MOLECULAR BILOGY AND GENETIC ENGINEERING

The section is working with three major objectives viz. Sugarcane improvement through transgenic approach, genes and promoter discovery and DNA marker technology and molecular breeding.

The following is the brief account of the work done in these areas during the period under report.

Group-I: Sugarcane improvement through transgenic approach

Agrobacterium mediated and biolistic methods of transformation for borer resistance and drought tolerance

Axillary buds and callus rose from young leaf sheath rolls and immature inflorescence from CoC671, Co86032 & CoVSI9805 were used in transformation studies. Transformation methods like Biolistic and *Agrobacterium* mediated were employed. *Agrobacterium* strain EHA105 having *Cry*1*Aa*3 with CaMV promoter and Kanamycin as selectable marker (*npt*II) for developing early shoot borer and internode borer resistance and *NDPK2* gene with hygromycin resistance as selectable marker for development of drought tolerance were used in this study. Prior to *Agrobacterium* infection, tissue was bombarded with tungsten particle without gene to injure the tissue and then it was infected with *Agro*-suspension. *Agro*-disinfected tissue was sub-cultured for direct regeneration. After transformation, cell lines were selected in the presence of suitable selectable marker for transformation events. Assessment was done for these putative transgenic sugarcane lines in T_0 and T_1 generations for expression of the target genes by various methods. The results of the T_0 generation has been reported in the last annual report of 2008-09.

T1 generation

All the seven plants for *Cry*1*Aa*3 and six plants for *NDPK*2 that were found positive by PCR in T_0 were taken for further generation. 125 plants from *Cry*1*Aa*3 and 99 plants from *NDPK*2 were generated by set planting in poly bags. These plants were further analyzed by PCR and ELISA.

Molecular analysis of T_1 plants

All the plants in T_1 were subjected to PCR and ELISA tests. Expression of foreign protein was found very low as compare with the T_0 generation and this needs to be further characterized.

Dipstick Analysis

Dipstick (X-press strip) analysis for the all the putative transgenic plants was carried out using Desigen Diagnostics, Mahyco, Jalna dipsticks. But none of the sample were positive for Cry1Aa3 protein may be the level of the protein is less than the detectable level.

Insect bio-assay

Insect larvae of *Chilo infuscatellus* were collected from the sugarcane fields. The larvae collected were in different developmental stages. The youngest larvae (around second instar larvae) were taken for infestation to sugarcane plants (untransformed as control plants) in green house. One larva per plant was release for infestation. Next day observed for infestation. None of the plants exhibited resistance, however in 30% of the plants observed delay of 4-5 days in formation of dead hearts.

Development of chloroplast transformation system in sugarcane

Chloroplast transformation was initiated in order to overcome the problems associated with nuclear transformation, where site-specific integration and high level of expression can be obtained. Optimization of tissue culture conditions and selection procedures, antibiotic sensitivity tests for Streptomycin (250-1000 mg/L) and Geneticin (25-100 mg/L) tested for chloroplast transformation in CoC 671 using leaf roll discs as explant. Transformation of organogenic and embryogenic callus of CoC 671 with the chloroplast transformation vectors pZE27 (*aadA*) (Streptomycin) and pZE29 (*nptII*) (Geneticin) were carried out and transformed calli were shifted to selection medium. Selection of chloroplast transformation cell lines and regeneration of transplastomic plants and subsequent molecular analysis of transplastomic plants by PCR using primers for *aadA* gene is in the process.

Around 60% lethality of the leaf discs were observed at 25 mg/L (Geneticin) and 250 mg/L (Streptomycin) concentrations. Most of the regenerating plants growing on streptomycin gradually turned into albino plants. Four plants out of 12 tested by PCR exhibited positive for *aadA* gene. Out of 11 plants tested, none of them were positive for *npt*II gene.

Group II: Genes and promoter discovery

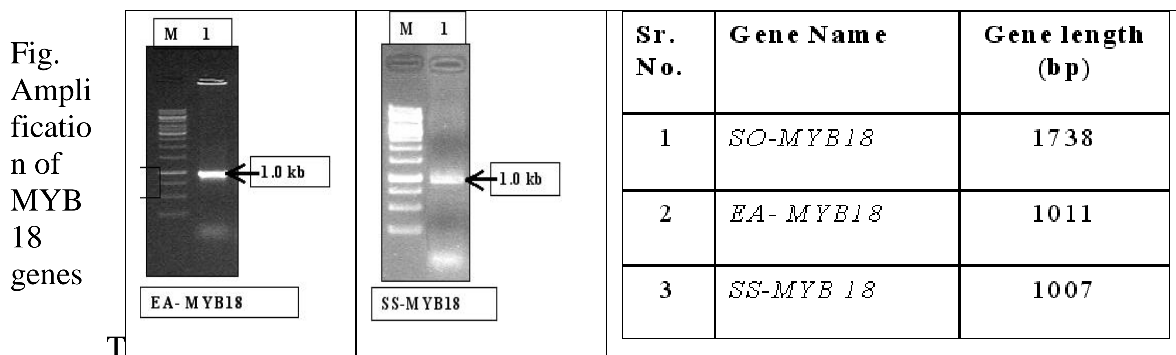
Sugarcane Grassy Shoot Phytoplasma Genome Analysis.

Approximately 60% genome of Sugarcane Grassy Shoot (SCGS) Phytoplasma, the first Asiatic phytoplasma from '*Ca.* P. oryzae' group was isolated and analysed by genomic SSH approach. The partial SCGS genome represented altogether 50,692 nucleotides, , comprising approximately 400 predicted ORFs. Initial comparative genome analysis with AY-WB and OY-M genome showed that it contains 67 rRNAs, 13 tRNAs, four RNaseP and 322 predicted ORFs with a minimal size of 30 amino acid residues. The 48% SCGS genes have not shown significant match, are potential species-specific genes and are being analyzed further towards deciphering the molecular basis of virulence.

For understanding the host-pathogen relationship and studying the mechanisms through which sugarcane perceives and responds to this biotic stress. the cDNA-SSH approach implemented. This allowed us to explicate the transcriptional regulatory mechanisms of sugarcane in response to SCGS infection. Under this *SoMYB*18 gene belonging to *R2R3-MYB* protein family was from *S. officinarum* hybrid. These MYB genes are involved in plant-specific regulatory processes and play important roles in the regulation of secondary metabolism, cellular morphogenesis, cell cycle signal transduction, SCGS infection and other environmental factors. Therefore these are being studied in detail and these will be available for developing SCGS resistant transgenic sugarcane in near future.

Sugarcane Genomics for Abiotic Stress Tolerance

Suppression subtractive hybridization (SSH) and RACE-PCR approaches were attempted to elucidate the transcriptional regulatory mechanisms and to isolate the target genes from sugarcane during water stress condition. The genome walker approach was used for isolating the promoters from sugarcane. Nearly 181 water stress responsive cDNAs were identified and their functional categorization was done, of which one *MYBAS1* gene was isolated, cloned in pET vector and protein studies were in progress. One sugarcane multiple stress inducible promoter was isolated and was being studied for its usefulness in transgenic technology**.** MYB genes are known for their role in the control of plant-specific processes and response to the changing environments. *MYB*18 genes were isolated from wild relative species of sugarcane such as *Erianthus*, *(EA-MYB* 18*)* and *Saccharum spontenum, (SS-MYB*18) and their expression studies were being carried out.



Sr. No.	Gene Name	Gene length (bp)
1	*SO-MYB18*	1738
2	*EA- MYB18*	1011
3	*SS-MYB 18*	1007

Fig. Amplification of MYB 18 genes

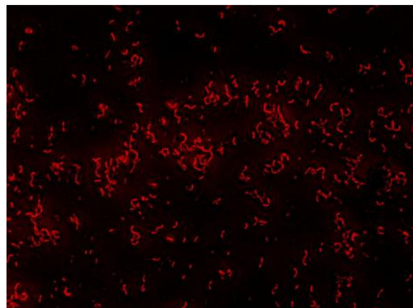
Towards assessing the sugarcane response to salinity stress cDNA-SSH libraries of leaf, root and recovered plants were being constructed. After analysis of all three libraries a thorough comparative analysis will be carried out. Further important salt stress inducible genes will be isolated and candidate genes markers will be developed which can be further used for evaluating available germplasm.

Successful elucidation of pathway has been worked out in sugarcane genomics for abiotic stress induced during water-deficit and salinity stress alleviation in sugarcane. Isolated three transcription factor genes and one stress inducible promoter from sugarcane and it's wild relative species which will be useful in transgenic technology and IPR related issues.

Characterization of genes from *Bacillus* spp. isolated from sugarcane endo-rhizosphere for salinity and pH tolerance

For confirmation of presence of PHA granules in *Bacillus sp.*, Nile blue staining of the same culture was done. *E. coli* clones having *Bacillus* plasmid genes were sequenced, BLAST analyzed and submitted to the NCBI and the accession numbers were obtained. Effects of varied stress conditions like 3%, 5% and 7% each of $MgCl_2$, $CaCl_2$, KCl, KH_2PO_4 and NaH_2PO_4; 4.5, 5.5 and 9pH were checked on the growth of these *E. coli* clones.

Nile blue staining of the culture in LB with 7% NaCl proved that *Bacillus* does produce PHA under stress conditions **(Fig1).** BLAST analysis showed the presence of PHA synthase III gene in one of the clones. Clones RSCl028, RSCl030, RSCl031, RSCl032, RSCl044 and RSCl045 showed growth under all the above-mentioned stress conditions.



scence microscopic photo showing PHA positive *Bacillus spp.*

Characterization of Fructosyl-transferases (FTFs) for their utilization for development of byproducts from sugarcane wastes.

The deduced full length sequence was analyzed using Bioedit software and NCBI database. The sequence revealed 97% similarity with bacterial FTF (also known as levansucrase) encoding sacB gene of *Bacillus subtilus* (X02730.1) and complete glyco_hydro_68 protein domain specific for FTF family proteins. On functional characterization of the enzyme, the enzyme exhibited pH optima of 7 at 37 ^{o}C and temperature optima at 37 & 40 ^{o}C. Effect of important metal ions on enzyme activity was analyzed. It was seen that addition of Ca ions enhanced enzyme activity. The total enzyme activity was restored by ~ 81% after EDTA treatment in presence of Ca^{+2} ions. Na^{+} ions restored ~ 44% of the total enzyme activity after EDTA treatment. Mn^{+2} reduced enzyme activity by ~61% where as Na^{+}; K^{+} & Mg^{+2} reduced enzyme activity by ~24%. EDTA treatment blocked enzyme activity completely. 5mM Fe^{+3}, Hg^{+2} & Zn^{+2} ions inhibited enzyme activity completely. The enzyme activity increased with increase in sucrose concentration linearly up to 200mM and thereafter remained constant. It was seen that the enzyme was most stable at pH 7 in the absence of substrate, where as its activity reduces at higher pH. Hence, these factors of enzyme can be utilized for fructan production on relatively large scale using sucrose as substrate. Enzyme activity utilizing various sucrose-rich sugarcane wastes for efficient production of fructan will be further studied.

Group III: DNA marker technology and molecular breeding

Molecular analysis of cytoplasmic genome diversity

The PCR amplification of the chloroplast and mitochondrial genomes of sugarcane with universal mitochondrial primers and gene-specific primers for chloroplast and mitochondria

yielded PCR product of same size. No diversity could be revealed so far with the use of available set of primers.

Two sets of hybrids were selected wherein the female parent was *Saccharum officinarum* (2N = 110) and the male parent was *Erianthus* (2N = 60). The expected number in the F1 hybrids was 55 + 30 = 80. But the chromosome number observed in the progeny was 60. Also, progeny resembled the male parent (*Erianthus*) in its morphological characteristics and banding pattern.

In vitro **Mutagenesis for Genetic Improvement of salinity tolerance in sugarcane varieties Co 86032, Co 740 and CoVSI 9805**

Embryogenic calli were subjected to gamma irradiation (0, 10, 20, 30, 40, 50, 60, 70 and 80 Gy) and grown in *in vitro* condition for regeneration under different levels NaCl concentrations (50, 100, 150, 200, 250 and 300 mM). Under preliminary study of different doses of the radioactivity to the embryogenic calli, it was found that, 20-30 Gy for Co86032 and 40-50 Gy for Co740 were observed 50% lethality. From the initial screening, around 45 plants are found promising with their parents in terms of the brix percentage, girth and number of canes. These plants were subjected to rod-row trial in the field for further evaluation.

Developing molecular markers for sugar related traits and utilization of MAS for identification of high sucrose genetic stalk

Sugarcane varieties having high and low sucrose contents at the maturity level were selected. PCR amplification of genomic DNA was carried out using EST based microsatellite or EST-SSR. Out of 30 primers tested, 23 showed amplification. 21 primers showed monomorphic banding pattern and rest two exhibited polymorphism.

Persons visit to abroad

- Dr. R. M. Devarumath working from 28th Sept. 2009 to 22nd June 2010 as visiting fellow on deputation for nine months under the guidance of Dr. Robert Henry, Director and Dr. Peter Bundock, Centre for Plant Conservation Genetics, Southern Cross University, Lismore, 2480 NSW, Australia. The Overseas Associateship for Specialized Training of YOUNG SCIENTISTS IN NICHE AREAS OF BIOTECHNOLOGY: for a period of nine months (Funding from: Department of Biotechnology, Govt. of India, New Delhi)
- Ms. Madhuri C. Pagaria, Kulkarnai P.A., Prabu G.R., Devarumath R.M. and Kawar P.G. Transcriptomic identification of candidate genes involved in sugarcane responses to salt stress based on cDNA - SSH analysis. Plant and Animal Genome (PAG) XVIII Conference held at San Diego, California, USA, 09-13 January 2010,P04.
- Viniti Vaidya and D. Theertha Prasad (2010). Molecular evaluation of bacterial fructosyltransferase for production of fructan: a low calorie sweetener**.** Proceedings of 6th

International conference on ‘Functional foods for chronic diseases: Diabetes and Related Diseases’ 33, 118-120.

- Edkie G.N. and Prasad D.T. (2009). Evaluation of salinity inducible PHA genes from *Bacillus* spp. Proceedings of 6th International conference: ‘Functional Foods for Chronic diseases’ at Texas Women’s University (USA), December 4-6, 2009. 15, 67-69.

Publications

- Kawar, P.G., Devarumath, R.M., Nerkar, Y.S. (2009). Use of RAPD markers for assessment of genetic diversity in sugarcane cultivars. Indian Journal of Biotechnology 8: 67-71.
- Raviraj M Kalunke, Archana M. Kolge, K Harinath Babu and D Theertha Prasad (2009). *Agrobacterium* mediated transformation of sugarcane for borer resistance using *Cry 1Aa*3 gene and one-step regeneration of transgenic plants. Sugar Tech 11(4): 355-359.
- Sehgal D, Raina SN, Devarumath RM, Sasanuma T, Sasakuma T, (2009) Nuclear DNA assay in solving issues related to ancestry of the domesticated diploid safflower (*Carthamus tinctorius* L.) and the polyploid (*Carthamus*) taxa, and phylogenetic and genomic relationships in the genus *Carthamus* L. (Asteraceae). Molecular Phylogeny and Evolution 53(3): 631-644
- Vaze a, Nerkar G., Pagariya M., Devarumath RM and Theertha Prasad (2010) Isolation and PCR amplification of Genomic DNA from Dry leaf samples of sugarcane. International Journal of Pharmaceutical and Bio Science V1 (2010).

D. Sequences Submitted to Genbank Database

- Kawar, P.G., Pagariya, M.C., Prabu,G.R., Kulkarni, P.A., Dixit, G.B. and Theertha Prasad, D. Response of sugarcane genes to Sugarcane Grassy Shoot Phytoplasma infection. Submitted 65 ESTs; Accession numbers: Year- 2010 GW316416 to GW316480
- Kawar, P.G., Prabu, G.R., Pagariya, M.C., Dixit, G.B. and Theertha Prasad, D. Identification and isolation of SCGS phytoplasma gene specific fragments by ribotyping. Submitted 04 SCGS ESTs; Accession numbers: Year- 2010 GW316412 to GW316415
- Kawar, P.G., Pagariya, M.C., Prabu, G.R., Dixit, G.B. and Theertha Prasad, D**.** Sugarcane Grassy Shoot phytoplasma geneome analysis by SSH approach
  Submitted 17 sugarcane genomic fragments; Accession numbers: Year- 2010 GS887677 to GS887693
- Pagariya, M.C., Kulkarni, P.A., Prabu, G.R., Devarumath, R.M. and Kawar,P.G. Sugarcane genes expressed in response to salt stress. Submitted 51 ESTs; Accession numbers: Year- 2009 GT982643 to GT982693
- Kawar, P.G., Pagariya, M.C., Prabu, G.R., Dixit, G.B. and Theertha Prasad D. Sugarcane Grassy Shoot Phytoplasma genome analysis by SSH approach. Submitted 83 SCGS genomic fragments; Accession numbers: Year- 2009 GS635186 to GS635265 and GS883114 to GS883116.
- Vaidya, V., Kawar, P. and Prasad, D.T**.** Bacillus subtilus levansucrase gene complete cds. Accession No. Year-2009 FN599519.
- Gargi E.N., Dharade K., Prasad D.T. RFLP of Bacillus subtilis BSp1 plasmid. Accession number: Year 2010 GS887704 – GS887749.

Project proposals submitted for funding/ sanctioned

- Project proposal entitled 'Induced Mutagenesis: Selection for Salinity tolerance and molecular characterization of tolerant lines in sugarcane', sanctioned by DAE-BRNS (BARC), Mumbai (BRNS Letter No.2009/37/51/BRNS/3306, Dt. 04.03.10). Total project amount is Rs.17.28 Lakhs for 3 years.
- Submitted the revised project proposal entitled "Sugarcane Improvement: Conventional breeding and Biotechnology approaches", to Sugar Development Funds, Govt. of India. Rs. 2.85 crores. Dr. D. Theertha Prasad, Principal Investigator, Co-investigators: Dr. K. Harinath Babu, Dr. R. M. Devarumath, Dr. R. S. Hapase and Mr. Prashant Kawar
- Submitted project proposal entitled 'Development of Chloroplast transformation system in sugarcane' to DBT, New Delhi for funding. Total estimated cost of the project is Rs.41,99,800/- for three years. PI: Dr. K. Harinath Babu, Co-PI: Dr. V. S. Ghole and Mr. Prashanth Kawar.

Meetings

- 12th Institutional Bio-Safety Committee (IBSC) meeting was held on 14th December 2009 in the Molecular Biology and Genetic Engineering Laboratory of VSI.
- Eighth Research Advisory Committee (RAC) meeting of the MB & GE of VSI was held on March 13, 2010 in the MB & GE lab, VSI
- The International Consortium for Sugarcane Biotechnology (ICSB) meeting was held during 16-17 November 2009.