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Transgenic Cotton: An Overview

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ABSTRACT

The first transgenic cotton having insect or herbicide resistance was released to the field in 1996 in the United States. Since then the rapid increase in transgenic cotton acreage within 10 years attests to the overall success of agricultural biotechnology. This review article provides an overview of genetically modified cotton and its application in agricultural production. We first critically review cotton tissue culture as the basic work of biotechnology. Then, three main transformation methods, namely, *Agrobacterium*-meditated, particle bombardment and pollen tube-pathway are described in this paper. The performance of transgenic cotton plants engineered for insect, disease, herbicide resistance and fibre improvement is reviewed from a perspective of the benefits and limitations. Finally, recent progress in plastid engineering research of cotton is briefly mentioned. Cotton genetic engineering shows great potential to enhance breeding programs by introducing novel traits that have eluded more traditional plant improvement methods and therefore will likely play an increasingly important role in the genetic improvement of cotton in the future.

Keywords: *Gossypium*, plastid engineering, tissue culture, transformation

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INTRODUCTION

Cotton is worldwide one of the most important commercial crops and consequently plays a vital role economically, politically, and socially. Chiefly a fiber crop, it has been estimated to contribute US $15-20 billion to the world's agricultural economy, with over 180 million people depending on it for their livelihood (Benedict and Altman 2001).

There are 51 diverse species in the genus *Gossypium*. Four are cultivated, *G. hirsutum* L. and *G. barbadense* L., which are tetraploid (2n = 4x = 52), and *G. arboretum* L. and *G. herbaceum* L., which are diploid (2n = 2x = 26). The species most widely grown around the world is *G. hirsutum*. More than 95% of commercial cotton is upland cotton (G.

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Invited Review

hirsutum), while long staple cotton, *G. barbadense*, occupies a small area of less than 5%. Two species of diploid cotton, *G. arboretum* and *G. herbaceum,* more tolerant to drought stress, are grown in restricted areas of Asia and Africa.

The traditional breeding methods use sexual hybridization to introduce desirable agronomic traits, such as high yield, good quality and disease resistance, into new breeding lines which may be released after several years of field testing. Significant progress has been made through different breeding programs. The yield increase contributed by genetic improvement was 7-10 kg/ha/year for the USA (Wilkins 2000), 23kg/ha/year for Australia (Constable *et al*. 2001), and 8-10 kg/ha/year for China (Kong *et al*. 2000). Conventional breeding contributed a lot to cotton improvement, but progressed slowly recently because of a shortage of germplasm and creative breeding tools.

Molecular breeding provides a new way which allows genes to be selectively and effectively reshuffled into the most desirable combinations. This may include the introduction of foreign genes into the plant genome that confer novel traits that enhance food and fiber quality directly, or through providing protection against biotic and abiotic stresses in the environment (Wilkins 2000). Since initial commercialization in 1996, global planted area of biotech crops has soared by more than fifty-fold from 1.7 million hectares in six countries to 90 million hectares in 21 countries in 2005 (James *et al*. 2005).

The first generation of transgenic cotton genetically engineered to provide insect or herbicide resistance was released into commercial production in 1996 on 12% of cotton production acreage in the U.S. The overall success of transgenic cotton was soon apparent in the dramatic increase in total hectares committed to transgenic cotton within the past 20 years in the world. The focus of this paper is to present an overview of cotton genetic transformation as it currently stands in terms of improving field and fiber quality for the benefit of society and the environment.

COTTON CELL AND TISSUE CULTURE

Somatic embryogenesis and plant regeneration

A rapid, simple and efficient plant regeneration protocol is a prerequisite for genetic manipulation *in vitro*. Somatic embryogenesis *via* a callus phase (indirect method) has been reported in several cotton species. The first report on induction of somatic embryogenesis was from a wild species of cotton by Price and Smith (1979); however, somatic embryos could not develop into plantlets. The first successful regeneration of whole cotton plant *via* somatic embryogenesis was obtained by Davidonis and Hamilton (1983). However, the method had limitation due to long incubation period of callus for induction of proembryoids and low efficiency of embryo formation. In a different study, Shoemaker *et al*. (1986) evaluated seventeen *G. hirsutum* L. cultivars for induction of somatic embryogenesis and plant regeneration. Approximately 40% of the somatic embryos underwent normal germination and the procedure was simple and rapid. To the same year, somatic embryogenesis from callus cultures of mature leaf and petiole explants from six cotton varieties has been reported by Gawel *et al*. (1986).

Trolinder and Goodin (1987, 1988a, 1988b) found that induction of somatic embryogenesis in cotton is genotype dependent. Finer (1988) also reported plant regeneration from somatic embryogenic suspension cultures established from cotyledons of cultivar Coker 310. Some Coker varieties have been reported to have the highest regeneration potential compared to other varieties. However, plant regeneration in China cultivar of Upland cotton YZ-1 through somatic embryogenesis was first reported by Jin *et al*. (2006a), which showed predominant ability of somatic embryogenesis over Coker lines with a high ratio of somatic embryogenesis within two months and produced higher number of somatic embryos from one gram of embrogenic calli.

Many factors can influence the efficiency of a regeneration procedure. The main factors determining the tissue culture response in cotton include genotypes, donor plant

Table 1 Studies on somatic embryogenesis and plant regeneration in cotton.

Genotype	Explant used	Mode of regeneration	Reference
G. klotzschianum A	H	C-SE	Price and Smith 1979
G. hirsutum L.	COT	C-PE -PT	Davidonis and Hamilton 1983
G. klotzschianum A	ST, P, LD	C-SE	Finer and Smith 1984
G. hirsutum L.	H, IE	C-SE-PT	Rangan *et al*. 1984
G. hirsutum L.	H	C-SE-PT	Shoemaker *et al*. 1986
G. hirsutum L.	LD. P	C-SE-PT	Gawel *et al*. 1986
G. hirsutum L.	H	C-SE-PT	Umbeck *et al*. 1987
G. hirsutum L.	H	C-SE-PT	Tolinder and Goodin 1987
G. hirsutum L.	H	C-SE-PT	Tolinder and Goodin 1988a
G. hirsutum L.	H	C-SE-PT	Tolinder and Goodin 1988b
G. hirsutum L.	COT	C-SC-SE-PT	Finer 1988
G. hirsutum L. *G. barbadense* L. *G. arboreum* L.	H	C-SE	Trolinder and Chen 1989
G. hirsutum L.	P	C-SE	Gawel and Robacker 1990
G. hirsutum L.	H	C-SE-PT	Zhang *et al*. 1991
G. hirsutum L.	H	C-SE-PT	Voo *et al*. 1991
G. hirsutum L.	COT, H	C-SE-PT	Firoozabady and de Boer 1993
G. hirsutum L.	H	C-SE-PT	Kumar *et al*. 1998
G. hirsutum L.	COT, H	C - SE-PT	Zhang *et al*. 2000b
G. barbadense L.	H	C-SC-SE-PT	Sakhanokho *et al.* 2001
G. hirsutum L.	H	C - SE-PT	Mishra *et al*. 2003
G. klotzschian A	H	C-SE-PT	Sun *et al*. 2003
G. hirsutum L.	H	C-SE-PT	Kumria *et al*. 2003
G. hirsutum L.	H	C-SE-PT	Chaudhary *et al*. 2003
G. arboretum L.	H	C-SC-SE-PT	Sakhanokho *et al*. 2004
G. hirsutum L.	COT, H	C-SE-PT	Wu *et al*. 2004
G. hirsutum L.	H	C-SE-PT	Ganesan and Jayabalan 2004
G. hirsutum L.	H, P, SA	C-SE-PT	Aydin *et al*. 2004
G. hirsutum L.	H	C-SE-PT	Jin *et al*. 2005a, 2005b
G. davidsonii, *G. raimondii*, *G. stocksii*, *G. aridum*, *G. klotzschianum*	H	C-SC-SE-PT	Sun *et al.* 2006

C: callus; COT: cotyledon; H: hypocotyl; IE: immature embryo; LD: leaf disc; P: petiole; PE: pro-embryo; PT: plantlet; SA: shoot apex; SC: suspension culture; SE: somatic embryo; ST: stem

and culture systems (Trolinder and Chen 1989; Zhang *et al*. 1991; Zhang *et al*. 1997). An in-depth study of such factors would enable the development of genotype-specific culture methods to better enhance the tissue culture response of the recalcitrant crops. Wu *et al*. (2004) developed a new protocol for the highly efficient somatic embryogenesis and plant regeneration of ten recalcitrant Chinese cotton cultivars. The protocol was initially developed using *Gossypium hirsutum* L. cv. 'Coker 201', then applied to recalcitrant cultivars from China. It was achieved by regulating IBA (indole-3-butyric acid)/KT (kinetin) regimes, KNO_3 levels and L-asparagine (Asn)/L-glutamine (Gln) at different stages of the culture process. Studies on somatic embryogenesis in cotton so far are listed as **Table 1**.

Shoot and meristem culture of cotton and its application

Though studies on somatic embryogenesis have been successfully implemented for many years, the method has been reported to generate undesirable somaclonal variations in cotton (Stelly *et al*. 1989; Firoozabady and de Boer 1993). An extensive seed-to-seed variability in *in vitro* regeneration has been observed among Coker lines (Trolinder and Chen 1989; Gawel and Robacker 1990). Maintenance of callus and cell cultures for longer periods often results in plants that are morphologically abnormal and functionally sterile. Such variations pose a serious problem for maintenance of genetic uniformity in plants regenerated *in vitro*.

Several reports on plant regeneration *via* pre-existing meristems in cotton have been published (**Table 2**). Bajaj and Gill (1986) first obtained plant regeneration by using shoot tips from field-grown plants of *G. hirsutum*, and followed by Agrawal *et al*. (1997), Gupta *et al*. (1997), Zhang *et al*. (1996), Hemphill *et al*. (1998) and Hazra *et al*. (2000).

Among the explants for meristem culture, the embryo axis explant has many advantages as that the shoot regeneration process was relatively simple and not prone to somaclonal variations and chromosomal abnormalities (Saeed *et al*. 1997). The process of *in vitro* plant propagation from pre-existing meristems mainly consists of three steps: i) induction of shoot buds and their multiplication, ii) elongation of shoot buds into shoots and iii) *in vitro* or *ex vitro* rooting of shoots to form plantlets.

The size of the explant shows a tremendous difference in the frequency of plants recovered (Gould *et al*. 1991). Although the number of plants regenerated from shoot apex explants is higher, rooting is still problematic and variable, suggesting that rooting is highly dependent on the genotypes (Gould *et al*. 1991; Hemphill *et al*. 1998). One potential way to surmount the rooting problem in culture is the efficient grafting of shoots to seedling rootstocks (Luo and Gould 1999; Jin *et al*. 2006b). Most recently, the induction of multiple shoots (3.4 to 8.3 shoots/axis) from dormant axillary buds by cytokinin (Agrawal *et al*. 1997; Gupta *et al*. 1997; Saeed *et al*. 1997; Hemphill *et al*. 1998; Morre *et al*. 1998) offers a promising means for increasing regeneration efficiency, especially during selection in transformation experiments.

Table 2 Studies on plant regeneration in cotton *via* pre-existing meristems.

Species	Explant used	Response	Reference
G. arboreum L.	M, ST	Adventitious buds, multiple shoots	Bajaj and Gill 1986
G. hirsutum L.			
G. hirsutum L.	SA	Single shoot	Gould *et al*. 1991
G. barbadense L.			
G. hirsutum L.	SA	Single shoot	Zhang *et al*. 1996
G. hirsutum L.	CN, SA	Multiple shoots	Agrawal *et al*. 1997
G. hirsutum L.	SA, 2C	Multiple shoots	Gupta *et al*. 1997
G. arboreum L.	SA, 1C; SA, 2C		
G. hirsutum L.	ST	Single shoot	Saeed *et al*. 1997
G. hirsutum L.	EA	Single shoot	Agrawal *et al*. 1997
G. hirsutum L.	PM	Multiple shoots	Hemphill *et al*. 1998
G. hirsutum L.	CA	Multiple shoots	Morre *et al*. 1998
G. hirsutum L.	SA	Single shoot	Zapata *et al*. 1999
G. hirsutum L.	CN, SCN, ST	Multiple shoots	Hazra *et al*. 2000
G. arboreum L.	PB		
G. hirsutum L.	EA	Single shoot	Hazra *et al*. 2002
G. hirsutum L.	EA	Multiple shoots	Banerjee *et al*. 2003

1C: one cotyledon; 2C: two cotyledon; CA: caulinar apex; CN: cotyledonary node; EA: embryo axis; M: meristem; PB: petiole base; PM: preexisting meristem; SCN: split cotyledonary node; SA: shoot apex; ST: shoot tip.

THE CHIEF APPROACHES TO GENETIC TRANSFORMATION OF COTTON

Agrobacterium-mediated transformation

Agrobacterium-mediated transformation is the most widely used method to transfer genes into cotton. Umbeck *et al*. (1987) for the first time described the transformation of cotton 'Coker' varieties, an easily regenerable genotype, by using hypocotyl sections as explants inoculated with *Agrobacterium*. The plasmid CMC 1204 used for transformation contained a gene for neomycin phosphotransferase (NPTII) and chloramphenicol acetyltransferase (CAT). Three transgenic plants were identified that expressed NPTII and CAT. Firoozabady *et al*. (1987) used *Agrobacterium* strain LBA4404 to transform cotyledon pieces from 'Coker 201' in the same year, and obtained 15 transformants. The whole process from infection to transfer of transgenic plants to soil took 6-8 months. Perlak *et al*. (1990) developed transgenic cotton of *Bacillus thuriengiensis cry1A (b)* and *cry1A (c)*. Under high insect pressure with *Heliothis zea* (cotton bollworm), these transgenic *Bt* plants showed effective boll protection. Bayley *et al*. (1992) introduced 2, 4-dichlorophenoxyacetic acid (2, 4-D) resistance into cotton cultivar 'Coker 312' by transferring the 2,4-D monooxygenase gene, *tdf*A, *via Agrobacterium*-mediated transformation. The transgenic plants were tolerant to three times the field level of 2,4-D used for wheat, corn, sorghum and pasture crops. Cotton is very sensitive to 2,4-D, even spraying it with a small amount can cause serious damage. 2,4-D resistance prevents cotton from 2,4-D damage when 2,4-D is applied to other adjacent crops.

In another case, herbicide-resistant transgenic cotton plants carrying mutant forms of a native acetohydroxyacid synthase (AHAS) were developed by Rajasekaran *et al*. (1996). Meanwhile expression of protease inhibitor gene in cotton cultivar 'Coker 312' was also reported (Thomas *et al*. 1995).

More recently, our laboratory developed a reliable and high-efficiency system of transforming embryogenic callus (EC) of an upland cotton cultivar YZ-1 *via Agrobacterium tumefaciens* in cotton (Jin *et al*. 2005a). The effects of *Agrobacterium* strains, acetosyringone (AS), co-culture temperature, co-culture duration, *Agrobacterium* concentration and physiological status of EC on stable transformation were evaluated. An overall scheme for producing transgenic cotton was presented, through which an average transformation rate of 15% was obtained.

Although cotton has been transformed *via Agrobacterium*-mediated methods, *Agrobacterium*-mediated transformation has been associated with a few potential problems. One such problem is that *Agrobacterium*-mediated transformation of cotton has been limited to those specific cultivars that can be regenerated in tissue culture. To overcome this difficulty, Zapata *et al*. (1999) used the shoot apex as an explant for *Agrobacterium*-mediated transformation in cotton. Out of a total of 1010 *Agrobacterium*-treated shoot apices, eight plants grew on kanamycin selection at 100 mg/L and were transferred to soil. Progeny obtained by selfing were germinated in the green house. Evidence for integration of the *GUS* gene was observed in two successive generations from the regenerants.

Cotton transformation based on the shoot apex method has the advantage of being more genotype independent than somatic embryogenesis, and potentially allows for a speed-

ier recovery of transgenic lines. However, apart from the rooting problems, the advantages are easily offset by the low frequency of stable germline transformation events (reviewed by John 1997).

The second issue of concern is that *Agrobacterium* continues to persist on tissue following transformation, resulting in what is called a systemic infection (Matzke *et al*. 1996). However, while this is most serious in clonally propagated species, it is not a major concern in seed-propagated species such as cotton (Wilkins *et al*. 2000).

Regardless of the transgenic methods employed, successful transformation was dependent on root formation for recovery of transgenic plants. For 'Coker' lines, only 5-6% of somatic embryos root sufficiently to allow recovery of regenerated plantlets (Wilkins *et al*. 2000). Surmounting the rooting bottleneck would undoubtedly increase efficiency and decrease production costs of plant regeneration *via* somatic embryogenesis and genetic transformation in cotton.

Although cotton is cultivated as a herbaceous plant annually throughout the world, the genus is both perennial and woody and induction of shoot and root organogenesis is characteristically difficult (Gould *et al*. 1991). Especially, the loss of regeneration potential due to failure to form strong root systems during the *Agrobacterium*-mediated transformation resulted in very low efficiency of transformation and plant regeneration (Jin *et al*. 2006b). It is well known that transformation protocols calls for the use of many components that potentially provide a stress to the cells grown. It has been shown that β-lactam antibiotics interfere with growth, rooting and shoot regeneration of cultured cells and tissues (Montserrat *et al*. 2001; Teixeira da Silva *et al*. 2003). Recently, we developed an efficient grafting system for transgenic plant recovery in cotton (*G. hirsutum*) (Jin *et al*. 2006b). Using strong seedling rootstocks was the first important step to obtain high rate of successful grafts. Scion size >0.6 cm and seedling rootstock at age of 6-12 days were appropriate for grafting. The successful grafting ratio was higher when using hypocotyls without a radicle. Shoot-tips and shoot stem with axillary buds were also suitable for *in vitro* grafting. An over 90% successful grafting ratio could be obtained with the above optimized conditions which represented a significant improvement over currently available methods for recovery of cotton plantlet from somatic embryogenesis after transformation.

Particle bombardment-mediated transformation

There are two main types of explants used in particle bombardment-meditated transformation of cotton. One is the embryo meristem (shoot apex) and the other is embryogenic cell suspension cultures. The shoot apical meristem (SAM) is a population of cells located at the tip of the shoot axis. The shoot apex is divided into three layers (reviewed in Wegner 2006). Layer 1 (L1) is a single layer of cells that generally only undergoes anticlinal divisions, and gives rise to the epidermis. Layer 2 (L2) is also a single layer, and gives rise to ground tissue, while the innermost layer (L3) forms the body of new tissues, including vasculature and germline tissue. Only transformation events that occur in the L3 layer will result in germline transformation. Transformation that occurs in the L1 and L2 layers will result in chimeric phenotypes. The advantage of using the embryo meristem as an explant is that it allows genotype-independent transformation and the relatively rapid recovery of transgenic progeny (John 1997). The disadvantage of using embryonic meristems is that the preparation of shoot tip meristems is an extremely tedious, labor-intensive task, which involves the surgical removal of leaf primordia to expose the meristem, followed by the careful excision of meristem explants from imbibed seeds. Also, the stable transformation rate is very low (0.001 to 0.01%).

The first report on particle bombardment-mediated transformation in cotton was published by Finer and McMullen (1990). They bombarded embryogenic cell suspensions of 'Coker 310' with hygromycin genes as a selecting marker. Hygromycin resistant transgenic plants were developed *via* somatic embryogenesis, five months after bombardment. Three years later, McCabe and Martinell (1993) described a protocol for variety-independent transformation in cotton. They bombarded meristems (embryo axes) using the electric discharge gun for gene transfer. Integration of the *GUS* gene was demonstrated in R_0 and R_1 transformants. Progeny analysis showed transmission of the transgene in a Mendelian fashion. After that, transformation of meristems *via* particle bombardment were reported by Chlan *et al*. (1995) and Keller *et al*. (1997). Rajasekaran *et al*. (2000) achieved high frequency stable transformation of cotton by particle bombardment of embryogenic cell suspension cultures. They observed an increased stable transformation frequency of 4% compared to 0.7% in an earlier report by Finer and McMullen (1990). The high efficiency of stable expression was due to the multiple bombardment of rapidly dividing cell suspension.

Reports on cotton transformation *via A. tumefaciens* and particle bombardment mediated techniques were listed in **Table 3**.

Transformation via pollen-tube pathway

Introduction of exogenous DNA into a plant embryo through the pollen tube pathway after pollination was first reported by Zhou *et al*. (1983) in cotton. Hu and Wang (1999) reviewed the procedures used with this approach, the confirmation of the results, and the field performance of the transformed plants. The theory of this technique can be briefly described as follows: after pollination, the nucellar cells form a pathway to allow the pollen tube passage to the embryo sac, by removing the stigma and applying a DNA solution on the severed style after pollination, the exogenous DNA could presumably reach the ovary by flowing down the pollen tube and integrating into the just fertilized but undivided zygotic cells. The transformed seeds could be obtained directly without protoplast preparation, cell culture, and plant regeneration. By introducing ^{3}H-labeled DNA from bluish dogbane (*Apocynum venetum*) into cotton, Gong *et al*. (1988) indicated that the route of the exogenous DNA into the embryo sac was through the pollen tube and the exogenous DNA was randomly taken up by the eggs, zygotes, synergid and polar nucleus. However, those results did not provide clear evidence indicating that the DNA introduced was successfully integrated into the genome and expressed in the progeny of plants.

With the *GFP* gene as a reporter gene, the transgenic embryos and seeds of cotton (*G. hirsutum*) were obtained by the methods of pollen tube pathway with the plasmid pBIN35s-mgfp (Huang *et al*. 1998; Huang *et al*. 2001). Sothern blotting analysis proved the foreign gene had inserted into the cotton genome. Green fluorescence was detectable and screenable in cotton tissue by fluorescence microscopy and a hand-held ultraviolet lamp. These studies strongly confirmed the feasibility of pollen tube pathway method for cotton transformation.

Genetic transformation through pollen tube pathway offers several advantages, namely: The technique can overcome the host-range limitations of *Agrobacterium*, and nearly all genotypes can be transformed. Furthermore, transformation protocols are simplified, since complex bacteria/plant relationships varying with each system are eliminated. This is why this method is very popular in China especial for cotton genetic transformation. Many useful genes have been delivered into cotton genome using pollen tube pathway, such as the *Bt* gene (Xie *et al*. 1991), *Api* gene (Huang *et al*. 2001), *ipt* and *gus* gene (Yu *et al*. 2000), *Cpti+bt* gene (Guo *et al*. 1999), *Chi* and *GNA* gene (Liu *et al*. 2002). To date, more than half the successful genetic transforma-tions of cotton in China were made by pollen tube pathway method. More and more results showed success of pollen-tube pathway transformation in cotton, but stable transformation, independent experiences and molecular evidence

Table 3 An overlook for approaches to cotton genetic transformation.

Transgenic trait	Introduced gene	Method	Explant	Reference
Selectable markers or	*NPT*II and *OCS*	AT	COT	Firoozabady *et al*. 1987
reporter gene	*NPT*II and *CAT*	AT	H	Umbeck *et al*. 1987
	HPT	PB	ECS	Finer and McMullen 1990
	GUS	PB	ZEM	Chlan *et al*. 1995
	*NPT*II	AT	ST	Zapata *et al*. 1999
	*NPT*II and *GUS*	PB	ECS	Rajasekaran *et al*. 1996, 2000
	GFP	AT	H	Sunilkumar and Rathore 2001
	*NPT*II and *GUS*	AT	H	Satyavathi *et al*. 2002
	GUS gene without promoter	AT	EC	Jin *et al*. 2005a, 2005b
Pathogen resistance	Bean chitinase gene	AT	H	Tohidfar *et al*. 2005
	Antimicrobial peptide	AT	H	Rajasekaran *et al*. 2005
	Andochitinase	AT	H	Emani *et al*. 2003
	Antisense *AV2* gene	AT	ST	Sanjaya *et al*. 2005
Insect resistance	*CrylAc*	AT	ST	Perlak *et al*. 1990
	Proteinase inhibitors	AT	H	Thomas *et al*. 1995
	Bromoxynil tolerance	AT	Cot	Fillati *et al*. 1989
	CrylAc and *API*-B	AT	EC	Wu *et al*. 2005
Herbicide tolerance	*tfdA* for 2,4-D resistance	AT	H	Bayley *et al*. 1992
	tfdA for 2,4-D resistance	AT	H	Lyon *et al*. 1993
	tfdA for 2,4-D resistance	AT	H	Chen *et al*. 1994
	CP4 (CP4 EPSPS) for glyphosate tolerance	AT	H	Nida *et al*. 1996
	Mutant AHAS for sulfonylurea tolerance	AT	H	Rajasekaran *et al*. 1996
	BAR	AT	SA	Keller *et al*. 1997
Stress tolerance	Mn superoxide dismutase	AT	H	Payton *et al*. 1997
		PB	ECS	
Fiber genes	E6 antisense RNA	PB	ZEM	John 1996
	E-6 promoter +pha	PB	ZEM	John and Keller 1996
	FbL 2A promoter +pha	PB	ZEM	Reinhardt *et al*. 1996
	acsA and *acsB* genes	AT	P	Li *et al*. 2004
	ACTIN	AT	H	Li *et al*. 2005

acsA and acsB: *Acetobacter sylinum* celluloses synthase; AHAS: acetohydroxy acid synthase; API: Arrowhead proteinase inhibitor; AT: *Agrobacterium tumefaciens*; BAR: phosphinothricin acetyltransferase; CAT: chloramphenicol acetyltransferase; COT: cotyledon; EC: embryogenic callus; ECS: embryogenic cell suspension; EPSPS: 5-enolpyruvylshikimate-3-phophate synthase; GFP: green fluorescent protein; GUS: β-glucuronidase; H: hypocotyl; HPT: hygromycin phosphotransferase; NPTII: neomycin phosphotransferase II; OCS: octopine synthase; PB: particle bombardment; pha: polyhydroxyalkanoate synthase; SA: shoot apex; SE: somatic embryo; ST: shoot tip; ZEM: zygotic embryo meristem.

are needed for further research.

GENETICALLY MODIFIED COTTON

Transgenic cotton for insect resistance

About 100 species of insects are known to be associated with cotton. The most serious pests of cotton are bollworms: *Heliothis zea* Boddie and *H. armigera* Hubn. These bollworms are the caterpillars of several species of moths. The caterpillar feed in the boll damaging lint and seeds and cause a considerable reduction in yield and quality. Since the end of the 1980s, cotton production has decreased due to a decline in both yield and coverage area. The decline in yield of 15 to 30% has mainly been caused by bollworm (*Helicoverpa armigera* Hubner) infestation. In 1992 and 1993, outbreaks of cotton bollworm infestation in China caused direct economic losses of about $630 million. Furthermore, farmers were discouraged from growing cotton. As a result, the national growing area decreased by 10-15%, and there is a tendency for cotton production to move from relatively favourable areas towards marginal regions (Zhang *et al*. 2000a).

Control of insect pests in cotton cultivation depends mainly on the use of chemical insecticides that are under serious public debate for reasons of human safety and environmental pollution. Scientists have been looking for new strategies to control cotton insect pests. An attractive alternative is the production of proteins with insecticidal activity by the cotton plant itself. Numerous laboratory and field tests confirm that the most efficient and cheapest method for protecting cotton from pests is the utilization of transgenic cotton for insect resistance. The most widely favoured genes thought to be most useful for cotton are the *Bt* toxin genes which contains a crystalline protein toxin.

Genetic manipulation of Bt

Bacillus thuringiensis, commonly known as *Bt*, is a bacterium that occurs naturally in the soil. It has been used for more than 50 years as a biological insecticide (Qaim and Zilberman 2003). A critical factor following transformation is the desired expression of the insecticidal gene. *Bt* genes are Adenine-Thymine (A-T) rich while plant genes tend to have a higher Guanine-Cytosine (G-C) content. The expression of insecticidal proteins has been enhanced by increasing GC content of their encoding genes (Perlak *et al*. 1991). The first results on transfer of *Bt* genes in tobacco and tomato were published in 1987 (Fischhoff *et al*. 1987). Since then *Bt* genes have been transferred to many crops including cotton, maize, rice and potato. Perlak *et al*. (1990) produced insect resistant cotton by introducing the *Bt* gene into the cotton genome. The *Bt* coding sequence was modified to increase the levels of both *cry1A (b)* and *cry1A (c)* insect control protein expression to 0.05-0.1% of the total soluble proteins. These truncated forms of the insect control protein genes *Bt* provided effective pest control. The plants with the modified *cry 1A (b)* gene had a 10-100-fold higher level of insect control protein compared with the wild type gene. Similar results were obtained with the *cry 1A (c)* gene (Perlak *et al*. 1991).

Chinese scientists began modification of *Bt* genes in 1991. Since then, tremendous progress has been made in this field. The effects of different degrees of gene modifycation were investigated in the *cry1A* genes. The results indicated that removal of the polyadenylation sites and ATTTA sequences, and changes to a total of 353 of the 615 codons, raised the levels still higher (up to 0.2-0.3% of total soluble protein) – 100-fold higher than the level for unmodified genes (Zhang *et al*. 2000a).

Field testing of *Bt* cotton

The first field trial with genetically engineered plants expressing Bt toxin was conducted in 1986 with tobacco. Since then, many transgenic crops have been tested in field in USA, Argentina, Australia and China. In 2005, *Bt* crops occupied 16.2 million hectares in the world (James 2005).

The first field trials with *Bt* transgenic cotton were conducted in USA in 1988 (Jenkins *et al*. 1991). The cry1A proteins expressed in *Bt* cotton and *Bt* corn have been extensively tested for toxicological analysis in the laboratory and field. In China, the development of transgenic cotton that expresses Cry1A insecticidal proteins from Bt spp. *kurstaki* has resulted in new varieties or lines with improved resistance to key lepidopteran insect pests. Cotton plants expressing modified *cry1A* gene sequences have demonstrated excellent control of pests such as bollworm, tobacco budworm and pink bollworm in greenhouse and field experiments. Meanwhile, transgenic *Bt* cotton did not affect the natural enemies. Numerous field experiments showed that the total labour for pest control workdays could be decreased by 57% by planting *Bt* transgenic cotton varieties, of which the bollworm controlling labour workdays were decreased by 70% compared with planting regular cotton varieties, the total pest controlling input was reduced by 70%, of which the bollworm controlling input was reduced by 90% (Zhang *et al*. 2000a). Thus, Chinese breeders and farmers have more interest in the breeding and commercialization of transgenic *Bt* cotton.

Other insecticidal proteins

Apart from *Bt* genes, other genes for insect resistance such as those for proteinase inhibitors, α-amylase inhibitor, chitinases and lectins are also being used to produce transgenic insect-resistant cotton plants. The discovery of non-Bt insecticidal proteins from a host of plant and microbial sources offers a wealth of opportunity to significantly extend the range of insect pests that can be effectively controlled in transgenic crops.

Proteinase inhibitor genes

The presence of antimetabolic proteins, which interfere with the processes of digestion in insects, is a strategy for defense that plants have used extensively (Thomas *et al*. 1995).

Proteins can occur in tissues that are particularly vulnerable to attack, such as seeds, or can be induced by mechanical wounding in tissues attacked by chewing insect pests, such as leaves.

The first gene of plant origin to be successfully transferred to another plant species resulting in enhanced insect resistance was isolated from cowpea encoding a trypsin inhibitor (*CpTi*) (Hilder *et al*. 1987). Scientists at the Institute of Genetics of China also cloned the cowpea trypsin inhibitor (*CpTi*) gene. This gene was successfully engineered into cotton plants by both *Agrobacterium*-mediated transformation and the pollen tube pathway. The molecular data confirmed the stability of this gene and transgenic plants had increased resistance to cotton bollworm (Li *et al*. 1998).

The presence of serine proteinase inhibitors in plants can reduce insect attack. They are of interest because of their potent inhibitory activities against proteolytic enzymes of insects. Analysis of the effects of dietary proteinase inhibitors has shown that these are detrimental to the growth and development of insects, from a variety of genera including *Helicoverpa*, *Spodoptera* and *Diabrotica* (Wu *et al*. 1997).

More recently, fourteen different cDNA fragments encoding serine proteinases were isolated by reverse transcription-PCR from cotton boll weevil (*Anthonomus grandis*) larvae. Using a combination of 50 and 30 RACE, the full-length sequence was obtained for five of the cDNAs. Northern blotting analysis showed that for 2 genes, expression is induced upon feeding and is concentrated in the gut of larvae and adult insects. Reverse northern analysis of the 14 cDNA fragments showed that only two trypsin-like and two chymotrypsin-like were expressed at detectable levels. Under the effect of the serine proteinase inhibitors soybean Kunitz trypsin inhibitor and black-eyed pea trypsin/chymotrypsin inhibitor, expression of one of the trypsin-like sequences was upregulated while expression of the two chymotrypsin-like sequences was downregulated (Oliveira-Neto *et al*. 2004).

Lectin genes

Lectins are carbohydrate binding proteins and are abundant in seeds and storage tissues of some plant species. Lectins such as those purified from snowdrop or garlic are toxic to insects but not to mammals. The most effective protein tested is the lectin from snowdrop (*Galanthus nivalis agglutinin*, GNA), which gave approximately 80% mortality at a concentration of 1 g/L in the diet, when used in assays with first and third instar nymphs. GNA also had antimetabolic effect on brown plant hopper (BPH), and green leafhopper pests of rice (Powell *et al*. 1995). The *gna* is the first transgene to exhibit insecticidal activity towards sap-sucking insects in crop plant.

Recent interest has mainly concentrated on the lectin from snowdrop (GNA) in China, because it has shown activity against aphids, which are the third most important pests of cotton in China. Scientists in China have transformed the *gna* gene into cotton plants using the *Agrobacterium*-mediated method. Results of laboratory experiments indicated that GNA increased the resistance of cotton to aphids. Apart from the *gna* gene, scientists at the Institute of Genetics of CAS obtained transgenic cotton plants carrying the pea lectin (*P-Lec*) gene, which showed some resistance to cotton bollworm (Liu *et al*. 2002).

Second and third generation insect resistant cotton

Although constitutive expression of insecticidal transgene products has provided high levels of resistance in crop plants, tissue-specific or inducible expression might be desirable under some circumstances. Because the epidermal cells are the first to be attacked by insects, defence genes expressed under epidermal cell-specific promoters (e.g. CER6, an enzyme for cuticular wax production) might be useful. Phloem-feeding insects can be targeted using the root phloem-specific promoter AAP3 (Okumoto *et al*. 2004), the phloem-specific pumpkin promoter PP2. Progress is being made with chemically inducible promoters, including those induced by ethanol, tetracycline, copper, glucocorticoid steroid hormones, and steroidal and nonsteroidal ecdysone agonists (Padidam *et al*. 2003). Creating a 'within plant refuge' is a novel application of using inducible promoters whereby the transgenic plant or parts thereof can serve as a refuge plant as long as either the expression of the insecticidal gene is not induced or the induction wears off (Christou *et al*. 2006).

Theoretical models predict that plants expressing two dissimilar Bt toxin genes are likely to have the potential to delay resistance in target insect populations more effectively than single toxin-containing plants (Christou *et al*. 2006). The simultaneous introduction of three genes expressing insecticidal proteins, *Cry1Ac*, *Cry2A* and *Gna*, into indica rice to control three major pests, rice leaf folder (*Cnaphalocrocis medinalis*), yellow stemborer (*Scirpophaga incertulas*) and the brown planthopper (*Nilaparvata lugens*), has been reported (Bano-Maqbool *et al*. 2001). The *Bt* genes target the leaf folder and the stem borer, and the *Gna* gene targets the plant hopper. Triple transgenic plants were more resistant compared with their binary counterparts.

Transgenic cotton line SGK321 expressing two insecticidal proteins (*Cry1A* and *CpTI*) was commercialized in

northern China and demonstrated increased insecticidal activity on *H. armigera* relative to single gene *Bt* cotton (Liu *et al*. 2005).

Comparison of three different transgenic *Bt* cotton populations containing either the single *Cry1Ac* or *Cry2Ab* gene, or both genes, for fruit penetration and damage by a feral and *Cry1Ac*-selected strain of cotton bollworm revealed that transgenic cotton containing two *Bt* genes performed better (Jackson *et al*. 2004).

Developing some non-conventional sources of insecticidal novel proteins was another significant characters of the next generation insect resistant cotton. Second generation insect-resistant transgenic plants with increased potential for durable resistance might result from the deployment of plants expressing multiple insecticidal novel proteins such as the VIP (vegetative insecticidal proteins) produced by *B. thuringiensis* during its vegetative growth. These have insecticidal activity towards a wider spectrum of insect pests, yet they have little sequence homology with the more conventional Cry proteins (Yu *et al*. 1997). Transgenic cotton expressing such a VIP is expected to be released commercially in the USA.

Photorhabdus and *Xenorhabdus* bacteria are symbionts of entomopathogenic nematodes. Unlike Bt toxins, proteins produced by these two bacteria are not acutely toxic when ingested by the insect. Instead they cause septicaemia in the insect, the insect is killed and its tissues are used as nutrients by the nematode (Chattopadhyay *et al*. 2004). Considerable progress has been made in the identification of several toxin genes from these two bacteria (Williamson and Kaya 2003). These genes encode large insecticidal toxin complexes with little homology to other known toxins. Arabidopsis plants expressing toxin A gene from *Photorhabdus luminescens* showed strong insecticidal activity against one lepidopteran and moderate activity against a coleopteran pest (Liu *et al*. 2003).

Transgenic cotton for disease resistance

Diseases are another important factor which causes huge yield loss. The most common diseases are bacterial blight, leaf spots, grew mildew, wilts and root rot. Fusarium wilt, caused by *Fusarium oxysporum*, is a soil-borne fungal disease. This disease causes death or stunting of the plant with yellowing and wilting of leaves. Verticillium wilt caused by *Verticillium alboatrum* is another soil borne and most common diseases in the world. The disease is aggravated by cold wet weather and irrigation. Stunting, chlorotic, mottling and shedding of the leaves, squares and bolls are the symptoms of this disease. Besides the two wilt diseases, nematodes also cause considerable losses in cotton yield and quality (Goodell 1993), but this disease is not serious in China.

Mycoparasitic fungi are proving to be rich sources of antifungal genes that can be utilized to genetically engineer important crops for resistance against fungal pathogens. Emani *et al*. (2003) transformed cotton and tobacco plants with a cDNA clone encoding a 42 kDa endochitinase from the mycoparasitic fungus, *Trichoderma virens*. Plants from 82 independently transformed callus lines of cotton were regenerated and analysed for transgene expression. Several primary transformants were identified with endochitinase activities that were significantly higher than the control values. Homozygous T2 plants of the high endochitinase-expressing cotton lines were tested for disease resistance against a soil-borne pathogen, *Rhizoctonia solani* and a foliar pathogen, *Alternaria alternata*. Transgenic cotton plants showed significant resistance to both pathogens.

Fertile, transgenic cotton plants expressing the synthetic antimicrobial peptide, D4E1, were produced through *Agrobacterium*-mediated transformation (Rajasekaran *et al*. 2005). *In vitro* assays with crude leaf protein extracts from T_0 and T_1 plants confirmed that *D4E1* was expressed at sufficient levels to inhibit the growth of *Fusarium oxysporum* f. sp. *vasinfectum* and *Verticillium dahliae* compared to extracts from negative control plants. *In planta* assays with the fungal pathogen, *Thielaviopsis basicola*, which causes black root rot in cotton, showed typical symptoms such as black discoloration and constriction on hypocotyls, reduced branching of roots in GUS negative control T_1 seedlings, while transgenic T_1 seedlings showed a significant reduction in disease symptoms and increased seedling fresh weight, demonstrating tolerance to the fungal pathogen.

Rencently, Tohidfar *et al*. (2005) introduced the *chi* gene into the cotton genome. Integration of the *chi* gene into the genome of putative transgenic plants was confirmed by Southern blot analysis. Western immunoblot analysis of leaves isolated from T_0 transformants and progeny plants (T_1) revealed the presence of an immunoreactive band with MW of approximately 31 kDa in transgenic cotton lines using anti-chitinase-I polyclonal anti-serum. Chitinase specific activity in leaf tissues of transgenic lines was several folds greater than that of untransformed cotton. Crude leaf extracts from transgenic lines showed *in vitro* inhibitory activity against *Verticillium dahliae*.

Cotton leaf curl virus (CLCuD) is one of the many important threats for cotton productivity and has emerged as a serious disease of cotton in the world. Cotton transgenics for resistance against cotton leaf curl disease using antisense movement protein gene (AV2) were developed in an Indian variety (F846) via *Agrobacterium*-mediated transformation using shoot-tips as explants (Sanjaya *et al*. 2005). A binary vector pPZP carrying the antisense *AV2* (350 bp) gene along with the *npt*II gene was used. Transgenic nature of the putative transgenics was confirmed by molecular analysis. Shoots were induced on selection medium and subcultured on rooting medium containing IBA and 75 mg/L kanamycin. Transgenic plants were recovered in 12–16 weeks from the time of gene transfer to establishment in pots. Preliminary analysis of the field-established plantlets was conducted by PCR. T_1 plants were obtained from T_0 seeds, the presence of the *AV2* and *npt*II genes in the transgenic plants was verified by PCR and integration of T-DNA with *AV2* into the plant genome of putative transgenics was further confirmed by Southern blot analysis. Several T1 lines were maintained in the greenhouse. Progeny analysis of these plants by PCR analysis showed a classical Mendelian pattern of inheritance.

Transgenic cotton for herbicide resistance

Glyphosate [(*N*-phosphonomethyl) glycine] is a nonselective, postemergent, foliar-applied systemic herbicide most commonly marketed under the trade name Roundup® and Glyphomax®. Glyphosate-tolerant cotton was widely available commercially in the U.S. for the first time in 1997. It was immediately accepted and has increased in total acres planted and in market share each year since its release. It is estimated that glyphosate-tolerant cotton planted accounted for 87.6% of the total U.S. cotton crop in 2005 (Data source from the USDA National Agricultural Statistics Service, http://www.usda.gov/nass/pubs/).

One of the most commonly used herbicides to control broadleaf weeds is 2,4-D. 2,4-D and several related phenoxy compounds have been used extensively for more than 50 years. It is a post-emergence, translocatable herbicide and specific only to broadleaf plants. Soil organisms that degrade 2,4-D were identified more than forty years ago and the multi-enzyme pathways for 2,4-D degradation have subsequently been demonstrated in several bacterial genera. The first gene (*tfdA*) involved in the 2,4-D degradation pathway of soil organism *Alcaligenes eutrophus*, encodes 2,4-D monooxygenase enzyme, which converts 2,4-D into less toxic 2,4-dichlorophenol and glyoxylate by cleavage of the aliphatic side chain. Transgenic plants resistant to 2,4-D have been developed in tobacco as a model system. Cotton has also been engineered for 2,4-D resistance by *tdfA* isolated from *Alcaligenes eutrophus* plasmid pJP5 (Bayley *et al*. 1992; Lyon *et al*. 1993). Herbicide resistant transgenic

cotton harboring a single copy of the *tdfA* gene is released for field trials (Bayley *et al*. 1992). Transformants containing the *tfdA* gene were also verified to exhibit 50- to 100-fold greater tolerance to 2,4-D compared with untransformed controls by Chinese scientists (Chen *et al*. 1994).

Acetolactate synthase (ALS) is a central enzyme in the biosynthesis of the branched chain amino acids leucine, isoleucine and valine in plants. Herbicides targeting this enzyme belong to four structurally distinct classes: sulfonylureas, imidazolinones, pyrimidine sulfonamides and pyrimidinylsalicylates. These herbicide classes offer effective selective control of grass weeds. ALS, as an herbicide target site, is the most widely studied enzyme in terms of kinetics and genetics. It is the target that is reported to have the highest incidence of developing resistance to herbicides. A large number of mutations have been characterized; most of them are due to single amino acid sequence changes that do not affect the enzyme function but easily induce herbicide resistance in plants where they occur. This has resulted in the emergence of a great number of weeds with resistance to the sulfonylurea and imidazolinone herbicides classes (Tranel and Wright 2002). Genetic engineering methods were used to generate crops with resistance to ALS inhibiting herbicides including transformation with genes coding for modified forms of ALS. Gene transfer has been used to incorporate sulfonylurea resistance into several commercially important crops including cotton (Rajasekaran *et al*. 1996).

Resistance to bialaphos, a non-selective herbicide, was introduced into cotton through genetic engineering (Keller *et al*. 1997). A gene encoding phosphinothricin acetyltransferase (*bar*) from *Streptomyces hygroscopicus* was inserted into elite varieties of cotton through particle bombardment. Herbicide (Basta®) tolerance up to 15,000 ppm was demonstrated in greenhouse trials. The above studies demonstrate the potential for introducing commerically important genes directly into elite varieties of cotton.

Genetic engineering of cotton fiber

Over the last several decades, significant improvement has been made in the physical properties of cotton fiber through classical plant breeding. However, to make cotton fiber more versatile for textiles, there is a need to improve not only its strength and length but also its dye binding, thermal, wrinkle and shrinkage resistance properties. Recombinant DNA technology and improved transformation methods may enable production of new and improved fibers.

To improve the insulating characteristics of cotton fibers, engineered *phaB* (acetoacetyl-CoA reductase) and *phaC* (polyhydroxyalkanoate synthase) genes from *Alcaligenes eutrophus* were used for transformation. As a consequence, the rate of heat uptake and cooling was slower in transgenic fibers, resulting in higher heat capacity (John and Keller 1996). In another report, production of thermostatic polymer polyhydroxybutyrate (PHB) in cotton fibers was obtained through particle bombardment mediated plant transformation with tissue-specific and developmental regulation of cotton gene *FbL2A* (Rinehart *et al*. 1996).

Two cotton fiber cDNAs (GhMyb7 and GhMyb9) and their corresponding genes, encoding R2R3-MYB proteins, have been isolated from the allotetraploid cotton (*G. hirsutum* L. cv. 'DES119') and characterized. Northern blot analysis showed that GhMyb7/9 is expressed in flowers and fibers, and its expression in fibers is developmentally regulated. Auxin treatment increases transcript levels of GhMyb7/9 in fiber cells in an in vitro ovule culture system. In an *in vitro* DNA–protein-binding assay, suggesting that GhMYB7/9 may play a role in the transcriptional regulation of the *Ltp3* gene during fiber development. The overexpression of GhMyb7 in both transgenic tobacco and *Arabidopsis* plants causes a pleiotropic effect on plant development, including dwarf, abnormal leaf shape, and retarded root development (Hsu *et al*. 2005).

The *acsA* and *acsB* genes, which are involved in cellulose synthesis in *Acetobacter xylinum*, were transferred into pollen grains of brown cotton with the aim of improving its fiber quality by incorporating useful prokaryotic features into the colored cotton plants (Li *et al*. 2004). Fiber strength and length from the most positive transformants was 15% greater than those of the control (non-transformed), a significant difference in cellulose content was found between the transformed and control plants.

Li *et al*. (2005) characterized 15 *G. hirsutum* ACTIN (*GhACT*) cDNA clones using single-celled cotton fiber (*G. hirsutum*), a unique experimental system, to study cell elongation. RNA gel blot and real-time RT-PCR analysis revealed that *GhACT* genes are differentially expressed in different tissues and plays an important role in fiber elongation but not fiber initiation. As the functional genes were explored from cotton fiber, engineering cotton fiber according to market demand would be practical.

CHLOROPLAST GENETIC ENGINEERING OF COTTON

Chloroplast genetic engineering offers a number of unique advantages, including a high-level of transgene expression, multi-gene engineering in a single transformation event, transgene containment via maternal inheritance, lack of gene silencing, position and pleiotropic effects, and undesirable foreign DNA (Grevich and Daniell 2005).

Thus far, over forty transgenes have been stably integrated and expressed via the tobacco chloroplast genome to confer important agronomic traits, as well as express Industrially valuable biomaterials and therapeutic proteins. Highly efficient plastid transformation has been recently accomplished *via* somatic embryogenesis using species-specific chloroplast vectors in soybean, carrot, and cotton. Recent advances in plastid engineering provide an efficient platform for the production of therapeutic proteins, vaccines, and biomaterials using an environmentally friendly approach.

The complete chloroplast genome sequence of *Gossypium hirsutum*

Recently, Daniell's research group first reported the complete cotton chloroplast genome map, which will provide useful information for chloroplast genetic engineering (Lee *et al*. 2006).

The complete cotton chloroplast genome is 160,301 bp in length, with 112 unique genes and 19 duplicated genes within the IR, containing a total of 131 genes. There are four ribosomal RNAs, 30 distinct tRNA genes and 17 intron-containing genes. The gene order in cotton is identical to that of tobacco but lacks rpl22 and infA. There are 30 direct and 24 inverted repeats 30 bp or longer with a sequence identity ≥90%. Most of the direct repeats are within intergenic spacer regions, introns and a 72 bp-long direct repeat is within the psaA and psaB genes. Cotton chloroplast genome lacks rpl22 and infA and contains a number of dispersed direct and inverted repeats. RNA editing resulted in amino acid changes with significant impact on their hydropathy. Phylogenetic analysis provides strong support for the position of cotton in the Malvales in the eurosids II clade sister to *Arabidopsis* in the *Brassicales*.

Development of cotton chloroplast genetic engineering

Cotton plastid transformation has been extensively attempted using vectors containing species-specific cotton chloroplast vectors and different selectable markers. Spectinomycin had a lethal affect on cotton cultures that prevented the selection of transgenic lines. After identification of suitable selectable markers, the concept of a "double barrel" vector for plastid transformation was used for the first time. This concept employs a vector containing two selectable marker

genes (*aphA-6* and *npt*II) to detoxify the same antibiotic, in which they function around the clock in green and non-green plastids (Kumar *et al*. 2004). This is vital as most of the bombarded calli or suspension cultures are non-green tissues containing proplastids instead of chloroplasts. Gene expression and gene regulation systems in proplastids are quite different from green chloroplasts. Transformed proplastids should develop into mature chloroplasts and transformed cells should survive the selection process during all stages of development in the light and the dark. Following bombardment, transgenic callus cultures were multiplied on higher concentrations of kanamycin containing media in order to increase the amount of transgenic chloroplasts. Transgenic somatic embryos were matured, elongated into plantlets, and transferred to growth chambers for subsequent flowering and seeding. Additionally, several crosses between wild-type and chloroplast transgenic lines of cotton were performed to confirm maternal inheritance, lack of pollen transmission, and the Mendelian segregation of transgenes. As expected, no germination was seen of seeds from the F_1 cross of a wild-type female × male transgenic pollen, whereas, all self-pollinated transgenic seeds germinated on kanamycin plates (Daniell *et al*. 2005). Using this "Double barrel" plastid transformation vectors combining with an efficient regeneration system via somatic embryogenesis, cotton plastid transformation were achieved for the first time by Kumar *et al*. (2004). The"Double barrel" plastid transformation vectors is at least 8-fold (1 event/2.4 bombarded plates) more efficient than the 'Single Gene/ Single Selection (SGSS)' vector (aphA-6, 1 event per 20 bombarded plates). Chloroplast transgenic lines were fertile, flowered and set seeds similar to untransformed plants. Transgenes stably integrated into the cotton chloroplast genome were maternally inherited and were not transmitted via pollen when out-crossed with untransformed female plants.

The successful transformation of the cotton chloroplast genome marks a major advance in plastid genetic engineering. Plastid engineering offers solutions for concerns about transgene containment and the development of resistant insects, hopefully increase the public acceptance of GM cotton.

CONCERNS ABOUT THE SAFETY OF BIOTECH COTTON

Biotech cotton has multifarious advantages, and most papers and reports that have been published on this technology are favorable. The technology, however, does carry some risks, and unfortunately the negative aspects of biotechnology have not been properly covered in the scientific publications.

Unstable expressing of *Bt* gene and resistance to Bt toxins

Transgenic *Bt* cotton has been widely adopted in the world. It offers satisfactory control of major *lepidopteran* insects including cotton bollworm and pink bollworm. However, with millions of hectares of transgenic *Bt* cotton grown yearly, the possibility of insects developing resistance to Bt toxin needs to be addressed to ensure the sustainable use of *Bt* cotton (Gould 1998). One important principle of existing resistant management plans for *Bt* crops is that the *Bt* plants express the toxin at high and consistent levels, referred to as a 'high-dose' (Gould 1998). However, He *et al*. (2006) found that survival of the Asian corn borer (*Ostrinia furnacalis*) increased as the plants aged. This phenomenon was also observed for the cotton bollworms *H. armigera* (Zhang *et al*. 2001; Wu *et al*. 2003) and *H. zea* (Greenplate 1997; Lambert 1997). Increased survival in each species may be attributed to the decline in protein expression as the growing season progresses (Greenplate 1999; Zhang *et al*. 2001). Chen *et al*. (2005) evaluated the effect of high temperature on the insecticidal properties of *Bt* Cotton in China. The results showed that high temperature may result in the degradation of soluble protein in the leaf, with a resulting decline in the level of the toxin CryIA. It is believed that this may be the cause of the reduced efficacy of *Bt* cotton in growing conditions in China, where temperatures during the boll period often reach 36-40°C.

Some have predicted that Bt-insect-resistant crops would be of limited durability because mutations present at low frequency in 'wild' pest populations would be selected and give tolerance to the toxins. When a *Bt* gene is inserted into a cultivar, the Bt toxin is produced throughout the cotton plant during the entire growing season. Consequently, target pests are exposed to high levels of the toxin continuously, a situation likely to elicit resistance faster than intermittent exposure to conventional insecticides.

Selection for resistance to Bt toxin is akin to the development of resistance to chemical insecticides but the process is subtle. Most models of resistance to Bt toxin assume to be recessive, autosomal and controlled by a single diallelic gene with Mendelian inheritance (Gould 1998; Tabashnik *et al*. 2000; Liu *et al*. 2001). The Bt toxin is assumed to kill susceptible heterozygous (*Rr*) and homozygous (*rr*) individuals causing rapid selection for the few homozygous resistant (*RR*) survivors.

Constant presence of Bt protoxin in transgenic plants and the planting of *Bt* crops on a broad scale is thought to make the development of resistance more likely (Gould *et al*. 1997; Gould 1998; Hilder and Boulter 1999; Tabashnik *et al*. 2000). Although resistance has not developed in the field (Tabashnik *et al*. 2003, 2005), resistance has been demonstrated in several cotton pests in the laboratory (Tabashnik *et al*. 2000).

The Bt toxin could harm natural enemies indirectly

A major tactic of Integrated Pest Management (IPM) is to preserve natural enemies associated with crop pests (Bates *et al*. 2005). Natural enemies of pest species include generalist predators such as carabid beetles or specific parasites such as parasitoid wasps (Vojteck *et al*. 2005). Although insect-resistance factors expressed in crops might not have a direct effect on natural enemies of pests, indirect effects are almost inevitable.

The insect-resistant biotech cotton varieties are specific to a group of insects that includes most bollworms and budworms but excludes natural predators and parasites. The active toxin binds to receptors in the insect's midgut cells. The binding creates pores in the wall of the insect's gut, allowing ions to equalize, ultimately causing the gut to lose its digestive function (Gutierrez *et al*. 2006a). Once the binding has taken place after ingestion, the insect's gut is paralyzed, forcing it to stop eating. After the stomach is immobilized, the cells break open and the pH of the stomach decreases as its fluids mix with the lower-pH blood. A lower pH allows the spores to germinate and colonize the rest of the insect's cells. The bacteria spread throughout the rest of the host by the bloodstream until complete paralysis of the insect occurs (Gutierrez *et al*. 2006b). This process takes anywhere from an hour to a week to kill the insect. Beneficial insects might feed on insects that have taken up the toxin but have not died yet, or might digest by-products of insects such as honeydew that are contaminated with toxin. No data show that biotech toxin could kill beneficial insects, but the toxin could harm beneficial insects indirectly in the two ways described above.

Ponsard *et al*. (2002) examined the effect of Bt-cotton and of *lepidopteran* prey (*Spodoptera exigua* Hübner) that had ingested it on the adult survivorship of four important heteropteran predators of cotton pests. Longevity significantly decreased for *Orius tristicolor* White and *Geocoris punctipes* Say (by 28 and 27% of the control value, respectively), whereas no effect was found for *Nabis* sp. and *Zelus renardii* Kolenati.

Feeding on plant material expressing Bt proteins were shown to affect the growth and development of a carabid

beetle, with early instars being more sensitive than later instars and adult beetles (Meissle *et al*. 2005). To assess the ecological effects of Bt-cotton cultivars, the development of *Spodoptera litura* on transgenic Bt-cotton, the intake of Bt toxins, and the effects of Bt-cotton reared *S. litura* on young larvae of *Propylaea japonica* (a predator) were evaluated. The results suggested that the Cry1Ab/Ac fusion toxin had no direct effect on young larva of *P. japonica*, and a combined interaction of poor prey quality and Cry1Ac toxin may account for the negative effects observed on *P. japonica* development when fed NuCOTN 33B-reared *S. litura* (Zhang *et al*. 2006).

So, it can be concluded that all measures to protect crops against insect pests will reduce the numbers of available prey for predators and parasites, even if there is no direct effect (Schuler *et al*. 2003). This could be true particularly in cotton for the third and later generations towards crop maturity, when the amount of toxin is reduced and not all the target larvae will be killed.

Increased use of herbicide in stead of insecticides

U.S. data show that on average insecticides were applied to cotton 3 times per season to control the largest insects before the adoption of *Bt* cotton varieties in 1996. Five years later (2000/01), the *Bt*-planted area increased to 72% of the total cotton area, and insecticide use was reduced to 0.77 sprays per season against the target insects (Benbrook 2001). *Bt* cotton definitely reduced insecticide use. However, the introduction of herbicide-resistant biotech varieties in cotton has the potential to increase herbicide use. Herbicide tolerance, both in cotton varieties in the United States and in crops elsewhere in the world, is the most-used trait in biotechnology so far. International statistics show that of the total area of 9000 million hectares planted to biotech crops in 2005, 71% were under herbicide-resistant varieties (James 2005). Herbicide-resistant varieties make it possible for farmers to give up other control measures and rely on selected post-emergence herbicides as the backbone of weed management systems in cotton and other crops. Otherwise, long-time-use of herbicide may cause environmental problems, such as polluting the soil and underground water.

Non-target pests of Bt toxin may emerge as major pests

Bt cotton is effective against a variety of budworms and bollworms, but it is not effective in controlling many secondary pests requiring insecticides use for their control (Mahaffey *et al*. 1995; Gianessi and Carpenter 1999; Ru *et al*. 2002; Gutierrez *et al*. 2006b).

Effectiveness of transgenic cottons with *B. thuringiensis* (*Bt*) *cry1Ac* gene along with non-transgenic commercial cultivars of *G. hirsutum* and *G. arboreum* for the management of cotton bollworm, *Helicoverpa armigera* was evaluated (Sharma and Pamapathy 2006). The results showed that Bollworm damage in squares and bolls was significantly lower in the transgenic hybrids than in the nontransgenic ones, especially, the larval numbers were significantly lower on the transgenic hybrids during rainy season under high infestation. However, there were no differences between the transgenic and non-transgenic hybrids in their relative susceptibility to cotton jassid, *Amrasca biguttula* and serpentine leaf miner, *Liriomyza trifolii*, white fly, *Bemisia tabaci*, green bug, *Nezara viridula*, ash weevil, *Myllocerus undecimpustulatus*, and red cotton bug, *Dysdercus koenigii*.

Experience in China (Mainland) shows that populations of secondary pests such as aphids, mites, thrips, lygus bugs, whitefly, and leaf hopper, increased in Bt cotton fields after the target pests – budworms and bollworms – had been controlled (Xue 2002). Supporters and opponents of biotech varieties agree that Bt genes provide good control of target pests. But once the targets pests are controlled, minor and non-target pests may emerge as major pests. When minor pests become major ones, they may change the pest complex situation, and pests that are more difficult to control than the target pests may emerge as major pests, bringing new and difficult problems.

CONCLUSIONS

In summary, the first generation of transgenic cotton with bollworm or herbicide resistance, or both, contributed obviously to increase cotton production. Efficient transformation methods have been established meeting the needs of cotton breeding programs, but the functional genes available are still limited. In the long run, how to improve both pest and disease resistances, together with fiber quality simultaneously, should be the most important objectives in future. As novel genes are cloned and transformed, more transgenic cottons with diversified input traits will be seen in the cotton field.

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