Abiotic stress tolerance transgenic plant production in cotton by overexpression of SiNAC transcription factor gene from foxtail millet (*Setaria italica* L.) through *Agrobacterium tumefaciens* mediated transformation

DBT RESEARCH PROJECT

PROJECT COMPLETION REPORT (2011-2014)

(RESUBMISSION)

Ref. No.: BT/PR15256/AGR/02/791/2011. *dated*: 30.12.2011.

Submitted to

Government of India
Ministry of Science & Technology
Department of Biotechnology
New Delhi - 110 003.

Submitted by
Dr. N. JAYABALAN
Principal Investigator

Dr. T. Senthil Kumar
Co-Principal Investigator

Department of Plant Science
School of Life Sciences
Bharathidasan University
Tiruchirappalli - 620 024.
Tamil Nadu, India.

October 2015

Scientific and Technical Progress Report (STPR) [*]

(R&D projects)

Section - A: Project Details

A1	File No. & Project Title	:	**"Abiotic stress tolerance transgenic plant production in cotton by overexpression of *SiNAC* transcription factor gene from foxtail millet (*Setaria italica* L.) through *Agrobacterium tumefaciens* mediated transformation".**
A2	DBT Sanction Order No. & Date	:	BT/PR15256/AGR/02/791/2011, Dated: 30.12.2011
A3	Date of Project Initiation (If this date is different than date mentioned in A2, please clarify the reason	:	30.12.2011
A4	Date of Project completion	:	29.12.2014
A5	Name of Project Coordinator/ Principal Investigator/ Co-PI/Co - Investigator	: :	Dr. N. Jayabalan (Principal Investigator) Dr. T. Senthil Kumar (Co- Principal Investigator)
A6	Institute(s)	:	Bharathidasan University Tiruchirappalli-620 024. Tamil Nadu. India.
A7	Address with Contact Nos. Landline Mobile Email	: : : :	Dr. N. Jayabalan UGC-BSR Faculty Fellow Department of Plant Science Bharathidasan University Tiruchirappalli-620 024. 0431- 2351788 09443601166 jayabalan5419@gmail.com
A8	Total Approved Cost (including additional cost, if any)	:	38,17,434/-
A9	Approved Duration	:	30.12.2011 to 29.12.2014.
A10	Rational and background information of project (in brief Maximum 500 words)		Annexure I
A11	(i) Approved Objectives of the Project	:	To transfer the SiNAC Transcription factor gene on cotton by using *Agrobacterium tumefaciens* mediated transformation. To find out the impact of Si NAC gene involved in the production of plant cells from salinity and water

		:	deficit stresses. To check the efficiency of the transgenic plant in different abiotic stress condition. To identify the stress responsiveness of protein in transgenic and non transgenic plant. To analyze the productivity and desirable progeny for agriculture programme.
	(ii) Details of approved work Plan and milestone	:	**<u>1- 6 - months</u>** ▪ Development of technology to transfer the gene through using *Agrobacterium tumefaciens.* **<u>7- 12 - months</u>** ▪ Regeneration of transgenic plants in lab condition. **<u>13- 18 - months</u>** ▪ Regeneration of transgenic plants in growth chamber and green house conditions. **<u>19- 24 - months</u>** ▪ Molecular analysis to confirm the presence of target gene by PCR, Southern Blot. **<u>25- 30- months</u>** ▪ Protein identification and characterization of transgenic and non-transgenic cotton plant **<u>31- 36- months</u>** ▪ Efficiency of the new transgenic plant productivity in different abiotic stress conditions.
A12	Specific Recommendation(s)/ suggestion(s) made by the DBT committee/ Task Force at the time of project sanction/ or earlier progress review (if any)	:	The task Force noted that the project was taken up to improve abiotic stress tolerance in cotton using SiNAC transcription factor gene. Method to induce transformation through microinjection was standardized. The studies on T_0 and T_1 plants indicated there are few events which have reported an improved tolerance under drought and salt conditions under pot culture experiments. The copy number of the five events has been studied through Southern analysis and two single copy events identified. However physiological characterization of the transgenic events is inadequate. The protein analysis has captured the expression of the SiNAC protein (?) alone and being the Transcription factor its influence on other stress response related proteins has not been studied. Overall the Task Force noted that project progress is not up to the mark. Completion report is not accepted in its present form. Investigator must re-characterize the material (molecular characterization) and resubmit the revised report for review and acceptance.

	Works done additionally based on the recommendations made by the Task Force at the time of earlier progress review		**Physiological characterization** 1. Shoot and root length 2. Dehydroascorbate reductase 3. Hydrogen peroxide 4. Malondialdehyde 5. Relative water content **Molecular characterization** 1. RT-PCR and Northern blot 2. Two Dimensional gel electrophoresis

Annexure I

Rational and Background information:

Genetic manipulation of crop plants for enhanced abiotic stress tolerance holds great promise for sustainable agriculture. Abiotic stress has been shown a quantitative character and thus they are controlled by multiple genes. However, there is number of instances where single gene transfers have lead to the development of tolerance plants. The increasing problem associated with rise of salinity in soil and water is a major threat to agriculture productivity world wide along with the cold stresses. In order to avoid these problems, a new emerging trend is by transferring a new well characterized and validated *SiNAC* transcription factor gene from foxtail millet to cotton thereby producing a new transgenic plants with good tolerant towards the water deficit and salinity stress. The overexpression of *SiNAC* transcription factor gene as well as other two well characterized transcription factors are the drought-responsible element (DRE) recognized by DREB or CBF transcription factors and the abscisic acid (ABA) responsible elements (ABRE) recognized by bZIP domain transcription factors. In this case, the overexpression of *SiNAC* transcription factor gene from foxtail millet has to be transferred into cotton cultivar SVPR-2.

The good quality cotton production with more yields requires controlling the biotic and abiotic stresses. Plant growth and productivity is under constant threat from environmental challenges in the form of various biotic and abiotic stresses. Plants are frequently exposed to abiotic stress conditions such as low temperature, salt, drought, flooding, heat, oxidative stress and heavy metal toxicity. All these stress factors pose a challenge to farmers. Transgenic approaches offer new opportunities to improve tolerance beside abiotic stresses, so the present investigations to be carried out the abiotic stress tolerance on cotton by overexpression of *SiNAC*

transcription factor gene from foxtail millet through *Agrobacterium tumefaciens* mediated transformation.

Section-B: Scientific and Technical Progress

B1. Progress made against the Approved Objectives, Targets & Timelines during the Reporting Period:

Introduction

Genetic engineering in the first instance addresses improvement of breeding material by introducing special valuable genes (resistance, quality, tolerance, etc.,) from different germplasm or other sources. Arid and semiarid regions are characterized by a very stochastic environment, where biotic and abiotic factors exert determinant influences on ecosystem structure, function, restrict distribution and productivity of plants. Among these environmental factors salinity, water availability, cold, heat and heavy metal plays major role in determining changes in productivity and community compositions. Cold, salinity, drought and heavy metal stress are considered as major factors in limiting plant development and productivity, mainly of important crops. Today there is increasing use of saline water in agriculture in arid and semiarid regions, where the availability of fresh water is scarce. Water-deficit stress, cold stress and salinity stress are major causes of plant growth inhibition due to reduction in water availability, sodium ion accumulation and mineral imbalances and these are leading to cellular and molecular damage. Regarding this reason, we would like to transfer SiNAC gene which transcribe characterized protein which contains a NAM (Number of Apical Meristem), ATAF (*Arabidopsis thaliana* Transcription Factor) and CUC (Cup Shaped Cotyledon) transcription factor with trans activation activity. Several reports describe and support the hypothesis that the endogenous functions of these transcription factors regulate their target gene expression through binding to the cognate cis-elements in the promoters of the stress-related gene. NAC genes showed a strongly localized expression in guard cells by drought stress, on investigation, the response of stomata under drought stress. More stomatal pores were closed significantly in rice transgenic plant than in the WT under both normal and drought-stressed conditions (Hu *et al.,* 2006).

Approved Objectives of the Project:

- To transfer the SiNAC Transcription factor gene on cotton by using *Agrobacterium tumefaciens* mediated transformation.
- To find out the impact of SiNAC gene involved in the protection of plant cells from salinity and water deficit stresses.
- To check the efficiency of the transgenic plant in different abiotic stress condition.
- To identify the stress responsiveness of protein in transgenic and non transgenic plant.
- To analyze the productivity and desirable progeny for agriculture programme.

Objectives completed:

- The SiNAC Transcription factor gene on cotton by using *Agrobacterium tumefaciens* mediated transformation was done.
- The impact of SiNAC gene involved in the protection of plant cells from salinity and water deficit stresses was done.
- Checked the efficiency of the transgenic plant in different abiotic stress conditions.
- Identified the stress responsiveness of protein in transgenic and non transgenic plant.

Objective 1

- **To transfer the SiNAC Transcription factor gene on cotton by using *Agrobacterium tumefaciens* mediated transformation.**

Materials and methods

I. Genetic transformation through microinjection:

Cotton Seeds SVPR-2

↓

Delinted with conc. H_2SO_4 and washed in running tap water

↓

Surface sterilization of two days germinated seeds with 5% Teepol and washed in running tap water for 30 min.

↓

In vitro sterilization with 0.1 % mercuric chloride for 4 to 5 min.

↓

Rinsed several times with sterile distilled water

↓

Microinjection of overnight culture of SiNAC (OD_{600} = 0.2 - 1.0) taken in a syringe and needle

↓

Injected (0.5-1.0 mm depth) for average of 5 times into the ESAM of seeds

↓

Excess culture was removed by sterile filter paper (Whatman No.1)

↓

Explants were kept on MS medium (half strength) for co-cultivation (2-3days)

↓

After co-cultivation the seedlings were washed with Cefotaxime (200 mg/L)

↓

The co-cultivated seedlings were transferred to selection medium for 10-15 days (Hygromycin B- 20 mg/L)

↓

GUS Histochemical analysis was performed in putative transgenic plants

↓

PCR Analysis was performed to confirm the presence of transgene

Isolation of Total RNA

Total RNA was isolated from Non-transgenic plant (WT) and transgenic plant (CT1, CT2 and CT3) leaves by using spectrum plant total RNA isolation kit (Sigma, USA). Materials used for the RNA isolation were treated with DEPC water and autoclaved. The total RNA isolation protocol was followed as per the instruction manual. After, that quality of RNA was checked on the 1.2% denatured formaldehyde agarose gel in MOPS buffer. The samples were loaded on the each well and the high range RNA ladder (*Thermo Scientific, USA)* was loaded to determine the size of the fragment and the gel was run at 60 V for approximately 40 min and viewed under UV transilluminator. Total RNA samples were estimated by Nanodrop 1000 spectrometer (Thermo Scientific, USA). About 1 µL of sample was used for the quantification.

Complementary DNA (cDNA) synthesis and Semi Quantitative RT-PCR analysis

Specific primers for *SiNAC* gene

SiNAC - Forward primer : 5′- AACACTCGCATTGTGGGGAT - 3′ (20 bp)

SiNAC - Reverse primer : 5′- AGTCGCCTGCTGCTAAAGAA - 3′ (20 bp)

Amplicon size : 970-bp

The PCR reagents, stock and working concentrations

Reagents	Stock Concentration	Final Concentration	Volume (µL)
Nuclease free water	-	-	19.5 µL
PCR mixture	10X	1X	2.5 µL
cDNA (1/10 dilution)	1089.98 ng/µL	100 ng	1.0 µL
Forward primer	10 µM	0.4 µM	1.0 µL
Reverse primer	10 µM	0.4 µM	1.0 µL
		Total volume	***25 µL***

cDNA was synthesized from total RNA samples of transgenic and WT cotton plants. The protocol was followed as per the instruction of manual Transcriptor First Strand cDNA synthesis Kit (Roche Diagnostics GmbH, Germany). Transcriptor single stranded cDNA was analyzed by agarose gel electrophoresis and the expression of *SiNAC* gene in transformed cotton was confirmed by semi RT-PCR. The PCR conditions were as follows: 90 °C for 3 min, initial denaturation; 30 cycles of 94 °C for 40 s, 60 °C for 40 s, and 72 °C for 60 s, and final extension at 72 °C for 10 min, and finally hold at 4 °C for storage.

Northern blot

Mini gel electrophoresis

Total RNA was quantified by Nanodrop spectrophotometer (Eppendorf). An equal volume of 2X RNA loading dye contains 0.025% ethidium bromide (Fermentas) was added to RNA sample (about 1 µg/1 µL). Then the sample was thoroughly mixed by vortex and incubated at 65 °C for 10 min and then kept for Ice. The RNA samples were loaded in well and run in mini gel to check the presence of 16S and 25S rRNA along with other RNAs in the total RNA isolation from transgenic and WT cotton plants.

Denaturing Formaldehyde agarose gel electrophoresis

Once checked the quality of total RNA mini gel, fifteen micrograms of total RNA was resolved on a 1.2 % formaldehyde denaturing gel and transferred to the nitrocellulose membrane (Hybond-N^+, Amersham Biosciences, UK) using diethylpyrocabonate (DEPC) treated 20X SSC. 3.6 g agarose was added to the 300 mL of 1X MOPS buffer and boiled for dissolving. After

cooling the contents to about 45 °C, 9 ml of 37% formaldehyde was added and mixed by gentle swirling. The molten agarose was poured in gel casting tray and kept for solidification. Denatured RNA sample mixture was loaded in wells and gel was run in 1X MOPS at constant voltage (40-60V) for separation.

Transfer to solid support and Immobilization

For Northern transfer, the gel was rinsed by several times with MilliQ water to remove the formaldehyde. A piece of nylon membrane was cut for the size of gel, rinsed in distilled water for 5 min and kept in 20X SSC (DEPC) at least for 5 min before use. Capillary blotting was performed in 20X SSC for 14-18 h. The gel was lifted along with nitrocellulose paper and position of each well was marked on nylon membrane using pencil. The membrane was rinsed with 2X SSC and dried by keeping Whatman 3 mm filter paper above it. The blotted membrane exposed UV cross linking for about 2 min to fit the total RNA properly into the membrane.

Pre-hybridization and hybridization with probe preparation

Pre-hybridization and hybridization were performed with ULTRAhyb® Ultrasensitive hybridization buffer (Ambion, USA) according to the manufacturer's instructions. The blot was hybridized at 42 °C with α [^{32}P]dCTP labeled 970-bp *SiNAC* fragment labeled with mega prime DNA labeling system (Amersham Biosciences, UK).

Blot washing and Autoradiography

The blot was washed with following solutions in the given order till background counts were reduced to 2-5 counts per second as detected by Mini-Monitor GM tube. The blot washed 5 min for 2 times with 2X SSC and 0.1 % SDS and 15 min for 2 times in 0.1X SSC and 0.1 % SDS at 42 °C. The membrane was removed from hybridization bottle, air dried and wrapped with saran wrap. The blot was exposed to X-ray films (Kodak Biomax MR) in cassettes with intensifying screen at -70 °C. The autoradiogram was developed after 1-2 days depending on the counts. The X-ray films develop set up was already mentioned in the chapter 4.2.6.3.1 was followed.

Results and Discussion

Microinjection of *A. tumefaciens*

Embryonic shoot apical meristem of cotton seeds (cv.SVPR-2) was used as an explant for gene delivery using microinjection of *A. tumefaciens* (Fig 1). 100µl culture of *A. tumefaciens* at different OD_{600} values (0.2, 0.4, 0.6, 0.8, and 1.0) was microinjected 5 times to evaluate the optimal bacterial density for efficient gene transfer. After co-cultivation, the explants were transferred to selection media supplemented with 1/2 MS + B5 Vitamins + 1% sucrose + Hygromycin-B (20 mg/L) (Table 1). Five days old and 15 days old seedlings were subjected to GUS assay (Fig 2 a & 2c) and allowed to grow in selection media (1/2 MS+BAP) upto shoot and root development (Fig 2d).

Baskaran *et al.,* (2011) described a method of gene transfer to elite *indica* rice cultivar by microinjecting the **embryonic shoot apical meristem (ESAM)** of seeds with *Agrobacterium.* Microinjection of ESAM has also been achieved in tomato by our research group (Vinoth *et al.,* 2013). This technique was followed for cotton (SVPR 2) genetic transformation and successfully got regeneration of the transformed cotton plants (Please see fig 1 & 2).

In the present study microinjection of cotton seed ESAM was performed with different concentration of acetosyringone and *Agrobacterium* which favored the penetration of bacteria into cotton shoot meristems. *Agrobacterium* after microinjection can transfer its T-DNA to the cells of apical meristems. Various densities of *Agrobacterium* and Acetosyringone concentrations were evaluated for gene transfer efficiency. The efficiency varied in different concentrations of *Agrobacterium* & acetosyringone in co-cultivation media. The efficiency of 20% was achieved with a density of *Agrobacterium* 0.6 at OD_{600} and containing 100mM acetosyringone. Table - 1 represents the optimal concentration of *Agrobacterium* for efficient gene transfer. Increasing or decreasing concentration of *Agrobacterium* (OD_{600} > 0.8 < 0.4) reduced the efficiency of transformation (Table-1).

GUS assay

The seedlings derived from microinjected cotton seeds were subjected to histochemical GUS assay to check the expression of uidA gene after 4 days post co-cultivation according to the procedure described by Jefferson *et al* (1986). For analysis of the transformants, seedlings that were tested and found free of residual *Agrobacterium* were used. The seedlings were incubated overnight at 37°C in a solution containing 0.1 M phosphate buffer, pH 7.0, 2 mM X-Gluc, 5mM each of potassium ferricyanide and ferrocyanide and 0.1% Triton X-100. The seedlings were later soaked with 75% ethanol to clear chlorophyll. The gene transfer efficiency was calculated as the average number of ESAM showing GUS staining. Also the efficiency was expressed as the number of Hm-resistant and GUS positive SAM/initial number of ESAM inoculated × 100%.

Transient expression of GUS in the absence and presence of acetosyringone (0-200) was evaluated after co-cultivation. The percentage of ESAM showing GUS expression with increasing acetosyringone till 100mM followed by a decrease at higher concentrations.

Screening for transformed plants using PCR

Genomic DNA was isolated from the young leaves of T_0 plants grown in plant growth chamber. The isolated genomic DNA was also used for PCR amplification with SiNAC gene - specific primer (5'- 3') F - AACACTCGCATTGTGGGGAT and R - AGTCGCCTGCTGCTAAAGAA. PCR was performed in 20µl (total volume) reaction mixture containing 1.5 µl template DNA , 10 µl PCR master mix, 1.0 µl each of forward and reverse primer and 6.5 µl sterile distilled water. PCR conditions for SiNAC gene 94°C for 5 min, 94°C for 1 min, 64°C for 1 min, 72°C for 1 min for 30 cycles and 72°C for 10 min. The amplified products were resolved on 0.8% agarose gel.

The genomic DNA of the T_0 plants was tested for the presence of the GUS gene by using specific primers and PCR products yielded a 1.0 kb amplicon. The presence of SiNAC gene was also confirmed by amplifying the gene with specific primer and the expected **970bp** fragment was observed as sharp bands.

Southern blot analysis

Sothern blot hybridization was performed to confirm the integration and copy number of the SiNAC gene using genomic DNA isolated from PCR-positive (T_0) and non-transformed cotton plants. 10µg of genomic DNA from PCR-positive and non-transformed cotton plants was digested with the enzyme EcoRI that has a single restriction site in the T-DNA region of the pCAMBIA 1304-SiNAC plasmid. Digested genomic DNA was resolved on 1% agarose gel and transferred to a Hybond N+ membrane (GE healthcare, Buckinghamshire, UK) according to the method described by Sambrook *et al.,* The PCR amplified **970 bp** SiNAC gene amplicon was labeled with Alkphos Direct labeling kit (GE healthcare) according to the manufacturer's instructions and used as a probe in the hybridization. The hybridization membrane was washed at 55°C and subjected to chemiluminescent development using CDP-star as a substrate (GE healthcare), and then exposed to X-ray film (Kodak, India). The binary vector pCAMBIA 1304-SiNAC and genomic DNA from non-transformed cotton plants served as positive and negative controls respectively.

RT-PCR analysis

Total RNA was isolated from WT and T_1 cotton plants using spectrum total RNA isolation kit (Sigma, USA) and it was checked on 1.2 % agarose denatured formaldehyde gel prepared by MOPS buffer. Total RNA samples were estimated by Nanodrop 1000 spectrometer at the wavelength of 260/280 and 260/230 (Thermo Scientific, USA). cDNA was synthesized from isolated RNA samples using Transcriptor First Strand cDNA synthesis Kit (Roche Diagnostics GmbH, Germany). The cDNA samples were analyzed on 1.2 % agarose denatured formaldehyde gel electrophoresis and Nanodrop spectrophotometer. RT-PCR analysis showed the expression of *SiNAC* transcript and it specifically amplified 970-bp fragment using gene specific primers in all the transgenic except WT control (Figure 6a).

Northern blot analysis

Total RNA was isolated from WT and three T_1 transgenic cotton plants using spectrum total RNA isolation kit kit (Sigma, USA) as per manufacturer's instructions. Fifteen micrograms of total RNA was resolved on 1.2 % formaldehyde agarose gel electrophoresis and processed gel was transformed to nylon membrane for an overnight. The blot was hybridized at 42 °C with α

[^{32}P]-dCTP labelled 970-bp *SiNAC* fragment using with mega prime DNA labeling system (Amersham Biosciences, UK). The expression of *SiNAC* transcripts in transgenic cotton was confirmed by Northern blot analysis which showed expression of *SiNAC* transcript in all the three single copy southern positive transgenic plants (CT1, CT2 and CT3) whereas no expression was observed in WT as expected (Figure 6b).

Optimization of Hygromycin B and Microinjection

In this study the ESAM of cotton seed was used as an explant for gene delivery. Successful gene delivery was assessed by GUS analysis and growth in the presence of inhibitory concentrations of Hm-B. Different concentrations of Hm-B (0, 5, 10, 15, 20 mg/l) and Cefotaxime (200 mg/l) was tested to find out the optimal conditions which would allow the efficient selection of tissue that was microinjected. Among different concentrations of Hm-B (20 mg/l) was found to be suitable concentration for efficient selection of transformed plants. Germination of cotton seeds reduced or totally stopped above 20 mg/l concentration. High concentrations of Hm-B (> 20 mg/l) caused the tissue without any survival. Thus 20 mg/l Hm-B was concluded to be the minimum concentration which presented seed germination in cotton. Therefore this concentration was taken for selection of transgenic plants.

Table - 1. Transformation efficiency in five times microinjected embryonic shoot apical meristem of cotton seeds (cv.SVPR-2).8iu

Conc. of *Agrobacterium* (OD_{600})	Conc. of Acetosyringone (mM)	Number of co-cultured embryonic shoot apical meristem yielding	
		Hm-B resistant (percent)	GUS positive (percent)
0.2	0	0/100 (0%)	0/100 (0%)
0.2	50	0/100 (0%)	0/100 (0%)
0.2	100	2/100 (2%)	0/100 (0%)
0.2	150	1/100 (1%)	0/100 (0%)
0.2	200	0/100 (0%)	0/100 (0%)
0.4	0	0/100 (0%)	0/100 (0%)
0.4	50	3/100 (3%)	0/100 (0%)
0.4	100	8/100 (8%)	2/100 (2%)
0.4	150	4/100 (4%)	0/100 (0%)
0.4	200	1/100 (1%)	0/100 (0%)
0.6	0	6/100 (6%)	1/100 (1%)
0.6	50	12/100 (12%)	3/100 (3%)
0.6	100	20/100 (20%)	8/100 (8%)
0.6	150	15/100 (15%)	4/100 (4%)
0.6	200	8/100 (8%)	2/100 (2%)
0.8	0	2/100 (2%)	0/100 (0%)
0.8	50	6/100 (6%)	1/100 (1%)
0.8	100	12/100 (12%)	3/100 (3%)
0.8	150	8/100 (8%)	2/100 (2%)
0.8	200	3/100 (3%)	0/100 (0%)
1.0	0	0/100 (0%)	0/100 (0%)
1.0	50	0/100 (0%)	0/100 (0%)
1.0	100	0/100 (0%)	0/100 (0%)
1.0	150	0/100 (0%)	0/100 (0%)
1.0	200	0/100 (0%)	0/100 (0%)

Figure: 1. Effect of hygromycin on seed germination. Seeds were germinated and grown on ½ strength MS basal medium containing 0, 5, 10, 15 and 20 mg/L hygromycin for 10 days.

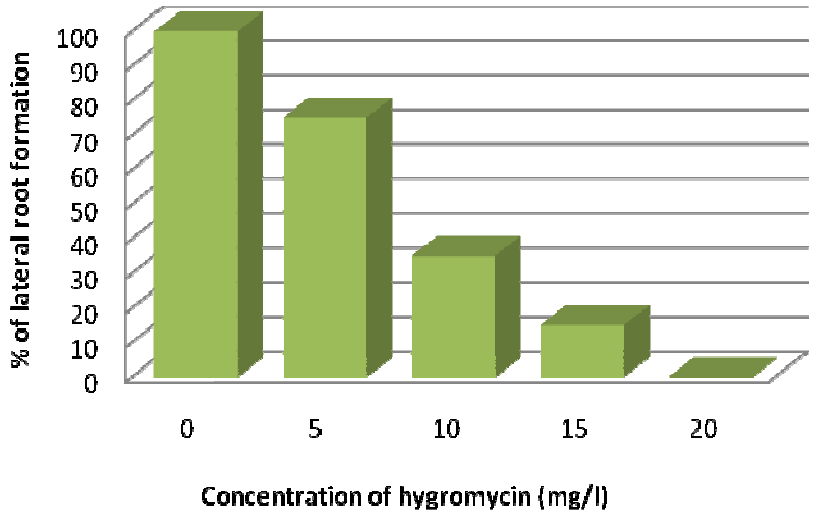


Fig. -1a.

Figure: 2

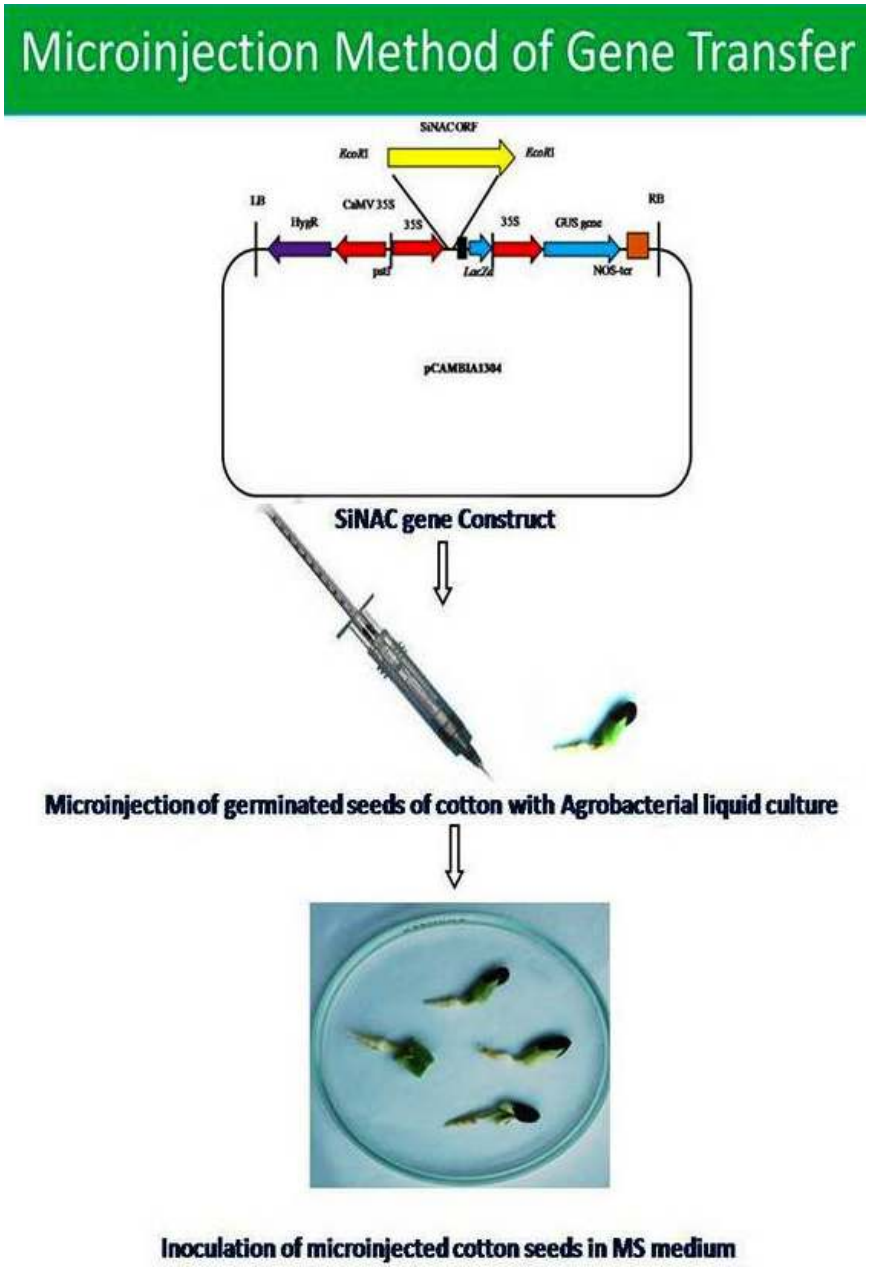
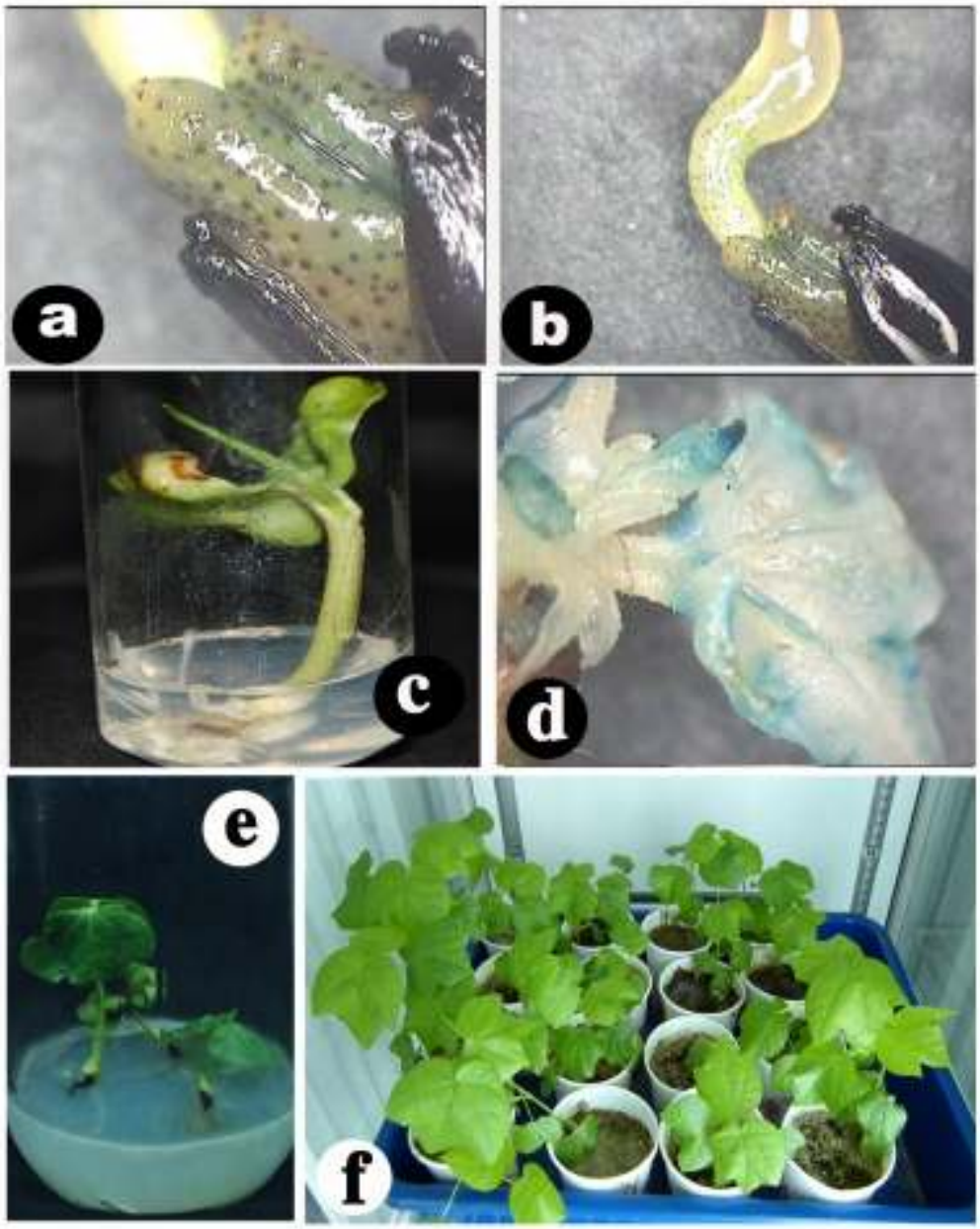
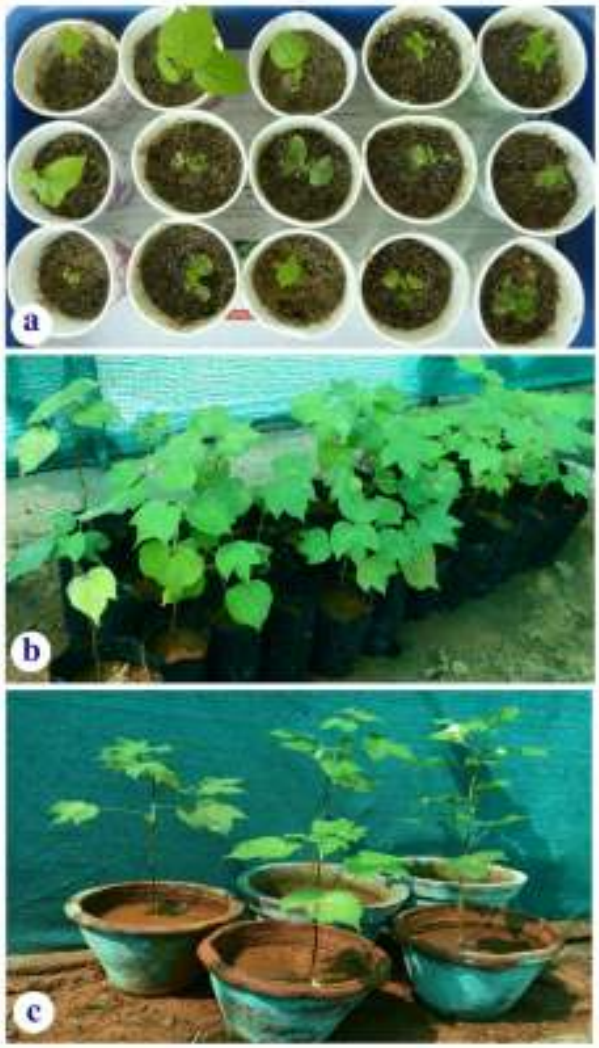

Microinjection Method of Gene Transfer
SiNAC ORF
EcoRI
EcoRI
LB
CaMV 35S
HygR
35S
35S
GUS gene
RB
pstI
Lac Zα
NOS-ter
pCAMBIA1304
SiNAC gene Construct
Microinjection of germinated seeds of cotton with Agrobacterial liquid culture
Inoculation of microinjected cotton seeds in MS medium

Figure: 3 Transformation of embryonic shoot apical meristem from seed



a, b. GUS expression in after co-cultivation 3 days old seed, c. Rooted Plant, d. GUS expression leaf, e. Hm positive plant, Hardened plant in growth chamber

Figure: 4 Putative transgenic lines



a. Transgenic plants in growth Chamber condition,
b. Transgenic plants growth in Green house condition, c. Putative transgenic plants

Figure: 5 PCR & Southern blot analysis

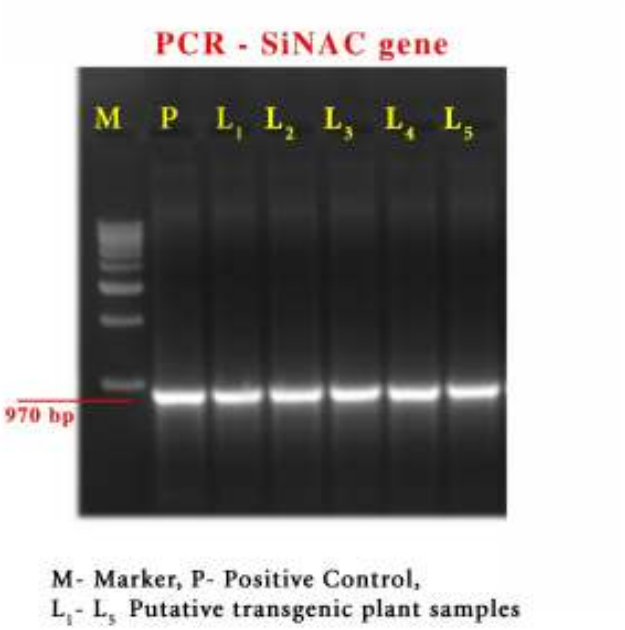
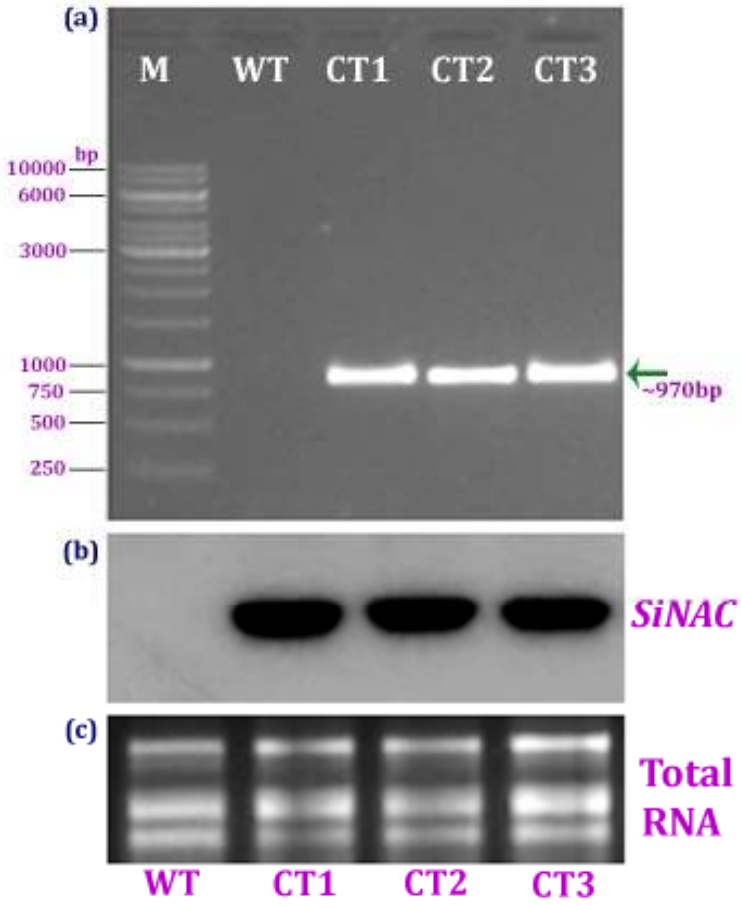


Fig.6 RT-PCR and Northern analysis



(a). Reverse transcriptase (RT)-PCR, (b). Northern blot, (c). Total RNA isolation

Objective 2

2. **To find out the impact of SiNAC gene involved in the production of plant cells from salinity and water deficit stresses.**
3. **To check the efficiency of the transgenic plant in different abiotic stress condition.**
4. **To identify the stress responsiveness of protein in transgenic and non transgenic plant.**

Analysis of Transgenic Cotton Plants for Abiotic Stress Tolerance

Three independent southern-positive transgenic cotton lines were obtained through *Agrobacterium*-mediated genetic transformation. The independent transgenic lines (totally 12 plants) were denoted as CT1, CT2 and CT3 respectively and the wild type cotton plants were designated as WT. Seeds were collected from three independent lines (T_0). The collected seeds were subjected to salt tolerance assay under *in vitro* condition. Then the T_1 plants were evaluated under abiotic stress conditions to analyze their tolerance level.

Seed Germination assay under salt stress

For seed germination studies, WT and T_1 transgenic cotton seeds were surface sterilized and germinated on 90-mm-Petri dishes containing half strength MS agar medium supplemented with 200mM NaCl. Germination assay was carried out in three replicates, and the germination rate was scored after 3 days. Tolerance to NaCl stress was judged based on the ability of the seeds to germinate on sterile cotton and grow on MS medium. The number of germinated seeds was expressed as a percentage of the total number of seeds sown, and the performance of germination was compared to that of WT seeds.

Leaf Disc Assay

Leaf discs were excised from healthy and fully expanded cotton leaves of one month old WT and T_1 transgenic plants (CT1, CT2 and CT3) using a cork borer. Leaf discs were floated on 20 ml solution of different concentrations of NaCl (50,100,150,200 and 250mM) for salinity stress. The experiment was carried out in three replicates. The plates were placed in the culture room for 4 days and subsequently the number of greenish and yellowish leaf discs were scored.

Assessment of transgenic cotton plants under water deficit stress

WT and T_1 transgenic cotton plants grown in the glass house were subjected to water deficit stress under green house condition by withholding water continuously for 20 days. The phenotype of the WT and T_1 plants were observed at regular interval after withholding water. Plants were allowed to recover by supplying water for 7 days after 20 days of water withholding and the recovery in WT and transgenic plants was observed morphologically.

Assessment of transgenic cotton plants under salt stress

Three independent transgenic lines (T_1 plants) were evaluated for stress tolerance assay. To assess the relative salinity tolerance of transformed cotton plants, both transformed (T_1) and wild type (WT) were transferred into plastic pots containing equal amount of potting mixture (vermicompost and sand; 1:1 v/v). Initially for 1 week, the plants were watered without NaCl and thereafter the cotton plants were watered once in 2 days with different concentrations of NaCl (0, 50, 100, 150, 200, 250, and 350mM). Each treatment comprised 3 pots in replicates of three.

Biochemical and physiological analysis of transformed cotton plants under salt stress

For all biochemical assays, 1-month-old T_1 and WT cotton plants which treated with different concentrations of NaCl (0, 50, 100, 150, 200, 250, and 350mM) for 2 weeks were used. Shoot and root length of the WT and T_1 plants were measured after salt treatment. Free proline concentration was measured using the method of Bates *et al.,* (1973). The total chlorophyll content was estimated spectrophotometrically according to the method described by Arnon (1949). Ascorbate peroxidase (APX) activity was measured according to the method described by Chen and Asada (1989). Catalase (CAT) activity was measured by following the method of Aebi (1984). Superoxide dismutase (SOD) activity was assayed by following the method of McCord and Fridovich (McCord and Fridovich 1969). Dehydroascorbate reductase (DHAR) Miyake and Asada (1992), respectively. Hydrogen peroxide (H_2O_2) level was determined according to Velikova et al. (2000). Malondialdehyde (MDA) content was evaluated using the method developed by Heath and Packer (1968). The relative water content (RWC) in the leaves

of cotton plants was determined by following the method of Turner (1981). Each analysis comprised 3 samples in replicates of three.

Statistical analysis

Each experiment was repeated thrice and the control was maintained at every step of analysis. The statistical significance of the data obtained from this study was determined by one-way analysis of variance (SPSS version 17). The mean values were compared by Duncan's multiple range test ($p<0.05$).

Analysis of protein samples from salt treated plants

Proteins were extracted from leaf samples of both WT and transgenic plants (Mirellie et al) and SDS-PAGE was performed to check the expression of stress induced SiNAC protein and other stress related proteins.

Two dimensional Gel Electrophoresis

The resultant pellet was resuspended 100 µl of lysis buffer consisting of 7 M urea, 2 M thiourea, 4% (w/v) CHAPS and 40mM Tris, 1% (w/v) dithiothreitol and then centrifuged at 12,000×*g* in 4 °C for 10 min. The supernatant was stored at -80°C and total protein concentration was quantified using Bradford's method (Bradford, 1976). Briefly, 100 µg proteins were dissolved in 125 µl rehydration buffer (7 M urea, 2 M thiourea , 2% CHAPS, 40 mm DDT, and 0.5% IPG buffer pH 3–10). The protein solution was loaded onto an immobilized pH gradients strip (IPG strip, 7 cm, NL 3–10,GE health care, Sweden) and kept it for overnight for passive rehydration of IPG strip. Isoelectric focusing (IEF) was performed as follows: S1: step: 250 V for 30 min; S2: Grad: 4,000 V for 1.30 mints and S3: Step: 4,000 V for 5 h and a total of 23,000 Vhr (voltage multiplied by hours). The current did not exceed 50 mA per strip. After IEF, the strips were equilibrated in equilibration buffer I (50 mM Tris-HCl, pH 8.8, 6mol/L urea, 30% glycerol, 2% SDS, and 1% DTT) for 15 min and then in buffer II (replaced 1% DTT with 2.5% iodoacetamide) for another 15min. The equilibrated gel strip was loaded onto a 12 % SDS-polyacrylamide gel in 1.5-mm-thick under the conditions described above. SDS-PAGE was performed at 15°C using minigel from Bio-Rad at constant power of 70V/gel for 1 h followed by 100V/gel until the Bromophenol blue reached the bottom of the gel. The gel was stained for 2 h or overnight in 0.1 % of Coomassie brilliant blue R-250 solution (Sigma) in 40 % methanol and

10% glacial acetic acid. Then the gel was rinsed thrice with milliQ for a minute and the remain stain was removed using destaining solution containing 10% glacial acetic acid and 40% methanol in milliQ until clear protein spots were visualized. Then the gel was stored in 10% ethanol at 4°C if for long times. Molecular mass of protein spots were calculated using protein ladder (PageRuler^{TM} Plus Prestained Protein Ladder, Thermo Scientific, USA).

Results and Discussion

Seed Germination under salt stress

To test the ability of seeds to germinate under salt stress, seeds from WT and transgenic cotton plants were surface sterilized and placed on MS agar medium supplemented with 200mM NaCl. The germination rate was observed (Fig.6), and the percentage of germination was calculated after 4 days of germination. Higher percentage of germination was observed in the case of transgenic seeds (CT1, CT2 and CT3) as compared to the WT grown in MS media supplemented with NaCl whereas no significant differences were observed between transgenic seedlings and WT plants grown in MS medium without any stress.

Leaf disc assay

The WT and transgenic cotton plants were exposed to salt stresses, and their response was examined by leaf-disc senescence assay under salt treatments. The leaf discs excised from mature leaves of WT and T_1 transgenic cotton plants were used for the stress analysis. During the 4 days period of incubation in NaCl solution, the leaf discs from WT plants bleached drastically compared to the transgenic leaf discs. Leaf discs treated with distilled water devoid of NaCl appeared green in WT plants (Fig.7).

Evaluation of transgenic cotton plants under water deficit stress

The tolerance level of both WT and transgenic cotton plants was evaluated under water deficit stress. Water irrigation was withheld continuously for 20 days. WT plants could not survive beyond seven days of water withholding and could not recover after water irrigation. They exhibited extensive withering and defoliation after 10 days. On the other hand, transgenic cotton plants were able to grow upto 10 days of water withholding. Transgenic plants also

showed defoliation and withering after 10 days but they partially recovered soon after the water was irrigated (Fig.8).

Evaluation of transgenic cotton plants under salt stress

To find out whether the expression of SiNAC enhances salinity tolerance of transformed (T_1) cotton plants, the performance of transformed plants against NaCl-induced salinity stress was tested. One month old WT plants and T_1 plants were watered with different concentrations (0, 50, 100, 150, 200, 250 and 350mM) of NaCl. Under normal conditions (0mM NaCl), no phenotypic differences were observed between the T_1 and WT plants. However, under increased salinity stress, the WT plants exhibited chlorosis, necrosis, leaf burning, defoliation, less shoot, root length and completely withered above 150mM NaCl. In contrast, the T_1 plants showed healthy growth even at 250mM NaCl and did not show any of the above symptoms. Beyond 250mM NaCl, even the T_1 plants were unable to survive and completely perished (Fig.9).

Biochemical and physiological evaluation of transformed cotton plants under salt stress

Under salinity stress, shoot and root length of T_1 plants were relatively higher when compared to WT plant (Fig.10.i&ii). The transformed (T_1) cotton plants survived up to 250 mM of NaCl, whereas WT plants survived up to 150mM NaCl and beyond that WT plants were perished. Hence, biochemical and physiological analyses were carried on transformed (T_1) and WT cotton plants up to 250 mM and 150mM, respectively (Fig.10).

Effect of salinity stress on proline and chlorophyll content

Under normal growth conditions, the concentration of free proline in leaves of T_1 plants was similar to that of WT plant. When the T_1 and WT plants were treated with increased concentrations of NaCl, the T_1 plants accumulated 40–70% higher proline than WT plant and T_1 plant showed highest proline content at all tested NaCl concentrations (Fig.11.i). The leaf chlorophyll content gradually decreased with increased concentration of NaCl. However, under same concentration of NaCl, the chlorophyll loss in T_1 plants was significantly less noticeable than that of the WT plant. The chlorophyll loss was 10–35 % in WT plant, whereas 7.5–18.6 % in T_1 plants (Fig,11.ii).

Effect of salinity stress on activities of antioxidant enzymes and other biochemicals

Under standard growth conditions, the T_1 and WT cotton plants accumulated similar amount of APX, CAT, and SOD. The antioxidative enzyme activities gradually increased with the increased concentration of NaCl in both T_1 and WT cotton plants. However, the T_1 plants maintained 70–220 % APX, 80–90 % CAT, and 10–80 % SOD activity than their counterparts. Among the three T_1 plants analyzed, CT2 plant showed the highest APX, CAT, and SOD activities than the remaining two T_1 cotton plants at all tested NaCl concentrations (Fig.11.iii,iv & v). When the T_1 and WT cotton plants were watered with different concentrations of NaCl, the activity of DHAR enzyme increased up to 150 mM and thereafter decreased. However, the T_1 cotton plants accumulated 70–260 % DHAR than WT cotton plants (Fig.11.vi). Among the three T_1 cotton plants, plant showed highest activity than remaining two T_1 cotton plants at all tested NaCl concentrations.

There were no noticeable differences in H_2O_2 content between the T_1 and WT cotton plants under normal growth conditions. When the T_1 and WT cotton plants were watered with different concentrations of NaCl, the T_1 cotton plants accumulated less amount of H_2O_2 than WT plant (Fig.11.vii).

Under normal growth conditions, the T_1 and WT cotton plants showed similar concentration of MDA. The MDA concentration gradually increased in both T_1 and WT plants with increased concentration of NaCl. However, under all tested NaCl concentrations, the WT plant showed higher MDA content than T_1 plants (Fig.11.viii). This implies that the degree of lipid peroxidation in T_1 plants is lower than that in the WT plant.

Under normal growth conditions, the RWC in T_1 and WT cotton plants were similar**.** Even though the RWC gradually decreased in WT and T_1 plants under different concentrations of NaCl, the T_1 plants maintained higher RWC than the WT plant (Fig.11.ix).

Expression of SiNAC protein under salt stress

SDS-PAGE analysis of transgenic cotton plants (CT1, CT2 and CT3) under salt stress showed a protein band at ~48kDa which corresponds to SiNAC protein and other stress related proteins whereas WT plants produced no protein band which represents the absence of SiNAC protein (Fig.12). Two dimensional electrophoresis of T_1 plant gave five protein spots which were not present in the WT plant sample. This confirms the expression of SiNAC and other stress related proteins in salt treated T_1 plants (Fig.13). Since there is no availability of antibody for SiNAC protein we could not proceed further.

Fig. 7 Seed Germination under salt stress

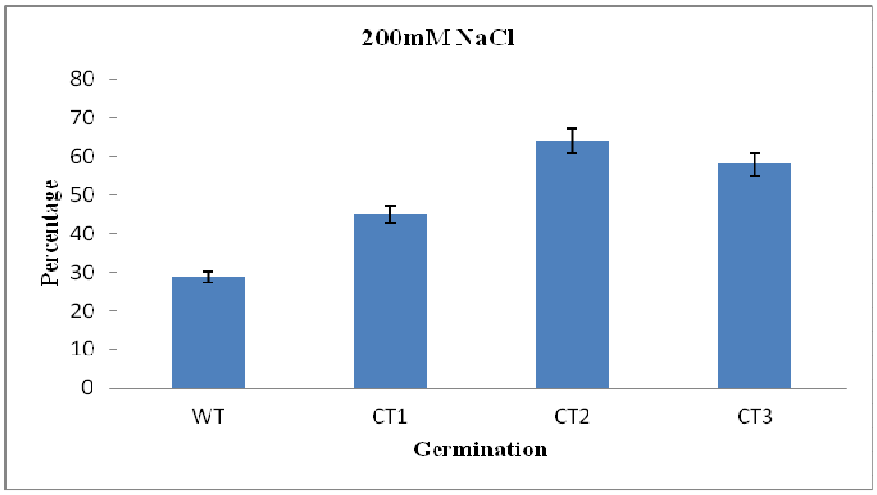


Fig. 7 Percentage of seed germination under 200mM Nacl in sterile cotton saturated with ½ strength MS liquid medium

Figure: 8 Leaf Disc Assay

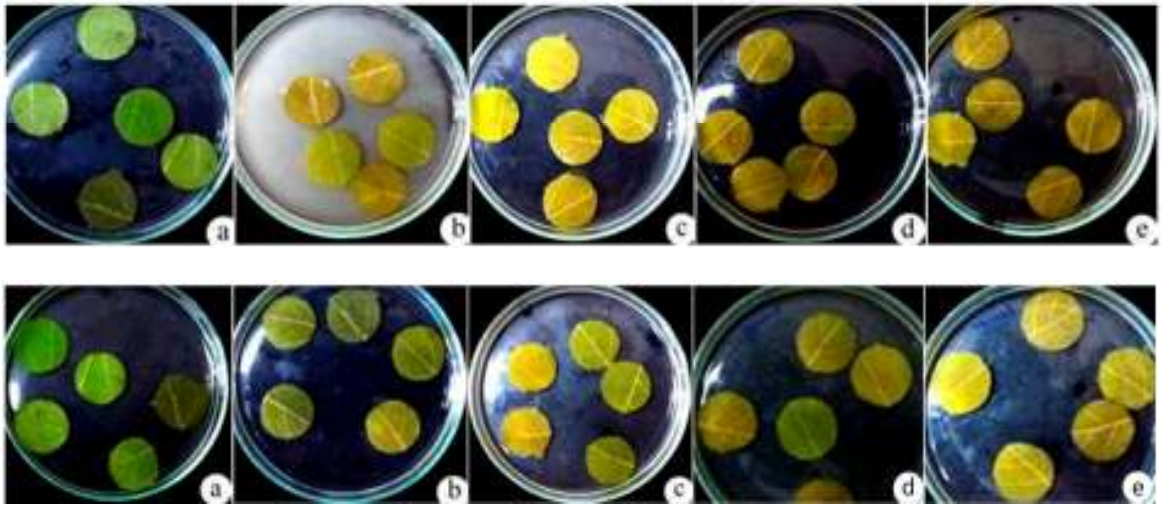


Fig. 8 (i) and (ii). Phenotypic difference in the salt stress promoted senescence in WT and transgenic cotton plants expressing SiNAC gene

i (a)	-	Discs floated in water served as control
i (b-e)	-	Leaf discs of WT plants incubated in 50, 100, 150 and 250mM NaCl respectively for 4 days
ii (b-e)	-	Leaf discs of Transgenic plants incubated at 50, 100, 150 and 250mM NaCl respectively for 4 days.

Fig. 9 Evaluation of cotton plants under water deficit stress

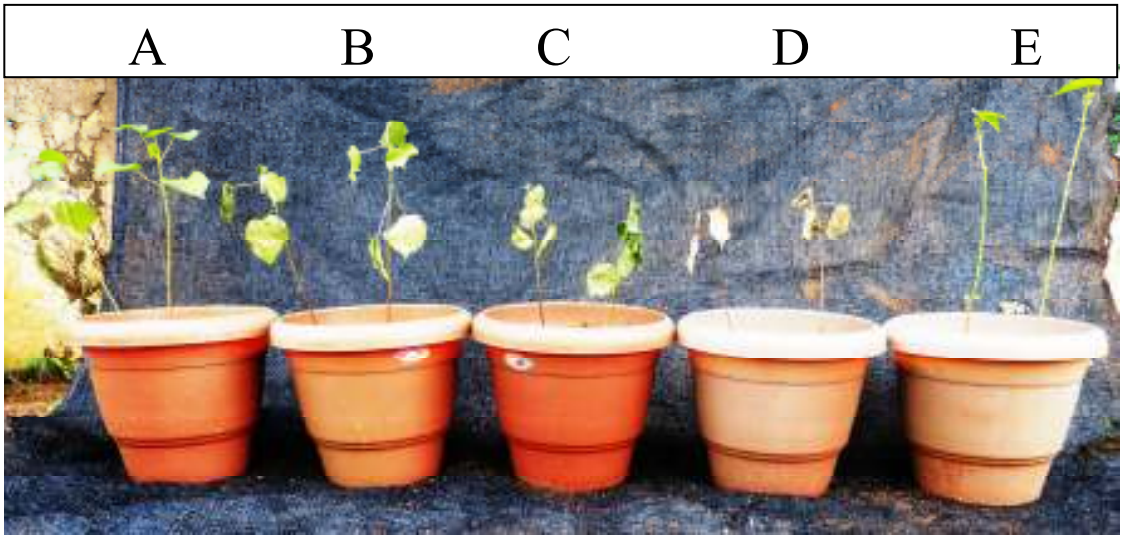


Fig. 9 **A – Day 1**

B – Day 7

C – Day 15

D – Day 20

E – Recovery of transgenic plant after resuming water irrigation

Fig. 10 Evaluation of cotton plants under salt stress

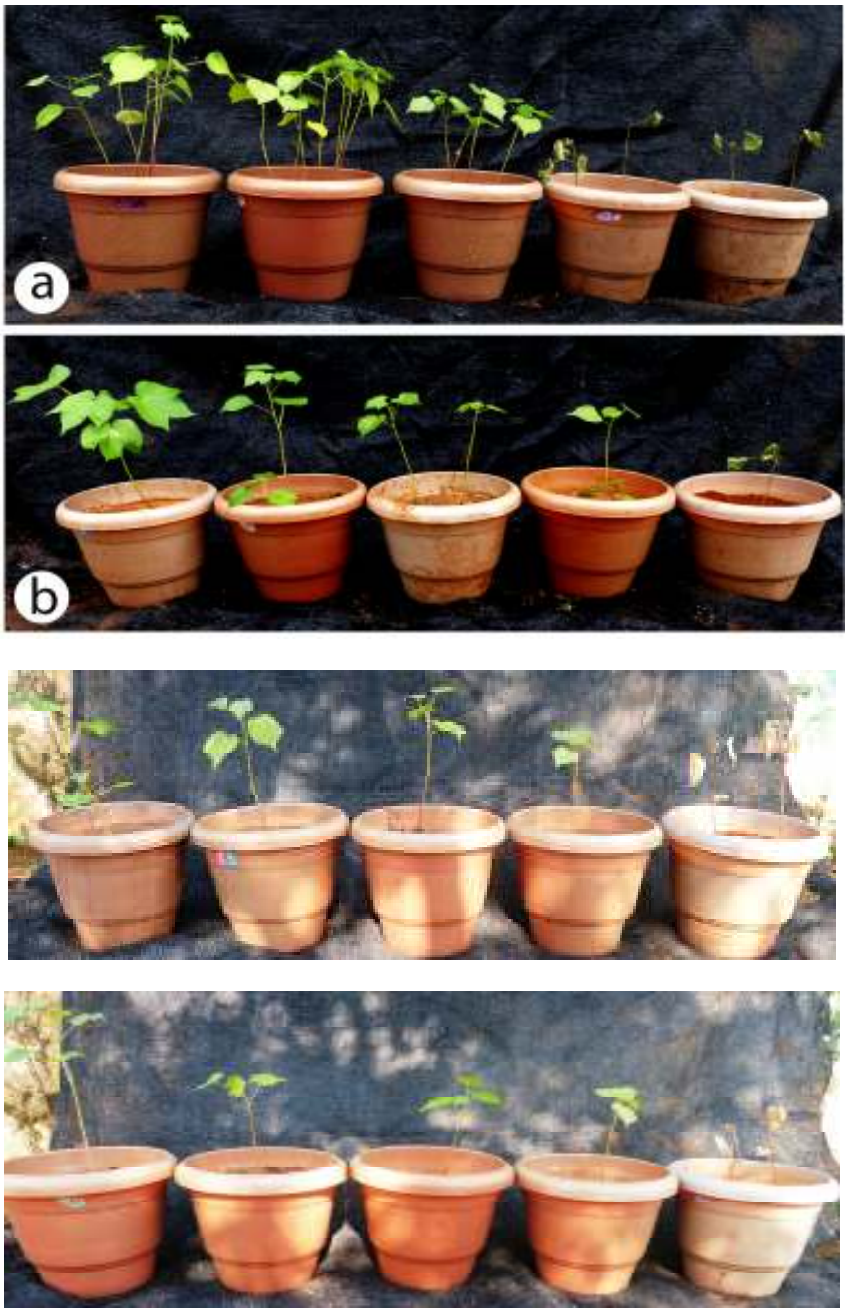
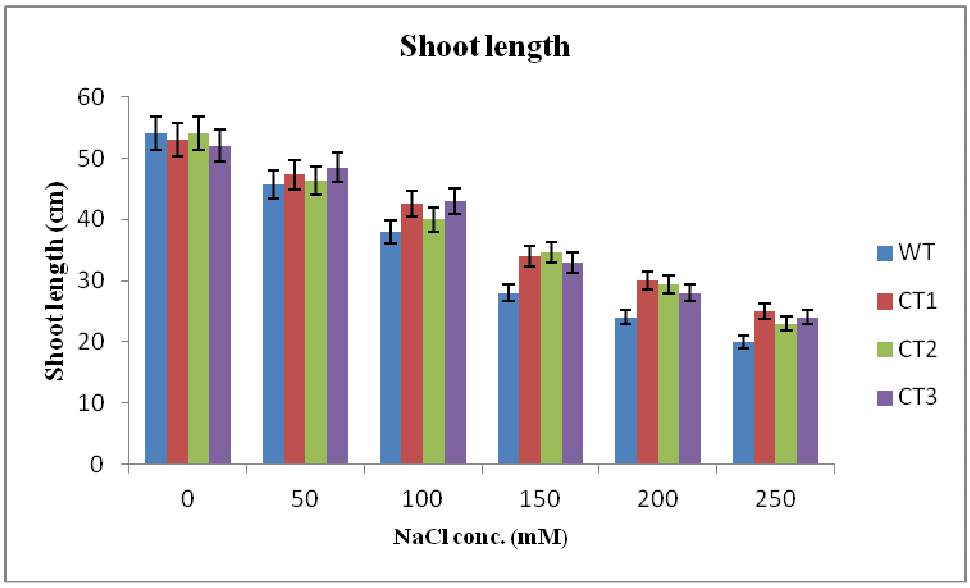


Fig. 10 (a). WT plants under green house condition at 50,100,150,200 and 250mM NaCl

(b, c & d). Transgenic plants (CT1, CT2 and CT3) under green house condition at 50,100,150,200 and 250mM NaCl

Figure: 11 Shoot and root length of WT and T_1 plants under salt stress

(i)



(ii)

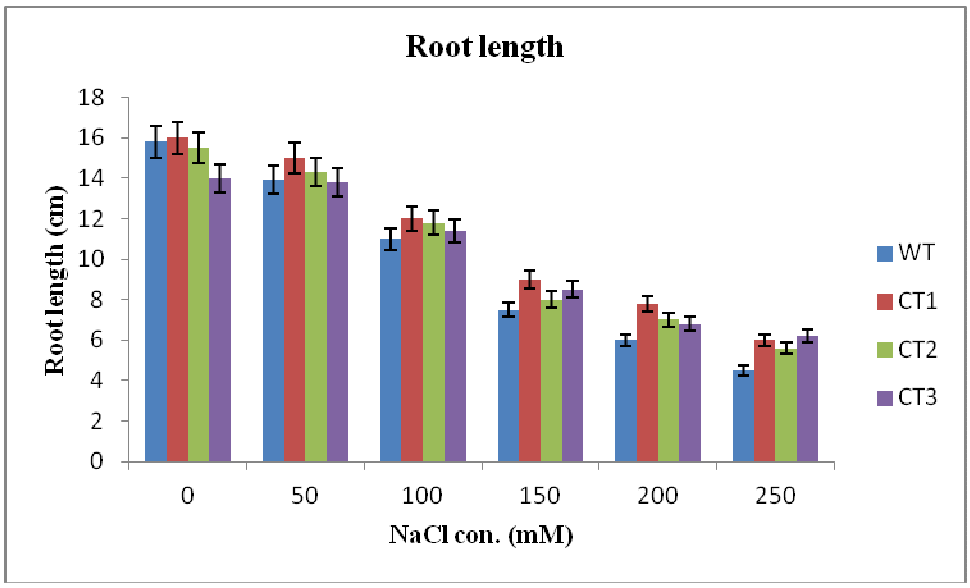
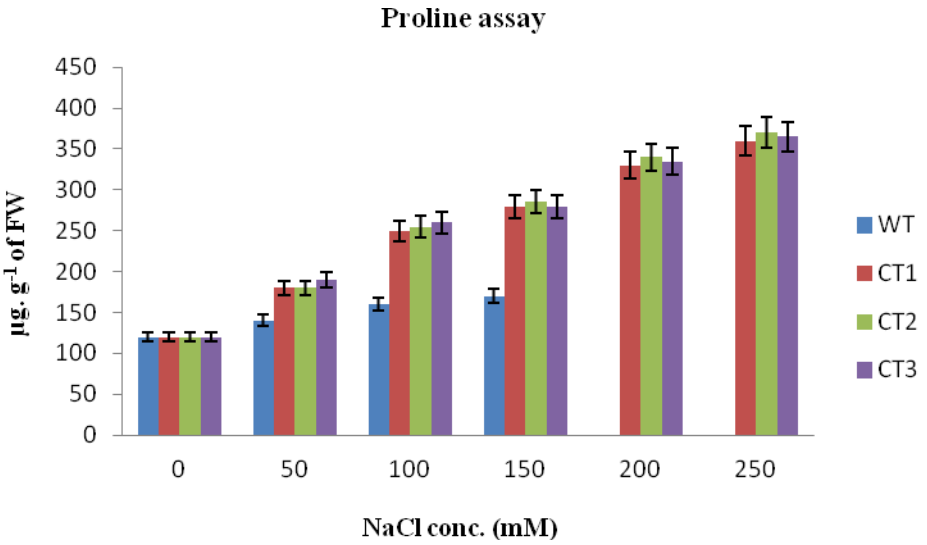
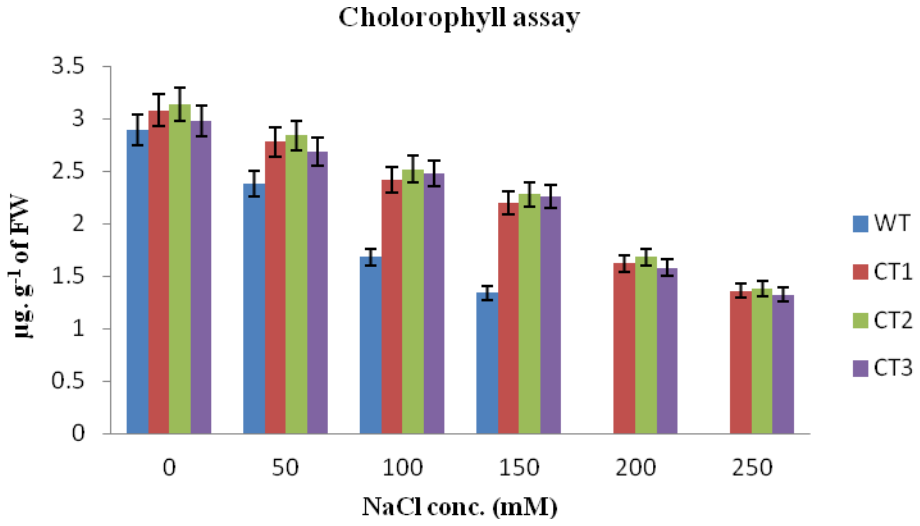


Figure: 12 Biochemical analysis of WT and T_1 plants

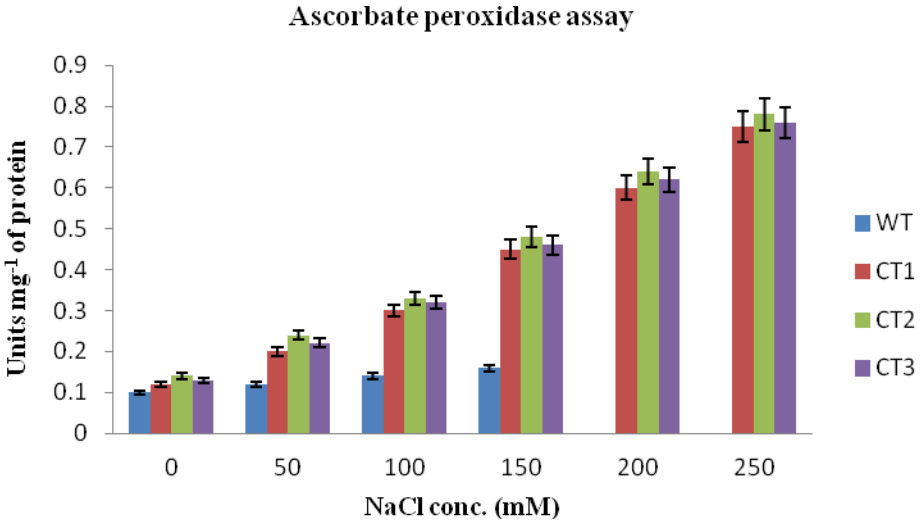
(i)



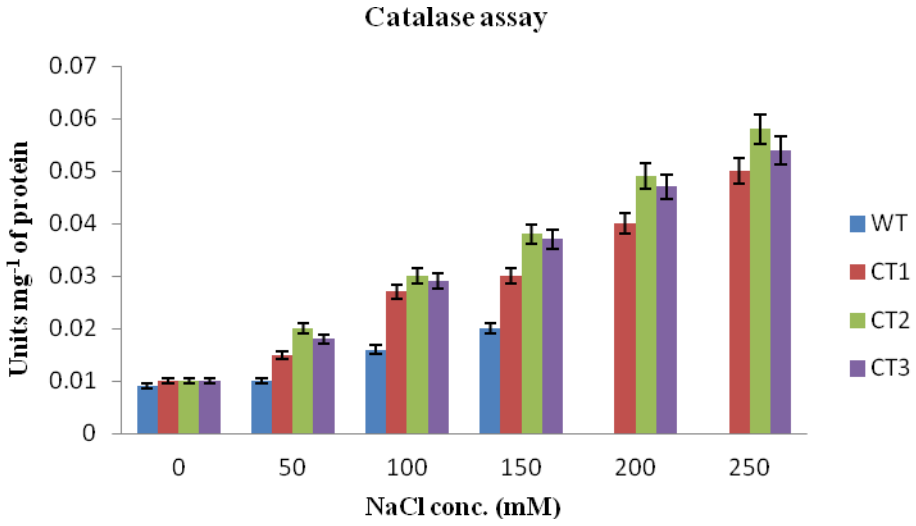
(ii)


Cholorophyll assay
3.5
3
2.5
2
1.5
1
0.5
0
µg. g-1 of FW
0
50
100
150
200
250
NaCl conc. (mM)
WT
CT1
CT2
CT3

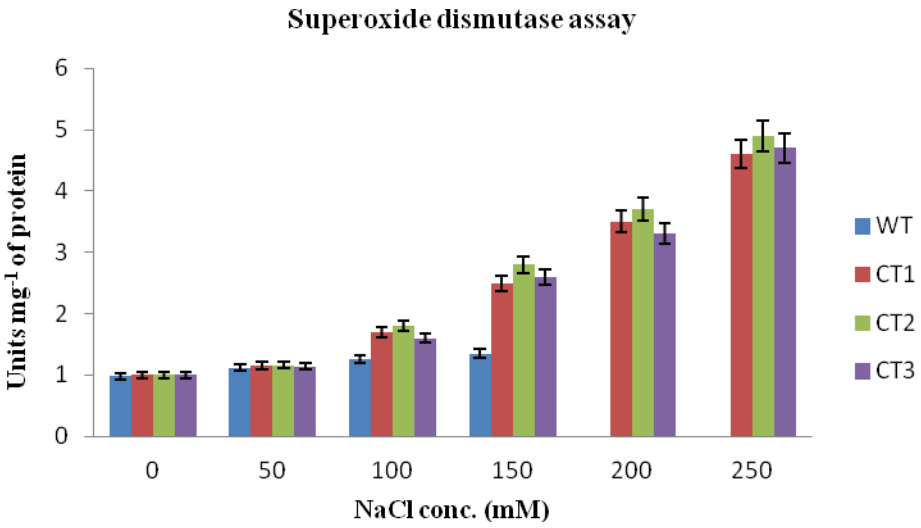
(iii)


Ascorbate peroxidase assay
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
Units mg-1 of protein
0
50
100
150
200
250
NaCl conc. (mM)
WT
CT1
CT2
CT3

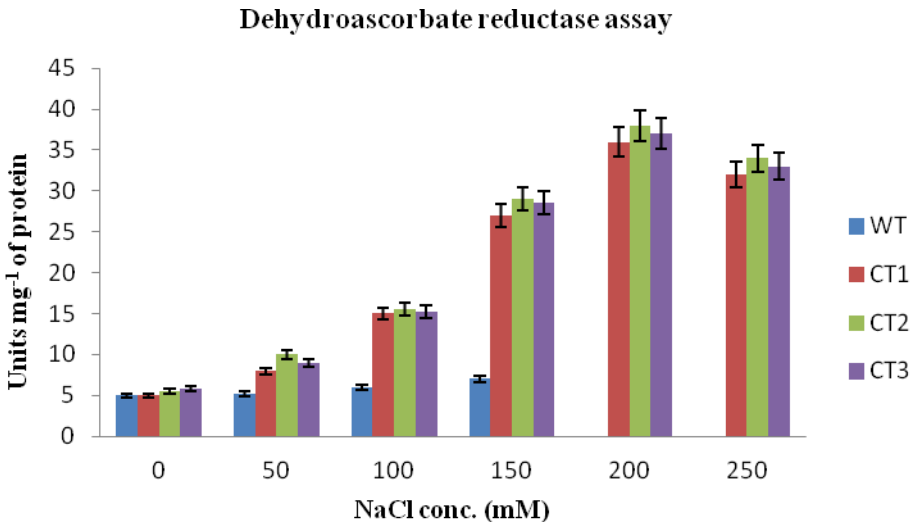
(iv)


Catalase assay
Units mg-1 of protein
0.07
0.06
0.05
0.04
0.03
0.02
0.01
0
0
50
100
150
200
250
NaCl conc. (mM)
WT
CT1
CT2
CT3

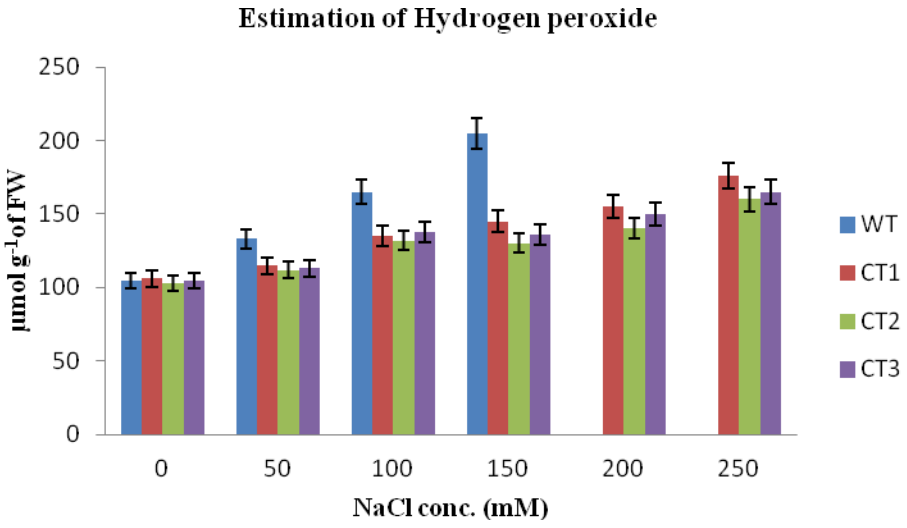
(v)


Superoxide dismutase assay
Units mg-1 of protein
6
5
4
3
2
1
0
0
50
100
150
200
250
NaCl conc. (mM)
WT
CT1
CT2
CT3

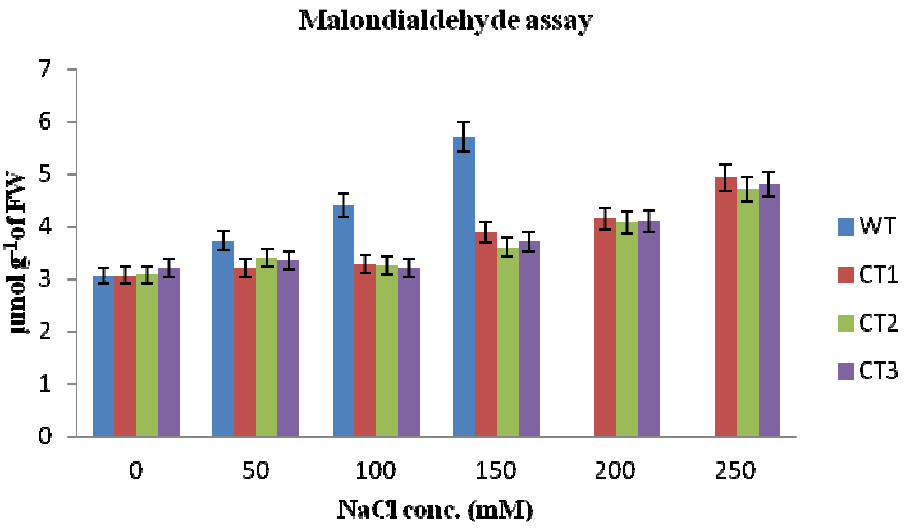
(vi)


Dehydroascorbate reductase assay
Units mg-1 of protein
45
40
35
30
25
20
15
10
5
0
0
50
100
150
200
250
NaCl conc. (mM)
WT
CT1
CT2
CT3

(vii)


Estimation of Hydrogen peroxide
µmol g-1of FW
250
200
150
100
50
0
0
50
100
150
200
250
NaCl conc. (mM)
WT
CT1
CT2
CT3

(viii)


Malondialdehyde assay
μmol g-1of FW
0
1
2
3
4
5
6
7
0
50
100
150
200
250
NaCl conc. (mM)
WT
CT1
CT2
CT3

(ix)

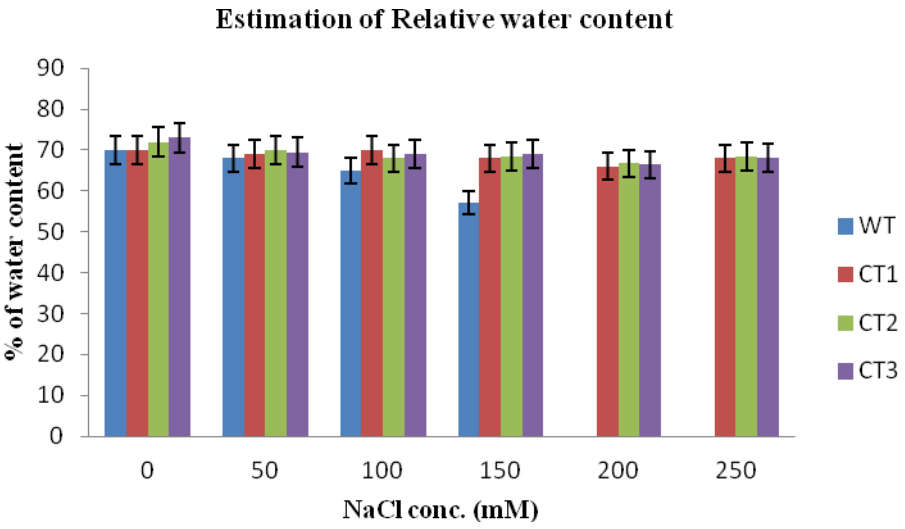
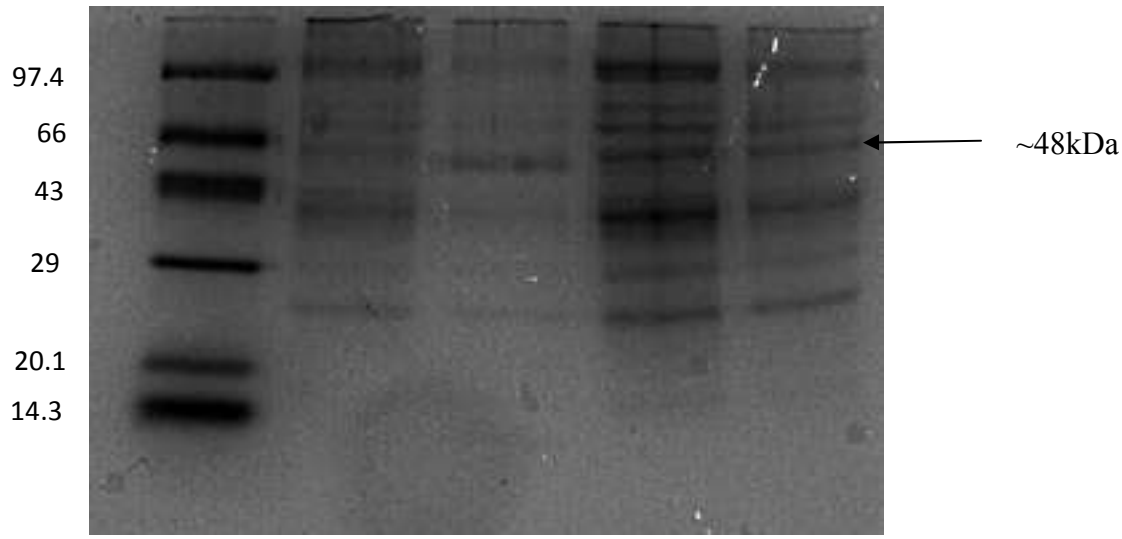

Estimation of Relative water content
% of water content
0
10
20
30
40
50
60
70
80
90
0
50
100
150
200
250
NaCl conc. (mM)
WT
CT1
CT2
CT3

Fig. 12 Response of cotton plants exposed to various concentrations of NaCl for 2 weeks under green house conditions

Figure. 13 Expression of SiNAC protein under salt stress

M WT CT1 CT2 CT3



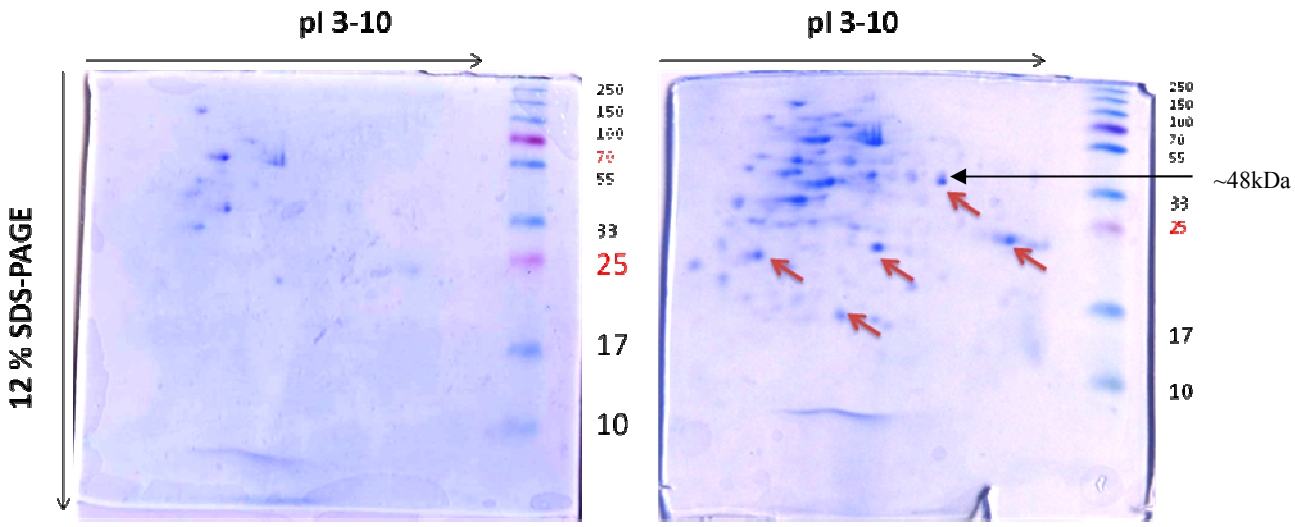
M – Protein Marker (kDa)

WT – Absence of SiNAC protein band

CT1,CT2 & CT3 – Presence of ~48kDa SiNAC protein

Figure. 14 Two Dimensional Gel electrophoresis

(a) **(b)**



WT Plant **T_1 plant (CT2)**

Abbreviations

BAP	-	6, Benzyl Amino Purine
B_5	-	Gamborg's Medium (1969)
Cef	-	Cefotaxime
2,4-D	-	2,4-Dichloro phenoxy acetic acid
EASM	-	Embryonic shoot apical meristem
g/l	-	Gram per liter
IBA	-	Indole-3-acetic acid
Kin	-	Kinetin
µl	-	Micro liter
Mm	-	Millimeter
Mg	-	Milligram
MS	-	Murashige and Skoog's Medium

NAA	-	Naphthalene acetic acid
OD	-	Optical density
%	-	Percentage
PCR	-	Polymerase chain reaction
Hm	-	Hygromycin

References:

Abu Sefyan I. Saad, Xu Li, He-Ping Li, Tao Huang, Chun-Sheng Gao, Mao-Wei Guo, Wei Cheng, Guang-Yao Zhao and Yu-Cai Liao (2013). A rice stress-responsive NAC gene enhances tolerance of transgenic wheat to drought and salt stresses. Plant Science 33-40.

Aebi H (1984) Catalase *in vitro*. Methods Enzymol 105:21–126

Arnon DI (1949) Copper enzymes in isolated chloroplasts. Polyphenoloxidase in *Beta vulgaris*. Plant Physiol 24(1):1–15.

Baskaran, P. and Indranil Dasgupta (2011). Gene delivery using microinjection of *Agrobacterium* to embryonic shoot apical meristem of elite indica rice cultivars, Journal of Plant Biochemistry and Biotechnology 2: 268-274.

Bates LS, Waldren RP, Teare JD (1973) Rapid determination of free proline for water stress studies. Plant Soil 39:205–207.

Chen GX, Asada K (1989) Ascorbate peroxidase in tea leaves: occurrence of two isozymes and the differences in their enzymatic and molecular properties. Plant Cell Physiology 30:987–998.

Doulis AG, Debian N, Kingston-Smith AH, Foyer CH (1997) Differential localization of antioxidants in maize leaves. Plant Physiology 114:1031–1037.

Goh CJ, Nathan MJ, Kumar PP (1995). Direct organogenesis and induction of morphogenic callus through thin section culture of *Heliconia psittacorum,* Scientia Horticulturae 62: 113-120.

Gurusamy Dhandapani, Mogilicherla Kanakachari, Kethireddy Venkata Padmalatha, Mullapudi Lakshmi Venkata Phanindra, Vivek Kumar Singh, Sanagala Raghavendrarao, Narayanasamy Jayabalan, Azhagiyamanavalan Lakshmi Prabha and Polumetla Ananda Kumar (2015). A Gene Encoding Cold-Circadian Rhythm-RNA Binding-Like Protein (CCR-Like) from Upland Cotton (*Gossypium hirsutum* L.) Confers Tolerance to Abiotic Stresses in Transgenic Tobacco. Plant Molecular Biology Reporter 33:22-42.

Heath RL, Packer L (1968) Photoperoxidation in isolated chloroplasts. I. Kinetics and stoichiometry of fatty acid peroxidation. Archives of Biochemistry and Biophysics 125:189–198.

Honghong Hu, Minigqiu Dai, Jialing Yao, Benze Xiao, Xiangha Li, Qifa Zhang and Lizhong Xiog (2006). Overexpressing a NAM, ATAF and CUC (NAC) transcription factor enhances drought resistance and salt tolerance in rice. Proceedings of the National Academy of Sciences 103: 12987-12992.
Jefferson RA, Burgees SM and Hirsh D (1986). β - Glucuronidase from *Escherichia coli* as a gene-fusion marker. Proceedings of the National Academy of Sciences 83: 8447-8451.

Kondeti Subramanyam, Muthukrishnan Arun, Thankaraj Salammal Mariashibu, Jeevaraj Theboral, Manoharan Rajesh, Narendra K. Singh, Markandan Manickavasagam and Andy Ganapathi (2012). Overexpression of tobacco osmotin (Tbosm) in soybean conferred resistance to salinity stress and fungal infections. Planta 236: 1909-1925.

McCord JM and Fridovich I (1969) Superoxide dismutase: an enzymatic function for erythrocuprein (hemocuprein). J Biol Chem 244: 6049–6055.

Mireille Faurobert, Esther Pelpoir and Jamila Chaib (2011). Phenol extraction of proteins for proteomic studies of recalcitrant plant tissues. In H. Thiellment, M. Zivy, C. Damerval and V. Mechin (Eds.), Methods in Molecular Biology (pp. 9-14). New Jersey: Humana Press.

Miyake C, Asada K (1992) Thylakoid-bound ascorbate peroxidase in spinach chloroplasts and photoreduction of its primary oxidation product monodehydroascorbate radicals in the thylakoids. Plant Cell Physiology 35:539–549.

Kumria R, Sunnichan VG, Das DK, Gupta SK, Reddy VS, Bhatnagar RK and Leelavathi S (2003). High-frequency somatic embryo production and maturation into normal plants in cotton (*Gossypium hirsutum*) through metabolic stress. Plant Cell Reports 21: 635-639.

Tanveer Khan,Vanga Siva Reddy, Sadhu Leelavathi (2010). High-frequency regeneration via somatic embryogenesis of an elite recalcitrant cotton genotype (*Gossypium hirsutum* L.) and efficient *Agrobacterium*-mediated transformation. Plant Cell Tissue and Organ Culture 101: 323-330.

Turner NC (1981) Techniques and experimental approaches for the measurement of plant water stress. Plant Soil 58:339–366.

Velikova V, Yordanov J and Edreva A (2000) Oxidative stress and some antioxidant systems in acid rain treated bean plants. Protective role of exogenous polyamines. Plant Science 151:59–66.

Vinoth S, Gurusaravanan P and Jayabalan N (2013). Optimization of factors influencing microinjection method for *Agrobacterium tumefaciens*- mediated transformations of tomato. Applied Biochemistry and Biotechnology 169: 1173-1187.

B2. Summary and Conclusions of the Progress made so far:

Microinjection

Microinjection of ESAM (Embryonic Shoot Apical Meristem) of germinated cotton seeds is an efficient method in which the gene is delivered directly into the meristematic cells through *Agrobacterium tumefaciens*-mediated transformation. Various parameters such as bacterial density (OD_{600} - 0.6), Hygromycin concentration (20mg/L), concentration of acetosyringone (100 mM) were optimized to get high transformation efficiency.

GUS assay

GUS assay was performed to confirm the expression of the GUS reporter gene in leaves from putatively transformed plants surviving on Hygromycin selection medium. Leaves showing blue colour formation after incubating with X-Gluc substrate were considered as GUS-positive.

PCR analysis

Genomic DNA was isolated from the young leaves of T_0 plants grown in plant growth chamber. The genomic DNA of the T_0 plants was tested for the presence of SiNAC gene was also confirmed by amplifying the gene with specific primer and the expected **970 bp** fragment was observed as sharp bands.

Southern blot analysis

Sothern blot hybridization was carried out to confirm the stable integration and copy number of the SiNAC gene in the genome of PCR-positive plants (T_0). The copy number of SiNAC gene integration into the individual cotton genome was revealed by number of hybridization signals.

RT-PCR analysis

Total RNA was isolated from WT and T_1 cotton plants and it was checked on 1.2 % agarose denatured formaldehyde gel. cDNA was synthesized from isolated RNA samples using Transcriptor First Strand cDNA synthesis Kit (Roche Diagnostics GmbH, Germany). RT-PCR

analysis showed the expression of SiNAC transcript and it specifically amplified 970-bp fragment using gene specific primers in all the transgenic except WT control.

Northern blot analysis

Total RNA was isolated from WT and three T_1 transgenic cotton plants and the expression of *SiNAC* transcripts in transgenic cotton was confirmed by Northern blot analysis which showed expression of SiNAC transcript in all the three single copy southern positive transgenic plants (CT1, CT2 and CT3) whereas no expression was observed in WT as expected.

Analysis of transgenic cotton plants for abiotic stress tolerance

Seed germination of the T_1 plants under saline condition (200mM NaCl) was 60% when compared to WT plants with 25% which indicates the tolerance ability of the T_1 plants under *in vitro* condition.

In leaf disc assay, WT plants showed extensive yellowing and chlorosis in leaves after 4 days of incubation whereas leaf discs of transgenic cotton plants remained green upto 3 days and 30% of the leaf discs showed the above said morphological changes after 4 days. This observation clearly indicates the tolerance of the T_1 plants upto 250mM.

Under water deficit conditions, WT plants exhibited extensive withering, defoliation and unable to recover after resuming water irrigation. On the other hand, transgenic cotton plants were able to grow upto 10 days of water withholding. Transgenic plants also showed defoliation and withering after 10 days but they partially recovered soon after the water irrigation.

Biochemical and physiological evaluation of transformed cotton plants under salt stress

Under salinity stress, shoot and root length of T_1 plants were relatively higher when compared to WT plant. The transformed (T_1) cotton plants survived up to 250 mM of NaCl, whereas WT plants survived up to 150mM NaCl. Beyond 150mM NaCl and beyond that WT plants were perished. Hence, biochemical and physiological analyses were carried on transformed (T_1) and WT cotton plants up to 250 mM and 150mM, respectively.

Effect of salinity stress on proline and chlorophyll content

The concentration of free proline in leaves of T_1 plants was similar to that of WT plant under normal growth conditions. T_1 plants accumulated higher proline than WT plant and T_1 when the T_1 and WT plants were treated with increased concentrations of NaCl. The T_1 plants showed highest proline content at all tested NaCl concentrations. The leaf chlorophyll content gradually decreased with increased concentration of NaCl. However, under same concentration of NaCl, the chlorophyll loss in T_1 plants was significantly less noticeable than that of the WT plant. The chlorophyll loss was in WT plant, whereas in T_1 plants.

Effect of salinity stress on activities of antioxidant enzymes and other biochemicals

The T_1 and WT cotton plants accumulated similar amount of Ascorbate peroxidase, Catalase, and Superoxide dismutase, Dehydroascorbate reductase under standard growth conditions. The antioxidative enzyme activities gradually increased with the increased concentration of NaCl in both T_1 and WT cotton plants. However, the T_1 plants maintained higher APX, CAT, and SOD activity than their counterparts. Among the three T_1 plants analyzed, CT2 plant showed the highest APX, CAT, SOD and DHAR activities than the remaining two T_1 cotton plants at all tested NaCl concentrations.

There were no noticeable differences in Hydrogen peroxide content between the T_1 and WT cotton plants under normal growth conditions. When compared to WT plants, the T_1 plants accumulated less amount of H_2O_2 under increasing concentration of NaCl.

The Malondialdehyde concentration of both T_1 and WT plants gradually increased with increased concentrations of NaCl. However, the WT plant showed higher MDA content than T_1 plants under all tested NaCl concentrations. This result suggests that the degree of lipid peroxidation in T_1 plants is lower than that in the WT plant.

The Relative water content of T_1 and WT cotton plants were similar under normal growth conditions**.** The T_1 plants maintained higher RWC than the WT plant whereas it gradually decreased in WT and under different concentrations of NaCl.

The overall results suggests that T_1 cotton plants were able to sustain upto 250mM NaCl and withered at 350mM concentration and activity of antioxidant enzymes in transgenic cotton plants increased with increasing concentrations of NaCl. This reveals the salt tolerance ability of the transgenic cotton plants due to the expression of SiNAC transcription factor. These morphological and physiological characters indicated the stress tolerance ability of T_1 plants. CT2 transgenic line showed higher activity in all antioxidants and biochemical assays when compared to CT1 and CT3 lines.

Presence of protein band (~48kDa) from salt stress treated transgenic plants showed the expression of SiNAC along with expression of other stress related proteins.

Two dimensional electrophoresis of T_1 plant (CT2 line) gave five protein spots which were not present in the WT plant sample. This confirms the expression of SiNAC and other stress related proteins in T_1 plants. Since there is no availability of antibody for SiNAC protein we could not proceed further.

B3. Details of New Leads Obtained, if any: Nil

B4. Details of Publications & Patents, if any: Publications - Two

1. Subiramani Sivakumar, Ganesan Premkumar, Govindarajan Siva, Mogilicherla Kanakachari, Manickam Vigneswaran, Sadhasivam Vinoth, Thiruppathi Senthil Kumar and Narayanasamy Jayabalan. Optimization of factors influencing microinjection method for *Agrobacterium*-Mediated transformation of Embryonic Shoot Apical Meristem in Cotton (*Gossypium hirsutum* L. cv.SVPR-2). International Journal of Current Biotechnology. 2014, 2(12):35-40.

2. G. Prem Kumar, Sivakumar Subiramani, Siva Govindarajan, Vinoth Sadasivam, Vigneswaran Manickam, Kanakachari Mogilicherla, Senthil Kumar Thiruppathi, Jayabalan Narayanasamy. Evaluation of different carbon sources for high frequency

callus culture with reduced phenolic secretion in cotton (*Gossypium hirsutum* L.) cv. SVPR-2. Biotechnology Reports. 2015, 7:72-80.

3. Papers presented in seminars/conferences

   i. Presented a paper entitled **"Gene transfer using microinjection of SiNAC to embryonic shoot apical mersitem of cotton (*Gossypium hirsutum* L.) cv.SVPR-2"** in National Symposium on Himalayan Biodiversity: Prospects and Challenges held during 20th and 21st March 2014 at Centre for Advanced Studies in Botany, North-Eastern Hill University, Shillong, Meghalaya.

   ii. Presented a paper entitled **"Effect of carbon sources on phenolic secretion in callus culture of cotton (*Gossypium hirsutum* L. cv.SVPR-2)"** in Third Indian Biodiversity Congress 2014 held on December 18-20, 2014 at SRM University, Chennai, Tamil Nadu.

Section - C: Details of Grant Utilization

C1. Equipment Acquired or Placed Order with Actual Cost:

(Rupees figures given in table are shown in Lakhs)

Name and Details of Equipment/Asset Acquired	Sanctioned Cost	Actual Cost	Balance/Amount to be reimbursed
A. Sanction Equipment			
i). Thermo mixer comfort	2.76668	2.72085	0.04583
ii). Biophotometer plus	3.34210	3.16898	0.17312
iii). Refrigerated Micro Centrifuge	2.89456	2.86704	0.02752
iv). Ice Flaker Machine	1.49990	1.49993	**(-)** 0.00003
v). Accupipets	1.72710	1.63630	0.09080
B. Equipment procured without sanction			
--	--	--	--
Total	**12.23034**	**11.8931**	**0.33724**

C2. Manpower Staffing and Expenditure Details:

(Rs. In Lakhs)

Year of the Grant	FINANCIAL YEAR	SANCTIONED POSTS	NUMBER	SCALE OF PAY	ANNUAL OUTLAY	OUTLAY FOR THE ENTIRE PERIOD	REVISED SCALE, IF ANY	REVISED ANNUAL OUTLAY	REVISED PROJECT OUTLAY	ACTUAL RELASES BY DBT	ACTUAL EXPENDITURE	BALANCE
I	1st April 2011 – 31st March 2012	JRF	2	0.12000 + 20% HRA	3.456	10.944	--	--	--	3.456	0.278	3.178
	1st April 2012 – 31st March 2013						--	--	--	3.178	2.794	0.384
II	1st April 2013 – 31st March 2014	JRF	2	0.12000 + 20% HRA	3.456		--	--	--	3.114 (2.73 + 0.384)	3.048	0.066
III	1st April 2014 – 29th December 2014	SRF	2	0.14000 + 20% HRA	4.032		--	--	--	+ 0.066	- 2.646	- 2.58

C3. Details of Recurring Expenditure:

(Rupees figures given in SE are shown in Lakhs)

S. No.	Budget Heads	Unspent balance carried forward from previous financial year **2013-2014**	Grants received from DBT during the year **2014-2015**	Details of other receipts/interest earned on the DBT grants	Total grant available **(Sum of Column 3+4+5)**	**Expenditure** incurred during the financial year, **2014-2015 (3rd year grant Due from DBT)**	Balance amount available **(Column 6-7)**	Remarks (If any)
1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**
i. Non-Recurring								
1.	Equipments	0.33724	--	--	0.33724	--	0.33724	
ii. Recurring								
2.	Human Resource (Manpower)	0.066	--	--	0.066	2.646	0.066	
3.	Consumables	0.00003	--	--	0.00003	3.0	0.00003	
4.	Travel	0.00163	--	--	0.00163	0.43131	0.00163	
5.	Contingency	0.00080	--	--	0.00080	0.5	0.00080	
6.	Overheads (if applicable)	--	--	--	--	1.0	--	
7.	Other grant released (if any)	--	--	--	--	--	--	
8.	Details of other receipts/interest earned on the DBT grants	--	--	0.80500	0.80500	--	0.80500	
	Grand Total	**0.4057**	**--**	**0.80500**	**1.21070**	**7.57731**	**1.21070**	

Expenditure incurred during the financial year, 2014-2015 = (-) 7.57731

Balance available shall be adjusted in the further grant release (3rd Year – Financial year 2014 to 2015) = (+) 1.21070

Due from DBT (3rd year grant - Financial year 2014 to 2015) = (-) **6.36661 Lakhs**

C4. Financial Requirements for the Next Year with Justifications (If applicable):

Due from DBT Financial year 2014 -2015 (3rd year grant)

		Rupees in Lakhs
1. Human Resource	:	2.58000
2. Consumables	:	2.99997
3. Travel	:	0.42968
4. Contingency	:	0.49920
5. Overheads	:	1.00000
Total	**:**	**6.36661**

(ALL RUPEE FIGURES SHOULD BE GIVEN IN LAKHS)

Budget Heads	**SANCTIONED OUTLAY**	**Releases made by DBT**				**Expenditure as per Statement of Expenditure (s)**				**BALACNE (Column 6-10)**	**3rd Year 1.4.14 - 29.12.14 Expenditure**	**Due from DBT Financial year 2014 -2015 (3rd year grant) (11-12)**
		Details in Financial Year wise manner				**Details in financial year wise manned**						
		1st	**2nd**	**3rd**	**TOTAL**	**1st (1.4.12 - 31.3.13)**	**2nd (1.4.13 - 31.3.14)**	**3rd**	**TOTAL**			
1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**	**13**
Equipment	**12.23034**	12.23034	--	--	**12.23034**	11.8931	--	--	**11.8931**	0.33724	--	+ 0.33724
Manpower	**10.944**	3.456	2.73	--	**6.186**	3.072	3.048	--	**6.12**	0.066	2.646	- 2.58
Consumables	**9.0**	3.0	3.0	--	**6.0**	2.99997	3.0	--	**5.99997**	0.00003	3.0	- 2.99997
Travel	**1.5**	0.5	0.5	--	**1.0**	0.49837	0.5	--	**0.99837**	0.00163	0.43131	- 0.42968
Contingency	**1.5**	0.5	0.5	--	**1.0**	0.49920	0.5	--	**0.99920**	0.0008	0.5	- 0.4992
Overhead	**3.0**	1.0	1.0	--	**2.0**	1.0	1.0	--	**2.0**	--	1.0	- 1.0
Other Cost, if any	--	--	--	--	--	--	--	--	--	--	--	--
Interest earned/ any other receipt	--	--	--	--	--	--	--	--	--	0.805	--	+ 0.805
Total	**38.17434**	**20.68634**	**7.73**	--	**28.41634**	**19.96264**	**8.048**	--	**28.01064**	**1.21070**	**7.57731**	**- 6.36661**

[Signature(s) of all Project Coordinator/ Principal Investigator/Co-PI/Co-Investigator of the project]