CHAPTER : 6 DISCUSSION

The significant loss in crop productivity in response to abiotic stress forms the principal rationale of the present research, which is based on hypothesis that the drought resistant genes from *Pennesitum glaucum* could lead to the development hybrid crops with enhanced productivity under drought conditions .When plant experiences environmental stresses such as drought, salinity, temperature, cold etc, they activate a diverse set of physiological, metabolic and defense processes to survive and sustain the growth. Tolerance and susceptibility to these stresses are very complex. Water deficit is a factor which limits plant quality and sustenance. Therefore drought tolerance plant (*Pennisetum glaucum*) requires less water for maintenance and the growth. Drought tolerance or susceptibility is a mutagenic trait and is therefore difficult to control. Transcriptomics, proteomics, subtractive hybridization and gene expression approaches are commonly used to identify the activation and regulation of several stress-related transcripts and proteins, which are generally classified into two major groups. One group is involved in signaling cascades and in transcriptional control, whereas members of the other group operate for the membrane protection, as osmoprotectants, as antioxidants and as ROS scavengers (Shinozaki and Yamaguchi-Shinozaki, 1997).

Subtractive hybridization is a powerful technique that enables researchers to compare two populations of mRNA and obtain clones of genes that are expressed in one population but not in the other. In the present study, a drought stress responsive subtractive cDNA library was constructed from 22 days old pearl millet seedlings (*Pennisetum glaucum* cv. PPMI741and breeding line (652-657)) subjected to drought stress at room temperature by 30% PEG 6000 for different time period (30 min , 2hr, 4hr, 8hr, 16hr, 24 hr and 48hr). Plants with high capacity for water retention can better survive drought stress. During 0 to 48 hours of a 30% PEG600 treatment, *Pennesitum glaucum* PPMI741 samples were observed to have high relative water content (RWC) and showed a low water loss rate (WLR). RWC values indicated the drought tolerance ability of pearl millet which decreased with the increasing period of drought stress exposure

Water deficit condition is induced by different methods and addition of PEG media has been observed to be very effective. Molecules of PEG of 6000 and above molecular

weight cannot enter the pore of plant cell (Oertli, 1985) and thus causes cytolysis rather than plasmolysis. Therefore, PEG is suitable solute for imposing low water stress conditions and this is analogous to the water condition in soil. Our study shows that pearl millet seedling had highest RWC (95.76%) in control (no water stress condition) and gradually with increasing duration of low water stress with conditions, RWC decreased with lowest (56.2%) at 48 hr of drought induction. However, it was observed that at 8 hr of low water stress condition, RWC of seedlings dropped to as low (48.6%).It is quite possible that plant system tried to rebound back to water deficit stress and thus RWC values increased after an early stage of water stress condition (8hrs).

This result was correlated by the presence of many genes involved in reactive oxygen species (ROS) detoxification in the subtracted cDNA library. These include genes that code for various proteins including glutathione peroxidase (EST ID: Plate 6F_B5_Pg_SSH), Adenylate kinases proteins (EST ID: Plate_6F_O3_ Pg_SSH), citrate synthase(EST ID: Plate_7F_O7_ Pg_SSH) and chloroplast 30S ribosomal protein S7(EST ID: Contige_33_ Pg_SSH).A direct result of stress-induced cellular changes is the enhanced accumulation of toxic compounds in cells that include reactive oxygen species (ROS). Enzymes like glutathione peroxidase have been shown to be involved in ROS scavenging pathways in plants which reduce the amount of ROS produced due to oxidative stress linked to drought and related conditions. Transgenic plants over-expressing glutathione peroxidase were found to be more tolerant than wild-type plants to a combination of drought stress (Yoshimura *et al.,* 2004).Adenylate kinases proteins (EST ID: Plate_1F_O3_ Pg_SSH) have been found to be involved in signal transduction and the regulation of gene expression. It is probable that these proteins play a regulatory role in the plant drought stress response. Usually such proteins include phosphatases (EST ID: Plate_6F_G2_ Pg_SSH), transcription factors, and enzymes in phospholipid metabolism (EST ID: Plate_7F_E12_ Pg_SSH) (Thomashow,1998; Park *et al*., 2001; Seki *et al*., 2001; Singh *et al*.,2002; Shinozaki *et al*., 2003).

Genes encoding amino transferase protein (EST ID: Plate_3F_G11_ Pg_SSH) was one of the genes obtained in our SSH library. It is known to be functionally expressed in protein modification process. It is also reported that ten differentially expressed phospho-proteins are functionally active in rice leaves in response to heat

stress. Heat stress induced the dephosphorylation of RuBisCo and the phosphorylation of ATP-b, decreasing the activities of RuBisCo and ATP synthase. ATP-citrate synthase (EST ID: Plate_contgi_50_ Pg_SSH) is a key enzyme in the acetyl coenzyme A (CoA) pathway (Hynes and Murray, 2010) and catalyzes the ATP-dependent reaction of citrate and CoA forming acetyl-CoA and oxaloacetic acid (Fatland et al., 2005). Glutamine synthetase (EST ID: Plate_9F_G10_ Pg_SSH) ⁄ glutamate synthase is involved in GS ⁄GOGAT cycle, actively performed in the conversion of a-ketoglutarate into glutamate by the action of glutamate dehydrogenase (GDH). Although an increase in the aminating GDH activity has been detected in salt-stressed rice roots and wheat leaves (Lutts *et al*., 1999; Wang *et al*., 2007; Brugiere *et al*. 1999) pointing towards a central role for cytosol GS in the biosynthesis of Proline via a constant pool glutamate under stress conditions.

RAD23 DNA repair proteins (EST ID: Plate_5F_B11_ Pg_SSH) was another contigs observed in our SSH library. RAD23 has been observed to be expressed under heat and drought stress conditions. It has also been reported that gene RAD23 had tissue specific expression in arabidopsis as observed by RT –PCR analysis in different tissues of rosette leaves, roots, bolt stems, cauline leaves, unopened flower buds, and mature opened flowers (Zhang,P.G. *et al*.,2010).

The Zinc finger protein (EST ID: Plate_6F_E11_ Pg_SSH) observed in our library is a C3HC4-type RING finger whose expression was found to be induced by drought. This protein has been reported to have crucial roles in the growth, differentiation, transcription, signal transduction and on cogenesis (Reddy *et al.,* 1992) and also involved in the ubiquitin-mediated protein degradation pathway (Lorick*et al.*, 1999). Hsp70 proteins(EST ID: Plate_contegi_45_ Pg_FSSH) are the predominant forms of chaperones expressed under high temperature stress conditions: Hsp70 chaperones, along with their co-chaperones (e.g. DnaJ/Hsp40 and GrpE)make up cellular machines that interact with a wide range of proteins in almost all cellular compartments. *Arabidopsis* genome contains at least 18 genes encoding members of this family, of which 14 belong to the DnaK subfamily and four to the Hsp110/SSE subfamily (Wang *et al.,*2004). Presence of *Hsp* 70 in our library further confirms the role of this gene in drought stress response. The metallothionein(EST ID: Plate_8F_F2_ Pg_SSH) genes respond not only to heavy metals but to many biotic and abiotic stress factors, so their

gene products should be considered as general stress proteins (Hassinen *et al*.,2011). The role of metallothionein in ROS scavenging in *G. hirsutum* in response to drought, results in the over expression of this metallothionein, which consequently increased tolerance to drought through reduction of the hydrogen peroxide level. Brosche *et.al*.,(2005) observed a high level of metallothion in expression in *Populus* trees growing in dry areas. Berta *et al*., (2009) found that transcription of metallothionein in leaves and the cambial zone of *Populus alba* depended on changes in water status and suggested the involvement of metallothionein in protection of plant cells during low water stress condition. Metallothionein was also observed to be expressed in our SSH library.

Members of the SR protein gene family play a significant role in the regulation of alternative splicing, an important means of generating proteome diversity and regulating gene expression (Duque, 2011). A gene homologous to the serine/arginine rich (SR) (EST ID: Plate_contegi_26_ Pg_FSSH) protein called SC 35 which has a RNA recognition motif was also identified in the library. Plant glycine-rich, RNA-binding proteins are small, approximately 16–17 kDa .The gene of the drought inducible glycine-rich RNA-binding protein (EST ID: Plate_2F_D9_ Pg_SSH) (GR-RBP) was isolated and characterized to determine its role in the response of apple (*Malus prunifolia*) seedlings to drought stress using leaves from plants under water stress (Wang *et al.,*2011).Its presence in the differentially expressed library indicates its prime role in maintaining the efficient functioning of photosynthesis during drought stress in pearl millet. Other proteins which help in maintaining photosynthetic activity were identified in the library. Some of these like the P700 chlorophyll apoprotein A2 (EST ID: Contige _6_Pg_FSSH), photosystem II D2 protein (EST ID: Plate_7F_G6_Pg_FSSH), Photosystem II 44 kDa reaction center protein (EST ID: 5F_B2_Pg_FSSH) are part of the photosynthetic apparatus and may be produced in the drought stress.

The ESTs also showed presence of a few novel and uncharacterized genes with no homology matches to any of the major public databases. Their role in drought stress can be investigated in further studies. These results suggest that representation of housekeeping and non-target genes were substantially reduced while at the same time differentially up-regulated stress responsive genes were enriched in our drought stress responsive *Pennesitum glaucum* EST collection.

Relative gene expression quantification by quantitative RT-PCR confirmed that the SSH library contained differentially expressed genes like Abscisic stress ripening protein(EST ID: Contig_12_ Pg _FSSH), Ascorbate peroxidase (EST ID: Contig_23_Pg_FSSH), Glyoxalase (EST ID: Contig_24_Pg_FSSH), Rab7(EST ID: Singlet_223_Pg_FSSH), Aspartic proteinase Oryzasin (EST ID: Contig_37_Pg_FSSH-P), Ubiquitin-Conjugating enzyme E2-7 (EST ID: Contig_27_ Pg_FSSH), DnaJ-like protein (EST ID:4F_E10_Pg_FSSH), Calmodulin-like protein (EST ID: Singlet_204_Pg_FSSH) ,Putative beta-1,3-glucanase (EST ID: Contig_53_Pg_FSSH), and Inosine-5'-monophosphate dehydrogenase(EST ID: Contig_11_Pg_FSSH). Few genes expressed in the EST library were randomly selected for differential expression analysis by qPCR in *Pennesitum glaucum* seedlings under drought stress for different time durations (30 min, 2hrs, 4hrs, 8hrs, 16 hrs 24hrs and 48 hrs).The quantitative up-regulation of the selected genes for their expression in response to drought stress clearly indicated that the subtractive cDNA libraries constructed in this study were substantially enriched for stress responsive genes.

Plant growth and productivity are adversely affected by various biotic and abiotic stress factors, while drought is one of the major abiotic stresses that adversely affect crop growth and yield (Jaleel C.A. *et al* 2009).The *Asr* gene family (Abscisic acid, Stress and Ripening), classified as a new group of Late Embryogenesis Abundant (LEA) (Caramelo *et al.,* 2008; Battaglia *et al.,* 2008), has been reported to be induced under water stress (Chang *et al.,* 1995) and involved in adaptation to dry climates (Frankel *et al.,* 2003). It is also reported to be involved in abscisic acid signalling and has been used to develop transgenic *Arabidopsis* with enhanced drought and salt tolerance. The gene was up-regulated during drought and salt stress in transgenic Arabidopsis. (Yang *et al.,* 2005).Our investigation also showed that *Asr* gene was gradually up-regulated to 10.0 fold change after 24 hr drought stress.

Ascorbate peroxidase (APX) has been found in higher plants, algae, and some cyanobacteria, but not in animals (Shigeoka et al., 2002). In higher plants, APX isozymes are distributed in at least four distinct cellular compartments: stromal APX (sAPX) and thylakoid membrane-bound APX (tAPX) in chloroplasts, microbody (including glyoxysome and peroxisome), membranebound APX (mAPX), mitochondrial membrane-

bound APX (mitAPX) and cytosolic APX (cAPX) (Kawakami *et al*., 2002). In a previous study, the effect of flooding time on the expression of APX gene in egg plant was observed. The maximal increase (7.83) was found at 48 h of flooding treatment and APX gene transcript of leaf was affected during the time course of flooding. The highest (8.48) and lowest (1.85) levels were observed at 12 h and 0 h of waterlog treatment, respectively (Kuan-Hung, 2007).In our investigation, peroxidase (ESTS ID: Contig_23-FSSH-Pg) gene was found in drought responsive library of *Pennesitum glaucum*. The transcript level was gradually increased to 5.42 fold at 24hr after drought treatment but decreased after 4hr and 48hr. The gene was not over expressed in response to drought stress at 4 hr and 48 hr interval indicating that the gene has transient expression.

Expression profiling of Calmodulin-like protein gene suggested the up-regulation up to 3.38 fold and down-regulation up to level of 0.087 fold at 2hrs and 24hrs respectively of treatment times. Previous studies have shown that Calmodulin-like protein plays positive role in plant stress tolerance. Calmodulin-like protein has been shown to regulate some stress-response genes in plants (Liu *et al.,* 1998). Expression of Calmodulin-like protein enhanced the salt and drought tolerance in transgenic rice plants (Mallikarjuna *et al.,*2011). Calmodulin-like protein gene has been isolated from various plant species and characterized for expression under abiotic stress. In rice, qRT-PCR analysis was performed to monitor the expression levels of Calmodulin under high salt stress. Expression profiling of the gene showed it to be up-regulated up to 6.0 fold and a down-regulation up to a level of 2.0 fold at 1 and 12 hrs respectively of treatment times (Guoyun *et al.,*2013). Calmodulin-like protein (ESTS ID: Singlet_204-FSSH-Pg)in our study displayed a high level of up-regulation up to 3.38 fold at 24hrs of drought stress treatment. However the gene was down regulated up to a level of 0.087 fold at 2hrs of stress. Our results showed calmodulin-like protein gene to be involved in late drought stress response in *Pennisetum glaucum.*

Proteins containing inosine-5'-monophosphate dehydrogenase (IMPDH) domain (PF00478) along with cystathionine binding synthase (CBS) domain has been classified in this subgroup. Only one CBSIMPDH gene has been identified in *Arabidopsis thaliana* and *Oriyza sativa.* In previous studies, under the condition of osmotic stress, expression of the gene was found to be up regulated in shoots of *Arabidopsis* at 1 hr and 24 hr of

osmotic stress. Under salt stress, IMPDH genes were found to be up regulated at 1, 6 and 12 hr time points in roots, while in shoots these genes were expressed more in 1 hr and 24 hr of salt stress. The analysis of expression of these genes gives an idea that CBS domain containing proteins might play an important role in drought, salt and osmotic stress response/tolerance. Oxidative stress, arising from an imbalance in the generation and removal of reactive oxygen species (ROS) such as hydrogen peroxide (H_2O_2), is a challenge faced by all aerobic organisms (Finkel and Holbrook,2000; Allen and Tresini,2000). In present research, inosine-5'-monophosphate dehydrogenase (ESTS ID: Contig_11-FSSH-Pg) showed higher expression at 2hr and 24 hr of drought stress (30%PEG6000) in whole seedling of *Pennesitum glaucum*.

The Glyoxalase pathway has been identified in a wide range of organisms including plants (Maiti *et al.,* 1997; Norton *et al.,* 1990), yeast (Bito *et al.,* 1999) and protozoa (Rhee *et al.,* 1986). Analysis of expression patterns using Gene vestigator (Zimmermann *et al*., 2004) showed that GLX2-1 transcript levels are elevated during several abiotic stresses, including: anoxia, hypoxia, drought, light, osmotic and salt. In earlier studies, GLX2-1 transcript levels increased two-fold after 24 hour recovery period following salt stress in *Brassica juncea* **(**Alam *et al*., 2013**).** Exposure of the plants to salt stress led to increase in GLX2-1 transcript levels of 15 fold increase in 24 hour (Devanathan *et al.,* 2014). These results confirm that GLX2-1 is induced during abiotic stress and suggest that it may have a role in stress tolerance. In the present study, Glyoxalase gene (ESTS ID: Contig_24-FSSH-Pg), showed higher expression level after 30 min and 2hr. However, after 2hr this gene down regulated in drought stress.

DNAJ-like protein are mostly used in model plants and crops and do not always maintain stable expression levels among different tissues, experimental conditions and species (Die *et al.,* 2010; and Zhu *et al.,*2013). Systematic validations of reference genes have mainly focused on models and important crop species such as *Arabidopsis* (Czechowski, 2005), rice (Jain *et al* 2006), wheat (Paolacci *et al*., 2009), barley (Janska *et al.,* 2013). DNAJ were the most stable genes in PEG treatment. DNAJ-like protein is known to be up regulated in heat stress but down regulate in salt stress at different time duration in *Ammopipathus mangolicus* (Yan *et al.,* 2014). In the present study, DNAJ-

like protein was up regulated gradually at 4hr, 8hr, 16hr and 24 hr but it was observed to be highly expressed at 2hr in drought stress (30% PEG treatment).

The role of *Rab7* in abiotic stress tolerance, such as salinity and drought has been validated and earlier studies have shown over expression of *AtRab7*, *PgRab7* and *PjRab7* in *A. thaliana* and tobacco under conditions of NaCl stress (Mazel and Levine,2002).The *Rab*7 proteins are important component of the vesicle trafficking system in all eukaryotes (Zerial and McBride, 2001).The role of At*Rab*7 protein in eukaryotes seems to be associated with the late endocytosis, where it functions in the fusion of late endosomes to lysosomes or vacuoles.

Plant beta -1, 3-glucanase (ESTS ID: Contig_53-FSSH-Pg) one of the typical PR proteins, can catalyze the hydrolysis of βb-1, 3-glucans, which are a major component of the cell wall of most fungi although very little has been found in higher plants so far (Stone and Clarke 1992; Leubner-Metzger and Meins, 1999).

Aspartic Proteinase Oryzasin gene (ESTS ID: Contig_37-FSSH-Pg) classified in aspartic proteinases and metallo proteinases are present at later stages expressed in salt, heat and drought stress in rice, barley and *Coffea arabica* (Dominguez and Cejudo, 1996). The said gene is expressed under drought stress in our investigation with up regulated expression at 2hr to 24 hr of drought stress (30% PEG6000) and down regulation at 30 min and 48 hr indicating that the gene is not responding during early stress conditions as well as during late stress conditions.