Construction and analysis of SSH library of early heat stress induced genes of *Vigna aconitifolia* (Jacq.) Marechal

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Abbreviations and symbols

BLAST	-	Basic Local Alignment Search Tool
B-ME	-	β-mercaptoethanol
bp	-	Base Pair
cDNA	-	Complementary DNA
CTAB	-	Cetyl Trimethyl Ammonium Bromide
°C	-	Degree centigrade
Conc.		Concentration
d	-	Day
DEPC	-	Diethyl Pyrocarbonate
dH_2O	-	Distilled water
ddH_2O	-	Double distilled water
DNA	-	Deoxyribonucleic acid
DNase	-	Deoxyribonuclease
dNTPs	-	Deoxynucleotide triphosphates
ds	-	Double stranded
EDTA	-	Ethylene Diamine Tetraacetic Acid
EST	-	Expressed Sequence Tag
EtBr	-	Ethidium Bromide
F	-	Forward
g	-	Gram
hr	-	Hour
IPTG	-	Isopropyl β-D-Thiogalactoside
kb	-	Kilo Base
LA	-	Luria Agar
LB	-	Luria Broth
mg	-	Milligram
mM	-	Milimolar
ml	-	Millilitre

M	-	Molar
MS medium	-	Murashige and Skoog (1962) medium
MAS	-	Marker Assisted Selection
min	-	Minute
MOPS	-	Morpholino Propane Sulfonic Acid
mRNA	-	Messenger RNA
NCBI	-	National Centre for Biotechnology Information
nm	-	Nano meter
OD	-	Optical density
PCR	-	Polymerase Chain Reaction
Pmol	-	Pico mole
RNA	-	Ribonucleic acid
RNase	-	Ribonuclease
rpm	-	Rotation per minute
RT-PCR	-	Reverse Transcriptase-Polymerase Chain Reaction
sec	-	Second
SNP	-	Single Nucleotide Polymorphism
ss	-	Single stranded
Taq	-	Thermus aquaticus
TBE	-	Tris-borate EDTA
TE	-	Tris-EDTA
Tm	-	Melting Temperature
Tris	-	Tris (hydroxymethyl) amino methane
µg	-	Microgram
µl	-	Microliter
V	-	Volts

1. INTRODUCTION

Abiotic stresses are the primary cause of crop losses worldwide, reducing average yield of major crop plants by more than 50 percent (Boyer, 1982; Bray *et al.,* 2000). Stress due to increased temperature is an agricultural problem in many areas in the world. Heat stress often is defined as where temperatures are hot enough for sufficient time that they cause irreversible damage to plant function or development. In addition, high temperatures can increase the rate of reproductive development, which shortens the time for photosynthesis to contribute to fruit or seed production. High day temperatures can have direct damaging effects associated with hot tissue temperatures or indirect effects associated with the plant-water-deficits that can arise due to high evaporative demands. Evaporative demand exhibits near exponential increases with increases in day-time temperatures and can result in high transpiration rates and low plant water potentials. Combining with drought stress, the elevated temperature often results in yield loss and reduces the quality of crops (Hall, 2001; Boyer, 1982; Bray *et al.,* 2000).

Plant species and genotypes vary for tolerance level to elevated temperatures during their growing period. Acquiring thermo-tolerance has been considered to be an active process by which considerable amounts of plant resources are diverted to structural and functional maintenance to escape damages caused by heat stress. Heat tolerance of plant is a complex trait most probably controlled by multiple genes. In order to cope with heat stress, plants implement various mechanisms, including maintenance of membrane stability, scavenging of ROS, production of antioxidants, accumulation and adjustment of compatible solutes, induction of mitogen-activated protein kinase (MAPK) and calcium-dependent protein kinase (CDPK) cascades, and, most importantly, chaperone signaling and transcriptional activation (Bray *et al.,* 2000; Wahid *et al.,* 2007).

Moth bean (*V.aconitifolia* (Jacq.) Marechal) is a hot-weather, drought resistant legume that grows throughout the tropic, sub-tropic and warm

temperate areas of different countries. Despite of its multiple uses for food, feed and agriculture, it is cultivated on marginal lands, under rain fed conditions mainly in the arid and semi-arid zones. It adapts to extremes or uncongenial ecological niches particularly, in areas receiving scanty rainfall with erratic distribution. Due to its harsh natural habitat it has evolved a few morphological and physiological features imparting tolerance to extreme drought and heat stress (Kumar and Singh, 1988). An inducible nature of thermo-tolerance in moth bean with genetic variations for level of inductions has been observed in our laboratory. Identification of over-expressed genes in heat tolerant genotype will help us to understand the overlying mechanism of heat tolerance.

Each cell-type within an organ or tissue is defined by a specific transcriptional, protein, and metabolic profile that determines its function and response to stress, and molecular role of various transcription factors such as drought responsive element binding protein (DREB), late embryogenesis abundant protein (LEA), heat shock factors (HSF), apetela/ethylene responsive factor (AP2/ERF) etc., has been well characterized by now; yet we have not succeeded in the development of an abiotic stress tolerant variety; suggesting that still many signaling molecules and transcription factors imparting heat tolerance needs to be identified.

Functional genomics approaches have been used in recent years to understand the stress responsive mechanism in plants. Candidate genes involved in heat tolerance mechanism have been identified, characterized, and assessed for their comparative transcriptional activity by using whole genome sequencing or expressed sequence tag (EST) libraries; in many plant species but not in moth bean. Suppression subtractive hybridization (SSH) technique is based on selective amplification of differentially

expressed sequences, hence overcomes the technical limitations of the traditional subtraction methods i.e. requirement of several rounds of hybridization, difficulties in identification of rare transcripts (Sargent *et al.,* 1983; Hedrick *et al.,* 2000; Dugid *et al.,* 1990) and hence been widely used for identification and isolation of differentially regulated cDNA pools in tissues under different conditions. Thus the use of EST based markers could lead to genetic mapping of a gene that directly affects the trait or a specific sequence could be targeted due to its predicted function based on sequence comparison.

Understanding the mechanisms involved in the response of plants to adverse environmental conditions is, without a doubt, the first step in the generation of crops with higher tolerance to stress. Research at the level of genes (genomics), proteins (proteomics), metabolites (metabolomics), individuals (physiology, systemic-biology) and communities (ecology) has been fundamental in the current understanding of the response of plants to stress. In particular, a huge development in the field of genomics in the last 20 years has led to a deeper understanding in areas such as gene expression, organization and its relationship to stress tolerance (Perez-Torres *et al*., 2008). So many technologies are available for the analysis of gene expression such as Real-time PCR, Microarrays, semi quantitative RT-PCR and RNA interference etc.

Keeping in view the above facts and a necessity to track early heat induced genes mainly the signaling genes the present study entitled "Construction and Analysis of SSH Library of early heat induced genes of *Vigna aconitifolia* (Jacq.) Marechal" was contemplated with the following objectives:

- Screening of genotypes for their heat tolerance levels.

- To perform suppression subtractive hybridization (SSH) of unstressed with heat stressed isolated RNA of *Vigna aconitifolia* (Jacq.) Marechal.
- Cloning of SSH cDNA products into a suitable vector and sequencing.
- In-silico sequence analysis of above made SSH cDNA library after proper editing using various bioinformatics software.
- Validation of the SSH cDNA library through Semi-Q RT PCR assay.

2. REVIEW OF LITERATURE

Moth bean (*Vigna Aconitifolia* (Jacq.) Marechal) is grown in drought-prone regions and is an important pulse crop of the Indian subcontinent (Kamble, 2003). Moth bean has been identified as one of the potential protein food source. It is rich in protein (23.6 g); calcium (202 mg) in it can make it an excellent supplement to cereal diet (Asha, 2005). It is reported to be extremely drought tolerant and highly salt sensitive pulse crop (Sharma and Kakani, 2002). In India, it is cultivated mainly in the arid and semi-arid zones on marginal lands, under rain fed conditions. Moth bean is considered to be a primitive crop, harboring wild characters viz. Spreading nature, flower and pod dropping, pod shattering, comparatively small seed and pod size etc. In spite of low yielding potential, it covers a considerable area in the western arid zone of India owing to its adaptation to harsh and hostile arid situations.

Moth bean occupies 13.52 lac ha area with production of 2.91 lac tones and average yield is 215.26 kg ha^{-1}. Rajasthan ranks first in area and production contributing 85 per cent area (12.28 lac hectares) and 78 per cent production (1.49 lac tones) at the country level (anonymous

2010). Moth bean is known for higher proportion of albumin and glutamine fractions of protein, is a good source of lysine and leucine amino acids and its fodder is valuable pasture (Sudarsan *et al.*, 2002). Realizing its importance, crop improvement efforts were initiated as early as seventies. Being a tolerant crop to various abiotic stresses including high temperature, drought and high irradiance, efforts to understand abiotic stress tolerance mechanism at molecular level in moth bean have not been made. Therefore, the understanding developed in various crops would be reviewed here.

It has been estimated that potential yield of annual crops is lost due to abiotic stress every year. Plant productivity is severely affected by abiotic stress factors as a sequel to it, physiological and biochemical responses in plants vary and cellular aqueous and ionic equilibriums are disrupted. Also hundreds of genes and their products respond to these stresses at transcriptional and translational level (Cushman and Bohnert, 2000; Sreenivasulu *et al.,* 2006 a). Environmental cues are perceived and transmitted by a myriad of plant signal transduction pathways that, by turning on specific transcription factors in the nucleus, lead to the activation of genes encoding effector proteins that enable adaptation to environmental challenges (Angers *et al.,* 2010; Madlung and Comai, 2004). Hence it is very important for researchers to know the effect of any stressed conditions on the expression of a particular gene in plant system.

2.1) Effects of heat stress on plants:

A sudden increase of the temperature above the optimal physiological range is considered heat stress, and usually interferes with normal cell homeostasis by producing changes in membrane fluidity and

permeability, in protein folding and in the metabolic pathways which, if unchecked, can lead to cell death. All living organisms are constantly exposed to rapid temperature changes that can cause acute or chronic stresses. Therefore all living organisms and primarily the plants that are sessile have developed during evolution special adaptations essential for survival and development in a stressful surrounding environment, subjected to temporary and rapid temperature changes (Morimoto, 1998; Krishna, 2003; Kotak *et al.*, 2007; Guy *et al.*, 2008).

Heat stress due to increased temperature is an agricultural problem in many areas in the world. Heat stress affects plant growth throughout its ontogeny, though heat-threshold level varies considerably at different developmental stages. For instance, during seed germination, high temperature may slow down or totally inhibit germination, depending on plant species and the intensity of the stress (Abrol and Ingram, 1996). High temperatures can cause considerable pre and post harvest damages, including scorching of leaves and twigs, sunburns on leaves, branches and stems, leaf senescence and abscission, shoot and root growth inhibition, fruit discoloration and damage, and reduced yield (Guilioni *et al.,* 1997; Ismail and Hall, 1999; Vollenweider and Gunthardt, 2005). Growth chamber and greenhouse studies suggest that high temperature is most deleterious when flowers are first visible and sensitivity continues for 10–15 days. Reproductive phases most sensitive to high temperatures are gametogenesis (8-9 days before anthesis) and fertilization (1-3 days after anthesis) in various plants (Foolad, 2005). Sensitivity of photosynthesis to heat mainly may be due to damage to components of photo system II located in thylakoid membrane of the chloroplast and membrane properties (Al-Khatib and Paulsen, 1999). The adverse effects of heat stress can be mitigated by developing crop plants with improved thermotolerance using various genetic approaches. For this

purpose, however, a thorough understanding of physiological responses of plants to high temperature, mechanisms of heat tolerance and possible strategies for improving crop thermotolerance is imperative.

2.1.1) Physiological and biochemical attributes of heat stress:

As sessile organisms, plants are particularly sensitive to thermal insults, primarily when they grow in field conditions in warming periods. An increase in the temperature above the physiological optimum is sensed by the plants as heat stress, and can cause several injuries at cellular level. These heat stress injuries are dependent on the temperature intensity, duration and rate of temperature increase. At very high temperatures severe cellular injuries may occur within minutes, and even cause plant cell death due to catastrophic collapse of the cellular organization. At moderately high temperatures, cell injuries and death are mostly dependent on the long term exposure. Typically, an excess of high temperatures causes several pre- and post-harvest damages such as sunburns on leaves, branches and stems, leaf senescence/ abscission, shoot and root growth inhibition, flower abortion and drop, fruit discoloration and damage, and reduced yield (Wahid *et al.,* 2007).

Heat stress accelerates the kinetic energy and movement of molecules across membranes thereby loosening chemical bonds within molecules of biological membranes. This makes the lipid bilayer of biological membranes more fluid by either denaturation of proteins or an increase in unsaturated fatty acids (Savchenko *et al*., 2002). The integrity and functions of biological membranes are sensitive to high temperature, as heat stress alters the tertiary and quaternary structures of membrane proteins. Such alterations enhance the permeability of membranes, as evident from increased loss of electrolytes. The increased solute leakage, as an indication of decreased cell membrane thermo-stability (CMT), has

long been used as an indirect measure of heat-stress tolerance in diverse plant species, including soybean (Martineau *et al.*, 1979), potato and tomato (Chen *et al.,* 1982), wheat (Blum *et al.*, 2001), cotton (Ashraf *et al.*, 1994), sorghum (Marcum, 1998), cowpea (Ismail and Hall, 1999) and barley (Wahid and Shabbir, 2005).

Effects of heat stress on plasmalemma, shows more fluidity of lipid bilayer under stress. This leads to the induction of Ca2+ influx and cytoskeleton reorganization, resulting in the up regulation of mitogen activated protein kinases (MAPK) and calcium dependent protein kinase (CDPK). Signalling of these cascades at nuclear level leads to the production of antioxidants and compatible osmolytes for cell water balance and osmotic adjustment. Production of ROS in the organelles (e.g., chloroplast and mitochondria) is of great significance for signalling as well as production of antioxidants (Bohnert *et al.,* 2006). For example, generation and reactions of activated oxygen species (AOS) including singlet oxygen, superoxide radical (O^{2-}), hydrogen peroxide (H_2O_2) and hydroxyl radical (OH^-) are symptoms of cellular injury due to high temperature (Liu and Huang, 2000).

AOS cause the autocatalytic peroxidation of membrane lipids and pigments thus leading to the loss of membrane semi-permeability and modifying its functions (Xu *et al.*, 2006). Superoxide radical is regularly synthesized in the chloroplast and mitochondrion and some quantities are also produced in microbodies. The scavenging of $O^{2-}$by superoxide dismutase (SOD) results in the production of H_2O_2, which is removed by APX or CAT. However, both $O^{2-}$and H_2O_2 are not as toxic as the (OH^-), which is formed by the combination of O^{2-} and H_2O_2 in the presence of trace amounts of Fe^{2+} and Fe^{3+} by the Haber-Weiss reaction. The OH^- can damage chlorophyll, protein, DNA, lipids and other important

macromolecules, thus fatally affecting plant metabolism and limiting growth and yield (Sairam and Tyagi, 2004). The antioxidant defense mechanism is a part of heat stress adaptation, and its strength is correlated with acquisition of thermotolerance (Maestri *et al.,* 2002). High temperature stress leads to increased antioxidant enzyme activity in crop plants (Sairam *et al.,* 2000). Increase in peroxidase enzyme activity was noted at high temperature (Gullen and Eris, 2004).

High temperature influences the photosynthetic capacity of C_3 plants more strongly than in C_4 plants. It alters the energy distribution and changes the activities of carbon metabolism enzymes, particularly the rubisco, thereby altering the rate of RuBP regeneration by the disruption of electron transport and inactivation of the oxygen evolving enzymes of PSII (Salvucci and Brandner, 2004).

2.1.2) Molecular basis of thermotolerance in plants:

The immobility of the plants limits the range of the responses to environmental factors and places a strong emphasis on cellular and physiological mechanisms involved in the adaptation and protection. Plants have evolved complex mechanisms to cope with daily changes in the environment. At a molecular level, this is illustrated by the thousands of transcriptional changes observed in seedlings, leaves, roots, and pollen as plants reprogramme cellular processes to adapt to hot or cold temperatures (Fowler and Thomashow, 2002; Kreps *et al.,* 2002; Busch *et al.,* 2005; Larkindale and Vierling, 2008; Frank *et al.,* 2009). In response to heat stress, plants manifest different morphological adaptations, including short-term avoidance or acclimatization mechanisms involving leaf

orientation, transpiration cooling, or alteration of membrane lipid compositions (Wahid *et al.,* 2007).

A common feature of the plant response is that an initial exposure to moderately elevated temperature provides resistance against a subsequent usual lethal dose of heat stress. This phenomenon is referred to as acquired thermo-tolerance (Kumar *et al.,* 2007). Even when plants grow in their natural distribution range, they may experience high temperatures or diurnal fluctuations that would be lethal in absence of this rapid acclimatization. Therefore, the acquisition of the thermo-tolerance may reflect a more general mechanism that contributes to the homeostasis of the metabolism on a daily basis (Hong *et al.,* 2003). The heat shock response involves the accumulation of molecular chaperones or heat shock proteins (HSPs) that stabilize partially unfolded proteins (Beniwal *et al.*, 2004; Kotak *et al.,* 2007).

2.3) Heat Stress networks and signaling pathways:

Signaling pathways have to be regarded as complex networks. The signal transduction network is characterized by multiple points of convergence and divergence that enable signal integration at different levels, and provide the molecular basis for the appropriate downstream responses that characterize them. At the molecular levels, heat stress response in plant cells is characterized by the rapid perception of the stress signal that triggers a cascade of downstream signaling events and transcriptional controls, which in turn activate stress responsive mechanisms to re-establish homeostasis and protect and repair damaged proteins and membranes (Kotak *et al.,* 2007). The accumulation of unfolding proteins occurs in specific cellular compartments such as the endoplasmic reticulum (ER) (Ron and Walter, 2007). These responses involve the activation of specific chaperones as the Hsps which are

transcriptionally induced by the Hsf genes (Morimoto *et al.,* 1998). Hsf activation in plant cell is still not completely understood and two general models were developed to explain their induction in the heat stress response. In the first models the changes in protein homeostasis, due to the heat stress, disturbed the interaction between Hsp and inactive form Hsfs (Beniwal *et al.*, 2004). The release of the Hsfs from the Hsp interaction allows them to trimerize by forming the activated transcriptional factor required for inducing heat stress target genes.

In the second model, the changes in the membrane fluidity activate specific Ca+2 permeable channels and, in turn, Ca+2 influx in the cytoplasm results in the up-regulation of calcium dependent proteins such as CDPK, the calmodulin CaM and calmodulin binding protein kinase CBK (Vigh *et al.,* 2007; Liu *et al.,* 2008; Saidi *et al.,* 2009). The specific calcium dependent kinase can phosphorilate the Hsf and induce its activation. The two models are not exclusive to one another, but both of them can induce the activation of the Hsfs, and at nuclear level lead to a rapid reprogramming in gene expression. This results in the production of antioxidants and compatible osmolytes for cell water balance and osmotic adjustments, protection against the reactive oxygen species (ROS) in the organelles and elevated synthesis of Hsps (Miller and Mittler, 2006; Wahid *et al.,* 2007 and Kotak *et al.,* 2007).

A new transcriptional pathway, involved in the activation of heat stress genes, has been described more recently by Gao *et al.,* 2008. A putative MTTF-bZIP28 protein (Membrane-Tethered Transcriptional Factor bZIP28 localized to the ER, is maintained in an inactive state by associating with the membrane under non heat stress conditions. Heat stress induces a signal that triggers the cleavage of the bZIP28, followed by its redistribution from the endoplasmic reticulum to the nucleus to activate the expression of heat stress genes (Gao *et al.*, 2008). How this

distinct mechanism is connected to the classical Hsf-dependent pathway is still poorly understood.

2.4) Techniques for identification of Stress-associated genes by various methods:

Various techniques have been used for the detection of gene expression in plants. These techniques are categorized into two types. The first type involves detection of hybridization signal intensity derived from Northern blotting or a microarray, which measures relative intensity of a signal than the absolute value of the signal. The second type is based on the direct count of the individual RNA that are present in the sample, which can be achieved by "Massively Parallel Signature Sequencing" (MPSS) (Brenner *et al.,* 2000), Expressed Sequence Tag sequencing" (ESTs) or "Serial Analysis of Gene Expression" (SAGE) (Velculescu *et al.,* 2000).

Gene isolation and cloning through molecular biology research can be based on RNA or protein expression, differential screening, differential display technique, DNA insertions such as transposon or T-DNA insertions, map based cloning and methods of random cDNA sequencing and genome sequencing. The recent upsurge in activities concerned with identifying genes with unknown functions through research on ESTs and sequencing of total genomes is a boon for stress work. Certain techniques have been employed to identify the gene whose expression is differentially regulated in response to various environmental stresses in higher plants. Such methods include differential display polymerase chain reaction (DDPCR) (Liang and Pardee, 1992), suppression subtractive hybridization (SSH) (Diatchenko *et al.,* 1996), SAGE (Velculescu *et al.,* 2000), DNA-chip and microarray (Schena *et al.,* 1995) and cDNA-amplified fragment length polymorphism (AFLP) (Vos *et al.*, 1995). Subtractive hybridization is

largely used to identify stress responsive genes. It is a powerful technique that enables researchers to compare two populations of mRNA and obtains clones of the genes that are expressed in one population but not in the other. A brief review of genes identified for various mechanisms associated with abiotic and biotic stress tolerance by SSH have been presented below.

2.5) Suppression subtractive hybridization for differential expressed gene identification:

The SSH procedure developed by Diatchenko *et al.*, in year 1996 has the additional advantage that it exploits the suppression PCR effect, eliminating the need for physical separation of single and double-stranded cDNAs (Gurskaya *et al.*,1996). Using SSH (Mahalingam *et al.*, 2003) characterized the stress/defense transcriptome of *Arabidopsis* and identified *Arabidopsis* genes that are differentially expressed in response to ozone, bacterial and oomycete pathogens and the signaling molecules salicylic acid (SA) and jasmonic acid. They identified a total of 1,058 differentially expressed genes from eight stress cDNA libraries.

Fernandez *et al.*, (2003) constructed enriched organ-specific cDNA libraries in a small scale sequencing project in sunflower. Differential organ-specific ESTs were generated from leaf, stem, root and flower bud at two developmental stages (R1 and R4). Organ-specificity ranged from 75 to 100% of non-redundant sequences in the different cDNA libraries. The R4 flower cDNA library was the less redundant library with 62% of unique sequences. Out of a total of 919 sequences that were edited and annotated, 318 were non redundant sequences. Comparison against sequences in public databases showed that 60% of non redundant sequences showed significant similarity to known sequences. The number of predicted novel genes varied among the different cDNA libraries,

ranging from 56% in the R4 flower to 16 % in the R1 flower bud library. Comparison with sunflower ESTs on public databases showed that 197 of non redundant sequences (60%) did not exhibit significant similarity to previously reported sunflower ESTs. This approach helped to successfully isolate a significant number of new reported sequences putatively related to responses to important agronomic traits and key regulatory and physiological genes. This work is to evaluate alternative strategies to high-throughput sequencing projects for the identification of novel genes differentially expressed in sunflower as a source of organ specific genetic markers that can be functionally associated to important traits.

Zhang *et al*., (2005) constructed three subtractions were conducted between samples of the two genotypes of Fescue collected after 12 h of exposure to 39, 42, and 44^0 C. A total of 2495 ESTs were generated, of which 1800 clustered into 434 contigs and 656 were singlets. The putative functions of ESTs were predicted by BLASTX. Nearly 30% of the contigs and 39% of the singlets had no similarity to GenBank sequences. Differentially expressed genes selected by subtractions were classified into 10 broad categories according to their putative functions generated by BLAST analysis. Under heat stress conditions, cell maintenance, chloroplast associated and photosynthesis, protein synthesis, signaling, and transcription factor related genes had higher expression levels in the heat-tolerant genotype. Genes related to metabolism and stress had higher expression in the heat-sensitive genotype. The expression of 17 selected gene transcripts were examined by RT-PCR using plant tissues of the two genotypes grown under heat stress and under optimal temperature conditions (24^0 C) for fescue. Results from RT-PCR confirmed the differential expressions of these transcripts. The differential expressions of at least 11 of these genes were attributable to heat stress rather than to differences in the genetic backgrounds of the genotypes.

Gazendam and Oelofse, (2006) identified and isolated the drought responsive genes conferring drought tolerance in cowpea. A cDNA library enriched for cowpea genes expressed specifically during responses to drought was constructed. The library consists of 4, 160 individual clones. Preliminary sequencing results identified two clones known to be stress-related plant genes (GST (glutathione-*S*-transferase) and PR-1 (pathogenesis-related protein-1). SSH was used to construct sterility and fertility cDNA libraries, which included differentially, expressed clones between fertile and sterile buds of the A/B line 'AB01'. The positive clones were randomly selected by polymerase chain reaction amplification (PCR) and 25 high quality sequences (22 from the fertile-tester library and three from the sterile-tester libraries) were generated.

2.6) Role of bioinformatics in EST sequence annotation:

In recent years, Bioinformatics accelerated the understanding of evolutionary relationships between species as well as structure function relationships of proteins. Various tools have been designed by National Center for Biotechnology Information (NCBI) for nucleotide and protein sequence analysis. VecScreen is a system for quickly identifying segments of a nucleic acid sequence that may be of vector origin. NCBI developed VecScreen to minimize the incidence and impact of vector contamination in public sequence databases. VecScreen searches a query for segments that match any sequence in a specialized non-redundant vector database (UniVec). The search uses BLAST with parameters preset for optimal detection of vector contamination. Those segments of the query that match vector sequences are categorized according to the strength of the match, and their locations are displayed.

The Basic Local Alignment Tool (BLAST) finds region of local similarity between sequences. The program compares nucleotide or protein sequences to sequence databases and calculates the statistical significance of matches. BLAST can be used to infer functional and evolutionary relationships between sequences as well as help identify members of gene families.

The ever growing general and non redundant biological databases posses the increasing potential to search the functionality in the hypothetical proteins by scavenging homologous sequences using specific online web tools like CDD-BLAST, INTERPROSCAN, PFAM and COGs (Anandkumar *et al.,* 2008; Gore and Raut, 2009). These bioinformatics web tools can search the orthologous sequence in biological sequence databases for the target sequence, resultant assist in classification of target protein in particular family (Edward *et al*., 2000; Anandkumar *et al.,* 2008; Gore and Raut, 2009). Along with those, with the advent of protein structure prediction methodology, several online web servers are available which can construct three dimensional structures based on target sequence information using best scored orthologous template protein structures. The strategy involved in such a structure building is aligning the target sequence with orthologous sequence whose three dimensional structure is known (Chen *et al*., 2006; Gore and Raut, 2009).

Blast2GO (B2G), a research tool designed with the main purpose of enabling Gene Ontology (GO) based data mining on sequence data for which no GO annotation is yet available. B2G joints in one application GO annotation based on similarity searches with statistical analysis and highlighted visualization on directed acyclic graphs. This tool offers a suitable platform for functional genomics research in non-model species. B2G is an intuitive and interactive desktop application that allows monitoring and comprehension of the whole annotation and analysis process (Conesa *et al.,* 2005).

2.7) Semi-Q RT PCR assay for Expression Analysis:

Quantitative measurement of specific gene expression is a critically important tool in understanding basic cellular mechanism and effects of various agents on cell Function. Three methods are extensively used for transcript quantification: Northern blotting, microarray analysis, and RT-PCR (reverse transcription polymerase chain reaction) analysis. The combined use of reverse transcriptase followed by polymerase chain reaction (RT-PCR) is a powerful technique for quantification of the mRNA levels.

Two kinds of RT-PCR are commonly applied in published studies: semi-quantitative RT-PCR and quantitative RT-PCR (qRT-PCR or real-time RT-PCR). In semi-quantitative RT-PCR, a target cDNA species is amplified using the same number of cycles for all investigated samples. After electrophoretic separation in a gel and staining with EtBr (or some other nucleic acid dye), the expression rate of the target gene is assessed by measuring the intensity of the band corresponding to the generated amplicon. The band's intensity reflects the number of copies of the target cDNA (i.e. of the target mRNA) at the beginning of the PCR, and thus the level of expression of the target gene in the sample. To ensure that the analysis yields reliable results, the concentration of total cDNA must be the same in all of the samples analyzed. Each cDNA sample is, therefore, initially diluted until the intensity of the band corresponding to a reference gene (which is supposedly representative of the total amount of cDNA) obtained from each sample is the same after a defined number of PCR cycles. In such semi-quantitative RT-PCR, the intensity of a given band is only correlated to the level of expression of the corresponding gene during the exponential phase of the PCR, which spans a window of just a few cycles that differs both between genes and between samples according to

the amounts of transcripts originally present i.e. Semi-quantitative PCR, the amounts of PCR products are measured during the exponential (i.e. log) phase of the PCR reaction, which occurs before saturation is reached (Siebert 1999; Thellin *et al.,* 1999). This is performed for both, the housekeeping and the target genes.

4. RESULTS

Present investigation involving heat tolerant pulse crop *V. aconitifolia* for development of stress related gene data bases showed promising results enhancing our understanding towards mechanism of heat tolerance at molecular level and a good source to isolate and use genes. The experiments were conducted at Plant Biotechnology Centre, SKRAU, Bikaner and National Research Centre on Plant Biotechnology (NRCPB), IARI, New Delhi.

4.1) Phenotypic characterization of genotypes for tolerance to heat stress conditions:

A total of 100 genotypes (core collection identified under National Agricultural Innovation Project "Bioprospecting for genes and allele mining for abiotic stress tolerance") of moth bean were evaluated for heat tolerance at seedling stage under the *in vitro* conditions. All genotypes of moth bean were grown in growth chamber and were watered with measured amount of water (50ml/pot) at every fourth day under in vitro conditions 27^0C and 12/12 hour flesh light. Treatment was given at 47^oC for 3 h which was predetermined for screening through experiments in the laboratory. Most of the seedlings showed stressful characteristics like desiccation of leaf and stem, leaves either dried completely or curled at margins or tips, the stem lodged or wilted completely. Based on visual

effects like leaf drying, curling, stem lodging and number of plants showing wilting symptoms under stressful conditions genotypes were categorized into four different categories viz., tolerant, moderately tolerant, moderately Susceptible and susceptible phenotypes (fig 4.0). The RMO-40 was showing tolerant phenotype, not having any symptoms of stress whereas PLMO-30, PLMO-65 and PLMO-198, showed susceptible phenotype. In this study, it was found that among 100 moth bean accessions 9% were tolerant accessions, 28% were moderately tolerant accessions, 46% were moderately susceptible and 17% were observed as susceptible (table 4, fig 4.1). The genotypes followed normal distribution for tolerance level; however it was skewed towards susceptibility.

Table 4: Classification of *V. aconitifolia* accessions for tolerance based on phenotypic symptoms developed when exposed at 47 C for 3h. Where S= susceptible, MS= moderately susceptible, MT= moderately tolerant and T= tolerant.

S.No.	Level of Tolerance	Genotypes
1.	Suceptible	IC-39728, IC-472232, PLMO-211, IC-35937, IC-129208, IC-285166, IC-39846, IC-16218, IC-472147, PLMO-15, PLMO-30, IC-39704, PLMO-198, IC-140635, IC-472162, IC-120986, PLMO-65
2.	Moderately Susceptible	IC-472158, IC-39808, IC-121051, JAWALA, IC-39706, IC-36394, IC-311396, IC-20992, RMO-3, IC-52150, PLMO-82, IC-36477, IC-129196, IC-36573, IC-36096 PLMO-26, IC-36649 ,IC-333125, IC-415139, IC-9100,IC 36189 IC-129194, 36376, PLMO-6, IC-129243, PLMO-78, IC-121015-1, IC-39735, IC-140716, IC-36557,IC-140677, IC-36161, VDV-6172, IC-36537, VDV-6172, IC-36537, IC-39653, IC-36523, IC-120986, IC-36482, IC-140660, PLMO-132, PLMO-8, IC-472236, PLMO-207, IC-140635

3.	Moderately Tolerant	IC-472257,IC-36521, IC-36462,IC-35934,IC-36539, IC-472202, IC-52147, IC-10141, IC-472189, IC-103016, IC-405139, 225-9-9-17-1-2, IC-39693, IC-36392, IC-36487, IC-120973, IC-30639, PLMO-134, IC-39742, IC-472177, IC-9083, IC-129216, IC-39648, IC-129190, IC-472185, IC-35864, PLMO-44, IC-472173,
4.	Tolerant	RMO-40, IC-129179, IC-36592, IC-36017, IC-472179, IC-39839, IC-36366, IC-472196, IC-36150

TOLERANT

MODERATELY TOLERANT

MODERATELY SUSCEPTIBLE

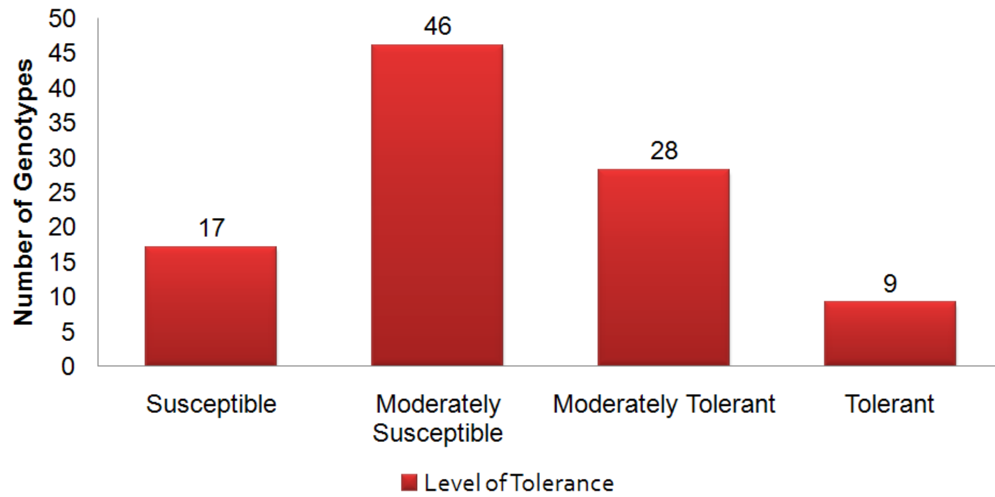


Figure 4.1: Frequency distribution of susceptible, moderately susceptible, moderately tolerant and tolerant genotypes among 100 genotypes of *V. aconitifolia.*

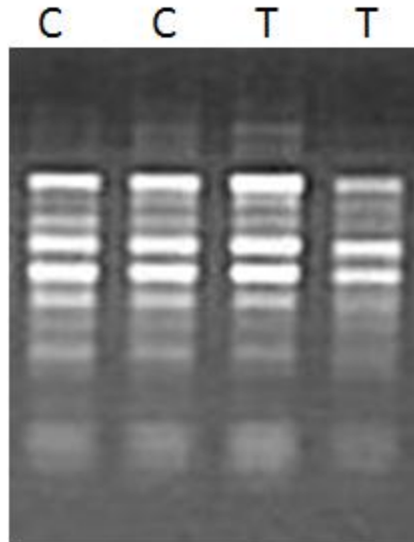


Figure 4.2: High quality RNA. Lane 1-4: RNA isolated from control (C) and treated (T) plants under study

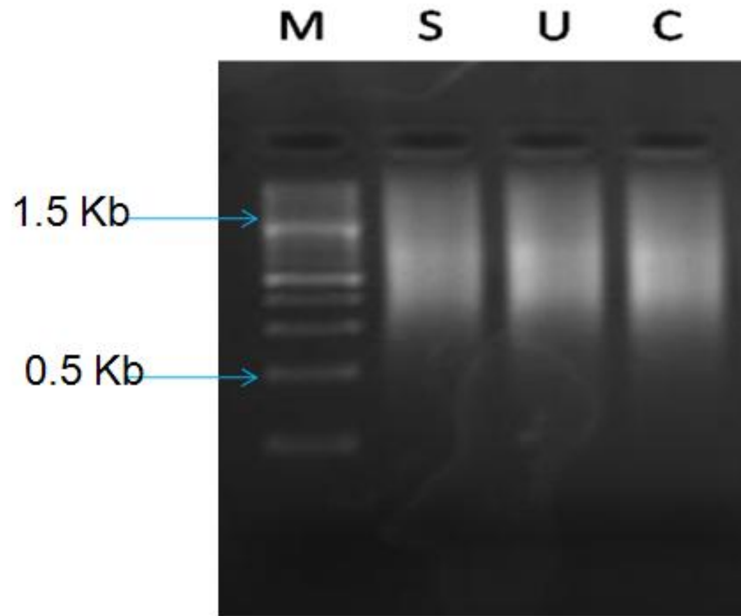


Figure 4.3: Agarose gel (1.2%) electrophoresis of PCR amplified subtracted cDNA. M = 100 bp DNA ladder, S = forward subtracted Secondary PCR product, U = Unsubtracted cDNA product, C = Skeletal Muscle Control.

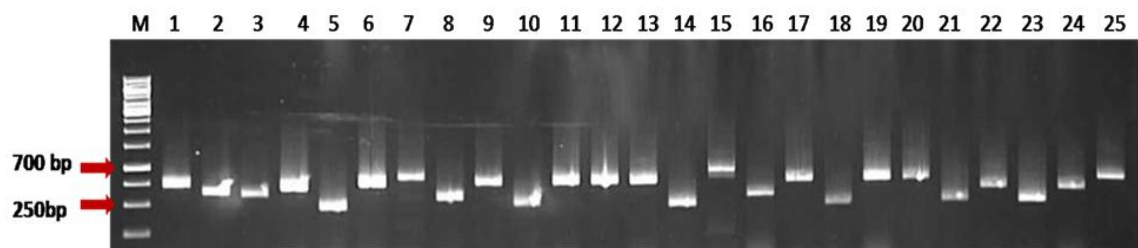


Figure 4.4: The PCR analysis of partial clones in the subtracted library. Lane 1-25: PCR products from different clones. Lane M: DNA size markers.

4.2) mRNA preparation:

The mRNA was extracted from the total RNA of both driver and tester samples separately using the magnetic mRNA isolation kit (New England BioLabs, USA) and was quantified by nanodrop spectrophotometric quantification assay (Thermo Scientific NanoDrop 8000). The concentration of mRNA of driver and tester samples was 813 ngµl and 825 ng/µl respectively. The RNA was of high quality as evident by the gel analysis where clear two bands for rRNA (18S and 28S) are visible along with a few more faint bands (fig 4.2).

4.3) Suppression Subtractive Hybridization (SSH) cDNA Library:

SSH provides a powerful method to analyze global gene expression profiles. Forward subtraction was conducted between leaves from heat stressed and unstressed seedlings of RMO-40. The final SSH forward subtracted PCR products (0.5-1.2 Kb, fig 4.3) were cloned into pJET1.2 vector and transformed into DH5α cells. In total 900 clones was obtained and subsequent colony PCR showed that the size of these inserts ranged from 250 to 700 bp (fig 4.4). Thus we successfully constructed a putative heat stress specific forward subtracted cDNA library of *V.aconitifolia* var. RMO-40. A clear smear of size in range of 0.5-1.2 Kb is a symptomatic of successful SSH.

4.4) Sequence analysis

A total of 900 clones were randomly selected for sequencing from this subtracted cDNA library and 768 clones were turned out to be positive, fit for sequencing. Screening for vector sequences further revealed that all the clones had vector sequence contamination ranging from 1 to 3 fragments. The sequences were further analysed as follows:

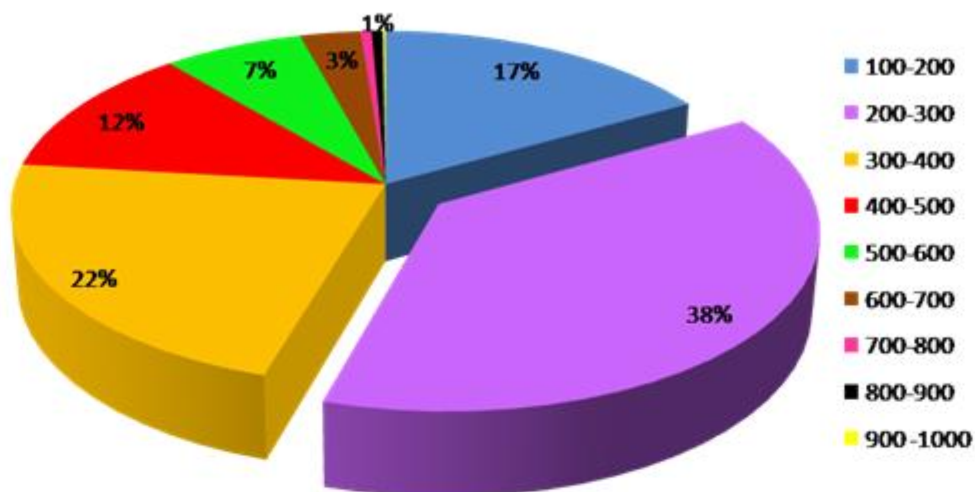


Figure 4.5: A graphical representation of the sequence length: Out of 738 ESTs, maximum sequences were of 200-300bp size.

4.4.1) EST assembly

A total 900 clones were generated from the current SSH library, of which 768 ESTs were sequenced. After a quality check, 738 high quality ESTs (sequences having length >100bp) were obtained (see figure 4.5). A total of 488 unigenes (114 contigs and 374 singletons), were derived by sequence alignment of 738 ESTs and each contig had 2-44 ESTs with an average length of the 347 bp. The majority of contigs (70%) contained 3 or fewer ESTs whereas only 1.47% contigs were made from 10 or more ESTs indicating high degree of normalization and substraction efficiency. All EST sequences have been submitted in the dbEST division of GenBank (JK265689-JK266422, JK226915). Out of 206 ESTs (28%) of unknown proteins 160 ESTs (14%) were found to be novel to moth bean. Only 578 ESTs (78%) showed significant BLASTX similarity (<1E-06) in the NCBI non-redundant (nr) database. Gene ontology functional classification terms (BLASTX results and GO terms), were retrieved for 479 (65%) sequences, and 339 sequences were annotated with 165 enzyme commission (EC) codes and were mapped to 68 different KEGG pathways. 452 ESTs were further annotated with InterProScan (IPS) and no IPS were assigned to 153 ESTs.

Amongst EST sequences we found only 5 chaperons viz. JK265946 (heat shock coagnate-70 Kda protein 1 ATP binding isoform 2 (hsc-70)); JK266046 (DnaJ-like protein) and 3 unknown chaperon like proteins to moth bean (JK265750, JK265751 and JK265912). Our study tracks 29 different nucleic acid and protein binding proteins associated with heat tolerance. Some ESTs such as JK266327 (phosphatidylinositol-4 kinase), JK266052 (WRKY transcription factor), JK266237 (DREB 2), JK265696 (nac-domain ipr003441) and JK265804 (zinc finger ccch domain containing protein), had single representations in SSH library.

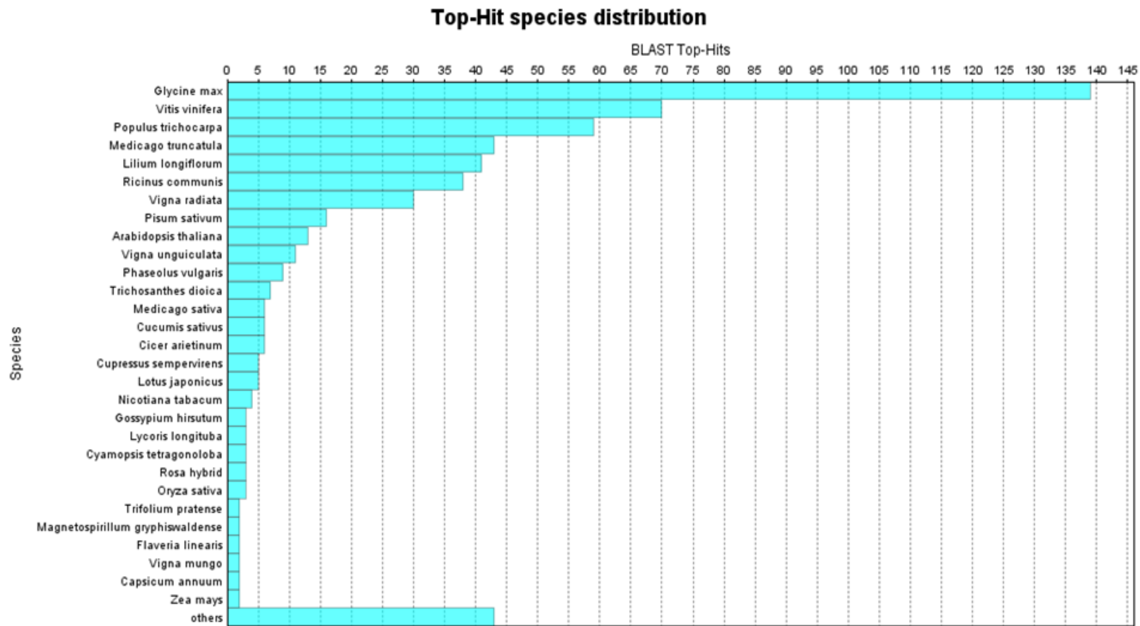


Figure 4.6: A graphical representation of the top hit species distribution: The transcripts analyzed show maximum homology to the Glycine max, followed by Vitis vinifera and Populus trichocarpa. No homology was found with V.aconitifolia as very few transcripts available in the databases.

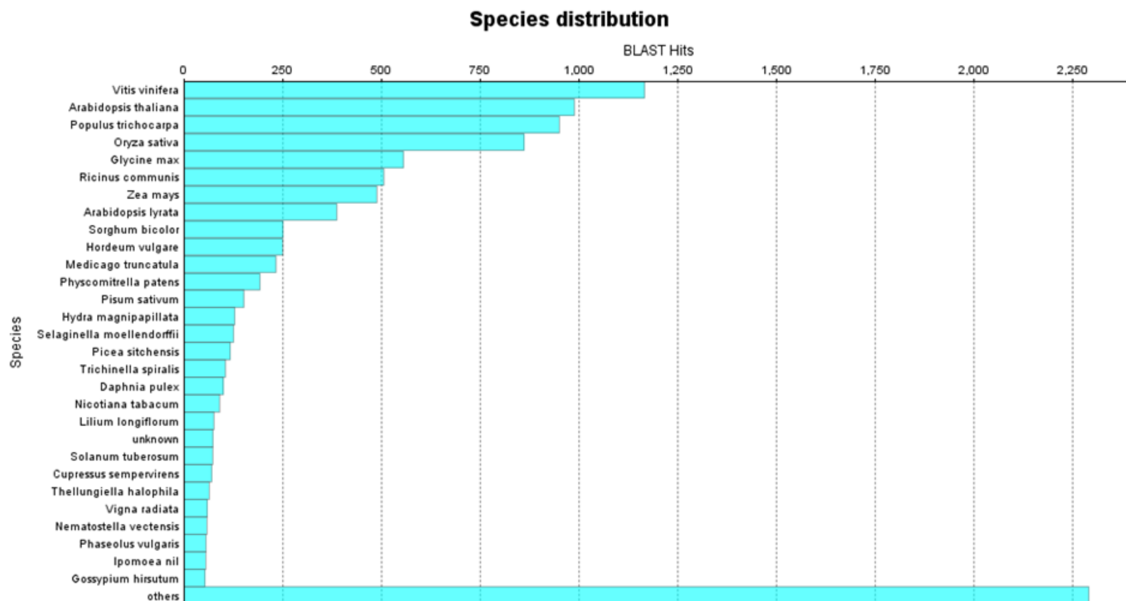


Figure 4.7: A graphical representation of the species distribution: Species distribution chart of V.aconitifolia after blastX to NCBI nr. Very less homology with Vigna radiata is due to limited avaibility database on vigna species.

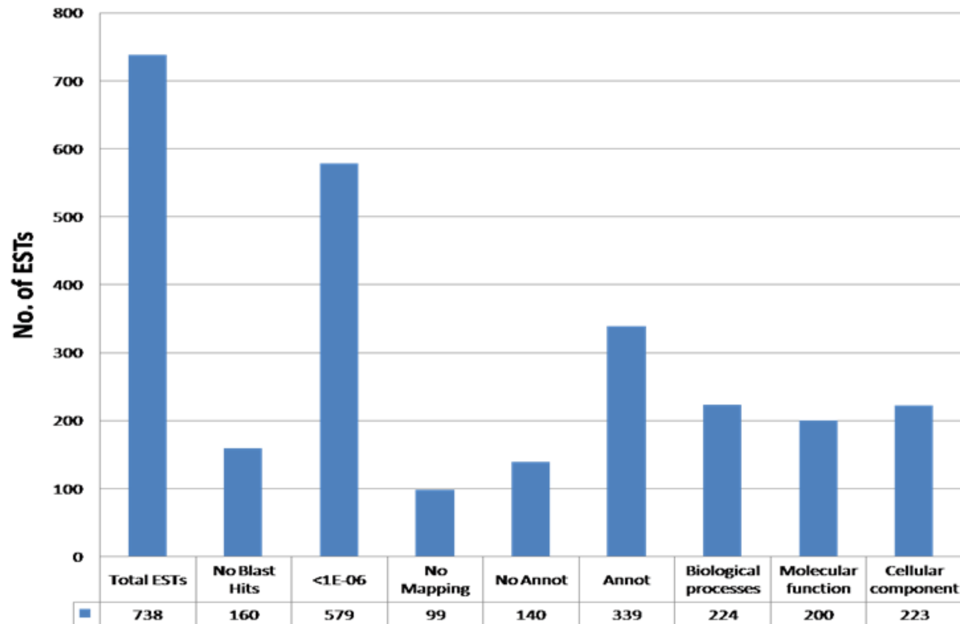
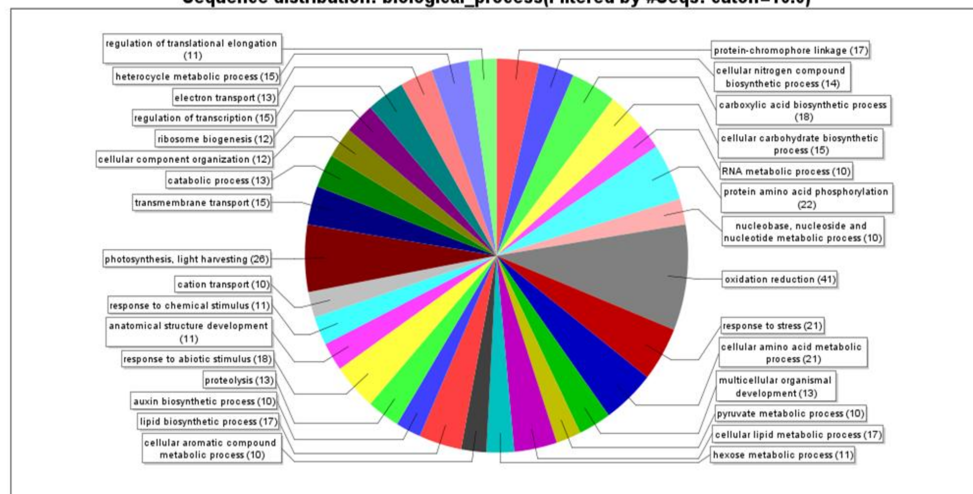


Figure 4.8: A graphical representation of annotation statistics of the present library: the total number of ESTs, ESTs with no blast hits, number of ESTs annotated as known protein with an E-value threshold of E-06, total number of ESTs not mapped, total number of ESTs mapped but not annotated, the total number of ESTs annotated with at least one category of Gene Ontology (GO) and the number of genes annotated in each of the three major GO categories, biological process, molecular function and cellular component.

Figure 4.9: A graphical representation of Sequence distribution based on biological process: pie chart showing

Sequence distribution: biological_process(Filtered by #Seqs: cutoff=10.0)



distribution of the ESTs annotated to various biological processes filtered by sequence cutoff=10. A large number of ESTs belongs to oxidation-reduction and photosynthesis processes.

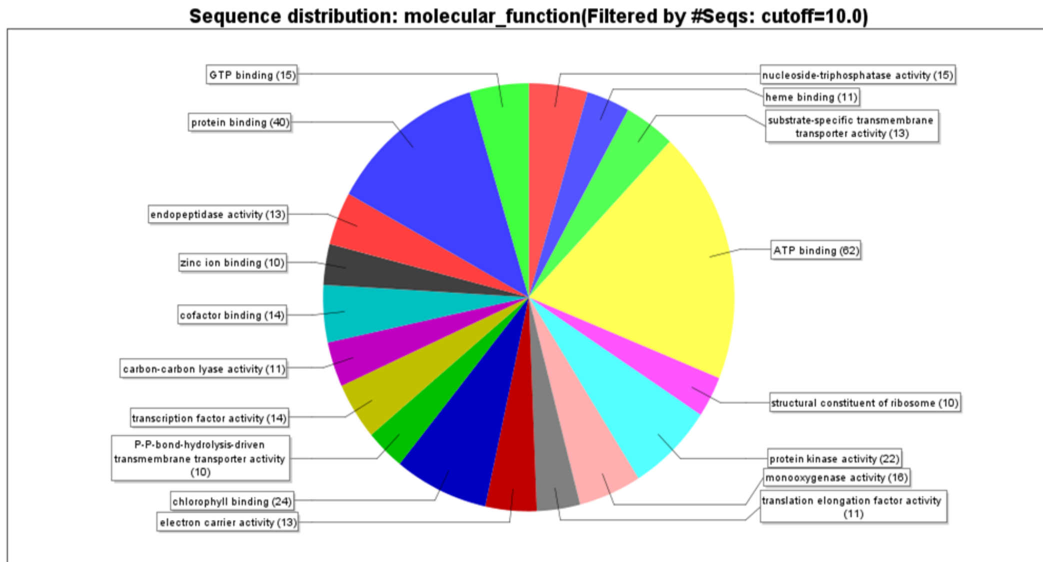
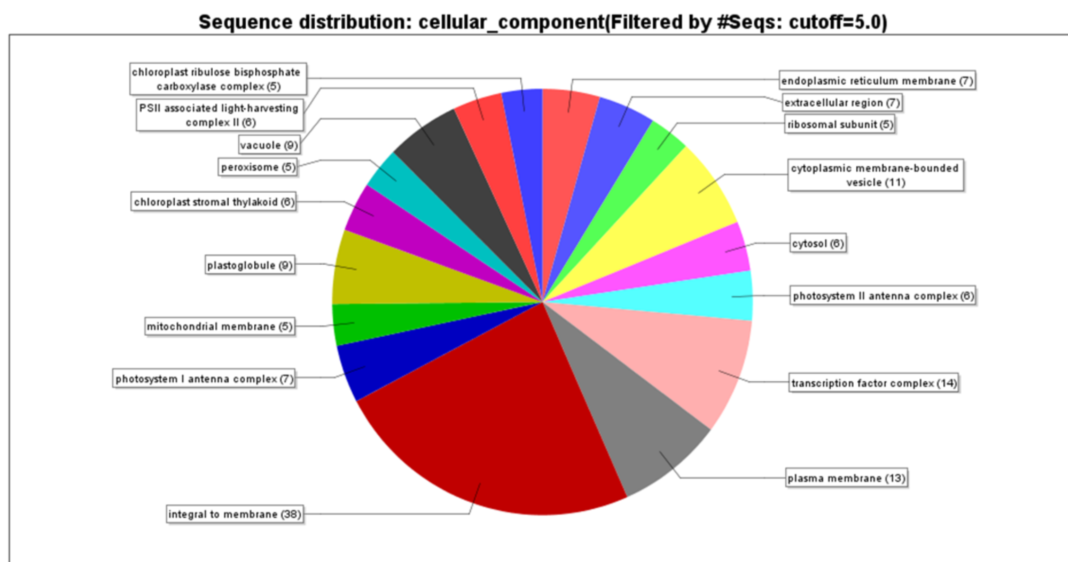


Figure 4.10: A graphical representation of Sequence distribution based on molecular function: pie chart showing distribution of the ESTs annotated to various molecular functions filtered by sequence cutoff=10. A large number of ESTs shows ATP binding and protein binding activities.

Figure 4.11 : A graphical representation of Sequence distribution based on cellular component: pie chart showing distribution of the ESTs annotated to various cellular components filtered by sequence cutoff=5.



Majority of ESTs are integral to membrane and part transcription factor complexes.

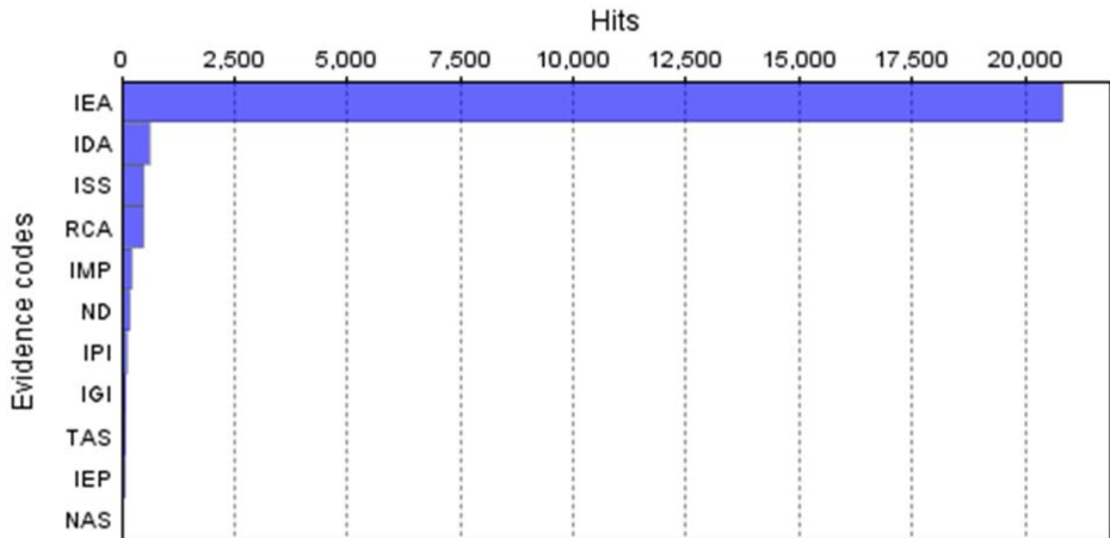


Figure 4.12 : A graphical representation of Evidence code distribution for BLAST hits: A chart showing the distribution of the different evidence codes throughout the GO terms per BLAST hit.

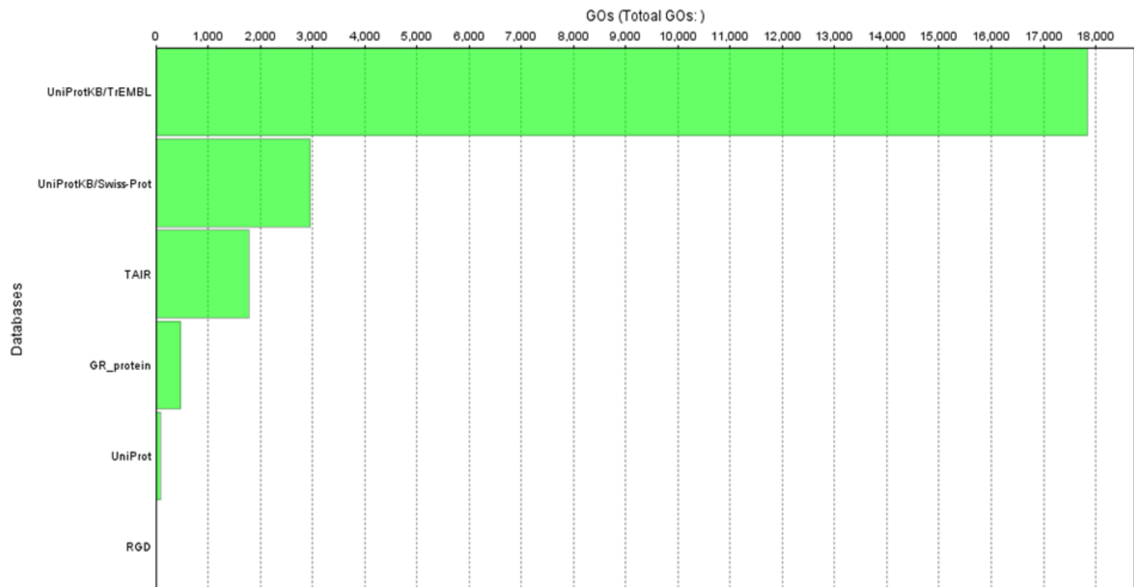


Figure 4.13 : A graphical representation of Mapping Database sources: shows the databases from which annotations had been taken. Maximum annotations in the present study are taken from UniProtKB/TrEMBL.

4.4.2) Functional characterization of the moth bean unigene and EST dataset

BLASTX analysis of 488 unigenes showed 410 total hits against NCBI non-redundant (nr) database with E-value < 1E-06. A majority of top Blast hits matches were from proteins of legume species, with maximum hits from *Glycine max* and *Vitis vinifera* (figure 4.6) *and* no match was found with *Vigna aconitifolia* indicating the novelty of the moth bean unigene dataset. However, majority of matches were with proteins of non-legume species like, *Vitis vinifera, Arabidopsis thaliana* and *Populus trichocarpa* (see figure 4.7)*.* The availability of the whole genome and predicted proteins of these species and limited sequence information of legumes in the database may have led to the highest homology of moth bean sequences with these non-legume genomes. Functional annotation of ESTs by Blast2GO resulted in gene ontology functional classification terms for 639 (86.5%) sequences of which 339 (45.9%) ESTs were functionally annotated (GO consensus and EC number) and 140 (18.9%) sequences were mapped but not annotated by Blast2GO because of their high e-value scores (see figure 4.8). Gene ontology functional classification terms (BLASTX results and GO terms), were retrieved for 479 (65%) sequences, and 339 sequences were annotated with 165 enzyme commission (EC) codes and were mapped to 68 different KEGG pathways. 452 ESTs were further annotated with InterProScan (IPS) and no IPS were assigned to 153 ESTs. Transcripts with no IPS assigned might be either an unknown isozyme or a new family member of known protein super families.

These results demonstrate that a large number of moth bean genes are not characterized and are attributing to the high heat tolerance level of moth bean where major efforts in functional analyses are needed. Based on GO annotation, EST sequences were divided into three organizing

principal GO categories: cellular locations, molecular functions and biological processes. Some ESTs were annotated with the three categories simultaneously. A gene product might be associated with or located in one or more cellular components; it is active in one or more biological processes, during which it performs one or more molecular functions. At the second level GO, 224 sequences were assigned to biological process category, 200 sequences to the molecular function category and 223 sequences to the cellular component category. In biological processes, cellular and metabolic processes were the dominant term (55%). In the molecular function category binding (27%) was the most dominant term followed by catalytic activity (24%). In the cellular compartments, cell part (30%) was the most represented term, followed by intercellular organelle (25%) (figure 4.9-4.11).

We were able to assign nearly three-quarters of the genes to functional groups based on sequence similarity with known gene motifs. Although functional assignment based only on sequence homology needs experimental verification, it nonetheless provides a measure of the diversity of the genes in the stress cDNA collection.

4.4.3) Pathway classification and analysis of transcripts

About 339 sequences were annotated with 165 enzyme commission (EC) codes (see figure 4.12, 4.13) and were mapped to 68 different KEGG pathways. Of the 68 pathways 45 (66%) were contained within the metabolism category (metabolic pathways). KEGG metabolic pathways well represented by ESTs were biosynthesis of steroid, phenylpropanoid and terpenoids (7 enzymes), amino acid metabolism (17 enzymes), biosynthesis of purine and pyrimidine (7 enzymes), photosynthetic pathway (5 enzymes), oxidative phosphorylation (2 enzymes), starch and

sucrose metabolism (3 enzymes), signaling pathway (4 enzymes), biosynthesis of vitamins such as riboflavins, nicotinic acid, pantothenic acid and retinol

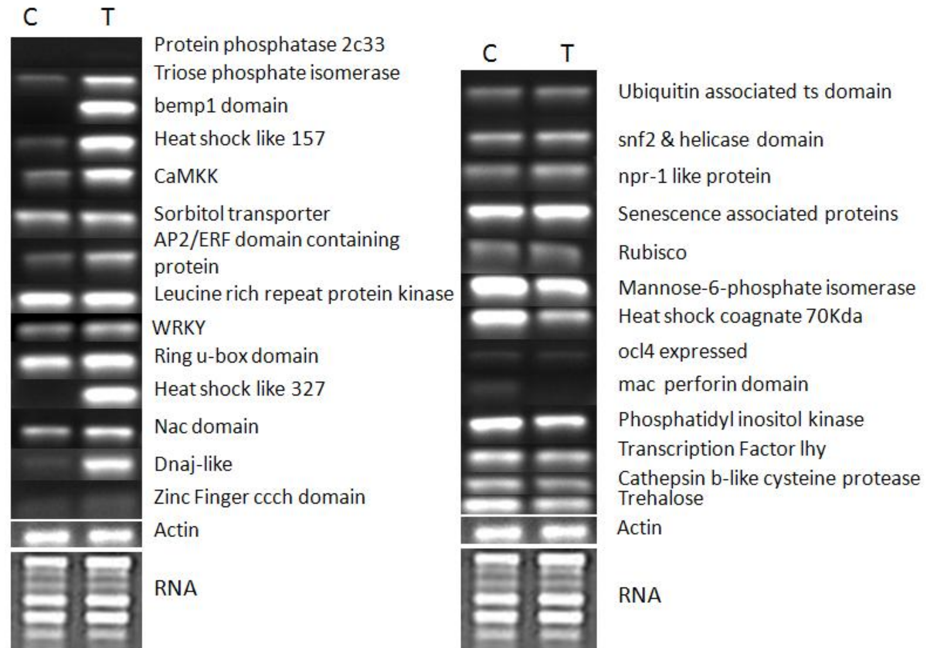


Figure 4.14: Expression analysis through semi-quantitative RT-PCR: Lane 1-2: shows differentially expressed ESTs between control (C) and treated (T) plants of RMO-40 under study.

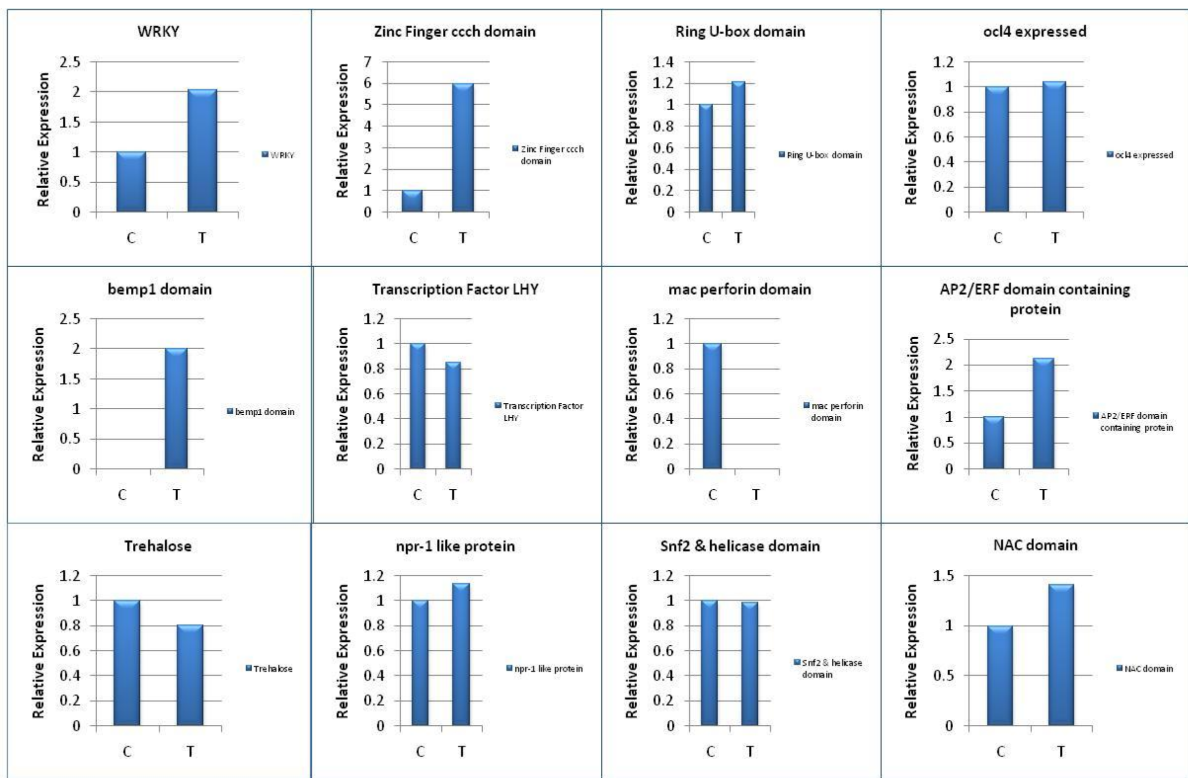


Figure 4.15: A graphical representation of Relative Expression levels observed: graphs showing relative expression levels of differentially expressed genes under stressed (C) and unstressed (T) conditions.

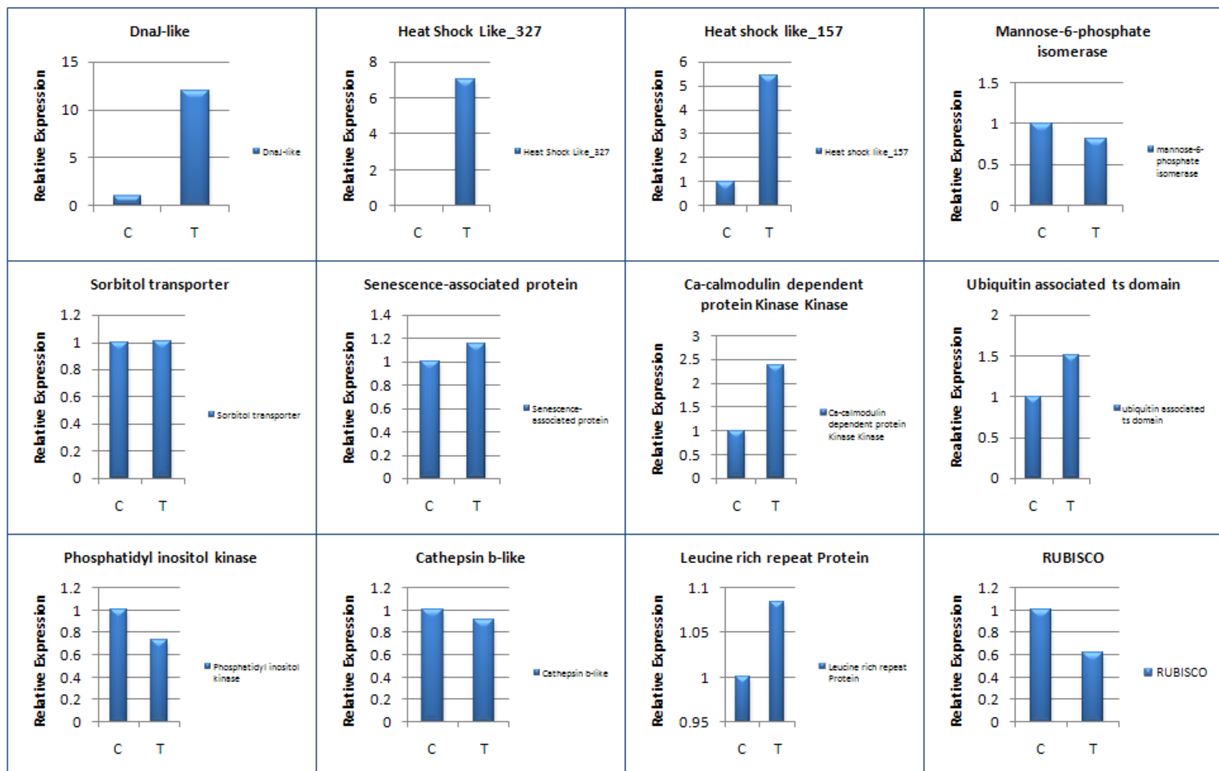


Figure 4.16: A graphical representation of Relative Expression levels observed: graphs showing relative expression levels of differentially expressed genes under stressed (C) and unstressed (T) conditions.

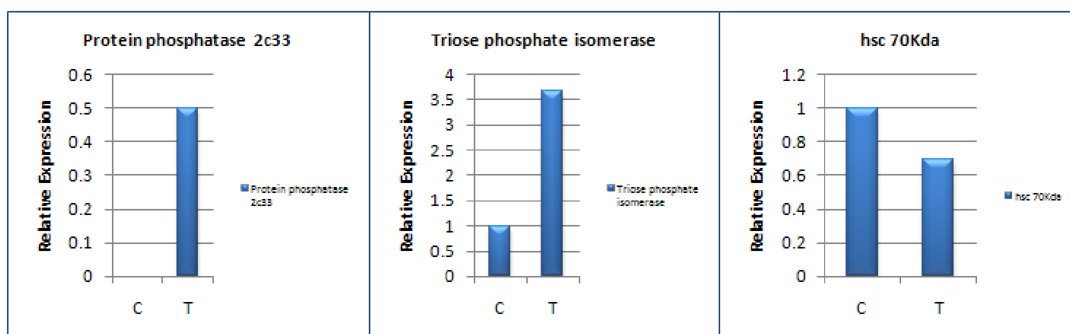


Figure 4.17: A graphical representation of Relative Expression levels observed: graphs showing relative expression levels of differentially expressed genes under stressed (C) and unstressed (T) conditions.

(4 enzymes), lipid metabolism (5 enzymes), biosynthesis of other secondary metabolites (7 enzymes). ESTS coding several enzymes that are involved in the synthesis of serine (JK266289), proline (JK266411), leucine (JK265743) and methionine (JK265944) were found in the present library. Moreover we had tracked 5 enzymes related to the cysteine and methionine metabolism pathway with their EC numbers as 2.5.1.48, 2.5.1.6, 1.14.17.4, 3.1.3.77 and 42.1.109.

4.5) Differential expression analysis of ESTs under heat stress through semi-quantitative RT-PCR assay

An expression level of 27 ESTs generated from the above SSH cDNA library was studied through semi-quantitative RT-PCR assay (see figure 4.14-4.17). The genes under study were up-regulated (16 ESTs) or down-regulated (7 ESTs) under Heat stress in treatment (T) as compared to the control (c). Though forward SSH library was constructed to clone up regulated genes, normalization through suppression process at times results in cloning of down regulated genes also.

The expression levels of sixteen genes were found to be up regulated in 5 min of heat stress. These transcripts are Protein phosphatase 2c33, NAC-domain, Triose phosphate isomerase, Leucine

rich repeat containing protein, Ubiquitin associated ts domain, npr-1 like protein, AP2/ERF domain containing protein, bemp1 domain containing protein, WRKY, senescence associated protein, ca-calmodulin dependent protein kinase kinase, Ring u-box domain containing protein, Zinc finger ccch domain, Dnaj-like protein, Heat shock like_327 and Heat shock like_157. Although an increase in transcription level was observed for all the above mentioned genes, a much higher up regulation was observed in Dnaj-like protein, Heat shock like_157, Heat shock like_327, bemp1, zinc finger ccch domain and protein phosphatase 2c33.

The expression levels of three transcripts viz. Sorbitol transporter, snf2 and helicase domain containing protein and ocl4 expressed protein was found to be almost equal under both stressed and unstressed conditions. Constitutive expression of stress related protein sorbitol transporter could be attributed to the in-build tolerance level of moth bean owing to its evolution under such harsh climatic conditions. However the expression level of ocl4 expressed gene was slightly up regulated whereas that of snf2 helicase domain containing protein was slightly down regulated.

Amongst 27 transcripts eight were found to be down regulated under stressed conditions, theses transcripts are - RUBISCO, Heat shock coagnate 70Kda, mannose-6-phosphate isomerase, mac-perforin domain containing protein, phosphatidyl inositol kinase, transcription factor lhy, cathepsin b-like cysteine protease and trehalose phosphate synthase. Transcript for mac perforin domain containing protein was found to be highly downregulated under stress conditions.

5. DISCUSSION

Heat stress due to increased temperature is an agricultural problem in many areas of the world. Transitory or constantly high temperatures causes an array of morphological, anatomical, physiological and biochemical changes in plants, which affect plant growth and development and may lead to drastic reduction in economic yield (Wahid *et al.,* 2007). The plants normally growing under physical water stresses have developed certain constitutive characteristics in addition to inducible mechanism to deal with harsh climate and change occurring in the environment. For example plants growing in water deficit and high temperature conditions have developed constitutive characters like leaf modification leading to leafless nature, fleshy nature with polysaccharides holding water and morphology minimising the exposure to sun rays sunken stomata to reduce evapo-transpiration etc. In addition there are ephemerals tuning there lifecycle with the availability of the water. Their lifecycle and metabolic process seems to be controlled by the availability of water and temperature of the environment. Moreover heat stress is often associated with the inappropriate functioning of various metabolism related enzymes and various modification taking place at molecular level drifting plants towards senescence, owing to this plants have developed certain tolerance mechanism in order to carry on with their normal metabolisms even under stressed conditions. Such traits are expected to be controlled by inducible genes.

Moth bean falls into the latter category of ephemerals also has constitutive traits like long tap root system and indeterminate growth habit whereas its flowering induction is found to be affected by moisture content and temperature of the environment. Its seedling have been found to be resistant to high temperatures reaching up to 48°C in July, immediate after sowing in Bikaner region of Rajasthan. Looking at its inducible nature of heat tolerance and capacity of withstanding such a high temperature

stress it is fruitful to identify the genes working at molecular level imparting such a high level of heat tolerance to this vary crop specie.

Our own experiment conducted on seedlings grown at 27^0C has shown that these seedlings can efficiently sustain rise in temperature up to 42^0C. The inbuilt capacity of tropical crops to sustain higher temperature has also being indicated by many workers (Hall, 2001). However, varietal differences have also being observed, RMO-40 being more resistant at 47^0C over other varieties. The genotypic effect on the ability to sustain temperature stress has been indicated in the literature (Ottaviano *et al.*, 1991; Joshi *et al.*, 1997; Mullarkey and Jones, 2000). Moreover it has also been reported that land races have more tolerance to the temperature stress compared to improved genotypes. In this study, it was found that among 100 moth bean accessions 9% were tolerant accessions, 28% were moderately tolerant accessions, 46% were moderately susceptible and 17% were observed as susceptible accessions.

Plants exposed to excess heat exhibit a characteristic set of cellular and metabolic responses, including a decline or cessation of housekeeping genes and an accelerated accumulation of wide range of stress inducible genes. Varietal sensitivity or tolerance to heat stress has been also shown to depend on the ability to maintain activities of antioxidant enzymes (Rainwater *et al.,* 1996; Rizhsky *et al.,* 2002 and 2004; Vacca *et al.*, 2004). The heat tolerant genotype of fescue maintained higher transcripts of genes involved in cell maintenance, chloroplast associated and photosynthesis protein, contrast genes related to metabolism and stress had higher expression in the heat sensitive genotype (Zhang *et al.,* 2005). Comparison of expression data from different plant species experiencing heat stress under variable conditions, for example from different tissue types, developmental stage, growth

conditions, or applications and durations of stress treatments, shows related patterns of transcript accumulation, with the expression of about 2% of the genome being affected (Kotak *et al.,* 2007).

5.1 Construction of a SSH cDNA library and Sequence analysis of differentially expressed cDNAs.

Molecular control mechanisms for abiotic stress tolerance are based on the activation and regulation of specific stress related genes. These genes are involved in the whole sequence of stress responses, such as signalling, transcriptional control, protection of membranes and proteins, osmoregulation and free-radical and toxic-compound scavenging (Desai *et al.,* 2006).

The differentially expressed ESTs identified in our study provide a list of genes regulated in response to early heat stress in leaf tissue of moth bean. The SSH strategy can be used as an alternative and complementary transcript profiling tool to the gene chip micro-arrays, especially to identify novel genes and transcripts present in low abundance (Ishihara *et al.,* 2005). Thus the SSH technology will have more utility in a system where genome sequence information and microarray chip are not available for transcript profiling. cDNA libraries prepared from different tissues exposed to various stress conditions and developmental stages are valuable tools to obtain the expressed and stress regulated portion of the genome. The sequencing of cDNA gives direct information on the mature transcripts for the coding portion of the genome that can be subsequently used for gene identification and functional studies. As expected the present SSH library resulted into identification of mainly signalling genes (19%), responsible for initializing a molecular response under heat stress.

5.1.1 Sequence analysis

Unknown proteins were prominently identified approaching up to 206 ESTs (28%) of unknown proteins where 160 ESTs (14%) were found to be novel to moth bean. Well all such naive transcripts indigenous to moth bean needs to be characterised for their putative essential role under heat stress. Some of them might be up-regulated as a result of a signal generated due to down regulation of yet other known or unknown transcript, perhaps these known transcripts are the ones acting on the top level and inducing heat signalling cascades. Around 20 different types of signalling genes and 16 different transcription factors have been shown to be associated with early heat stress in moth bean for the first time. Very low predominance of heat shock proteins among up-regulated genes suggests an important role of only two chaperons (DnaJ, hsc-70 kda1) along with the signalling molecules in initiating response towards heat tolerance. Considerable number of the ESTs (9%) was representing photosynthesis related proteins and majority of ESTs (33%) belongs to the metabolism related protein category.

A plethora of physiological and metabolic adjustments occur during heat acclimatization and in response to other stresses. The regulation of genes involved in temperature, drought and salt stress is known to reflect a cross-talk between different signalling pathways (William *et al.,* 2007). Our analyses enabled us to position several genes in their respective metabolic pathway (amino acid and lipid), suggesting that these pathways are involved in stress responses.

Amino acid metabolism and the TCA cycle are the major pathways that generate precursors for various biological molecules. Proline is a proteinogenic amino acid with an exceptional conformational rigidity, and

is essential for primary metabolism. Since the first report on proline accumulation in wilting perennial rye grass (*Lolium perenne*) (Kemble and Pherson, 1954), numerous studies have shown that the proline content in higher plants increases under different environmental stresses. ESTS coding several enzymes that are involved in the synthesis of serine (JK266289), proline (JK266411), leucine (JK265743) and methionine (JK265944) were found in the present library. These amino acids are the precursors for the synthesis of several specialized metabolites and are directly involved in cellular osmotic adjustment.

Similarly, ESTs encoding lipid metabolism related proteins such as cinnamic acid 4-hydroxylase (JK266169), lipoxygenase (JK266262) acyl oxidase acx3 (JK266024) and acyl protein thioesterase was found in moth bean data set. These results suggest that lipid degradation occurs concomitantly. On the other hand, ESTs encoding enzymes involved in lipid synthesis such as fiddle head like protein (JK265790) involved in very long chain lipid metabolism, udp-n-acetylglucosamine o- acetyltransferase domain containing protein (JK266279, an acyl-carrier protein) , acyl-binding domain 3 (JK265958), acetyl carboxylase (JK266242) and acyl synthase (JK266283) are more abundant in the moth bean dataset. ESTs corresponding to several enzymes involved in sterol metabolism such as bri1 (brassinosteroid insensitive 1) kinase inhibitor 1 (JK266299, negative regulation of brassinosteroid biosynthetic process) and cinnamyl alcohol dehydrogenase (JK266264, steroid biosynthesis process, EC:1.1.1.145) suggests lipid modifications in memberanes during heat acclimatization. Long chain fatty acid perhaps maintains memberane fluidity due to increased hydrophobic interactions between phospholipid bilayer.

Intrestingly, agronomically important sequences related to response and/or defence to pathogens such as callose synthase (JK266207), plant

disease resistance response protein family (JK266227), mac perforin domain (JK265891), bactericidal permeability increasing (JK266259), are few ESTs reported in the present database. One such defence related EST is for Rpp4 candidate 2 gene (JK265785, *resistance to Phakopsora pachyrhizi1*). The fungus *Phakopsora pachyrhizi* causes asian soybean rust (ASR); a formidable threat to world soybean (*Glycine max*) production (**Bromfield,** 2010). Like *V.aconitifolila, G.max* is also legume specie hence such defense related genes could be further explored for their potential role in the development of biotic stress resistant varieties.

5.2 Expression Analysis through semi-Q RT-PCR assay:

The induction of heat shock proteins (HSPs) when plants are exposed to elevated temperature has been well documented (Kotak *et al.,* 2007; Lee *et al.,* 1995; Ogawa *et al.,* 2007). HSPs function as molecular chaperones in maintaining homeostasis of protein folding and are related to the acquisition of thermo tolerance (Wang *et al.,* 2004). In the present moth bean database we found two uncharacterized like proteins whose GO annotations suggests them to be cytoplasm resident binding protein involved in protein folding in response to heat shock. We named them as heat shock like 327 and 157 respectively. Heat shock like 327 (JK265933, InterPro: PTHR24076 (PANTHER), seg (SEG))., was found to be expressed only in 5 min heat stressed plants viz. treated (T) and not in the unstressed plants viz. control (C). Similarly expression levels of heat shock like 157 (JK265771, InterPro: PTHR24076:SF72 (PANTHER), seg (SEG)) was highly up-regulated with heat stress.

The folding of proteins and the assembly of protein complexes within sub-compartments of the eukaryotic cell is catalyzed by different members of the Hsp70 protein family. The chaperone function of Hsp70 proteins in

these events is regulated by members of the DnaJ-like protein family, which occurs through direct interaction of different Hsp70 and DnaJ-like protein pairs that appear to be specifically adapted to each other (Cyr *et al.,* 1994). DnaJ-like proteins have a well conserved J-domain, which is responsible for their interactions with Hsp70 (Shuh-ichi *et al.,* 1997). In the present study DnaJ-like protein (JK266046, InterPro: IPR001305, PTHR 15852:SF2 (PANTHER)) was 10 folds more expressed in treated. The main role of DNAJ proteins is in protein folding as it is well known that the proteins produced, must be folded into the correct 3-dimensional shape to function properly. Proteins that help with this 3-dimensional folding are called chaperones. The role of a chaperone is to bind to unfolded or partially folded proteins, prevent misfolding, and assemble or disassemble multi-protein structures. DNAJ proteins are known as co-chaperones because they help another family of chaperones (DNAKs) with protein folding. DNAJ and DNAK proteins must work together to facilitate protein folding.

Transcription factors usually play important roles in signal transduction pathways and are the earliest group of gene to respond to abiotic and biotic stresses (Shinozaki *et al.,* 2003). Molecular and genomic studies have shown that various transcription factors are involved in the regulation of stress-inducible gene (Uno *et al.,* 1997). Transcription factors an attractive target category for manipulation and gene regulation is the small group of transcription factors that have been identified to bind to promoter regulatory elements in genes that are regulated by abiotic stresses (Shinozaki and Yamaguchi-Shinozaki, 1997). They activate cascades of genes that act together in enhancing tolerance towards multiple stresses. Individual members of the same family often respond differently to various stress stimuli. On the other hand, some stress responsive genes may share the same transcription factors, as indicated

by the significant overlap of the gene expression profiles that are induced in response to different stresses (Seki *et al*., 2003).

The zinc finger motifs, which are classified based on the arrangement of the zinc-binding amino acids, are present in many transcription factors and play critical roles in interactions with other molecules (Takatsuji *et al.,* 1998; Moore and Ullman, 2003). A large amount of zinc-finger transcription factors are implicated in important biological processes and many of them often share common characteristic to form a family. So far, several zinc finger families have been found in plants, such as RING-finger, Ethylene Responsive Factor, W-box binding transcription factor (WRKY) and DNA-binding with one finger (DOF) (Lijavetzky *et al.,* 2003; Arnaud *et al.,* 2007). However, most of them are identified as DNA-binding or protein-binding proteins, fewer function as RNA-binding proteins. We found RNA binding Zinc finger ccch domain containing protein (JK265825, InterPro: COIL, TMHMM) to be upregulated under stressed condition in moth bean. CCCH genes in plants perform a variety of functions in different tissues at multiple developmental stages. Genes in the CCCH family encode zinc finger proteins containing the motif with three cysteine and one histidine residues. They have been known to play important roles in RNA processing as RNA-binding proteins (Freemont*,* 1993; Kosarev *et al.,* 2002) in plants and animals.

APETALA2/ETHYLENE RESPONSE FACTOR (AP2/ERF) transcription factors regulate a number of biological processes including development, reproduction, responses to hormones, adaptation to biotic and abiotic stresses (Elliott *et al.,* 1996; Nakano *et al.,* 2006; Stockinger *et al.,* 1997). It includes all genes coding for at least one AP2 domain and can be further separated into the ERF, the AP2, and the RAV families (Liu *et al.,* 1998). The subdivision of the AP2/ERF group into families is based

on the number of AP2 domains present in the proteins, together with the presence of other DNA binding domains. The AP2/ERF family of transcription factor, especially the C-repeat-binding proteins/dehydration-responsive element-binding proteins (CBF/DREB) and ERF subfamily, has been extensively studied in response to drought stress (Yamamoto *et al.,* 1999). In the present study we report an upregulation in AP2/ERF domain containing protein (JK266265) under early heat stress showing symptomatic development of drought stress accompanied by heat stress. No IPS match was found for this transcription factor in this moth bean's library; and hence it could be a new family member of AP2/ERF transcription factor.

Proteolysis of important regulatory proteins is a key aspect of cellular regulation in eukaryotes (Ekaterini *et al.,* 2001; Shigetsugu *et al.,* 2001) and there is evidence that the ubiquitin-proteosome pathway is important in implementation of the plant defense response (Del Pozo and Estelle 1999; Wei *et al.,* 1994). The F-box containing proteins (JK265836) of the SCF complex constitute a family of E3 ligases, key components of the ubiquitin-proteasome pathway (Azevedo *et al.,* 2002), as are many RING finger proteins. U-box proteins are involved in regulated protein degradation. RING u-box domain containing protein (JK265875, InterPro: IPR013083, PTHR12183:SF2 (PANTHER)), in our study showed two fold upregulation under stressed condition. The U-box domain has been suggested to be a modified RING finger motif where the metal-coordinating cysteines and histidines have been replaced with other amino acids (Austin *et al.,* 2002). Known U-box-containing proteins have been implicated in the ubiquitin/proteasome system as its RING finger motif is thought to mediate protein-protein interactions and E3 ligase complex assembly.

WRKY (JK266052) is an upregulated gene in our study (InterPro: IPR003657). WRKY transcription factors (TFs) are key regulators of many plant processes, including the responses to biotic and abiotic stresses, senescence, seed dormancy and seed germination (Chen *et al.,* 2011). Modification of the expression patterns of WRKY genes and/or changes in their activity contribute to the elaboration of various signalling pathways and regulatory networks. WRKY25, WRKY26, and WRKY33 positively regulate the cooperation between the ethylene-activated and heat shock proteins-related signalling pathways that mediate responses to heat stress; and that these three proteins interact functionally and play overlapping and synergetic roles in plant thermotolerance (Li *et al.,* 2011).

An upregulated NAC (JK265717) family domain (InterPro: IPR003441), is one of the largest plant transcription factor families, only found in plants to date (Riechmann *et al.,* 2000). Cbs octicosapeptide phox bemp1 domain-containing protein (JK266056) (InterPro: IPR000270; G3DSA:3.10.20.240 (GENE3D), PTHR11911 (PANTHER), PTHR11911:SF10 (PANTHER), SSF54277 (SUPERFAMILY)) expressed only under stress in the present study. The Phox/Bemp1 (PB1) domains are present in many eukaryotic cytoplasmic signaling proteins. They are dimerization/ oligomerization domains present in adaptor or scaffold proteins and kinases that serve to organize platform that ensure specificity and fidelity during cellular signaling (Hemant *et al.,* 2009; Gilmour *et al.,* 2004).

Calcium is a universal signaling molecule in animal and plants, the transient increase of calcium level during stress is well documented (Heather, 1999). Ca^{2+} signals are core regulator of plant cell physiology and cellular responses to the environment. The channels, pumps, and carriers that underlie Ca^{2+} homeostasis provide the mechanistic basis for

generation of Ca^{2+} signals by regulating movement of Ca^{2+} ions between sub-cellular compartments and between the cell and its extracellular environment. In stomatal guard cells, variation in the timing of stimulus-induced Ca^{2+} oscillations has been correlated with the intensity of both the stimulus and the resultant end response, with alterations in the signature associated with loss of aperture closure (McAinsh *et al.,* 1998; Allen *et al.,* 2000). External Ca2+ or oxidative stress elicited Calcium calmodulin dependent protein kinase kinase i.e CaMKK (JK265783) showed an upregulation under early heat stressed condition. The Ca^{2+}-calmodulin-dependent protein kinase (CaM kinase) cascade includes three kinases: CaMKK; and the CaMKI and CaMKIV, which are phosphorylated and activated by CaMKK. Heat shock triggers cytosolic Ca^{2+} bursts, which is tranduced by Ca^{2+} binding proteins (CBP) such as calmodulin (CaM) and CaM related proteins and then upregulates the expression of HSPs (Liu *et al.,* 2003). No IPS match was found for CaMKK indigenous to this moth bean data set suggesting that this domain might be a naive member of CaMKK family.

Leucine-rich repeats are short sequence motifs present in a number of proteins with diverse functions and cellular locations. All proteins containing these repeats are thought to be involved in protein-protein interactions. Leucine rich repeat containing protein kinase (JK265743, InterPro: seg (SEG)) showed an upregulation. GO annotations says it to be mitochondrial memberane protein having transmemberane receptor protein kinase activity. It has been found to be regulating biological processes such as auxin biosynthesis and leaf and inflorescences morphogenesis.

Expression levels of transcription factors like outer cell layer 4 (ocl4) (JK266321, no IPS match found) controlling the differentiation and

maintenance of epidermal cell fate are members of the HD-ZIP IV class family of plant-specific transcription factors, most of which are specifically expressed in the epidermis of tissues (Vernoud *et al.*, 2009), Sucrose NonFermentable/Snf2 and helicase domain (JK265940, InterPro: PTHR10799 (PANTHER), PTHR10799:SF179 (PANTHER), seg (SEG)), nonexpressed pathogenesis related/npr-1 like protein (JK265775, InterPro: PF11900 (PFAM), SignalP-NN(euk) (SIGNALP)) and sorbitol transporter (JK265951, InterPro: IPR005828; IPR016196; IPR020846; PTHR24063 (PANTHER), PTHR24063:SF85 (PANTHER), tmhmm (TMHMM)) was found to be almost equal under both stressed and unstressed conditions. Constitutive expression of such stress related genes might be ascribed as an eternal adaptation of mothbean.

Trehalose phosphate synthase (JK266275, InterPro: IPR003337, IPR023214, PTHR10788:SF6 (PANTHER) which converts trehalose-6-phosphate to free trehalose was found to be downregulated in treated compared to control along with cathepsin b-like cysteine protease (JK265900, InterPro: IPR000169, IPR000668, IPR13128, G3DSA:3.90.70.10 (GENE3D), SSF54001(SUPERFAMILY)), ribulose bisphosphate carboxylase (JK266105, InterPro: IPR000894), phosphatidyl inositol 4-kinase (JK266348, InterPro: IPR000403; IPR015433; IPR018936; G3DSA:3.30.1010.10 (GENE3D), seg (SEG)) and heat shock coagnate 70Kda (hsc 70, JK265967, InterPro: seg (SEG), SignalP-NN(euk) (SIGNALP), tmhmm (TMHMM)). Heat shock cognate 70 is a family member of Hsp70 which is constitutively expressed in cytosole and is an interactive protein that was found to mobilize hsp70 through plasmodesmata and regulate nuclear import of karyophyrins. It interacts with NLS (nuclear localizing signal)-containing proteins in the cytoplasm before their nuclear import, moreover, mammalian cells transfected with siRNAs (small interfering RNAs) targeted to Hsc70 showed greatly decreased HSF1

activation with expression of HSF1 target genes being dramatically reduced; loss of Hsc70 expression in cells resulted in an increase in stress-induced apoptosis indicating that Hsc70 is a necessary and critical regulator of HSF1 activities (Sang-Gun Ahn, *et al.,* 2005).

However, downregulation of above stress related genes in present study is unexpected but probably their role starts after initial signal transduction where they are involved in osmoregulation and maintenance of cellular function or even before 5 min. Moreover, there exists some possibility of cloning of down regulated ESTs in forward SSH library due to normalization process. This is also possible that certain genes are expressed for very brief period and may show variations in abundance among two RNA preparations.

Thus SSH method used in this used in this study has been proven to be a powerful tool in cloning and identification of differentially expressed genes under given abiotic stress. However, transgenic, RNA interference (RNAi) or some other technique should be used to verify the functions of the above expressed genes. Further characterisation of these genes would be useful to develop novel strategies for heat tolerant crops.

6. SUMMARY AND CONCLUSION

The present investigation carried out to understand early heat response in moth bean at molecular level at seedling level. Heat tolerance level was determined for 100 genotypes of moth bean, where seeds grown for 15 d at 27°C, 2300 lux, 12/12 h photoperiod in plastic pots containing sterilized vermiculite soil saturated with 1/4th MS media, were subjected to a heat stress of 47°C for 2.5 hr, in an oven. Seeds of *V. aconitifolia* var. RMO-40 were grown *in vitro* on filter paper bridge in test tubes containing 1/4 MS media at 27°C under 12/12 h light/dark

photoperiod for 15 days. Seedlings were later on transferred to growth chamber for heat treatment at 42°C for 5 minutes (tester). SSH cDNA library was constructed by using the Clontech PCR-Select^{TM} cDNA subtraction kit, starting with 2μg of mRNA from tester and driver samples, according to manufacturer's instructions. The experiments carried out to has generated good information, as summarised below.

1. Amongst 100 genotypes accessed for their heat tolerance level 9% were tolerant accessions, 28% were moderately tolerant accessions, 46% were moderately susceptible and 17% were susceptible. RMO-40 showed high degree of tolerance towards heat stress.

2. In total 900 clones were obtained and subsequent colony PCR showed that the size of these inserts ranged from 250 to 700bp. Thus we successfully constructed a putative heat stress specific subtracted cDNA library of *V.aconitifolia* var. RMO-40.
3. All EST sequences have been submitted in the dbEST division of GenBank (JK265689-JK266422, JK226915).

4. A total of 488 unigenes (114 contigs and 374 singletons), were derived by cluster assembly and sequence alignment of 738 ESTs. The majority of contigs (70%) contained 3 or fewer ESTs whereas only 1.47% contigs were made from 10 or more ESTs indicating high degree of normalization and subtraction efficiency.

5. 578 ESTs (78%) showed significant BLASTX similarity (<1E-06) in the NCBI non-redundant (nr) database.

6. Out of 206 ESTs (28%) of unknown proteins 160 ESTs (14%) were found to be novel to moth bean.

7. Gene ontology functional classification terms (BLASTX results and GO terms), were retrieved for 479 (65%) sequences, and 339 sequences were annotated with 165 enzyme commission (EC) codes and were mapped to 68 different KEGG pathways. 452 ESTs were further annotated with InterProScan (IPS) and no IPS were assigned to 153 ESTs.

8. A majority of top Blast hits matches were from proteins of legume species, with maximum hits from *Glycine max* and *Vitis vinifera* (see figure 4) *and* no match was found with *Vigna aconitifolia* indicating the novelty of the moth bean unigene dataset.

9. At the second level GO, 224 sequences were assigned to biological process category, 200 sequences to the molecular function category and 223 sequences to the cellular component category.

10. In biological processes, cellular and metabolic processes were the dominant term (55%). In the molecular function category binding (27%) was the most dominant term followed by catalytic activity (24%). In the cellular compartments, cell part (30%) was the most represented term, followed by intercellular organelle (25%).

11. Amongst EST sequences we found only 5 chaperons viz. JK265946 (heat shock coagnate-70Kda protein 1 ATP binding isoform 2 (hsc-70)); JK266046 (DnaJ-like protein) and 3 unknown chaperon like proteins to moth bean (JK265750, JK265751 and JK265912).

12. Our study tracks 29 different nucleic acid and protein binding proteins associated with heat tolerance along with 20 different types of signalling molecules.

13. An expression level of 27 ESTs generated from the above SSH cDNA library was studied through semi-quantitative RT-PCR assay, where 16 ESTs were upregulated, 8 ESTs were downregulated and 3 ESTs showed constitutive expression.

14. In future expression profile of heat linked genes using real time PCR and micro array can be generated.

SSH library constructed using heat tolerant genotype of *Vigna aconitifolia* var. RMO-40 resulted in sequencing, assembly, and annotation of 738 high-quality early heat responsive EST sequences. Several transcripts coding for known stress-related proteins, novel genes (160) with unknown functions that may have a potential role in heat stress tolerance in moth bean were also identified. The up regulation of several ESTs was confirmed by semi-quantitative analysis. The EST dataset and the information about transcription of several genes can be useful for the research community and help identify potential candidate genes for heat tolerance in moth bean. Our study can also serve as an important resource for developing functional markers, full length gene isolations, TILLING, and heat-responsive promoter isolation and in heat functional genomics.

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Construction and Analysis of SSH Library of early heat induced genes of *Vigna aconitifolia* (Jacq.) Marechal

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ABSTRACT

Amongst 100 genotypes accessed for their heat tolerance level 9% were tolerant accessions, 28% were moderately tolerant accessions, 46% were moderately susceptible and 17% were susceptible. RMO-40 showed high degree of tolerance towards heat stress and was used for SSH cDNA library construction. Heat induction was carried out by exposing 14 d old seedlings to elevated temperature of 42°C for 5 min. A total of 488 unigenes (114 contigs and 374 singletons), were derived by cluster assembly and sequence alignment of 738 ESTs; out of 206 ESTs (28%) of unknown proteins 160 ESTs (14%) were found to be novel to moth bean. Only 578 ESTs (78%) showed significant BLASTX similarity (<1E-06) in the NCBI non-redundant (nr) database. Gene ontology functional classification terms (BLASTX results and GO terms), were retrieved for 479 (65%) sequences, and 339 sequences were annotated with 165 enzyme commission (EC) codes and were mapped to 68 different KEGG pathways. 452 ESTs were further annotated with InterProScan (IPS) and no IPS were assigned to 153 ESTs. Around 20 different types of signalling genes and 16 different transcription factors have been shown to be associated with early heat stress in moth bean for the first time. In addition, expression status of 27 ESTs in response to heat stress was evaluated through semi-quantitative RT-PCR assay.

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APPEDICES

List of solution used for experimental analysis

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1. **TBE 5x Stock**

   Tris base – 54 gms

   Boric Acid – 27.5 gms

   0.5M EDTA – 20 ml

   Make Volume to 1 litre and Autoclave

2. **Chloroform : Isoamyl alcohol (24: 1)**

   To make 500 ml add 480 ml of Chloroform and add 20 ml of Isoamyl alcohol.

3. **70%/80%/95% Alcohol**

   70%- Mix 70 ml of Absolute alcohol with 30 ml of ddH_2O.

   80%- Mix 80 ml of Absolute alcohol with 20 ml of ddH_2O.

   95%- Mix 95 ml of Absolute alcohol with 5 ml of ddH_2O.

4. **0.5 M EDTA (pH – 8.0)**

   186.1 gms of Disodium Ethylene Diaminetetra Acetate. 2 H_2O to 800 ml of H_2O. Shake vigoursly on a magnetic stirrer for several hr. Adjust the pH to 8.0 with NaOH. Dispense into aliquots and sterilize by autoclaving.

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5. **1 M Tris**

Dissolve 121.1 gms of Tris in 800 ml of H_2O. Adjust the pH to desired value by adding concentrated HCl.

PH	HCl
7.4	70 ml
7.6	60 ml
8.0	42 ml

Adjust the volume of the solution to 1 litre with ddH_2O. Dispense into aliquots and sterilize by autoclaving.

6. **T_{10} E_1 pH – 8.0**

1 ml of 1M Tris (pH – 8.0) added to the 200µl 0.5 M EDTA – pH – 8.0 and make the volume 100 ml.

7. **0.1 % DEPC treated water**

5 ml of DEPC mixed with 5 lt MQ water and stirred it with spatula for 5-10 ml.

8. **ß – mercaptoethanol (BME)**

It is obtained as 14.4 M solution. Store in a dark bottle at 4°C.

9. **20 X MOPS**

41.9g MOPS
6.8g Sodium Acetate
2.6g EDTA
400ml DEPC treated MQ water.

All contents were mixed and pH was adjusted to 7.0 with NaOH.
Final volume=500ml with DEPC water.

10. **Ethidium Bromide (10 mg/ml)**

Added 1 gm of Ethidium Bromide to 100 ml of H_2O. Stirred on

magnetic stirrer for several hr to ensure that the dye has dissolved. Wrap the container in aluminum foil or transfer the solution in dark bottle and store at room temperature.

11\. **Agarose 1.5 %**

1.5 gms in 100 ml of 1 x TBE

12\. **Gel loading dye (10X)**

0.25 gms of Bromophenol Blue

0.25 gms of Xylene cyanol

50% glycerol

Make 100 ml final volume with 50% glycerol

13.		242 gm
	50X TAE buffer (per litre)	57.1 ml
	Tris base	100 ml
	Glacial acetic acid	
	0.5M EDTA(pH 8.0)	
14.	**20 X RNA dye**	
	1X	MOPS
	50%	Glycerol
	0.1%	Bromophenol Blue
15.	**100ml formaldehyde gel**	
	1g	Agarose
	1g	20X MOPS
	0.48ml	37% formaldehyde
	28ml	DEPC MQ water
16.	**Ampicillin**	100 mg/ ml