrandt 1972), but the process is still being improved to make it faster, more efficient, and more genotype independent. Several reviews of cotton biotechnology and transformation were published at the turn of the millennium (Wilkins et al. 2000; Sakhanokho and Chee 2002; Kumria et al 2003a), which are recommended for additional information on the state of cotton transformation. The present work is intended to cover the literature gap over the past decade and to outline recent trends in cotton transformation. Only refereed literature is considered for this review, but a number of interesting patents and patent applications are worth examining when one is working with cotton.

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4.2 Cotton Tissue Culture

Work on the basic protocol for most cotton tissue culture began in 1972 with Schenk and Hildebrandt (1972) and culminated in the first report of plant regeneration from a cultured cotton tissue by Davidonis and Hamilton (1983). It was established that MS medium (Murashige and Skoog 1962; Smith et al. 1977), with the addition of 100 mg L^{-1} myo-inositol, 0.4 mg L^{-1} thiamine HCl, glucose to reduce tissue browning, and a combination of an auxin and a cytokinin, e.g. 2 mg L^{-1} naphthaleneacetic acid (NAA) and 0.5 mg L^{-1} benzyladenine (BA) (Smith et al. 1977), could induce callus from cotyledon and hypocotyl tissues. Other cotton tissues, such as leaves and petioles (Gawel et al. 1986; Trolinder and Goodin 1988a; Zhang et al. 2000; Kumria et al. 2003b), immature zygotic embryos (Hussain et al. 2004), and roots (Sun et al. 2005a), have been successfully used, but the vast majority of cotton work has been done with cotyledons or hypocotyls. Once ample callus was developed, the tissue could be moved to an embryogenic-callus induction medium lacking the auxin and cytokinin. After 3–4 months of culturing, proembryo masses would develop, which would in turn, develop into embryos and eventually, into plants when placed on a low salt medium (Stewart and Hsu 1977) with reduced or no NH_4^+, elevated KNO_3, and 5 g L^{-1} glucose in the medium (Davidonis and Hamilton 1983). Although the vast majority of cotton tissue culture and plant regeneration is via somatic embryogenesis, Khan et al. (2006) have reported organogenic plant regeneration when cotyledon or hypocotyl-derived callus was cultured on medium supplemented with only BA.

The general protocol outlined above has worked for producing cotton tissue cultures that could regenerate plants, but overall, it was and continues to be a very labor intensive process that requires 10–12 months to complete (Mishra et al. 2003; Sun et al. 2006). The length of time in culture inherently presented potential problems of somaclonal variation, increased difficulty of plant regeneration, and plant infertility. Furthermore, although early reports suggested that a wide range of cotton varieties could be cultured (Shoemaker et al. 1986), the varieties from the Coker family of cultivars have been the most prolific in plant regeneration or easily cultured (Firoozabady and DeBoer 1993). These limitations of culture duration and restricted cultivar selection have given rise to a plethora of research aimed at improving the basic culture process.

In screening a wide number of regional varieties using the typical cotton protocol previously described, researchers have found varieties useful to their programs that regenerated plants from callus cultures comparable to Coker varieties (Sakhanokho et al. 2004a; Chinchan et al. 2005). A number of culture modifications have been reported, however, aimed at placing into culture a researcher's favorite non-Coker cotton variety. Some of these culture modifications were as simple as choosing N^6-(2-isopentenyl)-adenine (2iP) (Gonzalez-Benito et al. 1997; Kumar and Pental 1998) or zeatin (Zhang et al. 2000; Zhang et al. 2001) instead of BA for a cytokinin; or choosing 2,4-dichlorophenoxyacetic acid (Trolinder and Goodin 1988b; Kumria et al. 2003b; Wilkins et al. 2004; Sun et al. 2006), picloram

(Ganesan and Jayabalan 2005) or indolebutyric acid (Wu et al. 2004; Jin et al. 2005) instead of NAA as an auxin. The addition of 0.5 mg L^{-1} putrescine to culture medium also was reported to increase somatic embryo production 2–53-fold in non-Coker upland cotton cultivars.

For other cultivars more complicated medium modifications have been reported. Divya et al. (2008) introduced into culture three Indian cultivars by replacing MS vitamins with Nitsch vitamins (Nitsch and Nitsch 1969) and adding ethylene-inhibiting silver nitrate and phenolic-binding activated charcoal to their medium. Diploid cotton species were cultured and plants regenerated from multi-step treatments of raising and lowering cytokinin concentrations, changing carbon sources from glucose to a combination of glucose, sucrose, and maltose followed by plant rooting on activated charcoal medium (Sun et al. 2006). Sakhanokho et al. (2004b) also cultured diploid cotton with a simpler series of media but supporting the tissues with filter paper in the later stages of the process to keep them dry.

Even with Coker cultivars researchers have been improving the culture process to reduce the time from placing an explant into culture to regenerating plants from it and to facilitate genetic transformation. Many of these medium modifications were similar to those used to culture non-Coker varieties. In addition to using Nitsch vitamins (Divya et al. 2008), Trolinder and Goodin (1987), Finer (1988), and Ganesan and Jayabalan (2005) replaced MS vitamins with Gamborg's B5 vitamins (Gamborg et al. 1968). The addition of glutamine (Finer 1988) or a combination of aspargine and glutamine (Wu et al. 2004) was reported to increase embryo development in the later stages of cotton embryogenesis. Umbeck et al. (1987), Firoozabady, and DeBoer (1993) showed that 30 °C was a better culture temperature than the 28 °C typically used to culture cotton. To increase shoot growth gibberellic acid has often been added to the late stages of the culture process (Davidonis and Hamilton 1983; Trolinder and Goodin 1988b; Firoozabady and DeBoer 1993; Satyavathi et al. 2002; Kumria et al. 2003b; Ganesan and Jayabalan 2005; Ikran-ul-Haq 2005; Sanjaya et al. 2005). Other modifications were less common, like the addition of hemoglobin to culture medium to control browning and to increase callus fresh weight gain and embryoid production (Ganesan and Jayabalan 2004).

Stress has also been suggested to increase somatic embryogenesis in cotton as indicated by the high phenolic content seen in cotton lines that can regenerate plants compared to that in those that cannot produce plants (Kouakou et al. 2007). Kumria et al. (2003b) reported that a one-fifth strength MS medium increased embryogenesis twofold to threefold. Embryogenesis was also reported to be improved by using dark grown and etiolated hypocotyl tissue (Kouadio et al. 2004), as well as tightly sealing Petri dishes of tissue with Parafilm™ (Kumria et al. 2003b; Kouadio et al. 2004; Leelavathi et al. 2004). A myo-inositol starvation of embryogenic cotton callus for one sub-culture was suggested by Kumar and Tuli (2004) to synchronize embryogenesis for efficient plant regeneration. To increase embryo development and plant recovery a slow drying with ventilation was suggested by Wu et al. (2008).

An early modification that would impact many aspects of cotton tissue culture and transformation in the future was the application of liquid culture to various

stages of the process. Suspension cultures of cotton typically are begun by inducing callus on a gelled medium using the standard protocol described previously or a modification of it and then placing callus in a non-gelled embryogenic-callus induction medium (Trolinder and Goodin 1987; Finer 1988). The liquid cultures are typically grown in the dark on a rotary shaker at between 120 and 150 rpm. One major advantage to adding a liquid culture step to the process is a reduction in overall time to produce somatic embryos (Sakhanokho et al. 2004b; Ganesan and Jayabalan 2005). Often the liquid culture was passed through a sieve or series of sieves from 10 to 100 mesh (Trolinder and Goodin 1987; Cousins et al. 1991; Mishra et al. 2003; Wilkins et al. 2004; Sakhanokho et al. 2005). This sieving mechanically synchronized embryonic masses and separated cream-colored, fast-growing embryogenic callus from the typically phenolic-laden cotton callus (Wu et al. 2008).

Protoplast production has been accomplished on a limited basis from callus, cell suspensions, and whole plant tissues of cotton using a combination of cellulases, pectinases, and hemicellulases in various combinations (Peeters et al. 1994; Sun et al. 2005a, b). Plants were also regenerated via somatic embryogenesis from protoplasts (Sun et al. 2005a, b) but little else seems to have been done with this technology.

In addition to callus cultures and protoplasts, the micro-propagation of cotton and the development of organogenic shoot cultures have been demonstrated. Hemphill et al. (1998) reported the regeneration of shoots from pre-existing meristems of shoot apices and nodal buds from 14 to 28 day old sterile seedlings. Ozyigit and Gozukirmizi (2008) similarly showed that the cotyledon node and shoot tip of germinated cotton seedlings could be cultured to form complete rooted plants. By treating the cotyledon node and shoot tip with BA, Gupta et al. (1997) induced multiple shoots from the pre-existing meristems. Ganesan and Jayabalan (2005) took this shoot induction process a step further by making an organogenic callus culture from cultured shoot tips exposed to 2.0 mg L^{-1} BA and 1 mg L^{-1} kinetin in MS medium.

4.3 Transformation

The transformation of cotton was first reported by Umbeck et al. (1987) and Firoozabady et al. (1987). Both groups, working a few miles apart in Madison, Wisconsin, used similar tissue culture and transformation techniques. Umbeck et al. (1987) transformed cotton hypocotyl segments from Coker 312 while Firoozabady et al. (1987) used cotyledon pieces from Coker 210. Both used the LBA4404 strain of *Agrobacterium tumefaciens* containing the neomycinphosphotransferase II gene (*nptII*) that confers resistance to the antibiotic kanamycin. Both exposed their tissues to a bacterial solution for a few seconds (Firoozabady et al. 1987) or overnight (Umbeck et al. 1987) and then co-cultured the bacterium and cotton tissues for 3 days without antibiotics to control bacterial growth. At the end of the

3 day co-culture period the tissues were placed on MS medium containing up to 50 mg L^{-1} kanamycin for selecting transgenic events and up to 500 mg L^{-1} carbenicillin (Firoozabady et al. 1987) or 500 mg L^{-1} cefotaxime (Umbeck et al. 1987) to control bacterial growth. Both groups induced embryogenesis by removing the growth regulators from the culture medium while still maintaining the kanamycin and the carbencillin or cefotaxime. Plants were regenerated and rooted on Stewart and Hsu's (1977) low salt medium.

Many research labs have successfully used the same protocol as did Umbeck et al. (1987) and Firoozabady et al. (1987) with an occasional culture modification, as those mentioned previously, to reduce the time to produce transgenic events or to transform a favorite non-Coker cultivar. Other modifications focused on the actual transformation. For instance, the *A. tumefaciens* strain LBA4404 has commonly been used for cotton transformation but EHA101 (Song et al. 2000; Tohidfar et al. 2005), EHA105 (Sunilkumar and Rathore 2001; Burke et al. 2008), AGL-1 (Cousins et al. 1991; Meng et al. 2007), GV3101 (Ikram-Ul-Haq 2005), and GV3111 (Bayley et al. 1992) have also been used. In addition, cotton being a dicotyledonous plant that produces a huge amount of phenolic compounds has been typically grown and used in transformations without the addition of reagents to induce virulence. Gould and Magallanes-Cedeno (1998), Sunilkumar and Rathore (2001), Balasubramani et al. (2005), Jin et al. (2005), Yuceer and Koc (2006), and Wu et al. (2008) reported that 10 μg L^{-1}–50 mg L^{-1} acetosyringone could significantly increase cotton transformation. Another difference from the basic Umbeck et al./Firoozabady et al. transformation has been in the gene used to convey resistance for selecting transgenic events. Besides using the *nptII* gene, the gene for hygromycin phosphotransferase (*hpt)* that confers resistance to the antibiotic hygromycin (Meng et al. 2007), a glyphosate resistant 5-enolpyruvyl-3-phosphoshikimate synthase (EPSP) that confers tolerance to glyphosate the active ingredient of RoundUp® herbicide (Zhao et al. 2006), the phosphinotricin acetyltransferase gene (*pat*) that confers resistance to phosphinotricin herbicides (Beringer et al. 2004), and a heat shock protein 101 (Burke et al. 2008) have been reported as useful in developing transgenic cotton.

An alternative to the Umbeck et al./Firoozabady et al. embryogenesis approach to cotton transformation was to use organogenesis as reported by Balasubramani et al. (2005) and Yuceer and Koc (2006). Balasubramani et al. (2005) transformed the embryonic axis from cotton seeds germinated for 48 h by exposing them for 30 min to *A. tumefaciens* strain LBA4404 containing the *hpt* and the β-gucuronidase (*gus*) gene. The tissues were subsequently cultured on MS medium containing B5 vitamins, 2 mg L^{-1} BA, 0.5 mg L^{-1} kinetin, 250 mg L^{-1} cefotaxime, and 50 mg L^{-1} hygromycin. Multiple shoots were induced from the tissue with the addition of 1.0 mg L^{-1} Thiadiazuron to the BA and kinetin mixture. Yuceer and Koc (2006) used meristem tissues from 5 to 10 day old seedlings. The meristems were inoculated for 15 min with strain EHA101 containing the *nptII* and *gus* genes. Selection and multiple shoot regeneration were accomplished on MS medium with 0.5 mg L^{-1} kinetin.

Although the Umbeck et al./Firoozabady et al. transformation process has been successfully used to produce transgenic cotton plants the cultivar limitations and

length of time in culture have significantly restricted the use and ease of cotton transformation. These restrictions have never really been reduced by improving tissue culture methods. Instead researchers have tried other options to avoid these problems. The simplest approach to, at least, reducing the time between the introduction of DNA into cotton cells and the regeneration of transgenic plants has been to use established cotton cultures instead of beginning the transformation with freshly isolated plant tissue.

In general, the *Agrobacterium*-mediated transformation of established embryogenic callus has been similar to the transformation of the initial explants. Prior to transformation several subcultures of the initial embryogenic callus were done to produce a rapidly growing, friable, loose white to yellow callus having little if any phenolic browning (Leelavathi et al. 2004; Wu et al. 2005, 2008; Jin et al. 2005). The vigorously growing embryogenic callus was exposed to *A. tumefaciens* with (Jin et al. 2005; Wu et al. 2008) or without (Leelavathi et al. 2004; Wu et al. 2005) acetosyringone induction of the bacteria. The embryogenic callus was then exposed to kanamycin as a selection agent for several subcultures followed by embryo induction and maturation.

Inserting DNA into cultures of established embryogenic callus was reported to reduce the time required to produce transgenic plants by, at least, 6 months (Wu et al. 2005) because the callus induction step was now not part of the transformation process. However, as the same old tissue culture technique was used to create the embryogenic callus for transformation, the overall time that the tissues were in culture or the effort required to produce the callus or transgenic events was not reduced. Consequently, issues such as somaclonal variation, loss of plant regeneration, and plant infertility due to extended time of cells in tissue culture still persisted. To deal with some of the potential regeneration issues from prolonged time in culture, a technique was developed to graft transgenic plants onto cotton seedlings to rescue events that did not root (Jin et al. 2006; Zhu et al. 2006). Beringer et al. (2004) also suggested that cryopreservation of established cultures could reduce some of the work involved in the overall process and very good cultured tissues could be maintained for multiple transformation projects. Although the drawbacks to cotton tissue culture of length of time from explants excision to plant regeneration and limited cultivar range persist, the transformation of embryogenic callus is a huge step forward in cotton transformation.

Callus is also amenable to other transformation techniques. Finer and McMullen (1990) used DNA-coated tungsten particles to transform, by particle bombardment, an embryogenic suspension of Coker 310, which resulted in hygromycin resistant cotton plants. Similarly, Beringer et al. (2004) transformed embryogenic suspensions of cotton cultivar GC510 by vigorously agitating the suspended pro-embryo masses with silicon carbide micro-fibers (WHISKERS™) coated with DNA containing the PAT gene. Selection was done using the herbicide Herbiace™ (gamma-(Hydroxymethylphosphinyl)-L-alpha-aminobutyryl-L-alanyl-L-alanine sodium salt). The authors argued that the WHISKERS™ technique was easier to do and to scale up to large industrial needs than was particle bombardment or *Agrobacterium*-mediated transformation.

An extension of the particle bombardment of callus was the transformation of cotton plastids reported by Kumar et al. (2004). Using both the *nptII* gene and the 3′-aminoglycoside phosphotransferase type VI (*aphA*-6) in plastid expression cassettes, transgenic cultures resistant to kanamycin were identified and plants regenerated. The authors argued that plastid transformation would eliminate the risk of transgene escape from predominantly self pollinating cotton. More importantly, expression levels of transgenes may be very high considering the large number of plastids in each cell of a cotton plant. The elevated expression levels may be critical for insect or disease resistance.

A final approach to transformation using callus is a chemically induced direct transformation. Sawahel (2001) exposed an embryogenic cotton suspension to MS medium containing 0.25 M spermidine and the polycation hexadimethrine bromide and DNA containing the *hpt* and the *gus* genes. Selection using hygromycin was later employed and transgenic cells expressing GUS were identified.

Although the transformation of embryogenic callus reduces the length of time from the introduction of DNA into cotton tissue to when a plant is regenerated, it does little to increase the number of cotton cultivars that can be transformed. To increase the cultivar range, avoiding tissue culture by using whole plant tissues has been attempted. McCabe and Martinell (1993) soaked cotton seed in a fungicide solution for 24 h. Mature embryos, with cotyledons removed, were excised from the softened seed, and oriented on a gelled medium with their meristems pointing up. DNA containing the *gus* gene precipitated onto gold particles was bombarded into the cotton meristems using the electrical discharge gun (Accell™). The bombarded embryos were moved to McCown's woody plant medium (Lloyd and McCown 1981) to germinate. Because the bombardment randomly transformed meristem cells, the transformed plants had a high probability of being chimeric. Consequently, the authors sampled leaves for GUS expression and pruned each plant to produce plants with predominately transformed sectors. The probability of a plant having transformed germ-line cells was on the basis of GUS patterns in petiole vascular tissue (Christou and McCabe 1992) and confirmed by germinating progeny seeds.

Similar meristem bombardment transformations have been reported using the Bio-Rad PDS/1000/He gene gun and either the *npt*II gene with kanamycin selection (Chlan et al. 1995) or a modified acetohydroxyacid synthase (*ahas)* gene that confers resistance to Imazapyr herbicide (Aragao et al. 2005; Rech et al. 2008). The use of selection greatly decreased the labor involved in cotton meristem transformation. Although this transformation protocol holds promise for transforming a wide number of cultivars it is very inefficient with a reported transformation efficiency of 0.5% (Rech et al. 2008).

An alternative to transforming the apical meristem from mature seed by particle bombardment is the transformation of apical meristems with *A. tumefaciens*. Meristems isolated from 5 to 7 day old cotton seedlings cultured on a MS medium containing 0.1 mg L^{-1} kinetin for 3–5 days were either cut along their sides or longitudinally through their centers prior to inoculation with *A. tumefaciens* strain EHA101 containing the *nptII* and *gus* genes (Gould and Magallanes-Cedeno 1998).

A modification of this wounded-meristem method was to keep the seedling intact, spread the cotyledons apart, stick a sewing needle into the meristem and inoculate the wounded area with *A. tumefaciens* LBA4404 containing the *nptII* and *gus* genes for 60 min (Keshamma et al. 2008). In both cases the meristems were grown into mature plants and progeny positively assayed for GUS activity

Meristems have also been transformed with *A. tumefaciens* LBA4404 containing the *nptII* and *gus* genes by placing it on the un-injured excised meristem for 15 min followed by 1–2 months of culture as shoots on kanamycin containing selection medium (Zapata et al. 1999). Modifications of this procedure included a 5 min vacuum infiltration of the shoot tips with the bacteria (Katageri et al. 2007) and the inoculation of the shoot tips by submerging them in a bacterial solution for 20 min (Satyavathi et al. 2002; Sanjaya et al. 2005).

As with particle bombardment of mature seed meristems, both of these *A. tumefaciens*-meristem transformation approaches yield plants that are chimeric and potentially not transformed in germ-line cells. Thus far the efficiency of these techniques is low (Rech et al. 2008) and in many cases time consuming and labor intensive. The advantage of these methodologies, in general, is their potential of using any cotton cultivar for transformation.

Transforming a cotton gamete would be one means to avoid the chimeric issues associated with meristem transformations and may possibly be the most rapid means to produce transgenic plants from a wide variety of cotton. One approach to transforming cotton gametes has been through the so called pollen-tube pathway (Zhou et al. 1983; Huang et al. 1999; Zhang et al. 2009). In this procedure DNA is injected into a cotton boll 10–24 h after the flower is pollinated. The flower may be treated with gibberellic acid to reduce the likelihood of floral abscission (Zhang et al. 2009). The bolls are left to mature, and the transgenic progeny are harvested, germinated, and tested for gene expression. Zhang et al. (2009) reported that large doses of DNA, up to 400 mg mL^{-1}, were needed to facilitate the transformation and that not all flowers on a plant would be useful for yielding transgenic plants. Overall, on a transgenic event per injected flower basis transformation efficiency ranged from 0.5% to 1%. Although this protocol seems promising, Kohel RJ and Yu (2001) reported that when using the protocol to develop herbicide tolerance in cotton they could detect the presence of their plasmid DNA in progeny seedlings but they could not detect gene expression as indicated by herbicide tolerance.

An alternative to the pollen-tube pathway transformation is the direct transformation of pollen itself (Li et al. 2004). Li et al. (2004) reported that *A. tumefaciens* strain GV3101 containing the *hpt* and *gus* genes was mixed in pollen germination medium with 50 mg of pollen collected from freshly dehisced anthers and placed under vacuum for 30 min. Following release of the vacuum the pollen was collected as a pellet by centrifugation and used to pollinate emasculated flowers. There were 1,038 flowers pollinated in this work from which 30% formed bolls and set seed. Of the seeds produced, 1.6% were hygromycin resistant, of which, 65% were also expressing the GUS gene.

4.4 Conclusion

Cotton has been cultured in vitro for 37 years and yet there are still today some of the same culture difficulties seen when it was first cultured, those being a very long culture time and a limited number of cultivars that can be cultured. These limitations have not been corrected by the large number of reported modifications to the basic tissue culture protocol outlined by Smith et al. (1977) and Davidonis and Hamilton (1983) The limitations inherent to the tissue culture process have made the transformation of cotton an arduous process. The transformation of embryogenic callus was a major step towards reducing the time required to produce a transgenic event, but it still has not solved the underlying issues of cotton tissue culture, the method just shortens the time required to produce a transgenic event. The initial success with transforming seed-derived apical meristems holds promise that the major drawbacks to cotton tissue culture and transformation may be overcome. It will be critical to the routine use of meristem transformation and in particular *Agrobacterium*-mediated meristem transformation that the efficiency and ease of performing the process be increased dramatically.

Pollen or floral-type transformations of cotton require significant space for growing mature cotton plants and for isolating them from other plants during and after a transformation. In addition to the space needs there are the time and labor issues involved in the transformation of many flowers on large plants in the warm conditions in which cotton grows. For pollen or floral-type transformations to be considered as practical options for routine production of transgenic cotton plants, they too will need to become easier and much more efficient processes.

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Chapter 5
New Tools and Traits for Cotton Improvement

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5.1 Introduction

The tools of modern biology are impacting cotton production significantly. Biotechnology is already an important tool for cotton growers around the globe. Since the first products were introduced in 1996, adoption has grown rapidly. After the initial product launches in the United States and Australia, adoption spread so significantly that by 2008, ten countries on five continents had approved commercial cultivation of cotton improved through biotechnology (Table 5.1; Agbios and James 2008).

On a trait basis, insect protection and herbicide resistance are the approved trait categories for cotton production. There are multiple product offerings in each of these trait categories. The products have matured to the point that second generation product offerings are already available in both of the trait classes. For insect protection, products in cotton deliver control of lepidopteran pests (Fig. 5.1) primarily through the employment of genes from *Bacillus thuringiensis* (Bt), a natural entomopathogen, and the products are commonly referred to as "Bt cotton" (Betz et al. 2000; Benedict and Altman 2001; Perlak et al. 2001). In the herbicide-resistant category, there are multiple product offerings. These products are designed to provide cotton with resistance to one or more agricultural herbicides. This allows farmers to apply the herbicide post-emergence, providing effective weed control without damage to the cotton plant. Increasingly, cotton varieties contain both insect protection and herbicide-resistant traits, commonly referred to as stacked traits. Economic, environmental, and social benefits of the current trait offerings have been realized and accrue to growers, the industry, and to society as a whole (Brookes and Barfoot 2008; James 2002; James 2007; James 2008; Purcell and Perlak 2004; Wakelyn et al. 2004).

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Table 5.1 Cultivation approvals for cotton biotech products (from Agbios and James 2008)

Year	Herbicide resistance	Insect protection
1996		United States, Australia
1997	United States	China, Mexico
1998		Argentina, South Africa
1999		
2000	Australia, South Africa	
2001	Argentina	
2002		India
2003		Colombia
2004	Colombia	
2005		Brazil
2006		
2007		
2008	Brazil	Burkina Faso

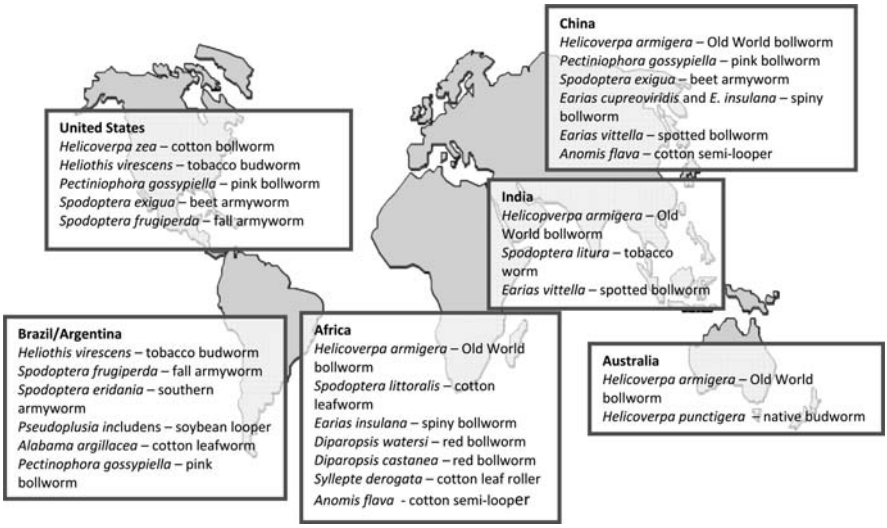

Fig. 5.1 Lepidopteran Insect Pests in Cotton (From: Matthews and Turstall 1994; Sun and Chen 1999)

In the future, biotechnology product offerings will broaden within the current trait categories and expand to new trait classes. New products for insect protection and herbicide-resistance will be available, and additional stacked offerings combining herbicide-resistance and insect protection will also enter the marketplace. New trait areas (Fig. 5.2) being explored include cotton tolerant to other agronomic pests, such as nematodes and fungi, cotton tolerant to abiotic stresses, cotton with improved fiber quality, and cotton with improved cottonseed and oil characteristics (Wakelyn et al. 2004; Rathore et al. 2008).

The tools of modern biology are also greatly impacting advances in cotton breeding. The International Cotton Genome Initiative (http://icgi.tamu.edu) was

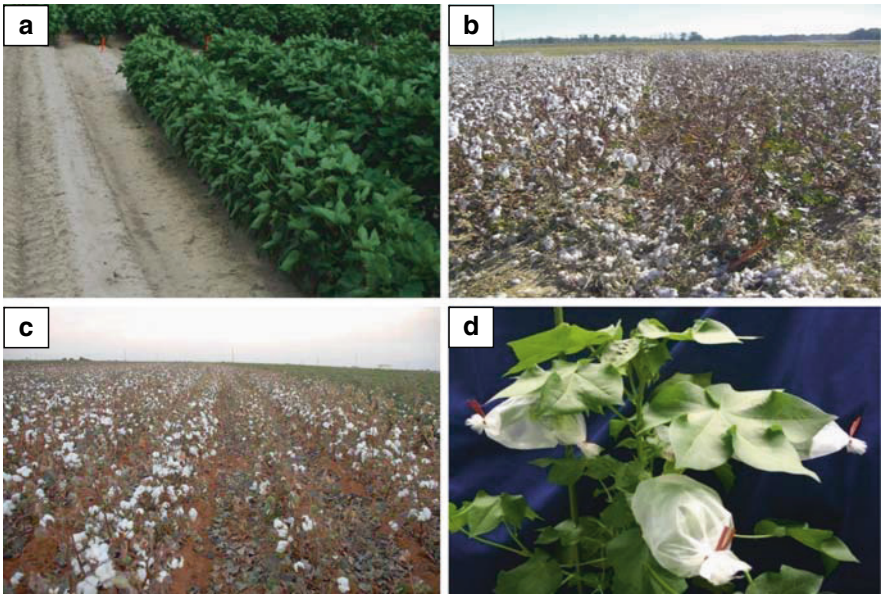

Fig. 5.2 New cotton traits in development. New biotechnology traits such as (**a**) Dicamba/glufosinate resistance; (**b**) Improved lepidopteran resistance; (**c**) Drought tolerance and (**d**) Lygus (plant bug) resistance are all being actively pursued and tested in greenhouse and field trials

launched in 2000 with one of the high priority goals of developing a large number of effective DNA markers in the complex tetraploid upland cotton genome (Brubaker et al. 2000). Fortunately, today a wide array of DNA markers can be deployed along with conventional breeding to accelerate the rate of genetic gain for important traits in a commercial cotton breeding program (Zhang et al. 2008). The combination of high throughput genotyping and marker assisted methods developed in maize and soybean can be applied to improvement programs of self pollinated crops such as cotton (Cantrell and Xiao 2008). Molecular breeding tools do not replace conventional breeding and selection but are an important complement in commercial breeding of cotton on a global scale.

5.1.1 Insect-Protected Cotton

Cotton has been improved through biotechnology to ward off damage from certain lepidopteran insect pests. Insect-protected cotton produces insecticidal proteins based on those found in the naturally occurring soil bacterium Bt (Benedict and Altman 2001; Perlak et al. 2001). Several lepidopteran active proteins from Bt have been employed in Bt cotton varieties. These include crystal endotoxin proteins, such as Cry 1Ab, Cry 1Ac, Cry 2Ab, and Cry 1F, as well as vegetative proteins, such as Vip3Aa (Agbios). In some cases, products employ combinations of two Bt

proteins to expand the spectrum of insects controlled while aiding in insect resistance management strategies (Agbios). There is one product approved in China that employs *Bt* technology, as well as a second insect-control protein, the cowpea trypsin inhibitor (Pray et al. 2001). Major lepidopteran pests are controlled effectively by Bt cotton in diverse geographies (Fig. 5.1). Lepidopteran cotton pests come mainly from the Noctuidae. Represented in this taxonomic family are the genera *Helicoverpa*, *Heliothis*, *Spodoptera*, *Earias*, and *Diparopsis*; most of the economic damage done to cotton globally can be traced to these genera (Matthews and Tunstall 1994). Especially important are *Helicoverpa zea* in the US and *Helicoverpa armigera* in sub-Saharan Africa and throughout Asia (Matthews and Tunstall 1994; Sun and Chen 1999). Another cosmopolitan lepidopteran pest is *Pectinophora gossypiella* (Lepidoptera: Gelechiidae), which can be found in most cotton growing regions globally and can be locally devastating to cotton production (Matthews and Tunstall 1994). To some degree, all of the Bt proteins mentioned above have some activity against all lepidopteran pests, although the potency of each varies among pest species. Overall potency of a Bt cotton will depend upon the Bt proteins expressed and their levels within the plant (dose). In general terms, Cry 1Ac and Cry 1Ab are very effective against most genera except *Spodoptera;* Cry 2Ab is effective against all genera; Cry1F has strong activity that includes *Spodoptera, H. virescens*, and *Pectinophora*; and Vip3A also has excellent activity against *Spodoptera* with moderate activity against several other genera (MacIntosh et al. 1990; Estruch et al. 1996; Sims 1997; Sivasupramaniam et al. 2000; Green et al. 2003; Adamczyk and Gore 2004; Bird and Akhurst 2007). These complementary activities are reflected in Bt-expressing cotton products either in the marketplace or approaching commercialization. Globally, Bt cotton provides effective control of lepidopteran pests and delivers benefits to the grower such as increased income and a more convenient system while reducing pesticide sprays (James 2002; Purcell and Perlak 2004; Brookes and Barfoot 2006, 2008).

Multiple stacked insect-protected traits with improved activity against a broad range of lepidopteran pests will be important for insect resistance management. Stacked trait offerings are already in the marketplace and in the future, additional products with multiple insect-control proteins will be available for lepidopteran control. Expansion of insect-control products to include additional pests beyond the Lepidoptera is being pursued. Transgenic solutions for plant bugs and other piercing and sucking insect pests are possibly the next generation of traits to be developed for cotton production. The attention these pests have received has greatly increased in recent years. In fact, for the first time since records have been kept, the piercing-sucking pest Lygus (plant bug) was identified as the number one pest affecting US cotton production in 2008 (Williams 2008). Key global targets in this large cotton pest complex include members of the orders Hemiptera and Homoptera. Members of these orders appear as significant pests in all agronomic settings and, as such, are present in every important cotton-growing region (Matthews and Tunstall 1994). Hemiptera, or true bugs, include members of the families Mirdae (Lygus, Mirids, and relatives) and Pentatomidae (stinkbugs and shieldbugs). Hemipteran pests damage cotton by piercing plant cells in vegetative and fruiting

tissues, injecting powerful digestive enzymes and then sucking out the liquefied cell contents (Wheeler 2001). Important homopoteran pests include aphids, whiteflies, and jassids; these pests insert mouthparts into plant vascular tissue and directly remove plant sap (USDA 2008a). Aphids and whiteflies, especially, cause additional problems in cotton associated with secondary fungal growth on plants and cotton lint because of the high sugar content of their excreta, or honeydew (USDA 2008a). Whiteflies also transmit geminiviruses leading to cotton leaf curl disease which leads to serious economic losses. The greater notice piercing-sucking pests have received lately has been, perhaps, predictable. Even though there have been claims that transgenics have directly caused piercing-sucking pest outbreaks (Cornell Chronicle Online), most IPM (Integrated Pest Management) experts consider the increase in visibility of these pests to be a logical by-product of the reduced-spray environment enabled by effective transgenic Lepidoptera control. Previously, heavy pesticide use targeting the major yield destroying Lepidoptera served also to control, to a very great extent, secondary piercing-sucking pest complexes in cotton. The growing importance of piercing and sucking insects globally makes this a key area of research to monitor over the coming years as solutions using biotechnology are discovered and developed.

5.1.2 Herbicide-Resistant Cotton

Herbicide-resistant cotton allows for effective in-crop use of certain agricultural herbicides. Cotton plants resistant to glyphosate, glufosinate, and bromoxynil herbicides are all available in the marketplace (Agbios; CaJacob et al. 2004; James 2008). Glyphosate targets the shikimate pathway of aromatic acid synthesis by inhibiting the key enzyme, 5-enolpyruvyl shikimate 3-phosphate synthase. Protection can be achieved by using a glyphosate-insensitive form of the enzyme. Glufosinate (also called phosphothrinocin) inhibits glutamine synthetase, an enzyme used for nitrogen assimilation. Resistance to this herbicide is through the expression of phosphothrinocin acetyl transferase, which detoxifies the herbicide. Bromoxynil inhibits electron transport by binding to chloroplastic D1 protein. In this case, cotton plants are protected by expressing bromoxynil nitrilase, an enzyme that detoxifies the herbicide. Herbicide-resistant cotton has allowed farmers to dramatically alter the manner in which they control weeds. Rather than having to rely on pre-emergent herbicides or use only herbicides with minimal risk of crop injury, herbicide resistance allows farmers to apply the herbicides post-emergence, providing effective weed control without damage to the cotton plant. These products have increased farmer income, provided greater flexibility and allowed for the adoption of favorable practices such as reduced tillage (Culpepper and York 1998; Kalaitzandonakes and Suntornpithug 2001; Gianessi et al. 2002; Brookes and Barfoot 2006, 2008).

Weed control will be an ongoing need in cotton. Cotton is in development that will be resistant to additional herbicides. These include resistance to auxin herbicides like 2,4-D and dicamba, as well as resistance to herbicides that inhibit

4-hydroxyphenylpyruvate dioxygenase (CaJacob et al. 2004). Cotton varieties with multiple herbicide-resistance traits will be employed to fit into diverse production systems. These multiple herbicide-resistance products will provide the farmer with greater flexibility and aid in controlling difficult to control species or weeds that have developed resistance to a particular herbicide.

5.1.3 Disease and Nematode Pathogens

Disease and nematode pathogens of cotton can negatively impact yield and cause severe losses. Major fungal and bacterial pathogens include seedling diseases (e.g. *Rhizoctonia solani, Pythium ultimum, Thielaviopsis basicola, Fusarium oxysporum* and *Fusarium solani,* and other *Pythium*, and *Fusarium* species), fungal wilt diseases (e.g. Verticilium and Fusarium wilt), root rots (e.g. *Phymatotrichum omnivorum, T. basicola*, and Pythium species), and foliar diseases (e.g. *Xanthomonas campestris* pv. *Malvacearum)* (Bell 1999). The most important nematode pathogens are *Meloidogyne incognita* (root-knot nematode) and *Rotylenchulus reniformis* (common reniform nematode) (Robinson 1999). While the importance of these pathogens is well recognized, efforts to develop biotechnology products to protect against these pathogens have not yet yielded commercial products. Breeding for resistance against these pathogens is a major focus of breeding programs around the globe. There has been some recent progress on biotechnology solutions but these are still early in development.

Production of anti-fungal peptides and proteins in crop plants has been one strategy to protect crop plants from fungal infections. Emani et al. (2003) expressed an endochitinase gene from *Trichoderma virens* and demonstrated enhanced fungal resistance in cotton. Chitinases have been employed as anti-fungal proteins as they can degrade the chitin commonly found in the cell walls of fungi. In the Emani study (2003) the endochitinase was successfully expressed in cotton and then tested for resistance against a seedling pathogen (*R. solani)* and a foliar pathogen (*Alternaria alternata)*. For *R. solani*, 67% of the transformed seedlings survived after 2 weeks while 98% of control seedlings died. For the foliar resistance, a reduction in necrotic symptoms was demonstrated. The gene encoding an anti-fungal protein from the traditional Chinese medicinal herb *Gastrodia elata* has also been expressed in cotton and analyzed for resistance to cotton wilt (Wang et al. 2004). Transgenic cotton plants expressing the gene did show an increased level of resistance to *Verticilium dahlia* that was stable to the R_3 generation although the levels of resistance did vary among the lines (Wang et al. 2004). Attempts have also been made to use glucose oxidase to reduce *V.dahliae* infections in cotton but the enzyme had severe phytotoxic effects (Murray et al. 1999). A synthetic anti-microbial peptide has been employed to demonstrate resistance against a major root rot pathogen. Rajasekaran et al. (2005) expressed a gene encoding the synthetic anti-microbial peptide D4E1 and demonstrated they could reduce disease symptoms and increase seedling weight compared to controls in plant assays with

the fungal pathogen *T. basicola*. Other investigators are attempting to use gene-silencing as a means of controlling pathogens. RNAi based gene-silencing has evolved into a powerful tool with multiple potential applications. Huang et al. (2006) used the technology to silence the parasitism gene 16D10 in root knot nematode. The investigators demonstrated effective silencing of the gene and a resultant reduction in nematode infectivity with suppression of nematode development in *Arabidopsis thaliana*. While all of these approaches are still early in testing, it is encouraging that some progress is being made to discover and develop biotechnology solutions to address fungal and nematode pathogens of cotton.

5.1.4 Abiotic Stresses

Abiotic stresses can also significantly impact cotton yields. Example of abiotic stresses include water, salt, heat, and cold stress. Both transgenic and modern breeding techniques are being explored to address these challenges. Both technologies will be important in producing potentially successful products. From a biotechnology perspective, there are many studies being conducted to identify genes of interest that could become potential commercialization candidates. A few examples are presented here. Yan et al. (2004) overexpressed *Arabidopsis* 14-3-3 protein GF14λ (a cellular regulatory protein) in cotton and tested the effect under water-deficit conditions. Cotton plants expressing this gene had a "stay-green" phenotype and wilted less than non-transgenic control plants under water stress conditions. He et al. (2005) have looked at engineering salt tolerance by increasing sodium uptake into vacuoles. They expressed the *Arabidopsis* vacuolar sodium/proton antiporter gene *AtNHX1* in cotton and reported higher salt tolerance as evidenced by increased biomass and fiber yield. Another area of active research is exploring how hydrogen peroxide scavenging and the antioxidant defense system may be employed to protect cotton leaves subject to low temperature photoinhibition. Genes encoding a number of enzymes related to these mechanisms have been expressed in cotton to attempt to elucidate these effects (Payton et al. 2001; Kornyeyev et al. 2001, 2003a, b; Logan et al. 2003; Light et al. 2005) but effective long-term protection to stress tolerance has not been demonstrated. Efforts to identify genes that will convey stress tolerance in cotton are in their early stages but there is much work ongoing in this area in both the public and private sector. Hopefully, there will soon be reports of significant enough progress to result in potential products entering a commercial development phase.

5.1.5 Quality Traits

On the quality side, cotton with higher fiber quality or improved cottonseed and oil characteristics are potential areas for biotechnology improvements (Wakelyn et al. 2004; Rathore et al. 2008).

Cotton is the single most important natural fiber, making up 35%–40% of the world fiber usage. Cotton is primarily grown for fiber whether it is for yield and/or quality. The cotton fiber is a single differentiated epidermal cell that can be defined as a plant hair or trichome. Twenty-five percent of the epidermal cells on a cotton seed's surface differentiate and elongate into a cotton fiber (Bowman et al. 2001). Fiber development has been divided into four processes: differentiating/initiation, elongation/expansion, secondary cell wall synthesis, and maturation (Basra and Malik 1984; Wilkins and Jernstedt 1999). Efforts have been made targeting biotechnology and genetic mechanisms that regulate or control fiber development, and elongation that enhances fiber yield quality.

Fiber quality characteristics that are currently measured include fineness, strength, length, fiber length uniformity, elongation, fiber maturity, color, and reflectance. The High Volume Instrument or HVI is commonly used to estimate these values. Micronaire, resistance of the cotton fiber to air flow, is the measure used by the HVI to estimate fineness. HVI measurements of fiber characteristics are used to determine the quality of the cotton fiber and the price paid per pound or kilogram. Targeting improvement or modification to these traits through biotechnology has been a major point of discussion among private and public cotton improvement programs. Biotechnology solutions to such properties as dye binding, wrinkle resistance, shrinkage resistance, vapor absorption, and other yet to be determined fiber characteristics' could be implemented to improve, diversify, and/or create new opportunities for the cotton fiber.

Biotechnology and genomic solutions have been aimed at identification and modification of fiber genes, modification of growth hormones levels, identification and manipulation of promoters and gene regulatory regions, and the modification or addition of metabolic pathways (John 1999). There has been considerable work on identifying EST's that are found during the different phases of fiber development. A detailed discussion and review of fiber genes, their regulation, expression patterns, and effects on fiber development is provided by Wilkins and Jernstedt (1999). Several thousands of genes are expressed in the fiber cells but they are also expressed in other organs and tissues of the plant. This area of research is very complex and will continue to develop during the next decade (John 1999).

Attempts to modify metabolic pathways have led to limited success. In the best example, John (1999) describes the development of transgenic cotton plants that produce and target poly-D(-)-3-hydroxybutrate (PHB) to the cotton fiber lumen. This work demonstrated that the synthesis of novel materials could be engineered. The quantities of PHB expressed by these plants were very small but did have an effect on some of the key fiber qualities (John 1999).

Cottonseed oil improvement is another area of emphasis for quality traits. One example is the work to develop nutritionally improved high oleic and high stearic cottonseed oils through gene silencing techniques (Liu et al. 2002). In this work, RNAi-mediated gene silencing was employed to reduce linoleic acid content while increasing oleic acid levels by sixfold or stearic acid levels by as much as 20-fold. Cottonseed as a protein source has the potential for greater use but has been underutilized because of the presence of gossypol within seed glands. Glandless

cotton varieties exist but have limitations because of increased vulnerability of such varieties to pathogen and insect attack. Recently, however, innovative work has been reported where tissue specific reduction of gossypol in the seed has been achieved, thus opening up the possibility of greater utilization of cottonseed as a nutritional source (Sunilkumar et al. 2006).

5.2 Breeding Technology

Population growth, climate change, and increasing fiber demand dictate that the rates of production gains accelerate without plateau. Current rates of genetic gain for lint yield under normal plant densities range from 7.1 to 8.7 kg ha^{-1} yr^{-1} (Schwartz and Smith 2008). The majority of this genetic gain has arisen from conventional breeding, selection, and biotechnology (Rathore et al. 2008). Conventional breeding cannot sustain maximum levels of genetic gain without building upon supporting technologies. Breeding technology is a key component of commercial R&D, bridging biotechnology and commercial breeding. Breeding technology is defined as supporting technologies such as high throughput DNA marker analysis, biometric support, biotech trait integration, and plant pathology (Eathington et al. 2007). Careful integration of these technologies can help cotton breeders realize the goal of direct genotypic selection rather than depend solely on phenotype-based selection. DNA markers can identify useful alleles on a global scale and be utilized in molecular breeding to accumulate favorable alleles in elite germplasm.

Breeding technology is based on the discovery of DNA markers and implementation of high throughput genotyping assays. The upland cotton genome (AADD tetraploid, $2n = 4x = 52$) is large and complex with an estimated DNA content of approximately 2,400 Mbp (1C) or 5.09 pg (2C) (Hendrix and Stewart 2005). Genome sequencing of the tetraploid AADD genome has been proposed but is not currently underway (Chen et al. 2007). Without extensive genome sequence information, cotton DNA marker discovery has focused on isolating DNA microsatellites or simple sequence repeats (SSRs) from random genomic clones, EST collections, or BAC-end sequence collections (Blenda et al. 2006).Over 9,000 SSRs are available in CMD database (http://www.cottonmarker.org/) and CottonDB (http://cottondb.org/). The next generation of DNA markers will be single nucleotide polymorphisms (SNP) based on rapid detection of single bp changes or insertion/deletions (indels) (Rafalski 2002). The transition to high throughput SNPs will be revolutionary in cotton because of the power to detect groups of physically adjacent SNPs in linkage disequilibrium. These groups of SNPs are referred to as a haplotype. Haplotype diversity can more accurately detect allele diversity in cotton than an individual SSR marker. As was recently demonstrated in *Eucalyptus spp*., SNP discovery in complex polyploid genomes is no longer intractable (Novaes et al. 2008).

Adequate genetic diversity is essential for breeding technologies to be successfully implemented. Large numbers of DNA markers reveal that the genetic distance between any two typical elite cotton inbred cultivars is less than 0.2. Figure 5.3 reveals a principle component analysis of genetic distances, estimated from approximately 400 DNA markers, of typical elite commercial upland germplasm relative to public global germplasm pool. The limited DNA marker diversity in elite commercial material is evident. The mean genetic distance in public germplasm pools is approximately 0.3. The challenge of breeding technology is to expand the diversity of the elite commercial germplasm pool by utilizing global germplasm collections.

Breeding methodology may need to be modified to fully leverage molecular breeding and DNA markers. One such innovation is marker assisted recurrent selection (MARS) where diverse biparental populations are created, progeny phenotyped, and genotyped. The data are utilized to build a MARS model to accumulate favorable marker alleles by subsequent cycles of recurrent selection (Eathington et al. 2007).The advantage of this strategy is to achieve genetic gain from markers in elite commercial germplasm and accumulate haplotype and trait association data across diverse populations for future predictive breeding programs. Genetic gains from early cotton MARS experiments have exceeded conventional pedigree selection from 2% to 25%. This compares lines derived from MARS to traditional phenotypic selection

Marker assisted selection (MAS) is becoming more feasible in cotton, especially for traits that are expensive and difficult to screen phenotypically. A good example of such a trait in cotton is resistance to the root knot nematode (*Meloidogyne incognito*) where resistance is quantified by destructive sampling of root galling or egg counts of progeny that must be grown under RKN infested conditions (Zhang

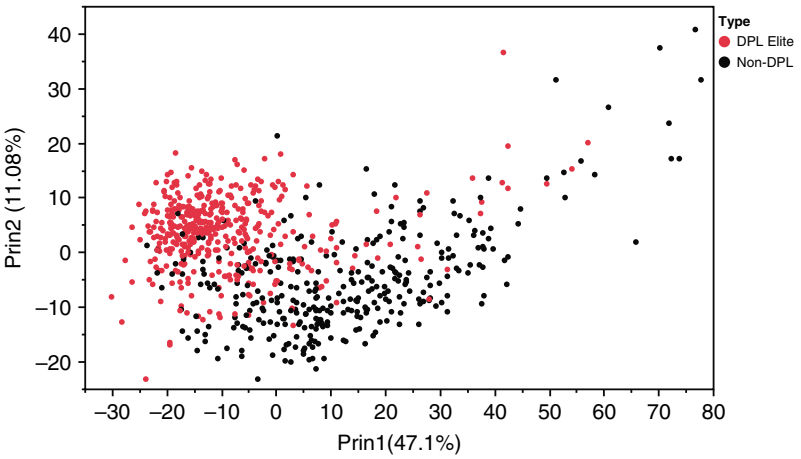

Fig. 5.3 Genetic diversity based on principle component analysis of DNA markers of elite commercial breeding lines (O) compared to global public cotton germplasm collection (X)

et al. 2006). DNA markers have been discovered that are linked to resistance to RKN (Niu et al. 2007; Wang et al. 2008). In addition to cost savings, MAS is a powerful tool to disrupt negative associations that may cause yield drag from certain resistant genetic sources. MAS can be conducted on segregating populations independent of the growing environment and most importantly, RKN infection is not required.

5.3 Advances in Yield

Cotton yields have increased significantly over the past several decades, averaging 8 kg ha^{-1} or 2% increase per year over the past 30 years on a global basis (ICAC 2007). Conventional breeding programs play a critical role in increasing yields (see Rathore et al. 2008 and Schwartz and Smith 2008 for discussions on genetic gains). Along with traditional breeding, novel breeding technologies will play an increasingly important role in driving yield gains (as discussed above). Improved agronomic practices have also contributed to yield gains. Examples of such practices are the use of plant growth regulators and the implementation of the boll weevil eradication program in the United States. More recently, adoption of biotechnology has led to an acceleration of yield gains. Yields increased dramatically over the first 10 years of biotechnology in cotton. Average global yields in 1996/1997 (the first year of biotechnology products) were 575 kg ha^{-1} but by 2006/2007 were well over 700 kg ha^{-1} with biotechnology being a major contributor to this unprecedented gain in yield (ICAC 2007, 2008). In the US, yield gains since 1996 have been dramatic, coincident with increased adoption of biotechnology (Fig. 5.4). On a trait basis, insect protected cotton increased yields in the U.S. by 9%–11% and in China by 8%–10% (Brookes and Barfoot 2006). Yield gains in India have also been dramatic in the years coinciding with the launch and increased adoption of Bt cotton. It took 15 years (1982–1997) for national yields in India to climb from 200 to 300 kg ha^{-1} but yields have climbed from 300 kg ha^{-1} to well over 500 kg ha^{-1} in just 6 years since the introduction of Bt cotton in 2002 (Gruere et al. 2008; ICAC 2007, 2008; Cotton Corporation of India 2009). Population growth, climate change, and increasing fiber demand dictate that cotton production gains need to continue to accelerate over the coming decades. Ideally, these gains will be achievable with reduced inputs per unit produced and bring about continuing benefits to farmers, neutral to farm size (Monsanto 2009).

5.4 Conclusions

Worldwide cotton production is changing. Today's cotton grower faces rising energy and nitrogen costs, increased competition for water, less available labor, and a highly competitive marketplace. Recent biotechnology trait introductions, new varieties, and production plans have significantly contributed to improved efficiencies and yields around the world. Dependence on current technology

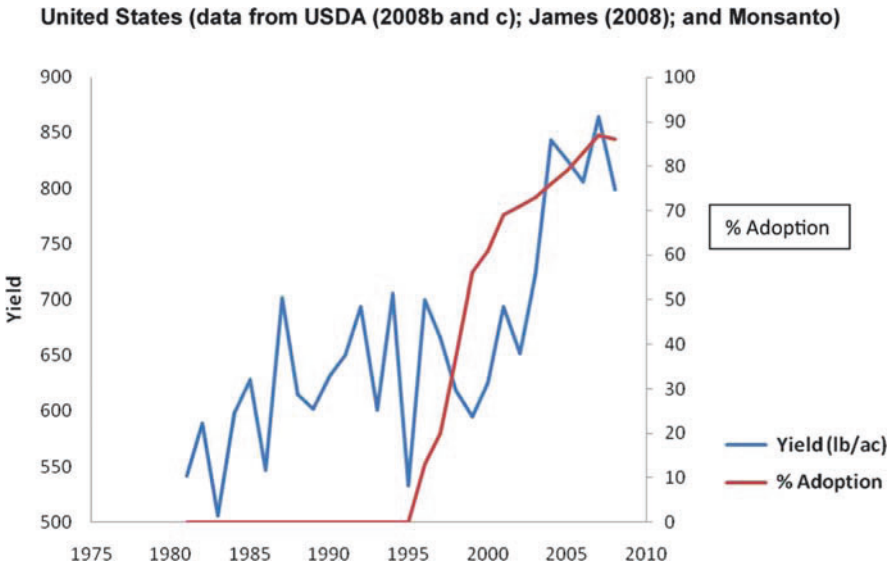

Fig. 5.4 Average cotton yields and adoption rates of biotechnology in the United States

introduced within the last decade will not provide the cotton industry the security and improvements required by a changing world and environment. It is essential that new and improved products be developed to protect cotton production through insect and weed resistance development in current cotton production systems.

Shifts in the US of cotton-growing regions to more diversified agriculture such as corn and soybeans, driven by global demand, will continue to readjust prior conceptions of cotton production. This diversification will eventually benefit growers with crop rotation schemes to help productivity and reduce dependence on a single crop. Flexibility and simplicity in cotton production to adjust to rapidly changing economic demands will become essential for grower profitability. Production in other world areas continues to grow, and technology is a major contributor. The productivity gains in India through the adoption of insect-protected cotton are an excellent example of how technology adoption can greatly improve productivity. A review of several studies of the impact of Bt cotton in India concluded that Bt cotton significantly lowers bollworm infestation, reduces insecticide sprays, and provides higher yields, thus resulting in an economic advantage for Bt cotton (APCoAB 2006).

The world population continues to grow. Agriculture has a responsibility to continue to provide the world's food, feed, materials, and clothing. It is essential that we get more produced from our available acres and resources. We must continue to increase our productivity and efficiency. Biotechnology is already playing a key role in providing valuable products to increase productivity in cotton. A number of innovative products are in development to expand the base of current traits and provide new traits of value to the industry. The future health of the cotton

industry is linked to investment in improvements in existing technology and the development of these new products. Governments, growers, and the agricultural industry continuing to respect patent and Plant Variety Protection (PVP) laws already in place will help to provide the incentive to invest in cotton research and the development of new traits and varieties. The future of the cotton industry will be bright and secure as the products in the cotton research and development pipeline reach the marketplace, providing value to stakeholders throughout the industry.

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Chapter 6
Insect Tolerant Cotton in India

S. Parimi, B.R. Char, R.K. Goravale, and C.B. Chaporkar

Cotton is a major agricultural and industrial crop that provides employment and income for hundreds of millions of people involved in its production, processing and marketing in more than 60 countries. The genus *Gossypium* consists of 50 wild and cultivated species. *G. hirsutum* is widely cultivated in the world followed by *G. barbadense*. Both the species are allotetraploids with AADD genomes and belong to the New World. A detailed description of cotton genomes is given in the chapter written by Dr. Khadi.

In India, cotton is grown under rain fed, as well as irrigated conditions. There are mainly three cotton producing zones in India

- North Zone includes Punjab, Haryana, the northern part of Rajasthan and part of Uttar Pradesh (*hirsutum* and *arboreum* zone)
- Central Zone includes Maharashtra, Gujarat, Madhya Pradesh and the southern part of Rajasthan (*hirsutum*, *arboreum, herbaceum* and partly *barbadense*)
- South Zone includes Andhra Pradesh, Tamil Nadu and Karnataka (*hirsutum*, *barbadense, arboreum* and *herbaceum*)

However, the productivity of cotton in India is low when compared to that in other cotton growing countries of the world despite the fact that India has the largest cotton growing area. The cotton crop also provides edible oil and seed byproducts for livestock. Cotton has an impact on our lives beyond recognition, and biotechnology has the potential to further improve the growth and quality of cotton. The cotton plant is a perennial tree but has been domesticated for fiber requirement.

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6.1 Insect Pests of Cotton

The limitations to cotton production in India include the damage caused by insect pests, and diseases and weeds, insect pests being a major constraint. Morphological and physiological attributes such as presence of nectaries, flowers which are heavy in pollen, and succulent plants with rich nutrients attract insects to feed on plants, or even to take up residence/shelter in/on its tissues. In an initial comprehensive attempt, as many as 109 species of insect and mite pests were recorded in India (Nangpal 1948), the number rising to 130 with the intensification of cotton cultivation (Dastur et al. 1960) prior to the introduction of hybrids, and the last report by Panwar (1995) reported that a total of 166 insect and mite species attack cotton crops. Records of the American serpentine leaf miner, *Liriomyza trifolii* (Burgess) (Srinivasan et al. 1995), the spiraling whitefly, *Aleurodicus dispersus* Russel, (Mani et al. 2001), the mealy bug, *Phenacoccus* spp. from Punjab (http://www.ncipm.org.in/), and the Mirid bug, *Creontiodes biseratense* (Distant) from Karnataka (Patil et al. 2006) are the new additions to the earlier report of Panwar (1995). The important lepidopteran insect pests of cotton in India include the American bollworm, (*Helicoverpa armigera* Hübner), the Pink bollworm (*Pectinophora gossypiella* Saunders), the spotted bollworm (*Earias vittella* Fb.), and the spiny bollworm (*Earias insulana* Boisd) (Table 6.1). The Tobacco caterpillar, *Spodoptera litura* Fb, also a lepidopteran insect pest, often causes serious damage to cotton.

During the 1960s, the incidence and the extent of losses caused by insects, as well as diseases were quite low. Consequent to the intensification of cultivation, the diversity of high yielding varieties/hybrids, and the increased use of manures and chemical fertilizers, there was an enormous increase in the spectrum, as well as intensity of pest damage. A survey during the 1950s revealed that the percent yield losses due to insect pest attack varied from 10%–17%, going up to 25%–35% during bad years (Sethi 1963). To the contrary, a survey conducted during the 1990s revealed that the yield loss in cotton had increased to a greater extent and was estimated to be around 50% because of insect pests alone (Dhaliwal and Arora 1996).

The Pink bollworm and the leaf hopper, *Amrasca biguttula biguttulla* Ishida were major pests till the 1970s. With the introduction of American cottons (*G. hirsutum* and *G. barbadense*) and hybrids, plant protection became an important part of cotton cultivation, as these varieties/hybrids were highly prone to insect pest and disease attacks (Joshi 1997). Interspecific hybrids involving *barbadense* parentage were more prone to pest damage compared to intraspecific hybrids. The cost of pest management in hybrid cottons rose to almost 40%–50% of the cost of cultivation. Among various insect pests, the American bollworm, (*H. armigera*), is the most dominant and destructive. It is equally devastating on legumes, tomato and several other crops. The annual losses caused by this pest alone were estimated to be about US $300 million (Rs. 1,200 crores) (King 1994). *H. armigera* took a major toll of cotton in the northern zone (Haryana and Punjab) during 1983–1984 and in southern state of Andhra Pradesh during 1987–1988 where nearly 66% yield

Table 6.1 Major insect pests of cotton in India

Sl No.	Common name	Scientific name
1	Leafhopper (Jassid)	*Amrasca biguttula biguttula* (Ishida)
2	Whitefly	*Bemisia tabaci* Gennadius
3	Thrips	*Thrips tabaci* Lind.
4	Tobacco caterpillar	*Spodptera litura* Fb
5	Old world (American) bollworm	*Helicoverpa armigera* Hübner
6	Spotted bollworm	*Earias vittella* Fb.
7	Pink bollworm	*Pectinophora gossypiella* Saunders
8	Termites	*Odontotermes obesus* Holmgren
9	Aphid	*Aphis gossypii* Glover
10	Spiny bollworm	*Earias insulana* Boisd
11	Stem weevil	*Pempherulus affinis* (Faust)
12	Shoot weevil	*Alcidodes affaber* Fb.
13	Mealy bug	*Phenacoccus* spp.
14	Mirid bug	*Creontiodes biseratense* (Distant)

loss was reported. Whitefly, hitherto a pest of minor importance in cotton assumed greater importance in the southern states (Andhra Pradesh, Tamilnadu and Karnataka) and Maharashtra during 1987–1988 (Panwar 1995).

Chemical control has been the most popular and often the only approach to suppress these insect pests. In fact, no other crop receives as much insecticides as cotton. Even though the cotton area in India amounted to a mere 5% of the gross cropped area, the amount of pesticide usage was as high as nearly 50% of the total pesticide consumption of the country, which was worth US $600 million (Rs. 2,800 crores) (Ghosh 2006). Insecticides of different classes and modes of action are being deployed for the management of bollworm complex (*H. armigera, P. gossypiella, Earias* spp.), *S. litura* and leafhoppers, followed by whiteflies and aphids to a lesser extent. The most commonly used insecticides included monocrotophos (22% of cotton insecticide market) followed by endosulfan, chlorpyriphos, cypermethrin, fenvalerate and acephate.

The heavy and indiscriminate use of all categories of insecticides leads to the development of resistance in pests to most of the commonly used insecticides in the country. The cotton plant protection entered a disastrous phase of pest management with the development of insecticide resistance in the most destructive pest, *H. armigera*, to DDT, endosulfan, organophosphates, carbamates and synthetic pyrethroids (Mc Caffery et al. 1989, Armes et al. 1996, Kranthi et al. 2001, 2002). Deployment of synthetic pyrethroids for the bollworm management, specifically, monocrotophos resistant *H. armigera* lead to the outbreak of whitefly, *Bemisia tabaci* Gennadius. Cotton farmers opted for insecticide applications as prophylactic measures to avert crop damage, which increased the costs of crop protection (10–16 sprays for bollworm management) and production besides environmental pollution and health hazards. In addition, the programs on breeding hybrids for resistance to destructive bollworm pest(s) did not yield any success.

In view of increasing public awareness about the undesirable side effects of chemical pesticides, emphasis is placed on “Integrated Pest Management” (IPM)

where non-chemical crop management practices are used in coordination with selective insecticides for effective insect pest control. The available information on the biology, behavior, habitat, alternate/collateral hosts and insecticide resistance in bollworm complex helped in designing and disseminating IPM strategies through the Technology Mission on Cotton and All India Coordinated Cotton Improvement Project of ICAR in collaboration with ICAR Institutes, State Agricultural Universities, Krishi Vignan Kendras besides many private and non-governmental organizations. The IPM practices included seed treatment for sucking pest management, growing marigold and/or okra as trap crop(s) (*H. armigera,* sucking pests); use of pheromone, light and sticky traps for insect monitoring and decision making based on thresholds; release of egg parasitoid, *Trichogramma chilonis* Ishi; predator, *Chrysoperla carnea* Stephens; use of microbial pesticides such as *Bacillus thuringiensis* based formulations and nuclear polyhedrosis virus (NPV) for *H. armigera*; neem and other plant derived insecticides; need based application of selective insecticides upon survey and surveillance of pests and natural enemies; rotation of insecticide molecules etc. According to Sundaramurthy and Basu (1993); Jayaswal and Sundaramurthy (1992), the IPM package in cotton reduced the number of insecticide applications and amount of insecticide active ingredient.

The cotton IPM technology was accepted by the farming community till these inputs and technical know-how were supplied to them by the government/institutional agencies. However, there were a number of limitations such as the timely availability of quality inputs/components like *Trichogramma* egg cards, NPV; lack of community approach; awareness and implementation among farmers, awareness on insect scouting and monitoring, environmental and ecosystem limitations such as high temperature; etc.

6.2 Development of Bt Cotton in India

As an effective and environmentally superior approach to control the bollworm complex and certain defoliators, a genetically modified cotton plant was developed that is protected from damage by many of the lepidopteran insect pests of cotton. This cotton plant, hereafter referred to as Bt cotton, produces an insect control protein Cry1Ac derived from the naturally occurring soil bacterium, *Bacillus thuringiensis* subsp. *kurstaki* (*B.t.k.*). Producing the Cry1Ac protein in the cotton plant itself provides effective season-long protection of the cotton plant against lepidopteran insect pests (Wilson et al. 1994). Microbial formulations of *B. thuringiensis* that contain the insecticidal proteins have been registered in numerous countries worldwide, including India, and have been safely used for control of lepidopteran insect pests for more than 30 years (Luthy et al. 1982). The Cry1Ac protein produced in Bt cotton is nearly identical in structure and activity to that found in nature and in commercial *B.t.k.* microbial formulations. *B. thuringiensis* and *B.t.k.* microbial formulations have been shown to be very specific to the target

insect pests, and do not have any deleterious effects to non-target organisms such as beneficial insects, birds, fish, and mammals, including humans (EPA 1988).

In India, first Bt cotton was introduced by a joint effort between Maharashtra Hybrid Seeds Company Ltd. (Mahyco), Mumbai and Monsanto Company, St. Louis. Work on the development of Bt cotton started in 1995. In 2002, Bt cotton (Bollgard®) was approved for release in India. Subsequently, in 2006, Bt cotton expressing two Bt genes, (Bollgard II®, *cry1Ac* and *cry2Ab* genes) was approved for release in the country. Two other events of Bt cotton have been approved for cultivation in the country, *cry1Ab* + *cry1A* gene (GFM Event) by Nath Seeds Ltd., India, and *cry1Ac* gene (Event 1) by JK Agri Genetics, India.

6.2.1 Adoption of Bollgard II® Cotton

Due to improved yield and effective control of bollworms, adoption of Bollgard II®, second generation insect tolerant technology has increased fourfold since its commercial launch in 2006. As per an estimate, approximately 4 million farmers cultivated Bollgard® and Bollgard II® cotton on 17.2 m acres equivalent to 76% of India's total 22.5 m acres cotton in the season of 2008. The acreage has steadily increased from 8.7 m acres in 2006 to 14.4 m acres in 2007. The farmers have a choice from over 250 Bollgard® and Bollgard II® cotton hybrid seeds within diverse genetic background, fiber quality and staple length sold by more than 25 companies. Bollgard II® acreage has increased to 4.5 m acres during the ongoing crop season (2008) as compared to 1.22 m acres during the last season (2007). Similarly, Bollgard® continued to be widely adopted on 12.7 m acres. The adoption of Bollgard II® is increasing as farmers realize that apart from protecting against bollworm attacks, Bollgard II® also gives additional protection against *S. litura* and Cotton semilooper. Within 6 years of the launch of Bollgard® cotton in 2002, India's cotton production has doubled, making it the second largest producer, and second largest exporter of cotton in the world.

6.2.2 Chronology of Bt Cotton Development

The approval for commercial release of three Bollgard® cotton hybrids viz., MECH-12 Bt, MECH-162 Bt and MECH-184 Bt, was given by the Genetic Engineering Approval Committee (GEAC) in March 2002. The sequence of events that lead to the release of Bt cotton hybrids from different events over the period of past 15 years is shown in Table 6.2.

The various regulatory authorities involved in the application approval process include Department of Biotechnology (DBT), Genetic Engineering Approval Committee (GEAC), Review Committee for Genetic Modification (RCGM, constituted by DBT) and MEC = Monitoring and Evaluation Committee (constituted by GEAC and RCGM).

Table 6.2 Year wise summary of regulatory processes leading to commercial release of Bt cotton hybrids in India

Years	Studies taken
1995–1996	Application and permit for importation of Bt cotton seed containing *cry1Ac* gene
1996–2000	Greenhouse breeding for integration of the c*ry1Ac* gene in to Indian germplasm, seed purification, and stock increase
1996–2000	Limited field trials to study the potential of pollen escape, aggressiveness and persistence of *cry1Ac* gene
1998–2000	Biochemical and toxicology studies
1998–2001	Multilocation field trials: agronomic and entomology performance of first generation Bt cotton hybrids, conducted by Mahyco and State Agril Universities.
2000–2001	Soil rhizosphere evaluations and protein expression analyzes from multilocation trials
2000	I. Application and permit for importation of Bt cotton seed containing *cry1Ac* and *cry2Ab* genes II. Greenhouse breeding for integration of the c*ry1Ac* + *cry2Ab* genes in to Indian germplasm, seed purification, and stock increase
2001	Advanced stage multilocational field trials of first generation Bt cotton hybrids, conducted by Indian council of Agricultural research
2002	I. Submission of final Biosafety, environmental safety, gene efficacy and performance documentation II. Commercial release of first-generation Bt cotton hybrids in South and Central zones of India III. Limited field trials for potential of pollen escape of *cry1Ac* + *cry2Ab* genes
2005	Commercial release of first-generation Bt cotton (Bollgard®) hybrids in North zone
2006	I. Commercial release of Bollgard II® technology based cotton hybrids in Central and South zones II. Commercial release of Bt cotton hybrids with *cry1Ab* + *cry1A* (GFM event) genes in all three zones (North, Central and South) III. Commercial release of Bt cotton hybrids with *cry1Ac* (Event 1) gene in all three zones (North, Central and South)
2007	Commercial release of Bollgard II® technology based cotton hybrids in North zone

6.3 Biosafety Assessment of Bt Cotton in India

As a genetically-modified crop, the deregulation of Bt cotton required environmental clearance under the rules and procedures notified by the Ministry of Environment and Forests, Government of India in 1989. The equivalence of Bt cotton to non-Bt cotton varieties prevalent in India in composition and agronomic performance was required to be demonstrated. Further, it was important to show that the Bt protein expressed, causes no adverse effect when consumed by domestic or wild animals and beneficial insects. The bio-safety assessment included environmental safety assessment and food and feed safety assessment. Environmental safety considerations include phenotypic characteristics of the crop, plant behavior, gene transfer to near relatives, and the effect on non-target

organisms. Food and feed safety studies encompass analysis of the expressed protein as well as a broad range of animal model studies for toxicity, allergenicity and unintended effects.

6.3.1 Environmental, Food and Feed Safety Assessment

The potential environmental effects of Bt cotton have been carefully evaluated before commercialization in India. A review of all available information including extensive field test results, safety studies and independent scientific research indicates that the commercial use of this cotton will not result in any adverse effects to the environment. The environmental safety was established by demonstrating:

- Lack of plant pest or weediness traits in the field
- No potential for gene transfer to other plant species
- Low levels of gene expression, low environmental exposure to expressed proteins
- Safety of Cry1Ac protein to non-target organisms (insects, birds, fish, mammals)
- Rapid degradation of the Cry1Ac protein in the soil

The food and feed safety of Bt Cotton in India is being evaluated on the principles/concept of "substantial equivalence" in which the composition of the Bt cotton, is compared to an appropriate counterpart that has an accepted standard of safety (WHO 1991, FAO 1996). The assessment process for substantial equivalence involves evaluation of agronomic and compositional parameters of the genetically modified crop and its traditional counterpart, taking into account the limits of natural variation; and characterization and evaluation for the potential to cause harm to human health, animal health or the environment. The parameters tested were protein, oil, ash, carbohydrate, moisture, gossypol, fatty acid profile.

The human safety assessment of *Bt* protein (Cry1Ac/Cry2Ab) and NPTII protein was done through protein characterization studies, establishing digestive fate in simulated gastric and intestinal fluids, and testing acute oral toxicity in mice. The feed safety assessment was made through allergenicity and toxicity studies done in different animal model systems. Additional discussion on studies carried out is presented in the Chapter by Dr. KK Tripathi.

6.4 Insect Resistance Management in Bt Cotton

Bt crops provide season long in-built protection against specific target insect pests and offer protection that is very effective and selective. Such an effective pest control technology raises a concern of possibility of resistance evolution in target

insect pests to the Bt protein(s), which needs to be addressed for the sustainability of the Bt crop(s). Most widely used, viable and effective resistance management approach is the use of high dose of Bt protein with a refuge, which is acceptable to the farmers, technology providers and regulators (Roush 1997, Gould 1998). This strategy should be used in conjunction with other management methods generally followed in an IPM module/package and effective resistance monitoring programs; besides developing new products.

Insect resistance management (IRM) is a proactive management package for delaying the development of resistance to Bt protein(s). The components of the IRM strategy are aimed at minimizing the frequency of resistance in the pest population of individuals carrying resistance genes to Bt protein by minimizing the exposure of target insect pest(s) to the Bt proteins, providing a population of susceptible individuals that can mate with any resistant individuals and thereby dilute any potential resistance evolution. IRM strategy to be deployed in a region is specific to the region, crop and target insect; and is based on information related to cropping patterns, regional/local agricultural practices, target insect pest biology, farmer preferences and the possibility of integration into existing management tactics (http://www. gmsciencedebate.org.uk/topics/forum/pdf/0076a.pdf).

6.4.1 *Insect Resistance Management strategy in India*

The key components of the any IRM strategy currently deployed in Bt crops is planting of a "refuge," either a structured or an unstructured refuge, for producing Bt susceptible moths, resistance monitoring programs, refuge compliance and technology adoption. The conditional approval of the first three Bt cotton hybrids in India was given by GEAC in their 32nd meeting held on March 26, 2002 (http://www. environ.nic.in/divisions/csurv/geac/bgnote.html) with the following refuge recommendation – "*Every field where Bt cotton is planted shall be fully surrounded by a belt of land called 'refuge' in which the same non-Bt cotton variety shall be sown. The size of the refuge belt should be at least five rows of non-Bt cotton or shall be 20% of total sown area whichever is more*" (Fig. 6.1). In order to facilitate this, the technology provider (company) packs the required amount of Bt and non-Bt seed in two individual packets in a box (pack in a box) with information on pest management, sowing information, agronomic management etc. The technology provider was also asked to monitor the susceptibility of bollworm populations collected from fields. The non-Bt cotton variety was mandated to be the Bt variety/hybrid's counterpart in 2002 which was later changed and approved to as "any non-Bt variety/hybrid of same species, maturity and fiber length as that of Bt variety/hybrid" or "a non-Bt popular hybrid," as approved in the 71st Meeting of the (GEAC) held on 11.10.2006 (http://www.envfor.nic.in/divisions/csurv/geac/geac-71.pdf).

Bollgard II® hybrids were introduced into India in 2006 with a view to manage any resistance evolution and also to provide the farmers a better choice of cotton

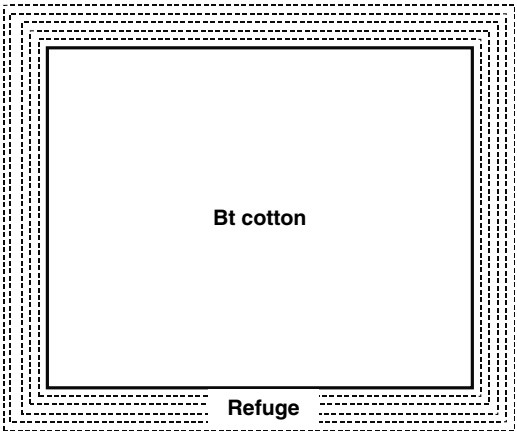

Fig. 6.1 Pictorial representation of mandated refuge design and placement in Bt cotton in India

hybrids. The refuge recommendation was mandatory and remained the same as followed for Bollgard® hybrids since 2002. Currently, single gene Bollgard® hybrids and second generation Bollgard II® hybrids are grown in all cotton growing regions of India.

As the Bt cotton hybrids' area increases, alternate hosts as natural refuge play an important role in providing a susceptible pool of moths for mating with survivors from Bt cotton areas. In India, *H. armigera*, *E. vittella* and *S. litura* have a number of alternate host plants like chickpea, pigeon pea, tomato, sunflower, maize, sorghum, chili, castor, soybean, okra besides non-Bt cotton, which support enough populations of bollworms, serving as natural refuge. (Manjunath 2005; Ravi et al. 2005; Vijaykumar et al. 2007) reported that most of these crops supported *H. armigera* populations during the cotton growing season and so, could be effective natural refuges for Bt cotton. Extensive studies are underway to further validate this data and provide the regulators in the country for consideration of a natural refuge in Bollgard II® cotton. A natural refuge will allow planting of Bt cotton in the mandated 20% refuge area and henceforth helps in net increase in production.

Additionally, planting of Bt cotton in India is followed by planting of either wheat, pigeon pea, or vegetables such as okra, tomato and chili. The temporal rotation of these crops with Bt cotton provide source of "off-season habitats" for pests such as *H. armigera*, *E. vittella* and *S. litura*; and aid in dilution of resistance. Vegetables such as tomato, okra, eggplant, chili, gram (black gram, green gram), sunflower, and soybean are grown in South India after the cotton crop is harvested. In the state of Gujarat, which has the highest Bt cotton area, peanut, potato, maize and tobacco, are grown besides other host crops. Pigeon pea, castor, Gram (black gram and green gram) and peanut are grown in irrigated and rain fed situations in Maharashtra and Madhya Pradesh. Keeping in view the cropping systems, a natural refuge is a viable strategy for resistance management in India.

6.4.2 Bt Cotton Adoption and Grower Compliance

The adoption rate of Bt cotton hybrids and compliance for refuge planting among the farmers influence the success and sustainability of the *Bt* crops. The technology provider or the company, in India, provides the refuge seed with the Bt seed in separate packets in a box (pack in a box). The literature related to agronomy and pest management is provided with the seed packets. The grower has to plant the seed appropriately following the resistance management plan mandated by the regulatory authority (GEAC).

The adoption rate of Bt cotton hybrids is unprecedented and increased from a meager less than 5% in the year of introduction (2002) to approximately 67% in 2007, which is expected to cross 80% of total cotton acreage in 2008–2009. This increase was also observed in the number of hybrids planted and number of farmers who cultivate Bt cotton. In 2002, there were three hybrids (MECH12, MECH162 and MECH 184) in the market and in 2008, the estimated number of Bt cotton hybrids available in the Indian market is more than 250 from >25 companies with four events.

Grower compliance for refuge planting varies and may be encouraged through focused education programs, rigorous monitoring and appropriate rewards for compliance. Considering the refuge compliance and faster adoption rates it is imperative that a strong resistance monitoring program is essential to monitor and report any shifts in the susceptibilities of target insect populations.

6.4.3 Resistance Monitoring Program

Resistance monitoring program requires baseline susceptibility of each of the target pest species to the relevant Bt protein present in the transgenic crop as part of the overall IRM strategy. The baseline susceptibility information will help in the estimation of diagnostic concentration or dose for resistance monitoring (Sims et al. 1995). Diagnostic dose ensures 99% or more mortality of susceptible insects in a population. Diagnostic monitoring is an efficient method to screen a large number of populations within a given time. Resistance monitoring program aims at collecting field populations annually from regions of maximum product sales and estimate the changes in the susceptibilities.

The susceptibility of old world bollworm, *H. armigera*, to the Cry1Ac protein demonstrated 67-fold variability, with the highest mean lethal concentration (LC_{50}) being 0.67 μg/mL (Kranthi et al. 2001). In another study by Jalali et al. (2004), the baseline-susceptibility of Indian populations of *H. armigera* to the insecticidal protein Cry1Ac, was determined in 1999 and 2001. Populations were collected from cotton fields of nine major cotton growing states in India, (Punjab, Haryana, Rajasthan, Madhya Pradesh, Gujarat, Maharashtra, Andhra Pradesh, Karnataka and Tamilnadu. All populations were susceptible to Cry1Ac in 7 days bioassays and the

LC_{50} demonstrated five- to sixfold variability and MIC_{50} (molt inhibitory concentration) ranged from 0.05 to 0.27 μg/mL of Cry1Ac protein. These baseline values are being used as benchmarks for monitoring resistance to Cry1Ac in *H. armigera* populations. The Cry1Ac susceptibility in *H. armigera* demonstrated 15-fold and 29-fold differences in LC_{50} and MIC_{50} values, respectively, among 21 populations collected from cotton and vegetable growing regions of India. Similarly, Cry1Ac was found to be highly toxic to the spotted bollworm *E. vittella* (Kranti et al. 2004). Baseline susceptibility data demonstrated that the variability in the LC_{50} values was 27-fold. Shukla et al. (2005) reported a 5-fold (LC_{50} values ranged between 0.006–0.0031 μg/mL) interpopulation variation in the *E. vittella* susceptibility to the Cry1Ac protein indicated by mortality, whereas there was tenfold variation in growth inhibition.

The resistance monitoring reports from 2002–2003 to 2007–2008 submitted by Central Institute of Cotton Research, Nagpur, India (2003–2008) report that the results of Cry1Ac susceptibility monitoring done over the past 6 years did not indicate any onset of resistance in the bollworm populations. The determination of baseline susceptibility of bollworms and *S. litura* to the Cry2Ab protein is in progress. The geographical variability in *Helicoverpa armigera* susceptibility levels to Cry2Ab protein expressed by transgenic maize was determined by Kranthi et al. (2009) through bioassays conducted on populations collected across India, prior to Bollgard II® commercial release. The LC_{50} values ranged from 6.0 to 28.6 μg Cry2Ab/mL of diet and the susceptibility data of 2007–2008 did not show any significant changes 2 years after cultivation of Bollgard II® in India.

6.4.4 Bt Cotton – an Integral Part of Cotton IPM Programs

The crop protection systems in cotton was classified by Smith (1969) into subsistence phase – crop is grown as a part of subsistence agriculture, exploitation phase – where new varieties are planted in new markets by using insecticides for pest management, crisis phase – where the continuous use of insecticides resulted in pest resistance, pest resurgence, secondary pests leading to increased control costs, disaster phase – where cotton production was no longer a viable option for markets and finally, integrated control phase. The integrated control in cotton was aimed at optimizing the available control methods and not maximizing them, which brought in the concept of "pest management." Stern et al. (1959) at the University of California described the concept of "integrated control" in their landmark publication. Besides documenting the principles of ecological balance in agriculture, population control by natural enemies and the use of artificial control mechanisms, they put forth the theory of "Economic Injury Level" where control costs should be justified as a sound financial practice. In simplest terms, IPM is an effective and environmentally sensitive approach to pest management that relies on a combination of common-sense practices (http://www.epa.gov/pesticides/factsheets/ipm.htm). IPM programs use the available pest biology information and pest control

methods to manage pest damage by the most economical means, and with the least possible hazard to people, property, and the environment.

The introduction of *Bt* crops caused a paradigm shift in cotton pest management programs. *Bt* cotton is effective against specific caterpillars (bollworm complex and *S. litura*) which reduced the broad spectrum insecticide use in cotton ecosystems due to which biological control organisms benefited significantly (Romeis et al. 2008). Insecticide use in cotton targeted against the bollworm complex was 50% of the total volume of the insecticides used in agriculture (Fitt 2008) and similar trends were observed in India (Gujar et al. 2000). Deployment of *Bt* varieties/hybrids caused a global reduction of 19% in the insecticide active ingredient (a.i.) applied (Brookes and Barfoot 2006). The reduction in insecticide applications was 42%–70% in the early years of commercialization in India (Qaim and Ziberman 2003). However, there was variation in the reduction of insecticide use among different states as reported by Raney (2006). In a study conducted by Bennett et al. (2004) in 9,000 farmer's fields, the reduction in insecticide sprays for bollworms was reduced by approximately 62%–76%. However, in both the studies there was no change in the number of sprays applied for sucking insect pest management. Reduction in the insecticide sprays during reproductive stage of *Bt* cotton, has been associated with resurgence of minor pests such as mirid bug (*C. biseratense*) in Karnataka during 2007 season, mealy bug (*Phenacoccus* spp.) during 2006 and 2007 and whiteflies in the northern cotton growing regions, and other sucking insect pests such as thrips and leafhoppers. It is likely that the reduction in the earlier use of broad spectrum insecticides may be the reason for this resurgence. During the 2008 cropping season, no major incidence of any of the above resurgent pests or new pests was reported in India.

The reduction in insecticide use caused an increase in beneficial insect activity. The enhanced survival of naturalist predators and parasitoids in *Bt* cotton crops than in conventional sprayed cottons and the absence of negative effects of Bt protein on non target Lepidoptera and beneficial insects was reported by several authors (Naranjo et al. 2005; Marvier et al. 2007; Romeis et al. 2006; Fitt and Wilson 2002; Wu and Guo 2003). There were no studies reported from India where concerted efforts were put to study the changes, if any, related to predators or parasitoids. However, our observations during the past couple of years revealed 4%–34% larval parasitism in target insects such as pink bollworm in populations collected from North and central India. The availability of selective insecticides for bollworm in refuge cotton (spinosad, indoxacarb, emamectin benzoate, chlorfenapyr) and sucking pest management (neonicotinoids, chlorfenapyr), also assist in conserving and enhancing beneficial insect populations. The use of these selective insecticides in Indian *Bt* cotton system, is not based on any decision support systems or economic threshold levels. The threshold levels being followed in *Bt* cotton hybrids in India are the same that were earlier used in conventional cottons. Growers spray these insecticides whenever necessary or when they observe insect populations in the fields, compromising the life of the available selective chemistries. This was one of the reasons for the resurgence of whiteflies and subsequently cotton leaf curl disease in North India during the earlier years of *Bt* cotton

commercialization. Later on, *Bt* cotton hybrids with tolerance or resistance to whiteflies were developed specifically for North India. In fact, GEAC mandated that varieties/hybrids for North Indian cotton region should be tolerant or resistant to cotton leaf curl disease. This emphasizes the necessity of incorporating host plant resistance (HPR) traits into commercial *Bt* cotton hybrids wherever necessary. Introgression of *Bt* genes into the sucking pest tolerant germplasm is the way forward for *Bt* cotton hybrids to effectively fit into an IPM program.

6.5 General Scheme of Transgenic Breeding of Bt Cotton

The best stable and promising inbred lines with transgenic trait (here the trait is *cry1Ac* or *cry1Ac* + *cry2Ab* genes) are selected for conversion. The cross is made between the recurrent parental line and donor parent. The progeny of the cross i.e., F_1 is grown and is backcrossed with the recurrent parent at least 3–4 times. Afterwards the pedigree method is followed and stable generation for transgenic trait as well as morphological trait is obtained. During each step transgenic trait is tracked using molecular and/or biochemical markers. The stability of selected progeny is confirmed and treated as nucleus seed source. The average time required to obtain nucleus seed source of a particular line is a minimum of 7–8 years.

The nucleus seed is further used to produce breeder's seed, which in turn is used to produce foundation seed at every stage of parental seed multiplication. The genetic purity achieved at various stages of seed production follow the Indian minimum seed certification standards existing for cotton.

6.6 Quality Assurance in Bt Cotton

Seed being a primary input in agriculture, its quality is of prime importance in attaining high degree of crop performance. The Bt cotton seed quality testing is done as per Internationally accepted procedures to evaluate the seed quality attributes in seed lots such as physical purity, genetic purity, gene (trait) purity, viability, vigor, germination ability, freeness from pests, disease and weed seeds which determines the planting value of the seed and subsequent crop performance.

The performance of the product depends on the genuineness/genetic purity of the seed supplied and this is tested by conducting grow out test (GOT) where in seed lots are evaluated for its unique morphological characters. The genuineness of the seed lot is examined by raising the crop with the required plant standing in replicates. The plots may be of any convenient size that will provide enough plants for the determination with high degree of accuracy. The spacing and crop management is carried out as required by the crop species under test to allow the development of the trait specified for the product. Observations are made by comparing individual plants against designated morphological characters specified for the

product at an appropriate crop stage. The results are expressed as a percentage of true to type as well as other species, other varieties or any aberrant found.

The success of Bollgard cottons is based on the trait purity in the product. The tools available for determining the level of trait purity in Bt cotton in India have been applied at breeding stages and before the commercial sale of F1 hybrid seed. At breeding stages, polymerase chain reaction (PCR) diagnostic tests are used to determine the purity of foundation seed lots. These PCR tests are designed to be event-specific, in that one primer resides in sequence flanking the point of insertion of the T-DNA. If available, a number of other cotton event PCR tests are also carried out with the expectation that the results would be negative, to establish purity for the event of interest. The trait purity standard for foundation seed lots is at the highest possible level (i.e., as close to 100% as possible) since this forms the core source for the commercial seed material to follow.

PCR is also used extensively at earlier breeding and backcross stages to establish purity during the selection process, and also to differentiate between heterozygotes and homozygotes at each generation. This latter test is achieved by identifying flanking sequence on both sides of the point of insertion. A 3-primer PCR is designed with flanking sequence primers and an internal T-DNA primer, such that in chromosomes where the insert is present, an amplicon of one size is obtained, whereas from a chromosome with no insert, a distinct amplicon is obtained from the flanking primers. Thus, heterozygotes for the T-DNA insertion would allow amplification of both PCR products, while homozygous positive and null plants would give single amplicons. These strategies have been routinely used in breeding programs for the development of new hybrids in Bt cotton.

At commercial seed testing stages, a testing approach using protein expression as a criterion of selection has been utilized. The most accurate and cost-effective method is ELISA (Enzyme-Linked Immuno-Sorbent Assay) which is carried out in multi-well plates with which high-throughput levels can be achieved. The ELISA method uses antigen-antibody specificity to enable detection of the protein of interest in crude plant extracts. Trait purity standards can be set at the desired confidence level and statistical sampling from seed lots carried out in an appropriate manner. The ELISA can be adapted to a quantitative format for estimating trait protein levels *in planta*. This has been widely used in India for Bt cotton regulatory submissions. A second type of immuno-test that has been in use is the protein-detection "strip," a lateral flow device which can give a qualitative result in field conditions. The test can be completed in 10–15 min with a basic field kit.

6.7 Conclusions and Future

Bt cotton (Bollgard® and Bollgard II®) technologies have enabled farmers to produce improved quality cotton with fewer sprays thereby reducing the cost of production and risk of exposure to toxic insecticides. Technologies such as

Roundup Ready flex (RRF) cotton, which reduce the cost of labor for weeding operations has already entered the regulatory testing system in India and is likely to be made available to farmers by 2011. Going forward, the farming community will be looking at breeders and technology providers for developing elite second generation Bollgard® hybrids with resistance or tolerance to sucking pests and disease complex. Proteins such as plant lectins and bacterial toxins, with novel modes of action against sucking pests and/or lepidoteran caterpillars, are being investigated in research programs in the private and public sector in India. Utilization of molecular markers (marker assisted breeding) for introgression of desirable traits from wild species will be another approach to overcome some of the biotic and abiotic stresses such as drought and sucking pests. Improvements are also expected in fiber properties in order to match the current spinning machines. Mechanized farming in cotton which includes all the basic operations such as sowing, spraying, and picking will be the requirement in coming years and breeding programs need to focus on these aspects to meet present day market requirements.

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Chapter 7
Insect Resistance Management for Transgenic Bt Cotton

G. Head and T. Dennehy

7.1 Introduction

The first cotton varieties with insect resistance traits introduced through biotechnological methods were commercialized in 1996 in the USA (Mendelsohn et al. 2003; James 2006). These products contained lepidopteran-active insecticidal proteins derived from the bacterium *Bacillus thuringiensis* (Bt) and were targeted at bollworms, like the tobacco budworm *Heliothis virescens* and the cotton bollworm *Helicoverpa zea*, that are difficult to control using conventional insecticides. Subsequently, a suite of comparable products containing other Bt proteins have been introduced. Increasingly now, cotton farmers are purchasing varieties with combinations of these insect resistance traits and herbicide-tolerance traits for improved weed control (James 2006; Brookes and Barfoot 2007). Bt cotton products represent a huge success story in agriculture, with unprecedented levels of adoption in the USA and around the globe, significant documented economic benefits to farmers, and substantially realized environmental benefits. Furthermore, the future will bring stacked trait products built upon a base of lepidopteran and weed control but with additional traits related to environmental stress tolerance and yield enhancement.

These prospects are exciting, but they also highlight the need to ensure that Bt cotton technologies are sustained in the marketplace. The primary concern in this respect is the potential evolution of Bt resistance in the target insect pests. Anticipating this concern, biotechnology companies have been working with academic scientists and regulators to design and implement proactive insect resistance management (IRM) programs (EPA 1998) for Bt cotton products. It is a testament to

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these programs that no instances of field-failures of Bt cotton related to resistance have occurred globally, despite a decade and more of high adoption by farmers. In this chapter, we will describe the nature of these programs, and their evolution, and discuss the reasons for their success.

7.2 The Nature of Current Commercial Bt Cotton Products

Thus far, the transgenic insect-protected cotton products that have been released commercially have all contained insecticidal proteins derived from Bt and all have been targeted at lepidopteran pests of cotton. These products are summarized in Table 7.1. All of these products have come from the private sector. The first commercial Bt cotton product, and still the most widely used product, contained a Cry1Ac protein and was marketed as Bollgard® cotton by Monsanto. It was first approved for use in 1996 in the USA, and has since been released commercially in Mexico, Colombia, Argentina, Brazil, Australia, China, India and South Africa. Monsanto subsequently released a second generation Bt cotton product called Bollgard® II cotton that contains two Bt proteins: Cry1Ac and Cry2Ab2. This product has begun to replace Bollgard cotton and is being grown commercially in the USA, Australia, India and Burkina Faso. In addition, Dow has released a product containing a Cry1Ac and a Cry1F protein that is marketed as WideStrike® cotton. As of 2009, it has been released for commercial use only in the USA but activity is underway to introduce it into other countries. One other product also will soon be available for commercial use in the USA; Syngenta has produced a product containing a Cry1Ab and a VIP3A protein (the latter protein is Bt-derived but is produced at a different point in the life cycle of that bacterium than Cry proteins), and this product has recently received regulatory approvals for commercial use.

Other Bt cotton products have been produced in China through a variety of Chinese companies, some linked to government research laboratories. Therefore these products are all functionally similar to Bollgard cotton. All of these products contain Cry1Ac variants of some sort, in one case combined with the cowpea trypsin inhibitor. Attempts have been made to introduce some of these products into India.

Table 7.1 Commonly-used commercial Bt cotton products

Commercial name	Event name and company	Insecticidal Bt protein/s
Bollgard	MON 531/Monsanto	Cry1Ac
Bollgard II	MON 15985/Monsanto	Cry1Ac and Cry2Ab2
WideStrike	TC1507/Dow	Cry1Ac and Cry1F
VipCot	COT102 × COT67B/ Syngenta	Cry1Ab and VIP3Aa

7.3 The Basics of IRM for Bt Cotton Technologies

The rate at which insect resistance may evolve to Bt cotton will be determined by the same factors that affect resistance evolution to conventional insecticides. These factors can be divided into: (1) the nature of the product, its performance, and how it is used (pattern of Bt expression in the crop plant and penetration of the product into the market); (2) the genetics of insect resistance (initial frequency of the resistant allele, degree of dominance of that allele, and fitness costs of resistance); and (3) aspects of insect behavior that mediate how the product affects the target insects (insect movement and mating). Examination of these factors, together with the IRM programs discussed in following sections, helps to explain the slow rate of resistance evolution to Bt cotton products over the past decade.

7.3.1 Product Performance

The pattern of Bt protein expression in a Bt cotton product, and particularly the magnitude and consistency of that expression, will affect the rate of resistance evolution. The preferred expression pattern is a season long, and the level sufficiently high to be able to control target insects that are heterozygous for any resistance genes (= "high dose"; EPA 1998), and this has been achieved in most current commercial Bt cotton products. By delivering a dose that kills all or nearly all of the susceptible heterozygous insect pests, resistance is made functionally recessive and slow to evolve. In addition, only a few resistance mechanisms will be sufficient to enable survival of target pests on these products, meaning that the frequency of resistant alleles relevant to Bt cotton will be very low.

7.3.2 Genetics of Insect Resistance

The degree of dominance of a resistant allele will be determined by the nature of the resistance allele itself and the efficacy of the product, and higher dominance will tend to lead to more rapid resistance evolution. In many but not all cases of Bt resistance studied so far, resistance is partially to completely recessive (Tabashnik et al. 2003). The only published cases of dominant or incompletely dominant resistance led to low levels of resistance (e.g., Huang et al. 1999), meaning that such alleles would not be sufficient to allow survival on a high expressing Bt product. The failure of these resistant colonies to survive on Bt crops has been confirmed (e.g., Li et al. 2007). These results suggest that resistance to Bt cotton likely will be inherited as a partially recessive trait. Furthermore, the high dose

nature of most Bt cotton products increases the likelihood that resistance alleles are functionally recessive (see above). Work on the fitness of Bt-resistant insect colonies derived from cases of field resistance, and from laboratory selection experiments designed to create colonies that might survive on Bt cotton, also indicates high fitness costs of resistance to Bt cotton (Gassmann et al. 2009), which tend to slow the rate of resistance evolution.

7.3.3 Insect Behavior

The way in which insects move between Bt and non-Bt plants determines insect exposure to the Bt toxin (Hoy et al. 1998). Both larval interplant movement, and longer range adult movement, have important effects on resistance evolution, by affecting the selection pressure for resistance, the likelihood that resistant individuals will mate with susceptible insects, and the rate at which resistance may spread after arising. For example, studies of the biology of the major cotton pests such as heliothines indicate that they tend to disperse relatively long distances (e.g., EPA 1998). This makes it likely that pockets of resistant insects will be diluted out by susceptible immigrants.

7.4 Components of IRM Programs for Bt Cotton

Wherever Bt cotton products have been commercialized, they have been released with an associated IRM program. These IRM programs for Bt cotton employ multiple components and reflect the input of academic, industrial and regulatory experts. Standard components of these programs include:

1. Consistently high levels of expression of Bt protein in all important cotton tissues
2. Refuge for susceptible target pest insects
3. Monitoring and remedial action plans
4. Use of alternative control measures (placement into an IPM context)
5. Development of subsequent products with different insecticidal mechanisms

The concept of creating a refuge for susceptible insects is an IRM strategy that is unique to Bt crops. The refuge is a source of large numbers of susceptible target insects that is located close enough to the Bt cotton field that any resistant insects emerging from the Bt cotton are likely to mate with susceptible insects from the refuge. The value of this approach has been demonstrated through mathematical modeling (e.g., Roush 1994, 1998) and limited field experiments (Shelton et al. 2000). Typically the refuge is an area of non-Bt cotton that must be planted by the farmer within a certain distance of the Bt cotton field. However,

Table 7.2 Minimum refuge size requirements for Bt cotton products in various countries, expressed as a percentage of the total cotton area except in the case of Australia

Country	Refuge options
USA[a]	Bollgard II/WideStrike: no structured refuge required for heliothine pests
	Bollgard: 5% unsprayed non-Bt cotton, 20% sprayed non-Bt cotton
Australia[b]	Bollgard II: 100% sprayed non-Bt cotton, 10% unsprayed non-Bt, 5% unsprayed pigeonpea, 15% sorghum, or 20% corn
Brazil	Bollgard: 20% sprayed non-Bt cotton
China	Bollgard: no structured refuge required

[a]In the USA, no structured refuge is required for Bollgard II and WideStrike cotton in the cotton-growing area from west Texas to the east coast because heliothines are the key target pests throughout this region. In the southwestern USA, the pink bollworm – a non-heliothine pest – is a key target and required different management practices

[b]For Australia, refuge options are expressed as a percentage of Bollgard II area

in some cases, the refuge may consist of non-cotton crops planted by the farmer that are suitable hosts for the target pests. Table 7.2 describes the refuge requirements in place for Bt cotton products in various countries. Suitable refuge sizes and designs will vary depending on pest biology, agronomics and many other factors. For example, the placement of the refuge must anticipate the level of larval and adult movement; where adult movement is greater, the Bt cotton crop and refuge can be separated, while still allowing interbreeding between insects emerging from the two areas.

Resistance monitoring is another important element of the IRM programs that are in place for Bt cotton products. Resistance monitoring involves regularly collecting populations of target pests from areas deemed as having a high potential for resistance evolution, testing the susceptibility of these insects to the Bt proteins present in the Bt cotton products being grown, and comparing the measured susceptibility to historical measures of susceptibility prior to the introduction of Bt cotton (Sivasupramaniam et al. 2007). Resistance monitoring provides early warning of resistance evolution and therefore insights as to the effectiveness of IRM programs. Care must be taken to include relevant controls in the testing and to relate the results back to the performance of the products in question. That is, variation in susceptibility measured in the laboratory is not a demonstration of field-relevant resistance; the laboratory measurements must be linked to changes in product performance in the field. When such a linkage is not evident, interpretation of monitoring results will be difficult and will lead to a variety of conclusions. For example, claims have been made, based on laboratory assays of susceptibility, that cotton bollworm have evolved resistance to Cry1Ac in the USA (Tabashnik et al. 2008), but these claims have been disputed on the grounds that appropriate controls were not used in the assays and no change in product performance was observed in the field (Moar et al. 2008). Regardless, the reality is that the marketplace in the USA has moved away from Bt cotton containing only Cry1Ac to products with multiple Bt genes, which will have effectively addressed any Cry1Ac resistance that may have been present (see Sect. 1.6).

7.5 Adapting IRM Practices to Local Needs

All of the scientific and economic elements important in designing IRM strategies vary considerably among crops and regions, so IRM strategies have to vary accordingly. Differences in target pest biology, agricultural practices, and product performance across regions will affect optimization of IRM strategies. This means that the size and design of refuges should vary geographically, as should educational approaches and implementation tactics. The differences among countries, and among products within countries, in refuge requirements for Bt cotton products reflect this variation (see Table 7.2). For example, the agronomic practices of growers in a particular crop will shape the IRM needs and constrain the IRM strategies that can be used. This is true for both gross differences and at subtler levels. Extreme differences exist, for example, between the way in which cotton is grown in the USA versus India and China, with far smaller average farm sizes and more diverse cropping systems in the latter countries. In the USA, with large, frequently contiguous, areas of Bt cotton, the risk of resistance is relatively high compared to the Asian cases and the use of structured refuges becomes more important. At the same time, coordinating structured refuges may not even be possible in the case of many small farms and millions of individual growers, hence the absence of structured refuge requirement for Bt cotton in China (see also Sect. 1.7). Similarly, in India there are approximately 200 million ha of farmed land with over 14 major crops each accounting for at least 1 million ha, and many others also accounting for substantial areas (Indian Department of Agriculture statistics: http://agricoop.nic.in/Agristatistics.htm). In any one region, many crops are grown, and most crops are grown in multiple climatic zones with varying agronomic practices. Most Indian cotton farms are small in size, typically less than 2 ha in size. Clearly any IRM approach must take into account both the large number of farmers involved in this case and the differences in their circumstances.

7.6 The Role of Pyramided Bt Cotton Products in IRM

As described in Sect. 1.2, there is a steady trend toward replacing the first generation of Bt cotton products containing a single Bt protein with products containing two Bt proteins (so-called pyramided products). The primary reason for introducing pyramided products is the IRM value they bring. Combining insecticidal proteins for target pest control is a strategy that can dramatically delay the evolution of resistance if the target insects are not able to develop a single mechanism of resistance that confers tolerance to both proteins simultaneously, and empirical studies with biotech crops have confirmed the value of this strategy. For example, transgenic broccoli engineered to produce Cry1C has been shown to control diamondback moth that is resistant to Cry1Ac (Cao et al. 1999; Zhao et al. 2003). Theoretical and empirical studies have demonstrated, however, that the strategy of

pyramiding multiple Bt proteins in a single product against the same insects pests is most effective if certain conditions apply. Mathematical modeling by Tabashnik (1989), Roush (1994, 1998) and Caprio (1998) predicted that transgenic plants expressing two Bt proteins could effectively delay resistance relative to plants producing either of the single proteins if:

1. Cross-resistance between the Bt proteins is low
2. The mortality of susceptible insects caused by each of the individual proteins is high

These models compared alternating insecticidal products, sequential use of the same products, and combining (pyramiding) the proteins. For transgenic crops, the most effective strategy was to pyramid insecticidal proteins within a single product. For example, with a 5% refuge, combining Bt proteins in a single product can delay resistance approximately eight times longer than deploying the same proteins sequentially (Roush 1998, Fig. 7.1). Alternatively, a pyramided product with a 5% refuge can more effectively delay resistance than a single-protein product with a 20% refuge. This means that a pyramided product potentially can be released with lower refuge requirements than products with a single Bt protein (or products with multiple proteins that represent only a single efficacious mode of action).

To understand the value of any particular pyramided product for managing insect resistance, it is important to consider whether it fulfills the conditions described above. That is, it is important to demonstrate that: (1) the probability of

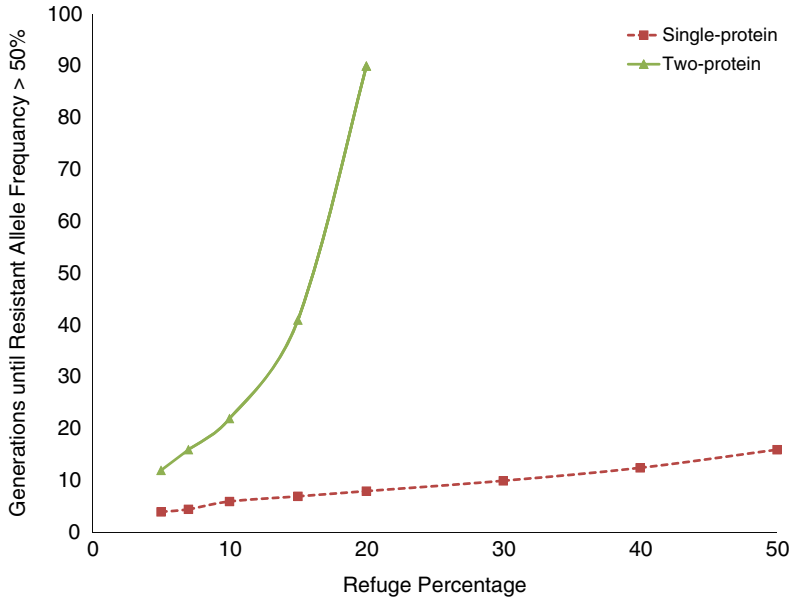

Fig. 7.1 Effect of refuge size on the rate of Bt resistance evolution to a product with two Bt proteins compared with a product with one Bt protein (derived from Fig. 7.2 in Roush 1998)

cross-resistance between the individual proteins is low; and (2) the level of the individual Bt proteins is sufficient to confer a high level of control of susceptible target pests. Consider the example of Bollgard II cotton. All available data indicates that the Cry1Ac and Cry2Ab2 proteins in Bollgard II cotton have very different insecticidal modes of action and therefore a very low likelihood of cross-resistance. For example, the Cry2Ab2 protein in Bollgard II cotton has been shown to control Cry1Ac-resistant strains of target pests (e.g., Tabashnik et al. 2002), while the Cry1Ac in Bollgard II effectively controls Cry2Ab-resistant target pests (e.g., Mahon et al. 2007). Furthermore, both of these proteins are highly active against key lepidopteran pests like tobacco budworm and pink bollworm (Greenplate et al. 2003). Therefore, Bollgard II cotton meets the desired criteria *Pectinophora gossypiella* for an effective pyramided product.

7.7 The Role of "Natural Refuge" in IRM

Highly polypagous target pests permit a slightly different approach to IRM for Bt cotton. If the target pests are utilizing a wide variety of host crops that are commonly grown in the vicinity of cotton, and they are not being controlled using Bt on these other hosts, then structured refuges for Bt cotton may not be necessary under these conditions. In these cases, both cropping practices and the degree of polyphagy of the target insect species will be important. Even in relatively homogeneous cropping areas like parts of the southern USA, alternative hosts can be an important source of refuge with respect to Bt cotton for highly polyphagous insects like the cotton bollworm (see Sect. 1.8). In countries like India where cropping systems are far more heterogeneous, alternative hosts will be more important still, particularly for polyphagous species like the cotton bollworm *Helicoverpa armigera*. Other than cotton, this species can be found on many of the major pulse crops, vegetables and tomatoes, and both dicotyledonous and monocotyledonous species. In particular, pulse crops like chickpea and pigeonpea, major hosts of this species, are preferred to cotton, and are present on comparable areas to cotton. Therefore, these alternative host crops will act as a "natural refuge" for Bt cotton in India (Ravi et al. 2005).

In general, for this approach to be realistic, four conditions should hold:

1. The target pest species must utilize multiple host plant species that overlap in both space and time.
2. The performance on the different host plant species must be comparable to allow the different alternative hosts to produce sufficient susceptible insects at the right time to interbreed with any resistant insects emerging from the Bt crop.
3. The distribution of these different host plant species must overlap at a sufficiently fine scale and consistently enough to act as a functional refuge in all relevant areas.
4. The pest insects must move freely between the different host plant species.

The natural refuge approach has been adopted in China, where alternative non-cotton hosts of the key target pest of Bt cotton, *H. armigera*, are abundant and support large pest populations throughout the growing season (Wu et al. 2002). Consequently, farmers growing Bollgard and other Bt cotton products in China are not required to plant structured non-Bt cotton refuges. These products have been grown on large areas for approximately a decade in China without resistance appearing in the field, suggesting that this approach can work (Wu et al. 2006). Based on the available evidence, a similar approach probably could be applied to Bollgard II cotton in large parts of Asia, Africa and Latin America. Agricultural systems in all of these regions are dominated by smallholders and are highly diverse in their cropping patterns. However, it should also be remembered that, if these alternative hosts are essential as a refuge for insects susceptible to Bt, then introducing similar Bt genes into these crops could threaten the durability of Bt cotton. This will be a challenge for companies and public institutions that are developing Bt crop technologies in the future, particularly in countries like India and China.

7.8 Combining Pyramided Products and Natural Refuge: Bollgard II Cotton in the USA

As described in Sect. 1.5, pyramided Bt cotton products potentially require smaller refuges to be sustainable than single Bt products. Where the targeted pests are relatively polyphagous (Sect. 1.6), it becomes particularly plausible that alternative non-cotton hosts may be a sufficient source of refuge to remove the need for planting non-Bt cotton refuges. In the last section, this possibility was discussed in the context of the diverse cropping systems of China and India. However, these concepts apply to polyphagous pests of Bt cotton in any region, particularly if those pests are highly mobile, so that different host crops can act as refuge for Bt cotton even when they are planted some distance away. In 2007, the US Environmental Protection Agency (EPA) – the regulatory agency charged with considering IRM issues for Bt crops in the USA – determined that a natural refuge approach would work for pyramided Bt cotton products (Bollgard II and WideStrike cotton) in the central and southeastern USA. They determined that the amount of natural refuge present for the two key target pests of Bt cotton in this region, cotton bollworm and tobacco budworm, is sufficient to preserve the durability of pyramided Bt cotton products. The underlying rationale was as follows:

1. A multi-state, 2-year study on cotton bollworm examined the distribution and abundance of its alternative hosts through aerial mapping, and the productivity of these hosts through surveys of larval populations and trapping of adult moths. The moths were analyzed for their host origin, using stable carbon isotope analysis, which distinguishes between moths from C3-type hosts, that could include cotton, and C4-type hosts that could not (Gould et al. 2002). The overall

conclusion of the study was that a substantial majority of adult cotton bollworm in all regions of the USA cotton belt originate from C4-type alternative hosts (particularly corn) and that a number of C3 alternative hosts (including soybean and peanuts) also are highly productive (Jackson et al. 2008; Head et al. 2009). Throughout the season and in all regions, a significant portion of the cotton bollworm adult population is derived from alternative, non-cotton hosts. These moths disperse large distances in search of suitable oviposition and feeding sites. Based on these results, the natural refuge for cotton bollworm in all generations and regions was found to be more than sufficient to ensure that pyramided products like Bollgard II cotton will provide durable control of cotton bollworm.

2. A comparable study for tobacco budworm in the eastern and central parts of the USA cotton belt (Orth et al. 2007) demonstrated that a significant portion of adult tobacco budworm populations in all regions of the USA cotton belt originates from alternative non-cotton hosts. Throughout the season and in all regions, the natural refuge present for tobacco budworm generally exceeded 5%. In the eastern states like Georgia and North Carolina, cotton appears to be a relatively minor contributor to tobacco budworm populations (Fig. 7.2). In the south-central states, the cotton contribution is higher but some amount of natural refuge exists in all portions of the USA cotton belt throughout the season.

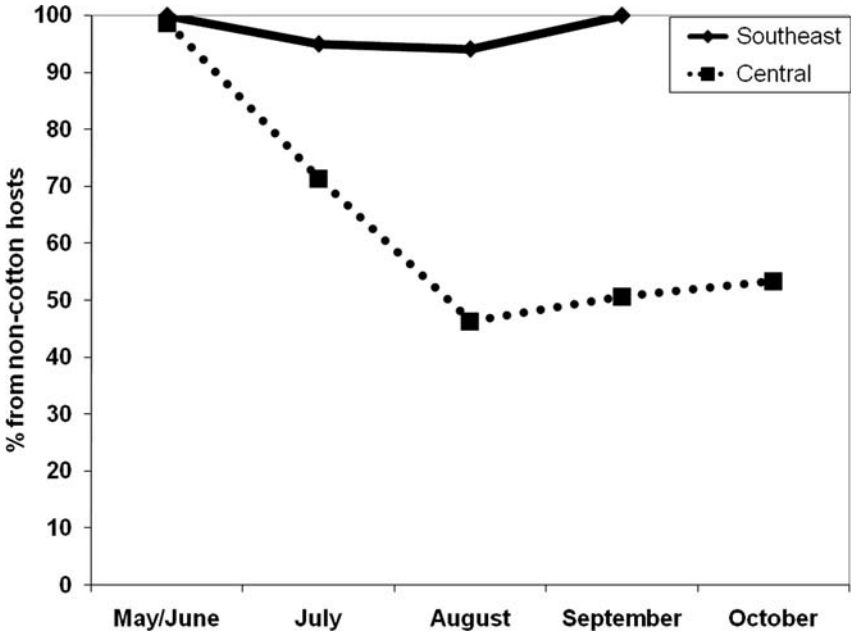

Fig. 7.2 Percentage of tobacco budworm derived from non-cotton hosts over the course of the cotton-growing season in two regions of the US Cotton Belt, based on gossypol assays (Orth et al. 2007) on moths collected in 2004

Mathematical modeling demonstrated that this amount of natural refuge could ensure the durability of pyramided Bt cotton products.

Using the existing natural refuge, in place of structured refuge options for Bollgard II and WideStrike cotton, has a number of benefits for farmers and society as a whole. Farmers can realize significant economic benefits by not having to plant a structured refuge. These economic benefits increase the rate of adoption of pyramided Bt cotton products in preference to single-Bt cotton, thereby reducing the selection for Bt resistance in target pests. The increased adoption of pyramided Bt cotton, and removing the need to plant and manage a structured refuge of non-Bt cotton, also reduces broad-spectrum insecticide use in cotton, resulting in reduced insecticide exposure and potential environmental and human health benefits. This reduced use of conventional insecticides in cotton reduces the risk of resistance evolving to these insecticides (particularly pyrethroid insecticides), preserving their utility for the control of lepidopteran pests in other crops.

7.9 Conclusions

IRM programs for Bt cotton must have a strong science-based framework because the underlying questions are scientific in nature; but scientific considerations must be balanced with an understanding of grower economics, and how these economics affect behavior. In addition, there must be a recognition that all of these factors vary among crops and across geographic regions, and that IRM plans must vary accordingly. Only this can achieve what all stakeholders in this technology want – a lasting, effective IRM strategy. Looking forward, it is heartening that no instances of field-failures attributable to resistance evolution to Bt cotton have yet been observed. However, in the future, the number of countries adopting Bt cotton will continue to increase, and the diversity of Bt cotton products will also increase. This will require (and facilitate) more diversity in IRM approaches. Overall, IRM for Bt crops has been a success story, but significant scientific and logistical challenges will continue to arise, demanding ongoing investment and innovation.

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Chapter 8
Opportunities for Engineering Abiotic Stress Tolerance in Cotton Plants

R.D. Allen

8.1 Introduction

Abiotic stresses, particularly water-deficit, salinity, and temperature extremes, are the primary factors limiting crop productivity, accounting for a reduction of more than 50% in yields worldwide (Boyer 1982). Approximately 22% of agricultural land is saline (Food, Agriculture Organization of the United Nations (FAO) 2004), areas affected by drought are expanding, and this trend is expected to increase (Burke et al. 2006). The rapid rate of world population growth, combined with a general increase in global prosperity and decrease in arable land, is creating increasing demands for food, fiber, biomaterials, and sustainable agriculture (Ragauskas et al. 2006). More than 80% of the available fresh water is consumed by agriculture (Delmer 2005). Drought is a perennial environmental constraint, affecting an estimated 25% of all crops worldwide at enormous cost. For example, West Texas dry-land cotton crops are decimated by drought on a regular basis. Estimates of the value-added worth of cotton with increased photosynthetic and water use efficiencies, enhanced flowering, and improved seed qualities exceed $200 million per year in Texas, $1 billion per year in the USA, and $5 billion per year globally (Wilkins et al. 2000). Ultimately, increasing food and fiber quality and quantity through biotechnology for improved stress tolerance and biomass production has the potential to impact the complex and interrelated issues of globalization, poverty, hunger, population growth, climate change, energy, biodiversity, and environmental degradation. The task of identifying gene functions and developing effective strategies to use these functions for crop improvement is huge, and much more knowledge is needed to achieve the promises of plant biotechnology.

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Drought induces a variety of plant responses, including changes in gene expression, accumulation of the phytohormone abscisic acid (ABA), production of osmotically active compounds, and synthesis of protective proteins that scavenge oxygen radicals or act as molecular chaperones (Wang et al. 2003). These responses are controlled by molecular networks that activate stress responsive mechanisms to re-establish homeostasis and to protect and repair damaged proteins and membranes (Ramachandra-Reddy et al. 2004). Abiotic stress responses are genetically complex and therefore, difficult to manipulate. Strategies for engineering abiotic stress tolerance in plants have relied primarily on the expression of genes that encode protective molecules, such as dehydrins, antioxidant enzymes or enzymes involved in the synthesis of functional and structural metabolites (see Park et al. 2004; Payton et al. 2001; Kornyeyev et al. 2001; Roxas et al. 2000 for examples). More recently, strategies to use genes that are involved in signaling and regulatory pathways for engineering for plant stress responses have been developed and show great promise (Umezawa et al. 2006).

The development of drought-tolerant crops has been hindered by our limited knowledge of the precise physiological parameters that reflect the genetic potential for improved productivity under water-limited and thermally stressful environments. The potential to identify key traits that limit yield under abiotic stress conditions hinges upon an understanding of the crop at the physiological and molecular levels. This indirect approach, combined with traditional empirical breeding strategies, will hasten yield improvement (Araus et al. 2002). Moreover, our understanding of physiological processes that result in crop yield is paramount for accurate identification and introgression of candidate genetic material for yield improvement. However, due to the lack of a complete understanding of the functions of the underlying genes, the introgression of quantitative trait loci (QTLs) involved in stress tolerance can also carry undesirable agronomic characteristics from the donor parents. Therefore, the development of transgenic plants by the introduction of selected genes provides a more focused approach to the development of plants with improved abiotic stress tolerance. In addition, use of transgenes allows for the transfer of genes from any source, including nonplant species. Transgenic technology also allows for the expression of the introduced gene to be precisely controlled both temporally and spatially. This capability can be critical if expression of a given gene is needed only at a specific developmental stage, in a specific organ or tissue, or in response to specific environmental conditions. Although promoters that are constitutively expressed at high levels are still widely used, they may not be appropriate for all transgenes. This is especially true for regulatory genes, which can sometimes have serious deleterious effects when constitutively expressed. Generation of transgenic plants that express gene cassettes controlled by stress-inducible promoters is now being tested. Therefore, transgenic modification provides a wide variety of options for the development of novel strategies for crop improvement.

Stress-responsive genes can be grouped into three general categories: (1) genes that encode enzymes or other proteins directly involved in stress acclimation, (2) genes that encode regulatory factors (protein or functional RNAs) involved in

controlling the expression of protective genes and, (3) genes of unknown function. Initial attempts to alter abiotic stress tolerance in transgenic plants focused on genes in the first group, which can be termed "single action genes" because they typically target specific metabolic pathways that are predicted to confer increased tolerance to stress. These include genes that encode water channel proteins, rate-limiting enzymes for biosynthetic pathways, detoxification enzymes, and transport proteins. A second "wave" of transgenic experiments has been initiated to evaluate "multi-functional genes', a second category of stress-induced genes, namely, regulatory proteins. Using these factors, many of the genes involved in abiotic stress responses can be regulated simultaneously by a transgene that encodes a single stress inducible transcription factor or other regulatory protein (Kasuga et al. 1999). This approach, which provides the potential to enhance tolerance to a variety of stresses or to combined stresses, depends on basic research to identify components of critical stress-responsive signal transduction pathways.

In addition to the technology used to generate transgenic plants that express their introduced genes in an appropriate way, it is also important to examine how these transgenic plants are evaluated to determine the effects of the introduced gene on stress tolerance characteristics. In most cases, transgenes have been tested only in model system plants, such as *Arabidopsis* or tobacco. While these "proof-of-concept" experiments can give important clues about the potential usefulness of specific genes in crop plants, such as cotton, in many cases the published work has depended on the assessment of transgenic plants under artificial environments that are unlikely to be faced by crops under field conditions. In addition, the physiological characterization in many of these studies does not extend beyond the evaluation of growth or survival under severe conditions. Therefore, a rigorous physiological evaluation of the tolerance of transgenic crop plants to abiotic stresses and the effects of specific transgenes on agronomic traits, such as yield and quality, are generally lacking. In this review, we briefly summarize recent progress in the application of transgenic technology for the improvement of abiotic stress tolerance in plants, with emphasis on drought, salinity and temperature stresses and, where possible, we outline the published and preliminary experiments in which transgenic modification of stress tolerance in cotton has been attempted.

8.2 Single-Action Genes

Initial attempts to improve plant stress tolerance using transgenic technology focused primarily on increased expression of protective proteins, including dehydrins, antioxidants, and production of osmolytes and neutral sugars. These types of genes can be called "single action genes", because they are usually targeted at the expression of a single gene product that is predicted to alter a specific biochemical pathway.

8.2.1 Antioxidants

Reactive oxygen species (ROS) are generated in cells of all aerobic organisms as a byproduct of normal metabolism, and the production of these toxic intermediates increases as a result of environmental stress. In plants, energetic electron transfer reaction in both chloroplasts and mitochondria produce ROS through single electron reduction of molecular oxygen to produce the superoxide anion (O_2^-) that is rapidly dismuted to hydrogen peroxide (H_2O_2). In the presence of iron or other metals, superoxide can react with H_2O_2 to produce the highly toxic hydroxyl radical; thus, effective scavenging of both superoxide and H_2O_2 are critical. The oxidative reactions of peroxisomes and glyoxysomes provide another important source of cellular H_2O_2. As with other aerobic organisms, plants have evolved effective ROS scavenging systems that are localized in virtually all cellular compartments. These antioxidant systems include a variety of enzymes and nonenzymatic metabolites that quench ROS and may also play a role in ROS signaling in plants (Allen 1995; Vranova et al. 2002).

Numerous attempts to improve stress tolerance in transgenic plants through increased ROS scavenging have been reported. These include transgenic plants that overexpress ROS scavenging enzymes, such as superoxide dismutase (SOD), ascorbate peroxidase, (APX), glutathione S-transferase/peroxidases, and catalase and enzymes involved in maintenance of reduced pools of low molecular weight redox regulators, such as glutathione reductase (GR) and monodehydroascorbate reductase. Transgenic tobacco plants that overexpress SOD in the chloroplast, mitochondria or cytosol were shown to have enhanced tolerance to oxidative stress induced by methyl viologen in leaf disc assays (Bowler et al. 1991; Sen Gupta et al. 1993; Van Camp et al. 1996), and overexpression of chloroplast Cu/Zn SOD in transgenic tobacco led to dramatic improvement in the photosynthetic recovery following severe photooxidative stress caused by exposure to chilling and high light (Sen Gupta et al. 1993) and potato plants (Perl et al. 1993). Transgenic alfalfa plants that overexpress chloroplast-targeted MnSOD showed improved tolerance to water deficit under both greenhouse and field conditions (McKersie et al. 1996).

Testing of transgenic cotton plants that express chloroplast-targeted forms of the antioxidant enzymes SOD, APX and GR has been carried out, and the results show enhanced recovery of photosynthesis in plants exposed to chilling temperatures and high light intensity (Payton et al. 2001). These authors have shown that overexpression of either APX or GR provided more effective protection from photooxidative damage than did SOD indicating that, under the conditions used, efficient removal of chloroplastic H_2O_2 was more critical than O_2^- scavenging. More focused physiological analysis indicated that leaves of these transgenic cotton plants maintained significantly higher photosynthetic rates and PSII activity during exposure to chilling temperatures than those of nonexpressing control plants, and these differences were found to be independent of the direct protection of photosystem II from photoinhibition (Kornyeyev et al. 2001). Protection was attributed to

Table 8.1 Lint yield and fiber quality measurements for transgenic cotton lines expressing increased level of the antioxidant enzyme ascorbate peroxidase

Genotype	Lint yield (lbs/acre)	Length (in)	Strength (g/tex)	Micronaire
Coker 312 null	1301	1.21	34.3	3.5
oeAPX 3–31	1695*	1.18	32.5	3.7
oeAPX 53–30	1701*	1.22	32.4	3.6

Two independently transformed transgenic cotton lines (oeAPX 3–32 and oeAPX 53–30) are shown along with a null, non-expressing line in the same genetic background (Coker 312 null). Plants were grown under rain-fed conditions in Lubbock, Texas during the 2004 growing season. *indicates statistically significant differences ($p < 0.05$). (E. Brechere and D. Auld, unpublished data)

the ability of these transgenic plants to maintain photosynthetic electron transport resulting in a lower reduction state of Q_A. Although small scale trials consistently showed higher yields from APX-expressing cotton lines compared to nonexpressing control plants under rain-fed conditions (Table 8.1), more recent results from larger scale field tests under different irrigation regimes failed to show significant differences in yield between transgenic and nontransformed plants under the conditions used (Gottula et al. 2009). However, transgenic plants did show significant differences in fiber properties.

Expression of a transgene that encodes glutathione S-transferase in transgenic tobacco plants resulted in substantial improvements in the tolerance of seedlings to both suboptimal and supraoptimal temperatures and salinity stress (Roxas et al. 1997). GST-expressing seedlings maintained higher levels of metabolic activity during stress treatments and showed reduced levels of lipid peroxidation (Roxas et al. 2000) Surprisingly, expression of the same transgene in cotton plants led to reduced seedling stress tolerance and increased levels of oxidative damage (Light et al. 2005). The reasons for this species-specific response to over-expression of GST are not completely understood but are likely to be related to differences in glutathione metabolism between the two species since total glutathione levels were increased in GST-expressing tobacco seedlings, relative to nonexpressing control seedlings while glutathione levels were lower in GST-expressing cotton seedlings.

8.2.2 Osmoprotectants

Single action genes include those involved in the biosynthesis of osmoprotectants, some of which also have antioxidant or other protective qualities that extend beyond their effects on cellular osmotic potential. Osmoprotectants include amino acids, such as proline; quaternary and other amines, such as glycine-betaine and polyamines; and a number of sugars and sugar alcohols, including mannitol, trehalose and galactinol that accumulate during osmotic adjustment (Vinocur and Altman 2005). Attempts to genetically engineer osmoprotection in plants have focused on the expression of genes that encode enzymes involved in the biosynthesis of specific

osmolytes, including proline (Delauney and Verma 1993; Nanjo et al. 1999; Zhu et al. 1998; Yamada et al. 2005), glycine-betaine (Ishitani et al. 1997; Lilius et al. 1996; Hayashi et al. 1997, 1998; Alia et al. 1998, 1999; Sakamoto and Murata 1998; Sakamoto et al. 2000; Holmstrom et al. 2000; McNeil et al. 2000), and "sugar alcohols", such as mannitol, trehalose, myo-inositol and sorbitol (Tarczynski et al. 1993; Yang et al. 1996; Shen et al. 1997; Abebe et al. 2003; Holmstrom et al. 1996; Zhao et al. 2000; Pilon-Smits et al. 1995, 1998, 1999; Garg et al. 2002; Cortina and Culiáñez Maciá 2005; Gao et al. 2000). Transgenic plants that overexpress polyamines have also been developed (Roy and Wu 2001, 2002; Kumria and Rajam 2002; Waie and Rajam 2003; Anderson et al. 1998; Capell et al. 2004).

In most of these cases, modification of the biosynthesis and metabolism of osmoprotectants is reported to result in improved stress tolerance. In addition to osmotic adjustment, the accumulation of compatible solutes may also help to protect plants from damage by quenching reactive oxygen intermediates, and by their ability to stabilize protein structure (Hare et al. 1998; Bohnert and Shen 1999; McNeil et al. 1999; Diamant et al. 2001). In some cases, negative pleiotropic effects have been observed, including growth retardation and reduced yield potential (Fukai and Cooper 1995) , and a review of the osmotic adjustment literature (Serraj and Sinclair 2002; Turner et al. 2007) indicates that increased osmotic adjustment has little if any, beneficial effect on yield under water stress. To our knowledge, attempts to increase the levels of osmoprotectants in transgenic cotton plants have not been reported.

8.2.3 Late Embryogenesis Abundant (LEA) Proteins

Members of this group of protective proteins accumulate during seed desiccation and in response to water stress (Galau et al. 1987). After their initial discovery in seeds, three major groups (numbered 1, 2 and 3) of LEA proteins were described in a range of plants and plant tissues. Homologues of group 1 and 3 LEA proteins have also been found in bacteria and in certain invertebrates. Group 3 LEA proteins, which are characterized by up to 13 copies of the amino acid motif TAQAAKEKAGE (Dure 1993), are predicted to act in the sequestration of ions during cellular dehydration. Over-expression of the barley group 3 LEA protein HVA1 in rice and wheat conferred increased tolerance to water deficit and salt stress (Xu et al. 1996; Sivamani et al. 2000; Rohila et al. 2002). Transgenic rice plants expressing either a wheat group 2 LEA protein (PMA80) or the wheat group 1 LEA protein (PMA1959) showed increased tolerance to dehydration and salt stresses (Cheng et al. 2002). A recent report demonstrates that over-expression of a group 4 LEA from *Brassica napus* also confers tolerance to salinity and water deficit stress in *Arabidopsis* (Dalala et al. 2009). Although LEA proteins were first discovered in cotton (Dure et al. 1981; Grzelczak et al. 1982), to our knowledge, development of cotton plants that over-express LEA proteins has not been reported.

8.2.4 Heat Shock Proteins

The heat stress response is a conserved stress defense mechanism found in all eukaryotic organisms. This system is activated by a wide variety of cytotoxic stimuli including elevated temperature, oxidative stress and chemical inducers (Vierling 1991; Waters et al. 1996). The response is characterized by the rapid reprogramming of gene expression leading to reduced expression of many genes and the accumulation of heat shock proteins (HSP). Many HSPs are molecular chaperones that are involved in the stabilization and/or refolding of denatured proteins (Schöffl et al. 1998). The central regulator of the heat shock response is the heat shock transcription factor (HSF), which specifically binds to palindromic DNA sequences known as heat shock elements (HSE), located upstream of HSP genes. Yeast and Drosophila express single HSFs while the *HSF* gene family in vertebrates is more elaborate with four members (*HSF1-4*). HSF1 and HSF3 are primarily responsible for general heat-induced HSP expression in these organisms.

The *HSF* gene family in plants is far more complicated than that in animals and yeast. Genes for more than 20 HSFs have been identified in the *Arabidopsis* genome and these have been classified by sequence similarity into three classes (A, B and C). While the class A and C HSFs are similar to those is animals and yeast, members of class B lack a conserved motif in the C-terminal activator domain that has been shown to be necessary for transcriptional activation. The tomato HSF system is characterized by cooperation between HSFA1 and HSFA2. HSFA1 is the master regulator of the heat stress response and is required for the heat-induced expression of HSFA2 (Mishra et al. 2002). During ongoing heat stress, HSFA2 accumulates to high levels and becomes the dominant HSF of tomato cells. Expression of the *Arabidopsis* ortholog of tomato HSFA2 is also strictly heat-inducible and it accumulates to high levels in cell cultures and leaves (Kotak et al. 2004). Attempts to enhance thermo-tolerance in transgenic plants by increasing the expression of HSPs have been carried out in a number of plant species (Malik et al. 1999; Li et al. 2003; Katiyar-Agarwal et al. 2003) and positive correlations between HSP expression and stress tolerance have been seen in some cases (Sun et al. 2001;Wang et al. 2005). However, development of transgenic cotton plants with altered expression of HSPs, either by direct expression of HSP genes or by expression of HSF genes, has not been reported.

8.2.5 Membrane Transporters

Restoration of ion homeostasis through modified expression of membrane ion transport proteins is an important strategy for the improvement of salt stress tolerance in transgenic plants. For example, transgenic tomato plants that express the yeast *HAL1* gene showed increased levels of salt tolerance (Gisbert et al. 2000). HAL1 is involved in the transport of K^+ and the transgenic plans were better able to maintain their K^+ levels during salinity stress than were wild-type plants.

The *Arabidopsis AtNHX1* gene, which encodes a vacuolar Na^+/H^+ antiporter, confers substantial salt tolerance when over-expressed in *Arabidopsis* and tomato plants (Apse et al. 1999; Quintero et al. 2000; Zhang and Blumwald 2001). Increased salt tolerance in these plants is likely due to the sequestration of Na^+ in vacuole. Over-expression of AtNHX1 in transgenic cotton plants was reported to confer tolerance to salt treatments as high as 200 mM NaCl (He et al. 2005). Under these conditions, AtNHX1-expressing plants maintained higher rates of photosynthesis, showed more rapid growth of both shoots and roots, and increased seed cotton yields relative to wild type plants. Surprisingly, these plants also produced greater seed cotton yields than wild type plants under field conditions with a significant increase in fiber length.

Over-expression of the *Arabidopsis AVP1* gene, which encodes a vacuolar H^+-pyrophosphatase (H^+-PPase), in both *Arabidopsis* and tomato has also been shown to increase tolerance to both salt and water deficit (Gaxiola et al. 2001; Park et al. 2005). AVP1 is critical for the maintenance of the proton gradient between the vacuole and cytosol to provide proton-motive force required by ion transporters such as AtNHX1. Expression of a homologous H^+-PPase from the halophyte *Thellungiella halophila* (TsVP) led to increased root development and improved salt stress tolerance in both tobacco (Gao et al. 2006) and cotton (Lv et al. 2008). The TsVP over-expressing cotton plants also showed increased tolerance to water deficit stress (Lv et al. 2009).

The plasma membrane Na^+/H^+ antiporter encoded by *Salt Overly-Sensitive 1* (*SOS1*) is essential for salt stress tolerance in *Arabidopsis* (Shi et al. 2000). Upregulation of the *SOSI* gene expression in *Arabidopsis* led to increased vigor and these plants maintained higher levels of photosynthetic quantum yield than wild-type plants when grown in the presence of supraoptimal concentrations of salt (Shi et al. 2003). These plants also accumulated less Na^+ in the transpiratory stream and in shoot tissues, indicating that they were more effective at recovering Na^+ from the xylem. Transgenic cotton plants that express an *Arabidopsis* SOS1 transgene have been developed in our laboratory (Y. Sun, J. Lee, and R.D. Allen, unpublished data) and preliminary evaluation of the effects of SOS1 over-expression on stress tolerance in cotton is now underway.

8.3 Multifunctional Genes

Much has been learned about the role of specific stress protective genes in determining stress tolerance phenotypes through experiments in transgenic plants and many of the transgenic plant lines produced show detectable increases in tolerance to specific or, sometimes, multiple stresses under laboratory conditions. However, in only a few cases have the effects of these genes been tested under field conditions in crop plants such as cotton. While promising, the levels of stress tolerance provided from the transfer of a single gene encoding a single specific stress protein may not reach the levels necessary to justify incorporation into a commercial

transgenic variety. Doubts about whether the resulting improvement in stress tolerance is of sufficient magnitude to provide an appreciable improvement in the performance of a certain crop under field conditions make it difficult to justify the huge financial investment necessary to bring a transgenic crop variety to market. Thus, efforts have more recently focused on functional evaluation of genes that play crucial roles in the regulation of native stress responses in plants. These experiments provide an important new understanding of the complex regulatory networks that plant cells use, to sense and respond to stressful conditions and provide additional opportunities for the use of transgenic strategies to alter plant stress responses that may provide stress tolerance traits that are sufficiently robust to justify commercial investment.

8.3.1 Transcription Factors

Genetic dissection of plant signal transduction has provided an important framework for the development of a more complete understanding of the complex signal transduction pathways that regulate plant responses to abiotic stress. Due to amenability of analysis, genetic analysis of plant stress responses has focused primarily on *Arabidopsis*. These efforts have identified diverse classes of transcription factors that have been associated with stress-responsive gene expression, including the bZIP, MYC/MYB, homeodomain leucine zipper (or HD-Zip), Zn finger, and ABI3/VP1 families.

Considerable overlap exists between the signal transduction events that occur during exposure to cold and drought stress (see Shinozaki et al. 2003; Zhang et al. 2004a for reviews). Some of these processes are regulated by ABA, while others are ABA independent (Shinozaki et al. 2003). For detailed reviews of the regulation of gene expression by ABA see Finkelstein et al. (2002); Himmelbach et al. (2003); Kuhn and Schroeder (2003). ABA response elements (ABREs) are located upstream of many ABA-responsive genes. These cis-acting elements interact with bZIP transcription factors. Promoters with optimal ABA responsiveness often contain a second *cis*-acting element (Shen and Ho 1995) that is similar to a C-repeat/dehydration-responsive element (CRT/DRE). Thus, ABRE-binding bZIPs and CRT/DRE-binding AP2 factors (CBF/DREB1) may interact to control gene expression in response to ABA, osmotic stress and cold temperatures (Narusaka et al. 2003) (Fig. 8.1).

Constitutive expression of the ABA-dependent bZIP transcription factors ABF3 or ABF4 in *Arabidopsis* was shown to result in upregulated expression of several ABA/stress-responsive genes including *RD29B, RA18*, *ABI1* and *ABI2*, leading to enhanced drought tolerance (Kagaya et al. 2002; Kang et al. 2002). Constitutive over-expression of ABF2 in *Arabidopsis* also resulted in other ABA-associated phenotypes including hypersensitivity to ABA and sugar and stunted growth (Kang et al. 2002). However, expression of the *Arabidopsis* ABF3 gene in rice and lettuce under control of the constitutive *Ubiquitin 1* promoter from maize resulted in

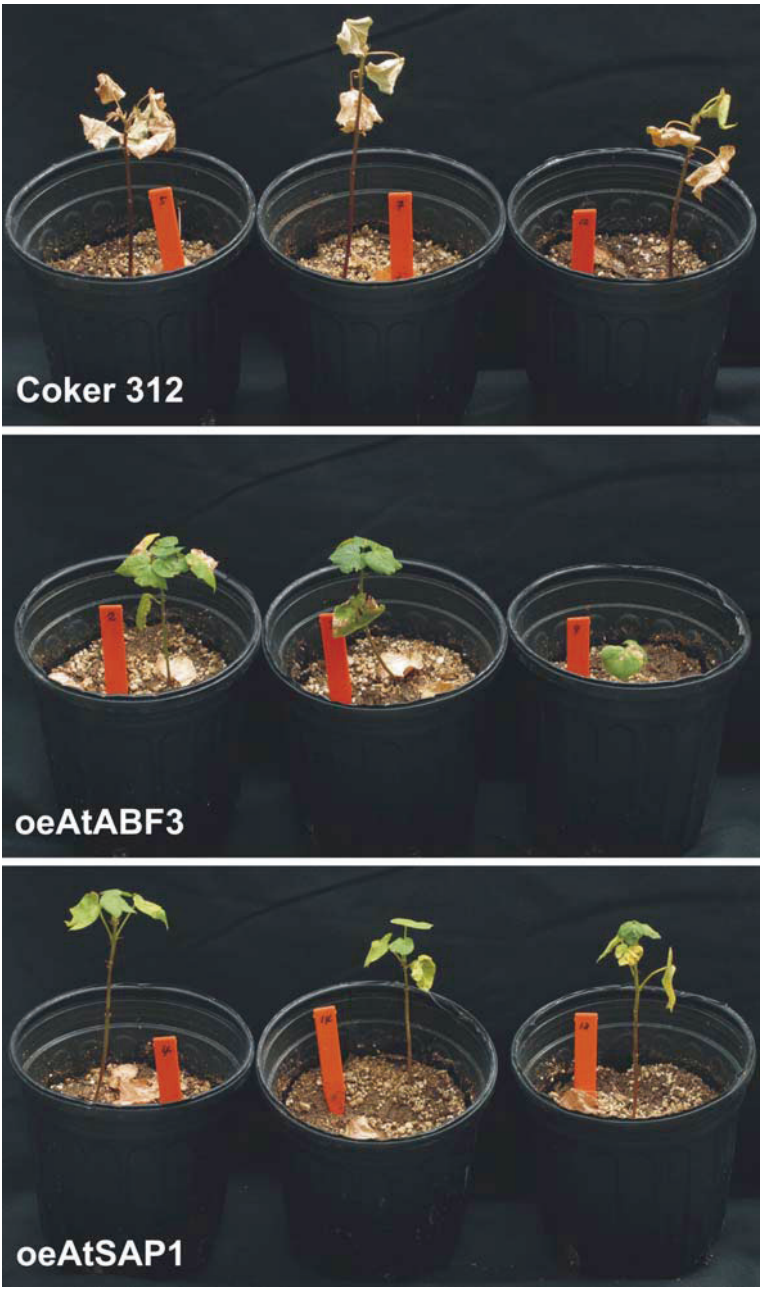

Fig. 8.1 Model for the transcriptional and post-transcriptional regulation of stress responsive gene expression in plant cells. Upstream regulatory sequences of many stress responsive genes, including *RD29A*, carry *cis*-acting sequences responsive to both ABA-dependent and ABA-independent transcription factors. ABA-dependent activation is mediated by ABF transcription

enhanced drought tolerance without negative effects on plant growth and development (Oh et al. 2005; Vanjildorj et al. 2005).

Based on these successful examples, transgenic cotton plants with increased expression of *Arabidopsis* ABF3 were created in our laboratory (L. Aleman, H. Abdel-Mageed, R.D. Allen, unpublished data). Our preliminary observations indicate that transgenic cotton plants that constitutively express ABF3 under control of the CaMV 35S promoter show enhanced expression of ABA-responsive genes under noninducting conditions, have more root development and show enhanced survival under severe water deficit (Fig. 8.1). However, these plants also exhibit deleterious side effects including slower vegetative growth and delayed flowering. Accelerated senescence and abscission of older leaves was seen in some transgenic cotton lines and the seeds produced by these plants failed to germinate under normal conditions. The relatively severe negative effects associated with constitutive expression of ABF3 in cotton led us to conclude that cotton plants respond too strongly to this transcription factor. Therefore, development of transgenic cotton plants in which *ABF3* expression is controlled by stress-responsive promoters may be required if this gene is to be used to improve stress tolerance without negative impacts on yield and other properties. Cotton plants that express these stress-responsive *ABF3* transgenes are now under development.

Cold acclimation in *Arabidopsis* involves the cold-responsive expression of a large number of genes, many of which are regulated by the *CBF*/*DREB1* regulon (Thomashow 2001). *CBF*/*DREB1* genes in *Arabidopsis* are expressed at low levels under normal growth conditions but their expression increases within several minutes after exposure to cold or drought stress. This gene family includes *CBF1*, *CBF2* and *CBF3* genes (Gilmour et al. 1998; Jaglo et al. 2001; Medina et al. 1999), also known as *DREB1B*, *DREB1C* and *DREB1A*, respectively (Liu et al. 1998). These genes encode transcriptional activators that bind to the conserved CRT/DRE DNA elements located in the promoters of certain cold-responsive genes (Baker et al. 1994; Gilmour et al. 1998; Stockinger et al. 1997; Yamaguchi-Shinozaki et al. 1994). Ectopic expression of these transcription factors in transgenic plants led to elevated freezing tolerance without prior cold treatment (Gilmour et al. 2000, 2004; Jaglo-Ottosen et al. 1998; Liu et al. 1998). Microarray analysis of *Arabidopsis* genes during cold acclimation indicated that the expression of about 500 genes was either up- or down-regulated in response to low temperature (Fowler and Thomashow 2002; Vogel et al. 2005). However, only about 15% of these genes were also responsive to CBF/DREB1 expression and could, therefore, be assigned

Fig. 8.1 (continued) factors such as ABF3 while ABA-independent responses are controlled by CBF/DREB 1 factors including CBF3. Under normal conditions, transcription of *CBF3* is suppressed by MYB15 and the zinc-finger transcriptional regulator ZAT10/STZ. Levels of the transcriptional activator protein ICE1 remain low under these conditions due to HOS1-dependent ubiquitination and degradation. Stress exposure leads to stabilization of ICE1 thorough SIZ1-dependent sumoylation. In addition, cold-responsive expression of the MBP-1-like transcriptional repressor LOS2 suppresses the expression of the *ZAT10*/*STZ* gene resulting in derepression of *CBF3* expression and activation of downstream stress protective genes

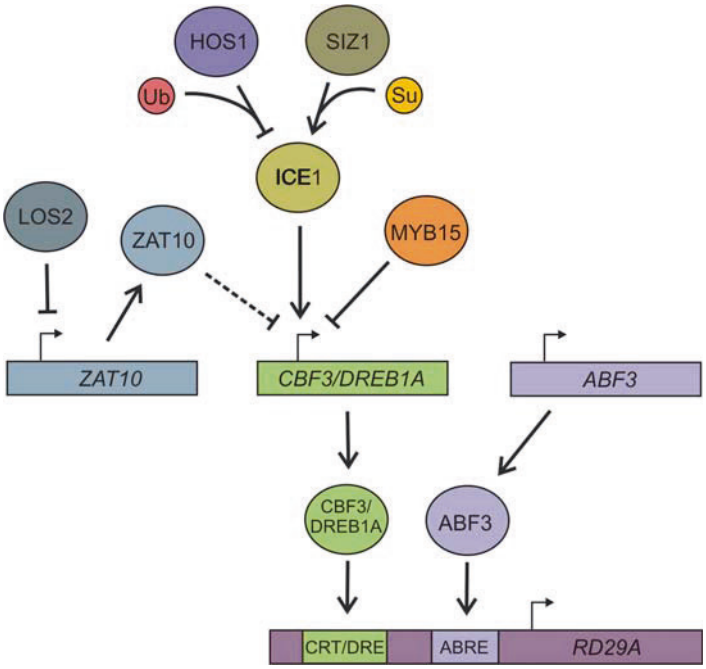

Fig. 8.2 Comparison of water deficit survival of greenhouse grown wild type cotton plants (Coker 312) and transgenic cotton plants that constitutively express either AtABF3 or AtSAP1. Plants were grown with ample water to the 5th leaf stage then water was withheld for 35 days. The plants were rewatered 1 day before they were photographed. Wild-type plants were dead after this severe water deficit exposure while plants of both transgenic genotypes survived and recovered from the stress treatment

to the CBF/DREB1 regulon. Therefore, although many of the genes that are most strongly induced in response to low temperature are regulated by CBF/DREB1, it is apparent that cold acclimation involves several low temperature-responsive regulatory pathways.

Transgenic *Arabidopsis* plants that constitutively over-express CBF1/DREB1B exhibited increased tolerance to freezing without negative side-effects on growth or development (Jaglo-Ottosen et al. 1998). Expression of cold responsive genes was shown to be activated in these plants at noninducing temperatures (Jaglo et al. 2001). Constitutive over-expression of CBF1/DREB1B in tomato plants also led to improved tolerance to chilling, drought and salt-stress but the growth of these plants was stunted and there was a reduction in fruit set and seed production (Hsieh et al. 2002). Over-expression of CBF3/DREB1A in transgenic *Arabidopsis* also leads to enhanced expression of target genes including *COR15a*, *RD29*, *KIN1*, *COR6.6*, and *COR47*/*RD17* (Liu et al. 1998; Kasuga et al. 1999; Maruyama et al. 2004) under noninducing conditions and these plants showed enhanced tolerance to freezing, drought and salt-stress. Constitutive expression of CBF3/DREB1A in transgenic rice plants resulted in substantially increased drought and salt-stress tolerance with less

improvement of tolerance to cold-stress (Oh et al. 2005) and these plants grew and developed normally. Subsequently, the over-expression of CBF3/DREB1A has been shown to improve the drought- and low-temperature stress tolerance in tobacco, wheat and groundnut (Kasuga et al. 2004; Pellegrineschi et al. 2004; Bhatnagar-Mathur et al. 2004) but the stress inducible *RD29A* promoter was used to minimize the negative effects of CBF/DREB1 over-expression in these plant species.

Due to severe negative consequences, regeneration of transgenic cotton plants that express *Arabidopsis* CBF3/DREB1A in our laboratory has proven to be very difficult. Initial attempts to regenerate cotton plants that constitutively express CBF3/DREB1A under control of the CaMV 35S promoter failed completely. Regeneration proceeded normally through somatic embryogenesis but the plantlets would not grow and most failed to develop roots (Y. Sun and R.D. Allen, unpublished data). Use of the stress-responsive *RD29A* promoter to drive stress-responsive CBF3/DREB1A expression allowed for the regeneration of plant that were able to thrive but these plants were severely stunted and virtually all were sterile (J. Lee, Y. Sun and R.D. Allen, unpublished data). Development of transgenic cotton plants that contain a CBF3/DREB1A transgene under a different stress-responsive promoter that does not contain a CRT/DRE element has been carried out and these plants grow almost normally and are fertile. Characterization of the stress tolerance characteristics of these plants is now underway.

Several lines of evidence indicate that signaling pathways in addition to those mediated by CBF/DREB1 transcription factors are involved in stress-adaptive responses, including cold acclimation. For example, *Arabidopsis* plants with mutations in the *eskimo1* gene showed substantial increases in freezing tolerance (Xin and Browse 1998). These plants accumulated high levels of the compatible osmolyte proline but did not exhibit increased expression of genes in the CBF/DREB1 regulon. Likewise, *ada2* mutants of *Arabidopsis* were more freezing tolerant than wild-type plants but did not show induction of cold-responsive gene expression indicating that the ADA2 may suppress the expression of a cold acclimation pathway that is distinct from the CBF/DREB1 regulon (Vlachonasios et al. 2003).

Two additional factors, HOS9 and HOS10, may negatively regulate genes that contribute to freezing tolerance (Zhu et al. 2004, 2005). Both genes were identified in genetic screens for mutants with altered regulation of a stress responsive luciferase reporter gene under control of the *RD29A* promoter. These constitutively expressed genes encode a putative homeodomain transcription factor and a putative R2R3-type MYB transcription factor, respectively. Mutations in these genes enhanced induction of *RD29A* and several other cold-responsive genes but did not alter cold induction of *CBF*/*DREB1* genes. Therefore, HOS9 and HOS10 may act independently of CBF/DREB1 to negatively regulate certain cold-responsive genes. Although expression of some cold-responsive genes was increased in *hos9* and *hos10* loss-of-function mutants, these plants were less freezing tolerant than wild-type plants both before and after cold acclimation treatments. Thus, HOS9 and HOS10 may regulate the expression of essential cold tolerance genes and loss of these activities in mutant plants may result in a compensatory increase in cold-induced gene expression.

Additional transcription factors from *Arabidopsis* that function downstream of CBF/DREB1 or in parallel signaling pathways have also been identified. The AP2 transcription factor DREB2 plays a role in drought adaptation in an ABA-independent manner (Liu et al. 1998; Nakashima et al. 2000). Expression of genes that encode two AP2 domain-containing proteins, RAP2.6 and RAP2.1 (Okamuro et al. 1997), is induced after the expression of CBF/DREB1. The *RAP2.1* promoter contains two CRT/DRE core sequence elements, and high levels of *RAP2.1* expression were seen in transgenic CBF/DREB1 over-expressing *Arabidopsis* plants, indicating that *RAP2.1* may be a target of the these transcriptional activators (Fowler and Thomashow 2002). Two transcription factors, RAV1 (AP2; Kagaya et al. 1999) and ZAT12 (zinc finger; Meissner and Michael 1997) have patterns of expression that are similar to those of CBF/DREB1 and, since neither RAV1 nor ZAT12 transcript levels were affected in CBF/DREB1 over-expressing plants, they probably operate in pathways that are parallel to the CBF/DREB1 regulon (Fowler and Thomashow 2002). The AP2-like genes *SHN* from Arabidiopsis and *WXP1* from *Medicago truncatula* activate the expression of genes involved in wax production. Constitutive expression of these genes under the control of the CaMV35S promoter in *Arabidopsis* (Aharoni et al. 2004) and alfalfa (Zhang et al. 2005) led to increased epicuticular wax accumulation on the leaf surfaces resulting in reduced water loss and enhanced drought tolerance.

LOS2 is critical for cold acclimation and for chilling tolerance under some conditions (Lee et al. 2002). Mutation of the *los2* gene in *Arabidopsis* specifically impairs the accumulation of transcripts from stress responsive genes. *LOS2* encodes a bifunctional enolase that can bind to a specific element within the promoter of the *ZAT10*/*STZ* gene. *ZAT10*/*STZ* encodes a Cys_2/His_2-type zinc finger transcriptional repressor involved in the regulation of some cold responsive genes. *ZAT10*/*STZ* expression is rapidly and transiently induced by cold and other stresses in wild-type *Arabidopsis* plants and this induction is stronger and more sustained in the *los2* mutant plants. Thus, LOS2 appears to participate in the regulation of cold responsive gene expression, at least in part, through its direct interaction with the *ZAT10*/*STZ* promoter. Although levels of CBF2/DREB1C were not altered in *los2* plants, expression of several CBF-regulated genes, including *RD29A,* was reduced in these plants, suggesting that LOS2 may affect the CBF/DREB1 regulon.

The role of ZAT10/STZ in the regulation of stress responsive gene expression is enigmatic. Like ZAT12, ZAT10/STZ contains a C_2H_2 zinc finger domain and an ERF-associated amphiphilic repression (EAR) motif (Kazan 2006), and both genes are expressed in response to drought, salt, cold and high light exposure (Sakamoto et al. 2000, 2004; Gong et al. 2002; Lee et al. 2002), yet there appears to be little if any functional overlap between these factors (Rossel et al. 2007). Although ZAT10/STZ negatively regulates gene expression (Sakamoto et al. 2000, 2004; Gong et al. 2002; Lee et al. 2002) it is required for the upregulation of cytosolic APX gene expression in *Arabidopsis* and both over-expressed and suppression of ZAT10/STZ expression in transgenic plants appears to lead to increased stress tolerance (Sakamoto et al. 2004; Mittler 2006).

Functional characterization of individual members of the WRKY transcription factor family is difficult due to the large number of genes that encode these factors in plants (Ülker and Somssich 2004). *Arabidopsis* contains 72 WRKY genes (Eulgem et al. 2000) and more than 100 of these genes exist in the rice genome (Wu et al. 2005). These proteins are characterized by the highly conserved WRKY domain that consists of approximately 60 amino acids including the invariant WRKYGQK core sequence plus a novel zinc-finger motif. WRKY proteins bind to promoter regions that contain the W-box sequence (T)TTGAC(C/T). WRKY transcription factors are known to be important regulators of biotic stress responses (reviewed in Eulgem et al. 2000; Dong et al. 2003; Eulgem and Somssich 2007) and they are also involved in certain developmental processes including embryogenesis, seed coat and trichome development, senescence, the regulation of certain biosynthetic pathways, and hormone signaling (Johnson et al. 2002; Lagacé and Matton 2004; Xie et al. 2005; Xu et al. 2004; Zhang et al. 2004a; Zou et al. 2004). Recently, WRKY transcription factor genes that are responsive to abiotic stresses have been identified (Gadjev et al. 2006; Jiang and Deyholos 2006; Ma et al. 2006; Mare et al. 2004; Miller et al. 2008; Pnueli et al. 2002; Zhou et al. 2008).

Transgenic over-expression of specific stress-responsive WRKY proteins in *Arabidopsis* has been show to increase tolerance to abiotic stress. For example, elevated expression of *Arabidopsis* WRKY25 led to increased tolerance to heat (Li et al. 2009) and salinity (Jiang and Deyholos 2009). Expression, in *Arabidopsis*, of three individual WRKY transcription factors from soybean led to distinct stress tolerance phenotypes (Zhou et al. 2008). Transgenic *Arabidopsis* plants that express *GmWRKY21* showed increased cold tolerance, while expression of *GmWRKY54* conferred increased tolerance to salinity and drought stress. Transgenic plants that over-express *GmWRKY13* were less responsive to ABA and were more sensitive to salinity and osmotic stress when compared with wild-type plants. These plants also showed an increase in lateral root development. Thus, individual WRKY proteins have unique regulatory footprints and confer distinct phenotypes. Transcription profiling experiments in cotton have shown that certain WRKY genes are expressed in developing cotton fibers and in regenerating protoplasts (Yang et al. 2006, 2008) and GaWRKY1 from *Gossypium arboreum* is reported to regulate genes involved in gossypol biosynthesis (Xu et al. 2004). However, the roles WRKY transcription factors in the regulation of abiotic stress responses or stress tolerance characteristics in cotton have not been characterized.

Nuclear Factor Y (NF-Y) transcription factors are apparently ubiquitous to all eukaryotes where they are involved in regulating the expression of diverse genes (McNabb et al. 1995; Edwards et al. 1998; Maity and de Crombrugghe 1998; Mantovani 1999). In mammalian cells, the NF-Y transcription factor complex consists of three unique subunits: NF-YA, NF-YB, and NF-YC. NF-YB and NF-YC initially form heterodimers in the cytoplasm that then translocate to the nucleus where NF-YA is recruited to generate mature, heterotrimeric NF-Y transcription factors (Frontini et al. 2004; Kahle et al. 2005). NF-Y binds to promoter elements with the core nucleotide sequence CCAAT, resulting in either positive or negative transcriptional regulation (Peng and Jahroudi 2002, 2003; Ceribelli et al.

2008). In most eukaryotic genomes, each NF-Y subunit is encoded by only one or two genes (Maity and de Crombrugghe 1998; Riechmann et al. 2000). However, as is the case with many transcription factor genes, the NF-Y subunit gene families in plants have expanded so that the genomes of both monocot and dicot plant lineages contain numerous genes for each NF-Y subunit. For example, *Arabidopsis* has ten genes that encode NF-YA, along with 13 genes for both NF-YB and NF-YC (Riechmann et al. 2000; Siefers et al. 2009). These 36 *Arabidopsis* NF-Y subunits could potentially combine to form nearly 1,700 putative transcription factors.

Although this complexity makes it difficult to determine the functions of specific NF-Y factors, the role of certain individual subunits is being revealed through genetic and transgenic analyses. Some NF-Y subunits are involved in developmental patterning; for example, *LEAFY COTYLEDON1* (*LEC1*), which encodes AtNF-YB9, and *LEC1-LIKE* (*L1L*), which encodes AtNF-YB6, are strongly expressed in developing embryos and are required for maintaining the identity of embryonic tissues (West et al. 1994; Lotan et al. 1998; Lee et al. 2003; Kwong et al. 2003). MtHAP2-1, an NF-YA subunit from *M. truncatula*, is expressed in a region of the root nodule meristem and is essential for nodule development (Combier et al. 2006). Other NF-Y subunits are involved in the regulation of abiotic stress tolerance. Over-expression of AtNF-YB1 leads to enhanced drought resistance in *Arabidopsis* (Nelson et al. 2007). Importantly, these authors also showed that transgenic maize plants that over-express ZmNF-YB2, the maize ortholog of AtNF-YB1, had increased stress tolerance resulting in improved yields when grown under drought conditions in the field. Over-expression of AtNF-YA5 also increased drought tolerance in *Arabidopsis* while *nf-ya5* mutant plants were more drought susceptible (Li et al. 2008). Although ESTs for NF-Y subunits exist in cotton, to our knowledge, the functions of these transcription factors have not been characterized.

8.3.2 Protein Kinases

Protein kinases represent another type of regulatory protein that has been used to improve stress tolerance in plants. Protein kinases initiate phosphorylation cascades that control downstream regulatory factors leading to altered stress responsive gene expression and tolerance to abiotic stress. An advantage of engineering signaling factors is that they can control the signal output involved in different aspects of homeostasis or damage prevention under abiotic stress (Verslues et al. 2006). One of these genes is the tobacco *NPK1* kinase and its *Arabidopsis* ortholog *ANP1*, which activates a mitogen-activated protein kinase (MPK3 and MPK6) signaling cascade that leads to enhanced tolerance to multiple environmental stresses in tobacco and maize (Kovtun et al. 2000; Shou et al. 2004). *NPK1*/*ANP1* acts upstream of the oxidative stress response pathway and can induce expression of HSPs, APX, GST and other stress responsive gene products. These proteins protect the photosynthetic machinery from damage caused by drought, thereby improving

agronomic traits such as yield in crops (Shou et al. 2004). Other kinases interact with proteins directly to confer a stress response. For example, SOS2 (salt overly sensitive 2) directly regulates the Na^+/H^+ antiporter SOS1 (Shi et al. 2000) that is known to be an important determinant of salt tolerance because of its role in ion homeostasis (Apse et al. 1999, 2003; Gaxiola et al. 1999). Although SOS2 is known to interact with the myristolyated calcium binding protein, SOS3, in a calcium-dependent manner, SOS2 appears to limit plant salt tolerance (Guo et al. 2004), and studies have shown that SOS2 is sufficient for activation of SOS1 and for increasing salt tolerance in plants. Cotton plants expressing *At*SOS2 under control of the CaMV 35S promoter that have recently been developed will be tested for increased salt tolerance (Y. Sun, J. Lee, and R.D. Allen, unpublished data).

8.3.3 Ubiquitin and SUMO Ligases

Post-translational modification of proteins via the attachment of a variety of small polypeptides such as ubiquitin and small ubiquitin-like modifier (SUMO) is an important regulatory mechanism in eukaryotic cells, including those of plants (Vierstra and Callis 1999; Melchior 2000; Hay 2001; Pickart 2001; Gill 2004; Kerscher et al. 2006). Ubiquitin ligases, which catalyze the attachment of ubiquitin to target proteins and thereby regulate their stability and/or activity, are involved in a wide range of regulatory pathways including those of virtually all phytohormones. Several of these are involved in stress signaling pathways; for example, transcriptional regulation of *CBF3*/*DREB1A* is modulated by post-translational modification of transcription factors while the stability of DREB2A is regulated by ubiquitination (see below). Furthermore, ring finger proteins such as SDIR and XERICO affect ABA signaling and biosynthesis in *Arabidopsis* (Zhang et al. 2007b; Ko et al. 2006), and a family of stress associated proteins (SAP) that contain A20-like zinc finger motifs are also involved in regulating stress responses (Mukhopadhyay et al. 2004).

Conjugation of the SUMO peptide to the target motif of protein substrates is known as sumoylation (Bernier-Villamor et al. 2002; Melchior et al. 2003; Schmidt and Müller 2003; Johnson 2004). Like ubiquitination, SUMO conjugation occurs in a series of biochemical steps mediated by E1-activating, E2-conjugating, and E3-ligation enzymes and the removal of SUMO from target proteins is catalyzed by SUMO proteases (Chosed et al. 2006). While ubiquitin is primarily responsible for the degradation of cellular proteins, ligation of SUMO to target proteins can interfere with ubiquitination, alter protein–protein interactions and subcellular localization, and modify transcription factor activity (Hochstrasser 2000, 2001; Gill 2003; Girdwood et al. 2004; Johnson 2004; Watts 2004). Sumoylation has been associated with the regulation of a wide range of cellular processes in eukaryotes including innate immunity, cell cycle progression, heat adaptation, DNA repair, nucleocytoplasmic trafficking, subnuclear targeting, and transcriptional regulation (Mao et al. 2000; Saitoh and Hinchey 2000; Freiman and Tjian

2003; Bohren et al. 2004; Dohmen 2004; Johnson 2004; Gill 2005; Hay 2005; Shuai and Liu 2005; Zhao and Blobel 2005; Hietakangas et al. 2006). The *Arabidopsis* sumoylation system is involved in many aspects of plant development and homeostasis. Levels of SUMO conjugates increase in response to a range of stresses and increased sumoylation levels correlate with increased expression of ABA- and stress-responsive genes (Kurepa et al. 2003; Lois et al. 2003; Miura et al. 2005, 2007; Yoo et al. 2006). An *Arabidopsis* SUMO E3 ligase, SIZ1 regulates the expression of genes involved in controlling plant responses to phosphate starvation and cold stress (Miura et al. 2005, 2007) and characterization of a *siz1* knockout mutant showed that loss of SIZ1 alters the expression of a set of genes in response to a water deficit (Catala et al. 2007). Recently, Miura et al. (2009) presented evidence that SIZ1 negatively regulates ABA signaling through sumoylation of ABI5. These authors demonstrate an epistatic genetic interaction between *SIZ1* and *ABI5*, and show that K391 in ABI5 is essential for SUMO1 conjugation. SUMO proteases encoded by the *OVERLY TOLERANT TO SALT1* (*OTS1*) and *OTS2* genes, regulate salt stress responses in *Arabidopsis*. Double mutants are sensitive to salt and they accumulate higher levels of SUMO-conjugated proteins than wild-type plants under both normal and salt-stress conditions (Conti et al. 2008). Over-expression of OST1 in transgenic *Arabidopsis* plants led to reduced levels of sumoylated proteins and increased salt tolerance compared to wild-type plants.

Ubiquitination and sumoylation can cooperate in the regulation of transcription factors involved in stress responsive gene expression. For example, transcriptional regulation of members of the *CBF*/*DREB1* gene family, which are transiently induced by low temperature (Liu et al. 1998; Gilmour et al. 1998; Medina et al. 1999) is controlled at both transcriptional and post-transcriptional levels (Fig. 8.2). Transcription of these genes is activated in response to low temperature by a constitutively expressed transcription factor ICE1 (for inducer of CBF/DREB1 expression 1). ICE1 is a MYC-like basic helix-loop-helix transcription factor that binds to canonical MYC *cis*-elements (CANNTG) in the *CBF3*/*DREB1A* promoter. This interaction induces expression of CBF/DREB1, which leads to induction of the CBF/DREB1 regulon (Chinnusamy et al. 2003; Lee et al. 2005). Negative transcriptional regulation of *CBF*/*DREB1* expression is mediated by MYB15, which binds to *CBF*/*DREB1* promoter regions and represses expression of *CBF*/*DREB1* genes and thus, the CBF/DREB1 regulon (Agarwal et al. 2006). While transcription of the *ICE1* gene is constitutive, the stability of the ICE1 protein is regulated by the RING-type E3 ubiquitin ligase HOS1 (Lee et al. 2001), which polyubiquitinates ICE1, targeting it to the 26 S proteasome for degradation (Dong et al. 2006). Since ubiquitination of ICE1 by HOS1 is induced by cold, HOS1 appears to be involved in the attenuation of plant responses to low temperatures. The activity of ICE1 is positively regulated via sumoylation by the SUMO ligase SIZ1 under cold conditions (Miura et al. 2007). ICE1 sumoylation interferes with HOS1-dependent polyubiquitination and suppresses expression of *MYB15*. Thus, under noninducing conditions, transcription of *CBF*/*DREB1* genes is repressed by MYB15 and the accumulation of ICE1 is inhibited by HOS1-mediated polyubiquitination. In response to stressful conditions, sumoylation of ICE1 by SIZ1 blocks its

ubiquitination, leading to stabilization and/or activation. The accumulation of active ICE1 leads to the repression of *MYB15* and the transcriptional activation of *CBF*/*DREB1*.

Unlike members of the *CBF1*/*DREB1* gene family, the function of *DREB2A* in the regulation of stress responses was less clear. Attempts to over-express DREB2A in transgenic plants failed to confer an altered phenotype and had no apparent affect on the expression of stress-responsive genes. However, deletion of a small region in the central part of the *DREB2A* coding sequence produced a constitutively active form called DREB2A-CA. Expression of DREB2A-CA in *Arabidopsis* resulted in dwarfed growth and increased stress tolerance (Sakuma et al. 2006), raising the hypothesis that DREB2A expression is subject to post-translational regulation. A recent search for protein factors that interact with the negative regulatory domain of DREB2A resulted in the identification of a unique RING-finger E3 ubiquitin ligase named DREB2A Interacting Protein 1 (DRIP1) (Qin et al. 2008). DRIP1 catalyzes the ubiquitination of DREB2A in vitro and mediates the stability of DREB2A in planta. Although E3 ligase genes such as HOS1 are expressed in response to stress and are thought to attenuate stress responses, DRIP1 is constitutively expressed and may be responsible for suppression of DREB2A expression under nonstressful conditions.

While the RING-finger E3 ubiquitin ligases HOS1 and DRIP1 negatively regulate plant stress responses, other members of the RING-finger protein family positively regulate stress responses. For example, over-expression of the RING-finger protein SDIR1 leads to increased drought stress tolerance and enhanced expression of several ABA-responsive genes (Zhang et al. 2007b) while over-expression of the RING-finger domain protein XERICO leads to increased ABA biosynthesis (Ko et al. 2006). Although the cellular targets of these ubiquitin ligases have not been specifically indentified, it is likely that they are transcription factors or other regulatory molecules that function to down regulate stress responses.

A family of stress responsive genes has been identified that encode proteins containing conserved A20-like and AN1-like zinc finger motifs at their N- and C-terminal domains, respectively. For example, expression of the *OsiSAP1* gene from rice is induced in response to a variety of environmental stresses including cold, salt, drought, anoxia, wounding and heavy metals and over-expression of this gene in transgenic tobacco plants conferred increased abiotic stress tolerance (Mukhopadhyay et al. 2004). Vij and Tyagi (2006) identified 18 genes encoding putative SAP1-like proteins in the rice genome and 14 genes of this type were identified in *Arabidopsis*. Like OsiSAP1, several of the rice *SAP1* genes were found to be stress responsive (Vij and Tyagi 2006) and analysis of public microarray data indicated that several of the *AtSAP1* transcripts are strongly induced by ABA and a range of abiotic stress treatments. Similarly, overexpression of the OsiSAP8 in both transgenic tobacco and rice conferred tolerance to salt, drought and cold stress at seed germination/seedling stage as reflected by the percentage of germination and gain in fresh weight after stress recovery. Transgenic rice plants were tolerant to salt and drought during anthesis stage without any yield penalty as compared to unstressed transgenic plants (Kanneganti and Gupta 2008).

Transgenic *Arabidopsis* plants that ectopically express one *AtSAP1* gene under control of the CaMV 35 S promoter as well as *Arabidopsis* plants with antisense suppressed *AtSAP1* expression and T-DNA knock-out mutants were developed and characterized in our laboratory (M, Kang, M. Fokar and R.D. Allen, unpublished data). The growth and development of these plants was indistinguishable from wild type plants under normal nonstressful conditions. However, when these plant were grown under chilling temperatures or exposed to water deficit, clear phenotypic differences became apparent. In general, AtSAP1 knockout and knock-down plants were more stress sensitive than wild type plants under chilling and osmotic stress, while transgenic *Arabidopsis* plants that over-express *AtSAP1* showed substantial increases in stress tolerance under water deficit conditions.

Development of transgenic cotton plants that ectopically express AtSAP1 has been completed in our laboratory and several independent transgenic lines that express this transgene were regenerated (H. Abdel-Magged, J. Lee, P. Payton and R.D. Allen, unpublished data). When compared with wild type plants, AtSAP1-expressing cotton plants showed increased survival under water deficit in greenhouse experiments (Fig. 8.1). No differences were seen between AtSAP1 cotton plants and wild type plants when they were grown under irrigated conditions in small-scale field trials. However, differences in stress tolerance were readily apparent in dry-land plots. When grown under these water deficit conditions AtSAP1-expressing plants showed increased vegetative growth, reduced wilting and chlorosis relative to nontransformed plants. While bolls of wild type cotton plants tended to open prematurely under these conditions, bolls of AtSAP1 over-expressing plants remained closed. Premature boll opening under drought stress results in production of immature, poor quality cotton fibers while the fiber produced by AtSAP1expressing plants was of higher quality. Thorough physiological evaluation and quantitative analysis of yield and fiber quality parameters from these plants is now underway.

8.3.4 MicroRNAs

MicroRNAs (miRNAs,) and small interfering RNAs (siRNAs) represent an additional mode of post-transcriptional regulation that is ubiquitous in eukaryotic cells. These small noncoding RNAs can silence gene expression by targeting specific mRNAs for degradation or by repressing their translation (Bartel 2004; Baulcombe 2004; Jones-Rhoades et al. 2006; Mallory and Vaucheret 2006). Although the function of miRNAs in developmental processes has been extensively studied (see Kidner and Martienssen 2005; Mallory and Bouché 2008 for recent reviews), the role of these regulatory molecules in the regulation of plant responses to abiotic stress is now beginning to emerge (Sunkar et al. 2007).

Jones-Rhoades and Bartel (2004) identified *Arabidopsis* miRNAs that were predicted to target genes involved in abiotic stress responses. For example, miR395, which is induced by sulfate starvation, targets transcripts for ATP sulfurylases and a

sulfate transporter AST68 (Jones-Rhoades and Bartel 2004; Allen et al. 2004). Sunkar and Zhu (2004) identified several stress-responsive miRNAs including miR393, which was strongly upregulated by cold, dehydration, salinity, and ABA treatments. miR393 catalyzes the cleavage of transcripts from several closely related F-box auxin receptor genes, including *TRANSPORT INHIBITOR RESPONSE1* (*TIR1*), which, targets AUX/IAA proteins for ubiquitination (Vierstra 2003). Thus, miR393-mediated inhibition of TIR1 down-regulates auxin signaling under abiotic stress conditions. Accumulation of miR159 is induced by ABA via the seed specific ABA-dependent transcription factor ABI3 (Reyes and Chua 2007). miR159 targets the cleavage of transcripts for the MYB transcription factors MYB33 and MYB101 that are positive regulators of ABA signaling. Thus, miR159 may act to attenuate ABA responses in plants. Genes for two closely related Cu-Zn superoxide dismutase genes (*CSD1* and *CSD2*) are transcribed under normal growth conditions but their mRNAs do not accumulate due to miR398-directed cleavage. miR398 is transcriptionally down-regulated in response to oxidative stress to allow for increased accumulation and translation of *CSD1* and *CSD2* transcripts (Sunkar et al. 2006) while sucrose-dependent induction of miR398 repressed expression of these mRNAs (Dugas and Bartel 2008). Furthermore, transgenic plants that express a transgene for a mutant *CSD2* that is resistant to miR398-mediated cleavage accumulate higher levels of CSD2 transcripts and these plants showed improved tolerance to oxidative stress conditions when compared to transgenic plants that express the miR398-susceptible CSD2 gene (Sunkar et al. 2006). The recent identification of conserved microRNAs in cotton (Zhang et al. 2007a) indicates that opportunities exist to use this important post-transcriptional regulatory system in future efforts to optimize abiotic stress tolerance in cotton.

8.4 Conclusions

Research made possible through the use of the wealth of genomic information and genetic resources available for model plant species such as *Arabidopsis thaliana* has led to a substantial increase in our knowledge about how plants respond to stressful environmental conditions. This leap in understanding has provided the basis for many new strategies for the optimization of stress tolerance in crop plants through both traditional and transgenic approaches. Although, to date, most of these strategies have been tested only in model plants, evaluation of some of them in important crop species, including cotton, is now underway. While much of the published research has focused on plant survival under severely stressful conditions, application of novel stress-tolerance technologies will require much more extensive physiological and agronomic evaluation to determine their ultimate applicability to crop systems. Stress tolerant plants that are simply capable of surviving severe conditions are of little value if they fail to produce yield improvement under moderately stressful conditions. Therefore, a broad-based evaluation strategy for candidate genes will be necessary to test their value under a wide

variety of field conditions. In cotton, this will include testing the effects of candidate genes on germination, seedling survival and stand establishment, vegetative growth and the transition to flowering, flowering period, boll maturation, and fiber development and quality. Though experiments in model species are valuable, experience shows that they do not guarantee similar results in a heterologous crop species. Our preliminary work in transgenic cotton with transcription factors such as ABF3 and CBF3 show that optimization of expression patterns using different promoters or other regulatory strategies will be necessary. The recent identification of complex post-transcriptional and post-translational regulatory mechanisms for stress responsive gene expression reinforces the need for direct testing of new technology in crop species. These mechanisms are able to fine-tune the expression of stress responsive genes by ensuring their suppression under nonstressful conditions and attenuating stress responses in the face of prolonged stress exposure. However, since these mechanisms can be sequence specific, we anticipate that, in some cases, different results will be achieved in plants depending on whether endogenous or heterologous coding sequences are used in a transgene.

The translational genomic approach in which the depth of knowledge gleaned from a model system is systematically translated to economically important crop plants that are less amenable for basic research is a powerful strategy for crop improvement. Although it may not yet be possible to accurately predict the outcome of any particular transgenic experiment, the ability to narrow the group of candidate genes based on experimental data from model species makes it possible to approach these experiments in a much more rational way. As our understanding of the complex regulatory networks that control stress responsive gene expression develops and the roles of these regulatory mechanisms in plant productivity are revealed, it is likely that substantial increases in crop yield under suboptimal conditions can be realized. It is critical that this research proceed if we are to have any hope of continuing to feed, clothe and house an ever-increasing human population in spite of diminishing resources and climatic instability.

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Chapter 9
Recent Advances in Molecular Biology Research on Cotton Fiber Development

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9.1 Introduction

As the world's leading natural fiber and the second largest oilseed crop, cotton (*Gossypium*) is a mainstay of global economies. In the later years of the twentieth century, biotechnology has advanced with unprecedented speed and ever-widening range of involvement of different disciplines and technologies. Transgenic insect-resistant Bt cotton was the first biotechnology crop grown in China, by which China has become the second country in the world in developing Bt cotton after USA (ISAAA report, 2007, from internet data). The rapid increase in transgenic cotton acreage in such a short period of time attests to the overall success of agricultural biotechnology.

Cotton fiber plays an irreplaceable role in the textile industry owing to its excellent natural properties. With people's increasing living standards and pursuit of returning to nature, good quality cotton textile is in great demand. Therefore, fiber modification has become one of the main objectives for cotton breeding. In the past decade, traditional and hybridization breeding has played a critical role in the improvement of cotton fiber quality. However, further progress can be difficult largely due to the long breeding cycle, insufficient germplasm resources, and negative correlation between fiber productivity and quality. In recent years, rapid development of functional genomics, genetic and analytic tools, especially comprehensive profiling of gene expression of cotton fiber cells, and application of model systems (such as *Arabidopsis*, tobacco, and yeast) has provided a new opportunity to improve the cotton fiber traits by genetic modification. As new generations of sequencing technologies such as Solexa (known as Illumina) and

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454 pyrosequencing (Roche) have drastically lowered the cost of DNA sequencing, international collaborations on cotton genome sequencing have become possible. Without doubt, genome sequence information will greatly deepen our understanding of the cotton crop, genome evolution, fiber cell development, cellulose and cell wall biosynthesis, and accelerate molecular breeding of new cotton cultivars (Chen et al. 2007).

9.2 Transcriptome of Cotton Fiber

Lint fibers of cotton are extensively elongated single epidermal cells that develop on the outer surface of ovules (outer integument). Cotton fiber initiates from 1 day preanthesis to 1 day postanthesis (DPA) and undergoes rapid elongation immediately after fertilization (Wu et al. 2006). The development of cotton fiber can be divided into four distinct but overlapping stages including fiber initiation, elongation, secondary cell wall deposition, and maturation (Basra and Malik 1984; Kim and Triplett 2001). Cotton fiber initiation and elongation are two important stages during cotton fiber development, as the initiation program determines the number of ovule epidermal cells that will differentiate into fiber cells and thus affects fiber yield, and the elongation stage is crucial for the final fiber length, a key trait of fiber quality. Cotton functional genomics studies related to fiber development focus on these two stages (Xu et al. 2007).

Transcriptome analysis, a powerful and high throughput tool to detect differentially expressed genes, has been extensively applied not only to reveal physiological states of cells, but also to identify gene functions. This approach is of great importance for cotton plants, as the complexity of cotton genome makes its investigation difficult and costly (Wilkins and Arpat 2005). An early attempt compared gene expression of 5–10-DPA ovules of a *fuzzless-lintless* (*fl*) mutant with those of ovules from the wild-type cotton (*Gossypium hirsutum* cv. Xu-142) using cDNA array containing 1,536 cDNA clones (Li et al. 2002a), which identified several genes whose transcripts were enriched in fiber cells, including *GhWBC1* (Zhu et al. 2003), *GhRDL1* (Wang et al. 2004), and *GhSAHH* (Li et al. 2008). Arpat et al. (2004) took a genomics approach to cotton fiber research by examining a large number of expressed sequence tags (ESTs) from elongating cotton fiber cells. They identified approximately 14,000 unique genes from 46,630 ESTs present in developing cotton fiber. The fiber transcriptome was estimated to represent 35%–40% of the genes in the cotton genome. Almost two-third of these annotated genes fell into three major categories: cell wall structure and biogenesis, cytoskeleton-related, and energy/carbohydrate metabolism (Arpat et al. 2004). Oligonucleotide microarrays revealed dynamic changes in gene expression between primary and secondary cell wall biogenesis, with more than 2,500 genes down-regulated at the end of the active elongation period and 81 genes preferentially upregulated during secondary cell wall synthesis. This study sheds new insight into how transcriptional activity defines different physiological and biochemical states in fiber cells at different

developmental stages and also provided numerous potentially interesting target genes for functional characterization (Arpat et al. 2004). In a later large-scale transcriptome analysis, a cDNA library from wild-type cotton ovules at fast elongation stage, which the cotton fiber elongate rapidly after initiation of elongation, was used, and a total of 12,233 unique sequences from 29,992 high-quality ESTs were obtained. Among them, 2,522 genes were significantly upregulated during elongation stage. In comparison with transcriptomes of the 3- and 10-DPA wild-type ovules and those of the *fl* mutant, 778 genes were fiber specific (Shi et al. 2006). More recently, gene expression in relation to metabolite changes of cotton fiber during cell elongation and secondary cell wall synthesis stages were analyzed, which showed that the genes involved in auxin signaling, cell wall-loosening, and lipid metabolism were highly expressed during fiber elongation stage. At secondary cell wall synthesis stage, genes related to cellulose biosynthesis were predominantly transcribed, whereas many other metabolic pathways were inactive. Transcriptional and metabolite profiling and enzyme activities were consistent with a specialization process of cotton fiber development toward cellulose synthesis (Gou et al. 2007).

Compared to the fiber cell elongation stage, the molecular feature of cotton fiber initiation stage remains largely mysterious. Fiber cell initiation is a complex process involving many pathways, including various signaling and transcriptional regulation components. Yang et al. (2006) generated an EST library, named GH_TMO ESTs library, using the ovules of earlier stages (−3 to 3DPA). In comparison with approximately 178,000 existing ESTs derived from elongating fibers and nonfiber tissues, GH_TMO ESTs show a significant enrichment of the genes encoding putative transcription factors, such as MYB and WRKY proteins and the genes encoding predicted proteins involved in auxin, brassinosteroid (BR), gibberellic acid (GA), abscisic acid (ABA) and ethylene signaling pathways. These data are consistent not only with the known roles of MYB and WRKY transcription factors in leaf trichome initiation in *Arabidopsis*, but also with the effects of phytohormones on fiber cell development documented by using in vitro cotton ovule culture system. Interestingly, most of the phytohormonal pathway-related genes were induced prior to the activation of MYB-like genes, implying an important role of phytohormones in cell fate determination (Yang et al. 2006). In another investigation, gene expression profiles of the 0-DPA ovules of six fiber development mutants were compared with those of the wild-type cotton, using cDNA microarray, which showed that 13 different genes were downregulated in some or all of the six mutants. Among them *GhMYB25*, which shows a high sequence identity to *Antirrhinum* MYB gene *MIXTA*, was upregulated in fiber initials relative to adjacent nonfiber ovule epidermal cells on the day of anthesis, suggesting a possible involvement of *GhMYB25* in cotton fiber initiation (Wu et al. 2006) (Table 9.1).

A comprehensive global cotton EST database was set up in 2006 with a total of 185,000 ESTs collected from more than 30 different cDNA libraries from various cotton tissues and organs. By sequence comparisons, 51,107 unique genes were identified and 33,665 of them represent partial or full-length nonrepeated coding

Table 9.1 Transcription factor genes related to cotton fiber development and their *Arabidopsis* homologs

Genes/Accession No.		Function in *Arabidopsis*	Reference
Cotton	*Arabidopsis thaliana*		
GaMYB2/AY626160	*AtGL1*/AT3G27920	MYB family transcription factor, trichome initiation	Wang et al. (2004)
GhMYB25/AY464054	*AtGL1*/AT3G27920	MYB family transcription factor, trichome initiation	Wu et al. (2006)
GhMYB109/AJ549758	*AtGL1*/AT3G27920	MYB family transcription factor, trichome initiation	Suo et al. (2003); Pu et al. (2008)
GhTTG1/AF530908	*AtTTG1*/AT5G24520	WD-repeat protein, trichome initiation	Humphries et al. (2005)
GhTTG3/AF530911	*AtTTG1*/AT5G24520	WD-repeat protein, trichome initiation	Humphries et al. (2005)
GhHOX1/AF530913	*AtGL2*/AT1G7984*0*	Homeobox protein, trichome initiation	Guan et al. (2008)
GhHOX2/AF530914	*AtGL2*/AT1G79840	Homeobox protein, trichome initiation	Guan et al. (2008)
GhHOX3/AY626159	*AtGL2*/AT1G79840	Homeobox protein, trichome initiation	Guan et al. (2008)
GhDEL65/AF336280	*AtGL3*/AT5G41315	bHLH protein, trichome initiation	Shangguan et al. (2008)
GhDLE61/AF336279	*AtGL3*/AT5G41315	bHLH protein, trichome initiation	Mandaokar et al. (2003)
GhCPC1/Contig16590	*AtCPC*/AT2G46410	Small MYB protein, root hair spacing	Taliercio and Boykin (2007)
GhCPC2/Contig17149	*AtCPC*/AT2G46410	Small MYB protein, root hair spacing	Taliercio and Boykin (2007)

regions (Udall et al. 2006). About 375,392 ESTs are now available for the *Gossypium* species in the Genbank database. A rich source of ESTs and cDNA sequences is invaluable not only for understanding the mechanisms regulating cotton fiber development, but also for cotton genomics studies and for generating new molecular markers for breeding. For example, from 489 primer pairs derived from EST-SSR, 123 polymorphisms were found. These markers were distributed over 20 chromosomes and six linkage groups in the cotton genetic map (Han et al. 2006).

9.3 Functional Identification of Genes Related to Cotton Fiber Development

9.3.1 Transcription Factors

Cotton fibers are seed epidermal hairs, which share many features with leaf trichomes, although the cotton fiber is unbranched. The models established for

Arabidopsis leaf trichomes may provide a framework for understanding fiber cell initiation and elongation (Table 9.1). MYB transcription factors are key regulators controlling *Arabidopsis* trichomes development. *GLABRA* (*GL1*) is a well-documented R2R3 MYB transcription factor regulating trichome fate determination, and *gl1* loss-of function mutants show a glabrous phenotype with only few trichomes on the edge of rosette leaves (Oppenheimer et al. 1991). AtMYB23 is also an R2R3 MYB transcriptional regulator and has partially overlapping functions with *GL1* (Kirik et al. 2005). Genetic and molecular evidence shows that GL1 forms a multimeric complex with TRANSPARENT TESTA GLABRA1 (TTG1), a WD40 protein (Walker et al. 1999), and GLABRA3 (GL3) or EGL3, a basic helix-loop-helix protein (Payne et al. 2000; Esch et al. 2003; Zhang et al. 2003). GL1-TTG-GL3 complex triggers trichome cell initiation probably by controlling the expression of *GLABRA2* (*GL2*), which encodes a homeodomain-leucine zipper protein (Ramsay and Glover 2005; Serna and Martin 2006). A small family of single-repeat MYB proteins, including CAPRICE (CPC), TRIPTYCHON (TRY), TRICHOMELESS1 (TCL1), ENHANCER OF TRY AND CPC1 (ETC1) and ETC2, negatively regulates trichome initiation and spacing by competing with GL1 for binding to GL3 (Kirik et al. 2004a, b; Wang et al. 2007).

Recent progress on isolation of transcription factors potentially involved in cotton fiber development has provided clues to understanding the early event of cotton fiber development. MYB genes were among the first group under investigation, with emphasis on their expression patterns and evolution in diploid and polyploidy cotton (Loguerico et al. 1999; Cedroni et al. 2003). *GaMYB2*/*FIF1*, a *GL1*-like gene of *G. arboreum*, is expressed early in developing fiber cells. When properly expressed in *Arabidopsis*, *GaMYB2* was able to restore trichome development to the glabrous *gl1* mutant, and its overexpression produced ectopic trichomes on the seed coat, strongly suggesting that *GaMYB2* participates in regulating cotton fiber development (Wang et al. 2004). Suo et al. (2003) reported the identification of 55 MYB genes which were expressed in ovules during fiber initiation. Among them *GhMYB109*, another homolog of *GL1*, was specifically expressed in cotton fiber initials and elongating fibers. *GhMYB25*, a homolog of *AmMIXTA*/*AmMYBML1* that controls conical cell and trichome differentiation in *Antirrhinum majus* petals (Martin et al. 2002; Perez-Rodriguez et al. 2005), was predominately expressed in ovules and fiber cell initials. Overexpression of *GhMYB25* in tobacco plants increased branches of leaf trichomes (Wu et al. 2006); whether it is involved in cotton fiber development is an interesting question to be answered. Two cotton single MYB repeat genes, *GhCPC1* and *GhCPC2*, were expressed in fiber cells at 1DPA, and one of them was downregulated in the fiber cells compared to ovule (Taliercio and Boykin 2007). Whether these putative CPC homologs of cotton play a critical role in fiber cell spacing awaits investigation.

Four putative *AtTTG1* homologs have been isolated from the ancestral D diploid genomes of tetraploid cotton *G. hirsutum*. All of them are widely expressed in various organs, including ovules and fibers. When expressed in *Arabidopsis*, *GhTTG1* and *GhTTG3* were able to restore trichome formation, seed coat pigmentation, mucilage production, and root hair positioning in the *ttg1* mutant (Humphries et al. 2005).

Three homeobox (HOX) genes, *GhHOX1*, *GhHOX2*, and *GhHOX3*, have been identified from cotton as well (Table 9.1). At the predicted amino acid sequence level, they show 66%, 34%, and 37% identities to *Arabidopsis* GL2, respectively. At least one of them, GhHOX1 that is closest to *Arabidopsis* GL2, may function in fiber development, as GhHOX1 was able to revert the glabrous phenotype of *gl2* mutant, indicating that this homeodomain-leucine zipper protein shares similar function with GL2 in controlling trichome development (Guan et al. 2008).

In the *Arabidopsis* trichome model, both the positive and the negative MYB regulators interact with the bHLH protein GL3. Both GaMYB2 and GhMYB109 contain the conserved amino acid signature for interaction with bHLH proteins (Serna and Martin 2006). Two *GL3*-like bHLH cDNAs from cotton ovule, *GhDEL65* (AF336280) and *GhDEL61* (AF336279), have been deposited in the Genbank (Mandaokar et al. 2003; Shangguan et al. 2008). It will be interesting to examine if they behave like GL3 during cotton fiber development.

Taken together, nearly all the molecular components involved in controlling *Arabidopsis* trichome development characterized so far have their counterparts in cotton ovule and fiber cells. The ability of cotton transcription factors like *GaMYB2*, *GhMYB109*, *GhTTG1*, *GhTTG3*, and *GhHOX1* to complement the respective trichome mutants of *Arabidopsis* suggests a similar molecular event triggering cotton fiber and *Arabidopsis* trichome formation.

9.3.2 Phytohormones

Plant hormones control almost all aspects of plant growth and development. Both the earlier in vitro studies and the recent functional genomics analyses have suggested phytohormones as critical regulators of cotton fiber development and boll retention. In the ESTs library of −3–3DPA (GH_TMO ESTs library), about 230 putative Abscisic acid (ABA)-, Brassinosteroid (BR)-, Gibberellic Acid (GA)-, ethylene- and auxin-related sequences were identified, indicating a role of phytohormones in early stages of fiber development (Yang et al. 2006).

9.3.2.1 Auxin and GA

For several decades, combinations of auxin and GA have been known to promote fiber cell development of in vitro cultured ovules. Exogenous application of indoacetic acid (IAA) and GA_3 to flower buds in planta or unfertilized ovules in vitro resulted in an increase of fiber cell number (Beasley and Ting 1974; Gialvails and Seagull 2001). The GH_TMO ESTs library revealed several genes involved in GA biosynthesis, such as *GA20ox*, *GA2ox*, *POTH1*, and *KO*, and signaling transduction components including *GA1*, *RGL2*, *RGL1*, *DPF1*, *PHOR1*, *RSG*, and *GAMYB*. Moreover, the library contained putative auxin-related genes, including those of auxin biosynthesis (YUCCAs, CYP83B1s, and NIT2), signaling (ARFs, AUX1,

TIR1, and PINs) and transport (AUX1 and PIN1) (Yang et al. 2006), further supporting the importance of auxin and GA in cotton fiber development.

In a cDNA array analysis, five putative auxin response genes were found to be highly expressed in fibers during fast elongation stage (6–12DPA), while their transcript levels were low both before and after the fast elongation stage (Gou et al. 2007). By contrast, expression of a putative auxin-repressed gene (homologous to AF336307) did not increase until the start of secondary cell wall synthesis. These data suggest a high level of auxin response present in rapidly elongating fiber cells and further support the classical assumption that auxin plays a role in promoting cotton fiber elongation (Gou et al. 2007).

9.3.2.2 BR and Ethylene

BR is required for normal plant growth and development. In general, BR has a similar effect as auxin in positively influencing fiber cell development. Application of low concentrations of brassinolide (BL) promoted fiber elongation whereas brassinazole (Brz), a brassinosteroid biosynthesis inhibitor, inhibited fiber development (Sun et al. 2004, 2005; Shi et al. 2006). Treatment of cotton floral buds with Brz resulted in complete absence of fiber differentiation (Sun et al. 2005), and this inhibitory effect could be reversed by simultaneous BL application, confirming that BR is required for fiber initiation and elongation.

The GH_TMO library is enriched with the ESTs corresponding to genes involved in BR biosynthetic and signaling pathways (Yang et al. 2006). BRI1 EMS SUPPRESSOR1 (BES1) is a downstream positive regulator in BR signaling pathway. Overexpression of *BES1* was able to promote stem elongation in *Arabidopsis*. Two putative cotton *BES1* homologs were present in the GH_TMO library, but not in the cDNA libraries derived from elongating fibers, suggesting an enhanced role of BR in fiber cell differentiation. *BR-INSENSITIVE 2* (*BIN2)* is presumed to be a negative regulator of BL signaling, and overexpression of *BIN2* delayed the development of *Arabidopsis*. The mRNAs of putative cotton *BIN2* homologs were highly accumulated during fiber cell elongation stage (Sun and Allen 2005). At the same time, the cotton homolog of *Arabidopsis BRASSINOSTEROID INSENSITIVE1* (*BRI1)*, which encodes a transmembrane BL receptor, was also expressed. Overexpression of cotton *BRI1* rescued the phenotype of *bri1* mutant of *Arabidopsis* (Sun et al. 2004). Expression of the fiber genes associated with cell elongation, including *XYLOGLUCAN ENDOTRANSGLUCOSYLASE/HYDROLASE (XTH)* and *EXPANSIN (EXP)*, *ACYL CARRIER PROTEIN (ACP)* and *ARABINOGALACTAN PROTEIN* (*AGP*) and *GhTUB1* (microtubule protein), was increased in ovules treated with BL and suppressed by Brz application, demonstrating that BR promotes fiber elongation by upregulating the expression of genes involved in cell expansion and cell wall reconstruction (Sun et al. 2005). DE-ETIOLATION 2 (DET2), a steroid 5α-reductase, catalyzes a major rate-limiting step of BR biosynthesis in *Arabidopsis*. A high level of *GhDET2* transcripts was detected during the fiber initiation and rapid elongation stages. Antisense-mediated

suppression of *GhDET2* inhibited both fiber initiation and fiber elongation (Luo et al. 2007). Therefore, the level BRs play a crucial role in the initiation and elongation of cotton fiber cells, suggesting that modulation of BR biosynthesis may improve fiber quality and yield.

Interestingly, physiology and gene expression studies by Zhu and his colleagues revealed an apparent role of ethylene in promoting fiber development (Shi et al. 2006). Addition of ethylene promoted fiber growth in the in vitro cultured ovules, whereas application of aminoethoxyvinylglycine (AVG), an ethylene inhibitor, inhibited fiber cell elongation. In addition, mRNA levels of three cotton 1-amino-cyclopropane-1-carboxylate (ACC)-oxidase (ACO) genes peaked during fiber elongation. They further demonstrated that saturated very-long-chain fatty acids (VLCFAs; C20:0–C30:0) may act upstream of ethylene to maximize the extensibility of cotton fibers. The lignoceric acid (C24:0) stimulated *ACO* gene expression rapidly that resulted in substantial elevation of ethylene production (Qin et al. 2007). In fact, molecular investigation of rice (*Oryza sativa*) and the marsh dock (*Rumex palustris*) also illustrated that ethylene was a major factor to promote underwater elongation of stems or leaves along with interactions with other hormones (Jackson 2007).

9.3.2.3 ABA and Cytokinin

ABA was proposed to play a negative role in cotton fiber growth, as application of ABA to the culture of unfertilized ovules caused retardation of fiber development (Beasley and Ting 1974). The inhibitory effect of ABA was partially compensated for by an addition of cytokinin, although cytokinin alone also showed an inhibitory effect on fiber growth (Lee et al. 2007). A notable feature of cotton fiber development is the overlap of elongation and second cell wall biosynthesis stages. It was shown that the high level of endogenous ABA occurred at 16DPA, when the content of cellulose was increasing dramatically, implicating a rise of ABA content as a signal of secondary cell wall biosynthesis, and that the ratio of ABA to auxin levels might be relevant to the regulation of secondary cell wall thickening (Yang et al. 2001).

9.3.3 Cytoskeleton Genes

Expansins are cell wall proteins that facilitate cell wall extension by disruption of noncovalent bonds between wall components. Six genes encoding expansins were isolated. Among them transcripts of *GhEXP1* were abundant in fiber cells (Harmer et al. 2002). In developing cotton fiber cells, four genes that belong to the α-expansin family were highly expressed during the fiber outgrowth and fast elongation stages, and were generally downregulated when cells entered the secondary cell wall synthesis stage (Gou et al. 2007).

Microtubules are considered dynamic structures that play a central role in many important processes, such as cell division, cell motility, intracellular transport, and cell shaping. In plants, microtubules with α- and β-tubulins as the major structural components are especially important for cell morphogenesis. Cotton fiber cells contain abundant amounts of tubulins. He et al. (2008) identified 795 cotton tubulin ESTs and cloned 19 β-tubulin (*GhTUB*) genes at cDNA level; of them nine *GhTUBs* were expressed at higher levels in the elongating fiber cells than in ovules of the *fl* mutants. The expression of *Gh-β-TubL* was correlated with the elongation pattern of fiber cells, and its mRNAs were barely detected in *fl* ovules. Overexpression of *Gh-β-TubL* in fission yeast promoted longitudinal growth by 1.74 fold (Ji et al. 2002). Another β-tubulin gene, *GhTUB1*, was also highly expressed in cotton fiber cells. Histochemical assay of the *GhTUB1* promoter fused to the *β-Glucuronidase* (*GUS*) reporter gene in transgenic cotton plants showed that high levels of GUS activities were located in young fiber cells, with weak or no GUS expression in other tissues (Li et al. 2002b).

Actin cytoskeleton plays an important role in cell morphogenesis and is essential for cell elongation and tip growth. Actins are encoded by a multigene family that comprises dozens or even hundreds of *ACTIN* (*ACT*) genes. In cotton, 15 *GhACT* genes have been identified, among them *GhACT1* was highly transcribed in elongating fiber cells from 8 to 14DPA; importantly, its transcript was barely detectable in other tissues. RNA interference (RNAi) of *GhACT1* substantially reduced the accumulation of its mRNA and protein, and disrupted the actin cytoskeleton network in fibers, resulting in a dramatic reduction of fiber length, but without significant inhibition on fiber initiation, suggesting that *GhACT1* has a critical function in fiber elongation (Li et al. 2005).

Profilin is an important actin-binding protein involved in regulating the organization of actin filaments. Expression of a cotton profilin gene (*GhPFN1*) is tightly associated with fast elongation of fiber cells. Overexpression of *GhPFN1* in transgenic tobacco cells was correlated with the formation of elongated cells that contained thicker and longer microfilament cables (Wang et al. 2005), suggesting that GhPFN1 may play a role in cotton fiber elongation by promoting actin polymerization.

9.3.4 Other Genes

From a physiological view, the process of fiber cell elongation is associated with strong cell turgor pressure and plasmodesmatal dynamics. During fiber cell development, plasmodesmata are opened from 0 to 9DPA, closed at 10DPA, and opened again at 16DPA. Rapid cell elongation is also associated with transporter activities, with a high expression level of sugar transporter gene during elongating stage (Ruan et al. 2001). *SuSy*, an encoding sucrose synthase, is highly expressed in initiating and elongating fiber cells, but not in adjacent normal epidermal cells. *SuSy* expression was dramatically reduced in the epidermis of *fl* mutant ovule, in

correlation with the lack of fiber initials (Ruan and Chourey 1998). Suppression of *SuSy* expression in transgenic plants resulted in reduced hexose levels in the ovules with a reduction of SuSy activity by 70% or more in ovule epidermis, leading to a fiberless phenotype (Ruan et al. 2003).

In the secondary cell wall synthesis stage, cellulose synthesis is a major event in cotton fiber. Cotton *ceLA1* and *ceLA2* were the first plant cellulose synthase (CesA) genes identified (Pear et al. 1996). At least five *CesA* genes showed increased expression levels during secondary cell wall synthesis (Gou et al. 2007). Expression of the gene encoding endo-1,4-/D-glucanase, a cell wall related enzyme, was decreased when cell elongation ceased (Shimizu et al. 1997), suggesting that it plays a specific role during fast elongation stage.

9.3.5 Fiber-Specific Promoters

Compared to the constitutive promoters, such as the CaMV *35S* promoter, a tissue-specific promoter can direct target gene expression in a specified tissue without altering pathways in other tissues, thus avoiding negative effects on plant growth. Promoters from a number of fiber-specific genes have been isolated and their activities were assayed using transgenic plants. Most of the fiber gene promoters examined show trichome- or fiber-specific (or preferential) activities and are of value in genetic engineering of cotton fiber traits. Promoter of *E6*, the first isolated fiber-specific gene, was the first one used for engineering cotton fiber quality (John and Keller 1996). *GhRDL1*, a gene highly expressed in cotton fiber cells at the rapid elongation stages, encodes a BURP-domain containing protein (Li et al. 2002a). *GaRDL1* promoter exhibited a trichome-specific expression pattern in transgenic *Arabidopsis* plants (Wang et al. 2004). *GhTUB1* transcripts accumulated preferentially in fiber cells and *pGhTUB1*::*GUS* fusion reporter was expressed at a high level in fibers, with only a much lower level in other tissues (Li et al. 2002b). Some other promoters are less tissue specific. For example, promoters of three cotton lipid transfer protein genes, *LTP3*, *LTP6,* and *FSltp4*, and other genes such as *GhGlcAT1* and *GhRGP1* were able to direct *GUS* gene expression in leaves and stems in transgenic tobacco plants (Hsu et al. 1999; Delaney et al. 2007; Wu and Liu 2006; Wu et al. 2007). A recent report (Shangguan et al. 2008) showed that the promoter of *GaMYB2* is active in various trichome cells. In cotton, *GaMYB2* promoter exhibited activities in developing fiber cells and trichomes of other aerial organs, including leaf, stem, and bract. In *Arabidopsis*, *GaMYB2* promoter was specific to trichomes. It is interesting that in tobacco plants, *GaMYB2* promoter directed *GUS* expression exclusively in glandular cells of the glandular-secreting trichomes. In addition to their application in genetic engineering, these promoters provide a valuable tool to dissecting the molecular mechanisms that regulate gene expression in leaf trichomes and cotton fiber cells.

9.4 Experimental Systems Used for Investigation of Cotton Fiber Genes

Cotton transformation is awfully tedious and the long process of tissue culture often induces phenotypic changes, particularly in T0 generation plants. Till now, only a few genes have been functionally characterized using transgenic cotton plants, including *SuSy* (Ruan et al. 2003), *GhACT1* (Li et al. 2005), *GhDET2* (Luo et al. 2007), and *GhMYB109* (Pu et al. 2008). To accelerate research, several exogenous systems including *Arabidopsis*, tobacco, and yeast have been employed for functional analyses of cotton genes. However, given the fact that *Arabidopsis* and *Gossypium* belong to different plant families (*Brassicaceae* and *Malvaceae*, respectively), and that *Arabidopsis* does not produce trichomes on seed, none of these "models" mentioned can replace cotton. Therefore, a more efficient and stable transformation procedure is required for cotton functional genomics. Very recently, application of virus induced gene silencing (VIGS) system on cotton has been reported (Tuttle et al. 2008). If the silencing effect can spread into ovules, VIGS can be greatly helpful for the molecular dissection of cotton fiber development.

9.5 Summary and Perspectives

In recent years, comprehensive analyses of gene expression profiles have provided a large number of candidate genes that are potentially involved in cotton fiber development and growth. However, how these genes are coordinately expressed and how their products can perform in concert remains largely unknown. There is no doubt that cotton genome research will provide enormous genomic data that the entire community of cotton scientists and breeders are waiting for. Improvement of technologies such as genetic transformation will further enable validation of function of many of these candidate genes. VIGS technology is also urgently needed for large scale functional genomics research of cotton.

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Chapter 10
Global Adoption of Biotech Cotton, 1996 to 2007

C. James and B. Choudhary

10.1 Introduction

This chapter provides an overview of the global adoption of biotech cotton, sometimes referred to as genetically modified (GM), or transgenic cotton, from its first commercialization in 1996–2007, a 12-year period. The adoption of biotech cotton in India, the largest cotton-growing country in the world, is documented in detail, along with reviews for each of the other three principal countries, China, USA, and Australia, each of which has grown more than 1 million ha during the first 12 years of commercialization.

10.2 Regulation and Approval of Biotech Cotton

All biotech crops are rigorously regulated and are subjected to detailed analysis by official national regulatory bodies before they are deregulated and approved for commercialization. Biotech crops typically take up to 10 years to develop, with the regulatory approval process costing up to $100 million per product (Burrill and Company 2008). The 10-year process starts with gene discovery followed by laboratory and contained facility testing to open field testing to generate a voluminous dossier for a submission request for deregulation and commercialization of the product. Furthermore, approval is required by each country prior to commercialization, making the process an extremely high cost and resource-intensive process. Some countries, for example the USA, do not require further approval for stacked

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events if the events have already been approved as single events. Other countries, for example South Africa and Argentina, require an additional approval of the stacked trait product even though the single traits in the stacked product have already been approved as single traits. This can lead to undue and costly delays of several years in approval of stacked products, representing a significant opportunity cost to countries because when commercialization is delayed, farmers are denied the advantages of their counterparts in other countries which do not require separate approval of stacked products.

A total of approximately 40 biotech cotton products have been approved between 1996 and 2008, and they fall into three categories (James 2007): resistance to lepidopteran insect pests, particularly cotton bollworm species (IR or Bt); herbicide tolerance (HT); and the stacked product of the two traits (IR/HT or Bt/HT). By far, the most prevalent biotech cottons have been Bollgard® and Bollgard®II (an improvement on Bollgard) for insect resistance; and Roundup Ready®(RR) for herbicide (glyphosate) tolerance. An improved RR product named RR® Flex was approved in the USA in 2005, and later in Australia, South Africa, and Mexico. Stacked traits are becoming more popular because they respond to the multiple yield constraints of farmers.

The first country to approve a biotech cotton was the USA in 1994 (2 years prior to commercialization), when it deregulated a BXN cotton, which was tolerant to the herbicide bromoxynil. In 1995, the USA approved two additional biotech cotton products: a Bt cotton Bollgard® that confers insect resistance and Roundup Ready® (RR® cotton), which confers herbicide tolerance. In 1996, the first year of commercialization of biotech cotton globally, only three countries — USA, Australia, and Mexico, commercialized single trait insect resistance products, with China following in 1997 with a fused Bt product developed by the Chinese Agricultural Academy for Science (CAAS). South Africa and Argentina approved an insect resistant product in 1997, India in 2002, Colombia in 2003, and finally Brazil in 2005. The pattern of introduction was as follows: Generally, the Bt insect resistance traits were the first to be approved, followed by herbicide tolerance, and the stacked traits of insect resistance and herbicide tolerance were approved last. Burkina Faso approved the BG®II trait for insect resistance in 2008, making it the tenth country to approve biotech cotton traits on a global basis.

10.3 Global Hectarage of Biotech Cotton, 1996–2007

The chronological order of the countries introducing biotech cotton is listed in Table 10.1. The USA, Australia, and Mexico were the first three countries to commercialize biotech cotton in 1996 followed by China in 1997, Argentina and South Africa in 1998, India and Colombia in 2002, Brazil in 2006, and finally, Burkina Faso in 2008. It is noteworthy that the nine countries commercializing biotech cotton span the principal continents of Asia, North and South America, and Africa. A cumulative total of 81.423 million ha of biotech cotton was grown

Table 10.1 First year of commercialization of biotech cotton, by country (1996–2008)

Year	Countries
1996	USA, Australia, and Mexico
1997	China
1998	Argentina and South Africa
2002	India and Colombia
2006	Brazil
2008	Burkina Faso

Source: Compiled by James (2008)

Table 10.2 Accumulated biotech cotton hectarage in nine countries (1996–2007)

Country	Millions of hectares	Percentage
More than 1 million ha		
USA	44.062	54
China	21.580	26
India	11.973	15
Australia	1.468	2
Subtotal	79.083	97
Less than 1 million ha		
Argentina	0.920	1
Brazil	0.620	1
Mexico	0.510	<1
South Africa	0.192	<1
Colombia	0.098	<1
Subtotal	2,340	3
Global total	81.423	100

Source: Compiled by James (2008) and ISAAA Annual Briefs on the Global Status of Commercialized Biotech/GM crops, 1996–2007

globally in the period 1996–2007. To put 81.423 million ha of biotech cotton into context, it is equivalent to two-thirds of the total arable land area of Germany (121 million ha), or about half the total arable land area of India (177 million ha), China (143 million ha), or the USA (165 million ha).

It is common knowledge that in some countries, biotech crops are sometimes grown by farmers prior to approval, and biotech cotton is not an exception. However, all the data in this chapter relate to biotech cotton that has been legally approved for commercialization in nine countries, seven developing countries, and two industrial countries; these are listed in Table 10.2 in decreasing order of millions of hectares of biotech cotton. The accumulated hectarage, totaling 81.423 million ha during the period 1996–2007, is shown in Table 10.2. The data in Table 10.2 indicate that the USA was the leading country in terms of planted biotech cotton during the first 12 years of commercialization and grew 44.062 million ha, equivalent to 54% of the global accumulated hectarage of biotech cotton during the period 1996–2007. China was ranked second and grew 21.580 million ha equivalent to 26%, India was third with 11.973 million ha equivalent to 15%, and Australia was fourth with 1.468 million ha or 2%. These top four countries, comprising two developing countries and two industrial countries, collectively

grew 97% of the global biotech cotton between 1996 and 2007, and each grew more than 1 million cumulative hectares.

The second group of five countries collectively grew a total of 2.34 million ha, equivalent to 3% of the cumulative global total of 81.423 million ha in the period 1996–2007. The five countries are all developing countries and include in decreasing order of biotech cotton hectarage: Argentina, planted 0.920 million ha equivalent to 1% of global; Brazil, 0.620 million ha or 1%; Mexico, 0.510 million ha equivalent to less than 1%; South Africa, 0.192 million ha at less than 1%; and finally Colombia with 0.098 million ha at less than 1% of the global total. Whereas the USA is the top biotech cotton-growing country with 54% of the world's total hectarage, there are only two industrial countries growing Bt cotton, the USA and Australia (which collectively grew 56%, equivalent to 45.53 million ha), compared with seven developing countries, led by China and India. The seven developing countries collectively grew 35.893 million ha, equivalent to 44% of the global total of 81.423 million ha of biotech cotton grown in 1996–2007.

Four self-explanatory graphs (Figs. 10.1–10.4) depict the global adoption of biotech cotton using two different parameters; hectares of biotech cotton planted, and the same data expressed as percentage adoption. The first two graphs, Figs. 10.1 and 10.2, exhibit the relative contribution to global adoption of the four principal countries USA, China, India, and Australia. In Fig. 10.2 the data from Fig. 10.1 are expressed as a percentage of the national total area of cotton planted in each country. The second two graphs (Figs. 10.3 and 10.4) show the relative contribution of the three categories of traits (Bt, HT, and Bt/HT) to global adoption, expressed in hectares (Fig. 10.3) and the same data expressed as percentage of the global area of cotton (Fig. 10.4). The following are the trends and highlights exhibited in Figs. 10.1–10.4.

Figure 10.1 indicates that the global hectarage of biotech cotton has climbed consistently every single year since its introduction in 1996 (831,000 ha) to 15 million ha in 2007; that is an18-fold increase over 12 years and equivalent to 44% of the

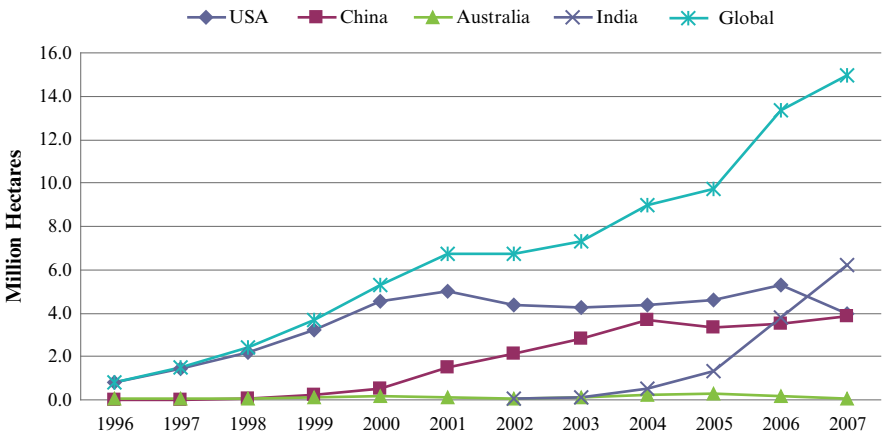

Fig. 10.1 Adoption of biotech cotton globally and in the four principal countries (1996–2007)

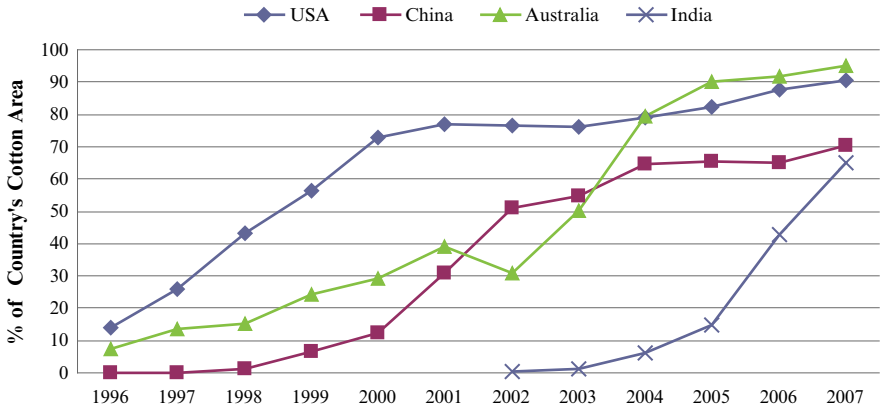

Fig. 10.2 Adoption of biotech cotton in four principal countries, expressed as percentage of cotton area planted in each country (1996–2007)

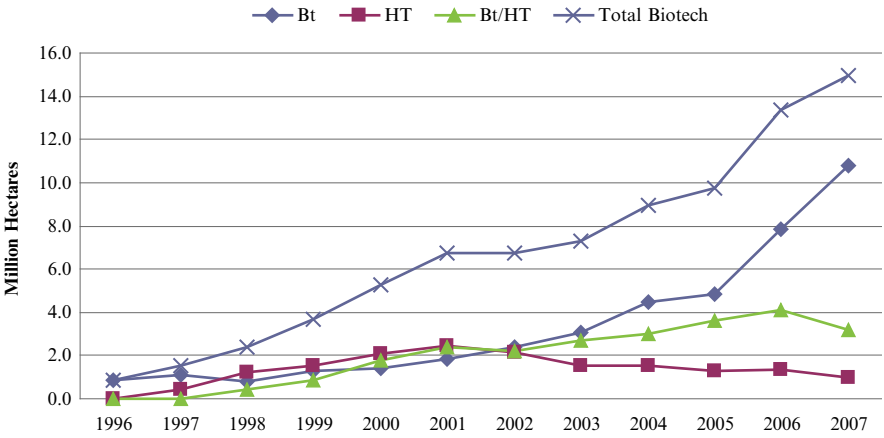

Fig. 10.3 Global adoption of biotech traits in cotton (1996–2007)

total global area of 34 million ha of cotton. Figure 10.1 also shows the dominance of the USA particularly in the early years, whereas China adopted at a more modest pace than the USA. India's very rapid rise from a few thousand hectares in 2002 to 6.2 million ha in 2007 is notable. Not surprisingly, Australia which grows only a tenth of the cotton hectarage of the USA is ranked as the lowest of the four countries in Fig. 10.1, when adoption measured in hectares of adopted biotech cotton. However, when adoption at the country level is measured in percent (Fig. 10.2), Australia has the highest adoption rate with over 90% of its total cotton hectarage planted to biotech cotton in 2007, marginally higher than the USA. Figure 10.2 clearly shows the early dominance of the USA in percent adoption until it was overtaken by Australia in 2004. China has consistently increased its percent adoption from a low

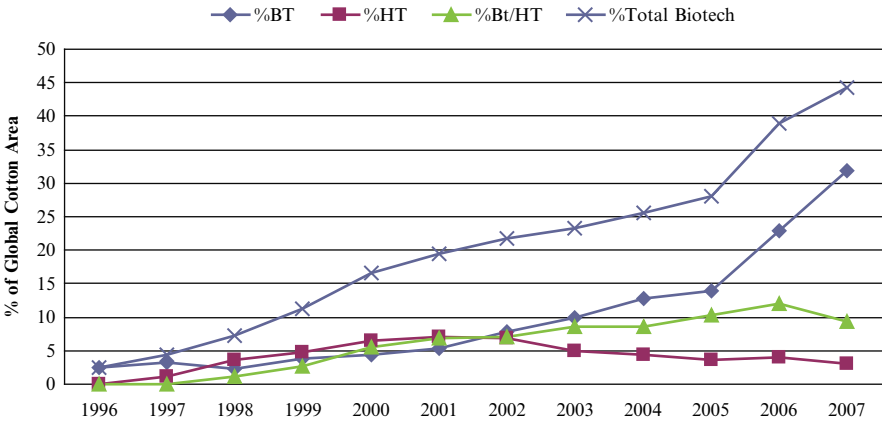

Fig. 10.4 Global adoption of biotech traits expressed as percentage of global cotton area (1996–2007)

of approximately 10% in 1997 to almost 70% in 2007. India's rate of adoption has been very high reaching 66% after only 6 years of adoption.

The data in Fig. 10.3 show the relative importance of the three categories of biotech cotton Bt, HT, and Bt /HT as measured in hectares. The dominance of the Bt category is evident, particularly from 2002 onward when India's contribution to the Bt category, added to that of China, results in increasingly large hectarages to the Bt category. For 2007, the relative hectarages of the three categories were Bt, 11 million ha or 75% of global; Bt /HT, 3 million ha or 20% of global, and HT 1 million ha, or less than 10% of global. The decrease in hectarage of Bt/ HT and to a lesser extent HT, exhibited in Fig. 10.3 between 2006 and 2007 is due to significant decreases in total plantings in cotton in both the USA and Australia, where the Bt/HT category is the major category in both countries, 85% in Australia and 78% in the USA. The trends in Fig. 10.3, based on hectares, are reflected in Fig. 10.4 data based on percent of global cotton hectarage. In 2007, 44% of the global hectarage of cotton was planted to biotech cotton, approximately 31% was in the Bt category, 10% was in the Bt/ HT category and only the balance of 3% in the HT category. The dominance of the Bt category is due to the large hectarages of Bt cotton in India (6.2 million ha in 2007) and China (3.8 million ha in 2007). The large Bt hectarages in India and China impact significantly on global trends despite the high percentage adoption rates of over 90% in Australia and the USA in 2007.

10.4 Country Case Studies

Country case studies for India, China, USA, and Australia are presented. The country case study for India, the largest cotton-growing country in the world, is presented in detail with overviews for China, USA, and Australia.

10.4.1 India Case Study

10.4.1.1 Introduction

India is highly dependent on agriculture, which generates almost one-quarter of its GDP and provides two-third of its people with their means of survival. India is a nation of small resource-poor farmers, most of whom do not make enough income to cover their meager basic needs and expenditures. Of the 90 million farmer households in India, approximately 85 million, which represent about 95% of all farmers, are small and resource-poor farmers who do not make enough money from the land to make ends meet (National Sample Survey India 2003). In the past, these included the vast majority of the 5.5 million Indian cotton farmers. India has a larger area of cotton than any country in the world, 9.4–9.5 million ha in 2007 cultivated by approximately 5.5 million farmers. Whereas India's cotton area represents 25% of the global area of cotton, it produced only 12% of the world production prior to the introduction of Bt cotton.

Approximately 65% of India's cotton is produced on dryland and 35% on irrigated lands. In 2006, hybrids occupied 80% (7.4 million ha) of the cotton area and 20% (1.8 million ha) were occupied by varieties. The percentage devoted to hybrids has increased significantly over the last few years, a trend that has been accentuated by the introduction in 2002 of high-performance Bt cotton hybrids, which have outperformed conventional hybrids. Cotton is the major cash crop of India and accounts for 75% of the fiber used in the textile industry. Cotton impacts the lives of an estimated 60 million people in India, including farmers who cultivate the crop, and a legion of workers involved in the cotton industry from processing to trading. India is the only country to grow all four species of cultivated cotton *Gossypium arboreum* and *G.herbaceum* (Asian cottons), *G. barbadense* (Egyptian cotton), and *G. hirsutum* (American upland cotton). *Gossypium hirsutum* represents 90% of the hybrid cotton production in India, and all the current Bt cotton hybrids are *G. hirsutum*. Potential losses due to insect pests of cotton are very high and estimated at 35%–40% globally (Oerke 2002). More specifically, Lepidopteran pests are very important; cotton bollworm (*Helicoverpa armigera*) particularly, is estimated to cause losses valued annually at $300 million in India alone (King 1994). The importance of Bt cotton is that it confers resistance to the most important insect pest of cotton in India and also affords some protection to some other lepidopteran pests of cotton.

10.4.1.2 Approval and Adoption of Bt Cotton in India

Following several years of successful field trials with Bt cotton, the Genetic Engineering Approval Committee (GEAC) of the Indian Government approved on March 26, 2002, the commercial cultivation of three Bt cotton Bollgard® hybrids containing *cry1Ac* Bt genes: Mech 12, Mech 162, and Mech 184, developed and registered by Mahyco (Maharastra Hybrid Seed Company) in India, in which

the technology was sourced from Monsanto (Luce 2002). The GEAC approval was for 3 years and required farmers to ensure a refuge of 20% or five rows, whichever is greater, and for Mahyco to provide the seed for refuge and to monitor the development of insect resistance, if any, by generation of base line susceptibility data (Hindu 2002; Ramachandran 2002). It is noteworthy that India is the only country in the world using hybrid Bt cotton seed extensively, whereas all other eight countries use Bt cotton varieties. For the Kharif season plantings in 2002, farmer demand for Bt cotton seed was very high. The seed was sold in packets containing 450 g of Bt cotton seeds and 120 g of non-Bt cotton seeds sufficient to plant 1 acre of Bt cotton and required refuge.

The adoption of Bt cotton in India over the period 2002–2007 is captured in Fig. 10.5 which provides annual information on three parameters: the total area planted to cotton, the hectares planted to Bt cotton; and the Bt cotton hectares expressed as a percentage of total cotton. The adoption rates for Bt cotton in India are unprecedented. In 2002, 54,000 farmers grew approximately 50,000 ha of officially approved Bt cotton hybrids for the first time. In 2003, the Bt cotton area doubled to approximately 100,000 ha. The Bt cotton area increased again fourfold in 2004 to reach half a million hectares. In 2005, the area planted to Bt cotton in India continued to climb reaching 1.3 million ha, an increase of 160% over 2004. In 2006, the record increases in adoption continued with almost a tripling of the area of Bt cotton to 3.8 million ha. This tripling in area was the highest percentage year-on-year growth for any country planting biotech crops in the world in 2006. Notably in 2006, India's Bt cotton area (3.8 million ha) exceeded for the first time, that of China's 3.5 million ha. In 2007, the Indian cotton sector continued to grow with a record increase of 63% in adoption of Bt cotton area from 3.8–6.2 million ha; this was the third consecutive year for India to have the largest year-on-year percentage growth of all biotech cotton growing countries in the world; a 160% increase in 2005, followed by a 192% increase in 2006 and a 63%

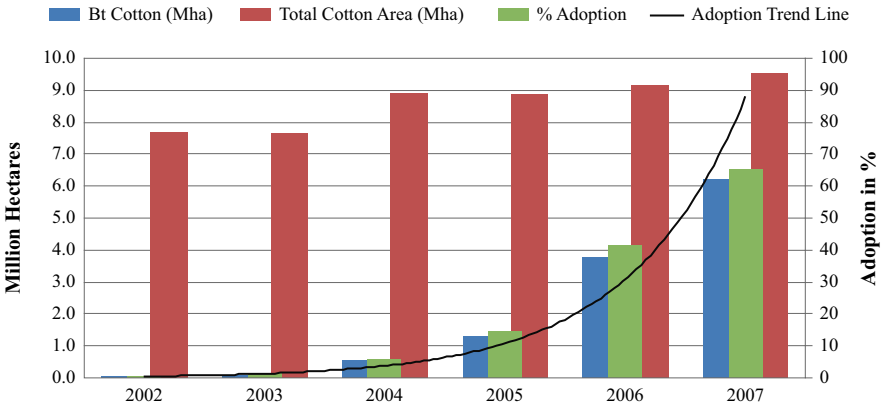

Fig. 10.5 Adoption of Bt cotton in India (2002–2007)

increase in 2007. In addition, in 2006/2007 India overtook the USA to become the second largest cotton producing country in the world, after China (FAS /FAS 2007).

Of the estimated 9.4 million ha of cotton in India, in 2007, 66% or 6.2 million ha were Bt cotton – a remarkably high proportion in a fairly short period of 6 years. Thus, there was an unprecedented 138-fold increase in area between 2002 (50,000 ha) and 2007 (6.2 million ha), which is the highest recorded adoption rate for any crop technology globally. This compares with a 67-fold increase globally for biotech crops over the 12-year period, 1996–2007 (James 2007); thus, the growth rate for India at 138-fold over 6 years compared with global growth at 67-fold over 12 years is approximately four times as fast. Of the 6.2 million ha of hybrid Bt cotton grown in India in 2007, 35% was under irrigation and 65% rain-fed. A total of 131 Bt cotton hybrids were approved for planting in 2007 compared with 62 in 2006, 20 in 2005 and only 4 Bt cotton hybrids in 2004. Over the years, India has also diversified deployment of Bt genes and hybrids, which are adapted to different agro-ecological zones and to ensure equitable distribution to small and resource-poor cotton farmers. The distribution of Bt cotton in the major growing states varies significantly. The major states growing Bt cotton in 2007 were: Maharashtra (2.8 million ha, equivalent to 46% of all Bt cotton in India); Andhra Pradesh (1.2 million ha or 19%); Gujarat (818,000 ha or 13%), Northern Zone (592,000 ha or around 10%), Madhya Pradesh (500,000 ha or 8%), and the balance in Karnataka and Tamil Nadu and other states.

It is conservatively estimated that approximately 3.8 million small and resource-poor farmers planted on average 1.6 ha of Bt cotton in 2007. The number of farmers growing Bt cotton hybrids in India has increased dramatically from 54,000 in 2002 to 80,000 in 2003, 300,000 small farmers in 2004 to 1 million in 2005, with over a twofold increase to 2.3 million farmers in 2006 and to 3.8 million farmers in 2007; this was the largest increase in number of farmers planting biotech crops in any country in 2007. The adoption of Bt cotton by 3.8 million small and resource-poor farmers represents around 70% of the total 5.5 million cotton farmers in India who are reaping significant benefits from Bt cotton.

Coincidental with the steep increase in adoption of Bt cotton between 2002 and 2007, the average yield of cotton in India, which had one of the lowest yields in the world, increased from 308 kg/ha in 2001–2002, to 560 kg/ha in 2007, with most of the increase in yield (up to 50% or more), attributable to Bt cotton. At a national level, Bt cotton is a major factor contributing to higher cotton production, which increased from 15.8 million bales (one bale weighs 170 kg or 375 lbs) in 2001–2002, to an estimated record 31 million bales (Cotton Advisory Board, India 2007). This quantum leap in cotton production since 2002–2003 has been triggered by improved seeds and particularly the ever-increasing plantings of improved Bt cotton in the nine cotton-growing states (Textile Commissioner Office, India 2007).

With the boom in cotton production in the last 5 years, India has become transformed from a net importer to a net exporter of cotton. Exports of cotton have registered a sharp increase from 0.92 million bales in 2004–2005 to 4.7 million bales in 2005–2006. The Cotton Advisory Board of the Government of India expects cotton exports to increase again to over 4.8 million bales in 2007–2008

(Lok Sabha India 2007). Concurrent with the boom in cotton production the Indian biotech and seed industry has also been growing at an unprecedented rate with high year-on-year growth because of the high adoption of Bt cotton by Indian farmers.

10.4.1.3 Approval of Gene Events and Bt Cotton Hybrids in India

The number of gene events, as well as the number of Bt cotton hybrids and companies marketing approved hybrids have all increased from 2002, the first year of commercialization of Bt cotton in India. The number of Bt cotton hybrids increased by more than twofold from 62 hybrids in 2006 to 131 hybrids in 2007. This has provided much more choice than previous years to farmers in the North, Central, and Southern regions, where specific hybrids have been approved for cultivation in specific regions (Fig. 10.6). A total of four gene events were approved for incorporation in a total of 131 hybrids offered for sale in 2007.

The first gene event, Bollgard®I (BG-I), featuring the *cry1Ac* gene was developed by Maharashtra Hybrid Seed Company Ltd. (Mahyco), sourced from Monsanto, and approved for sale for the sixth consecutive year in a total of 96 hybrids in 2007 for use in the North, Central, and South zones – this compares with 48 BG-I hybrids in 2006.

The second gene event, Bollgard®II (BG-II with event MON15985) also developed by Mahyco and sourced from Monsanto, featured the stacked genes *cry1Ac* and *cry2Ab*, was approved for sale for the first time in 2006 in a total of seven hybrids for use in the Central and South regions. This gene event was approved for commercial cultivation for the first time in the Northern region in 2007 and the number of hybrids for sale increased from 7 in 2006 to 21 in 2007 in the North, Central, and South regions.

The third gene event, marketed by JK Seeds featured the *cry1Ac* gene known as gene event 1, was sourced from IIT Kharagpur, India. The gene event was approved for sale for the first time in 2006 in a total of four hybrids for use in the North, Central, and South regions. Whereas this gene event was approved in four hybrids in 2006, the number doubled to eight hybrids in 2007.

The fourth and last gene event, the GFM gene event was developed by Nath Seeds, sourced from China, featured the fused genes *cry1Ab* and *cry1Ac* and approved for sale for the first time in a total of three hybrids in 2006, one in each of the three regions of India. In 2007, the number of hybrids doubled and six were offered for sale in three regions (Fig. 10.6).

In 2006, 15 companies offered 62 hybrids for sale in India. In 2007, both the number of hybrids and the number of companies increased significantly from 62 to 131 hybrids and the number of indigenous companies from 15 to 24 (Fig. 10.6). In 2007, the GEAC approved 69 new Bt cotton hybrids for commercial cultivation in the 2007 season, in addition to the 62 Bt cotton hybrids approved for sale in 2006, for a total of 131 hybrids. This has given farmers in India's three cotton-growing zones significantly more choice of hybrids to cultivate in 2007.

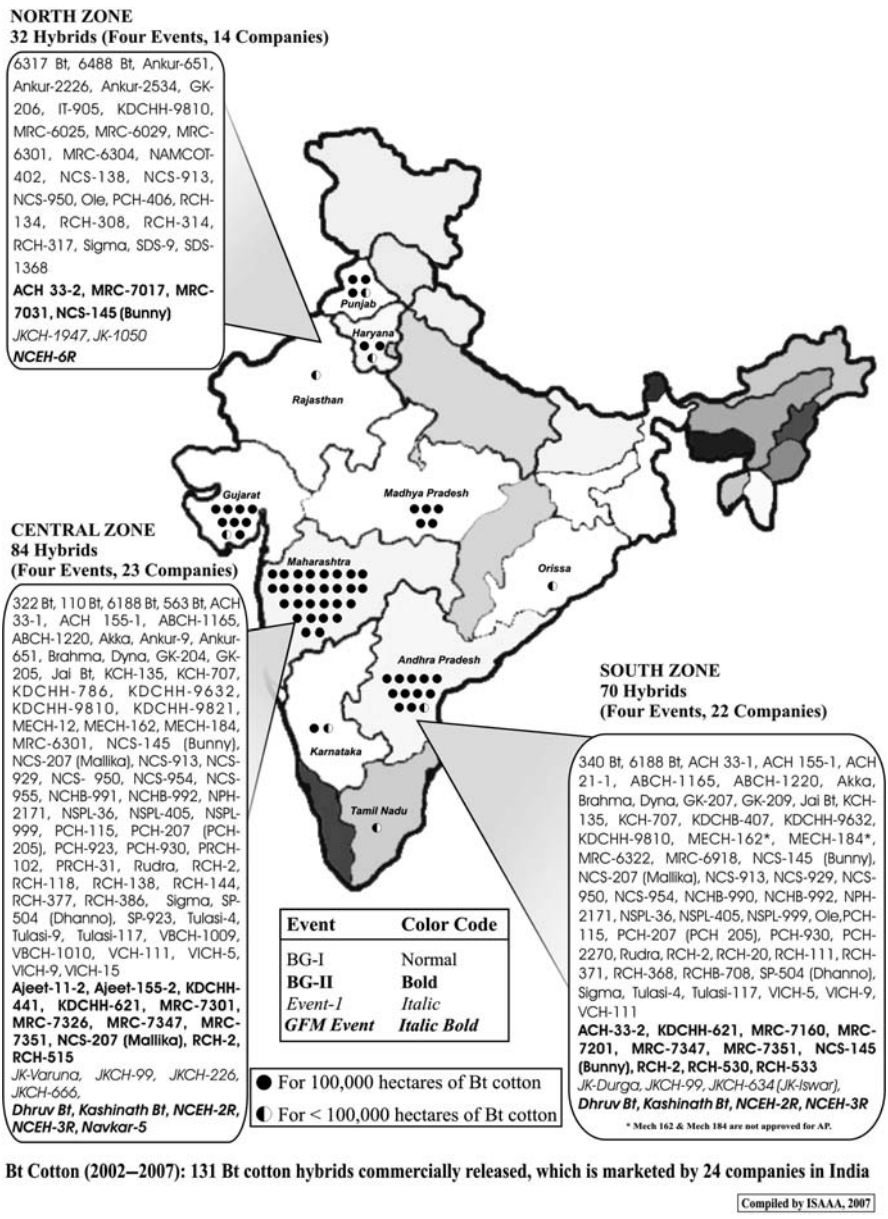

Fig. 10.6 Approval of gene events and Bt cotton hybrids in India, 2007

The adoption of Bt cotton in India is a remarkable experience, which has already benefited 3.8 million small and resource poor farmers, and the number is expected to continue to grow in 2008 and beyond. The significant increase in income of small

farmers growing Bt cotton has contributed to the alleviation of poverty of millions of small farmers and allowed insecticide applications to be reduced with significant positive environmental implications. A study by Gandhi and Namboodiri (2006) reported an average yield gain of 31%, a significant 39% reduction in the number of pesticide sprays, and an 88% increase in profit or an increase of US$250 per hectare for the 2004 cotton growing season. The cumulative benefit from biotech Bt cotton in India over the period 2002 to 2006 is estimated at $1.3 billion (Brookes and Barfoot 2008).

There is strong and growing political support for Bt cotton in India and in turn for other biotech crops. This is due to the remarkable progress that has been achieved in a relatively short period of time, with yields almost doubling in 5 years and multiple material and welfare benefits evident to farmers, the textile industry, exports, and at the national level. Leading politicians and policy makers have become advocates of biotechnology because of the multiple benefits it offers. Finance Minister P. Chidambaram has called for emulation of the cotton production success story, through the use of genetically modified Bt cotton, in the area of food crops to make the country self sufficient in its food needs. "It is important to apply biotechnology in agriculture. What has been done with Bt cotton must be done with food grains," Chidambaram said at the opening of the seventh edition of Bangalore's annual biotechnology event Bio-2007 on 7–9 June 2007 at Bangalore.

The approval and adoption of Bt cotton by the two most populous countries in the world, India (1.1 billion people) and China (1.3 billion people), can greatly influence the approval, adoption, and acceptance of biotech crops in other countries throughout the world, particularly in developing countries. It is noteworthy that both countries elected to pursue a similar strategy by first exploring the potential benefits of crop biotechnology with a fiber crop, Bt cotton, which has already generated significant and consistent benefits in China for over 10 years, with the same pattern emerging in India, the largest grower of cotton in the world.

10.4.2 China Case Study

10.4.2.1 Introduction

China, the second largest cotton-growing country in the world after India, planted 5.4 million ha of cotton in 2007. Cotton is the most important cash crop in China but is subject to very heavy damage by the insect pest, cotton bollworm (*Helicoverpa armigera*). In the past, the area planted to cotton in China was as high as 6.7 million ha, but severe damage due to cotton bollworm reduced this by 40%, to about 4 to 5 million ha in recent years. Loss due to cotton bollworm alone in 1992 (Jia 1998) was valued at the national level to be 10 billion RMB equivalent to US$1.2 billion (calculated at the official exchange rate in 2001 of 8.27 RMB = US$1.00).

In the 1970s and early 1980s, Chinese cotton farmers controlled bollworm and related pests with chlorinated hydrocarbons, such as DDT, until they were

superseded by organophosphates in the mid-1980s (Stone 1988). Cotton bollworms developed resistance to organophosphates in the 1980s and to pyrethroids in the early 1990s, leading to very heavy but ineffective use of insecticides. Eventually, overusage of insecticides resulted in unprofitability and led to a decline of cotton production in the more heavily infested bollworm areas in the Yellow River Valley. In the early 1990s, Chinese scientists initiated work on an alternative strategy of incorporating Bt as a transgene into cotton to confer resistance to cotton bollworm and related lepidopteran pests.

10.4.2.2 The Development and Adoption of Biotech Cotton in China

There have been two developers and suppliers of Bt cotton in China. The public sector Chinese Academy of Agricultural Sciences (CAAS) in collaboration with provincial academies and seed distribution organizations, and Monsanto/Delta and Pine Land from the international private sector. By 1996, CAAS had developed ten transgenic Bt cotton varieties. In 1997, the Biosafety Committee of the Ministry of Agriculture approved commercialization of the first CAAS Bt cotton which featured a modified Bt fusion gene, Cry1Ab/Cry1Ac. The CAAS Bt cotton was planted in the four provinces of Anhui, Shangdong, Shanxi, and Hubei (Jia 1998; James 1998). The cowpea trypsin gene, *CpTi*, with a different mechanism of resistance to Bt was also incorporated by CAAS in some Bt cotton varieties. By 1999, the CAAS single gene Bt cottons, and the Bt/CpTi cottons, designed to provide more durable resistance, were planted in nine provinces compared with four in 1998. It is estimated that at least 750,000 small farmers grew CAAS Bt cottons in 1999, most of which carried the single Bt gene. The single Bt cottons were planted in the nine provinces of Shangdong, Shanxi, Anhui, Jiangsu, Hubei, Henan, Hebei, Xinagjiang, and Lianoning. The CAAS cotton with the Bt/CpTi genes was planted in the four provinces of Shangdong, Shanxi, Anhui, and Hubei in 1999 (Jia 1999, personal communication). During 2000 and 2001, CAAS expanded its distribution and sales of Bt cotton varieties to all the cotton-growing provinces of China. Governmental institutions have also developed new Bt cotton varieties by backcrossing the CAAS and other Bt varieties with their own locally adapted germplasm and these are being distributed and sold in many provinces.

The other supplier of Bt cotton in China was Monsanto/Delta and Pine Land whose product was based on the variety 33B, which carries the *cry1Ac* gene. The product, which involved some collaboration with the Chinese, was approved for commercialization in 1997. This Bt cotton was initially grown in the province of Hebei, and later in Shandong, Henan, and Anuhi.

A multitude of public and private institutions, and companies are involved with Bt cotton development, distribution, and sales in China, making characterization of adoption a challenging task. In addition, many farmers save seed, with both formal and informal seed-sales compounding the challenge of generating estimates of adoption. In practice, annual rigorous surveys are the only practical means of generating an informative database to characterize adoption and assess the impact

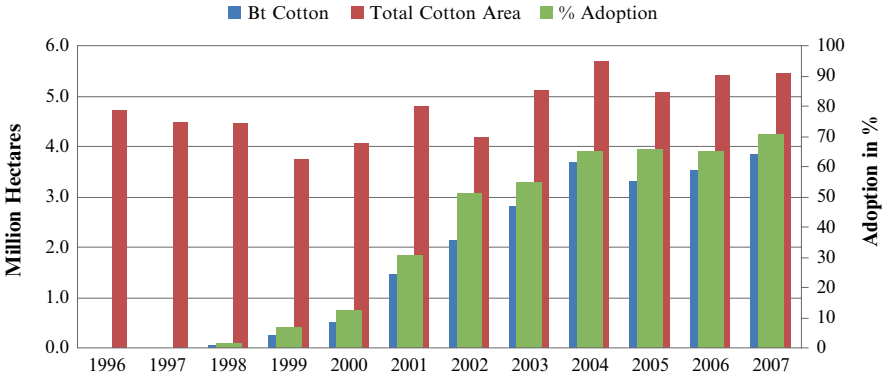

Fig. 10.7 Adoption of Bt cotton in China (1996–2007)

of Bt cotton on production. The first surveys were conducted in 1999 and a series of reports have been published (Huang et al. 2002; Pray et al. 2001) including a report on 5 years of experience, 1997 to 2001, with Bt cotton in China (Pray et al. 2002). The surveys started in Hebei and Shandong provinces, were expanded to include Henan Province in 2000, and further expanded to include Anhui and Jiangsu in 2001. In several of these provinces cotton can suffer significant damage from bollworm, and in provinces such as Hebei and Shandong, adoption rates for Bt cotton quickly soared to 97% and 80%, respectively, in 2000, following their introduction in 1997.

The data in Fig. 10.7 show that the total cotton area planted in China during the period 1996–2007 varies from a low of approximately 3.7 million ha in 1999 to a high of approximately 5.7 million ha in 2004. This significant variation in total cotton area impacts on the area of biotech cotton which has ranged from a low in 1998 of approximately 50,000 ha to an all time high of 3.8 million ha in 2007. Expressing the biotech cotton area as a percentage of total cotton area confirms a consistent increase in percent adoption of Bt cotton from 1997 to 2007, peaking at 69% in 2007. Adoption rate of Bt cotton in China is very high by any standard for any crop technology application. The small farmers who initially adopted Bt cotton in China in 1997 derived significant and multiple benefits from the technology and were very satisfied with the experience. They were keen to continue planting Bt cotton and were joined by other small cotton farmers, which in turn led to millions of small farmers adopting Bt cotton.

The number of cotton farmers in China fluctuates annually, depending on the planted area of the cotton crop. The estimated number of Bt cotton farmers in China has increased from a few thousand at its introduction in 1997 to a high of 7.1 million farmers in 2007. Thus, in 2007, Bt cotton was planted by 7.1 million small and resource-poor farmers on 3.8 million ha, which is 69% of the 5.5 million ha of all cotton planted in China. Based on studies conducted by the Center for Chinese Agricultural Policy (CCAP), it was concluded that, on average, at the farm level Bt

cotton increases yield by 9.6%, reduces insecticide use by 60%, with positive implications for both the environment and the farmers' health. Bt cotton also generates a substantial US$220 per hectare increase in income which makes a significant contribution to a better quality of life given that the income of resource-poor cotton farmers is around US$1 per day (James 2007). At the national level, it is estimated that increased income from Bt cotton is approximately US $800 million per year, projected to increase to US$1 billion per year by 2010. An important feature of Bt cotton in China is that it is produced by small farmers; the average farm is less than 1 ha and the cotton area, approximately 0.5 ha. Contrary to popular opinion, government no longer influences farm decisions regarding cotton production, and cotton quotas were discontinued by the government in 1998. Farmers themselves now decide whether or not to plant Bt cotton, and they buy seed and sell cotton in a competitive market where the price of cotton is not regulated by government. The Seed Law passed in 2000 allows private companies to conduct business directly with farmers. Thus, Chinese cotton farmers are no different to millions of small farmers who produce cotton in other developing countries like India, except that the farm size is smaller in China and their numbers are larger (Pray et al. 2002). The number of cotton farmers in China ranges from 9 to 13 million, whereas India has 5.5 million cotton farmers, approximately one-half of the cotton farmers of China. Cumulative benefit from biotech Bt cotton in China over the period 1998 to 2006 is estimated at $5.8 billion (Brookes and Barfoot 2008).

10.4.3 USA Case Study

10.4.3.1 Introduction

The USA has the third largest area of cotton in the world (4.2 million ha in 2007) after India (9.4 million ha) and China (5.4 million ha), and is the third largest producer after India and China in 2007. Cotton is the fifth largest crop in the USA by area and is produced by an estimated 30,000 farmers. Cotton is grown in the south and in the west in 16 states. Texas is the largest producer of cotton (approximately 30%), followed by Georgia. Cotton production systems range from low input rainfed cotton in Texas to the very intensive systems of Arizona and California there is an extensive literature on Bt cotton in the USA that includes several comprehensive reviews (Gianessi et al. 2002; Carpenter et al. 2002).

Bt cotton (*cry1Ac*) was introduced in 1996, principally to control the three major pests: tobacco budworm, cotton bollworm, and pink bollworm (James 1997). In the mid south and south east USA, cotton bollworm and tobacco budworm are the most prevalent pests, whereas pink bollworm is the most prevalent in the western states. Before the introduction of Bt cotton in 1996, 75% of the cotton area was treated with insecticides and an average of 2.4 sprays were specifically applied to control the bollworm/budworm complex which was estimated to cause a loss of 4%, despite

the application of insecticides (Carpenter and Gianessi 2001). In 1995, the year prior to the introduction of Bt cotton, tobacco budworm infestations were particularly high causing estimated losses of 29% in Alabama (Williams 1996). This was due to the development of resistance to the insecticides used.

10.4.3.2 Adoption of Biotech Cotton in the USA

Both Bt cotton and herbicide-tolerant (HT) biotech cotton were commercialized in the USA in 1996 and the stacked Bt/HT traits in 1997. The increase in adoption of biotech cotton in the USA (Fig. 10.8) indicates a very high rate of adoption starting from approximately 0.8 million ha in 1996 rising sharply to 5.0 million ha in 2001, modulated by slightly lower total USA cotton plantings from 2002 to 2005 and by a significant decrease in 2007, when cotton plantings plummeted with parallel decreases in biotech cotton hectarage. However, the percentage adoption data for biotech cotton in the USA between 1996 and 2007 show a consistent increase from approximately 15% in 1996 to a high of over 90% in 2007, reflecting the satisfaction of farmers with the technology.

Following the introduction of the stacked Bt/HT traits in 1997 they occupied a progressively higher proportion of the total biotech cotton reaching a high of approximately 70% in 2007. Notable new biotech cotton introductions in the period 1996–2007 included the Bollgard®II trait as a substitute for Bollgard® in 2002, RR®Flex in 2005 as well as stacked traits of the two products. Of the 4.3 million ha of upland cotton in the USA in 2007, 72% was occupied by the stacked traits of Bt and herbicide tolerance, 20% were herbicide tolerance, <1% was Bt, and only a balance of 7% was conventional. Cumulative benefit from biotech Bt cotton in the USA over the period 1998–2006 is estimated at $2.1 billion, plus $779 million for herbicide-tolerant cotton for a total of $2.9 billion (Brookes and Barfoot 2008).

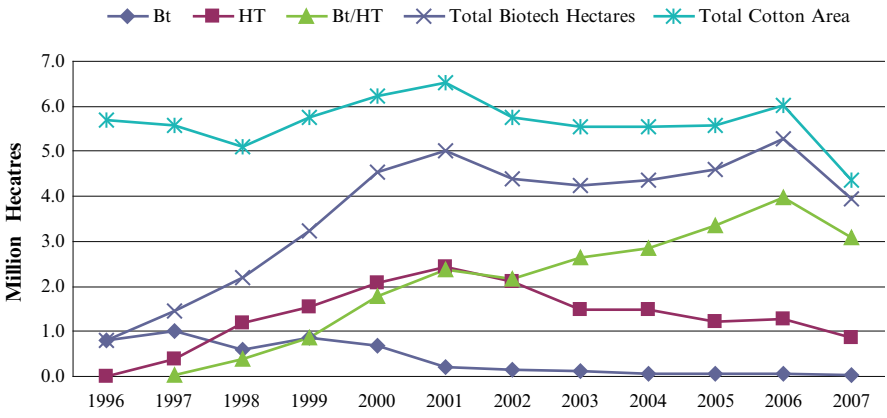

Fig. 10.8 Adoption of biotech cotton in USA (1996–2007)

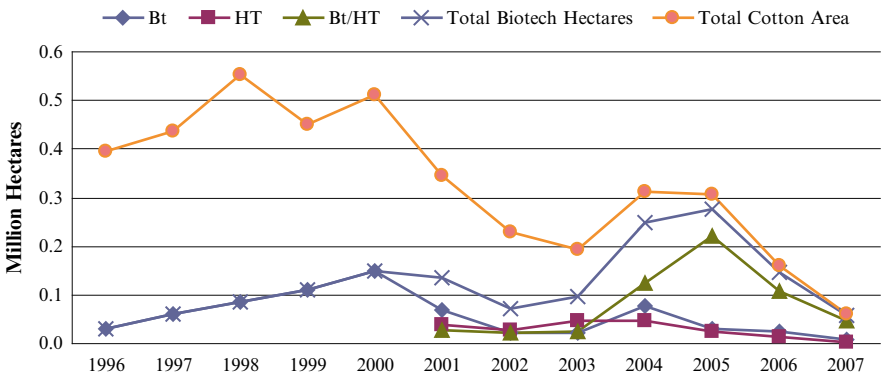

Fig. 10.9 Adoption of biotech cotton in Australia (1996–2007)

10.4.4 Australia Case Study

10.4.4.1 Introduction

Cotton production is highly mechanized in Australia and intensively managed with irrigation and other inputs, including fertilizer and insecticide. Data in Fig. 10.9 show that the planted area of cotton in Australia has fluctuated significantly from 400,000 to 500,000 between 1996 and 2000, decreasing to 200,000 in 2003 before recovering to 300,000 ha in 2005, and then plummeting to 60,000 ha in 2007; the latter sharp drop was due to a back-to-back 2-year period of the worst droughts in the history of Australia.

10.4.4.2 Adoption of Biotech Cotton in Australia

Australia has moved extremely fast, but responsibly, with the adoption of biotech cotton and has been justly rewarded with significant benefits. The first Bt cotton, INGARD (equivalent to Bollgard®), was approved in 1996, followed by RR cotton in 2000. Bollgard®II was approved in 2002 as well as BG®II x RR, and in 2006, RR®Flex was approved as well as RR®F x BG®II. Thus, Bt cotton, which was field-tested and commercially released in 1996/1997, was available in Australia in the same year as the USA. The *cry1 Ac* gene was incorporated in CSIRO varieties (INGARD®) and Bt cotton varieties are sold by Cotton Seed Distributors and Monsanto. The hectarage of INGARD Bt cotton increased from 30,000 ha in 1996–1997 to 165,000 ha in regulated annual step increases of 5% up to a maximum of 30% which was reached in 2000–2001 (Fig. 10.9 and Table 10.3). The Bollgard® area was held at 30%, or below, until the approval of BG®II and BG®II x RR® in 2002, after which the adoption of the stacked product was very fast, reaching 75% by 2005 with all biotech cotton occupying 95% of the total Australian cotton crop by 2007.

Table 10.3 Early adoption of Bollgard® Bt cotton in Australia

Year	Area Bt cotton (ha)	% of total cotton (ha)
1996–1997	30,000	8
1997–1998	60,000	15
1998–1999	85,000	20
1999–2000	125,000	25
2000–2001	165,000	30
2001–2002	146,000	30

Source: James (2002)

It is instructive, that from the outset, the registration of Bt cotton in Australia was conditional on the establishment of a resistance management strategy overseen by a committee with representatives from farmers and scientists from the public and private sectors. Resistance management is assigned a very high priority and the limit of 30% Bt cotton was designed to provide the other 70% of cotton as an additional refuge to the required regular refuge; the latter requires 10 ha of unsprayed cotton per 100 ha of Bt cotton, or 100 ha of sprayed conventional cotton, which was the preferred option of farmers.

The Australian biotech cotton program is extremely well managed and it is to the credit of Australia that it achieved complete substitution of the single Bt gene product (Bollgard®) with the dual Bt gene varieties (Bollgard®II) in only 2 years 2002–2003. This greatly accelerated and enhanced the stability of Bt resistance management, and simultaneously benefited from better and more reliable protection against the major insect pests. In 2002–2003, there was a limitation in place on the percentage of Bt cotton allowed to be planted in Australia. In 2003–2004, planting of Bollgard® was restricted to 15% on any farm in Australia and the combined area of the single and dual gene Bt products was restricted to a maximum of 40%. With the introduction of Bollgard®II, the deployment limitations of the single Bt gene product were lifted because concerns re deployment of resistance of the single Bt gene were no longer relevant.

Australia planted only 60,000 ha of cotton in 2007 (one-third of the area in 2006) because of the continuing severe droughts, the worst that Australia has experienced. As a result, irrigators were allocated limited volumes of water for cotton production and dry-land growers were completely dependent on late rains for planting. Of the 60,000 ha of cotton planted in 2007, the overall percentage adoption of biotech cotton was approximately 95% (Fig. 10.6), slightly higher than 2006. In 2007, about 80% of all cotton in Australia featured the stacked traits for herbicide tolerance and insect resistance (the dual RR® and Bt gene Bollgard®II) – this included RR®Flex; 12% with the dual Bt gene on its own, compared with 17% in 2006; 3% with a single gene for herbicide tolerance including some RR®Flex cotton, and the remaining 5% in conventional cotton, compared with 8% in 2006.

The cumulative benefit from biotech Bt cotton in Australia during the period 1998 to 2006 is estimated at $179 million (Brookes and Barfoot 2008).

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Chapter 11
Regulatory Systems and Requirements for Genetically Engineered Cotton from Lab to Land

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11.1 Biotech Crops

The global area of biotech crops continued to increase from 1996, and growth continued at a sustained double-digit growth rate of 12%, or 12.3 million ha (30 million acres) – the second highest increase in global biotech crop area in the last 5 years – reaching 114.3 million ha (282.4 million acres). Insect-resistant Bt cotton has been the fourth dominant crop with eight million ha and 63% growth in 2005, and is planted in nine countries. Cotton with stacked traits Bt/herbicide tolerance also occupied 4% of the global cotton area of 4.1 million ha in the USA, Australia, and Mexico. Herbicide-tolerant cotton grown in the USA, Argentina, Australia, Mexico, and South Africa on 1.4 million ha is equivalent to 1% of the global crop biotech area. In total, of the 35 million ha of global cotton, 38% or 13.4 million ha were biotech in 2006. In terms of value, it is estimated that biotech cotton amounts to 0.87 billion (14% of global biotech crop markets) (James 2007).

Despite this high adoption rate and future promises, there are concerns about the impact of genetically engineered (GE) crops on the environment. Regulatory approaches in Europe and North America are essentially different. In the EU, it is based on the process of making GE crops; in the US, on the characteristics of the GE product. In many other countries, which have a new regulatory system or are in the process of establishing one, the regulation is considered on either system or a mixture. Despite these differences, the information required for risk assessment tends to be similar. Each risk assessment considers the possibility, probability and consequence of harm on a case-by-case basis. (Nap et al. 2002).

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11.2 Beginnings of Biotech Regulation

Discussions on the possible hazards of cloning recombinant DNA molecules began in the early 1970s. The main concern was focused on laboratory practices needed to handle serious human and animal pathogens, possibility of creation of "hybrid organisms" with biological activities of an unpredictable nature, and the escape of "hybrid organisms" from the laboratory with unpredictable consequences. These concerns were examined by a committee of the National Academy of Sciences (USA) in 1974. As a consequence, the National Institutes of Health, USA (NIH), established the Recombinant Advisory Committee (RAC) in 1974. In February 1975, a historic international meeting was convened at Asilomar, California (Berg 2008). The conclusions from the conference were as follows: Certain experiments should be deferred; most of the work on recombinant DNA could proceed with appropriate safety measures; potential risks were assigned to different types of experiments; and safe bacteria and plasmids that could not survive in the environment if they escaped from the laboratory should be developed.

At the international level, the OECD report *Recombinant DNA Safety Considerations,* published in 1986, set out a concept called "Good Industrial Large-Scale Practice (GILSP)" applicable to intrinsically low-risk r-DNA organisms used in industrial production. The concept encompassed certain criteria which an r-DNA organism must meet in order to be given GILSP status. An internal survey carried out in 1988 in OECD countries on the use of the GILSP concept or its underlying principles showed that it had been adopted in the national guidelines in a number of countries.

The 1986 OECD report *Recombinant-DNA Safety Considerations* concluded that "assessment of potential risks of organisms for environmental or agricultural applications is less developed than the assessment of potential risks for industrial applications". It went on to say that "the means for assessing r-DNA organisms can be approached by analogy with the existing data base gained from extensive use of traditionally modified organisms in agriculture and the environment generally". The 1986 report also suggested that because of the "step-by-step assessment during the research and development process, the potential risk to the environment of the applications of r-DNA organisms should be minimised". The recommendations in this area noted that "considerable data on the environmental and human health effects of living organisms exist and should be used to guide risk assessments", and that "research to improve the prediction, evaluation, and monitoring of the outcome of applications of rDNA organisms should be encouraged". Any development of general international guidelines governing such applications was judged to be "premature" in 1986. It was recommended that "review of potential risks should be conducted on a case-by-case basis, prior to application. Case-by-case means an individual review of a proposal against assessment criteria which are relevant to the particular proposal; this is not intended to imply that every case will require review by a national or other authority since various classes of proposals may be excluded".

In 1988, the OECD's Group of National Experts on Safety in Biotechnology met to consider the need for a follow-up programme to the 1986 report. The group

decided that part of its programme would be to develop general principles that would identify a generic approach to the safety assessment of low – or negligible risk small-scale field research. The principles, labelled as "good developmental principles (GDP)", would be developed while countries continued to use the general case-by-case approach as defined in the 1986 report. At that meeting, there was agreement that GDP should apply equally well to both agricultural and other types of environmental testing (i.e. mineral leaching or waste degradation) and that a single document could appropriately describe principles for both these kinds of applications. Given the importance and complexity of the subject, and its widespread interest, an earlier version of this part was made available for discussion and public comment in 1990 and in 1992 a report on "Safety Considerations for Biotechnology" was published covering scientific principles for the design of small-scale field research with genetically modified plants and micro-organisms. The principles described, good developmental principles (GDP), are intended as scientific guides to the performance of low – or negligible risk small-scale field research, including basic and applied research. They are not intended to bypass or prejudice any regulatory action on field research with plants and micro-organisms. These principles would allow flexible national approaches to the design and conduct of small-scale field research.

These early developments in the countries with capacity for product and process development involving rDNA technologies, the discussions, debates and reports on safety concepts and assessment methodologies served as guidance for many countries promoting research, development and commercialisation of rDNA products. During 1986–1996, many countries in the North, few Asian countries in south put in place some form of regulatory System. However, as the Cartagena Protocol on Biosafety (CPB) was adopted in 2000 and entered into force on September 11 2003, a total of 147 countries have ratified the CPB as of 2008. The Global Environment Facility (GEF), as the financial mechanism to both the Convention on Biological Diversity (CBD) and its CPB, has played an important role in building the necessary capacity in biosafety since the adoption of the Protocol (Rao 2005). The GEF, together with UNEP, UNDP and the World Bank, assisted countries in developing and implementing National Biosafety Frameworks (NBFs). Today more than 120 countries in Africa, Asia, and Central/Eastern Europe have NBFs in place.

11.3 Components of Regulatory System/Framework

11.3.1 A Regulatory Regime

The regulatory regime comprises legislation, laws, acts, regulations, decrees, or guidelines that support and empower the administrative and institutional mechanisms for the decision-making process. In 1986, the leaders of the developed countries meeting in the OECD Council could state, on the basis of expert consensus, that "there is no scientific basis for specific legislation to regulate the use of recombinant DNA organisms". Perhaps due to this consensus many countries have

been addressing the risk assessment and management of GMOs by adaptation or extrapolation of existing legislation or through non-legislative means such as ministerial decree. For example new laws were passed to specifically address gene technology in Australia, South Africa, Japan, and at a regional level by European Union. Statutory instruments are utilised in India, USA, Canada, Argentina, and the Philippines within existing laws (Rao 2003).

In the Philippines, regulations of R&D in biotechnology are done through a National Committee on Biosafety (NCBP), created by Executive Order in 1990. NCBP is administratively under the Department of Science and Technology, but its members come from other agencies such as the Department of Agriculture, Health, and Environment. The Committee also consists of eminent scientists in biology, environment, physics, social sciences, and members from the community including NGOs. NCBP had developed biosafety guidelines for R&D: biosafety guidelines for small-scale laboratory work; biosafety guidelines for large-scale contained work and glasshouse trials; and biosafety guidelines for planned release of genetically modified organisms (GMOs) and potentially harmful exotic species (PHES).

On the other hand, the Indian Acts, rules and regulations as well as procedures for handling of genetically modified organisms (GMOs) and rDNA products have been formulated under the Environment (Protection) Act (EPA) 1986 and Rules 1989. The rules in general cover manufacture, use/import/export and storage of hazardous micro-organisms, genetically engineered organisms or cells and came into force from 1993. A set of rDNA guidelines were issued in 1990 covering genetically engineered organisms, genetic transformation of plants and animals, mechanism of implementation of biosafety guidelines and containment facilities under three risk groups. The guidelines have been revised matching with the needs of scientific knowhow in 1994 as "Revised Guidelines for Safety in Biotechnology". During 1998, to provide special review for genetically engineered plants, "Revised Guidelines for Research in Transgenic Plants and Guidelines for Toxicity and Allergenicity for Evaluation of Transgenic Seeds, Plants and Plant Parts" had come into force. In response to changing needs of transgenic technology and safety science globally, in 2008 another set of "Guidelines and standards for operating procedures (SOPs) for confined field trials of regulated genetically engineered (GE) plants".

In Australia, in June 2001, a new gene regulatory regime commenced operation. The regime is governed by new Commonwealth "gene technology act 2000". The intention was to create a more streamlined and certain pathway for industry and researchers seeking approval of GMOs that can be managing safety. The suitability of this approach in other countries should be based on merits and drawbacks in terms of complexity, timeliness, flexibility, and cost.

In Brazil, following two presidential decrees in 2003 and 2004 to approve the farming of farmer-saved biotech soybean seed for the 2003/2004 seasons, the Brazilian Congress passed a biosafety bill in March 2005 that provided for the first time, a legal framework to facilitate the approval and adoption of biotech crops in brazil. The Bill allowed, for the first time sale of commercial certified RR soybean seed and also for the first time, the approved use of Bt cotton (event BC 531) in the first registered variety DP98.

11.3.2 Administrative Systems

In general, the regulatory systems function through an administrative mechanism that includes the Competent Authority responsible for receiving and handling requests for permits (import, export, domestic use, including placing on the market, intentional introduction into the environment, field trials, contained use, transit, etc.), the system(s) for risk assessment and the system(s) for decision making. Not all countries have a single biosafety administration office. Some countries have biosafety administration responsibilities in several government departments. Others have centralised biosafety administration in one office for co-ordinated governance of biosafety issues over a number of government departments. The choice of framework reflects existing regulatory structures and the resources available for sustaining biosafety regulation (UNEP/GEF 2006).

For example in USA, in 1984, the White House Office of Science and Technology Policy (OSTP) published the "Coordinated Framework for Regulation of Biotechnology", a framework proposing that genetically engineered products would continue to be regulated according to their characteristics and novel features and not by their method of production. It also proposed that new biotechnology products be regulated under the existing federal statutory authority and regulation such as Food and Drug Administration (FDA), Animal and Plant Health Inspection Service (APHIS), Environmental Protection Agency (EPA). Similarly, in the *Philippines*, the administrative functions are carried out by different government agencies depending on the type of GMO activity: the Department of Agriculture deals with GMO activities related to plants and plant products, fish and aquatic resources, domesticated animals and animal husbandry; the Department of Science and Technology deals with GMO activities concerning research and development; and the Department of Environment and Natural Resources deals with GMO activities to do with bioremediation, forestry, and wildlife.

Decision-making process: In general, three important institutional mechanisms are often associated with approval or rejection of an application received for biosafety clearance: A national decision-making apex body that reviews data on proposed GM activities and approves or rejects them on the basis of the regulatory framework. A scientific advisory body that carries out or reviews risk assessments on GM activities and recommends what, if any, risk management measures may be needed to protect the environment and human health. This body may also advise on general biosafety issues. At research institution or university or private sector R&D level, Institutional Biosafety committees (IBSCs) operate to review all laboratory-based experiments.

For all operations at national level, a dedicated biosafety administration office that receives and processes applications for GM activities carries out daily biosafety administration; and coordinates public input, risk assessment, and decision-making activities of the NBF. This office is usually responsible for issuing biosafety communications (information about biosafety) and consultation with stakeholders about the processes. A separate inspectorate or monitoring mechanism that is

responsible for monitoring and ensuring compliance in cooperation with an enforcement regime is an indispensable component of the regulatory system.

In India, a regulatory set up oversees the development of GM (genetically modified) organisms including crops from the research stage to large-scale commercial use through a three-tier system. The *Institutional Biosafety Committee* (IBSC) operates at research-level approvals and *the Review Committee on Genetic Manipulation* (RCGM) reviews all approved ongoing research projects which are considered to be in the high-risk category and controlled field experiments. The Department of Biotechnology (DBT) under the Ministry of Science and Technology, Govt. of India, provides recognition to IBSC which is in the institution making the applications and also services RCGM for regulating research and limited field experiments. Finally, *Genetic Engineering Approval Committee* (GEAC) functions as an apex body under Ministry of Environment and Forests (MoEF) and is responsible for the approval of activities involving large-scale use of hazardous micro-organisms as well as recombinant products in research and industrial production from an environment angle or commercial use. At State Level, SBCC (State Biotechnology and co-ordination committee), DLC (district-level committee) inspect, supervise, and involve monitoring with the help of scientists from state and central government institutions.

Like in many countries, cotton with insect-resistant Bt gene was the first GM crop approved by the regulatory system for commercial release in March 2002. The Indian experience with wide-scale adoption of Bt cotton in the last 6 years provided several lessons: multiple agencies in regulation; prolonged time for each step of assessment and approvals; capacity f or scientific risk assessment, post-release management, dynamics of seed industry and markets; public perception and response; transparency and activist agenda; state of agricultural extension; inter-ministerial coordination, Centre-State relations, intellectual property and legal issues. In the final analysis three challenges – policy, technical and socio-economic – and their linkages in decision making were recognised. To address the policy challenges the government had decided to restructure the regulatory framework so as to establish a scientific, rigorous, efficient, predictable, and consistent regulatory regime articulated as autonomous "National Biotechnology Regulatory Authority" to provide effective single-window clearance mechanism.

11.4 Potential Risks and Benefits Associated with GM Crops

The commercialisation of *Bt* cotton had to pass through the rigorous process of ensuring environmental, food, and feed safety under the current biosafety regulations. Although genetic modification using selection and breeding has been carried out for centuries, the modern techniques of rDNA technology can obtain the same results by directly identifying the genes responsible for the desired character and transferring them into a variety of organisms. So, ideally, the risks associated with the introduction of GMOs should be the same as those with conventionally modified

hybrid crops and organisms. However, the main difference between the classical selection methods and improvement by rDNA technology is that the latter crosses the species barrier, so that a gene can be transferred within any living species such as micro-organisms, plants, and animals. Further, gene transfers are accomplished by manipulations outside the cells, which potentially allow for rearrangement and modification of genetic material before transfer, including the introduction of novel genes synthesised in the laboratory. Therefore, the transgenic approach of GMOs goes beyond the normal possibilities of nature, despite the fact that this does happen especially in case of horizontal gene transfers in nature, leading to safety concerns.

The application of GM technology involves a range of gene manipulation techniques to enhance, reduce or switch off the activity of specific unwanted genes. It also permits the introduction of new plant genes or genes from other organisms or enhancement of existing genes, to improve protein content, vitamins and nutrients, and starch or oil yield; modify oils or starches; and enhance fruit flavour, colour or nutrition. These also allow for providing insect tolerance, disease tolerance, other biotic and abiotic stress tolerances. It enables the development of rapid genotyping methods to accelerate conventional plant breeding, leading to the identification of genes responsible for the desired traits, and their transfer to other species. Thus, the potential benefits comprise increases in crop yields, decrease in the use of pesticides and herbicides, and improvement in nutritional content and storage characteristics.

The potential risks to humans, animals, and the environment need to be tested which could arise from the consequences of introducing new genes, such as the appearance of allergens in the pollen or the plant, and of toxins, and hazardous substances in GM food and GM feed; these possibilities also exist in naturally evolved or hybrids and varieties, which have been taken for granted as safe and do not undergo any safety tests. Ecological and other environmental risks may also arise from cross-pollination between GM crops and their wild relatives, potentially leading to loss of biodiversity and the emergence of new pests, diseases, and weeds that could acquire resistance to the gene engineered into the GM crops, and this must be tested to establish no harmful affect under the regulatory system.

It may be noted that there is no evidence of unique hazards either in the use of rDNA techniques or in the transfer of genes between unrelated organisms (Newell 2003). It is not true that GMOs are toxic or are likely to proliferate in the environment. However, all GMOs must undergo safety tests to establish safety. This means that the potential risks of GMOs could differ depending on the particular gene-organism combination and, therefore, each GMO product must undergo risk assessment.

11.4.1 Perceived Risk to Human Health

The perceived risks of GMOs to human health are related mainly to the toxicity and allergenicity of the new genes and organisms as well as their products.

11.4.1.1 Risk of Toxicity

The perceived risk of toxicity may be directly related to the nature of the product whose synthesis is controlled by the transgene or changes in the metabolism and composition of the organisms resulting from gene transfer. For example, in some cases the organism may contain inactive pathways leading to the production of toxic substances. The addition of new genetic material could reactivate these inactive pathways or otherwise increase the level of toxic substances within the plants. This could happen if the on/off signals associated with the introduced gene were located on the genome in places where they could activate the previously inactive genes (Jha et al. 2005). Further, the modified metabolism due to introduction of tolerance to chemical substances such as herbicides may also lead to the appearance of novel metabolites in the cell. In view of this, every GMO needs to be carefully evaluated for toxicity to human and animals and most of these perceived toxicity risks can be assessed using scientific methods, both qualitatively and quantitatively.

11.4.1.2 Risk of Allergies

The production of GMOs sometimes includes the introduction of a new protein from organisms which are not consumed as food. There is a perceived risk of such proteins becoming allergens since virtually most known food allergens are proteins. However, it may be noted that allergies can be developed by any individual to even common foods and substances such as wheat, milk, meat, eggs, herbs, cosmetics, drugs, plastics, rubber, wood, saw-dust, domestic and house dust, cotton, wool, carpet, pollen or other things, and there is no evidence that GM products pose more risk than conventional products consumed or used in daily life. The possibility of transferring allergens with genetic engineering came to light when a gene for a high methionine protein from the Brazil nut was incorporated into soybean to enhance its nutrient content (Tripathi 2002). The tests conducted on the soybean indicated the triggering of an allergic response in sensitive subjects, and this transgenic soybean was never released for sale in its country of development or any other place.

11.4.1.3 Antibiotic Resistance

The production of GMOs has in many cases involved the use of genes for antibiotic resistance as selectable markers. Early in the modification process in the laboratory, these markers help in selecting cells that have taken up foreign genes. Although they have no further use, the genes continue to be expressed in GMOs and become a permanent feature of the final product. The use of these antibiotic-resistance

markers has raised concerns that eating foods carrying these markers would reduce the effectiveness of the particular antibiotic to fight disease, as the antibiotic-resistant gene produces enzymes that can degrade or detoxify antibiotics. Therefore, theoretically if a transgenic tomato with an antibiotic-resistant gene is eaten at the same time with the antibiotic, it could destroy the antibiotic in the stomach. However, in many cases, high processing temperatures are seen to inactivate the enzyme in processed foods. Also there are apprehensions of the perceived risk of horizontal gene transfer, i.e. transfer of DNA from one organism to another outside of the parent-to-offspring channel. The transfer of the resistance gene from transgenic food to the micro-organisms that normally inhabit the stomach and intestines or to bacteria that are ingested along with food could help those micro-organisms survive an oral dose of antibiotic medicine. The resistance gene could also be transferred to human or animal pathogens making them impervious to antibiotics (Newell 2003). Although horizontal transfer of DNA does occur under natural circumstances and laboratory conditions, it has an extremely rare probability in the acid environment of the human stomach or even in the outside environment. It has been corroborated that horizontal transfer of DNA can occur at a rate of 1×10^{-8}, i.e. one transformant per 100 million cells, under strong antibiotic selection pressure (Nottingham 2002). The probability of such transfer would further decrease in natural circumstances.

11.4.1.4 Consuming Foreign DNA

There have been apprehensions about the dangers of eating the foreign DNA in GM foods, i.e. the pieces of DNA that did not originally occur in that food plant. DNA being present in all living things such as plants, animals, and micro-organisms is eaten by human beings with every meal. Most of it is broken down into more basic molecules during the digestion process, whereas the small amount that is not broken down is either absorbed into the blood stream or is excreted in the faeces. In an experiment of feeding mice with a harmless detectable DNA sequence, its progress was tracked through the gastrointestinal tract and the body. About 5% of the DNA was detectable in the small intestine, large intestine, and faeces up to 8 h after the meal; 0.05% was detectable in the blood stream up to 8 h; very small fragments could be detected in the liver and spleen for up to 18 h; and no foreign DNA could be detected after 42 h (Schubert et al. 1994). It is also well known that even if foreign DNA finds its way into the tissues of an organism, it is destroyed by the body's normal defence mechanism. There is no evidence that DNA from GMOs, including transgenic crops, is more dangerous to human health than DNA from conventional crops, animals, or associated micro-organisms that are normally eaten.

Thus, the DNA of the modified crop will usually be processed and broken down by the digestive system in the same way as that of conventionally bred or otherwise modified crops.

11.4.2 Risk to Environment

The introduction of GMOs in the environment necessitates a close examination for potential ecosystem disruption. Ecological perceived risks include the impact of introduced traits introgressing into other related species through out-crossing, potential build-up of resistance in insect populations to engineered insecticidal traits, unintended secondary effects on non-target organisms, and potential effects on biodiversity (Rissler and Mellon 1996).

11.4.2.1 Persistence of Transgene or of Transgene Products

The gene transferred into an organism or the resultant products can actually remain in the environment leading to environmental problems. For example, in the case of *Bt* crops it was suspected that insecticidal proteins can persist in the environment, but experiments have proved that these are degraded in the soil (Singh et al. 1999). There are also perceived concerns in the case of micro-organisms about their capacity to adapt to new environmental conditions and to persist in the environment as spores for longer times.

11.4.2.2 Interaction with Non-target Organisms

The intentional release of GMOs into the environment has led to an increase in the interest in possible interactions that may occur between other organisms in the environment.

11.4.2.3 Unpredictable Gene Expression or Transgene Instability

Unintended genomic changes can occur as a secondary consequence of genetic modification. Such changes can lead to the production of new proteins that may be toxic or allergenic, or may disrupt or alter metabolic pathways that play a role in making the GMO successful (FAO 2003a).

11.4.2.4 Gene Flow

Accidental cross-breeding between GMO plants and traditional varieties through pollen transfer can contaminate the latter with GMO genes resulting in the loss of the traditional varieties. Wind, rain, and insect pollinators can contribute to the spread of pollen resulting in the contamination of local varieties through cross-pollination/breeding (Lentini 2002). The consequences associated with such gene transfers may impact intellectual property, increase weediness if transferred to

compatible weedy relatives, or lead to the extinction of endangered varieties of the same genera. However, these risks can be anticipated easily and then evaluated experimentally prior to any commercial release as many factors influence the potential for such gene transfer from crop to crop. Some crops are highly outcrossing, with pollen carried to other fields by wind and by insects, whereas other species are highly self-pollinating with little potential for pollen transfer to neighbouring plants. Because of such differences, each case is evaluated individually for potential to contribute to the gene flow from transgenic to conventional crops.

11.4.2.5 Resistance/Tolerance of Target Organisms

The potential benefits of planting insect-resistant transgenic crops include decreased insecticide use and reduced crop damage. However, the innate ability of insect populations to rapidly adapt to environmental pressures poses a potential threat to the long-term efficacy of insect-resistant biotechnologies. Adaptation by insects and other pests to pest protection mechanisms can have adverse environmental and health impacts (FAO 2003b). For instance, adaptation by insect populations to an environmentally benign pest control technique could result in the use of chemical pesticides with higher toxicity. On similar lines, environmental concerns have been raised related to the development of virus-resistant transgenic crops. There are apprehensions about the development of stronger, i.e. more virulent viruses by either alteration in the process through which plant viruses are transmitted or widening of the range of hosts which can be infected by one particular virus. However, there are no detailed scientific studies on the actual existence of such effects and a case-by-case assessment is undertaken.

11.4.2.6 Increased Weediness

Weediness means the tendency of the plant to spread beyond the field where it was first planted. There are apprehensions about GM crops becoming weeds (FAO 2003b). For instance, if a salt-tolerant GM crop escapes into marine areas it could become a potent weed. There is also fear about the development of superweeds, i.e. a weed that has acquired an herbicide-tolerant gene due to genetic contamination with a herbicide-tolerance GMO through in-field cross-breeding to related species or through horizontal gene transfer. However, these perceived risks have not been proven with the existing weed science and scientists involved in developing technologies for weed control measures. There have been voices that weeds are important feeds for animals in developing countries, but this is also a fact that weeds destroy a considerable useful crop for feeding the society. It is another fact that unemployed youth do not want to work in farms in developing countries and the GM crops being developed as herbicide tolerant to control weeds have a great

resistance by the policy makers and activists. The small and marginal farmer does not have resources to control weeds from available herbicides or to defend his crop in the farm.

11.4.2.7 Loss of Biodiversity/Reduction of Cultivars

There have been concerns about the reduction in the genetic diversity in cropping systems (i.e. in situ) by the development and global spread of improved crop varieties. This genetic erosion has occurred as farmers have replaced the use of traditional varieties with monocultures. This is expected to further intensify as more and more transgenic crops are introduced which bring in considerable economic benefits to the farmers. The relative rate of susceptibility to any unforeseen infections or destructive situations increases when single varieties are used in cropping system in place of multiple varieties. However, it is argued (FAO 2003b) that there is always continuous and localised experimentation going on for the development of more effective crops, which helps in maintaining genetic diversity. As regards the conservation of traditional land bases, their germplasm should be maintained in seed banks (ex situ). In fact, biotechnology applications play a critical role in making seed banks storage more effective by accurately tracking genetic materials through molecular biology techniques.

11.4.2.8 Changes in the Soil Ecology

Many plants leak chemical compounds into the soil through their roots. There are concerns that transgenic plants may leak compounds different from the conventional plants, as the unintended consequence of their changed DNA (Viljoen 2005). There are speculations that this may change the ecology of the soil in terms of functional composition and biodiversity. The interaction between plants and soil micro-organisms is very complex, with the micro-organisms living around the plant roots also secreting chemical compounds into the soil (Kuhad et al. 2004). Research till date on the genes that, have been released, such as *cry1Ac*, shows that any such new proteins released by the plants are degraded and not taken up by micro-organisms.

11.4.3 Social and Ethical Concerns

Apart from concerns to environmental, human and animal health concerns from GMOs, there have been also the ethical, philosophical and spiritual concerns on the use of GMOs/LMOs. One of the perceived impacts of gene flow might be that genes from foreign species may be regarded by local community as a threat to the

natural integrity of natural crops. The ethical and spiritual aspect is very real to some, if not all indigenous people. Mexican and meso-American indigenous people have considered the maize as sacred. Local people of Mexico consider contamination of indigenous maize varieties as spiritual contamination. It has also been felt that greater corporate influence in public–private-partnerships (PPP), the interests of farmers in developing countries would be overlooked (FAO 2006). It is perceived that there would be no pro-poor research agenda by the corporate sector with a primary social cause involving ethical behaviour by the MNCs for the public at large. However, the Indian experience has been that all these perceptions have proven baseless after commercialisation of *Bt* cotton with various litigations on price, PIL (public interest litigations) on technology, its acceptability and adoption.

11.5 Regulatory Requirements: The Case of Bt Cotton Commercialisation in India: The First GM Crop of the Sub-Continent

The official release of *Bt* cotton involved putting in a lot of "labour" in the existing regulatory system before coming to the land for commercial cultivation. The hurdles put up by the opponents of GM technology resulted in a long time been taken in officially releasing the *Bt* cotton hybrids for the benefit of farmers.

Bt cotton, a transgenic plant, produces an insect-controlling protein *Cry1Ac*, the gene for which has been derived from the naturally occurring bacterium. *Bt.k.* (*Bacillus thuringiensis subsp. kurstaki*). The cotton hybrid containing the *Bt* gene produces its own toxin for bollworm attack, thus significantly reducing chemical insecticide use and providing a major benefit to the cotton growers and the environment.

Bt cotton being a transgenic crop required environmental clearance under Rules 7–10 of the Rules 1989 of EPA. Prior to the commercialisation of *Bt* cotton, large volumes of data and information were required to evaluate its performance and environmental safety. The assessment of the biosafety and environmental issues included molecular characterisation of the induced gene, biochemical characterisation of the expressed protein, estimation of the level of the expressed proteins in cotton, proteins in cotton products, safety of the expressed proteins to non-target organisms, environmental fate of the *Bt* protein, and agronomic, compositional and food and feed safety evaluation of *Bt* cotton compared to non-*Bt* cotton seed. The steps involved in the commercialisation of *Bt* cotton in India have been rigorous and detailed and match any biosafety system in the world.

The environmentally released *Bt* cotton contains the three genes inserted via GE techniques:

(a) *cry1Ac* gene encodes for an insecticidal protein and is derived from the common soil microbe *Bt.k.*;

(b) The *nptII gene*, derived from the prokaryotic transposon Tn5-, encodes the selectable marker enzyme NPTII (neomycin phosphotransferase II) and is used to identify the transformed cells that contain the *Cry1Ac* protein; and
(c) The *aad* gene isolated from transposon Tn7, which encodes the bacterial selectable marker enzyme AAD (aminoglycoside adenyltransferase), is used for the selection of bacteria containing the PV-GHBK04 plasmid on media containing spectinomycin or streptomycin.

NPTII and AAD proteins are used merely as selectable markers and have no pesticide activity and are not known to be toxic to any species.

The *Bt* transgene in the introgressed Indian inbred lines behaves as a single dominant Mendelian factor and is stably integrated in the plant genome. In the review of the data observed so far, the *Cry1Ac* protein produced in *Bt* cotton is 99.4% identical to the protein produced by the *Bt.k*. To be active against lepidopteron insects (Bollworm complex), the protein must be ingested by the bollworm complex of insects. In the insect gut, the protein binds to specific receptors in the mid-gut which activates the proteins, inserts into the membrane, and forms ion-specific pores. This disrupts the digestive processes and causes the death of the insect. The *Cry1Ac* protein produced in *Bt* cotton is considered non-toxic to non-lepidopteron insects, birds, fish and mammals as these species do not have receptors for the proteins on the surface of their gut cells. Also, the acidic medium in the gut of these organisms inactivates the *Cry1Ac* protein.

11.5.1 Steps Involved in the Commercial Release of Bt *Cotton*

The various stages in the release of *Bt* cotton in India are elaborated upon in the following paragraphs as per the chronology available in Annual Reports (1993–2004 to 2002–2003) of DBT.

In 1994, the company formed its IBSC and sought RCGM's permission to import transgenic *Bt*-cotton seeds from the USA. In 1995, permission was granted to import 100 g (gram) of *Bt*- cotton seeds of Coker-312. The company imported the same and initiated greenhouse experiments to incorporate the *Bt* gene into their elite cotton inbred lines as per biosafety guidelines. The procedure was further facilitated by adopting embryo rescue and tissue culture techniques. The *Bt* trait was thus successfully transferred into 60+ elite Indian cotton lines by the traditional back-crossing method of plant breeding. Inbreeding inter-specific crosses were also attempted to ascertain the risk of the transfer of the *Bt* gene into related *Gossypium* species. However, no seed setting was observed as a result of such crosses.

After the backcrossing with the Indian germplasm in greenhouse and converting them into *Bt* hybrids, the company sought permission from RCGM to conduct a limited contained field trial at one location in 1996 to conduct pollen escape experiments. After establishing the parameters for pollen escape in experiments conducted over a period of 2 years and subsequent to the examination of the data by

RCGM, the company sought permission for limited open field trials at five locations to further assess the pollen escape. These experiments were conducted during 1997–1998. In the meantime, the DBT put in place the 1998 guidelines for evaluating the toxicity and allergenicity of transgenic plants and plant parts. In the same year Mahyco started toxicological studies in the ruminant goat model and allergenicity studies in different animal models. RCGM permitted the company to conduct multi-location research trials (MLRT) at 25 + 15 locations to assess the efficacy of the *Bt* gene in the Indian elite germplasm. The data so generated were evaluated by MEC (Monitoring-cum-Evaluation Committee) and submitted to RCGM. The company was permitted to go ahead with further MLRT at 11 locations to assess the efficacy of the *Bt* gene in the Indian elite germplasm during 1999. After assessing the efficacy of *Bt* cotton against the bollworm complex, the gene flow, MLRT, and the toxicity and allergenicity data the company was asked to approach GEAC for conducting LST (large-scale field trials) to further test the efficacy of the *Bt* gene and the performance of *Bt*-cotton hybrids.

In 2000–2001, GEAC, based on the recommendations of RCGM, permitted Mahyco to conduct LST (large-scale field trials) in 100 ha to assess the efficacy of the *Bt* gene. During the same period the company was asked to generate various biosafety studies and approach ICAR for trials, which was supposed to be mandatory before the commercialisation of any seed. Also, during 2000–2001, the company was permitted to produce hybrid seeds of *Bt* cotton in 150 ha for further use, either for research or commercialisation, pending decision of GEAC. The LST data so generated in GEAC and ICAR trials were evaluated for the efficacy of the *Bt* gene and the agronomic properties of the *Bt* cotton hybrids, and the company was asked to repeat the large-scale trials to validate the data generated in the first large-scale field trials. During 2001–2002, Mahyco conducted a second round of large-scale field trials in 100 ha to assess the efficacy of the *Bt* gene and the performance of the *Bt* cotton hybrids. During this period, the company was also permitted to produce hybrid seeds in 300 ha as well as go ahead to repeat the ICAR trials for agronomic evaluations at 11 locations.

11.5.2 Biosafety Studies Conducted on Bt Cotton

11.5.2.1 Pollen Escape

Since cotton pollen grains are sticky and heavy, and form lumps, cross-pollination is feasible only through insects such as honeybees. Experiments were conducted in 1996, 1997, and at different locations to assess the out-crossing potential of the *Bt* trait from the company's varieties to the surrounding rows/islands of non-transgenic cotton. In the trials conducted in 1996 and 1997, out-crossing was observed only up to 2 m even by including honeybees as the pollinating agent. To reconfirm the low percentage of out-crossing at a short distance, a study was carried out in 2000 by using a modified protocol, approved by the MEC and set up by RCGM. Concentric rings of non-*Bt* cotton plants were replaced by 5 × 5 m

discrete blocks of non-*Bt* cotton with much open space to facilitate the flight of honeybees in all four directions around a solid block of *Bt* cotton plants. This arrangement of blocks allowed honeybees to travel a greater distance in search of pollen/nectar, and in turn increase the chances of cross-pollination and the dispersal of less than 2% of *Bt* pollen up to a distance of 15 m. Because of the genetic incompatibility exhibited among the cultivated cotton species of India, there is essentially no chance that the *Bt* gene will move from the cultivated tetraploid species to the cultivated diploid species, or any of the natural vegetation.

11.5.2.2 Aggressiveness and Weediness

To assess the weediness of *Bt* cotton, the rate of germination and vigour was compared by laboratory tests and in soil with the non-transformed parental line. The results demonstrated that there are no substantial differences between *Bt* and non-*Bt* cotton for germination and vigour. This also indicated that there was no substantial difference between transgenic *Bt* and control non-*Bt* cotton with regard to their weediness potential. In addition, the aggressiveness of the naturally shed seeds of *Bt* cotton compared to non-*Bt* control in 1997 was also studied. After harvesting the cotton plants, the area under planting for the 1997 season was left undisturbed and irrigated on a regular basis to allow for the germination of any seeds that may have remained in the ground after harvesting the main crop. The data so generated suggested that there were no significant differences in the germination rates of *Bt* cotton and non-*Bt* cotton, and that their aggressiveness properties were similar.

11.5.2.3 Soil Analysis

It was important to assess the possible risk of the accumulation and persistence of the plant-produced *Bt* proteins in the soil where the crop was grown repeatedly and plant residues such as roots were ploughed back into soil. To address the issue of the impact of the *Bt* protein released into the soil on soil organism studies were conducted at multilocation in the years 2000–2001 (five locations) and 2001–2002 (fourteen locations) by collecting soil samples from selected locations periodically, from 30 days after sowing to the post-harvest stage. The following observations were made.

Degradation Kinetics of Cry1Ac Protein in Soil

The level of the *Bt* protein in soil samples was determined by insect bioassays with *Helicoverpa armigera*. No *Bt* protein was detected in any of the soil samples at any point of time from the *Bt* cotton plots. These results were consistent with the

reported literature that the *Bt* protein is rapidly degraded in the soil and, therefore, there is no accumulation of the protein in the soil on which *Bt* cotton is grown.

Soil Microflora

Any impact of the *Bt* protein leached by the roots of the Bt cotton plant was assessed by culturing bacteria and fungi from the soil samples collected by the dilution planting method. ANOVA (analysis of variance) of the microbial population showed no significant difference between *Bt* and non-*Bt* soil samples. Similarly, no significant variation was observed in the population of soil invertebrates like earthworms and *Collembola*.

11.5.2.4 Effect of *Bt* Protein on Non-target Organisms

The *Bt* gene produces an insecticidal protein that is modelled on a naturally occurring soil bacterium with known insecticidal properties. Multilocational field trials were conducted during the years 1998–1999, 1999–2000, 2000–2001, and 2001–2002. The protocol adopted to conduct these trials had specific mention of the assessment of the effect of *Bt* cotton on non-target pests (sucking pests, secondary lepidopterans) and beneficial insects of cotton crop. The vast data collected over all these years from various locations showed that non-target sucking pest counts (aphids, jassids white fly and mites) did not vary significantly among *Bt* and non-*Bt* hybrids grown fields. The population of secondary lepidopteran pests – namely tobacco caterpillar, semi-looper, and leaf roller – remained negligible during all these years in the cotton crop and were not treated as serious pests of cotton. Beneficial insects, such as chrysopa, ladybird beetle, and spiders were also observed to be equally active in both *Bt* and non-*Bt* cotton crop fields. In all probability, with the reduction in the number of sprays on *Bt* cotton, the population of beneficial insect did go up.

11.5.2.5 Confirmation of the Absence of Terminator Gene

During the multiplication trials, certain anti-GM lobbies spread a rumour that Bt cotton under tests also contains genes that may inhibit germination of seeds in subsequent generations and farmers will be compelled to buy seed every year from companies. The experimental fields were also burnt in protest in certain places. The transgenic *Bt* cotton plant was developed by incorporating the *Bt* gene into it. Due to public apprehensions, as policy decision of the government rather than safety purposes, it was found desirable to assess that no other gene including the *cre recombinase* gene-integral component of the so-called Terminator technology was present in *Bt* cotton. A study was carried out by the Department of Genetics, University of Delhi (South Campus), Delhi, to check the presence/absence of such a gene in the *Bt* cotton. The PCR (polymerase chain reaction) analysis of

DNA samples isolated from individual seedlings derived from *Bt*-cotton hybrids showed that the *Bt*-cotton hybrid lines positive for *Cry1Ac* amplification did not show any amplification product using *cre* primers. This conclusively demonstrated the absence of the "terminator gene" in *Bt*-cotton hybrids.

11.5.2.6 Baseline Susceptibility Study

The Project Directorate of Biological Control, Indian Council of Agricultural Research(ICAR), Bangalore, carried out a baseline susceptibility study for *Helicoverpa armigera*, for 2 years in 2000–2001 and 2001–2002 at six and fourteen locations, respectively. Different geographical populations of American bollworm (*Helicoverpa armigera*) collected from six major cotton growing states of India (Madhya Pradesh, Gujarat, Maharashtra, Andhra Pradesh, Karnataka, and Tamil Nadu) were exposed to the insecticidal protein *Cry1Ac* through bioassays using probit analysis. The baseline data for the susceptibility of American bollworm to *Cry1Ac* protein were used as benchmark for monitoring the resistance in the bollworm pest to the *Cry1Ac* protein, in Indian hybrids.

11.5.2.7 Other Studies

There are three major cottonseed products: seed cotton, cottonseed oil, and cottonseed meal or cake. Seed cotton (non-delinted cotton seed) is primarily used as cattle feed. The defatted cottonseed meal is used almost extensively for animal feed, while refined cottonseed oil is a cotton product used for human consumption. During the refining process of cotton oil, the viscosity of the oil is reduced, proteins are coagulated and inactivated, gossypol is detoxified, and the microbial load gets decontaminated. The fundamental principle of substantial equivalence when applied to *Bt* cotton and its non-*Bt* counterpart has revealed that the *Bt* cotton seed is substantially equivalent in its composition to non-Bt cotton seed, and thus the food and feed derived from the *Bt* cotton seed will also be substantially equivalent to the food and feed derived from the non-*Bt* counterpart. In addition to compositional analysis, the wholesomeness of feed from *Bt* cotton was demonstrated in separate feeding studies with goats, rats, fish, chickens, cows and buffaloes.

Fish

The Central Institute of Fisheries Education, (ICAR), Mumbai, conducted this study in 2000–2001. The growth and the survival of Indian catfish fed a diet containing raw cottonseed meals derived from *Bt* cotton and its non-*Bt* counterpart were evaluated under Indian conditions. The results showed that the survival, growth rate, feed conversion ratio and body proximate composition was not significantly different for fish fed with *Bt* and non-*Bt* cotton seed (report submitted by the institute conducting the trials). The data from the study demonstrated that the

cottonseed meal from *Bt* and non-*Bt* cotton provided the same nutritional growth performance for Indian catfish.

Chicken

A study was carried out at the Central Avian Research Institute, (ICAR) Izatnagar, in the year 2000–2001 to compare chicken performance when fed on a diet containing *Bt* and non-*Bt* cotton for a period of 6 weeks. The results obtained demonstrated no significant difference in chicken performance in terms of body weight gain and feed intake for *Bt* and non-*Bt* groups. Similarly, the carcass characteristics in terms of dressing percentage, liver weight and heart weight were not significantly different between the treatments. Therefore, these findings concluded that the *Bt* cottonseed meal is nutritionally equivalent to the of non-*Bt* cottonseed meal when fed to chickens up to 6 weeks of age.

Cows and Buffaloes

Nutritional studies were conducted to analyse the effects of feeding cotton seed from *Bt* and non-*Bt* cotton on the feed intake, and production and composition of milk in lactating cows and buffaloes. The study for cows was conducted at the National Dairy Research Institute, (ICAR), Karnal, and for buffaloes at the G B Pant University of Agriculture and Technology, Pantnagar. The voluntary dry matter intake, 4.0% fat corrected milk, milk yield, and average fat content in the milk were similar in cows and buffaloes fed on a diet containing *Bt* and non-*Bt* cotton seed. Further, analysis of the milk and plasma samples in cows and buffaloes fed with *Bt* cotton did not indicate any presence of *Bt* protein, thus conclusively deriving that *Bt* protein soon disintegrates in the digestive tract of ruminants when they are fed with *Bt*-protein-containing seeds. Thus, *Bt* cotton seed is as wholesome and safe a feed for cows and buffaloes as non-*Bt* cotton seed.

11.5.2.8 Toxicological and Allergenicity Studies

Allergenicity Studies

For assessing the risks from the introduced protein in *Bt* cotton, studies were conducted in Brown Norway Rats to investigate any possible changes in the allergenicity of endogenous cotton seed proteins. Brown Norway Rats were used to assess the changes in endogenous allergens and inflammatory responses, which resemble those in humans. The protocol for this study was developed in consultation with expert scientists at the ITRC (Industrial Toxicology Research Centre), Lucknow, with approval from RCGM. No significant differences in feed consumption, animal weight gain, and general animal health were found between animals

fed with cotton seed and those with no cotton seed. At the end of the feeding period, the relative allergenicity of traditional cotton hybrids and *Bt* cotton was compared to *Bt* and non-*Bt* protein extracts in active cutaneous anaphylaxis assays. The results of the study concluded that there is no significant change in the endogenous allergens of *Bt* cotton seed compared to non-*Bt* cotton seed.

Toxicological Study

A goat-feeding study was conducted by the Industrial Toxicology Research Centre, Lucknow, in 1998. The treatment groups included goats fed with *Bt* cotton seed and control groups fed with non-*Bt* cotton seed. The feed analyses showed the similarity in nutrient and toxicant compositions between *Bt* and non-*Bt* cotton seeds, feed intake, weight gain, haematology and serum enzymes, measured for each animal during the feeding period of the study. At the end of this study, the animals were assessed for gross pathology and histopathology. It was concluded from the analyses that *Bt* cotton seed is as wholesome and safe for animal feed as non-*Bt* cotton seed. The differences observed across 48 goats in gross pathology and histopathology were attributed to being typical for any cotton seed feeding treatments on goats.

11.5.2.9 *cry1Ac* Gene and Protein in *Bt* Cottonseed Oil

A study was conducted by CICR (Central Institute for Cotton Research), Nagpur, to determine the presence of *Bt cry1Ac* protein and DNA fragment of *cry1Ac* gene in cottonseed oil derived from *Bt* and non-*Bt* cotton. Cottonseed oil derived from *Bt* cotton and non-*Bt* cotton showed no detectable levels of *cry1Ac* protein and DNA fragments of*cry1Ac* gene in the cottonseed oil derived from *Bt* cotton.

11.5.3 Agronomic Evaluation of Bt *Cotton*

To evaluate the efficacy of *Bt* cotton in controlling bollworm, Mahyco's R&D and product development division, under the guidance of regulatory bodies the RCGM and GEAC, conducted multilocation research trials regularly from 1998 to 2002. Also on the advice of GEAC, ICAR conducted field trials independently, using their own protocol, under the aegis of AICCIP (All India Co-ordinated Cotton Improvement Programme) during the seasons in the years 2000–2001 and 2001–2002. The large-scale field trials that were conducted included 15 replicated and 25 research trials during 1998–1999;11 replicated trials during 1999–2000; and concurrently with ICAR, 50 large-scale trials during 2001–2002; and 367 large-scale trials, 11 replicated, 14 research trials, and 16 North trials during 2001–2002. RCGM and GEAC approved the protocols and supervision of trials was conducted by the company and MEC. Teams of experts nominated by MEC regularly visited these trials and submitted their reports from time to time to RCGM and GEAC. These

trials were generally aimed at assessing the parameters of insect reactions – the number and species of bollworm complex, fruiting body damage (squares and bolls), rosetted flowers, locule damage and open boll damage, sucking pest infestation (aphids, jassids and whitefly) and beneficial insects; yield parameters – bolls per plant, seed cotton yield per hectare and fibre quality; insecticide usage – number of sprays for sucking pests based on ETL (economic threshold level), sprays for the bollworm complex based on ETL in *Bt*, non-*Bt* counterpart and check as well as for the economics of *Bt* cotton – savings on the number of sprays and yield benefit due to protection against bollworms.

Based on the overall assessment by ICAR, MEC, RCGM and GEAC, it was concluded that *Bt* cotton hybrid versions are superior to non-*Bt* cottons. Accordingly, based on their merits of higher yield realisation and resistance/tolerance to *Helicoverpa armigera*, *Bt* hybrids were recommended for their cultivation in India following the regulatory process during 2002. To date, more than 500 hybrids of *Bt* cotton have been released for the south, central and north zones of the country, and the list can be seen at http://www.igmoris.nic.inn, a web portal maintained by DBT.

11.6 Indian GMO Research and Future Expectations

Recognising the importance and potential of transgenic crops, extensive efforts have been initiated under the aegis of DBT for promoting R&D in this area. DBT supported the establishment of CPMB (centres for plant molecular biology) as early as in 1990. The seventh centre was established at the University of Delhi, South Campus, in 1997. Over the last 12 years, DBT has supported a large number of research projects that deal with the development of in vitro regeneration and genetic transformation protocols of important crop species grown in India and the development of transgenics with genes of agronomic importance. To further strengthen research in the area of crop biotechnology, a new institute, NIPGR (National Institute for Plant Genome Research), has been established in New Delhi with the mandate to strengthen plant biotechnology research in India. Thus, India has emerged as one of the leading countries in the world in promoting local R&D in agribiotechnology in general and GM crops in particular.

Several transgenic crops have been approved for conducting contained/confined limited field trials, including multilocation field trials and for replicated open-field confined trials have been approved till date. Research on more than 30 crops has been initiated in the country, and this has been well corroborated in the literature. By the year 2005, 22 Indian institutions and two international centres, spread over 16 cities, were actively involved in R&D work. Of these 24 public-funded institutions, five are in the union territory of New Delhi, and these account for a large share of the total grants committed by DBT and ICAR so far to GM-crops research. The 19 crops being researched are rice, wheat, cotton, potato, banana, tomato, oilseed rape, mustard, coffee, tobacco, eggplant or brinjal, cabbage, cauliflower, melon, citrus fruit, mung bean or blackgram, peanut, chickpea

and pigeon pea. While eight institutions are researching on two or more crops each, the others are concentrating on one crop each. Mainly four kinds of traits are being targeted of which two are resistant to attacks by insect pests and viral and fungal diseases, i.e. biotic stresses and tolerance of the abiotic stresses of drought, waterlogging and salinity. The other two traits include delayed ripening, leading to an increase in shelf-life, and thus improved storage properties, and an increase in the protein and micronutrient content. The important traits being targeted for the development of transgenics include insect resistance, herbicide tolerance, viral and fungal disease resistance, and stress tolerance. However, it is worthwhile to note that with the exceptions of the amaranth's gene isolated and used by the research team at JNU (Jawaharlal Nehru University) for protein enrichment of potato and rice, and the *Bt Cry1Ac* gene isolated at IIT (Indian Institute of Technology) Kharagpur, all the other transgenes originate from a few advanced public sector research institutions in some leading OECD countries, a couple of IARCs (International Agricultural Research Centres) in the CGIAR (Consultative Group on International Agricultural Research) -system and a few TMNCs. The transfer of these constructs to India is subject to the IPR issues of the transferring institutions and companies.

In general, the researchers, scientists, industry and other stakeholders opine that stringent biosafety regulations are necessary and they should be implemented effectively. However, a scientific analysis and practice of a decade of enforcing the prevailing biosafety regulatory system also reveal that many of the provisions are inappropriate and some of them are very difficult to comply with. The Indian regulatory Rules (1989) like in many other countries require renewal environmental/ commercial use approvals after 4 years of initial clearance. This condition seems impractical for the agribiotechnology sector because once an event is released in the environment the issue of its renewal is as redundant as the gene/event cannot be withdrawn from the environment.

The agribiotech industry also demands that the present regulatory system be based on process release rather than product release. There is a logic in this because when all the biosafety attributes of an transgenic event in a crop have been fully addressed, and it is considered safe for release in the environment, then a change in the genetic background in terms of hybrids of the same crop should not necessitate a repetition of the initial tests carried out at the time of release of the transgenic event. For example, the Bt gene events MON-531, MON 15985 which have been thoroughly tested for biosafety in initial cotton hybrids need not be tested again for biosafety in other cotton hybrids, and should only be addressed for agronomy issues and expression levels of the Cry protein.

11.7 Conclusions

Public acceptance of transgenic GE products is influenced by the perception of direct or indirect risks and benefits as well as the credibility of regulatory agencies that evaluate food, feed and environmental safety. For transgenic crops there is a

need for a more coordinated and transparent process that allows for greater public participation. For the society to benefit from GE crops, we must move away from the polarised positions that have defined the transgenic debate in the past to positions of mutual respect that allow a rational discussion of the technology's merits and risks. Opinions about the safety of GE crops/foods appear to be evenly split. GE foods are basically considered safe, but with the disagreement of some the controversy continues. So far no negative health effects due to GE foods have been documented in humans. However, unintended effects can arise from all forms of plant improvement, and therefore appropriate safety assessments are necessary. Consumers are far more comfortable with the GE of plants compared to the modification of other organisms. Our culture is in the grip of the "precautionary principle", and from agribiotechnology and biomedicine to geopolitics and international business, risk aversion has become a defining and paralyzing ethic of our times.

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Chapter 12
Socioeconomic Impacts of Bt (*Bacillus thuringiensis*) Cotton

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12.1 Introduction

Bt (*Bacillus thuringiensis*) cotton, which is resistant to different lepidopteran and coleopteran insect pests, was among the first genetically modified (GM) crops to be commercialized in the mid-1990s. In the US, Bt cotton was commercially approved in 1995. One year later, cotton farmers in Australia started using the technology, and in subsequent years, it was commercialized in China, Mexico, Argentina, South Africa, and India, and to a limited extent also in Indonesia. Very recently, Burkina Faso has approved Bt cotton as the first low-income country in Sub-Saharan Africa. In 2008, Bt cotton was grown on almost 15 million hectares (ha), which is over 40% of the total worldwide cotton area. India is now the country with the biggest Bt cotton area (7.6 million ha in 2008), followed by China (3.8 million ha), and the US (2 million ha) (James 2008). Most of these areas are cultivated with Monsanto's Bollgard I technology, involving the Cry1Ac Bt gene, but Bollgard II – with stacked Cry1Ac and Cry2Ab genes and a broader spectrum of target pests – has also been released in several countries. In addition to the Monsanto technology, the public sector has developed and commercialized Bt cotton varieties in China. In India, related public sector efforts are also at an advanced stage; the first public Bt cotton variety has recently been commercialized there.

The widespread and rapid adoption of Bt cotton over the last 14 years suggests that farmers are satisfied with this technology from an economic point of view. Numerous studies that have been carried out in different countries confirm that the

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socioeconomic benefits are sizeable. This chapter gives an overview of this body of literature, especially focusing on independent studies that have been published in peer-reviewed academic journals. While the experience with Bt technology in developed countries is also summarized, special emphasis is on developing countries for two reasons: First, developing countries are the main cotton producers, and they account for almost 80% of the worldwide Bt cotton area; and second, many of the cotton growers in developing countries are poor small-scale farmers, so that Bt technology might have important poverty implications. Indeed, apart from environmental and health concerns, socioeconomic impacts on smallholder farmers in developing countries are among the most contentious issues in the public GM crop debate, so that analyzing the factual evidence for Bt cotton is of particular interest.

The chapter is organized as follows. The next section discusses the agronomic, economic, and health impacts of Bt cotton at the farm level. Then, Sect. 12.3 analyzes household income and poverty outcomes in India, taking into account the technology's direct, as well as spillover effects at the village level. Section 12.4 discusses studies that analyze the impacts of Bt cotton from a macroeconomic perspective, while Sect. 12.5 concludes.

12.2 Farm Level Effects of Bt Cotton

12.2.1 Insecticide and Yield Effects

Bt cotton produces *Bt* proteins that are toxic to larvae of some lepidopteran and coleopteran insects. Therefore, Bt is a pest control agent that can be used as a substitute for traditional chemical insecticides. Following Lichtenberg and Zilberman (1986) and Zilberman et al. (2004), this can be expressed in a damage control framework:

$$Y = F(x)\ [1 - D(z\,, Bt\,; N)]$$

where Y is the effective cotton yield, and $F(\cdot)$ is the potential yield without insect damage, which depends on the variable inputs, x. $D(\cdot)$ is the damage function determining the fraction of the potential output being lost to insect pests; it can take values in the 0–1 interval. Crop losses depend on exogenous pest pressure, N, and they can be reduced through the application of chemical insecticides, z, and/or the use of Bt technology. If pest pressure is high and farmers use a lot of chemical insecticides in conventional cotton, Bt adoption should lead to substantial insecticide reductions.

However, Bt technology can also impact effective cotton yields. While the Bt gene does not affect potential yield, $F(\cdot)$, it can lead to a reduction in crop losses, $D(\cdot)$, when there is previously uncontrolled pest damage, thus leading to a higher Y.

Obviously, insecticide reduction and yield effects are closely related: farmers who use little amounts of insecticides in conventional cotton in spite of high pest pressure will realize a sizeable yield effect through Bt adoption, while the insecticide reduction effect will dominate in situations where farmers initially use higher amounts of chemical inputs. These linkages are visualized in Fig. 12.1 based on field trial data with Bt cotton in India. Figure 12.1 also demonstrates that Bt does not completely eliminate the need for insecticide sprays, as some crop damage still occurs when the technology is used. The reason is that the Bt toxin is very specific to certain pest species, while other insect pests, especially sucking pests, remain unaffected. Moreover, since Bt toxins usually do not cause 100% mortality, and toxin expression declines in aging cotton plants, insecticide sprays against Bt target pests are sometimes necessary based on economic threshold levels when there is heavy infestation.

What do the agronomic impacts of Bt cotton look like under practical farmer conditions? Table 12.1 confirms that both insecticide-reducing and yield-increasing

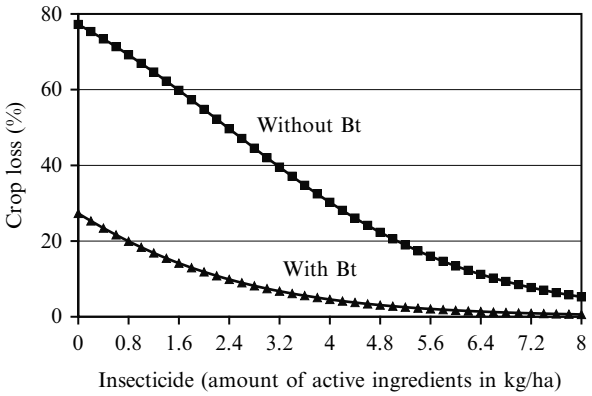

Fig. 12.1 Insecticide use and cotton crop losses with and without Bt in India
Source: Qaim and Zilberman (2003)

Table 12.1 Average agronomic effects of Bt cotton

Country	Insecticide reduction (%)	Increase in effective yield (%)
Argentina[a]	47	33
Australia[b]	48	0
China[c]	65	24
India[d]	41	37
Mexico[e]	77	9
South Africa[f]	33	22
USA[g]	36	10

Source: [a]Qaim and de Janvry (2003, 2005); [b]Fitt (2003); [c]Pray et al. (2002); [d]Qaim et al. (2006) and Sadashivappa and Qaim (2009); [e]Traxler et al. (2003); [f]Thirtle et al. (2003); [g]Carpenter et al. (2002) and Falck-Zepeda et al. (2000)
Note: For most countries, the numbers shown represent average results over several years of observations. The values are actually observed differences between adopters and nonadopters of Bt technology

effects can be observed internationally. On average, Bt yield effects are bigger in developing than in developed countries. This is not surprising, because pest infestation levels are often more severe in the tropics than in temperate climates. Moreover, farmers in developing countries often face greater technical and financial constraints. The positive yield effects of Bt cotton are highest in Argentina and India, ranging in a magnitude of 30%–40%. For Argentina, the explanation is simple: conventional cotton farmers underuse chemical insecticides – on average only about 2.5 kg are applied per season and ha – so that insect pests are not effectively controlled (Qaim and de Janvry 2005). In India, however, with an average of 10 kg/ha, insecticide use in conventional cotton is much higher than in Argentina (Qaim et al. 2006). This suggests that there are also factors other than insecticide quantity influencing damage control in conventional cotton and thus the yield effects of Bt technology. Among others, these factors include insecticide quality, insecticide resistance, and the correct choice of products and timing of sprays. The magnitude of the Bt yield effects is also confirmed in production function analyses with different econometric specifications (e.g., Huang et al. 2002; Thirtle et al. 2003; Qaim and de Janvry 2005; Qaim et al. 2006; Crost et al. 2007).

Insecticide reductions through Bt cotton range from 33% in South Africa to 77% in Mexico (Table 12.1). Worldwide, cotton is the biggest pesticide-consuming crop, so that these relative reductions also translate into huge reductions in absolute pesticide quantities, with concomitant positive effects for the environment. Brookes and Barfoot (2008) estimated that between 1996 and 2006 Bt cotton was responsible for a global saving of 128 million kg of pesticide active ingredients, reducing the environmental impact of total cotton pesticides by 25%.

How sustainable are such reductions in pesticides? In the first years of Bt crop deployment it was predicted that insect populations would soon develop Bt resistance, which would undermine the technology's effectiveness and lead to declining insecticide reductions, or even insecticide increases, over time. However, until now Bt resistance development has not been observed under field conditions, which might partly be due to successful resistance management strategies, including the planting of non-Bt refuges (Bates et al. 2005). But even in countries like China, where no deliberate resistance management strategy is implemented, Bt resistance has not yet been reported. Yet, there are also other factors that can lead to changes in Bt effects over time. In China, for instance, insecticide applications somewhat increased again after several years of Bt cotton use, in spite of the absence of Bt resistance. Wang et al. (2006) attributed this to secondary pests, which might have become more important through the Bt-induced reduction in broad-spectrum insecticides. Their analysis, however, was based on only 1 year of observations with increased insecticide applications and was disputed by others (e.g., Hu et al. 2006).

In India, Sadashivappa and Qaim (2009) also found variation in insecticide reductions over time. Using data from three rounds of a panel survey of farmers, they reported that insecticide quantities on Bt plots were reduced by approximately 50% in 2002 and 2004, while in 2006, average reductions were only 21% (Fig. 12.2). Yet, this lower reduction is not due to increases in insecticide sprays

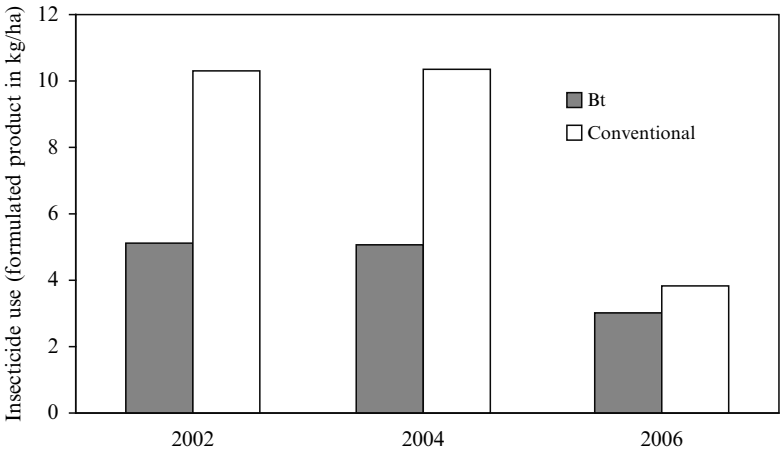

Fig. 12.2 Mean insecticide use on Bt and conventional cotton plots in India
Source: Sadashivappa and Qaim (2009)

on Bt plots, as one might expect when Bt resistance would emerge or secondary pests would gain in importance. On the contrary, sprays on Bt plots were further reduced, but sprays on conventional plots were reduced as well. This might be due to self-selection of farmers: by 2006, farmers who used to apply a lot of insecticides in conventional cotton had adopted Bt technology, so that the subsample of non-adopters now mostly consists of farmer who spray little anyway, either because of lower pest problems or because of lack of awareness of pest damage. Moreover, due to the wide dissemination of Bt cotton in India in recent years, there seems to be an overall suppression of Bt target pests, especially the American bollworm (Khadi et al. 2007). Similar effects have also been reported for some regions in the US (Carrière et al. 2003). Suppression of pests also on non-Bt plots can be interpreted as a positive externality of Bt technology. Still, further studies should be carried out, in order to better understand the long-term effects of Bt technology on insect populations in different environments.

12.2.2 Farmer Health Effects

The health hazards for farmers and farm workers applying pesticides have been analyzed in different countries (e.g., Sunding and Zivin 2000; Maumbe and Swinton 2003). Often, the problems are greater in developing than in developed countries, because environmental and health regulations are laxer, pesticides are mostly applied manually, and farmers are less educated and less informed about negative side effects. Against this background, Bt cotton could have positive health effects for farmers through significant reductions in pesticide use. Indeed, several authors have shown that Bt cotton leads to reductions especially in highly toxic

insecticides, belonging to toxicity levels I and II of the World Health Organization (WHO) classification (Qaim and Zilberman 2003; Qaim and de Janvry 2005).

More in-depth studies about the actual health implications for farmers have been carried out in China, where insecticide use in cotton is particularly heavy. Between 1999 and 2001, a series of surveys of small farmers was conducted in different provinces (Pray et al. 2002; Hossain et al. 2004). Randomly selected farmers were interviewed on cotton production aspects, including details of insecticide applications. In 2000, Bt cotton adopters had sprayed about 20 kg of formulated insecticides per ha, while conventional cotton growers had used 46 kg. Table 12.2 categorizes the insecticides used by chemical type. The most widely used insecticides were organophosphates and pyrethroids, and for these the Bt-related reductions were particularly pronounced. Furthermore, farmers were asked to give details on the frequency and type of insecticide poisonings experienced during sprays or immediately afterwards. Typical health symptoms include eye and skin irritations, headache, nausea, and breathing problems, among others. Table 12.3 demonstrates

Table 12.2 Average insecticide quantities used by cotton farmers in China (2000)

	Average quantity (kg/ha)		
	With Bt	Without Bt	Reduction (%)
Organophosphates	8.8	21.0	58
Pyrethroids	5.2	13.0	60
Organosulphates	2.8	6.0	53
Organochlorines	1.6	3.9	58
Amino-formicdacid esters	0.3	0.4	25
Other insecticides	0.8	2.1	64
Total	19.5	46.4	58

Source: Hossain et al. (2004)

Table 12.3 Type and toxicity levels of insecticides causing poisonings in a sample of 400 Chinese cotton farmers (1995–2000)

	WHO toxicity level	Poisoning cases
Organophosphates		
Chlordimeform	I	94
Parathion-methyl	I	65
Acephate	I	19
Carbofuran (furadan)	I	9
Phorate	I	9
Parathion	III	8
Monocrotophos	I	5
Pyrethroids		
Cypermethrin	II	12
Killingthrin 39	III	6

Source: Hossain et al. (2004)

that many of the poisoning cases reported are related to organophosphates and pyrethroids. While one-third of the conventional cotton growers had reported cases of poisoning in the 2000 season, the share among the Bt adopters was only 9%. Using econometric estimates of a health-production function, Hossain et al. (2004) could establish a clear causal relationship between Bt technology adoption and reductions in insecticide poisonings.

Bennett et al. (2003) have examined the health effects of Bt cotton in South Africa, and Kathage (2008) has done a similar analysis for India. Overall, they came to the same conclusion: with an increase in the uptake of Bt cotton among smallholder farmers, the rates of accidental insecticide poisoning have been declining. Given this consistent evidence, these results on positive farmer health effects of Bt technology are also transferable to other countries where cotton is grown by smallholders with heavy use of chemical insecticides.

12.2.3 Seed Prices and Profit Effects

Since most Bt cotton varieties and hybrids available to date have been commercialized by the private sector, a technology fee is charged. In some countries, like the US and Australia, the technology fee is displayed separately, while in many other countries, it is directly included in the seed price. In any case, the fee is associated with seed sales, so that seed costs for Bt technology adopting farmers increase. Table 12.4 shows average seed cost increases for Bt cotton in several countries.

The technology fee (or seed price markup), which companies can charge, depends on the value of the technology and the degree of market power in the national setting. Strong intellectual property rights (IPRs) and/or special seed sales

Table 12.4 Bt cotton seed cost increases and profit gains

Country	Seed cost increase (US$/ha)	Profit gain (US$/ha)
Argentina[a]	87	23
Australia[b]	151	66
China[c]	32	470
India[d]	43	135
Mexico[e]	58	295
South Africa (small farms)[f]	23	52
South Africa (large farms)[f]	47	129
USA[g]	79	58

Source: [a]Qaim and de Janvry (2003); [b]Fitt (2003); [c]Pray et al. (2002); [d]Qaim et al. (2006) and Sadashivappa and Qaim (2009); [e]Traxler et al. (2003); [f]Gouse et al. (2004); [g]Naseem and Pray (2004)

Notes: For most countries, the numbers shown represent average results over several years of observations. The values are actually observed differences between adopters and nonadopters of Bt technology

contracts limit competition and reduce farmers' options to save seeds. This is observed for Bt cotton in the US, Australia, Mexico, and Argentina. In India, Bt cotton seed prices were also relatively high during the first years of adoption, in spite of the fact that the technology is not patented there. The reason is that in India Bt is incorporated into cotton hybrids. Hence, there is a technical restriction for farmers to reproduce seeds, which also increases the seed companies' pricing scope. However, several state governments in India have issued maximum retail prices for Bt cotton seeds since 2006, which are well below the prices initially charged by seed companies. Such price interventions are attractive for farmers in the short run, but they reduce private sector incentives to commercialize new technologies in the future. In China, IPR protection is weak, and Bt is used in open-pollinated cotton varieties. Therefore, Bt seed costs are relatively low, and use of farm-saved seeds is widespread (Pray et al. 2001). In South Africa, Monsanto implements a system of price discrimination for Bt cotton seeds: small dryland farmers, which are dominant in the Makhathini Flats region, pay a significantly lower price than large-scale farmers (Gouse et al. 2004).

In spite of the technology fee, Bt cotton adopting farmers benefit in terms of higher average profits per ha (Table 12.4). That is, the economic advantages associated with insecticide savings and higher effective yields more than outweigh the technology fee charged on seeds. The absolute gains differ remarkably between countries. Apart from agroecological differences and unequal technology fees, this is partly due to dissimilar agricultural policies. In the US, China, and Mexico, the cotton sector is heavily subsidized, which encourages intensive production schemes and high overall yields. In Argentina, by contrast, farmers are not subsidized, but face world-market prices. World-market prices for cotton have been declining recently, which erodes the economic benefits resulting from technological yield gains.

Crosscountry differences notwithstanding, the profit gains for Bt technology adopting farmers are sizeable, especially in China, India, and Mexico. In many developing countries, cotton is primarily grown by smallholders with farm sizes of less than 2 ha; the analyses suggest that these small farms benefit from Bt cotton to the same extent as their larger counterparts (Qaim 2005). In China and South Africa, small farms were even shown to benefit more from Bt adoption than large farms (Huang et al. 2002; Morse et al. 2004).

India is now the country with the biggest Bt cotton area worldwide (James 2008), and reports on Indian farmers' experiences with the technology have featured prominently in the global public GM crop debate (Gruère et al. 2008; GRAIN 2004; Sahai and Rahman 2003). Therefore, a closer look at farm level economic impacts in India is particularly interesting. Table 12.5 shows cotton enterprise budgets with and without Bt technology for three growing seasons between 2002 and 2006. The data were collected from randomly sampled farms in four states of central and southern India (Qaim et al. 2006). For both technologies, cost and yield variations can be observed across the seasons, which are partly due to climate effects. We are particularly interested in the differences between Bt and conventional cotton. As expected, with Bt technology seed costs

Table 12.5 Crop enterprise budgets for Bt and conventional cotton in India

	2002		2004		2006	
	Bt	Conventional	Bt	Conventional	Bt	Conventional
Number of insecticide sprays	4.2	6.8	4.6	7.2	3.3	3.8
Insecticide use (kg/ha)	5.1	10.3	5.2	10.4	3.0	3.8
Yield of raw cotton (kg/ha)	1,628	1,213	1,836	1,362	2,080	1,458
Production cost (US$/ha)						
Seed	81.0	25.2	83.6	27.1	41.3	24.7
Insecticides	64.8	109.5	81.0	124.2	60.4	58.6
Fertilizer	96.9	85.4	96.9	85.7	100.5	75.5
Labor	150.3	116.0	178.1	151.2	236.9	209.4
Other cost	41.5	35.7	19.6	19.6	58.1	34.5
Total cost (US$/ha)	434.5	371.9	459.2	407.8	497.2	402.7
Revenue (US$/ha)	707.1	533.2	712.5	518.8	864.0	617.9
Profit (US$/ha)	272.5	161.3	253.3	111.0	366.7	215.2

Source: Qaim et al. (2006) and Sadashivappa and Qaim (2009)

are higher and insecticide costs are lower on average. Notable differences also occur for labor costs. In Indian cotton systems, insecticides are mostly applied manually with knapsack sprayers. Hence, spraying is labor intensive, and a reduction in the number of sprays is associated with labor savings. Yet, in the Indian context, these savings through Bt technology are offset by more labor being used for other operations, especially harvesting, so that overall more labor is used in Bt than in conventional cotton.[1]

Deducting production costs from sales revenues results in the profit per ha. Table 12.5 shows that profit gains through Bt have been large and increasing from $111/ha in 2002 to $152 in 2006. This increase is partly due to the Bt seed price caps introduced by state governments recently (Sadashivappa and Qaim 2009). It should be noted that the data in Table 12.5 mostly refer to adopters of official Bt seeds. There is also a black market in India for unapproved Bt seeds and F_2 hybrids. Bennett et al. (2005) showed that on average illegal Bt cotton hybrids generate higher profits than conventional hybrids, but lower profits than official Bt hybrids that are legally sold by seed companies.

[1]In India, cotton harvesting is primarily a female activity, so that Bt cotton technology especially improves employment opportunities for women. It should be noted, though, that the net labor effect of Bt technology is situation specific. In China, for instance, the reduction in the number of sprays is bigger and the yield increase is smaller than in India, so that overall Bt is labor-saving there (Pray et al. 2002).

12.2.4 Variability of Effects

While the results reported on agronomic and economic effects in previous subsections clearly underline the overall advantages of Bt cotton technology, they mask the fact that there can be significant impact variability. The suitability of insect-resistant Bt crops depends on local pest infestation levels, which can vary regionally and seasonally. In China, for instance, infestation levels of lepidopteran pests are highest in the northern and eastern parts of the country, so that the benefits of Bt cotton are most pronounced there. This is reflected in much higher adoption rates, as compared to western China (Pray et al. 2002). In the US, due to diverging pest infestation levels, Bt cotton adoption rates are lower in California than in other cotton-growing states (USDA 2008).

Table 12.6 displays regional variability of Bt cotton impacts in India. While Bt adopters in the states of Maharashtra, Karnataka, and Tamil Nadu realized significant net benefits in 2002, their colleagues in Andhra Pradesh suffered a loss in average incomes. Strikingly, most of the studies carried out by biotechnology critics during the early stages of Bt cotton diffusion in India placed heavy emphasis on observations from Andhra Pradesh (Sahai and Rahman 2003; GRAIN 2004). Overall, cotton in Andhra Pradesh is sprayed more often than in other states of India. Therefore, crop losses in conventional cotton are lower, and the expected Bt yield effect is small, especially in years with only moderate pest pressure. This small positive yield effect due to Bt technology itself was counteracted by a negative germplasm effect. In 2002, many farmers in Andhra Pradesh were affected by severe drought conditions, to which the hybrids carrying the Bt gene were not optimally adapted. Although the Bt gene itself does not alter the cotton plant's performance under water stress, the underlying germplasm was not particularly well suited for extreme drought situations. The number of Bt hybrids approved in India increased from three in 2002 to over 150 in 2008. Many of the new hybrids are also suitable for conditions in Andhra Pradesh, so that average farm level benefits there increased substantially (Kathage 2008; Sadashivappa and Qaim 2009). Nonetheless, the example demonstrates that Bt technology can only be successful when combined with locally adapted germplasm.

Also within a region, Bt crop impacts can vary, as has been shown by Bennett et al. (2006). Apart from agroecological factors, this can be due to differences in conventional pest control strategies and other farm and household characteristics. In the early stages of diffusion, farmers usually experiment with a new technology, and they reconsider their adoption decision based on personal experiences made

Table 12.6 Differences of Bt cotton effects in India by state (2002)

	Maharashtra	Karnataka	Tamil Nadu	Andhra Pradesh
Insecticide reduction (%)	46	62	78	34
Increase in effective yield (%)	32	73	43	−3
Gross margin increase (US$/ha)	92	270	247	−69

Source: Qaim et al. (2006)

Table 12.7 Adoption and disadoption of Bt cotton in India

	2002	2003	2004	2005	2006
Share of Bt adopters (%)	30	29	44	80	78
Share of adopters who disadopted after the season (%)	51	26	17	24	16
Share of disadopters who readopted in any of the following seasons (%)	38	14	3	21	–

Source: Sadashivappa and Qaim (2009)

(Qaim 2005). The adoption dynamics for Bt cotton in India are shown in Table 12.7 for a sample of typical farms in central and southern states of the country. Although adoption levels within the sample increased substantially over the first years of technological diffusion, the process was not unidirectional. After the first season in 2002, half of the adopters abandoned Bt technology, because they were not fully satisfied. Some also did not know how to use the technology properly and continued to spray substantially against bollworms. Also in subsequent seasons, some disadoption was observed, albeit the percentage of dropouts has been decreasing. Interestingly, a remarkable share of the disadopters readopted Bt technology after a break of 1 or 2 years. These patterns demonstrate that Bt crop adoption and disadoption are not irreversible decisions for farmers; they are part of a normal learning process.

12.3 Household Income and Poverty Effects of Bt Cotton

As discussed in the previous section, several recent studies have analyzed the impacts of Bt cotton on farm productivity in different countries. Many of these studies also looked into profit effects in the small farm sector of developing countries (e.g., Huang et al. 2002; Pray et al. 2002; Thirtle et al. 2003; Morse et al. 2004; Qaim and de Janvry 2005; Qaim et al. 2006). However, hardly any study so far has analyzed wider socioeconomic outcomes at the microlevel, including effects of Bt cotton on rural employment and household incomes. This dearth of broader microlevel research is probably also the reason for the ongoing controversy surrounding the poverty and rural development implications of GM crops (Lipton 2007; World Bank 2007; Friends of the Earth 2008; Gruère et al. 2008). The first comprehensive work in this direction is by Subramanian and Qaim (2009, 2010), who have examined direct and spillover effects of Bt cotton adoption in India, using a village modeling approach. Their research is summarized in the following.

12.3.1 Village Modeling Approach

In their analysis of the rural development effects of Bt cotton adoption in India, Subramanian and Qaim (2010) focused on one typical cotton-growing village in the

state of Maharashtra. This village is Kanzara in the district of Akola. In Kanzara, they carried out a census survey in 2004 to collect detailed data on household characteristics and economic activities. Of the total 305 village households, 102 are landless; the other 203 own land suitable for agricultural production. The average farm size of land-owning households in the village is 1.9 ha. All farm households cultivate at least some cotton, mostly next to a number of food and fodder crops for subsistence consumption and for sale.

For the analysis of income distribution effects, households were classified into poor, vulnerable, and rich, using local rural poverty lines. According to this classification, 48% of the households in Kanzara are poor, 38% are vulnerable, and 14% are rich. A social accounting matrix (SAM) was developed that considers 156 agricultural and nonagricultural activities. Agricultural activities include the cultivation of Bt and conventional cotton and numerous other crop and livestock enterprises. Nonagricultural activities include agricultural services, village production, retail trade, private services, government services, and transportation. In order to analyze the direct and spillover effects of increased Bt cotton adoption in the local village economy, a SAM multiplier model was developed (Subramanian and Qaim 2009) and used for different simulations. The construction and use of village SAMs and multiplier models is a common approach in the development economics literature to analyze the impacts of technologies and public policies (Taylor and Adelman 1996). But this approach had not been used previously in the context of Bt cotton or other GM crops.

12.3.2 Simulation Results

For the analysis of Bt cotton impacts at the village level in India, Subramanian and Qaim (2010) ran two scenario simulations – both considering an expansion in the village cotton area by a certain unit of land. The first scenario assumes that the additional area is cultivated with Bt cotton, while the second assumes that the additional area is grown with conventional cotton. Accordingly, differences between the two scenarios can be interpreted as the net impacts of Bt technology adoption. The impacts on returns to labor and household incomes are shown in Figs. 12.3 and 12.4, respectively. Both figures assume that the Bt and conventional cotton area expansion is 1 ha.

The cotton area expansion entails sizeable employment effects at the village level (Fig. 12.3). This is not unexpected, since cotton is a labor-intensive crop, and – apart from requiring agricultural labor – it also creates employment in nonagricultural sectors, such as transportation, trade, and other services. Strikingly, however, the overall changes in the returns to labor are higher in the Bt scenario, demonstrating that Bt cotton generates more employment than conventional cotton in the local economy. The difference is especially notable for hired female agricultural laborers, which is due to significantly higher yields to be harvested in Bt cotton. In the manual cotton production systems, hired women workers carry out most of the sowing,

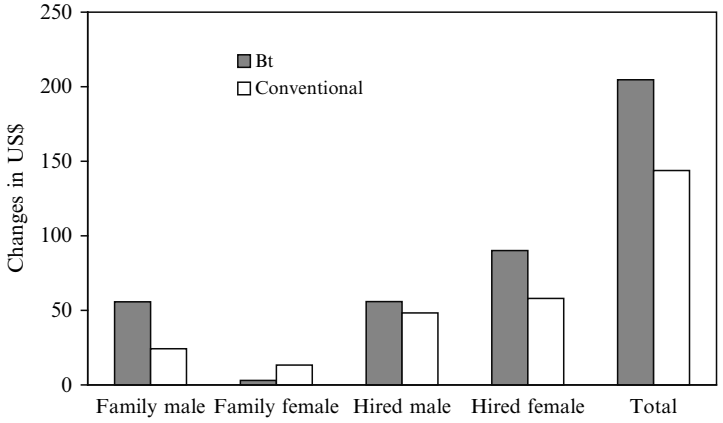

Fig. 12.3 Changes in returns to labor from increased Bt and conventional cotton production *Source*: Adapted from Subramanian and Qaim (2010)

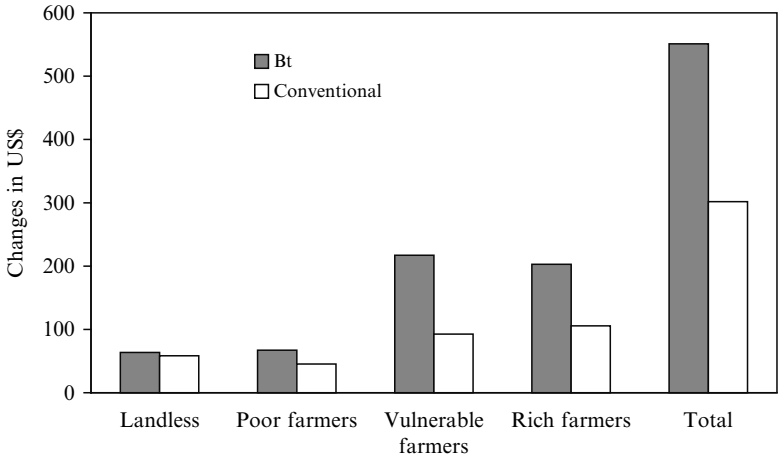

Fig. 12.4 Changes in household incomes from increased Bt and conventional cotton production *Source*: Adapted from Subramanian and Qaim (2010)

weeding, and harvesting operations, while men are mostly responsible for tillage, irrigation, and pest control. For male members of the farm families, returns to labor are also higher in Bt than in conventional cotton; this is largely driven by indirect effects. With reduced insecticide applications in Bt, some of the family male labor involved in pest scouting and spraying is saved, which means less employment in Bt cotton as a direct effect. However, the simulations show that this family labor saved in cotton production can be reallocated to other agricultural and nonagricultural activities, such that the overall returns to labor increase.

The impacts of a 1 ha Bt and conventional cotton area expansion on household incomes are shown in Fig. 12.4. These income effects occur due to changes in the

returns to the factors of production labor, capital, and land employed within the village. In addition, multiplier effects through spillovers to outside village markets and feedbacks are included. These are particularly important for a cash crop like cotton. For instance, higher cotton production and rising incomes within the village induce growth also in outside village sectors, which again leads to new employment and investment opportunities, including for village households. Again, the effects are bigger in the Bt cotton scenario. Total household income increases are 82% higher under Bt than under conventional cotton, implying a remarkable gain in overall economic welfare through Bt technology adoption at the village level. For landless households, the positive effects are relatively small. However, all types of farm households – including those below the poverty line – benefit considerably more from Bt than from conventional cotton. Strikingly, vulnerable farm households are the main beneficiaries, with additional income gains in a magnitude of 134%.

These results disprove the often heard argument that only wealthy farmers could benefit from Bt cotton. While the exact findings presented are specific to the study village, the social structure of the local economy is typical for smallholder cotton production in the semiarid tropics. Hence it is reasonable to conclude that Bt cotton produces similar benefits also in other parts of the developing world. The technology is net employment generating and causes income gains for all types of households, including those below the poverty line. This highlights that Bt cotton contributes to poverty reduction and rural development.

12.4 Macroeconomic Effects of Bt Cotton

So far, we have only looked at the microlevel effects of Bt cotton, neglecting wider impacts for markets beyond a rural village setting. Market impacts are important when analyzing the aggregate welfare outcomes of new technologies, which economists usually refer to as economic surplus effects. When only the market of one single crop is considered, so-called partial equilibrium models are used for the evaluation. Partial equilibrium models can only capture the first-round effects of a new technology. When indirect effects and spillovers to other markets and sectors of the economy shall be captured as well, general equilibrium models are used. General equilibrium models are also often employed to capture welfare effects of international trade. Both modeling approaches have been used for the evaluation of Bt cotton effects at the macrolevel. The related work is summarized in the following.

12.4.1 Partial Equilibrium Approaches

Whenever new crop technologies are adopted on a larger scale, the productivity increase will cause the crop's supply curve to shift downward. This is because the marginal cost of production decreases. This also holds true for Bt cotton

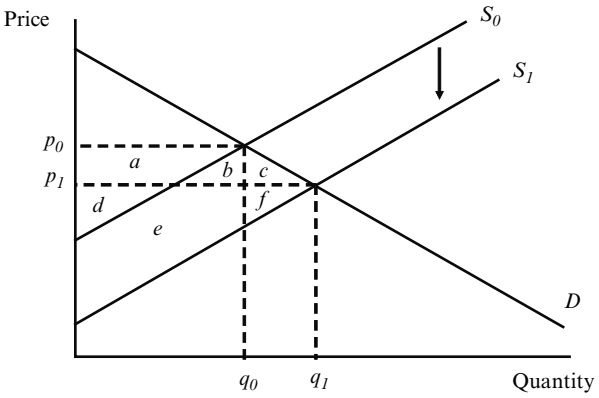

Fig. 12.5 Market impacts of Bt cotton technology

technology. When the cotton price is determined by market forces and the demand curve has the usual negative slope, the supply curve shift will lead to a lower equilibrium price. This is shown in Fig. 12.5, where D represents the cotton demand curve in a country, S_0 and S_1 are cotton supply curves before and after the introduction of Bt technology, and p_0 and p_1 are initial and new equilibrium prices. Cotton consumers clearly benefit from the price decrease; the gain in consumer surplus can be calculated as area (a+b+c). For farmers, the price decrease leads to a loss, which however is usually lower than their gain through the marginal cost reduction. The change in producer surplus can be calculated as area ($e + f$) minus area a. In addition to these consumer and producer surplus effects, the technology-developing company will capture an innovation rent through the technology fee charged on seed sales. Different authors have used such a partial equilibrium framework to evaluate the welfare and distribution effects of Bt cotton in different countries.

Price et al. (2003) estimated, that in the late 1990s, Bt cotton generated a total annual economic surplus gain of around $164 million in the US, of which 37% was captured by farmers, 18% by consumers, and 45% by the innovating companies. Similar results were also reported by Falck-Zepeda et al. (2000). Since Bt cotton adoption in the US has further increased since then, absolute surplus gains are higher today, but relative surplus distribution is still similar (Fernandez-Cornejo and Caswell 2006).

For Bt cotton in China, Pray et al. (2001) estimated economic surplus gains of about $140 million in 1999, with only 1.5% going to the innovating companies and the rest being captured by farmers. IPR protection in China is weak, and use of farm-saved Bt cotton seeds is widespread. Moreover, private sector Bt cotton varieties in China face competition from publicly developed Bt varieties. Under these conditions, it is difficult for companies to capture innovation rents, so that farmers are the main beneficiaries. Cotton consumers did not benefit in 1999, because the government controlled output markets thus preventing a decrease in the equilibrium price. Prices were somewhat liberalized more recently, so that

Chinese consumers now also benefit from Bt cotton technology. In India, Bt cotton surplus gains were projected at $315 million for 2005 (Qaim 2003). Cotton prices in India are not fully liberalized, so that consumer benefits were not considered. Farmers capture two-third of the overall surplus gains; the rest accrues to biotech and seed companies. As Bt cotton in India is commercialized in hybrids, use of farm-saved seeds is low. Thus the private sector innovation rent is higher than in China.

The surplus gains are proportional to the technology adoption rates in a country. Given that Bt cotton adoption has been increasing rapidly in recent years, the absolute gains today are still higher than those reported in the different studies. These results demonstrate that Bt cotton generates sizeable economic surplus gains, also beyond the individual farm level, whereby the surplus distribution effects depend on the particular situation. In developing countries, farmers seem to be the main beneficiaries of Bt cotton up till now. Yet, studies for Bt food crops in developing countries suggest that consumer benefits can be large, too (Krishna and Qaim 2008). In developed countries, private sector benefit shares are partly somewhat larger, because of more effective IPR protection. But even in the US and Australia, Bt cotton farmers realize significant positive welfare effects.

12.4.2 General Equilibrium Approaches

There are several recent studies that examine the economic growth effects of Bt cotton using general equilibrium modeling approaches. Most of these studies use the publicly available standard multiregion computable general equilibrium (CGE) model and database of the Global Trade Analysis Project (GTAP). Details of the general model as well as the underlying assumptions are described by Hertel (1997). Applications in a Bt cotton context are summarized in Table 12.8. These modeling approaches capture the vertical and horizontal linkages between cotton and all other markets, both within countries and across countries via trade flows.

At the global level, Bt cotton adoption entails welfare gains in a range of $0.7–1.8 billion/year. The differences across studies partly reflect different versions of the basic model used. The GTAP model is constantly updated with new data on

Table 12.8 Summary of Bt cotton welfare gains derived from general equilibrium modeling

Reference	Reference region	Reference year	Aggregate annual welfare gain (US$)
Frisvold and Reeves (2007)	Global	2005	1.4 billion
Elbehri and MacDonald (2004)	Global	2001	1.8 billion
Anderson et al. (2000)	Global	–	1.7 billion
Anderson et al. (2008)	Global	2001	0.7 billion
Huang et al. (2004)	China	2010	1.1 billion
Frisvold and Reeves (2007)	China	2005	0.6 billion
Elbehri and MacDonald (2004)	Africa	2001	0.1 billion

production, consumption, supply and demand elasticities, trade flows, tariffs, and other policies. More importantly, however, the assumed adoption rates for Bt cotton in different countries and regions matter a lot. Since Bt adoption is still increasing rapidly at the global level, the aggregate welfare gains are increasing, too.

Anderson et al. (2008) use the latest version of the GTAP model and result in a global welfare gain of $0.7 billion/year. Yet, this study also shows that global benefits could be further boosted through greater Bt cotton adoption in developing countries. With widespread adoption in the developing world, including in Sub-Saharan Africa, global annual welfare gains would rise to $2.3 billion. Apart from direct positive effects on farm profits, the agricultural labor saved through lower pesticide applications in Bt cotton would partly be channeled to other activities, including the production of food crops, resulting in higher labor productivity and household incomes. By contrast, with nonadoption of Bt cotton in Sub-Saharan Africa, that region would suffer an annual welfare loss of $0.1 billion, due to declining cotton prices and farm incomes (Elbehri and MacDonald 2004). Frisvold and Reeves (2007) show that when Bt cotton adoption is widespread and output expands, the global cotton price falls by 3%. Except for Africa, all other regions benefit, even if they do not adopt Bt cotton, since consumer welfare gains outweigh the losses of cotton farmers that have no access to the technology. Overall, nonadopting regions such as the European Union, which are net importers of cotton and textiles, gain $70.5 million/year through a positive terms-of-trade effect.

The biggest regional effects are observed in China, with an annual welfare gain of $0.6 billion (Frisvold and Reeves 2007); this is expected to further increase to 1.1 billion by 2010 (Huang et al. 2004). In China, cotton farmers benefit from Bt productivity and income gains, while consumers and the huge Chinese textile industry benefit from lower cotton prices. But India is soon catching up, as Bt adoption rates have been increasing there more rapidly than elsewhere. Anderson et al. (2008) estimate that widespread adoption of Bt cotton in India and other countries of South Asia will bring about additional regional welfare gains in a magnitude of $1.0 billion/year. These simulations clearly demonstrate that countries obtain greater economic welfare gains when they increase their Bt cotton adoption. And, it should be mentioned that the results presented here are lower-bound estimates of the overall benefits, because positive environmental and health externalities are not taken into account.

12.5 Conclusions

This chapter has analyzed the socioeconomic impacts of Bt cotton in an international context. There is ample evidence from many countries that Bt cotton significantly reduces insecticide applications. Apart from cost savings, these insecticide reductions are associated with positive effects for the environment and farmer health. Moreover, Bt cotton reduces pest-related crop losses, leading to higher effective yields. Such yield effects tend to be bigger in developing countries,

especially in the tropics, where pest infestation levels are often more severe than in temperate climates, and where farmers do not always control pests effectively through pesticides due to various constraints. These clear benefits for farmers come at the cost of higher seed prices. The magnitude of the technology fee charged by private companies on Bt seeds depends on the strength of IPR protection and enforcement in a country. Overall, the extra cost is lower than the benefits, so that farmers realize substantial gains in farm profits. However, given seasonal and regional variability in impacts, there are also cases where individual farmers did not benefit from Bt cotton in a particular year. Disappointed farmers tend to stop using the technology in the next year, but the rapid overall increase in adoption clearly indicates that the majority is satisfied with the technology.

Both small and large cotton growers benefit from Bt technology. In most developing countries, including in China and India, cotton is predominantly produced by smallholder farmers, who successfully improve their household income through Bt adoption. For India in particular, recent research demonstrates that all types of farm households – including those below the poverty line – benefit considerably from Bt cotton. Vulnerable farm households are even the main beneficiaries. Furthermore, Bt technology is employment generating, leading to higher returns to labor in rural areas. These results underline that Bt cotton contributes to poverty reduction and rural development. Macroeconomic modeling approaches with partial and general equilibrium models confirm that there are also sizeable aggregate growth effects associated with Bt cotton. Global annual welfare gains are in a magnitude of US $1 billion, and they are projected to more than double when adoption rates further increase in the developing world. These findings clearly suggest that GM crops can play an important role in promoting sustainable agricultural and overall development.

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