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Regeneration and Genetic Transformation of Cotton: Present Status and Future Perspectives

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Abstract

Genetically modified (GM) insects and herbicides resistant cotton crop was among the first commercial transgenics to be planted on a large scale. Some of released GM cotton varieties have proved to be commercially viable, demonstrated by the increasing acreage under transgenic cotton crop. Transgenic cotton has lowered the production costs, can be better managed and reduces the adverse impact of the extensive use of harmful chemicals. There are many more critical traits that can be improved through bioengineering to commercial advantage. Genes conferring tolerance to various biotic and abiotic stresses, or improving the yield and fibre quality have been isolated and characterised. The introduction of these genes into the cotton genome cotton is by no means an easy task. Most elite cotton varieties remain recalcitrant and not amenable to genetic manipulation to protocols so far developed. The present article provides a brief overview of the protocols reported for regeneration and transformation of cotton, the basic steps of engineering.

Introduction

Cotton crop plays a crucial role in the global economy as well as social and industrial infrastructure. Besides being the backbone of the textile industry, cotton and its byproducts are also part of the livestock feed, seed-oil, fertilizers, paper and other consumer products. Handling, processing and production of various consumer-based products of cotton also play an important role in the social and industrial structure. The genus *Gossypium* comprises 50

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species, four of which are cultivated. *G. arboreum* L. and *G. herbaceum* L. are diploid (2n = 26), while *G. barbadense* L. and the most widely grown species *G. hirsutum* L. are tetraploid with 2n = 52. *G. hirsutum* or upland cotton is grown in more than 95% of the worldwide acreage; the next important cultivar is *G. barbadense* or the long staple Egyptian cotton. Though cotton is a relatively stress tolerant plant, sub-optimal conditions adversely affect the boll retention, filling and fibre quality. It has been postulated that most crops including cotton realize only 50% of potential yield due to abiotic stresses (Boyer 1982). Cotton pests also constrain the profitability of cotton production. The traditional control of insect pests has been in operation by the extensive use of chemical pesticides, which have led to severe environmental problems (Benedict and Altman 2001). Though breeding procedures have been used for cotton improvement, with emphasis on improving fibre or lint quality, the yield potential has reportedly reached a plateau over the past 30 years. This is mainly due to narrow genetic base on account of non-availability of wild cotton relatives containing desired traits. The limited gene pool has made the crop susceptible to pathogen attacks as well as environmental stresses. GM cotton allows improvement by use of the genes of specific interest, making the crop sustainable both economically and environmentally.

The regeneration and transformation of cotton pose problems in the development of bio-engineered cotton as they are genotype dependent, and reproducible protocols have not been well worked out for most elite cotton varieties. The majority of these are recalcitrant and not amenable to genetic manipulation. The introgression of transgenes into the elite cultivars is time consuming and leads to the transfer of certain undesirable traits from the regenerable cultivars. On the other hand, germline transformation protocols have very low transformation efficiency, requiring (a) a very large number of transformations to obtain a reasonable number of transgenics, (b) screening for the trait of interest only in the T1 generation. Screening in the T1 generation is necessary in order to eliminate chimaeric plants.

The transformation and regeneration in the reportedly amenable cultivars also has the disadvantage of prolonged culture periods, leading to somaclonal variations. It is extremely laborious with a low efficiency of transformation and economically not very viable due to the high cost of production and a low success rate.

One of the most important contributions of plant biotechnology to the farmers has been the development of *Bt* cotton which also happens to be the first genetically modified (GM) crop, marketed successfully in the past decade and

is still in use. The cultivation of *Bt* cotton has reduced the amount of insecticide use by 30-50% in the USA and Australia alone (Benedict and Altman 2001). Despite disadvantages facing the cotton biotechnology, major efforts and significant advances have been made and several transgenics have been developed and introduced in the field. This article reviews the work so far reported in cotton regeneration and transformation.

Cotton Regeneration

In vitro regeneration of cotton has been a difficult goal to achieve, because morphogenic response is genotype dependent and most of the elite cultivars are recalcitrant to genetic manipulation. Somatic embryogenesis is the preferred method over organogenesis, because regenerants have a probable single cell origin and the somatic embryos have no vascular connections with the maternal tissue, indicating that they are more amenable to *in vitro* manipulations (Shoemaker et al. 1986). A variety of genotypes as well as culture media have been screened for cotton by various workers to search for reasonably morphogenic cultivars in Coker series, which would give rise to embryogenic calli in the presence of an auxin. There is a lot of variation between cultivars as well as within a cultivar for callus initiation, proliferation and regeneration potential. Though media and hormonal manipulations can be attempted to induce regeneration in a cultivar of interest, the inherent variation amongst seedlings of the same cultivar also needs to be addressed. A Coker 310 pure line was developed for high regeneration potential and the trait was further introduced in the F1 hybrids involving other recalcitrant varieties (Kumar et al. 1998). Similarly, highly regenerable lines of the elite Acala cotton have also been developed by successively selecting for the regeneration potential (Mishra et al. 2003).

Other than screening for regenerable cultivars and selecting for high regeneration potential during the development of a pure line, various culture techniques variations have been tried to improve cotton regeneration. The manipulation of protoplasts was thought to provide an answer to the genotype dependence of cotton regeneration. Cotton protoplasts were found to form micro-calli but no regeneration was observed (Bhojwani et al. 1977; Finer and Smith 1982; Firoozabady and DeBoer 1986; Saka et al. 1987). Peeters et al. (1994) have reported regeneration from protoplasts isolated from the hypocotyl-derived embryogenic cell lines of Coker 312.

Regeneration of cotton plants from apical meristems and embryonic axes has also been attempted to gain genotype independence and overcome the

genetic damage caused by prolonged culture period. These procedures have led to the induction of multiple shoots from transformed meristematic tissues, capturing all possible transformation events. Kinetin (Kn) along with naphthalene acetic acid (NAA) has been used to induce shoots regeneration from shoot meristems or exposed embryonic axes (Gould et al. 1991; Hemphill et al. 1998; Zapata et al. 1999; Saeed et al. 1997). The use of high concentration of BA (benzyl adenine) induced multiple shoots from the exposed embryonic axes (Moore et al. 1998).

Somatic embryogenesis was first reported in *G. klotzschianum* but mature plants could not be obtained (Price and Smith 1979). Later, Davidonis and Hamilton (1983) described plantlet regeneration after spontaneous embryogenesis from a two-year old callus culture derived from a highly polyploid (12x) cotyledonary tissues of *G. hirsutum* var. Coker 310 through somatic embryogenesis. Rangan (1993) and Mitten (1985) also described somatic embryogenesis and plant regeneration in cotton. This procedure involved a lengthy culture period and was difficult to repeat. Shoemaker et al. (1986) have tested 17 varieties of cotton for their regeneration potential on three different callus-induction media, containing glucose; the mature embryogenic calli were transferred to sucrose containing media for embryo germination in the two embryogenic varieties, namely, Coker 210 and 315. The three callus induction media contained Kn and IAA or NAA in a 1 : 2 ratio and the calli were allowed to mature in the presence of N6-isopentenyl-adenine (2iP). Trolinder and Goodin (1988a and b) have extensively studied the optimal hormonal requirement and the kind of explants for efficient somatic embryogenesis and plant regeneration in Coker 312. They have recommended the use of 2,4-D along with Kn for initiation and maintenance of the embryogenic calli.

A high nitrate containing media was used for proliferation and maturation of embryos. Several other investigators have worked extensively on plant regeneration through somatic embryogenesis from not only Coker (Finer 1988, Firoozabady and DeBoer 1993, Trolinder and Xhixian 1989, Choudhary et al. 2003) but also in Sicala, Siokara (Cousins et al. 1991, Rangan and Rajasekaran 1996), Chinese cotton Simian-3 (Zhang et al. 2001) and Acala varieties (Rangan 1993, Rangan and Rajasekaran 1996). Although regeneration efficiency via somatic embryogenesis has been improved, there are still some problems associated with cotton regeneration. These are: variation in the genotype dependent response, prolonged culture period, a high frequency of abnormal embryo development, low conversion rate of somatic embryos into plantlets and lack of shoot elongation.

The long-term cultures of cotton led to regeneration of morphologically abnormal and sterile plants; these somatic variations can be overcome by the use of freshly initiated or cryo-preserved calli for regeneration (Rajasekaran 1996). An embryogenic callus line with a high regeneration potential can be cryo-preserved and can be used from time to time for transformation and regeneration. Kumria et al. (2003) have reported a high frequency of accelerated production and development of somatic embryos that grew into normal plantlets within five to six months after callus initiation through manipulation of nutrition and microenvironment conditions. The embryogenic calli, initiated from hypocotyls or cotyledonary leaf sections on 0.1 mg/l 2,4-D, 0.5 mg/l Kn, and 3% maltose supplemented MS medium produced globular-stage somatic embryos upon transfer to hormone free MS medium containing high concentrations of nitrate. Subculture of globular structures on hormone free MS medium led to the development of torpedo and cotyledonary stage embryos at a low frequency (two - four per plate). The majority of these embryos either did not show further growth or entered dedifferentiation stage (Trolinder and Goodin 1988b). The protocol has significantly improved embryogenesis rate (two - three fold), when calli were cultured on 1/5 strength MS medium. The frequency of globular embryos developing into normal plantlets also increased to 20 - 24 per plate, when cultured on a filter paper placed on MS medium. More than 70% of cotyledonary embryos developed into normal plantlets, when cultured on full strength MS medium containing 0.05 mg/l GA_3.

Cotton Transformation

The transformation of cotton can be attempted, once an efficient and reproducible regeneration system has been established. The two basic methods employed for the introduction of the gene into cotton are direct gene transfer via particle bombardment and *Agrobacterium*-mediated transformation. The regeneration of a plant from the transformed cell is dependent on the nature of the explant as well as the genotype transformed.

The prolonged culture period and the genotype dependent response can be overcome by biolistic delivery of the transgene of interest. Transformation of embryogenic suspension cultures of cotton by particle bombardment was first reported by Finer and McMullen (1990); they recovered transgenic plants within a five-month period. The method was adopted to produce herbicide resistant Acala and Coker cotton (Rajasekaran et al. 1996). Further refinement of this technique increased the frequency. Refinement consisted of subjecting rapidly growing suspension cultures to multiple bombardments followed by gradually increasing the selection pressure. This procedure promotes the development of

stable transformed lines (Rajasekaran et al. 2000). Bombardment of meristems has been reported to completely bypass the somatic embryogenesis step. However, the protocol is extremely labour intensive, as it requires the removal of leaf primordial tissues around the meristems and their excision from the seeds or seedlings. A very low percentage (0.001 - 0.01) of the bombarded meristems were transformed. This is because stable transformation occurs, only when the germline cells in the L2 and L3 layers of the meristematic tissue are transformed. Penetration of particles in the epidermal layer is higher but they do not transmit the gene to advance generation (McCabe and Martinell 1993; Chlan et al. 1995). Splitting the meristem to expose the L2 layer, has been found to promote re-organization of the meristem layers, thereby increasing the selection pressure. Wilkins et al. (2000) tried this method to improve the germ-line transformation events. Pruning of meristems has been found to induce the development of lateral buds, giving rise to germline transformants. John (1997) reported that by this method the frequency of transformation improved (0.09%). The induction of multiple shoots from the meristem also increased the number of shoot-producing transformed meristematic cells (Agrawal et al. 1997, Gupta et al. 1997, Morre et al. 1998). Even though the germline transformation is genotype independent, it does not involve any tissue culture phase and takes less time than somatic embryogenesis-based protocols. Bombardment leads to multiple insertions of the transgene, of which a few could be truncated with or without re-arranged sequences. Thus such an event leads to gene silencing in subsequent generations, resulting in the loss of the trait. The procedure is highly skilled, labor intensive and transgenics can be obtained only in the T1 generation. Furthermore, chimaeras are frequently observed (Wilkins et al. 2000).

Direct gene transfer to cotton protoplasts has not been demonstrated as the isolation and culture of protoplasts is a difficult procedure with only a single report of regeneration (Peeters et al. 1994). Some of the transgenics produced via particle bombardments of suspensions or meristems are listed in Table 1.

The transformation of cotton via *Agrobacterium* is a simple and efficient method of choice. Cotton transformation via *Agrobacterium* was first reported by Firoozabady et al. (1987) and Umbeck et al. (1987). Since then eighteen US patents have been granted on cotton transformation and regeneration. Besides transformation of seedling explants and embryogenic suspensions, meristems have also been transformed via *Agrobacterium* (Gould and Magallanes-Cedeno 1998, Zapata et al. 1999). The transformed meristems were selected on media supplemented with Kn or BA and NAA to obtain well rooted plants or plants

that were grafted to a rootstock (Luo et al. 2001; Satyavathi et al. 2001). Since the meristems did not de-differentiate, the intervening callus phase and somatic variations were also absent. This method has the advantage of being genotype independent; it is rapid and also possesses a fewer number of transgene insertions. The main disadvantage is that it is labor intensive and the transformants are chimaeric.

Table 1. Some useful genes introduced via particle bombardment.

Variety	Explant	Gene introduced	Trait	Reference
Coker 315	Embryogenic cell suspension	Mutant AHAS gene	Tolerance to sulphonylurea and imidazolinone	Rajasekaran et al. 1996b
Coker 312, Delta Pine 50, Pima s-6	Embryo meristem	Bar	Bialaphos resistance	Keller et al. 1997
Delta Pine 50	Embryo meristem	Fibre-specific antisense RNA	To study the role of the gene in fibre production	John 1996
Delta Pine 50	Embryo meristem	Pha B and pha C	Fiber with altered thermal properties	John and Keller 1996
Coker 312, Delta Pine 50, Sea Island	Embryo meristem	Acetyl Co-A reductase	Fiber with altered thermal properties	Rinehart et al. 1996

Umbeck et al. (1987) reported that the *Agrobacterium*-mediated transformation of the hypocotyl explants of Coker 310 were selected on 50 mg/l of kanamycin, containing callus induction medium (0.1 mg/l Kn and 0.1 mg/l 2,4-D). The calli growing on the selection medium were subcultured for 4 - 6 weeks after callus initiation on a hormone-free selection medium. Repeated subcultures on the hormone free medium led to embryogenesis and embryos were found that germinated on Stewart and Hsu medium. Firoozabady et al. (1987) have utilized cotyledonary explants of Coker 210 for transformation as these have a larger surface area for infection and their regenerating surface remains in contact with the medium, reducing the number of escapes. The callus was induced on a medium containing 0.1 mg/l NAA and 5 mg/l 2iP for 2 - 3 weeks; and later for embryogenesis the calli were transferred to basal medium. Embryos germinated on a low ionic strength medium supplemented with 0.1 mg/l NAA and 0.1 mg/l of GA_3. About 85 - 90% of the explants produced calli on the selection medium, and of these 95 - 100% were kanamycin resistant (Firoozabady et al. 1987). Some of the important traits introduced in cotton via *Agrobacterium*-mediated transformation are listed in Table 2.

A detailed study of cotton transformation reveals that there are certain critical factors such as inclusion of acetosyringone in the co-culture medium, co-cultivation at low temperature (21_C), larger size of the callus pieces that contribute to better survival rate on the selection medium. Furthermore, a two-week pre-culture on a high cytokinin and low auxin medium was found to improve the survival of callus pieces, though this treatment delayed embryogenesis (Sunilkumar and Rathore 2001). The efficiency of transgene insertions and the response of the explants were much higher than that through meristem transformation. However, difficulties were experienced with respect to regeneration time, capacity and somatic variation that came

Table 2. Some useful genes introduced via *Agrobacterium*-mediated transformation.

Coker 312	Seedling explants	Cry1Ac	Resistance to lepidopteran insect	Perlak et al. 1990
Coker 312	Seedling explants	2,4-D mono-oxygenase	Herbicide resistance	Bayley et al. 1992 Lyon et al. 1993
Coker 312	Seedling explants	Protease inhibitor	Resistance to insects	Thomas et al. 1995
Coker 312	Seedling explants	EPSP synthase	Glyphosate tolerance	Nida et al. 1996
Acala- GC 510,B3991, CSC28, Royale	Embryogenic cell suspension	Mutant acetohydroxyacid (AHAS)	Tolerance to sulfonylurea and imidazolinones	Rajasekaran et al. 1996b
Coker 315	Seedling explants	Bromoxynil specific nitrilase	Bromoxynil tolerance	Stalker et al. 1988
-	Seedling explants	Mutant acetolactate synthase	Tolerance to sulphonylurea	Saari and Mauvais 1996
-	Seedling explants	Superoxide dismutase	Tolerance to oxidative stress and freezing	Allen 1995, Allen and Trolinder 1995
Coker 312	Seedling explants	Mn superoxide dismutase	Oxidative stress tolerance	Payton et al. 1997
Coker 315	Seedling explants	Glucose oxidase	Fungal resistance	Murray et al. 1999
Coker 310	Embryogenic calli	Cry1I a5	Resistance to lepidopteran insects	Leelavathi et al. 2003

to surface during prolonged culturing. Most of the cells on cut surfaces of explants got transformed, but very few of these were found to be competent for morphogenesis. Each micro-callus piece represented a single transformation event, requiring a separate culture for proliferation and the induction of somatic embryogenesis. Since the process of somatic embryogenesis is asynchronous, prolonged culturing is required to obtain a viable embryo that may germinate.

The regeneration potential is extremely variable within a genotype, restricting the ability of micro-calli to undergo somatic embryogenesis. Thus, the particular transformation event might not lead to regeneration. The germination of embryos and the rooting of the plants are both very inefficient, further lowering the over-all efficiency of the process.

The above-mentioned hurdles have been overcome partially by the use of embryogenic calli with a defined embryogenic potential as the explant source for transformation. The embryogenic calli, competent for regeneration were found to be a mixed population of meristematic cells, pre-embryogenic cells and globular embryos. Most of these *Agrobacterium*–mediated transformed calli cells can be regenerated into plants, given the proper environment of nutrients and microenvironment management.

Leelavathi et al. (2003) have reported the introduction of a *Bt* gene (*cry*1Ia5) into Coker 310 by means of transformation of embryogenic calli. The cotton calli with pre-embryogenic cells were transformed with *Agrobacterium* and cultured on Whatman filter paper placed on the selection medium (1/5 MS medium with 1% maltose) and sealed with the micropore porous tape. The low nutrition as well as desiccation induced by the filter paper and micropore tape caused rapid proliferation of the embryogenic micro-calli. Each callus piece, that developed on a selection medium, represented an independent transfor-mation event. Each of these micro calli proliferated on a similar medium and later the embryos germinated on the maturation medium (Basal MS with 1% maltose). Most of the germinating embryos formed well-rooted plants; the slow growing plants were grown on 0.05 mg/l GA_3 and rooted on 1.0 mg/l IAA. An average of 75 globular embryo clusters were observed on a selection plate. These immature embryos were cultured on a multiplication medium. They developed into cotyledonary embryos on the embryo maturation medium. An average of 12 plants was obtained per Petri plate of callus co-cultivated. About 83% of these plants have been confirmed to be transgenics by Southern blot analysis. An efficiency of 10 Kanamycin-resistant plants per Petri plate of embryogenic calli co-cultivated was recorded. This method is simple, reliable, efficient and least laborious than any other existing methods for cotton transformation.

The protocol described above demonstrates that the embryogenic calli offers a large population of cells competent for the *Agrobacterium* transforma-tion. The nutritional and dehydration stress given at the selection stage induces direct development of transformed somatic embryos, contributing to a high frequency of transformation. Such a high frequency of transformation is accompanied by the reduction of the period for culture to 3 - 5 months as

compared to the conventional transformation method. Since growing embryogenic calli lines form the starting material for transformation, even production of a single regenerable callus line in a commercial variety might be useful for parallel multiple transformation experiments.

Since all the transformation and regeneration protocols that have been worked out have their advantages, the protocol of choice for the introduction of a gene of interest would be according to the cultivar to be transformed and the protocol available for that cultivar. The use of particle bombardment over *Agrobacterium* or germ-line transformation instead of somatic embryogenesis would depend on the available expertise and economics of the laboratory (Table 3).

Table 3. Comparison of various transformation protocols of cotton.

Explant	Particle gun/ *Agrobacterium*	Tissue culture	Chimera	Initial analysis	Time* (months)
Meristem	Particle gun	None	Yes	T1	11 - 12
Embryogenic cell suspension	Particle gun	Yes	No	T0	10 - 12
Meristem	*Agrobacterium*	None/ multiple shoot induction	Yes	T0 or T1	11 - 12
Seedling explant	*Agrobacterium*	Yes	No	T0	10 - 12
Embryogenic callus	*Agrobacterium*	Yes	No	T0	5 - 6

*Time period required for obtaining stably transformed cotton plants ready for transfer to soil and subsequent breeding program.

Future perspectives

Cotton is an important renewable resource that shall continue to play an important role in the future world economy. Some major issues in cotton management are tremendous losses incurred by pest infestation and the expensive and environmentally harmful chemical control of these pests. Therefore, the thrust at present is on bioengineering insect resistant cotton varieties. In addition, there are several other traits that need to be addressed to further improve the cotton crop for enhancing its role in the future economies. Some of these traits are: lint yield and fibre quality with respect to colour, length, elasticity, dye retention, spinning quality, naturally coloured fibres or fibres with antimicrobial and heat resistant properties. The improvement of the seed oil content, reduction in gossypol content and bioengineering resistance

to fungal and viral infection as well as tolerance to low water conditions also are a priority. In the field of insect resistant cotton too, better strategies have to be evolved to avoid the development of resistance in the insect population. Some of the suggested steps are: the introduction of multiple *cry* genes, mutated *cry* genes, *cry* genes along with non-*Bt* insecticidal genes; tissue specific expression; enhanced expression or organelle specific expression like chloroplast expression, where the expression levels are very high. This will totally eliminate the use of pesticides, besides *Bt* crop management by crop rotation and maintenance of refuge plants.

Several molecular markers linked to the important traits have been developed for cotton. Furthermore, the molecular regulation of various vital processes is also under investigation through identification of differentially expressed genes. Some of these genes have been identified and characterized. Production of coloured fibre is another area of interest in transgenic cotton. The up regulation or down regulation of these genes can be engineered to achieve some of these goals. Engineering multi-gene regulated traits that are essential to improve yield and other qualities of cotton would require the isolation and characterization of tissue specific promoters for a regulated transgene expression. As for the transformation of cotton, a thorough study is required for organellar transformation of cotton, such that a high expression and maternal inheritance of the genes of choice is achieved. Varieties other than Coker have also been successfully transformed but most of the elite commercial varieties remain recalcitrant to tissue culture. It is of utmost importance that the elite varieties are transformed, so that the delay due to backcrossing and the retention of the undesirable traits from the regenerable cultivars can be avoided.

Though the basic engineering tools are available for this crop, cotton biotechnologists would have to face many more challenges before this crop, could be bioengineered for planting all over the subtropical world under diverse ecological conditions related to both biotic and abiotic stresses.

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