DEVELOPMENT OF TRANSGENIC EGYPTIAN COTTON VARIETIES USING BACTERIAL FRUCTOSYL TRANSFERASE GENE CODING FOR FRUCTAN ACCUMULATION

O. A. Momtaz[1], H. Z. Hassan[2], Hoda M. Barakat[2], Nahla A. El Sherif[1, 2], A. Bahieldin[1, 3] and M. A. Madkour[1]
[1]Agricultural Genetic Engineering Research Institute (AGERI)
Agricultural Research Center
Giza, Egypt
[2]Department of Botany, Faculty of Science
Ain Shams University
Cairo, Egypt
[3]Department of Genetics, Faculty of Agriculture
Ain Shams University
Cairo, Egypt

Abstract

Fructans are polyfructose molecules that function as non-structural storage carbohydrates in several plant species. Owing to their solubility, fructans may help cotton survive periods of osmotic stress induced by drought or cold. We developed transgenic Egyptian cotton plants (*Gossypium barbadense*), from long staple variety, Giza 85, and extra long staple variety, Giza 88 by introducing the bacterial *sacB* gene of *Bacillus subtilis* coding for fructosyl transferase enzyme fused to the vacuolar targeting sequence of the yeast carboxypeptidase Y (*cpy*) gene and the selectable marker gene *bar* encoding phosphoinothricin acetyl transferase enzyme which detoxifies Basta herbicide. The transformation was achieved by particle bombardment using shoot meristematic apices

R_0 plants acclimatized in the greenhouses were submitted to preliminary screening for chimeric transformants by painting the plant leaves with basta herbicide (200 mg/L) to confirm *Bar* gene integration. The *bar* gene was found to be incorporated into 30 % of transformed cotton plants. Further analyses on the basta positive- basta putatively transformed cotton plants were performed using Southern blot analysis. *cpy-sacB* chimeric gene was labeled and used as a probe. Polymerase Chain Reaction (PCR) revealed the presence of the intact *cpy-sacB* chimeric gene in 8 and 7 cotton plants from both Giza 85 and 88, respectively. Genomic Southern blot analysis confirmed the integration of *cpy-sacB* gene into the plant genome. *cpy-sacB* was found to be integrated into only two plants out of one thousand plants of each cotton variety.

Reverse transcriptase -polymerase chain reaction (RT-PCR) was carried out to permit the detection and quantification of RNA transcripts into the cotton plant genome. A 1.7 Kb cDNA fragment was detected using RT-PCR, which confirms the transcription of the chimeric *cpy-sacB* gene.

Polyfructose molecules were detected in plant cotton tissue by HPLC and the increase of dry weight in fructose level was determined in putatively transgenic plants transformed with the fructosyl transferase gene compared to non-transgenic ones.

Introduction

Cotton (*Gossypium barbadense*) represents one of the most important economical crops in Egypt. Egyptian cotton is an attractive crop for genetic engineering because of its worldwide known importance. One of the major problems facing Egyptian cotton is the environmental stress condition including salinity and drought factors.

Reprinted from the *Proceedings of the Beltwide Cotton Conference*
Volume 2:1423-1429 (2001)
National Cotton Council, Memphis TN

A major challenge in agricultural research is the utilization of the advances in recombinant DNA technology to improve the efficiency of crop production and stress tolerance. The emerging technology offers the potential to introduce foreign genes coding for desired agronomic traits into plants to improve their characteristics while maintaining the high level of productivity achieved by traditional plant breeding and current agricultural practices.

Several techniques to introduce foreign genes into plants have been developed. In 1983, the era of plant transformation was initiated when *Agrobacterium*-mediated gene delivery was used for producing transgenic plants **(Fraley *et al.,* 1983).** An obvious limitation of *Agrobacterium* was the apparent inability to transform monocots. Today, particle bombardment offers a rapid method for delivery of genes of interest to plant cells for both gene transient expression and stable transformation studies **(Klein *et al.,* 1987; Boynton *et al*., 1988).** The first report of microprojectile bombardment to deliver DNA to living cells was by **Klein *et al.* (1987).** Several subsequent publications described optimization of gun parameters and evaluations of gene expression have been reported **(Russell *et al.,* 1992).**

During the last few years, many transgenic plants were obtained resistance to insects **(Fischhoff *et al*., 1987; Chen *et al*., 1998; Altpeter *et al*., 1999)**, viruses **(Khalil *et al*., 1999)**, fungi **(Bliffeld *et al*., 1999)** and herbicides **(Comai *et al*., 1985; Datta *et al*., 1992)** as well as salt and drought tolerance **(Pilon-Smits *et al*., 1999; Sivamani *et al*., 2000).**

In plants, fructans are thought to be synthesized from sucrose in the vacuole by a concerted action of at least two fructosyl transferases **(Pontis and del Canpillo, 1985; Pollock and Cairns, 1991)**. Vacuolar fructans are soluble and hence may play a role in the osmotic adjustment of natural fructan accumulators in response to the environmental conditions changes via variation in the degree of polymerization (DP) of the fructan pool without having to alter the total amount of carbohydrates **(Pilon-Smits *et al.*, 1995)**.

Because no plant genes involved in the fructan metabolism have yet been isolated, a bacterial gene, the *sacB* gene from *Bacillus subtilis* encoding levansucrase was used in the present study.

To enhance fructan accumulation in cotton, a construct was created so that the fructosyl transferase gene of ***Bacillus subtilis* (*sacB*)** was fused to the vacuolar targeting sequence of the yeast carboxypeptidase Y (*cpy*) gene. Such vacuolar targeting signal was added to the upstream of the *sacB* sequence to cause accumulation of the synthesized fructan in the cell vacuole. The construct was placed under control of the double constitutive 35S promoter and transferred into cotton meristematic tissue so that the regenerated cotton plants can accumulate fructans.

The aim of this work is to introduce the *SacB* gene coding for the fructosyl transferase enzyme into the two Egyptian cotton varieties Giza 85 (long staple) and Giza 88 (extra long staple) to enhance fructan accumulation by the following steps:

1. Construction of a vector containing the bacterial *SacB* gene as well as the vacuolar targeting sequence, *cpy* gene and the *bar* gene as a selectable marker gene.
2. Transfer of the construct vector into the meristematic apices of mature cotton embryos (Giza 85 and Giza 88).
3. Regeneration of cotton plants through tissue culture.
4. Screening for the putatively transgenic plants in the greenhouse using basta as a selective herbicide followed by PCR, PCR-Southern and genomic Southern to confirm the integration of construct into the plant genome, as well as RT-PCR and HPLC to confirm *cpy-sacB* gene expression.

Materials and Methods

Seeds of the two cotton varieties Giza 85 and Giza 88 have been kindly supplied by the Cotton Research Institute, Agricultural Research Center (ARC), Ministry of Agriculture, Giza, Egypt.

pFrucI Vector Construction

The expression vector pFrucI used in the present study has been constructed as follows: The vector pRQ101B (provided by Rongda Qu, NCSU, USA) carrying the selectable marker *bar* gene was used. The gene is driven by the cauliflower mosaic virus 35S promoter and Adh1 sequences at the 5` terminus and NOS polyadenelation sequence at the 3` end. Digestion of pRQ101B vector with XmaI yielded two fragments of 1.9 and 3.39 Kb, the former fragment is the *bar* gene flanked with 35S promoter and Adh1 sequences at the 5` terminus and nos sequence at the 3` end. The *Xma*I *bar* fragment has been separated and eluted by agarose gel electrophoresis and then ligated to pKM5 (6.4 Kb) vector. The plasmid pKM5 (provided by Michael Ebskamp) carrying the *cpy-sacB* chimeric gene driven by the double 35S promoter at the 5` terminus and NOS sequence at the 3` end was dephosphorylated with calf intestinal phosphatase (CIAP) following restriction with *Xma*I enzyme to prevent the self ligation of the two sticky ends created. Ligation of the *Xma*I *bar* fragment with pKM5 generated pFrucI (8.1 Kb). This final construct (pFrucI) was used to transform cotton plants by the biolistic gun bombardment method.

Explant Preparation

Cotton seeds were delinted using 98% commercial sulfuric acid. The seeds and acid were mixed together until all the fuzz was removed. The seeds were then rinsed several times in water, and some sodium bicarbonate was added to neutralize any remaining acid. The seeds were then allowed to air dry completely. Delinted cotton seeds were surface sterilized by washing first in double distilled water several times, and then immersing in a solution containing 40% chlorox and one drop of detergent. They were mixed together on a magnetic stirrer for 20 min and then rinsed five times in sterile double distilled water and left in the final rinse overnight for the seed coats to soften sufficiently. Sterilized seeds were aseptically dissected to expose the meristem sections. The removed embryos with exposed meristematic tips were then placed carefully at the center of 100 X 15 mm petri dishes, with 25 - 30 embryos per plate. Bombardment was carried out within 2-3 h of preparation.

Cotton Transformation

Particle bombardment methodology was used to introduce genes into the commercial cotton varieties Giza 85 and Giza 88. Cotton seed axes were removed and bombarded with tungesten particles coated with pFrucI plasmid. The plasmid coated tungesten microprojectiles were prepared as described by **McCabe and Martinell (1993).** Microcarriers were applied to macrocarriers in ethanol, and allowed to air dry before use. The explants were bombarded using the following parameters; 0.5 µm tungsten powder, a chamber vacuum of 25 Hg, a pressure of 1100 psi, with a shooting distance of 6 cm.

Regeneration of Bombarded Tissue

After bombardment, plates were wrapped with parafilm and incubated overnight at 30°C in the dark to heal the bombarded tissues. The tissues were then transferred to sterile germination medium **(Shoemaker *et al.*, 1986).** Explants were cultured on this medium in a growth room adjusted at 28°C with a 16-hour photoperiod (90 mE $m^{-2}s^{-1}$). Two weeks following initial incubation, shoots began to appear and 1-2 weeks subsequently, the roots began to form. When an extensive root system had developed, plantlets were transferred to soil in 10 cm pots containing 1:1:1 mixture of sterile topsoil, sand and peat. The pots were placed in a greenhouse adjusted at a temperature of 28ºC with a 16-h photoperiod and 90% humidity, and covered initially with plastic bags until adaptation to soil was underway, and from 7 to 10 true healthy leaves were formed. The bags were then gently removed, and 2 weeks later, the plantlets were transferred to larger, 30 cm pots.

Screening of Putatively Transformed Tissues

The herbicide Basta was used for preliminary screening of putatively transformed cotton plants. Leaves from the putatively transformed and non transformed cotton plants were painted in the greenhouse with an aqueous solution of the herbicide Basta (200 mg/l). Another group of non-transformed cotton plants were painted with water and used as a negative control. The herbicide was applied by painting the terminal parts of the plant leaves from both sides. Plants showed resistance to leaf painting were subjected to the next molecular screening analyses.

Plant DNA Extraction

Genomic DNAs extraction from all basta positive cotton plants was performed separately using the CTAB method according to **Doyle and Doyle, 1987.**

Plant RNA Extraction

Plant RNA was extracted from cotton plants using the SV Total RNA Isolation System from Promega. (Technical manual No. 048).

Polymerase Chain Reaction

PCR was carried out using specific primers based on the coding sequences of the chimeric *cpy-sacB* gene. These primers have the following sequences:

1. Fruc-P
   5′-CCAGATCTAACAATGAAAGC
   ATTCCAGTTTAC-3′
2. Fruc-M
   5′ CCGATATCTTATTTGTTAACT
   GTTAATTGTCC 3′

Primers were designed and synthesized at the Agricultural Genetic Engineering Research Institute (AGERI), Egypt.

Genomic DNAs extracted from all Basta positive cotton plants was used separately for PCR amplification. The pFrucI vector was used as a positive control while DNA from non-transformed cotton plants was used as a negative one.

PCR conditions were optimized and the amplification reactions were made in volumes of 25 µl containing 1 X *Taq* DNA polymerase buffer (10 mM Tris-HCl, pH 9.0, 50 mM NaCl, 0.01 Triton X-100), $MgCl_2$ (2mM), 100 mM each of dATP, dCTP, dGTP, and dTTP, 0.2 mM of each primer, 30 ng of genomic DNA, and 5 units of *Taq* DNA polymerase. Amplification was performed in a Perkin Elmer Cetus DNA Thermal Cycler (Perkin Elmer) programmed for 35 cycles of 30 sec, denaturation at 94ºC, 1 min annealing at 50ºC, and 1 min extension at 72ºC. Amplification products were analyzed by electrophoresis in a 1% agarose gel.

PCR Southern Analysis

Southern blot analysis was carried out for amplified PCR products of the integrated *cpy-sacB* chimeric gene. Amplified products were run on a 1% agarose gel at 100 Volt. The probe used was the *Eco*RI/*Hin*dIII fragment of the *cpy-sacB* gene (1.7 Kb). Labelling and detection were carried out using the non-radioactive DIG system from Boehringer Mannheim.

Genomic Southern Analysis

Genomic DNA was isolated from cotton plant samples that gave positive PCR results and were digested using *Hin*dIII flanking restriction enzyme. The digested DNA were run at 40 Volts overnight on a 0.7% agarose gel then blotted onto positive nylon membrane and hybridized overnight with the non-radioactive *Eco*RI/*Hin*dIII fragment probe.

The DNAs in either PCR-Southern or genomic Southern were transferred onto B.M positive nylon membrane The prehybridization, hybridization and washing processes were conducted according to the manufacture's protocols (Boehringer Mannheim).

Reverse Transcription (RT)-PCR

RT-PCR was carried out using the Titan One tube RT-PCR system from Boehringer Mannheim to determine the presence or absence of RNA template of the *cpy-sac*B chimeric gene in the cotton genome background.

The reaction components were set up in two separate nuclease-free microfuge tubes placed on ice as described in the manual provided with the kit. 25 µl of each master mix 1 and master mix 2 were added to a 0.2 ml PCR tube on ice, mixed and centrifuged. The samples were placed at 50°C for 30 min. to eliminate any secondary structures. Samples were used directly in PCR. The temperature profile in the different cycles was as follows:-An initial strand separation cycle at 94°C for 2 min. This was followed by 33 cycles including a denaturation step at 94°C for 30 sec, an annealing step at 56°C for 30 sec and an elongation step at 68°C for 1.5 min. The final cycle was a polymerase or an extension cycle performed at 68°C for 7 min.

HPLC Analysis

Plants were grown in the greenhouse and leaves from both putatively transgenic and non-transgenic plants were cut off and hydrolyzed to determine the fructose level in each according to the FAO specifications (FAO, 1982) by boiling solutions of 0.5 g leaf tissues in 12.5 ml of 40% w/w (4 gm conc H_2SO_4+96 ml Distilled water) for 3 h. The hydrolyzed solution was neutralized by the addition of 2 g of barium carbonate to each sample. The neutralized hydrolysate was filtered to remove insoluble barium salts and the clear supernatants were injected into an Hewlett Packard 1050 HPLC. The samples were passed at a pressure of 1000 psi at flow rate of 0.5 cm^3/min through an Aminex HPX-87C column (Bio-Rad) operated at 85°C with water as eluent. The retention times of the separated fructose residues were detected by a refractive index detector HP 1047A. Fructose was used as an internal standard.

Results and Discussion

Vector Construction

The expression vector pFrucI was constructed for cotton transformation (Fig. 1). The vector carries both the chimeric *cpy-SacB* gene driven by the constitutive promotor Ca35S and the selectable marker *bar* gene. The 35S promoter has been used extensively and has been found to direct high level of gene expression in a wide variety of plants **(Odell *et al.,* 1985).** Duplication of the promoter, results in a 10-fold increase in expression **(Kay *et al*., 1987).**

Both pRQ101B vector (5.29 Kb) and pKM5 vector (6.4 Kb) have been digested with *Xma*I restriction endonuclease. *Xma*I is an isoschizomer of *Sma*I. The two enzymes identify the same restriction site CCCGGG. The only difference being that *XmaI* creates sticky ends whereas *Sma*I creates blunt ones. Digestion of pKM5 vector resulted in its linearization whereas digestion of pRQ101B vector yielded two fragments of 1.9 and 3.39 Kb, the former fragment is the *bar* gene flanked with CaMV 35 S promoter and Adh1 sequences at the 5` terminus and nos polyadenelation sequence at the 3` end. The *Xma*I *bar* fragment has been separated and ligated to the opened pKM5 (6.4 Kb) vector to generate pFrucI (8.1 Kb). This final construct (pFrucI) was used to transform cotton plants by the biolistic gun bombardment method. Selection based on the application of herbicides to transgenic crops has been shown to be practical and useful **(Bayley *et al*., 1992; Stalker *et al*., 1996; Keller *et al*., 1997**).

The transformation vector pFrucI was verified by restriction endonuclease digestion using restriction endonuclease enzymes; *Sac*I*, EcoR*I *and BamH*I (data not shown). Addition of all the lengths of the DNA fragments obtained after restriction endonuclease digestion of pFrucI plasmid with the above mentioned restriction enzymes yielded the length of the whole vector, 8.1 Kb, as indicated in the restriction site map (Fig. 1) which is a proof for its length and different restriction sites found on the map.

Biolistic Transformation of Embryonic Axes

Biolistic transformation of the meristematic tips of mature cotton embryos was carried out for both Giza 85 and Giza 88 varieties as described by **Momtaz *et al.* (2000).** There was no difference found in the transformability of these two varieties, similar to observations by **Rajasekaran *et al.* (2000).** The use of direct transformation of mature embryos has been found to be a simple and time saving procedure, circumventing the problems of tissue culture variations and allowing for the transformation of crops which otherwise prove difficult to regenerate through somatic embryogenesis. In other words, the particle bombardment method introduces the transgene directly into elite varieties, avoiding years of crossbreeding **(Keller *et al*., 1997).** It has been used to successfully transform a number of cotton cultivars (**John and Crow, 1992; McCabe and Martinell, 1993; John and Keller, 1996**), confirming that this methodology is reliable for cotton genetic engineering **(Keller *et al*., 1997)**. The practicality of particle bombardment for the generation of transgenic plants capable of transmitting the introduced DNA to progeny depends on the ability to identify and target either meristematic or embryogenic cells that will give rise to germ line tissue **(Finer and McMullen, 1991**). The main advantage of this method being that organized tissue can be used for transformation, thereby circumventing the difficulties of somatic embroyogenesis and avoiding somaclonal variation. In this study, the meristematic tips of mature embryos were used as explants for transformation. This type of transformation is labour-intensive because meristematic tissue is difficult to target and, without selection, a large number of plants must be regenerated and analyzed. In addition, the primary transgenic plants are most often chimeric **(Finer and McMullen, 1991**).

In this study, mature embryonic axes excised from seeds were used in the bombardment experiments and biolistic transformation of the meristematic tips was carried out. Physical parameters used in the transformation experiments, such as helium pressure, target distance, microprojectile type and size were previously optimized by using the reporter gene *GUS* **(Momtaz *et al*., 1993**). Using these conditions, the two Egyptian cotton varieties, Giza 85 and Giza 88 were transformed with the pFrucI construct.

Regeneration Procedure

Cotton is considered recalcitrant to *in vitro* proliferation and there has been many problems with somatic embryogenesis in cotton. Though it has been reported **(Davidonis and Hamilton, 1983; Finer, 1988),** the response is restricted to only a few cultivars **(Firoozabady and DeBoer, 1993**).

The shoot meristem-based method can be applied to plant transformation, either by particle bombardment **(Christou *et al*., 1991)** or *Agrobacterium*-mediated gene transfer **(Zapata *et al.,* 1999**). In our study, we used the particle bombardment method as a mean of transformation of cotton shoot apical meristems.

Recovery of Plants

Regeneration from the shoot apical meristem was direct and simple. Theoretically, each excised apex will develop into a rooted plant **(Gould *et al*., 1991).** However, the yield of shoots *in vitro* from isolated transformed apices depended on the incidence of contamination and rooting efficiency. The frequency of rooting may be cultivar dependent **(Gould *et al.,* 1991).** Problems with rooting were encountered in the germination of shoot apical meristems **(Gould *et al.,* 1991)** and in the germination of somatic embryos (Trolinder, personal communication); In this case, root primordia may have been present in many of the embryos. In our study, a regeneration

frequency of about 70% was observed across both transformed cultivars, Giza 85 and Giza 88 (data not shown).

Analysis of R_0 Putatively Transgenic Cotton Plants

Four different experiments have been carried out to confirm the presence of pFrucI construct within the genomic DNA of the putatively transformed cotton plants. These experiments are:

1. **Assay for Bar Gene Expression**

   Regenerated cotton plants obtained after bombardment (R_0) were assayed by painting the transformed plant leaves with 200 mg/l Basta herbicide to proof functional expression of the *bar* gene. A preliminary experiment has been done to determine the optimum basta concentration used for screening the positive *bar*-transformants. The concentration 200 mg/l was found to be the optimum one (Table 1). Such screening system exploited the ability of the enzyme phosphinothricin acetyltransferase (PAT) encoded by the *bar* gene to detoxify the herbicide Bialaphos (with commercial name of basta) as reported by **Wan *et al*. (1995) and Bommineni *et al*. (1997).**

   Two to three days after the herbicide application, cotton plants were scored and only the negative control plants showed necrosis. Visual inspection of the treated leaves, seven days post-treatment, clearly distinguished the resistant plants from the sensitive ones; positive control plants leaves had bleached dead leaves while putatively transgenic cotton plants showed green healthy tissue and were almost undistinguishable from the water-sprayed negative control plants. Out of 1000 bombarded regenerated cotton seedlings of either Giza 85 or 88 varieties only 300 and 280 respectively, showed resistance to basta treatments (Table 2). The putative transformed cotton plants did not exhibit any abnormal phenotypes. Furthermore, they reached maturity and gave flowers and seeds which confirms functional activity of the *bar* gene.

2. **PCR Amlification**

   The integration of the *cpy-sacB* chimeric gene into the genomic DNA from the putatively transformed cotton plants was detected by PCR amplification using primers based on the sequences from the coding region of the chimeric *cpy-sacB* gene. Then, the amplified DNA products were analyzed by electrophoresis in a 1% agarose gel (Fig. 2a).

   An amplified DNA fragment was detected in eight Giza 85 plants and seven Giza 88 plants with the expected size (1.7 Kb) of the *cpy-sacB* chimeric gene. The same amplified DNA fragment was also detected only in the positive control. This observation indicates the intact integration of the *cpy-sacB* chimeric gene in the putatively transformed cotton plants. The pFrucI was used as a positive control while DNA from non-transformed cotton plants was used as a negative one. Similar results were obtained by **Bahieldin *et al*., (2000)**, who reported the integration of intact *bar* and *uidA* genes in the wheat genome.

3. **PCR Southern Analysis**

   Southern hybridization analysis has been done to verify that the DNA fragments amplified with the above mentioned two specific primers were indeed *cpy-sacB* coding sequence. The 1.7 Kb *cpy-sacB* *Eco*RI/*Hin*dIII fragment from pFruc1 vector was used as a probe. The same plants that were PCR-positive were also found to be positive after Southern hybridization analysis (Fig. 2b).

4. **Genomic Southern Blot Hybridization Analysis**

   The results of the PCR analysis were further confirmed by Southern Blot hybridization of genomic DNA (digested with *Hin*dIII) using the chimeric *cpy-sacB* gene coding regions as radioactive probe. DNA extracted from two plants (Giza 85) and three (Giza 88) hybridized with *cpy-sacB* labeled probe.

   An autoradiogram of only four genomic DNA samples from putatively transgenic plants digested with *Hin*dIII are shown in Figure 3. The molecular weigths of the bands varied among the four transformants tested showing different insertion sites in the plant genome. This observation proves the integration of expression pFruc1 vector in the putatively transgenic cotton plant genome. Genomic Southern hybridization has been used by many authors to detect foreign gene integration within plant genome such as cotton **(Zapata *et al*., 1999)**, wheat (**Bahieldin *et al*., 2000**).

   It is difficult to know whether a plant or a branch originates from either a single or from multicellular parts in the bombarded shoot meristem. The presence of only one band in each of the four putatively transgenic cotton plant suggests the same pattern of chimeric gene insertion within each plant or at least each branch genome and subsequently confirm the one cell origin. This could be considered as a primary indication that the examined branches of the R_0 putatively transformed cotton plants were not chimeric. Further studies are needed, however, on R_1 and R_2 generations to confirm this suggestion.

5. **RT-PCR Analysis**

   The expression pattern of *cpy-sacB* gene was analyzed by RT-PCR method using total RNA from different leaf tissues of the putatively transgenic cotton plants. The two specific primers used were based on the coding regions of the *cpy sacB* chimeric gene (1.7 Kb). The RT-PCR products were analyzed on a 1% agarose gel. As shown in Fig 4, a 1.7 Kb band was detected, which corresponds to the expected size of the *cpy-sacB* cDNA. Such result confirms functional activity of the inserted gene into the cotton genome.

   RT-PCR method was used by, who used RT-PCR to detect the expression of a polygalacturonase gene in Kiwi fruit and **Wang *et al.* (2000),** who used RT-PCR to detect the expression of the potato U1 sn RNA in tobacco protoplast.

6. **Carbohydrate Analysis**

   Fructose (the structural unit of fructan) was purified on HPLC and used as a standard for comparing fructose levels in transformed and non-transformed cotton plants. The highest fructan (fructose polymer) accumulation was observed in transgenic cotton plants compared with the control or non-transformed ones; A slight increase in fructose accumulation was detected in leaves of transgenic cotton plants compared to non transgenic ones (data not shown).

   The success of the overall transformation process depends on the efficiency of the plant regeneration system. It was proven that plant genotype and the shoot inducing medium are the two main factors that affect plant regeneration efficiency **(Weeks *et al*., 1993; Cheng *et al*., 1997).** The transformation of shoot meristems has proven to be a suitable target in cotton **(Gould *et al*., 1991; Zapata *et al*., 1999)** since it evades problems with reduction of regeneration capacity, albinism

and loss of fertility, which often occurs after the transformation of regenerable callus cultures.

In conclusion, putatively transgenic cotton plants accumulating fructan pave the way for further studies on gene expression, gene inheritance and abiotic stress tolerance.

References

Altpeter, F.; I. Diaz; H. McAuslane; K. Gaddour; P. Carbonero and I. Vasil (1999). Increased insect resistance in transgenic wheat stably expressing trypsin inhibitor Cme. Mol. Breed., 5: 53-63.

Bahieldin, A.; R. Qu; W. Dyer; A. S. Haider and M. A. Madkour (2000). A modified procedure for rapid recovery of transgenic wheat plants. Egypt. J. Genet. Cytol. 29: 11-23.

Bayley, C.; N. Trolinder; C. Ray; M. Morgan; J. E. Quisenberry; O. W.; D. W. (1992). Engineering 2, 4-D resistance into cotton. Theor. Appl. Genet. 83: 645-649.

Bommineni, V. R.; P. P. Jauhar and T. S. Peterson (1997). Transgenic durum wheat by microprojectile bombardment of isolated scutella. J. Heredity 88: 474-481.

Boynton, J. E.; E. H. Gillham; J. P. Harris; A.. M. Hosler; A. R. Johnston; B. Ones; D. Randolf-Anderson L; D. Robertson; T. M. Klein; K. B. Shark and J. C. Sanford. (1988). Chloroplast transfer of *Chlamydomonas* using high velocity microprojectiles. Science 140: 1538-1541.

Chen, L.; S. Zhang; R. N. Beachy and C. Fauquet (1998;). A protocol for consistent large-scale production of fertile transgenic rice plants. Plant Cell Rep., 18: 25-31.

Cheng, M.; J. E. Fry; S. Pang; H. Zhou; C. M. Hironaka; D. R. Duncan; T. W. Conner and Y. Wan (1997). Genetic transformation of wheat mediated by *Agrobacterium tumefaciens*. Plant. Physiol. 115: 971-980.

Christou, P.; T. Ford and M. Kofron (1991). Production of transgenic rice (*Oryza sativa* L.) plants from agronomically important indica and japonica varieties via electric discharge particle acceleration of exogenous DNA into immature zygotic embryos. Bio/Technology 9: 957-962.

Comai, L.; D. Gaccioffi; W. R. Hoatt; G. Thompsen; R. E. Rose and D. Stalker (1985). Expression in plants of a mutant *aroA* gene from *Salmonella typhimurium* confers tolerance to glyphosate. Nature 317: 741-744.

Datta, S. K.; K. Datta; N. Soltanifar; G. Donn and I. Potrykus (1992). Herbicide-resistant Indica rice plants from IRRI breeding line IR72 after PEG-mediated transformation of protoplasts. Plant Mol. Biol. 20: 619-629.

Davidonis, G. H. and R. H. Hamilton (1983) Plant regeneration from callus tissue of *Gossypium hirsutum* L. Plant Sci: 89-93.

Doyle, J. J. and J. L. Doyle (1987). A rapid DNA isolation procedure for small quantities of fresh leaf tissue. Phytochemical Bulletin 19: 11-15.

Finer, J. J. (1988). Plant regeneration from somatic embryogenic suspension cultures of *cotton (Gossypium hirsutum* L.) Plant Cell Rep. 7: 399-402.

Finer, J. J.; McMullen, M. D. (1991). Transformation of soybean via particle bombardment of embryogenic suspension culture tissue. In Vitro Cell. Dev. Biol. 27P:175-182.

Firoozabady, E. and D. L. DeBoer (1993). Plant regeneration via somatic embryogenesis in many cultivars of cotton (*Gossypium hirsutum* L.). In Vitro Cell Dev Biol 29P: 166-173.

Fischhoff, D. A.; K. S. Bowdish and F. J. Perlack (1987). Insect tolerant transgenic tomato plants. Bio /Tech. 5: 807-813.

Fraley, R. T.; S. C. Rogers; R. B. Horsch; P. R Sanders; J. S. Flick; C. Fink; N. Hoffman and P. Sanders (1983). Expression of bacterial genes in plant cells. Proc. Natl. Acad. Sci. 80: 4803-4807.

Gould, J.; S. Banister; O. Hasegawa; M. Fahima and R. H. Smith (1991) Regeneration of *Gossypium hirsutum* and *G. barbadense* from shoot apex tissues for transformation. Plant Cell Rep 10:12-16.

John, M. E.; Crow, L. J. (1992). Gene expression in cotton (*Gossypium hirsutum* L.) fiber: cloning of the mRNAs. Proc.Natl Acad. Sci. USA 89:5769-5773.

John, M.E.; G. Keller (1996). Metabolic pathway engineering in cotton: biosynthesis of polyhydroxybutyrate in fiber cells. Proc. Natl Acad. Sci. USA 93:12768-12773.

Kay, R; A Chan; M Daly and J. McPherson (1987) Duplication of CaMV 35S promoter sequences creates a strong enhancer for plant genes. Science 230: 1299-1302.

Khalil, S. M.; H. El-Dawany and M. A. Madkour (1999). Production of transgenic squach plants resistance to Zucchini yellow mosaic potyvirus. Arab J. Biotech. (in press).

Klein T. M.; E. D. Wolf; R. Wu and J. C. Sanford (1987). High velocity microprojectiles for delivering nucleic acids into living cells. Nature 327: 70-73.

McCabe, D. E. and B. J. Martinell (1993). Transformation of elite cotton cultivars via particle bombardment of meristems. Biotechnology 11: 596-598.

Momtaz, O. A. and M. A. Madkour (1993). Transient expression of B-glucuronidase in fine extra long staple Egyptian cotton following the Biolistic bombardment. Proceedings of the Beltwide Cotton Conferences, Cotton Improvement Conference, New Orleans, IA, USA, pp. 624-627.

Momtaz, O. A., A. A. Diab and M. A. Madkour (2000). Development of transgenic Egypyian Cotton varieties from meristematic tissue. Proceedings of the Beltwide Cotton Conferen, Cotton Improvement Conference, San Antonio, Texas.(1) pp. 513-516.

Odell, J. T.; F. Nagy; N. H. Chua (1985) Identification of sequences required for activity of the cauliflower mosaic virus 35S promoter. Nature 313: 810-812 (1985)

Pilon-Smits, E. A. H.; N. Terry; T. Sears and K. Van Dun (1999). Enhanced drought resistance in fructan-producing sugar beet. Plant. Physiol. Biochem. 37: 313-317.

Pollock, C. J. and A. J. Cairns (1991). Fructan Metabolism in Grasses and Cereals: Annu. Rev. Plant Physiol. Plant Mol. Biol. 42: 77-101.

Pontis, H. G. and E. Del Campillo (1985). Fructans. In PM Dey Dixon, eds, Biochemistry of storage carbohydrates in green plants. Academic Press, London, pp 205-227.

Rajasekaran, K.; R. L. Hudspeth; J. W. Cary; D. M. Anderson; T. E Cleveland (2000). High-frequency stable transformation of cotton (*Gossypium hirsutum* L.) by particle bombardment of embryogenic cell suspension cultures. Plant Cell Reports 19:539-545.

Russell, J. A.; M. K. Roy and J. C. Sanford (1992). Physical trauma and tungsten toxicity reduce the efficiency of biolistic transformation. Plant Physiol 98: 1050-1056.

Shoemaker, R. C.; L. J. Couche; D. W. Galbraith (1986). Characterization of somatic embryogenesis and plant regeneration in cotton (*Gossypium hirsutum* L.). Plant Cell Rep. 3: 178-181.

Sivamani, E.; Bahieldin A.; J. M. Wraith; T. Al-Niemi; T. H. D. Ho; W. E. Dyer and R. Qu (2000). Improved water use efficiency under drought conditions in transgenic wheat constitutively expressing the barley *HVA1* gene. Plant J. (In Press).

Vaux, P.; F. Guerneau, R. Waugh and J. W. S. Brown (1992). Characterization and expression of Ulsn RNA genes from potato. Plant Molecular Biology 19: 959-971.

Wan, Y.; J. M. Widholm and P. G. Lemaux (1995). Type 1 callus as a bombardment target for generating fertile transgenic maize (*Zea maize* L.). Planta 196: 7-14.

Weeks, J. T.; O. D. Anderson and A. E. Blechl (1993). Rapid production of multiple independent lines of fertile transgenic wheat (*Triticum aestivum*). Plant Physiol. 102: 1077-1084.

Zapata, C.; S. H. Park; K. M. El-Zik, and R. H. Smith (1999). Transformation of a Texas cotton cultivar by using *Agrobacterium* and the shoot apex. Theor Appl Genet 98: 252-256.

Wan, Z. Y; Elspeth A. Machae; m. A. Wright; K. M. Bolitho, G. S. Ross and Ross G. Atkinson (2000). Polygalacturonase gene expression in Kiwi fruit: relationship to fruit softening and ethylene production. Plant Molecular Biology 42: 317-328.

Table 1. Determination of Basta herbicide concentration to monitor integrated *bar* gene in non-transformed Giza 85 and Giza 88.

Basta Conc.	Optimum lethal time (days)	Lethal effect estimate
100 mgs/L	22	Weak
200 mgs/L	7	moderate
500 mgs/L	3	high
1000 mgs/L	2	very high

Table 2. Screening levels of bombarded meristematic explants from G85 and G88.

Variety	# of explants	# of positives (*bar*)	# of positives (PCR)	G. S. positives	% of PTP
Giza 85	1000	300	8	2	0.002%
Giza 88	1000	280	7	2	0.002%

P.T.P = Putatively Transgenic Plants

G. S.= Genomic Southern

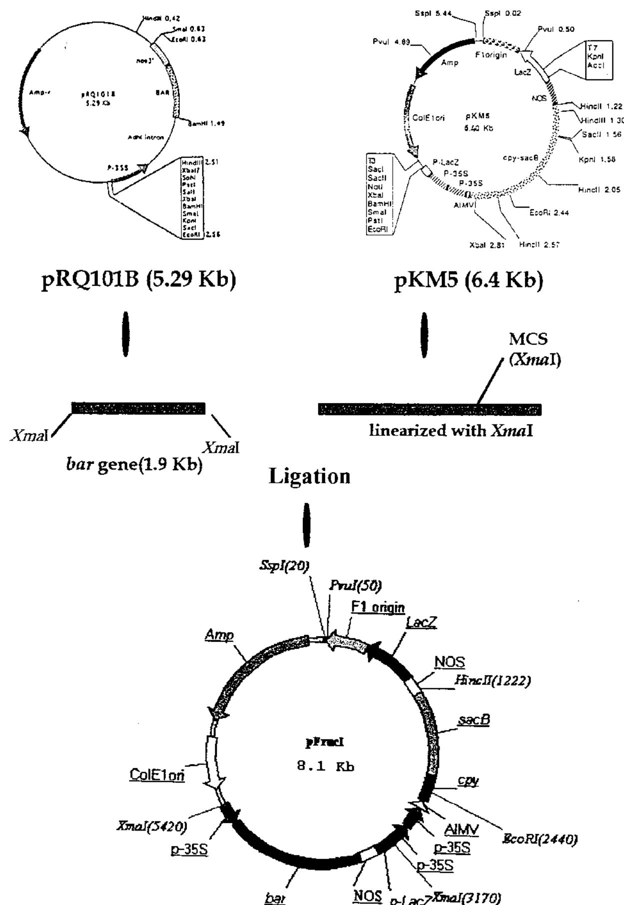
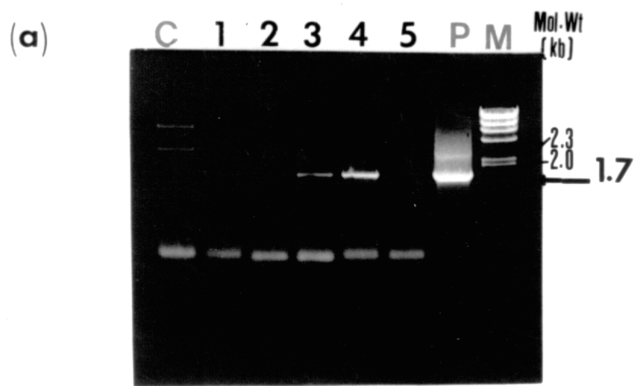


Figure 1. Construction of chimeric pFrucI.



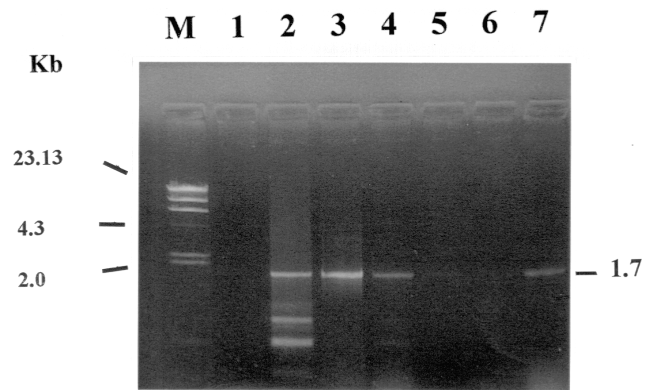


Figure 4. Identification of expressed chimeric *cpy-sacB* gene (1.7 Kb) integrated in putatively transgenic cotton plant genome using Reverse Transcription PCR from purified total RNA. (M) λ *Hind*III molecular weight DNA marker, lanes 1 to 7, amplified cDNA from RNA samples of putatively transgenic plants.

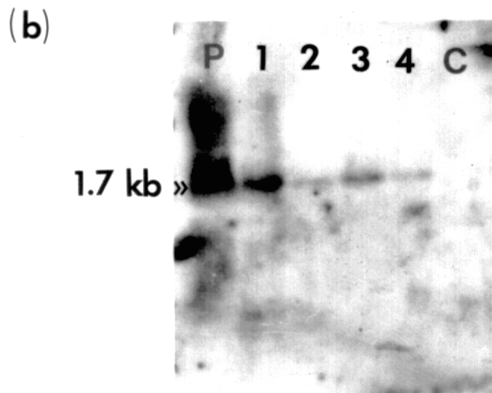


Figure 2. PCR and Southern analysis of selected putatively transformed cotton plants. (a). 1% agarose gel illustrating PCR amplified DNA from non-transformed (C), putatively transformed cotton plants (Lanes 1 to 5), amplified *cpy-SacB* recombinant gene (P) and λ *Hin*d III DNA molecular weight size marker (M). (b). Autoradiogram of Southern blot hybridization analysis of PCR product amplified from plant genomic DNA containing *cpy-SacB* recombinant gene. Lanes (1 to 4) Amplified DNA from 4 putatively transformed cotton plants. (P) Positive control (Amplified *cpy-SacB* recombinant gene). (C) Negative control (PCR analysis using genomic DNA of non transformed cotton plant.)

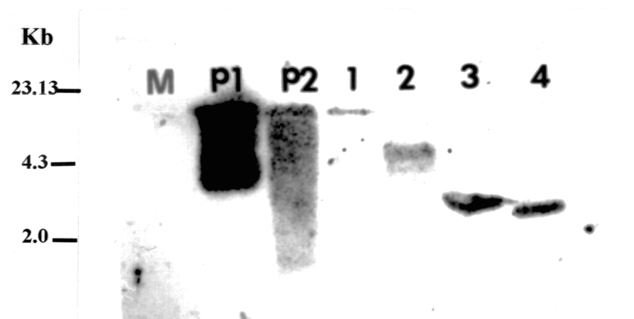


Figure 3. Genomic Southern hybridization analysis of putatively transgenic cotton plants. Genomic DNA samples isolated from four putatively transgenic plants were digested with HindIII restriction endonuclease flanking enzyme (Lanes 1 to 4), λ *Hind*III molecular weight size marker (M) and P1 and P2 indicating positive controls for pFrucI and pRQ101B respectively.