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Full Length Research Paper

Isolation and expression analysis of *Berberis chitria* Lidl. specific transcripts using subtractive hybridization technique

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The method of suppressive subtractive hybridization has been used to detect and clone differentially expressed genes during the development of *Berberis chitria* Lidl. in cold stress condition. For this, we used two different species, *B. chitria* Lidl. and *Berberis asiatica* Roxb., *B. chitria* containing high yield of berberine and *B. asiatica* has less. We used *B. chitria* Lidl. as tester and *B. asiatica* as an inducer of the starting material and were compared with mRNA-cDNA. We constructed cDNA library with insert size range from 100 to 600bp from *B. chitria* roots at developmental stage. The cDNA after subtraction were cloned and a total 28 clones were identified, which are unregulated in *B. chitria* root tissues, among these 8 were novel, while 20 showed homology with different genes in database. An explanation has been given regarding the role of these genes in the developmental process. Study of the genes identified in this report may enhance our understanding of the genetic circuit involved in the development of *berberis*.

Key words: Suppressive subtractive hybridization (SSH), *Berberis chitria* Lidl., ESTs.

INTRODUCTION

Berberis L. is an important medicinal genus of the family Berberidaceae (Bhakuni et al., 1968). About 500 species are available in temperate and subtropical regions of Europe, Asia, Africa, North America and South America. In India, *Berberis* are frequently distributed in Himalayan regions and maintained by the local peoples. The mixture of *berberis* root extract is yellowish called *rasaut*. It contains isoquinoline alkaloid berberine, which is important from the medicine point of view. British pharmacopeia patented a drug ‘orisol’ made by berberine. It was observed that percentage of the berberine differ from plant to plant and place to place. We have selected two species, *Berberis chitria* Lidl. and *Berberis asiatica* Roxb. *B. chitria* contained high yield of berberine (5%) and *B. asiatica* less (1.02%) in root tissues. Many genes are involved in beberine synthesis during plant development in cold stress. Gene expression played an important role during development and alkaloid synthesis in plants.

The present study revealed that the analysis of Express Sequence Tags (ESTs) is involved in the development and cold stress of *B. chitria* Lidl. Identification of genes is specially expressed when shrub is prepared for growth initiation, development and synthesis of bioactive compound. The products of genes are presumed to play major role in complex networks of development and biochemical process (Seki et al., 2001). The analysis of differences between two complex genome holds promise for the discovery of genes that are present or expressed

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specially in the one and absent or reduced numbers in other system (Diatchenko et al., 1996). We employed subtractive screening of root tissues in *B. chitria* (tester) and *B. asiatica* (driver) and characterize *B. chitria* specific transcripts. Many plants ESTs have been deposited in the public databases NCBI. This resource contains a vast amount of information that serve as a window for the array of gene expressed in higher plants, a different developmental stage and under a verity of environmental condition. Thus, EST development is a systematic means to catalog the transcriptome and transcript profiling with gene arrays allowing detailed studies of complex cellular process including metabolism (Girke et al., 2000; White et al., 2000) and mechanism of plant adaptation to variety of biotic and abiotic (Kawasaki et al., 2001).

MATERIALS AND METHODS

Plant material

Root tissues of both species (*B. chitria* Lidl. and *B. asiatica* Roxb.) were collected from cold and warm climatic conditions and immediately freezed in liquid nitrogen.

RNA isolation

Total RNA from the immediately freezed roots was isolated as described by Pawlowski et al. (1994).

mRNA isolation

Poly (A^+) RNA was isolated using Oligotex, according to manufacturer protocols (Qiagen, Germany).

cDNA synthesis

Single and double stranded cDNA were synthesized from the ultra pure mRNA using cDNA kit of Clontechs SMART PCR-synthesis kit Clontech, USA.

Suppression subtractive hybridization (SSH)

Suppression subtractive hybridization (SSH) was carried out using PCR-select cDNA subtraction kit according to manufacturers protocols (Clontech, USA). Tester and driver double stranded cDNA were prepared from 2 µg of *B. chitria* and *B. asiatica* using Clontechs SMART PCR-synthesis kit. Tester cDNA was digested with R*sa* I at 37°C for 1.0 h and then ligated to adaptors 1 and 2R in separate reactions at 4°C for overnight. After ligation, driver cDNA was added to each of the tester samples, which were subsequently resuspended in the hybridization buffer, heat denatured and then allowed to anneal at 68°C for 8 h. Then, two samples from first hybridization were mixed together and fresh denatured driver cDNA was added to the sample followed by incubation with two different nested primers performed to amplify and then inserted into the cloning vector $SK^{+/-}$ (Bangalore genei, India).

Dot blot analysis

Total 28 cDNA clones were obtained from subtractive cloning. Dot blots were performed on Hybond NX (Amersham Pharmacia Biotech. USA) with manual pin spotter / replicator device. The total cDNA probes were synthesized from *B. chitria* and *B. asiatica* root tissues and used for triplicate blots.

Northern hybridization

After dot blot analysis, screened clones were used for the confirmation of their expression. The RNA (5 µg) samples of *B. chitria* and *B. asiatica* was electrophoresed on a 1.4% agarose-formaldehyde gel and transferred on to Hybond NX (Amersham Pharmacia Biotech. USA). The membrane was hybridized with [α-^{32}p] dCTP-labeled probes at 65°C for overnight and washed twice under highly stringent conditions as described by Sambrook et al. (1989). The washed membrane was then exposed to X-ray film at 80°C.

Nucleotide sequencing and data analysis

The inserts were sequenced with M_{16} and T_7 promoter primers, which flank the multiple cloning site of $SK^{+/-}$ phagemid vector. DNA sequencing was performed with an automatic DNA analyzer (ABI prism 377). To investigate the possible identities of these Express Sequence Tags (ESTs), search against DNA and protein databases using the BLAST program at National Center for Biological Information (NCBI) was carried out (Altschul et al., 1997).

RESULTS

Express Sequence Tags (ESTs) analysis

A total of twenty clones were up regulated and eight in novel in the root tissue of *B. chitria*. To investigate the possible identities of these clones, each cDNA was sequenced and sequences were used to search homology. The obtained nucleotide sequence (all EST singletons and the long sequences) were manually scrutinized for using NCBI ORF-finder (http://www.ncbi.nim.nin.gov/gorf/gorf.html) and the BLAST analysis of the largest ORFs against the National Centre of Biotechnology Information (NCBI) database (NIH, USA) was performed. BLAST analysis of some of the nucleotide sequence against the NCBI database was performed. Any similarities with a score more than 20 or an e value ((Table 1).

An efficient of PCR-based cDNA subtraction method was employed for the isolation of developmental and cold stress responsive transcripts, which are present in *B. chitria* and absent or reduced in *B. asiatica* called difference cloning (Wieland et al., 1990).

An initial step towards the analysis of global gene expression during the development of *B. chitria* in cold stress condition is required. Here, a catalog of candidate genes was developed for regulated cold stress and development by selective, single pass sequencing of potential unregulated cDNA. Subtractive cloning of genes that are involved in the basic cellular mechanism during development of *B. chitria* in cold stress has been described in the present study.

The gene expression patterns obtained from this analysis are reasonably consistent among the triplicate blots. Based on conventional knowledge of development and stress-gene expression in plants, many of the genes that were expected to respond positively to the cold

Table 1. cDNA clones isolated by subtractive cloning of *B. chitria* Lidl.

Clone	Accession No.	Gene bank match	Annotation	*E*-value
Signal transduction and cellular communication				
BC1	EG949787	AY582736	Regulatory protein	9e-54
Cell division and DNA replication				
BC3	EG949789	Novel	ATPase	
BC28	EG949786	NM119579	Cyclin-dependant protein kinase (CDK)	5e-27
Metabolism				
BC5	EG949791	NM001006378	Amino acid permease	7e-20
BC18	EG949776	DQ356679	Arylsulfotransferase	2e-30
Stress related				
BC8	EG949766	AY372069	Heat shock protein	2e-76
BC22	EG949780	AB018117	Spermidine synthase	2e-72
BC23	EG949781	DQ336890	S-adenosyl-L-methionine synthetase	2e-41
Transcription factor				
BC4	EG949790	AY530925	Predicted protein GAL4	7e-04
BC6	EG949792	AP008215	G-protein coupled receptor	6e-49
BC9	EG949767	DQ537337	Arylphorin gene-specific binding protein-2	1e-43
BC12	EG949770	DQ776899	CBF1, Transcriptional activator	2e-36
BC19	EG949777	XM_504128	Ubiquitin	9e-37
Putative protein				
BC10	EG949768	X16469	7s seed protein	7e-30
Growth and development				
BC7	EG949765	DQ219417	Opie2 gag protein	1e-77
Hypothetical protein				
BC13	EG949771	DQ353752	Hypothetical protein	2e-106
BC16	EG949774	AY517651	Hypothetical protein	1e-24
BC17	EG949775	DQ846399	Hypothetical protein	6e-10
BC21	EG949779	AP008215	Hypothetical protein	7e-63
BC24	EG949782	NM008929	Hypothetical protein	3e-35
BC26	EG949784	D85726	Hypothetical protein	5e-59
Novel genes				
BC2	EG949788	CP000300	Novel	
BC11	EG949769	DQ226510	Novel	
BC14	EG949772	AJ633716	Novel	
BC15	EG949773	AY847099	Novel	
BC20	EG949778	AC151957	Novel	
BC25	EG949783	DQ916281	Novel	
BC27	EG949785	D16107	Novel	

treatments exhibited such patterns in the dot blots (Figure 1). After screening, 28 clones selected for dot blot analysis showed indication of induction development and cold stress expression ESTs. The developmental and cold stress related expression of these genes is further confirmed by northern-blot analysis (Figure 2).

As plant grows in natural habitat, many environmental and edaphic factors played an important role in their

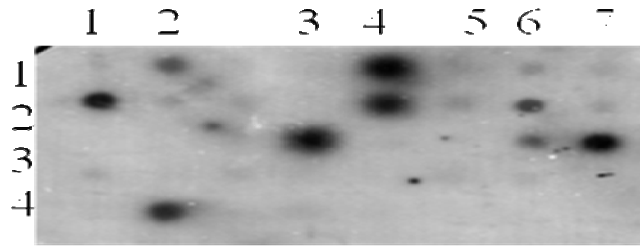

Figure 1. Dot blots analysis showing the positive expression of transcripts specifically for development and cold stress in *B. chitria* Lidl.

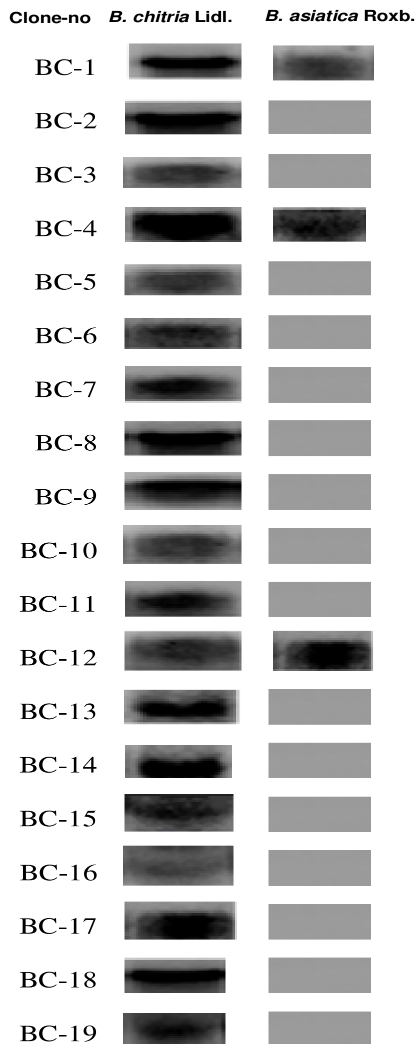

Figure 2. Northern blot analysis with *B. chitria* Lidl. and *B. asiatica* Rox. RNA , were confirmed by hybridized with subtracted probes. Transcripts obtained from the study specifically expressed in root tissues. *B. chitria* Lidl. showing all positive expression with twenty eight probes and *B. asiatica* Roxb also showing positive expression with three probes.

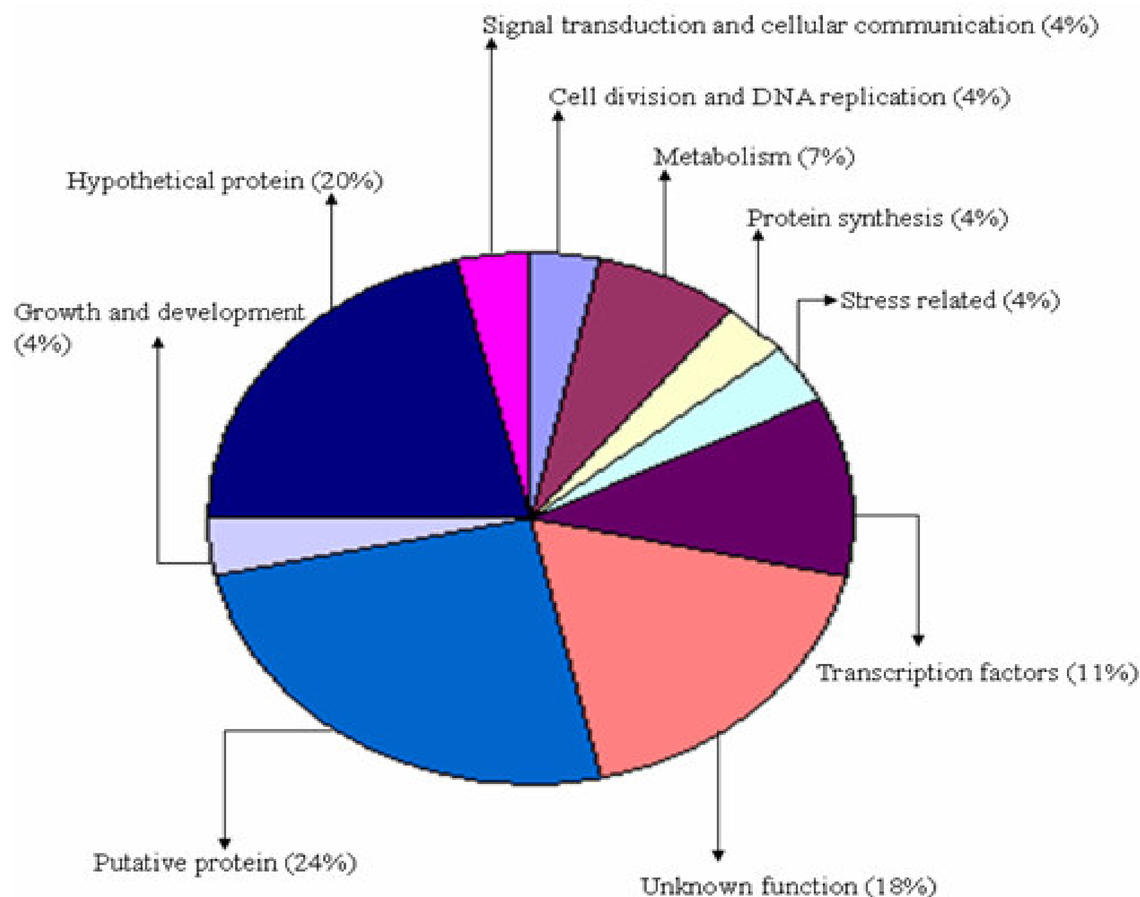

Figure 3. Percentage distribution of the redundant ESTs according to functional categories.

growth and development, such as cold temperature, humidity, light, soil, heat and soil rich minerals. These factors work as a stress-gene regulator affects their expression. We constructed cDNA library with insert size range of 100 to 600 bp from root (at developmental stage in cold stress) of *B. chitria.* We have used selective cDNA sequencing approach as a means to increase our probability of sampling the up regulated genes; we would expect a good presentation of the cellular process involved in the stress response mechanism and development of *B. chitria*. We have used all ESTs singletons and the long sequence to assign a putative function to each tag by comparison (Blast) with the non-redundant nucleotide and protein databases of the gene bank (Altschul et al., 1997). The ESTs were manually annotated using the information from the highest scoring matches (31%) returned by Blast search. The EST groupings according to functional categories are summarized in Figure 3. Of the 28 total redundant ESTs, 38% can be identified by significant similarity with known genes or proteins. Other 18% were classified as 'Unknown functions' or do not have significant similarity with known protein at the threshold e-valve. Members of this group include those that are similar only to other unknown plant ESTs (expressed proteins) or those that are similar only to hypothetical protein (20%) and 24% with putative protein.

As expected, these genes involved in basic cellular housekeeping are widely represented in the subtracted set. The result is indicated by the occurrence of transcripts involved in metabolism e.g. steroid biosynthesis, protein catabolism and ATP synthesis, growth and development (cell division and elongation, modification or synthesis of new cell-wall materials) DNA replication and repair, *de navo* protein synthesis, protein folding and destination (e.g. chaperones and transporters) and cellular communication. The transcript-tion of some of these housekeeping genes is integrated with the overall developmental and cold stress response mechanism, which is a question that might be addressed in the future through in depth analysis of the gene regulatory circuits.

The functional categories that can be directly associated with the development and cold stress related in gene expression patterns are those that encode for known or putative stress-related proteins and those with

putative roles in the regulation of developmental or stress related response (transcription factors, signal transduction and cellular communications). When combined, these categories constitute 49.9% of the total redundant ESTs-set indicating the high activity of these cellular processes at the specific developmental and environmental status of *B. chitria* used in this experiment. The majority of the genus under development and stress related category encode for proteins with known or putative roles in the prevention and or/ repair of cellular injuries caused by low temperature, ion toxicity ND light stress. Many of these genes are also known to be regulated by stress-response signaling hormones, such as abscisic acid (ABA) and ethylene (Shinozaki et al., 2000).

DISCUSSION

The present study has revealed the occurrence of genes that are involved in stress response and showed common physiological effects of cold stress and development in *B. chitria* Lidl. Clones obtained from the present study showed function in like; Cellular signaling is the primary event that leads to transcriptional activation of inducible genes. The process related to cellular signaling in the development of *B. chitria* in cold stress can be deduced from the ESTs encoding for proteins with known or predicted functions as membrane–bound receptors, cell cycle, cyclin dependant protein kinase, protein phosphates and mediator of protein-protein interactions. The integration of developmental and stress-related transcriptional machineries can also be inferred from the proteins with known or predicted functions as transcriptional regulator for both conditions. These ESTs were identified by similarities to of proteins containing signature DNA–binding motif including AP2 and zinc finger, because many of the known stress related transcriptional regulator in plants contain some of these signature domains (Stockinger et al., 1997; Liu et al., 1998; Chen et al., 2002). The current ESTs provide some insight on the possible range of regulators that function at low temperature and stress related response during development in natural habitat.

A total 28 positive clones were obtained after the subtractive hybridization between *B. chitria* and *B. asiatica*. The expression patterns of ESTs obtained from this analysis are reasonably consistent among the triplicate blots. The abundance of transcripts in *B. chitria* and *B. asiatica* were probed with radiolabeled probes of isolated clones. The developmental and stress related expression of these transcripts is further confirmed by Northern-blot analysis. The number of ESTs assembled in each contig allowed the identification of the most highly expressed genes in *B. chitria* in cold stress and three ESTs show affinity with *B. asiatica*. Explanation regarding the function clones is defined as; Cis-acting DNA regulatory elements in the plant (C-repeat/ dehydration response element (DRE)) that stimulates transcription in response to low temperature and water deficiency. A group of genes encoding seed maturation protein was up regulated in *B. chitria*. The nucleotide and corresponding amino acid sequence data revealed that up regulated clones isolated in this study were significantly homologous to transcription regulator that are involved in mechanism of DNA replication, Cell division, protein synthesis and cellular metabolism.

Heat shock protein is stress protein that is present in all cells. These appear when the cell is under heat stress or other stress. Its expression is increased when the cells are exposed to elevated temperatures and regulate their expression transcriptinally. The dramatic up regulation of heat shock protein induced mostly by heat shock factor (HSF) is a key part of the heat shock response. They are induced by various types of environmental stresses, such as cold, heat, oxygen deprivation and also monitors carrying of old proteins to the cells recycling bin and can help to newly synthesized protein fold properly. Heat shock proteins are also believed to play a role in the presentation of peptides on the cell surface to help the immune system to recognize diseased cells.

DNA binding protein binds in the 5' upstream region of the arylphorin gene and activates transcriptionally. Arylphorin proteins were found in two forms: ABP-1 and ABP-2 (Adachi et al., 1993). DNA binding activity of both ABP-1 and ABP-2 were found to bind same sequence in the arylphorin gene with the same affinity and stability. CBF1 (C-repeat / DRE binding factor) is dehydration response element and play important role in cold and dehydration of regulated gene expression in *Arabidopsis* (Stockinger et al., 1997). CBF bind and regulates the cold acclimation response transcriptionally. CBF amino acid sequence indicates that the protein has a potential nuclear localization sequence and a possible acidic activation domain. The transcript levels for CBF1, which appears to be a single or low copy number gene, did not change appreciably in plants exposed to low temperature or in detached roots subjected to water deficit. BC1 protein covers a wide variety of functions including adaptor/regulatory modules in signal transduction, pre-mRNA processing and cytoskeleton assembly. WD rich protein is another class of protein, which is up regulated in differentiating tissues. Such proteins are found in eukaryotic systems, which play an important role in regulation of wide range of functions, such as signal transduction, transcription and proliferation (Sompornpailin et al., 2002). One of such genes Msgb1, reported by Mckhann et al. (1997) belongs to this class of proteins, which is found to be express in young embryos. BC1 transcript has been found to be abundant in dividing cells and its function has been related to signal transduction in response to hormone mediated cell division. Arylsulfate sulfotransferase enzyme is occurring in eukaryotic intestinal bacterium. This enzyme catalyzed

the transfer of the sulfate group of phenylsulfate esters to phenolic compounds (Konishi-Imamura et al., 1994). One clone shown homology with ubiquitin is a small regulatory protein that is involved in post-translational modification by the covalent attachment of one or more ubiquitin monomers. Ubiquitin is a regulatory protein that is known as ubiquitous in eukaryotics. It is encoded with genes whose translation products are fusion protein. The basic function of ubiquitin is to mark other proteins for destruction, known as protolysis and also mark transmembrane proteins (receptors) and fulfill several signaling roles within the cells. Initial cell signaling is permitted to other proteins that contain ubiquitin binding domain in order to interact with the mono-ubiquitinated substrate. Ubiquitin is involved in many cellular processes in protein cyclin during the G_1 phase of mitosis and thus plays an important role in regulating the cell cycle and also involved in DNA repair, embryogenesis, transcription regulation and apoptosis. Another group of clone is encoded with polyamine that played important role in plant defense against environmental stress. Spermidine synthase (SPs) is an enzymes that play important role in plant defense against environmental stress (Bouchereau et al., 1999) and enhance tolerance to various stress including chilling, freezing and hyperosmosis. During exposure to chilling stress (5˚C) arginine decarboxylase activities have been increased. It consists of low-molecular weight aliphatic amines.

In plant, polyamines play important role in growth, development and reproduction (Kakkar et al., 2000; Martin-Tanguy et al., 2002). It has been shown that a high cellular level of polyamines correlates with plant tolerance to a wide array of environmental stress, such as low and high temperature (Roy et al., 1996; Shen et al., 2000). S-adenosyl-L-methionine synthetase (SAMS) has been found in two main clusters $SAMS_1$ and $SAMS_2$ distinguished by characteristic amino acid exchanges at specific position (Mckhann et al., 1997). Both genes are expressed in plant growth and various stresses (elicitor, nutritional down-shift, salt s-adnosy-L-methionine synthetase metabolites from the methyl cycle (L-homocysteine) or related pathways (L-ornithine, putrescine and spermidine). Cyclin-dependent protein kinase (CDK) encoded by CDC28 is a master regulator of cell division. CDC28 activity controls the timing of mitotic commitment, DNA replication, spindle formation and chromosome separation. Environmental stimuli and progress through the cell cycle are monitored through checkpoint mechanisms that influence CDC28 activity at key cell cycle stages. CDK are involved in regulation of the cell cycle and basal transcription by phosphorylating in animals (Umeda et al., 1998). Periodic accumulation and degradation of the CDK inhibitor in G_1, plays a role in setting a threshold of cyclin levels that is important in determining the length of the pre-short G_1 phase and in ensuring the correct order of cell cycle events.

Clone BC3 showed homology with ATPase that catalyzes the decomposition of ATP into ADP and a free phosphate ion. This dephosphorylation reaction releases energy. ATPase imports many of the metabolites necessary for cell metabolism and export toxins. Besides exchangers, other categories of transmembrane ATPase include co-transporters. Another clones encoded of amino acid permease enzyme catalyzes the stereo-specific transfer of amino acids, organic acids containing one or more amino substitutes across a biological membrane. Cellular metabolic needs are fulfilled by transport of amino acids across the plasma membrane. Transcription factors bind to DNA and enhance the gene expression during the development of cell. GAL-4 is an activation domain of DNA binding protein. It is associated with transcription regulation and chromatin dynamics. CBF transcription regulator is involved in of physiological functions including control of embryonic development, cell differentiation and homeostasis. BCE1 protein encoded signal transducers and activations of transcription. This protein activates transcription in response to extracellular signals. Signal-mediated activation of membrane receptor- kinase leads to auto-phosphorylation of the receptors. Histone protein binds to positive 5'CCAAT3' sequence, which is present in most eukaryotic promoter regions (CAAT Box). These activators bind DNA as heterodimers made by proteins CBF-A and CBF-B. Both subunits are required for DNA binding.

Clone BC-6 showed partial homology with ANF (Actrial natriuretic factors). ANF receptor is ligand binding domain based on structure of ligand receptor complex. It play important role during cell growth and cell differentiation and provide defense against pathogens. These types of receptors constitute an important super family of transcription regulators that are involved in widely diverse physiological functions, including control of embryonic development, cell differentiation and homeostatis. These receptors worked as dimeric molecules in nuclei to regulate the transcription of target genes in a ligand responsive manner. The ligand-binding domain acts in response to ligand binding, which caused a conformational change in the receptor to induce a response thereby acting as a molecular switch to turn on transcriptional activity.

With the documentation of differentially expressing clones in our study and other related studies (Gong et al., 2001; Kawasaki et al., 2001), it is possible to find newer avenues for engineering stress tolerance in plants. There are two major points emerging from this study that need further consideration. Firstly, it has been proposed that the transcriptional response against low stress is an early and transient event (Posas et al., 2000; Kawasaki et al., 2001). Secondly, the method adopted for the isolation of low stress-associated cDNA clones in this study showed several false clones. Theoretically, we expect that all the clones isolated in the present study should have higher level of transcripts in the tester as compared to driver

sample, but this was not true in all cases. For instance, northern analysis showed that transcripts corresponding to BC1, BC4 and BC12 were expressed strongly in the driver sample as well. It is possible that the transcripts corresponding to these clones were present in higher amounts in both in the driver and tester, which could not be subtracted employing one round of subtraction. It has been proposed that subtractive hybridization do not achieve sufficient enrichment of the sequence that occur only in the tester (the target), partly because of the high complexity, which prevents effective and complete hybridization and thus removal of the driver sequence.

Based on the present study, it is clear that there are several unknown component in the stress responses. The novel ESTs were found differentially regulated in tolerance and basic cellular mechanism, which need further characterization. The presence of considerable information in the public database offers an unmatched opportunity to characterize low stress response genes in the future. Such types of study were carried out by some other research groups on plants (Yucheng et al., 2007; Bo et al., 2007; Yamaura et al., 2010; Taro et al., 2010).

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