TRANSFORMATION OF EGYPTIAN COTTON VARIETIES USING A BACTERIAL *mtlD* GENE

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Abstract

Sugar alcohols and other low molecular weight compounds are known to accumulate in plants in response to some abiotic stress factors. A bacterial gene, the *mtlD*, that encodes mannitol-1-phosphate dehydrogenase, was used to transform Egyptian cotton. Two cotton varieties were used, Giza 86 and Giza 87, long and extra long staple, respectively. The transformation was carried out by particle bombardment of mature, dissected embryos using the Bio-Rad PDS/1000/He gun. The plant expression vector was constructed to contain the *mtlD* gene driven by the CaMV 35S promotor, and the selectable marker *bar* encoding phosphinothricin acetyl transferase. Use of mature embryos in this work allowed rapid and direct regeneration of plants without the difficulties of tissue culture induced variations, and circumvented the problems of somatic embryogenesis. Regenerated chimeric R_0 plants were submitted to preliminary screening by leaf painting using the herbicide basta at a concentration of 200 mg/L. Resistant plants were submitted to PCR and Southern analysis, which showed that the *mtlD* gene has been integrated into four of the regenerated plants. Assay of mannitol-1-phosphate dehydrogenase was successfully used to determine gene expression in transformed plants as compared to control plants.

Introduction

The functions of sugar alcohols in higher plants are not quite clear. A commonly held belief is that these compounds may confer beneficial traits on those species where they are found, rather than being simply intermediates of carbohydrate metabolism (Tarczynski *et al*., 1992). Suggested physiological roles of sugar alcohols include osmoregulation (Lewis and Smith, 1967), service as compatible solutes (Yancey *et al*., 1982), storage of reduced carbon and energy (Lewis and Smith, 1967), regulation of coenzymes (Lewis and Smith, 1967; Loescher, 1987) and neutralization of hydroxyl radicals (Smirnoff and Cumbes, 1989).

Genetic engineering is rapidly becoming one of the major tools used in field crop improvement and protection. Cotton is an especially attractive crop for genetic engineering because of its worldwide importance as a crop plant (Firoozabady *et al*., 1987). Various areas of interest are receiving attention, including fiber quality modification, stress tolerance, and herbicide and pest resistance (Pannetier *et al*., 1997). In Egypt, cotton is considered one of the major fiber crops and an essential economic asset. Transgenic cotton plants have been produced by biolistic bombardment of organized shoot tip meristems (McCabe and Martinell, 1993; Chlan *et al*., 1995; Keller *et al*., 1997) and regenerable embryogenic cell cultures (Finer and McMullen, 1990; Rajasekaran *et al*., 1996b) and by *Agrobacterium*-mediated transformation (Firoozabady *et al*., 1987). In this study, a bacterial gene encoding mannitol-1-phospahate dehydrogenase, previously used to transform tobacco, was used in the transformation of two Egyptian cotton varieties (Giza 86 and Giza 87) by particle bombardment with the aim of producing an accumulation of the sugar alcohol mannitol. Previous plant transformation experiments involving the *mtlD* gene have been carried out on tobacco. Tarcyznski *et al*. (1992) used the gene driven by a constitutive promotor to transform tobacco. The transgenic tobacco obtained could accumulate mannitol up to 6 mmol/g fresh weight. Karakas *et al*. (1997) produced transgenic tobacco plants with the *mtlD* gene. Experiments were conducted to determine whether mannitol provides salt and/or drought stress protection through osmotic adjustment or not. Non stressed transgenic plants were 20-25% smaller in size than non-stressed, non-transformed (wild type) plants under conditions of both salinity and drought. However, salt stress reduced dry weight in wild type plants by 44%, but did not reduce the dry weight of transgenic plants. The latter adjusted osmotically by 0.57 MPa, whereas wild type plants could not.

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Materials and Methods

Plant Material

Cotton seeds of two different varieties Giza 86 and Giza 87, long and extra long staple respectively, were obtained from the Cotton Research Institute, Agricultural Research Center (ARC), Giza, Egypt, and used in this work.

Vector Construction

The plasmid used in transformation was obtained as follows. The cab promoter of pcabmtlD, a 6.45 Kb vector constructed by Hans Bohnert and co-workers (Arizona University, Tucson, USA) was replaced by the CaMV 35S promoter derived from the plasmid pBI221. In order for this to be carried out, it was necessary to first construct a new plasmid, p35S, by removing the coding sequence of the CaMV 35S promoter from pBI221 using simultaneous digestion by PstI and BamHI. The resulting 0.8 Kb fragment was then cloned into pBluescript digested with the same two enzymes. The resulting plasmid was 3.76 Kb in length, and contained the desired promoter flanked by enzyme sites that were compatible with those in the original pcabmtlD. This permitted an easy ligation of the CaMV 35S promoter, taken as an EcoRI/BamHI fragment from the newly constructed p35S into pcabmtlD after removal of the cab promoter by digestion with both EcoRI an BamHI. The newly obtained plasmid was designated p35SmtlD. Following this, the *bar* gene from *Streptomyces hygroscopicus* was introduced as a 2.09 Kb HindIII fragment obtained from pAB_4 (an 8.55 Kb vector constructed by Ahmed Bahieldin, Ain Shams University, Cairo, Egypt) into the single HindIII site of the vector p35SmtlD. The final 7.34 Kb plasmid (figure 1) was designated pMann, and used as the plant expression vector for cotton transformation.

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 in double distilled water several times, and then placing 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 (figure 2a). 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 86 and Giza 87. Cotton seed axes were removed and bombarded with tungesten particles coated with pMann. The plasmid-coated tungesten microprojectiles were prepared as described by McCabe and Martinell (1993). The explants were bombarded using the

following parameters; 0.5µm tungsten powder, a chamber vacuum of 25 Hg, a pressure of 1100 psi, with a target distance of 6 cm.

Regeneration of Bombarded Tissue

After bombardment, plates were wrapped with parafilm and incubatedovernight at 30°C in the dark to heal the bombarded tissue. 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 (90mE $m^{-2}s^{-1}$). 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 28°C 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 (figure 2 b-d).

Screening of Putatively Transformed Tissue

The herbicide basta was used for preliminary screening of putatively transformed plants. A concentration of 200 mg/L was used to paint one third of each leaf. 200 mg/L was chosen for use in detection after assaying different concentrations of the herbicide on untransformed plants. The results showed that lower dilutions lead to a slow appearence of the symptoms of necrosis caused by basta, whereas higher concentrations speeded up the y appearance of symptoms, but a high death rate within only a few days. There was no difference in the lethal effect caused by herbicide leaf painting in Giza 86 and Giza 87. The results are summarized in table (1).

After 3-4 days, non-transformed control plants showed obvious signs of yellowing and necrosis in the painted areas. The plant parts that showed resistance to these symptoms for a whole week were selected for further molecular analyses. Around 200-300 mg of tissue per sample were harvested and quickly frozen in liquid nitrogen. The frozen tissue was ground to a fine powder and homogenized in 0.5 ml of preheated extraction buffer containing 2% CTAB, 20mM EDTA, 100 mM Tris-HCl and 1.4 M NaCl. Chloroform: isoamyl (24:1) was used to remove proteins and cell debris by centrifugation for 10 min at 14000 rpm. The extract was precipitated using ethanol. Both polymerase chain reaction (PCR), and Southern Blot analysis were carried out to test for positive integration of the gene.

Polymerase Chain Reaction

PCR was carried out using specific primers for the *mtlD* gene. The amplification reactions were made in volumes of 50 µl containing 1 X Taq DNA polymerase buffer with a final concentration of 10 mM Tris-HCl, pH 9.0, 50 mM NaCl, and 0.01 Triton X-100, also, $MgCl_2$ at a final concentration of 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 half a minute denaturation at 94°C, one minute annealing at 50°C, and one minute extension at 72°C. Amplification products were analyzed by electrophoresis in a 1% agarose gel.

Southern Blot Analysis

Southern blots were carried out using the non-radioactive DIG system. The probe used was the *mtlD* region taken as a gene-cleaned fragment from the vector pMann after double digestion using BamHI and HindIII. Genomic DNA was isolated from samples and tested using PCR. Positive samples were then blotted onto a positively charged nylon membrane, and hybridized overnight.

Mannitol-1-Phosphate Dehydrogenase Assay

This assay was carried out as reported by Su and Wu (1997). Protein was extracted from each of the test plants by freezing leaf tissue in liquid nitrogen and homogenizing it in 1 ml of extraction buffer (20 mM Tris-HCl, pH. 7.5, 1 mM DTT, 25 µg/ml PMSF). This was followed by incubation on ice for 40 min. The extracts were then centrifuged at 5,000 rpm for 5 min, and the resulting supernatents were centrifuged a second time, at 12,000 rpm for 10 min, and used for the assay. Then the following mixture was added: 100 mM Tris-HCl, pH 9.1, and 1 mM β-NAD. Incubation for one minute at room temperature then followed, and the reaction was started by the addition of mannitol-1-phosphate to a final concentration of 20 mM. Absorbance was recorded at 340 nm after 60, 90 and 120 seconds for each sample. The OD obtained in each case was compared to a standard curve of NADH.

Results and Discussion

Vector Construction

The expression vector pMann was constructed for transformation of cotton (figure 1). This contained the *mtlD* gene driven by the constitutive promotor CaMV 35S and the selectable marker gene *bar*. Choice of the CaMV 35S promoter was based on the comparative studies carried out by Tarczynski *et al*. (1992) in which promotor efficiency was tested depending on transcriptional efficiency. All constructs that were used in the process contained the unaltered *mtlD* coding sequence and an identical terminator element, the NOS terminator. Tobacco was transformed with plasmids containing the different constructions, and the level of mannitol in the resulting transgenic plants was tested. They found that the mannitol content of young leaves transformed with the CaMV 35S containing construct varied from < 1µmol/g to >6 µmol/g (fwt), whereas in the case of leaves transformed with the construct containing the NOS promoter, mannitol levels were generally either not detected or 2- to 3- fold lower than in transformants containing the CaMV 35S promoter (Tarczynski *et al*., 1992).

Choice of the *bar* gene as selective agent was based on the ease of application and the rapidity with which results are obtained. The gene originates from *S. hygroscopicus*, and encodes phosphinothricin acetyl-transferase, that acetylates the free NH_2 group of phosphinothricin, an inherent part of the herbicide basta, thereby preventing toxicity. In general, weed control 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).

Biolistic Transformation of Embryonic Axes

Conventional breeding programs have produced steady improvements in many agronomic traits, but the potential benefits of cotton genetic engineering have been slow in coming (Bayley *et al*., 1992; John and Stewart, 1992). This has been mainly due to the difficulty in transforming regenerable cotton plants. The technique originally developed to engineer cotton utilized *Agrobacterium tumefaciens* vectors and was applicable to only a few varieties (Bayley *et al*., 1992; Cousins *et al*., 1991; Firoozabady *et al*., 1987). The overriding problem each investigator faced was the necessity to regenerate fertile, healthy plants from the callus or suspension culture phase which their techniques required for both transformation and selection. The inability to use these protocols with the majority of elite cultivars has been a significant stumbling block for those who wish to expedite the use of genetic engineering to improve cotton (McCabe and Martinell, 1993). Therefore, the transfer of transgenes into elite commercial cultivars through *Agrobacterium* requires lengthy backcrossing. 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 exfor transformation. This type of transformation is labor-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 (see Finer and McMullen, 1991). McCabe and Martinell (1993) published their results on the transformation of cotton embryonic axes using particle bombardment. A number of positive transformants were achieved that gave a low copy number per genome, indicating chimeric germ line transformants. The segregation ratio of such plants is not Mendelian in the R_1 (consistent with chimeric transformants), but subsequent generations derived from transformed R_1 progeny indicated normal Mendelian inheritance.

In this study, mature embryonic axes excised from seeds were used in the bombardment experiments (figure 2a) 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 and Madkour, 1993; Momtaz *et al*., 2000). Using these conditions, the two Egyptian cotton varieties, Giza 86 and Giza 87 were transformed with the *mtlD* containing construct. 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. Transformation of regenerable tissue seems to be strongly restricted to certain genotypes due to low regeneration frequencies of most elite varieties (Christou, 1991), hence the importance of the particle bombardment method for transforming mature tissues. In our experiments, the marker gene *bar* was used only to facilitate screening of mature plants, but no selective agent was used in the culture media itself. Zhong *et al*. (1993) reported that the available selective agents are not efficient enough for a high frequency recovery of transformants, and that without using such agents transgenic material was in fact recovered during their experiments containing the reporter gene *GUS.* An important consideration in our work was the survival and maintenance of chimeric plantlets, from which transgenic progeny might be obtained. The number of successful transformation events was low, with only 0.2 % of the putatively transgenic plants obtained showing gene expression (table 2).

Leaf Painting with the Herbicide Basta

Preliminary screening of putative transformants was carried out by painting the leaves (around one third of the leaf area) of each plant with the herbicide basta at a concentration of 200 mg/L. Plants expressing the *bar* gene showed no symptoms for a week to ten days, whereas non-transformed plants and plants not expressing *bar* showed severe symptoms of leaf necrosis within three to four days. Herbicide sensitivity is known to vary according to the age and vigor of the plants, and this method is considered as a preliminary rapid screening technique for plant tissue. It is interesting to note that a relatively large number of plants showed resistance to the physical effects of herbicide spraying, yet when tested for the insertion of the *mtlD* gene, only a very limited number gave positive results. This could be due to the fact that although both the *mtlD* and *bar* genes were in a contiguous DNA, it may be the case that in certain transformants one of the genes is rendered inactive through breakage, or deletions (see Keller *et al*., 1997), which could be a possible result of the damage caused by the bombardment process.

Polymerase Chain Reaction and Southern Analysis

Plant genomic DNA was subjected to amplification by the polymerase chain reaction (PCR). The set of primers used was designed to amplify the full length of the *mtlD* gene. Standard PCR conditions were followed. PCR products were size-separated on a 1 % agarose gel (figure 3a).

Southern analysis of leaf DNAs from putatively transformed plants was carried out. PCR products were separated on a 1 % agarose gel, and blotted onto a positively charged nylon membrane. A Digoxigenin-labeled probe was extracted from pMann and used for hybridization. The specific fragment used was obtained by double digestion using a mixture of EcoRI and BamHI and genecleaning the relevant 1.8 Kb fragment containing the *mtlD* gene (figure 3b).

Mannitol-1-Phosphate Dehydrogenase Activity Assay

Plants that gave positive results at the DNA level were further tested to determine whether the *mtlD* gene was functional or not. A specific assay, designed to test mannitol dehydrogenase activity, was designed and carried out by Su and Wu (1997) during their work on transgenic rice transformed with the *mtlD* gene. Given that detection of mannitol produced in *mtlD*-transgenic plants is a time-consuming procedure, requiring high-performance anion-exchange chromatography coupled to pulsed amperometric detection (HPAE-PAD) and/or NMR spectrum analysis (Tarcyznski, 1992), this method is in contrast simple and straightforward. It relies basically on measuring the amount of NADH released by test tissue during set conditions in which the substrate, mannitol-1-phosphate, as well as β-NAD are added.

Results indicated that, out of the four tested plants, only 2 of the plants in which *mtlD* integration had been established showed marked enzymatic activity. Table (2) summarizes results achieved in the case of both Giza 86 and Giza 87 varieties.

Results showed that putatively transgenic plants (PTP) in the case of Giza 86 reached 0.2 %, whereas in the case of Giza 87, no transgenic plants expressing mannitol-1-phosphate dehydrogenase were recovered. This could be due to a variety of factors, which include end product inhibition. Clearly, more work is needed to determine the factors affecting gene expression, as well as to assay the effect of mannitol-1-phosphate dehydrogenase on increasing the tolerance levels of cotton to abiotic stress.

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Table 1. Lethal effect of different concentrations of basta herbicide on 8 week old leaves of Giza 86 and Giza 87 cotton plants.

Basta conc. (mg/L)	Lethal effect (days)
100	9
200	4
500	2
1000	1

Table 2. Illustration of screening of putatively transgenic cotton plants (Giza 86 and Giza 87) at different levels.

Variety	# of bombarded plants	# of basta positive plants	# of PCR positive plants	# of plants showing gene expression	PTP* (%)
Giza 86	1000	50	4	2	0.2
Giza 87	300	8	1	0	0

* PTP = Putatively transgenic plants

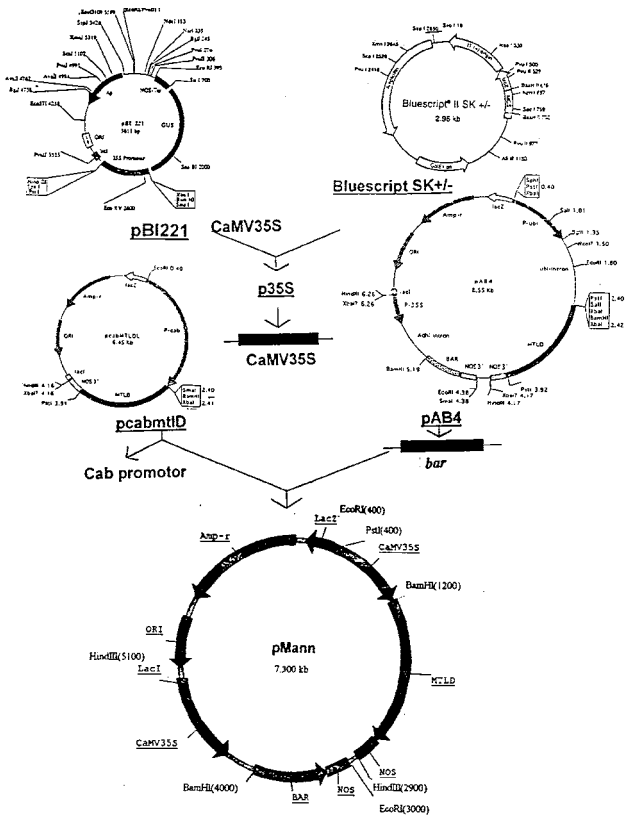

Figure 1. Schematic representation illustrating construction of pMann vector containing the *mtlD* gene driven by CaMV 35S promoter and the *bar* gene as a selectable marker.

Figure 2. Illustration of different developmental stages of putatively transgenic Giza 86 and Giza 87 cotton plants containing the *mtlD* gene. a) Shoot apices arranged at the center of a petri dish prior to bombardment, b) 30-day-old plantlets developed after bombardment, c) 2-month-old plants undergoing acclimatization in the greenhouse, d) Putatively transgenic mature cotton plant.

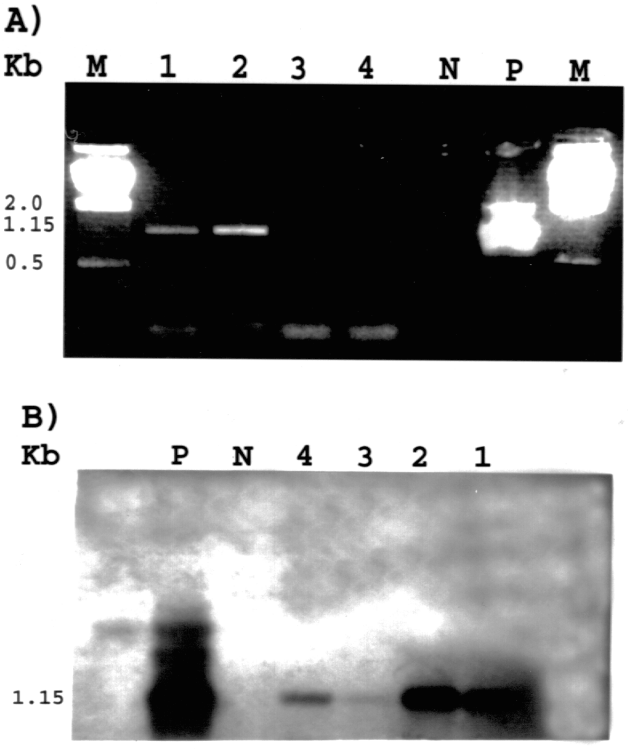

Figure 3. PCR and Southern blot analysis of DNA isolated from putatively transgenic cotton plants using *mtlD* specific primers. A) Agarose gel (1%) illustrating PCR products of four putatively transgenic cotton plants (samples 1-4). M indicates λ DNA digested with HindIII and used as a marker, P indicates pMann vector used as a positive control and N indicates an untransformed plant as a negative control. B) Southern blot analysis of PCR amplified DNA from four putatively transgenic cotton plants (1-4).

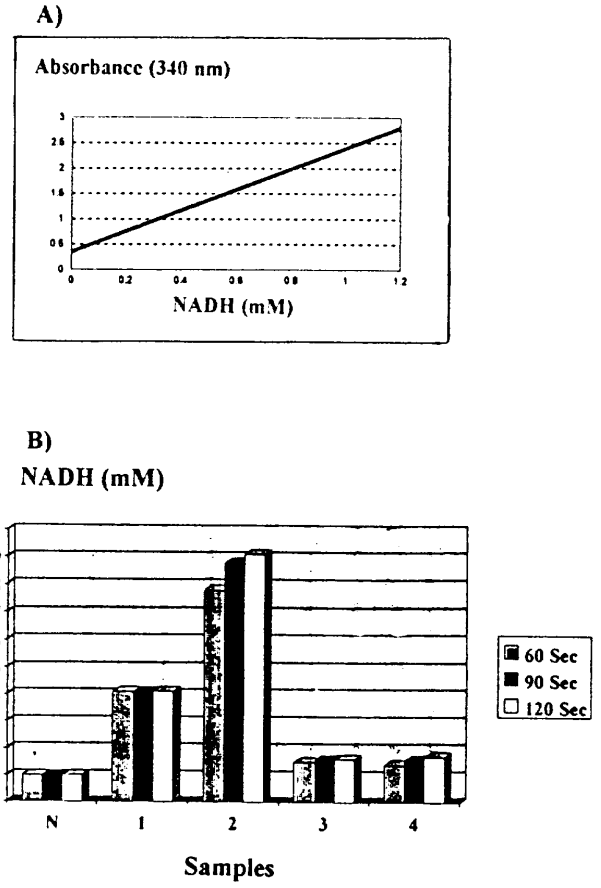

Figure 4. Mannitol-1-phosphate dehydrogenase assay for putatively transgenic cotton plants (Giza 86). A) Standard curve of NADH, where increasing concentrations of NADH (mM) are plotted against absorbance at 340 nm wavelength. B) Histogram illustrating mannitol-1-phosphate dehydrogenase activity represented by NADH (mM) present in four putatively transgenic cotton plants (samples 1-4). N represents a non transformed control plant.