Chapter 5: Summary
SUMMARY

Pennisetum is comparatively a hardy drought tolerant crop which usually grows at higher temperature with respect to other cereals and requires very low level of water. There is a high genetic similarity among various cereal genomes. Isolation and characterization of Pennisetum genes involved in various stress adaptation process will facilitate understanding their probable roles and transformation of its stress related genes to other crop plants can be used to make them stress tolerant. With this aim we started our experiments in identifying various differentially regulated genes under different abiotic stress conditions.

The work involved in this study can be summarized into following points.

+ With the aim of generating a differentially up-regulated EST data base for Pennisetum plant under different abiotic stress conditions, we constructed three subtracted shoot libraries under salt (NaCl), drought, low temperature (4°C) conditions and one normal root library. In this work we developed a novel approach for constructing subtracted cDNA libraries under different abiotic stress conditions. This strategy can be used in any other plant or animal systems to isolate differentially up-regulated or down regulated genes under any set of experimental condition.

+ For sequencing, ESTs clones were picked up from primary unamplified libraries to avoid the post amplification duplication for picking up the redundant clones. Random PCR amplification of the recombinant clones showed a good representation of cDNA inserts with sizes up to 3 kb with an average sizes more than 600 bp. Approximately 3000 recombinant clones were picked up and the cDNA inserts were sequenced with an average success rate of more than 80%. After discarding the bad quality sequences, we were able to submit 2494 EST clones to the databank (GenBank_Accn# CD724312 to CD726805). To our knowledge this is the largest number of ESTs submitted in the database for this plant and epitomize a substantial amount of ESTs isolated under abiotic stress conditions in different crop plants.
The subtracted strategy used here is biased towards isolation of differentially up-regulated genes. Our EST database also represents high enrichment of previously reported stress regulated genes and low level of house keeping genes. We did functional annotation of all the ESTs based on similarity with previously submitted nucleic acid and protein sequence in the data base by using BLAST 'X' Program from NCBI. Although these large groups of genes can be classified in many ways, we classified them into different group based on their probable functions. Functional classification showed wide range of proteins belonging to transcription factors, kinases, redox mechanisms, proteins involved in DNA damage and repair pathways and those involved in cellular growth metabolism. We also found a wide range of proteins of putative and unknown function, whose characterization will shed new light on stress tolerance mechanisms.

We have prepared cDNA microarray for Pennisetum by printing all the recombinant clones on to the glass slides. We performed microarray experiment under drought, salt, cold and high temperature conditions at an early and late time points to get a good representation of the genes that might be up regulating at different periods after stress treatments. Very stringent conditions were applied during normalization and data analysis procedures. The expression level of more than 1000 genes found to be up-regulated under these conditions.

However, we took only top 100 genes in each category for further analysis. Functional classification shows high up-regulation of different group of genes like those involved in (1) growth, metabolism, and various transport mechanisms (2) genes that modulate the action of other genes during various stress signal pathways like transcription factor, hormones and various calcium related proteins (3) genes whose products are involved in stabilizing and turn-over of various cellular macromolecules and several genes of unknown functions.

While analysing it was found nine genes are getting up-regulated under both salt and heat stress, five genes between heat and cold stress, twenty genes between salt and cold stress and four genes under salt, cold and heat stress conditions. This implies a significant cross talk among the above stress conditions to alleviate the stress induced damages. Detail characterization of these genes will clarify their
roles not only on the stress regulated mechanisms of this plant but also for other cereals.

- We found a wide range of cDNA clones representing different heat shock proteins and heat shock factors in our subtracted libraries. To check their detail expression profiles, real time RT-PCR analysis were done under all abiotic stress conditions like drought, salt, heat, cold, ABA and SA treatments at multiple time points. The real time data clearly shows the complex pattern of expression of these genes under these stress conditions. It was found that not only these Hsps are up-regulated under heat stress as according to their previous notion but it was found that the transcript level of almost all of them increased significantly under salt, drought and SA treatment. However, it was found that the transcript level of most of them was down regulated or remained unchanged during cold treatment.

- One of the cytoplasmic Hsp70 (CD726670) and Hsp82 (CD725542) was found to up-regulated significantly in all most all stress conditions except cold stress, making it a good candidate to make transgenic stress tolerant crop pants. Similarly, we found a single heat shock factor (CD725302) in all our differential libraries. This heat shock transcription factor was found to be up-regulated notably under different stress conditions emphasizing its importance in gene regulation during above stress conditions.

- We transferred *Pg* Hsp17.9 to *Arabidopsis* and raised transgenic plant. Preliminary analysis shows that *P. glaucum* Hsp17.9 was able to give tolerance against heat stress at the germination stage but not at the latter stage. It was found that after treating the seeds at 37°C for 72 hours more than 95% of wild type seeds were not able to germinate whereas transgenic plants more than 80% seeds were able to germinate. This implies its importance against heat stress conditions during germination stage. In microarray and real time analysis this gene was also found to be up-regulated during drought and salt treatments. Further detailed analysis requires to assess the probable role of *P. glaucum* Hsp17.9 under these stress conditions to establish its probable role.
We isolated full length cDNA clones of heat shock proteins by 5' and 3' RACE. Out of eight Hsps and one Hsf we have obtained full length cDNA clone for six Hsps and one Hsf (Hsp10, Hsp17.9, two Hsp70s, Hsp82, Hsp90 and the only heat shock factor). Nucleotide and deduced amino acid sequence analysis showed high homology of these Hsps with other plant homologs and most of them showed maximum homology to corresponding rice homolog than any other plant.

For further characterization, we expressed four recombinant P. glaucum Hsps (Hsp17.9, two Hsp70, Hsp82) and the P. glaucum Hsf in E. coli and purified the proteins to near homogeneity.

We checked chaperonic activity of P. glaucum recombinant heat shock proteins. They were able to prevent the thermal aggregation of citrate synthase under in vitro conditions. It was found that both the cytoplasmic Hsp70, Hsp82 and Hsp17.9 were able to prevent thermal aggregation of citrate synthase efficiently. It was also found that the prevention of thermal aggregation is dose dependent, however, we did not find any significant difference in their activity.