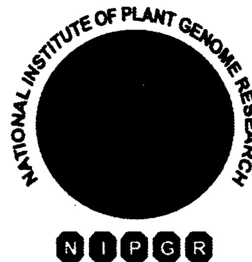
Transcripts profiling of a drought-tolerant chickpea (*C. arietinum*) cultivar under drought

Thesis submitted to
Jawaharlal Nehru University
New Delhi
For the award of the degree of

Doctor of Philosophy

By

Deepti Jain

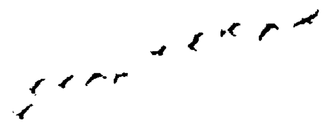


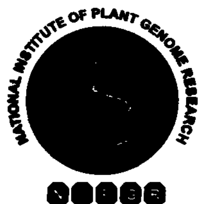
National Institute of Plant Genome Research
New Delhi-110067, INDIA

2009

Dedicated to
All those who taught me...

"Struggle for Existence & Survival of Fittest"

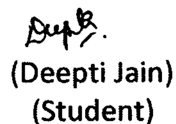




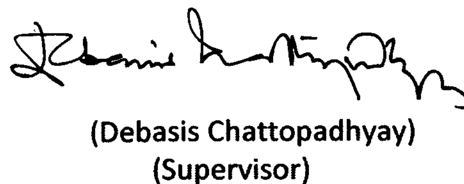
NATIONAL INSTITUTE OF PLANT GENOME RESEARCH
(Formerly known as National Centre for Plant Genome Research)
(An Autonomous Institution of the Department of Biotechnology, Ministry of Science and Technology, Government of India)
Aruna Asaf Ali Marg, Post Box Number 10531, New Delhi-110067

CERTIFICATE

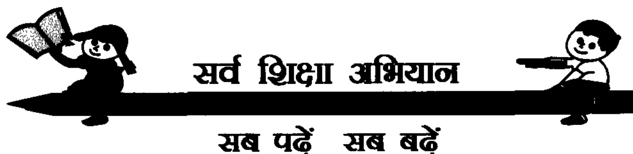
The research work embodied in this thesis "Transcripts profiling of a drought-tolerant chickpea (*C. arietinum*) cultivar under drought" has been carried out at the National Institute of Plant Genome Research, New Delhi. The work is original and has not been submitted so far in part or in full for any degree or diploma of any university.


(Deepti Jain)
(Student)

(Director)


(Debasis Chattopadhyay)
(Supervisor)

Ph. : 26741612, 26741614, 26741617, Fax : 91-11-26741658
E-mail : nipgr@nipgr.res.in; Website : http://www.nipgr.res.in



DECLARATION

I hereby declare that except where due acknowledgement has been made, the research work embodied in this thesis entitled **"Transcripts profiling of a drought-tolerant chickpea (*C. arietinum*) cultivar under drought"** has been carried out by me under the supervision of Dr. Debasis Chattopadhayay, at National Institute of Plant Genome Research, New Delhi, India. The work has not been submitted previously, in whole or in part, to qualify for any other academic award.

APRIL' 2009

DEEPTI JAIN
Ph. D Student
National Institute of Plant Genome Research
New Delhi-110067, INDIA

Acknowledgement

I know that people are always interested to read this part but I do not know really whether I will be able to express my intense and huge feelings within these pages in a short notice. It is sometimes difficult to express gratitude in words because the feelings go beyond them. These five years of my Ph.D. have changed the course of my thoughts and I have rediscovered myself with the help of some brilliant people I have been associated with.

Firstly, I offer my deepest gratitude and respect to my PhD supervisor, Dr. Debasis Chattopadhyay for his excellent guidance, mentoring, encouragement and support throughout the course of this study. My sincere gratitude and thanks for accepting me as his PhD student and guiding me all the way. His motivation in difficult times have enabled me to achieve all the milestones in time.

I would like to thank Prof. Akhilesh Kumar Tyagi, Director, NIPGR for providing world-class laboratory facilities and scientific environment during the tenure of my work.

I am highly indebted to our previous director Prof. Asis Datta for being kind and supportive during my research work. His ever-dynamic personality and energetic nature always inspired me, which never allowed feeling tired.

I am also thankful to Council of Scientific and Industrial Research, New Delhi for providing financial support.

I am also obliged to Dr. Nilanjan Roy and Dr. Natrajan, who advised me on designing the experiments of yeast study and also thank them for helping me in selection of genotypes and supply of vectors.

I acknowledge and pay my cordial respect and gratitude to all the faculty members of NIPGR, Prof. Sushil Kumar, Dr. Sabhyata Bhatia, Dr.Shubhra Chakarborty, Dr. Niranjan Chakraborty, Late Dr. Jayanti Sen, Dr. Praveen Verma, Dr. Alok Kumar Sinha, Dr. Manoj Majee, Dr. Gitanjali Yadav and Dr. Naveen Bisht for their help and support. I express my sincere thanks especially to Dr. Manoj Prasad and Dr. Aishverya Laxmi for their valuable suggestions and support at all times in various ways.

I also acknowledge all my loving labmates Parul, Rajeev, Smriti, Dhanya, Nazia, Mukesh, Neeraj, Swati, Pranav, Kajal and Charu for their pleasant friendship and lively atmosphere in the lab. I would like to take the opportunity to offer my special thanks to my seniors Rakesh Sir, Vineeta dee, Archana dee and Sarita dee for their elderly advices and friendly behavior.

I would also like to thank to Swagata dee, Bhumi dee, Raj Gaurav sir, Manikant sir, and Shalu dee for helping me in numerous ways. I shall never forget the advices and friendly company of my Pantnagar seniors, Lalit sir, Nasheeman mam and Aarti mam. I also would like to thank all my batchmates Sumit, Vinay, Prasad, Prabhakar, Susheel, Saurabh and Kundan for their help and support.

My special thanks to Aditi and Manjul for helping me in confocal studies and steromicroscopy work. They also taught me several techniques related to Arabidopsis, for which I will always be grateful to them.

Thanks also need to be given to CIF members Ashok ji and Surendra ji for Real-Time PCR analysis, Shobaram ji for microscopy, Anand ji for helping me in confocal microscopy and Arun ji for DNA sequencing facility. I am thankful to Bahadurjee, Vikas and Bikash for helping me in lab as well as maintaining the plants in green house.

My special thanks to Vibhu, brother Rahul, bhabhi Nupur, sister Sakshi, jijs Vishal, Vishu and Pooja for their immense patience and support to me. My nephew Aman and niece Cherrie for always trying to remind me of the existence of a life outside the lab. It would be like a drop in the ocean of words that can never reach their marks for my parents, who gave me the strength and courage to move ahead in life.

Acknowledgements are inherently endless and incomplete, and I wish indulgence from the many friendly and helpful people who might have slipped my mention but their help deserves the same gratitude.

Date: 23/4/09

Deepti.

Deepti Jain

Table of Contents

Abbreviations

CHAPTER 1 INTRODUCTION 1-4

CHAPTER 2 REVIEW OF LITERATURE 5-32

2.1 Abiotic Stress 6

2.1.1 Drought stress 6

2.1.2 Salinity stress 9

2.1.3 Cold stress 11

2.1.4 Heat stress 11

2.1.5 Oxidative stress and Reactive oxygen species (ROS) 11

2.2 Regulatory Proteins In Abiotic Stress Signaling 12

2.2.1. Sensory kinases 13

2.2.2. Ca^{+2}-dependent protein kinases (CDPKs) and CBL interacting protein kinases (CIPKs) 14

2.2.3. MAPK 15

2.2.4. Protein Phosphatases 15

2.2.5 Transcription factors 16

2.2.5.1 Transcription factor domains 16

2.2.5.2 Plant TFs 17

2.2.6 Other regulatory factors 21

2.3 Model Systems To Study Functions Of Regulatory Proteins 21

2.3.1 Model plants can be employed to study abiotic stress regulation 22

2.3.2 Yeast as a model employed to study abiotic stress regulation 26

2.3.2.1 Osmopathways in yeast 27

2.3.2.2 Study of regulatory factors in yeast 30

CHAPTER 3 MATERIALS AND METHODS 33-65

3.1 Materials 33

3.1.1 Plant materials 33

3.1.2 Yeast strains 33

3.1.3 Bacterial strains 33

3.1.4 Plasmid vectors 33

3.1.5 Chemicals and materials 34

3.1.6 Oligonucleotides used in the present study 34

3.1.7 Concentrations of various antibodies used in the present study 36

3.2 Methods 36

3.2.1 General Sterilization Procedures 36

3.2.2 Nutrient media 36

3.2.3 Plant growth conditions and maintenance 37

3.2.4 Recombinant DNA techniques for cloning and DNA analysis 37

3.2.4.1 Polymerase Chain Reaction 37

3.2.4.2 Reverse Transcription PCR (RT-PCR) 37

3.2.4.3 Separation of DNA on Agarose Gels 38

3.2.4.4 Elution of DNA from agarose gel 38

3.2.4.5 Purification of PCR products 38

3.2.4.6 T/A cloning of PCR Products 38

3.2.4.7 Restriction Digestion of DNA Molecules 39

3.2.4.8 Ligation of DNA Fragments 39

3.2.4.9 Preparation of Competent Bacterial Cells 39

3.2.4.10. Transformation 40

3.2.4.11. Confirmation for the presence of insert 40
3.2.4.12. Alkaline lysis midiprep of plasmid DNA 41
3.2.4.13 Purification of Plasmid by PEG Precipitation for Sequencing 41
3.2.4.14 Spectrophotometeric estimation of nucleic acid 41
3.2.5 Molecular analysis of gene expression 42
3.2.5.1 Treatments for RNA isolation 42
3.2.5.2 Isolation of RNA from chickpea seedlings 42
3.2.5.3 Isolation of RNA from yeast cells 43
3.2.5.4 RNA quantification 43
3.2.5.5 Denaturing formaldehyde gel for RNA electrophoresis 43
3.2.5.6 Transfer of total RNA on Nylon Membrane 44
3.2.5.7 Radioactive probe preparation, purification and hybridization 44
3.2.5.8 Washing and Autoradiography 45
3.2.6 Southern hybridization 45
3.2.6.1 Isolation of plant genomic DNA 45
3.2.6.2 Digestion of genomic DNA 46
3.2.6.3 Southern blotting 46
3.2.7 Construction of subtractive cDNA library 46
3.2.7.1 Plant materials and stress treatments 46
3.2.7.2 Isolation of high quality total RNA and mRNA 47
3.2.7.3 First strand cDNA synthesis 47
3.2.7.4 Second-strand synthesis 47
3.2.7.5 Rsa I Digestion 48
3.2.7.6 Adaptor Ligation to tester cDNA 48
3.2.7.7 First Hybridization 48
3.2.7.8 Second Hybridization 49
3.2.7.9 PCR Amplification 49
3.2.7.10 Secondary PCR 50
3.2.7.11 Cloning of amplified differential cDNA 50
3.2.8 DNA array/Macroarray 51
3.2.8.1 DNA array preparation 51
3.2.8.2 DNA array hybridization 51
3.2.8.3 Data analysis for DNA-array 51
3.2.9 FULL-LENGTH GENE ISOLATION BY 5′-RACE 52
3.2.9.1 First Strand cDNA Synthesis 52
3.2.9.2 Purification of cDNA 53
3.2.9.3 TdT tailing of cDNA 53
3.2.9.4 PCR of dC-tailed cDNA 53
3.2.9.5 Nested Amplification 54
3.2.10 Promoter isolation by Genome Walking 54
3.2.10.1 Determination of quantity and purity of genomic DNA 54
3.2.10.2 Digestion of genomic DNA 54
3.2.10.3 purification of DNA 55
3.2.10.4 Ligation of Genomic DNA to Genome-walker adaptors 55
3.2.11 DNA -Binding Assay (EMSA) 55
3.2.11.1 Preparation of recombinant protein 55
3.2.11.2 Over expression and purification of GST-CaZF 55
3.2.11.3 SDS-PAGE 56
3.2.11.4 EMSA 56

3.2.12 Yeast One-Hybrid Assay 57
3.2.12.1 Preparation of recombinant protein 57
3.2.12.2 Yeast Transformation 57
3.2.12.3 ONPG assay 58
3.2.13 Gene disruption and complementation of yeast (Saccharomyces cerevisiae) 58
3.2.13.1 Deletion of complete open reading frames in yeast 58
3.2.13.2 Deletion Strain Confirmation 59
3.2.13.3 Deletion Mutant Construction 59
3.2.13.4 Complementation of deletion mutants 59
3.2.14 Yeast Spot Assay 59
3.2.15 Functional analysis of CaZF in tobacco 60
3.2.15.1 Cloning of CaZF in a plant binary vector pBI121 60
3.2.15.2 Cloning of CaZF in a plant binary vector pCAMBIA1302 60
3.2.15.3 Transformation of Agrobacterium tumefaciens 60
3.2.15.4 Tobacco Plant Transformation 61
3.2.15.5 Selection and Plant Regeneration 61
3.2.15.6 Seed Plating in Culture Medium 61
3.2.16 Analyses of CaZFOX tobacco transgenic plants 61
3.2.16.1 Abiotic stress tolerance evaluation 61
3.2.16.2 Cellular localization of CaZF protein 62
3.2.16.3 Morphological analysis of CaZF overexpressing lines 62
3.2.16.4 Confocal imaging 62
3.2.16.5 Auxin Transport Assay 62
3.2.17 qReal-Time PCR 63
3.2.18 Functional analysis of 5' upstream region of CaZF in tobacco 63
3.2.18.1 Cloning of 5' upstream region of CaZF in pBI101.2 vector 63
3.2.18.2 Transformation of Agrobacterium tumefaciens 63
3.2.18.3 Tobacco Plant Transformation 64
3.2.18.4 Selection and Plant Regeneration 64
3.2.19 GUS assay 64
3.2.19.1 GUS histochemical assay 64
3.2.19.2 GUS spectrophotometric assay 64
3.2.20 Proline Assay 65
3.2.21 Chlorophyll estimation 65
3.2.22 Glycerol estimation 65
CHAPTER 4 COMPARATIVE TRANSCRIPTIONAL PROFILING OF DROUGHT TOLERANT AND SUSCEPTIBLE CHICKPEA CULTIVARS **66-90**
4.1 Introduction 66
4.2 Results 68
4.2.1 Differential drought tolerance in two chickpea cultivars 68
4.2.2 Construction of subtracted cDNA library and dfferential screening 69
4.2.3 Identification of drought responsive transcripts differentially expressed more in PUSABGD72 75
4.2.4 Comparative expression of ESTs in PUSABGD72 with respect to ICCV2 75
4.2.5 Monitoring expression profiles of high expressing genes 82
4.2.6 Macroarray data validation by northern analysis 86
4.2.7 Working pathway for drought tolerance mechanism 86
4.3 Discussion 87

CHAPTER 5 CLONING AND IN-VITRO CHARACTERIZATION OF *CAZF*, A C2H2 ZINC-FINGER PROTEIN FROM CHICKPEA 91-98
5.1 Introduction 91
5.2 Results 93
5.2.1 Isolation of a partial clone 93
5.2.2 CaZF encodes an EPF type C2H2 zinc finger protein 93
5.2.3 In silico analysis of CaZF encoding protein 93
5.2.4 Phylogenetic analysis of CaZF protein 94
5.2.5 CaZF binds in vitro to EP sequence repeat 94
5.2.6 CaZF activates transcription in yeast 94
5.2.7 Transactivation Domain mapping of CaZF in yeast 95
5.2.8 Genomic Organization of CaZF 95
5.2.9 Southern analysis 95
5.2.10 Cellular localization of CaZF 95
5.2.11 Expression analysis of CaZF under various stress treatments 96
5.2.12 Tissue specific expression of CaZF 96
5.3 Discussion 97
CHAPTER 6 FUNCTIONAL CHARACTERIZATION OF *CAZF* IN YEAST (*SACCHAROMYCES CEREVISIAE*) 99-110
6.1 Introduction 99
6.2 Results 101
6.2.1 CaZF expression in yeast is galactose inducible 101
6.2.2 CaZF provides osmotolerance to yeast 102
6.2.3 CaZF suppresses salt sensitivity of Calcineurin (CAN) and HOG pathway mutants 102
6.2.4 CaZF induces expression of HOG and Calcineurin regulated genes 103
6.2.5 CaZF induces glycerol accumulation 105
6.2.6 CaZF directly binds to STRE and activates CTT1 promoter 106
6.2.7 Requirement of CaZF C-terminal domain for salt tolerance 106
6.3 Discussion 107
CHAPTER 7 FUNCTIONAL CHARACTERIZATION OF *CAZF* IN CONSTITUTIVELY EXPRESSING TRANSGENIC TOBACCO PLANTS 111-129
7.1 Introduction 111
7.2 Results 113
7.2.1 Overexpression of CaZF in transgenic tobacco 113
7.2.1.1 Cloning of CaZF cDNA in plant binary vector pBI121 113
7.2.1.2 Agrobacterium mediated transformation of Nicotiana tabaccum 113
7.2.1.3 Confirmations of putative transgenic lines 114
7.2.2 Phenotypic analysis of putative transgenic lines 114
7.2.3 Auxin transport in CaZF overexpressing lines 115
7.2.4 Analysis of early auxin inducible genes 116
7.2.5 Effect of dehydration, salt and heat stress on tissue culture grown CaZF overexpressing lines 116
7.2.6 Effect of salt and drought stress on green house grown CaZFOX transgenic lines 117
7.2.7 Transcript analysis of the stress inducible marker genes in *Nicotiana tabacum* 117
7.2.8 Biochemical response of CaZFOX transgenic tobacco plants 117
7.2.8.1 Determination of proline content 117
7.2.8.2 Determination of chlorophyll content 118
7.2.9 Analysis of altered gene expression due to CaZF overexpression in transgenic N. tabacum 118

7.2.10 Cloning of upstream activating sequences (UAS) 123
7.2.11 Interaction studies of CaZF with CAP2 124
7.2.11.1 CAP2 binds to DRE in CaZF-UAS in gel shift assay 124
7.2.11.2 CAP2 activates CaZF promoter expression in yeast 125
7.2.11.3 CAP2 activates CaZF promoter expression in planta 125
7.3 Discussion 126
SUMMARY **130-133**
BIBLIOGRAPHY **134-152**
PUBLICATION

[illegible]	Abscisic acid
[illegible]	6-Benzylaminopurine
[illegible]	Bovine serum albumin
[illegible]	Complementary DNA
[illegible]	Cetyltrimethylammonium bromide
[illegible]	Diethyl pyrocarbonate
[illegible]	Deoxyribonucleic acid
[illegible]	Deoxy nucleotide triphosphates
[illegible]	Dithiothreitol
[illegible]	Ethylenediaminetetra acetic acid
g	Gram
[illegible]	(N-[2-Hydroxyethyl]piperazine-N[2-ethanesufonic acid])
h	Hours
[illegible]	Indole-3-acetic acid
[illegible]	Isopropyl -D-thiogalactopyranoside
[illegible]	Kilobases
[illegible]	Kilodalton
B	Luria broth
[illegible]	Multiple cloning site
[illegible]	2-(N-Morpholino)-ethanesulfonic acid
in	Minutes
[illegible]	Messenger RNA
N	Naphthaleneacetic acid
[illegible]	Open reading frame
[illegible]	Polyacrylamide gel electrophoresis
[illegible]	Phosphate buffered saline
[illegible]	Polymerase chain reaction
[illegible]	Polyethylene glycol
[illegible]	Phenylmethylsulfonyl fluoride
[illegible]	Tris- EDTA
[illegible]	N, N, N, N'-Tetramethylethylenediamine
[illegible]	5-Bromo-4-chloro-3-indolyl-D-galactopyranoside
[illegible]	5-Bromo-4-chloro-3-indolyl-D-glucopyranoside
[illegible]	Yeast Peptone Dextrose

Chapter 1

Introduction

Introduction

Plants are often exposed to various envioronmental stresses when grown in field and within a physiological tolerance limit, a mild abiotic stress induces an adaptive response in the plant, allowing it to grow with a greater tolerance to the same or different stresses (Boominathan et al., 2004). Abiotic stresses, such as drought, extreme temperature or high and fluctuating salinity, can severely impair plant growth and performance and are also responsible for significant yield reductions in cultivated areas worldwide (Oztur et al., 2002). Plants respond to stress by making adjustments at the levels of morphology, phenology, physiology and biochemistry (Zhang et al., 2000). At the cellular level, a part of this response results from cell damage, whereas the others correspond to adaptive processes. Adaptation to water deficit brings about changes in the metabolic processes and perhaps in the structure of the cell that allows the cells to continue metabolism at low water potential (Ingram and Bartels, 1996). Many laboratories have focused their efforts on the isolation and characterization of genes induced by stress. Understanding the regulation of expression patterns of specific stress responsive genes would play an important part in unlocking the mysteries of plant stress responses and adaptation.

Chickpea (*Cicer arietinum* L.), also known as bengal gram, channa, is an edible grain legume (pulse). Chickpea is the only widely cultivated species of the genus *Cicer* and belongs to the Fabaceae family (Kupicha, 1981). The crop is a self-pollinated diploid (2n = 2x = 16) with a relatively small genome size of 740 Mbp (Arumuganathan and Earle, 1991). Chickpea seeds provide an excellent source of protein, especially for vegetarians or vegans (Taylor and Ford, 2007). Furthermore, chickpea is an additional benefit to the farmers as it fixes a substantial amount of nitrogen for the subsequent crops and adds much needed organic matter that improves soil health, long-term fertility and sustainability of the ecosystems (Ahmad, 2005).

Chickpea, the third most important grain legume, loses more than 50% of yield globally due to abiotic stresses (Ahmad, 2005). Among the abiotic stresses, drought is almost ubiquitous to major chickpea growing regions. Worldwide, an increasing use of irrigation is exacerbating the problem of soil-salinity and it was predicted that by 2050, 50% of all the arable land would be salinized (Wang et al., 2003). Most of the legumes are known to be salt sensitive and die before maturity; therefore, it is important to produce cultivars tolerant to these abiotic stresses for sustainable increase in chickpea production. The cultivated chickpea has a high morphological but narrow genetic variation (Udupa et al., 1993) which makes it difficult for breeders to produce elite

cultivars with durable resistance to the major biotic and abiotic stresses. A major goal for chickpea improvement is the development of high-yielding plants that have a growth advantage in water-limiting conditions at all stages of development.

Mechanisms by which plants adapt to abiotic stresses need to be quantified at a physiological, molecular and genetic level (Bohnert et al., 1995). The first step towards this perspective is the rapid discovery of genes. Identification and functional characterization of important stress inducible genes will facilitate the understanding of the molecular mechanisms underlying the stress tolerance responses. This can be done through gene expression profiling of the whole transcriptome at specific stages of plant development and under specific growth conditions. Genes identified through whole genome sequencing projects or from expressed sequence tag (EST) libraries may be assessed for their comparative transcriptional activity in response to specific plant stresses to predict candidate genes for stress tolerance. This approach has a potential to assist molecular plant biologists in improving stress tolerance by gene selection and/or genetic manipulation. In order to obtain a complete picture of a plant's response to stress, it would be ideal to study the expression profiles of all the genes in its genome. Currently, this is only possible for model crops like *Arabidopsis thaliana*, *Oryza sativa*, *Medicago truncatula*, *Populus trichocarpa* whose genomes have been sequenced and there is huge expression database available. Until this is available for other crops, researchers have to rely on information generated by studying these model crops and explore the EST/cDNA sequences from the same or closely related species.

Functional genomics employs global transcriptional profiling coupled with the use of mutants and transgenics, to study gene function in a high-throughput mode (Vij and Tyagi, 2007). Functional genomics can be broadly divided into three different categories, *viz*., transcriptomics, proteomics and metabolomics. Transcriptomics involves generation and analysis of gene expression profiles of an organism in response to a particular physiological condition. Similarly, proteomics and metabolomics involve global expression profiling of the proteins or metabolites, respectively. The ultimate goal of functional genomic studies on abiotic stresses is to find suitable candidates that govern stress tolerance so that they can be directly selected or used through biotechnology approaches to improve crop performance. The functional validation of these genes can be done by overexpression or suppression of a candidate gene (s) within a transgenic plant/model system that is subsequently phenotyped for the associated stress tolerance. A large and increasing number of genes, transcripts and proteins have been correlatively

implicated in stress response pathways. It is important to analyze the functions of stress-inducible genes, not only to understand the molecular mechanisms of stress tolerance & the responses of higher plants, but also to improve the stress tolerance of crops by gene manipulation (Seki et al., 2002). Many stress-inducible genes include those that encode signaling molecules, such as enzymes involved in phospholipid metabolism, various protein kinases including mitogen-activated protein (MAP) kinases, calcium-dependent protein kinases (CDPKs), receptor-like kinases and histidine kinases (Shinozaki and Yamaguchi-Shinozaki, 2000; Seki et al., 2001) and regulatory proteins like transcription factors (TFs). Through the analysis of transcriptome several kinds of TFs responding to environmental stresses in *Arabidopsis* and other plant species, including AP2/EREBP transcription factors, bHLH proteins, NAC proteins, bZIP proteins and zinc finger proteins have been identified (Takatsuji, 1999; Shinozaki and Yamaguchi-Shinozaki, 2007; Nakashima et al., 2009). The roles for AP2/EREBP transcription factors such as CBF/DREB subfamily members were extensively studied, and the CBF/ DREB-pathway in response to abiotic stress has been well established (Novillo et al., 2004; Agarwal et al., 2006). The zinc finger proteins represent a large and important protein superfamily in eukaryotic organisms. Some zinc finger proteins have been shown to be involved in responses to environmental stresses (Kim et al., 2001; Mukhopadhyay et al., 2004; Sakamoto et al., 2004). Transgenic plants and T-DNA or transposon-tagged mutants have been analyzed to reveal the function of stress-responsive loci in *Arabidopsis*. The reverse genetics approach will be even more important in extending our understanding of regulatory factors in stress signaling. A combination of molecular, genomic and genetic analyses is presently used to elucidate the complex systems that regulate the responses of gene expression to abiotic stresses.

There is a tremendous amount of biological diversity among different plant species in the process of adaptation to drought stress. All stress adaptive mechanisms may not be operating in all plants to the same extent. The response and/or tolerance of a particular plant type to a given stress vary with its genotype and environment. These inconsistencies may be attributed to the biological differences among the genotypes used, plant growth conditions, stress treatment conditions and/or their detection methodologies. There is a great emphasis on identifying novel stress responsive genes from stress tolerant species. Tolerant species may have specific adaptive mechanisms and express novel stress responsive genes (Cushman and Bohnert, 2000; Umezawa et al., 2006). Therefore the changes in transcription profiles of different economically important crop plants are

needed to be monitored (VandenBosch and Stacey, 2003). In the past biologists also showed an interest in comparing the responses between tolerant and susceptible genotypes to a particular physiological condition. Genes expressed by a salt tolerant rice genotype (Pokkali) were compared to those expressed by a salt susceptible genotype (IR29) in response to salt stress. The two genotypes were found to be differ in the timing of gene expression upon stress (Kawasaki et al., 2001). In general, comparison of gene expression profiles between contrasting genotypes provides much information in understanding the spatial and temporal patterns of gene expression required for abiotic stress tolerance. The challenge remains is the identification of stress-responsive novel genes and elucidating their roles that can make the most efficient use of water and maintain acceptable yields when challenged with drought.

The purpose of this study is to identify a collection of putative genes that could be associated with the drought-tolerance phenomenon in chickpea by looking at the differences in gene expression between drought tolerant and susceptible varieties. The identification and characterization of genes controlling the drought-tolerance phenomenon in chickpea will provide scientists with a better understanding of drought tolerance. Temperate grain legumes such as pea, fava bean, lentil, chickpea, and others share similar gene arrangements (VandenBosch and Stacey, 2003). Therefore, we expect that our database will benefit the study of other legume plants by comparing the expression patterns of the transcripts.

Considering the gaps in knowledge regarding the mechanisms of abiotic stress tolerances in chickpea and the opportunities for study identified in this study, the aims of this study were to:

- Analysis of the physiological parameters of a drought-tolerant Chickpea cultivar under progressive water depletion. Construction of subtracted EST libraries from the Chickpea seedlings at different drought conditions. DNA Sequencing and annotation of the EST-clones.
- Monitoring expression profile (at transcript level) of drought inducible genes in different conditions relevant to dehydration with the help of DNA array and Northern analysis, and comparison of expression with a drought-sensitive Chickpea cultivar.
- Cloning and characterization of a gene differentially expressing in drought-tolerant and sensitive cultivar.

Chapter 2

Review of Literature

The mechanism through which plants perceive environmental signals and transmit them to cellular machinery to generate adaptive response is of fundamental importance to biology. Plants are often exposed to various environmental stresses when grown in field and within a physiological tolerance limit. A mild abiotic stress induces an adaptive response in the plant, allowing it to grow with a greater tolerance to the same or different stresses (Siminovitch and Cloutier, 1982; Lang et al., 1994; Mantyla et al., 1995; Knight et al., 1998). Plants express a number of genes in response to water deficit. At the cellular level, a part of this response results from cell damage, whereas the others correspond to adaptive processes. Adaptation to water deficit brings about changes in the metabolic processes and perhaps in the structure of the cell that allows the cells to continue metabolism at low water potential (Ingram and Bartels, 1996). Dehydration and other stresses cause rapid elevation in the cytosolic free calcium ion ([Ca^{+2}]cyt) concentration (Knight et al., 1991). As an adaptive response, the subsequent stresses show altered magnitude and kinetics of [Ca^{+2}]cyt, depending on the nature and intensity of the previous stress indicating an existence of a signal storage mechanism. Different stress-exposure alters cytosolic calcium-signature and calcium-regulated gene expression differently in the following stresses (Knight et al., 1996; Knight et al., 1998), suggesting that plants are able to discriminate different stimuli and then store the impression of individual stimuli in a unique way. The first step in switching on such molecular responses is to perceive the stress as it occurs and to relay information about it through a signal transduction pathway. These pathways eventually lead to physiological changes and results into the modification of molecular and cellular processes.

Drought, cold and high-salinity stresses generate complex stimuli that have different yet related attributes and may deliver quite different information to the plant cells (Xiong et al., 2002). These complex stimuli are governed by stress-responsive genes. Many genes respond to drought, salt and/or cold stress at the transcriptional level and the products of these genes function in the stress response and tolerance. Transcriptome analyses using microarray technology (Seki et al., 2001; Zhu, 2001) have identified several genes that are induced by abiotic stresses, and these genes have been classified into two major groups (Bray, 1993; Thomashow, 1999; Shinozaki and Yamaguchi-Shinozaki, 2000). One group encodes products that directly protect plant cells against stresses, whereas the products of the other group regulate gene expression and signal transduction in abiotic stress responses. Several different sets of cis- and trans-acting factors are known to be involved in stress-responsive transcription. Some of them are controlled by abscisic acid

(ABA) but others are independent of ABA, indicating the involvement of both ABA-dependent and -independent regulatory systems for stress-responsive gene expression (Bray, 1993; Thomashow, 1999; Shinozaki and Yamaguchi-Shinozaki, 2000; Xiong et al., 2002).

The development of genetically engineered plants by the introduction and/or overexpression of selected genes seems to be a viable option to hasten the breeding of "improved" plants. Intuitively, genetic engineering would be a faster way to insert beneficial genes than through conventional or molecular breeding. Also, it would be the only option when genes of interest originate from cross barrier species, distant relatives, or from non-plant sources.

2.1 Abiotic stress

Low temperature, drought, and high salinity are common stress conditions that adversely affect plant growth and crop production. Understanding the mechanisms by which plants perceive environmental signals and transmit the signals to cellular machinery to activate adaptive responses is of fundamental importance to biology. In this section, we will discuss some important environmental stresses encountered by plants in their life cycle (Figure 2.1)

2.1.1 Drought stress

A major abiotic stress factor that leads to reduction in crop production is drought or dehydration due to a water deficit. Drought shock causes metabolic and osmotic imbalance in plants that leads to turgor loss and closure of the stomata, followed by repression of cell growth and inadequate photosynthesis (Shinozaki and Yamaguchi-Shinozaki, 2007). A large number of genes are expressed in plants to protect the cells from stress damage and to restore the metabolic disorder caused by desiccation. These genes, can be classified into two functional groups, the first group encodes proteins giving improved stress tolerance. They code for proteins involved in the production of chaperons, water channels, transporters, detoxifying enzymes, all having a protective function in the cell. The second group includes different proteins having a regulatory role, such as transcription factors, protein kinases and phosphatases, enzymes involved in phospholipids metabolism and ABA biosynthesis (Chen and Murata, 2002; Yamaguchi-Shinozaki and Shinozaki, 2006; Shinozaki and Yamaguchi-Shinozaki, 2007) (Figure 2.2).

Most abiotic stresses tested have been shown to elicit rises in cytosolic free calcium levels $[Ca^{2+}]_{cyt}$ which is thought to be the primary stimulus-sensing event for several stresses (Bartels and Sunkar, 2005; Yamaguchi-Shinozaki and Shinozaki, 2006). If this is

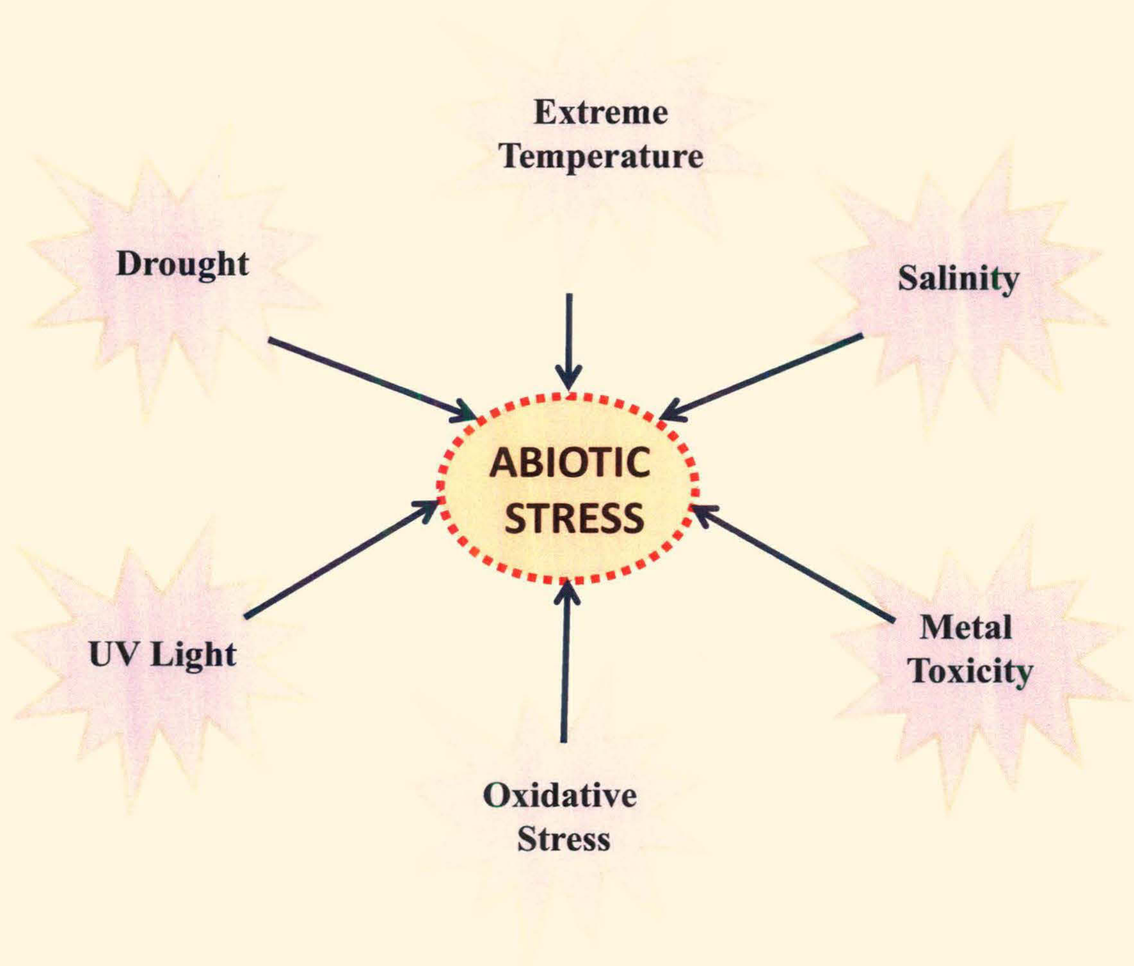


Figure 2.1. Abiotic stress. Different abiotic stresses encountered by a plant during its life cycle.

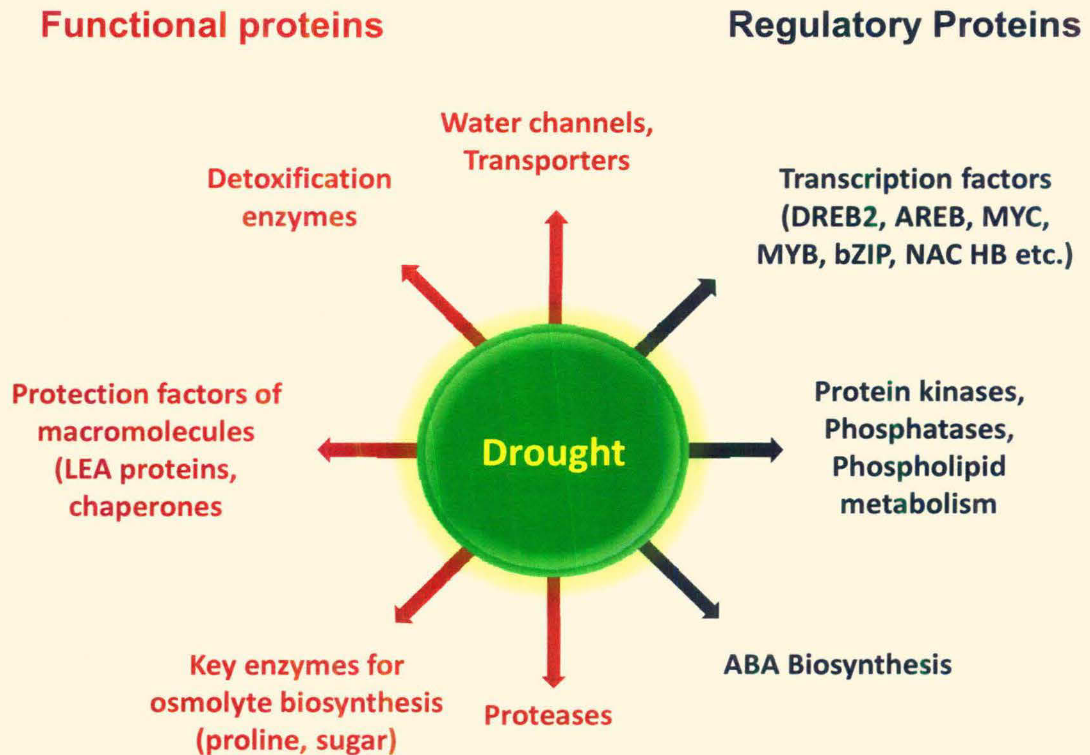


Figure 2.2. Functions of drought stress-inducible genes in stress tolerance and response. Gene products are classified into two groups. The first group includes proteins that probably function in stress tolerance (functional proteins), and the second group contains protein factors involved in further regulation of signal transduction and gene expression that probably function in stress response (regulatory proteins).

the case then mechanisms could exist for encoding the information that relates to the particular stress through the calcium signature. Alternatively, the stress might be sensed through other components either in parallel to or upstream of Ca^{2+} in the pathway. An additional level of regulation is achieved via the function of calcium binding proteins, also known as calcium sensors, which further strengthen the specificity in the calcium signalling pathway (Snedden and Fromm, 1998; Luan et al., 2002; Sanders et al., 2002). Ca^{2+} sensors are small proteins that apparently do not have any enzymatic activity themselves and function by interacting with their target proteins, thereby monitoring temporal and spatial changes in Ca^{2+} concentrations (Zielinski, 1998). Such sensors bind to Ca^{2+} and change their conformation in a Ca^{2+}dependent manner.

In plant cells, many Ca^{2+} sensors have been identified which include calmodulin (CaM) and calmodulin-related proteins (Zielinski, 1998; Luan et al., 2002), Ca^{2+}dependent protein kinases (CDPKs) (Harmon et al., 2000; Sanders et al., 2002), and the still recently discovered class of Ca^{2+} sensors, calcineurin B-like proteins (CBLs) (Luan et al., 2002). CBLs seem to be one of the major components in calcium signalling pathways in mediating response to abiotic stress. CBL proteins exert their function by interacting and regulating a group Ser/Thr protein kinases called CBL-interacting protein kinases (CIPKs) (Shi et al., 1999; Kim et al., 2000; Luan et al., 2002). CIPKs influence the expression pattern of number of stress inducible genes like *RD29A*, *KIN1* and *KIN2*, which are among the major genes imparting tolerance to plants (Cheong et al., 2003; Kim et al., 2003; Pandey, 2008). Thus, an important feature of the role of Ca^{2+} as a signal is the presence of repetitive Ca^{2+} transients. These transients may be generated both by first round second messengers and by signaling molecules such as abscisic acid (ABA) that may themselves be produced as a result of cascades of early Ca^{2+} signals.

Phytohormone ABA is the main plant hormone regulating stress-related gene expression, causing closure of the stomata and the subsequent expression of drought related genes (Zhu, 2002; Chinnusamy et al., 2004; Vinocur and Altman, 2005; Umezawa et al., 2006; Valliyodan and Nguyen, 2006; Xiong et al., 2006; Shinozaki and Yamaguchi-Shinozaki, 2007). Recent approaches especially the use of ABA-biosynthesis and ABA-insensitive mutants have shown that signalling of water stresses may be understood in two major pathways: abscisic acid (ABA) dependent and ABA-independent stress-response pathways (Bray, 1997; Thomashow, 1999; Shinozaki and Yamaguchi-Shinozaki, 2000). The ABA-dependent pathway may also have two different routes, i.e, requiring new protein synthesis or not (Ingram and Bartels, 1996; Bray, 2002; Shinozaki

and Yamaguchi-Shinozaki, 2007). In the route where new protein synthesis is not required, the promoter domain in all ABA-responsive genes contains an ABA-responsive element (ABRE, ACGTGGC). Several *rd* (responsive to dehydration) and *erd* (early responsive to dehydration) genes encoding a wide range of proteins are ABA dependent. When ABRE is bound with its corresponding bZIP family of TFs like EmBP-1, it can lead to ABA-induced gene expression. An example of this is the ABA-mediated induction of dehydration responsive *Arabidopsis rd29B* gene by two bZIP TFs, AREB1 and AREB2. In the route where new protein synthesis is required for the ABA-induced gene expression, *de novo* synthesis of new proteins is prerequisite. Such genes have no ABREs and its ABA-responsive elements combine with the MYC-family TFs. In the ABA-independent gene expression pathway some genes may be induced by ABA but ABA is not the essential condition (Ingram and Bartels, 1996; Bray, 1997). *Arabidopsis* gene *RD29A/COR78/LTI78* is one of such example (Kreps et al., 2002). This gene is induced in both an ABA-dependent and an ABA-independent manner. It has two regulative elements, of which one is ABA-responsive and the other one is non-ABA responsive. Non-ABA responsive cis-acting elements are known as DREs (dehydration responsive element, TACCGACAT) also referred as CRTs (C-repeat). DRE/CRTs are recognised by their DNA-binding transcription factors DREBs (DRE binding protein) or CBFs (C-repeat binding factor), respectively (Shinozaki and Yamaguchi-Shinozaki, 2000; Valliyodan and Nguyen, 2006). This element is essential for water stress induced gene expression but is not under the regulation of ABA (Ingram and Bartels, 1996). Several ABA deficient mutants namely *aba1*, *aba2* and *aba3* have been reported for *Arabidopsis* (Koornneef et al., 2004). ABA deficient mutants for tobacco, tomato and maize have also been reported (Liotenberg et al., 1999). Without any stress treatment the growth of these mutants is comparable to wild type plants. Under drought stress, ABA deficient mutants readily wilt and die if the stress persists.

In ABA-deficient (*aba*) or ABA-insensitive (*abi*) *Arabidopsis* mutants, some genes are also induced by both dehydration and low temperature, thereby demonstrating the inter-related nature of stress-responsive mechanism pathways. Besides these, there are many other regulatory genes that control expression in response to these stresses such as zinc-finger proteins, salt overly sensitive-2 (SOS2) like protein kinases, Ser/Thr protein kinase (PKS5), basic/helix-loop-helix (bHLH), the APETALA2/ethylene-responsive factor (AP2/ERF) domain-containing protein RAP2, and growth factor-like proteins (Bartels and Sunkar, 2005). These genes respond rapidly and transiently to drought

stresse and their expression peaks for several hours after stress and then decreases. This is followed by synthesis of function proteins like late embryogenesis abundant (LEA) proteins, detoxification enzymes, and enzymes for osmoprotectant synthesis whose expression increases gradually after stress (Yamaguchi-Shinozaki and Shinozaki, 2006). For details on the current understanding of gene regulation in response to these stresses see Figure 2.3.

2.1.2 Salinity stress

Salt stress in general reduces the water uptake capacity of the plant, which as a consequence reduces growth rate and metabolic activity. The presence of a higher salt concentration in the saline soil changes the osmotic potential of the cell, which inhibits the ability of the plant to take up water as well as necessary minerals and ions, such as K^+ and Ca^{2+}. Secondly, inhibition of growth and photosynthesis by Na^+ and Cl^- toxicity is the most common effect when plants are exposed to salinity. Metabolic toxicity occurs because Na^+ competes and substitutes the K^+ that is essential for many enzyme activities and protein synthesis (Hasegawa et al., 2000; Tester and Davenport, 2003; Munns et al., 2006). These primary damaging effects are followed by subsequent secondary stresses or inhibitory effects such as the production of ROS that cause oxidative damage to membrane lipids and proteins and eventually lead to programmed cell death (PCD). The ability to maintain the right cellular cytosolic sodium concentration is crucial for the growth of the plant in high salt concentration.

Most of the higher plants try to exclude Na^+ by Na^+/H^+ antiport at the plasma membrane. The potassium ion (K^+) is the most abundant cation and an important macronutrient in higher plants. Therefore, it is very important to maintain the right K^+/Na^+ ratio in the cytosol for the normal function of cells. Like potassium, the calcium ion (Ca^{2+}) also plays an important role in signaling cell metabolism responses to abiotic stresses including temperature stress, salt stress, oxidative stress and anoxia. Cellular calcium mediates the signaling that leads to an increased expression of abiotic stress responsive genes which may encode proteins having protective functions. A salt stress-induced calcium signal is most likely to induce the distinct Salt-Overly-Sensitive (SOS) pathways in plants in response to the ionic stress, particularly Na^+ stress.

SOS signaling appears to be relatively specific for the ionic aspect of salt stress and is calcium-dependent. The targets of this type of signaling are ion transporters that control ion homeostasis under salt stress. The input of the SOS pathway is likely excess extracellular or intracellular Na^+, which somehow triggers a cytoplasmic Ca^{2+} signal. The

outputs are expression and activity changes of transporters for ions such as Na^+, K^+, and H^+. The input for osmotic stress signaling is likely a change in turgor. Salt stress signal transduction consists of ionic and osmotic homeostasis signaling pathways, detoxification (e.g. damage control and repair) response pathways, and pathways for growth regulation. The ionic aspect of salt stress is signaled via the SOS pathway where a calcium-responsive SOS3-SOS2 protein kinase complex controls the expression and activity of ion transporters such as SOS1 (Zhu, 2002). Genetic analysis indicated that SOS1, SOS2 and SOS3 function in a common pathway in controlling salt tolerance. An important group of Ca^{2+} sensors in plants is the SOS3 family of Ca^{2+} binding proteins. A loss of function mutation in the *Arabidopsis* SOS3 gene renders the mutant plants hypersensitive to NaCl. There is genetic evidence for regulation of the *Arabidopsis* SOS1, a plasma membrane Na^+/H^+ anti-porter by a calcium-activated protein kinase complex composed of the SOS2 kinase subunit and of the SOS3 calcium-binding subunit to re-establish the cellular ionic homeostasis. This signaling pathway mediates salt induction of the SOS1 gene in *Arabidopsis* (Serrano, 1996; Xiong et al., 2002). Because the SOS pathway operates during ionic stress, it is thought that homologs of SOS3 and SOS2 may also function in the transduction of other stress or hormonal signals. In the case of abiotic stress signaling, evidence suggests that Ca^{2+}-dependent protein kinases (CDPKs) and the SOS3 family of Ca^{2+} sensors are major players in coupling Ca^{2+} signals to specific protein phosphorylation cascades. Protein phosphorylation cascade finally targets proteins directly involved in cellular protection or transcription factors controlling specific sets of stress-regulated genes. The products of these genes may participate in the generation of regulatory molecules like the plant hormones ABA, ethylene, and salicylic acid (SA).

Salt tolerant plants protect themselves from salinity by lowering the rate of Na^+ and Cl^- transport to leaves and compartmentalizing these ions to vacuoles other than cytoplasm or cell walls to avoid salt toxicity. During salt stress one of the complex molecular responses in the plant is to produce stress proteins and compatible osmolytes that may have protective and scavenging functions against cell damaging ROS molecules produced during the stress (Zhu, 2001). A major category of organic osmotic solutes consists of simple sugars (mainly fructose and glucose), sugar alcohols (glycerol and methylated inositols) and complex sugars (trehalose, raffinose and fructans) (Bohnert et al., 1995). Others include quaternary amino acid derivatives (proline, glycine betaine, β-alanine betaine, proline betaine), tertiary amines 1, 4, 5, 6-tetrahydro-2-mehyl-4-carboxyl pyrimidine), and sulfonium compounds (choline osulfate, sulfonium propironate) (Nuccio

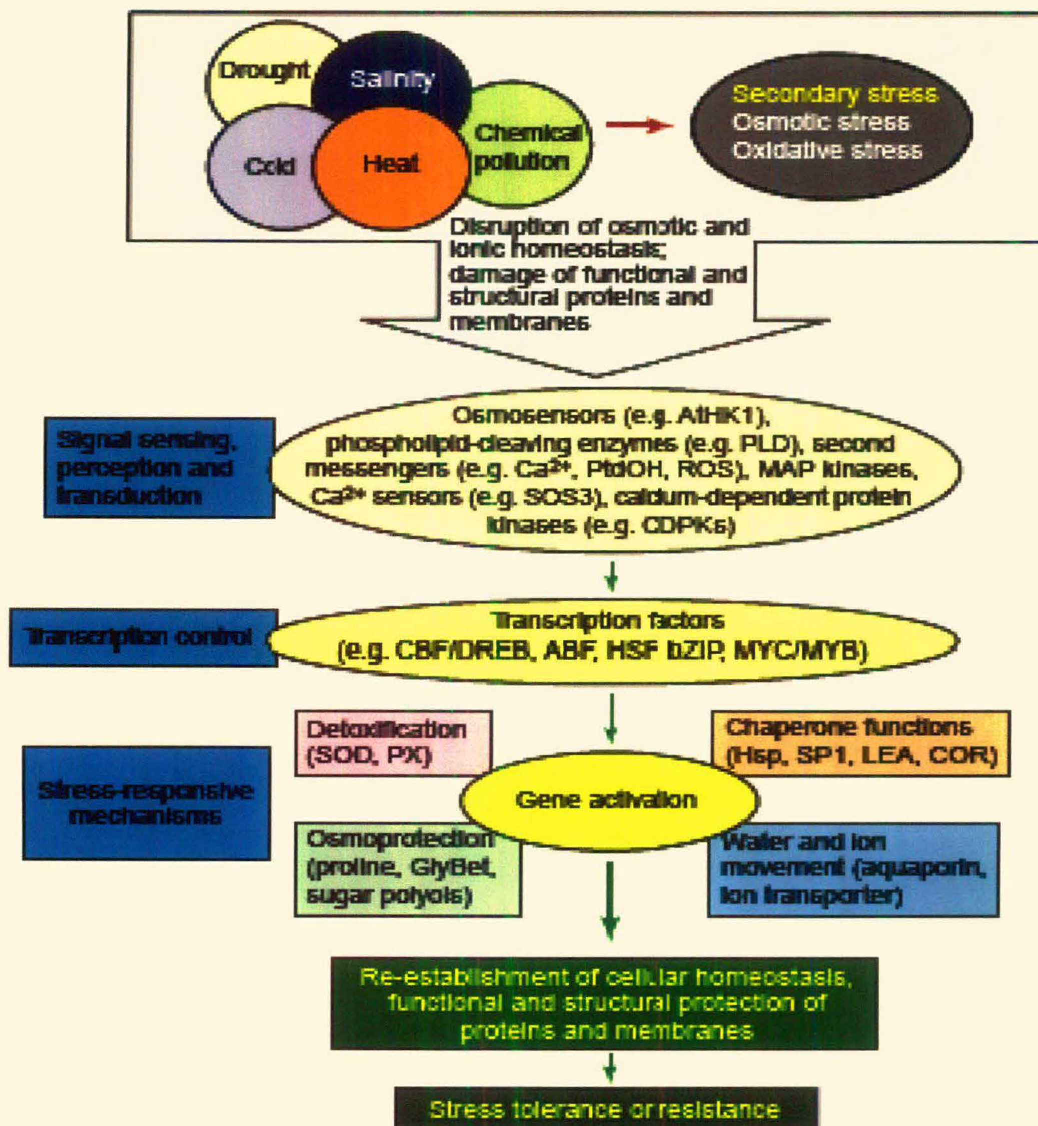


Figure 2.3. Complex model of the plant response to abiotic stress. Primary stresses, such as drought, salinity, cold and heat often cause cellular damage and secondary stresses, such as osmotic and oxidative stress. The initial stress signals trigger the downstream signaling process and transcription controls, which activate stress-responsive mechanisms to re-establish homeostasis and to protect and repair damaged proteins and membranes. Abbreviations: ABF, ABRE binding factor; AtHK1, Arabidopsis thaliana histidine kinase-1; bZIP, basic leucine zipper transcription factor; CBF/DREB, C-repeat-binding factor/ dehydration-responsive binding protein; CDPK, calcium-dependent protein kinase; COR, cold-responsive protein; Hsp, heat shock protein; LEA, late embryogenesis abundant; MAP, mitogen-activated protein; PLD, phospholipase D; PtdOH, phosphatidic acid; PX, peroxidase; ROS, reactive oxygen species; SOD, superoxide dismutase; SP1, stable protein 1.

et al., 1999). Furthermore, many of the osmoprotectants enhance stress tolerance of plants when expressed as transgene products (Bohnert and Jensen, 1996; Zhu, 2001) (Figure 2.4).

2.1.3 Cold stress

One of the most common environmental stresses is unfavorable change in the ambient temperature. Cold-induced desiccation can cause several physiological effects such as protein denaturation, precipitation of various molecules, membrane damage and lysis of the cells. The additional factor that causes cellular damage is the production of ROS during cold-induced injury (Thomashow, 1999; Smallwood and Bowles, 2002). Cold acclimation is the first line of defense and has been found to be involved in the stabilization of plasma membranes against cold-induced injury. Low temperature reduces the water absorption by roots and water transport in shoots and as a result desiccation shock and wilting of the plant occurs (Uemura et al., 1995; Thomashow, 1998, 1999; Smallwood and Bowles, 2002). Therefore, many of the functional proteins such as LEA and antifreeze proteins as well as regulatory proteins which are involved in drought signaling are also involved in cold or salt stress signaling (Thomashow, 1999; Novillo et al., 2004; Goyal et al., 2005; Shinozaki and Yamaguchi-Shinozaki, 2007). Cold acclimation or the application of ABA can induce the cascade of many cold-responsive (COR) genes (Mohapatra et al., 1988; Thomashow, 1999) which are homologous to cryoprotective proteins which protect the plasma membrane from cold shock and destabilization and lead to freezing tolerance (Steponkus et al., 1998).

2.1.4 Heat stress

Plants cope with extreme high temperatures by the induction of a group of genes called heat shock genes that enable the plant cell to survive in two ways. One group of heat shock proteins (HSPs) act as molecular chaperons that counteract protein denaturation and aggregation, and other HSPs, including ubiquitin and certain proteases, target non-native proteins for degradation (Gurley, 2000). *Arabidopsis* mutants that have a mutation in heat shock gene *Hsp101* are unable to acquire tolerance to high temperature (Queitsch et al., 2000; Hong and Vierling, 2001).

2.1.5 Oxidative stress and Reactive oxygen species (ROS)

In plants, a wide range of abiotic and biotic stress factors can induce oxidative stress. Salt, drought and heat are accompanied by the formation of ROS molecules such as superoxide anion O^{2-}, hydrogen peroxide H_2O_2, and hydroxyl ion OH^-, which damage membranes and macromolecules (Mittler, 2002; Blokhina et al., 2003). ROS production

is the very early response in the process of cell or tissue necrosis (Heath, 2000; Kwak et al., 2003; Patel et al., 2006). H_2O_2 might have a role as second messenger of the important plant defense signaling molecule SA to activate the induction of defense related gene expressions that lead to plants systemic defense or resistance response (Chen et al., 1995; Durner and Klessig, 1996; Ryals et al., 1996). Plants have developed several antioxidation strategies to scavenge these toxic compounds. The main way to reduce damage caused by ROS in plant cells is accomplished by several antioxidants (ROS scavengers) that include enzymes such as CAT, superoxide dismutase (SOD), APX and glutathione reductase, as well as non-enzyme molecules such as ascorbate, glutathione, carotenoids, and anthocyanins (Zhu et al., 1997; Mittler, 2002; Blokhina et al., 2003).

2.2 Regulatory proteins in abiotic stress signaling

Stress-induced gene expression can be broadly categorized into two groups: the first group includes proteins that most probably function in abiotic stress tolerance. These include molecules such as chaperones, late embryogenesis abundant (LEA) proteins, osmotin, antifreeze proteins, mRNA-binding proteins, key enzymes for osmolyte biosynthesis, water channel proteins, sugar and proline transporters, detoxification enzymes, and various proteases. LEAs have been implicated in detoxification and alleviation of cellular damage during dehydration. LEA proteins may also function as chaperone-like protective molecules to combat cellular damage (Umezawa et al., 2006). Water deficit, high osmolarity, and low temperature stress results in the accumulation of a group of LEA proteins. Such proteins may preserve protein structure and membrane integrity by binding water, preventing protein denaturation or renaturing unfolded proteins, and sequestering ions in stressed tissues.

LEA proteins and chaperones have shown to be involved in protecting macromolecules like enzymes, lipids and mRNAs from dehydration (Yamaguchi-Shinozaki et al., 2002). Both osmolytes and LEA proteins contribute to stabilization of membrane and protein structures by conferring preferential hydration at moderate desiccation and replacing water at extreme desiccation. Osmolytes also contribute to osmotic adjustment and act as hydroxyl radical scavengers (Serrano and Montesinos, 2003). One of the mechanisms that plants use to combat the detrimental effects of water loss is to synthesize compatible solutes, typically certain polyols, sugars, amino acids, betaines and related compounds (Bohnert et al., 1995). These solutes facilitate the maintenance of favorable turgor pressure during water stress and in addition may serve as protective agents by stabilizing proteins. Compatible solutes have also been shown to

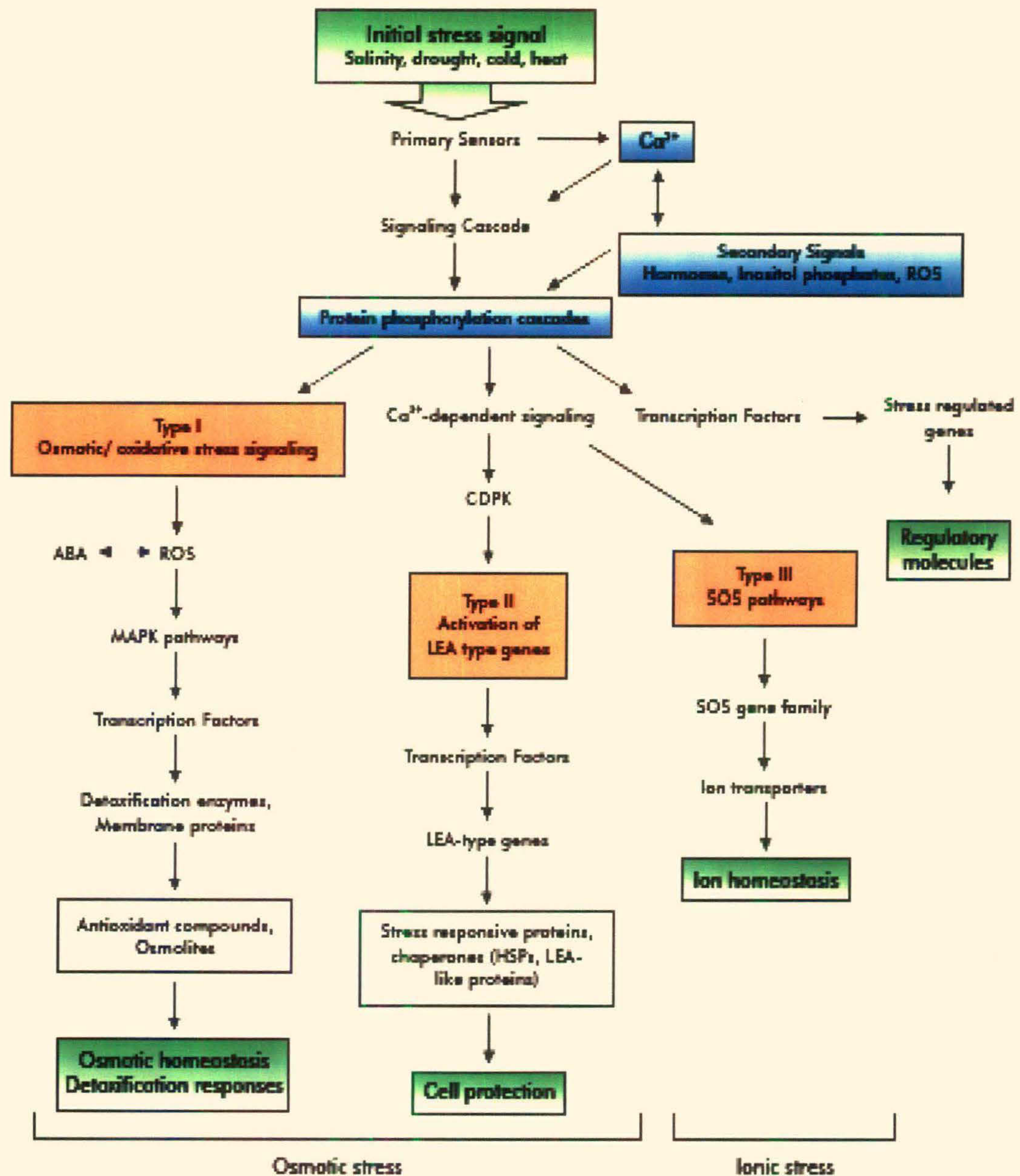


Figure 2.4. Schematic pathway for the transduction of osmotic and ionic stress in plants. A signal transduction pathway starts with signal perception, followed by the generation of second messengers (e.g., inositol phosphates and reactive oxygen species [ROS]). Second messengers can modulate intracellular Ca^{2+} levels, often initiating a protein phosphorylation cascade that finally targets proteins directly involved in cellular protection or transcription factors controlling specific sets of stress-regulated genes.

function as free radical scavengers, protecting DNA from the degradative effects of reactive oxygen species (Akashi et al., 2001).

Proline accumulation was correlated with improved plant performance under salt stress. Proline might confer a protective effect by inducing stress-protective proteins. Plant polyamines have been shown to be involved in plant response to salinity. Glycine-betaine is a widely studied osmoprotectant, the accumulation of which has been studied with respect to modifications of several metabolic steps. Trehalose, a rare, non-reducing sugar, is present in many bacteria and fungi and in some desiccation-tolerant higher plants. Trehalose is thought to protect biomolecules from environmental stress, as suggested by its reversible water-absorption capacity to protect biological molecules from desiccation-induced damage. Mannitol is another sugar alcohol that accumulates upon salt and water stress and can thus alleviate abiotic stress.

The second group is comprised of regulatory proteins, i.e. protein factors involved in further regulation of signal transduction and stress-responsive gene expression. These include various transcription factors, protein kinases, protein phosphatases, enzymes involved in phospholipid metabolism, and other signaling molecules such as calmodulin-binding protein. In this section we will review regulatory proteins involved in abiotic stress signaling.

2.2.1. Sensory kinases

Specificity in signaling is easy to envision if each stress signal has a sensor that can specifically transduce the signal to cellular targets. Two-component systems, consisting of a sensory histidine kinase and a response regulator, function as stress sensors in bacteria and yeast. For example, in yeast, an osmosensory histidine kinase, SLN1, senses osmotic stress and activates the HOG1 mitogen-activated protein kinase (MAPK) cascade (Maeda et al., 1994). Based on the stress-inducibility of its transcript and its ability to complement *sln1-ts*, *ATHK1* is thought to be a candidate osmosensor (Urao et al., 1999). Some receptor-like protein kinases have been implicated in abiotic stress responses. Transgenic analysis has shown that wound, salt and osmotic stresses induce *C7* (*NtC7*) in tobacco and that this putative membrane-localized receptor- like protein may play an important role in osmotic stress tolerance (Tamura et al., 2003). Determination of the *in vivo* role of higher plant putative sensory kinases will help us to determine the regulatory function of sensory kinases in abiotic stress signaling.

2.2.2. Ca^{+2}-dependent protein kinases (CDPKs) and CBL interacting protein kinases (CIPKs)

Changes in calcium concentration can be sensed by two kinds of proteins: the sensor relays, including CBL proteins, and the sensor responders, including CDPKs. Plants contain a large family of Ca^{2+}-dependent protein kinases (CDPKs). The involvement of CDPKs in osmotic signaling has been suggested by the transcriptional induction of CDPK genes in response to salinity, cold, or drought (Saijo et al., 2000; Chehab et al., 2004). The involvement of CDPKs in stress-induced gene transcription was demonstrated using a maize leaf protoplast transient expression system (Sheen, 1996). In these studies, a constitutively active form of an *Arabidopsis* CDPK (*AtCDPK1*) activated the expression of a barley ABA-responsive promoter fusion [*HVA1::LUC*; (Sheen, 1996)]. Ectopic expression of CDPK induced the expression of a rice stress-responsive gene, *RAB16* (Saijo et al., 2000) and *HVA1* in the maize protoplasts (Sheen, 1996). *RAB16* and *HVA1* have a G-box type ABA-responsive cis element (ABRE), which can be activated by a bZIP transcription factor (Leung and Giraudat, 1998). While many more CDPKs exist in the plant genome, little is known about the targets or specificity of most of these kinases. The non-enzymatic CBLs interact with kinase partners, the CIPKs, also called salt overly sensitive (SOS) 2-like protein kinases (PKSs) or SnRK3s.

A complex network of interactions between members of the CBL and CIPK families has been reported (Gong et al., 2004). The most-studied members of these families, SOS3 and SOS2, interact together in the SOS pathway, leading to Na^{+}-homeostasis in response to salt stress (Chinnusamy et al., 2004). The SOS2/SOS3 complex activates the SOS1, Na^{+}/H^{+} antiporter, by phosphorylation. On the other hand, SOS2 also regulates vacuolar transporters like the CAX1 (Cheng et al., 2004) and the AtNHX1 (Qiu et al., 2004). Concerning the other proteins of the families, the transcripts of the *CIPK3* gene accumulated in response to ABA, cold, and salt stress, and the corresponding protein was proposed to regulate the response pathways to these treatments (Kim et al., 2003). Overexpression of *AtCBL1* was shown to trigger an increase in drought and salt induction of stress genes correlated with an enhanced tolerance to both stresses (Albrecht et al., 2003; Cheong et al., 2003).

2.2.3. MAPK

Till date several MAPKs were shown to be activated by abiotic stresses in different plant species. The MAP kinase pathways are intracellular signal modules that mediate signal transduction from the cell surface to the nucleus (Robinson and Cobb, 1997). They

seem to be widely used as osmolarity signaling modules. The core MAPK cascades consist of three kinases that are activated sequentially by an upstream kinase. The MAP kinase kinase kinase (MAPKKK), upon activation, phosphorylates a MAP kinase kinase (MAPKK) on serine and threonine residues. This dual-specificity MAPKK in turn phosphorylates a MAP kinase (MAPK) on conserved tyrosine and threonine residues. The activated MAPK can then either migrate to the nucleus to activate transcription factor directly, or activate additional signal components to regulate gene expression, cytoskeleton-associated proteins or enzyme activities, or target certain signal proteins for degradation. It has been shown that different MAPK pathways may share common components; yet, activation of one pathway may not necessarily affect another pathway. The yeast HOG1 pathway is the best-studied MAP kinase pathway. In plants, MAPK cascades have been shown to participate in auxin and cytokinin signal transduction and cell-cycle regulation and are implicated in wound and pathogenesis responses as well as in environmental stress signal transduction (Jonak et al., 1999). In alfalfa plants, a MAPK was activated within 10 min of cold treatment. It was also activated by drought stress as well as mechanical stress, but not by heat, salt stress or exogenous ABA (Jonak et al., 1996), suggesting that this MAPK mediates drought and cold signaling via an ABA-independent pathway. Similarly, in tobacco cells, the SIPK (a MAPK) and another protein kinase, HOSAK, were activated by osmotic stress and this activation is independent of Ca^{2+} or ABA (Hoyos and Zhang, 2000).

2.2.4. Protein Phosphatases

Studies have been focused mainly on phosphatases involved in the regulation of MAPKs, which are transiently activated by phosphorylation. Several phosphatases, including protein Tyr phosphatases (PTP), dual-specificity protein Tyr phosphatase (DsPTP), and protein Ser/Thr phosphatase 2C (PP2C), were shown to down-regulate MAPKs that are involved in osmotic signaling. A mutant of the DsPTPMKP1 exhibits an enhanced salt resistance in comparison to wild-type plants, indicating that this phosphatase is a negative regulator of salt stress tolerance (Ulm et al., 2002). Interestingly, the alfalfa MP2C, a PP2C, is able to suppress the lethal phenotype induced by overexpression of a constitutively active STE11, which is the MAPKKK involved in the yeast hyperosmotic HOG1 MAPK cascade (Meskiene et al., 1998). This suggests that MP2C can also inactivate the plant MAPK cascade involved in osmotic signaling. The role of two *Arabidopsis* PP2Cs, ABI1 and ABI2, in the negative regulation of ABA signaling has been reported (Schweighofer et al., 2004). Thus the regulatory role of

protein phosphatases may be to bring the stress-activated MAPKs back to their basal activity level, allowing a new activation of the signaling pathway after a second stimulation.

2.2.5 Transcription factors

Regulation of gene expression is associated with most biological phenomena and is largely mediated through proteins that interact directly or indirectly with specific DNA sequences (*cis*-elements) in the promoter region of genes. Transcription factors play critical roles in all aspects of a higher plant's life. It is the programmed and regulated interactions between transcription factors and genomic DNA that bring a genome to its life and define many of its functional features (Grandori et al., 2000; Dimova et al., 2003; Kohler et al., 2003). Most of the known transcription factors can be grouped into families according to their conserved DNA binding domain (Boggon et al., 1999). The four largest families are the MYB super family, AP2/EREBP, basic helix loop helix (bHLH), and C2H2 zinc finger family. Several transcription factor families are found only in plants, some of which have been greatly amplified during evolution. These include the AP2/EREBP, NAC and WRKY families (Riechmann and Meyerowitz, 1998; Eulgem et al., 2000). In the activation of abiotic stress-responsive genes in plants, it seems that there is not a general rule regarding which class of transcriptional factors activates which class of stress-responsive genes. Instead, there could be several kinds of transcriptional factors regulating one group of stress-responsive genes, or even several transcriptional factors that can cooperatively activate the same gene.

2.2.5.1 Transcription factor domains

DNA-binding domains of plant transcription factors contain amino acid residues that contact DNA strands at *cis*-acting elements, and these determine the specificity of the protein (Aukerman et al., 1991). The functional domains of plant transcription factors are usually derived by comparing amino acid sequences deduced from cDNA clones with their animal counterparts (Liu et al., 1999). The plant transcription factors generally consist of a DNA–binding region, an oligomerization site, a transcription regulation domain, and a nuclear localization signal (NLS) (Goff et al., 1992; Washburn et al., 1997). Therefore, the classification of plant transcription factors (Table 1) depends on their structural features (Martin and Paz-Ares, 1997; Sainz et al., 1997; Guilfoyle et al., 1998). The base recognition residues are often highly conserved. Several plant transcription factors possess both specific and nonspecific DNA–binding domains, with the latter occasionally necessary for transactivation of target genes. Normally, each plant

transcription factor has only one type of DNA-binding domain, occurring in either single or multiple copies. For instance, most plant myb-related proteins have two Myb domains. One, two or three fingered DNA-binding motifs occur for C2C2, C2H2 and C2H3 zinc finger transcription factors (Dietrich et al., 1997; Meissner and Michael, 1997), and AP2 (APETALA2) factors may exhibit two DNA binding domains connected by a conserved sequence (Jofuku et al., 1994). Many plant transcription factors form hetero and/or homo-oligomers, affecting DNA binding specificity, the affinity of transcription factors for certain promoter elements (Katagiri and Chua, 1992; Guiltinan and Miller, 1994) and nuclear localization (Sainz et al., 1997).

Table 1: Structural feature of conserved domains that are used to classify plant transcription factors according to (Liu et al., 1999).

Domain name	Domain structure
Zinc finger	Finger motif (s) each maintained by cysteine and/or histidine residues organized around a zinc ion
bZIP	A basic region and a lucine- rich zipper like motif
Myb-related	A basic region with one to three imperfect repeats each forming a helix-helix-turn-helix
Trihelix	Basic, acidic and proline/glutamine-rich motif which forms a trihelix DNA binding domain.
Homeodomain	Approximately 60 amino acid residues producing either three or four alpha helicase or an N-terminal arm.
Myc b/HLH	A cluster of basic amino acid residues adjacent to a helix – loop-helix motif
MADs	Approximately 57 amino acid residues that comprise a long alpha helix and two ß strands
AT-hook motif	A consensus core sequence R (G/P) RGRP with the RGR region containing the mainor groove of A/T- rich DNA
HMG-box	L- Shaped domain consisting of three alpha- helices with an angle of about 80° between the arms.
AP2/EREBP	A 68 amino acid region with a conserved domain that constitutes a putative amphiphatic alpha-helix.
B3	A 120 amino acid conserved sequence at the C- termini of VP1 and ABI3
ARF	A 350 amino acid region similar to B 3 in sequence

2.2.5.2 Plant TFs

Plants devote a large portion of their genome capacity to transcription, with the *Arabidopsis* genome coding in excess of 1500 transcription factors (Riechmann et al., 2000). These transcription factors often belong to large gene families, which in some cases are unique to plants. There has been major progress in linking specific members of these families with plant stress responses.

2.2.5.2.1 ERF transcription factors

ERF proteins are a subfamily of the APETALA2 (AP2)/ethylene-responsive-element-binding protein (EREBP) transcription factor family that is unique to plants. ERF proteins share a conserved ERF domain that can bind to two similar *cis*-elements: the GCC box, which is found in several *PR* (*PATHOGENESIS-RELATED*) gene promoters where it confers ethylene responsiveness, and the C-repeat (CRT)/dehydration-responsive element

(DRE) motif, which is involved in the expression of dehydration- and low-temperature-responsive genes. The large size of the ERF family may be due in part to the wide range of stresses that family members have been linked to. The RNA levels of specific *ERF* genes are regulated by cold, drought, pathogen infection, wounding or treatment with ethylene, SA or jasmonic acid (JA). Post-translational control of ERF proteins has also been observed. Several studies showed upregulation of GCC or CRT/DRE-motif-containing genes and enhanced resistance to specific stresses in response to *ERF* overexpression (Liu et al., 1998; Kasuga et al., 1999; He et al., 2001; Berrocal-Lobo et al., 2002; Wu et al., 2002). ERF proteins from one plant species have been shown to function in other plant species, enhancing their potential utility in increasing the stress tolerance of plants (He et al., 2001; Jaglo et al., 2001; Gu et al., 2002; Wu et al., 2002).

2.2.5.2.2 bZIP transcription factors

bZIPs are a large family of transcription factors in plants. One class of bZIP proteins that is linked to stress responses comprises the TGA/*octopine synthase* (*ocs*)-element-binding factor (OBF) proteins. TGA/OBF factors may serve both negative and positive roles in plant stress responses (Pontier et al., 2001; Fan and Dong, 2002). Other bZIP proteins have been implicated in stress signalling, including UV lights and salt/drought stress signalling (Jakoby et al., 2002). The ABRE-binding factor (ABF)/AREB-proteins work through an abscisic acid (ABA)-dependent signal transduction pathway (Choi et al., 2000; Uno et al., 2000).

2.2.5.2.3 WRKY transcription factors

WRKY proteins are a novel family of transcription factors that are unique to plants. WRKY proteins contain either one or two WRKY domains, a 60-amino-acid region that contains the amino-acid sequence WRKYGQK and a new type of Cys2/His2-type zinc finger (CX4-CX22-23HX1H) motif. Specific WRKY family members show enhanced expression and/or DNA-binding activity following induction by a range of pathogens, defense signals and wounding (Eulgem et al., 2000). WRKY proteins bind to the W box, which is found in the promoters of many plant defense genes (Maleck et al., 2000; Chen et al., 2002). WRKY proteins also regulate the expression of regulatory genes such as receptor protein kinases (Ohtake et al., 2000; Asai et al., 2002).

2.2.5.2.4 Zinc finger transcription factors

Several classes of zinc finger motifs are present in transcription factors and function as parts of DNA-binding and protein-protein interaction domains (Takatsuji, 1998). Moreover, most of the functional motifs present in eukaryotic transcription factors have

their counterparts in the plant kingdom. A zinc finger represents a sequence motif in which cysteine and/or histidine residues coordinate a zinc atom (s) to form local peptide structures that are required for their specific functions. The zinc finger motifs, which are classified based on the arrangement of the zinc binding amino acids, play critical roles in interactions with other molecules. Some classes of zinc-finger motifs (e.g. TFIIIA- and GATA types) are, in most cases, parts of DNA-binding domains of transcription factors and have been shown to be directly involved in the recognition of specific DNA sequences. Other classes (e.g. LIM- and RING-finger types) are mostly implicated in protein-protein interactions. Most of the eukaryotic zinc-finger motifs have also been found in plants. Mutations in some of the genes coding for zinc-finger proteins have been found to cause profound developmental aberrations or defective responses to environmental cues.

2.2.5.2.4.1 TFIIIA type

In TFIIIA–type zinc finger (Miller et al., 1985), there are two cysteines and two histidines in a conserved sequence motif ($CX_2 FX_5 LX_2 HX_3 H$) tetrahedrally coordinate a zinc atom to form a compact structure that interacts with the major groove of DNA in a sequence-specific manner (Pavletich and Pabo, 1991). The first TFIIIA-type zinc-finger protein in plants (ZPT2-1, renamed from EPF1) was identified from petunia. It contains two canonical TFIIIA-type zinc-finger motifs. It is characterized by a long spacer between the two zinc fingers in contrast to clustered-type protein found in animals. Another structural feature of ZPT2-1 is that both the zinc-finger motifs contain a sequence, QALGGH, in the putative DNA-contacting surfaces. This sequence is quite highly conserved in many TFIIIA-type zinc-finger proteins in plants. The TFIIIA–type zinc finger family in plants has more than 30 proteins, like STZ. STZ in *Arabidopsis* complements the salt sensitive phenotype of a yeast mutant which is deficient in the phosphoprotein phosphatase calcineurin (Lippuner et al., 1996). The expression of the *STZ* gene increases with salt treatment in plants. These observations suggest the role of STZ in the regulatory processes associated with salt tolerance in plants. These proteins have either two or three TFIIIA-type zinc fingers, including the QALGGH sequence. Another TFIIIA-type, SUPERMAN protein contains only one zinc finger that is similar to those of the EPF family in petunia in terms of the presence of the QALGGH sequence. It should be noted that all the proteins mentioned above contain the QALGGH sequence in zinc-finger motifs. Interestingly, this conserved sequence motif has not been reported from organisms other than plants, suggesting that this type of zinc-finger protein, which

forms a major class of transcription factors in plants, might be involved in controlling the processes that are unique to plants.

2.2.5.2.4.2 GATA1-like

The GATA1 family forms one of the major families of the Cys2/Cys2-type zinc-finger transcription factors in eukaryotes. The DNA-binding domain consensus is CX2CX17CX2C and contains a zinc atom coordinated by the conserved four cysteines. The CONSTANS protein that promotes flowering in *Arabidopsis* has two repeats of GATA1-like zinc fingers in CX2CX16CX2C arrangement. Similar to the *STZ*, *AtSTO* (Lippuner et al., 1996) complements the salt-sensitive phenotype of the yeast mutant deficient in the phosphoprotein Calcineurin, which is implicated in the dephosphorylation of ion channels. In addition to reducing the growth of the mutant yeast in high-salt media, *STO* can partially compensate for the absence of Calcineurin in all tested processes: recovery from the growth arrest induced by a factor and increased tolerance to Mn^{2+}. Thus, *STO* might represent a component of the salt tolerance mechanism that is conserved in yeast and plants.

2.2.5.2.4.3 Dof family

The Dof (DNA binding with one finger) family proteins interact with the promoter sequences of several genes, whose expression is regulated tissue-specifically or in response to stress signals. Dof family proteins are characterized by the presence of a conserved domain (Dof domain) including a zinc-fingerlike motif, CX2CX21CX2C, followed by a basic region (Yanagisawa, 1996). Dof domain-like sequences are neither present in the yeast genome nor in animals.

2.2.5.2.4.4 RING-finger type

The RING finger is a bipartite asymmetric motif, C-X2- C-loop I-C-X-H-X2-C-X2-C-X2-C-loop II-C-X-C (loops I and II are variable in length), which has been found in many regulatory proteins throughout the plant, animal, fungal, viral and protozoan kingdoms. In these proteins, the RING-finger domain is considered more likely to mediate protein-protein interactions (Borden et al., 1995), but their direct involvement in DNA binding has not been ruled out. In plants, COP1 (*CONSTITUTIVE PHOTOMORPHOGENIC*1) is the best characterized regulatory protein containing the RING-finger motif.

2.2.5.2.4.5 PHD-finger type

PHD finger (Cys4-His-Cys3), is similar to the RING finger (Cys3-His-Cys4) in the arrangement of putative zinc-binding amino acids. The PHD finger (Plant Homeodomain

finger) is so called because this sequence motif was originally noted in two plant proteins containing homeodomains.

2.2.5.2.4.*6* LIM family

The LIM contains a cysteine-rich motif of CX2- CX17–19HX2CX2CX2CX16–20CX2–3C. Based on association with other functional domains, LIM-domain-containing proteins are categorized into three subclasses (Sanchez-Garcia and Rabbitts, 1994). One class of proteins contains a homeodomains (LIM-HD proteins). The second class has no homeodomain, but contains only LIM domains (LIM-only proteins). The third class has two LIM domains linked to a protein kinase domain. Many LIM-containing proteins have been implicated in the transcriptional regulation of cell differentiation and growth regulation. Recent evidences suggest that the LIM domain serves as the site for protein-protein interactions with itself (homodimerization) (Feuerstein et al., 1994), with helix-loop-helix type transcription factors (Johnson et al., 1997; Kong et al., 1997) or with protein kinases (Kuroda et al., 1996).

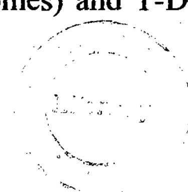
2.2.6 Other regulatory factors

Enzymes involved in ABA biosynthesis, are also useful for improving stress tolerance by regulating many stress-related genes in transgenic plants. ABA is synthesized de novo primarily in response to drought and high salinity stress. It was demonstrated that overexpression of the gene encoding 9-cisepoxycarotenoid dioxygenase (NCED), a key enzyme in ABA biosynthesis, improves drought stress tolerance in transgenic *Arabidopsis* plants (Iuchi et al., 2001).

2.3 Model systems to study functions of regulatory proteins

From the above outline of common themes in abiotic stress regulation, it can be seen that most of the research in plant environmental stress signaling is conducted by a candidate gene approach, i.e. identify candidate genes based on knowledge in heterologous systems and then characterize their expression or biochemical functions in plants under stress conditions. These studies are sometimes strengthened by altering the expression of these genes to result in phenotypic alterations in plants under various stress conditions. In order to reveal their putative functions involved in abiotic stress tolerance, various high throughput methods were developed for the confirmation and validation of gene function by gene inactivation. There are two main complementary approaches developed for identifying mutations in target genes, namely TILLING (Targeted Induced Local Lesions In Genomes) and T-DNA insertion mutant lines. Using these techniques

632.1 J193 T



TH-16455

various attempts were made in order to show the putative functions of abiotic stress-responsive genes in *Arabidopsis*, rice, maize and barley.

Heterologous complementation in yeast mutants is also reliable upto an extent. As has been demonstrated in a few cases in yeast, when one gene is knocked out in a mutant, another gene that does not function in that pathway in the wild type may fill in the position and complement the defect (Sprague, 1998). But, circumstantial evidence obtained from expression and biochemical studies in many cases requires the confirmation from genetic studies. However, until now, no mutations in abiotic stress receptors, phosphoinositol module, CDPKs and MAPK module as related to abiotic signaling have been reported in plants. Clearly, genetic studies still have many gaps to bridge in our understanding of abiotic stress signaling in plants.

2.3.1 Model plants can be employed to study abiotic stress regulation

A key to progress towards breeding better crops under stress has been to understand the changes in cellular, biochemical and molecular machinery that occur in response to stress. Modern molecular techniques involve the identification and use of molecular markers that can enhance breeding programs but the lack of a precise knowledge of the key genes underlying the QTLs made this technique less acceptable. Therefore, the development of genetically engineered plants by the introduction and/or overexpression of selected genes seems to be a viable option to hasten the breeding of ''improved'' plants. Intuitively, genetic engineering would be a faster way to insert beneficial genes than through conventional or molecular breeding. These sophisticated approaches for the molecular breeding of stress-tolerant crops will require the unraveling of additional stress-associated gene resources, from both crop plants and model plant species that are highly stress-tolerant. The genome sequences of two model plants, *Arabidopsis* and rice, have now been determined and have revealed the limitations of linear models for environmental signal transduction. Moreover most of the recent molecular studies on plant stress tolerance have been done with *Arabidopsis* as a model system.

Numerous signal or signal-like molecules have now been identified and presumed, through some evidence, to function in plants as mediators of osmotic adaptation. Some of these affect osmotic tolerance in genetically modified plants. Transcriptional modulation has always been predicted to play a major role in the control of plant responses to stress (Shinozaki and Yamaguchi-Shinozaki, 1996; Zhu et al., 1997). Transcription factors have been identified based on interaction with promoters of osmotic/salt stress– responsive genes. Since the promoters that are controlled by these transcription factors are

responsive to several environmental signals, it is not clear which transcription factors, if any, function only in osmotic/salt stress responses. ABA-deficient and -insensitive mutants have been used to delineate transcription factors as components of osmotic stress signal transduction pathways that either involve or are independent of the growth regulator (Shinozaki and Yamaguchi-Shinozaki, 1996). ABRE and DRE/CRT are cis-acting elements that function in ABA-dependent and ABA-independent gene expression, respectively, in response to abiotic stress. Several cDNAs encoding the DRE binding proteins, DREB1A and DREB2A have been isolated from *A. thaliana* and shown to specifically bind and activate the transcription of genes containing DRE sequences (Liu et al., 1998). DREB1/CBFs are thought to function in cold-responsive gene expression, whereas DREB2s are involved in drought-responsive gene expression.

The transcriptional activation of stress-induced genes has been possible in transgenic plants over-expressing one or more transcription factors that recognize regulatory elements of these genes. *DREB1A* overexpressing transgenic plants exhibited constitutive activation of stress-responsive genes and enhanced freezing, dehydration, and salt tolerance (Liu et al., 1998; Kasuga et al., 1999). Ectopic overexpression of *CBF1/DREB1B* in transgenic plants induces cold-responsive genes and enhances freezing tolerance (Thomashow, 1999; Jaglo et al., 2001). The DREB2 genes are induced by dehydration stress and may activate other genes involved in drought stress tolerance (Liu et al., 1998). The AREB/ABF proteins require an ABA-mediated signal for their activation, as indicated by their reduced activity in the ABA-deficient *aba2* and ABA-insensitive *abi1* mutants and their enhanced activity in the ABA-hypersensitive *era1* mutant of *Arabidopsis* (Uno et al., 2000). Overexpression of ABF3 or AREB2/ABF4 caused ABA hypersensitivity, reduced the transpiration rate, and enhanced drought tolerance in transgenic *Arabidopsis* plants (Kang et al., 2002).

Recently, transgenic plants expressing a phosphorylated form of AREB1 with multisite mutations displayed induction of many ABA-responsive genes without exogenous ABA application (Fujita et al., 2005; Furihata et al., 2006). MYC and MYB proteins are synthesized following accumulation of endogenous ABA, defining their role in later stage stress responses. Overexpression of both AtMYC2 and AtMYB2 not only resulted in an ABA-hypersensitive phenotype but also improved osmotic stress tolerance of the transgenic plants. Recently, a drought-inducible RD26 gene encoding a NAC transcription factor was identified (Fujita et al., 2004). Expression of this RD26 NAC transcription factor gene is induced by drought, high salinity, ABA, and JA treatments.

Thermo-tolerant *Arabidopsis* plants were raised by de-repressing the activity of ATHSF1, a heat shock transcription factor leading to the constitutive expression of heat shock proteins at normal temperature (Lee et al., 1995). Another transcriptional regulator, Alfin1, when overexpressed in transgenic alfalfa (*Medicago sativa L.*) plants regulated endogenous MsPRP2 (NaCl-inducible gene) mRNA levels, resulting in salinity tolerance, comparable, to a few available salt tolerant plants (Figure 2.5) ((Winicov and Bastola, 1999).

Constitutive overexpression of SCOF-1, a soybean protein, increased cold tolerance of transgenic *Arabidopsis* and tobacco (*Nicotiana tabacum*) plants (Kim et al., 2001). Similarly, transgenic plants developed by expressing a drought-responsive AP2-type TF, SHN1-3 orWXP1, induced several wax-related genes resulting in enhanced cuticular wax accumulation and increased drought tolerance (Zhang et al., 2000). Thus, clearly, the overexpression of some drought-responsive transcription factors can lead to the expression of downstream genes and the enhancement of abiotic stress tolerance in plants (Zhang et al., 2004). Cross-species complementation studies have indicated possible roles for several legume TFs. For example, the *Arabidopsis apetala1* (*ap1*) floral development mutant was rescued using a PIM cDNA clone, which they called PEAM4 (Berbel et al., 2001). Likewise, *PsPI*, a pea MADS-box gene homologous to the petal and stamen identity genes *PISTILLATA* (*PI*), from *Arabidopsis* and *GLOBOSA*, from snapdragon were characterized (Berbel et al., 2001). Similarly, overexpression of *PvNAP*, a kidney bean (*Phaseolus vulgaris*) NACTF homologous to *Arabidopsis AtNAP*, successfully complemented the leaf abscission phenotype of an *atnap* null mutant, indicating a possible role of NAP in bean leaf abscission (Guo and Gan, 2006).

In addition to transcription factors, genes involved in stress signal sensing and a cascade of stress-signaling in *A. thaliana* has been of recent research interest (Winicov and Bastola, 1997). Study of these signal transduction components is an approach to know how the sensitivity of cells can be reduced to stress conditions, or such that a low level of constitutive expression of stress genes is induced. Notable among these is the *SOS3* gene that encodes a Ca^{2+} binding CNB-like protein. Mutation of *SOS3* produces salt-sensitive plants with altered K^+ transport characteristics (Liu and Zhu, 1997; Liu et al., 1998). Another CNB-like protein, AtCLB1 is able to suppress salt sensitivity in CNB yeast mutants, but only in the presence of a mammalian CNA subunit (Kudla et al., 1999). The yeast *CNA/CNB* genes are also able to confer salinity tolerance when overexpressed in transgenic plants, further implicating these regulatory molecules in plant

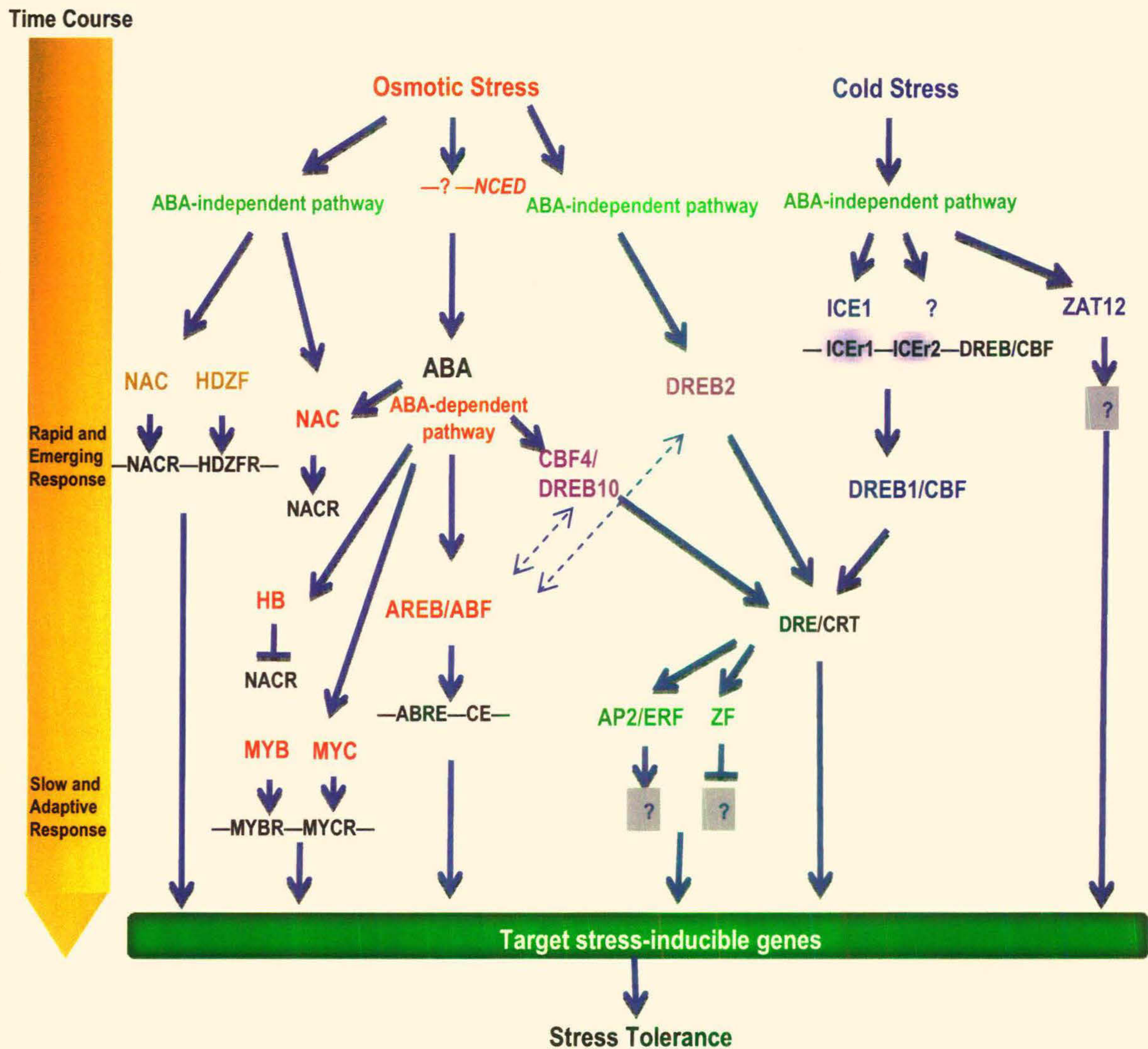


Figure 2.5. Transcriptional regulatory network of *cis-acting elements and transcriptional* factors involved in osmotic- and cold-stress responsive gene expression. Regulatory cascade of stress-responsive gene expression is shown from top to bottom. Early and emergency responses of gene expression are shown in upper part, and late and adaptive responses in the bottom. Thick arrows indicate the major signalling pathways and these pathways regulate many downstream genes. Broken arrows indicate protein-protein interactions.

stress signaling. It seems clear that a Ca^{2+} binding, CNB-like molecule plays an important role in osmotic stress responses in plants. Transgenic tobacco plants produced by altering stress signaling through functional reconstitution of activated yeast calcineurin not only opened-up new routes for study of stress signaling, but also for engineering transgenic crops with enhanced stress tolerance (Grover et al., 1999).

Overexpression of functionally conserved *At-DBF2* (homolog of yeast DBf2 kinase) showed striking multiple stress tolerance in *Arabidopsis* plants (Lee et al., 1999). Mutation of the *ABI1* gene that renders plants insensitive to ABA also causes an osmotic stress-sensitive phenotype (Finkelstein and Somerville, 1990), confirming the important role of ABA in stress signaling. Overexpression of an osmotic-stress-activated protein kinase, SRK2C resulted in a higher drought tolerance in *A. thaliana*, which coincided with the upregulation of stress-responsive genes (Umezawa et al., 2004). Similarly, a truncated tobacco mitogen-activated protein kinase kinase kinase (MAPKKK), NPK1, activated an oxidative signal cascade resulting in cold, heat, salinity and drought tolerance in transgenic plants (Kovtun et al., 2000; Shou et al., 2004). Although direct evidence has not been given to SLN1 homologue of *A. thaliana* histidine kinase (*AtHK1*), it is thought to be a candidate sensor, as it is upregulated during salt and low temperature stresses (Urao et al., 1999). Receptor-like protein kinase (*NtC7*) induced under abiotic stress response has been shown to confer osmotic stress tolerance in overexpressing transgenic tobacco plants (Tamura et al., 2003). In addition, activation of MAPK cascade in response to cold and salt stress has been identified in *Arabidopsis* by yeast two-hybrid interactions, complementation of osmosensitive yeast mutants and by overexpression studies in plants (Teige et al., 2004). Results have been suggested that MAPK cascade is an important convergent point for cross-talk between different abiotic stress responses. Interestingly, calcium-dependent protein kinase (*CDPK*) has been identified as an important component of osmotic signaling pathways. Rice *CDPK7* gene has been overexpressed and shown to be a positive regulator in triggering stress-responsive genes in response to salt/drought, however transgenic plants confer tolerance to cold, drought and salinity stress (Saijo et al., 2000).

Similar to the *Arabidopsis* findings, the products of stress inducible genes identified in rice can also be classified into functional proteins and regulatory proteins (Rabbani et al., 2003). Comparative analysis of regulatory genes in *Arabidopsis* with those in rice revealed a considerable degree of similarity in stress regulation between the two genomes at the molecular level. Rice homologues of CBF/DREB1 and DREB2, ten OsDREB1s

and four OsDREB2s, respectively, have been identified based on rice genome sequence analyses. The function of these genes in stress-inducible gene expression has been demonstrated in rice. Overexpression of OsDREB1A in *Arabidopsis* revealed a similar function of the rice genes in stress-responsive gene expression and stress tolerance (Dubouzet et al., 2003). Recently, overexpression of OsDREB1 or *Arabidopsis* DREB1 also improved drought and chilling tolerance in rice (Ito et al., 2006). Transgenic overexpression of *OsMAPK5* in rice (Xiong and Yang, 2003), increased tolerance to several abiotic stresses, including salt stress, probably by enhancing ROS detoxification. These data indicate that similar transcription factors function in abiotic stress tolerance between dicotyledonous and monocotyledonous plants.

Desiccation-tolerant plants (generally referred to as resurrection plants) have been widely used as model plants for dehydration studies (Bartels and Salamini, 2001). These studies suggest that desiccation tolerance in the vegetative tissues of *C. plantagineum* is unlikely to result from the presence of genes that are unique to resurrection plants, as the relevant genes are also present in the genome of non-tolerant plants. Thus, the difference between desiccation-tolerant and non-tolerant plants is likely to reside in the expression patterns and regulation of the genes (Deng et al., 2002).

Likewise, *Mesembryanthemum crystallinum* (ice plant) is a halophyte that turned out to be a favorable model plant. Salt stress response mechanisms in the ice plant were studied with respect to both the salt-stress-induced C3/CAM shift and through the characterization of Na^+/K^+ transporters (Su et al., 2003) and aquaporins (Vera-Estrella et al., 2004).

Thellungiella halophila (salt cress) is an extreme halophyte which was recently shown to be a most amenable model plant for investigating tolerance to salinity stress. *T. halophila* is closely related to *A. thaliana.* In contrast to *Arabidopsis,* however, *Thellungiella* tolerates extreme salinity, cold and drought. Furthermore, *T. halophila* shares many of the advantages of *A. thaliana* as an experimental system. Salt tolerance in *Thellungiella* is associated with specific features of ion transport, including high K^+/Na^+ selectivity of ion uptake into root cells and K^+/Na^+ exchange between leaf epidermal and mesophyll cells.

2.3.2 Yeast as a model employed to study abiotic stress regulation

The precise mechanism (s) by which plants perceive osmotic stress is still a matter of debate. As researchers try to dissect the complex patterns of molecular biology, the use of different model organisms has become one way to break down more difficult systems into

simpler ones. The rationale for using model organisms is the fact that the main pathways and regulatory systems have been strongly conserved between different organisms during evolution. Hence, understanding a simple organism can provide a low resolution map that is useful for understanding the more complex patterns of a higher organism. The yeast *Saccharomyces cerevisiae*, also called budding yeast or baker's yeast, is one of the oldest and most thoroughly studied model organisms due to its many genetic and practical advantages. However, yeast has the great advantage of being a eukaryote, and is therefore more similar to higher organisms such as animals and plants. It is therefore frequently possible to express recombinant proteins from higher eukaryotes which are functional in yeast. Compared to higher organisms, there are also fewer gene duplications. This, in combination with the fact that recombinant transformed DNA is easily integrated into the yeast genome by homologous recombination, makes it easy to study gene function by targeting specific yeast genes for disruptions (gene knockouts) or introducing other well-defined mutations into the genome. Especially the biological role of the stress activated pathways is a key focus for several research groups globally. In yeast, the ease through which genes encoding components of the pathways can be manipulated has greatly aided this endeavour.

2.3.2.1 Osmopathways in yeast

Increased and decreased expression of genes is controlled by signaling pathways that sense osmotic changes and transmit the signal to the transcriptional machinery. Changes in medium osmolarity have been shown to affect different signaling pathways in yeasts. In high osmotic condition, *S. cerevisiae,* initiates an efficient adaptive response, which maintains cellular Na^+/K^+ balance, retains turgor and repairs cellular damages. Salt stress signaling, especially the adaptation to the hyperosmotic stress, is mediated by a MAPK cascade (Maeda et al., 1994; Maeda et al., 1995).

2.3.2.1.1 HOG MAP Kinase Pathway

The HOG pathway is the best-understood osmo-responsive system in eukaryotes and hence serves as a prototype osmo-regulating signaling pathway. By far the best-characterized MAP kinase system is the HOG pathway. The inability of mutants with an inactive HOG pathway to adapt properly to high-osmolarity medium and the known function of genes whose expression is stimulated via the HOG pathway confirm that the cellular role of the HOG pathway is indeed to orchestrate a significant part of the transcriptional response of yeast cells to high osmolarity. The HOG pathway also mediates posttranscriptional effects. Sensor molecules, such as the high osmolarity sensor

SLN1 or the low osmolarity sensor SHO1, which are located on the plasma membrane, initiate the signaling pathway (Ota and Varshavsky, 1993).

The signal activates two apparently redundant MAPKKK's, Ssk2 and Ssk22 which phosphorylate and activate MAPKK, Pbs2 (Brewster et al., 1993), which in turn stimulating the MAPK, Hogl (Posas et al., 1996) (Figure 2.6). Activated Hoglp after moving to nucleus further induces downstream osmoresponsive genes through at least five transcription regulators. Msn2p, Msn4p (Martinez-Pastor et al., 1996; Schmitt and McEntee, 1996; Rep et al., 1999) are two functionally redundant C2H2 zinc finger proteins and activate STRE (Stress responsive upstream activator element) mediated induction of several general stress responsive genes *CTT1*, *HSP12*, *DDR2*, *TPS2* etc, required possibly for damage repair (Estruch, 2000; Rep et al., 2000; Mager and Siderius, 2002). Two other Hoglp-regulated transcription activators, Msnl and Hotlp regulate *GPD1*, *GPP2*, genes for glycerol biosynthesis enzymes (Rep et al., 1999).

Under osmotic stress Hoglp regulated transcription factors recruit activated Hoglp directly to osmoresponsive promoters (Alepuz et al., 2001; Proft and Struhl, 2002) that further stimulate recruitment of RNA Pol II (Alepuz et al., 2003) and Rpd3 histone deacetylase to promote transcription initiation (De Nadal et al., 2004). Skolp (Proft and Serrano, 1999), related to bZIP/ATF family of transcriptional regulators (Wilkinson et al., 1996), represses *ENA1* expression through CRE (Cyclic AMP Responsive Element) in unstressed condition (Figure 2.7). Under hypertonic stress Skolp is phosphorylated by Hoglp and converted into a transcription activator by recruiting SAGA histone deacetylase and SWI/SNF complex to promote chromatin remodeling (Proft and Struhl, 2002) and induce *ENA1* expression in conjunction with calcineurin (CAN)/Crzlp mediated pathway (Proft and Serrano, 1999). Skolp/Acrlp also controls expression of *HAL1*, which was isolated in a screen for genes that confer enhanced salt tolerance when overexpressed (Rios et al., 1997; Proft and Serrano, 1999). Taken together it seems that Skolp/Acrlp can function as both activator and repressor. An attractive speculation, based on recent findings with Hotlp as the recruiting factor for Hoglp and the ability of Hoglp to activate transcription when targeted to a promoter (Alepuz et al., 2001), would be that Skolp/Acrlp and Hoglp form a complex that activates transcription. Genetic evidence and gene expression data link additional transcription factors to HOG pathway function: Smplp (Posas et al., 2000), Sgdlp (Akhtar et al., 2000), and Gcn4p (Pascual-Ahuir et al., 2001).

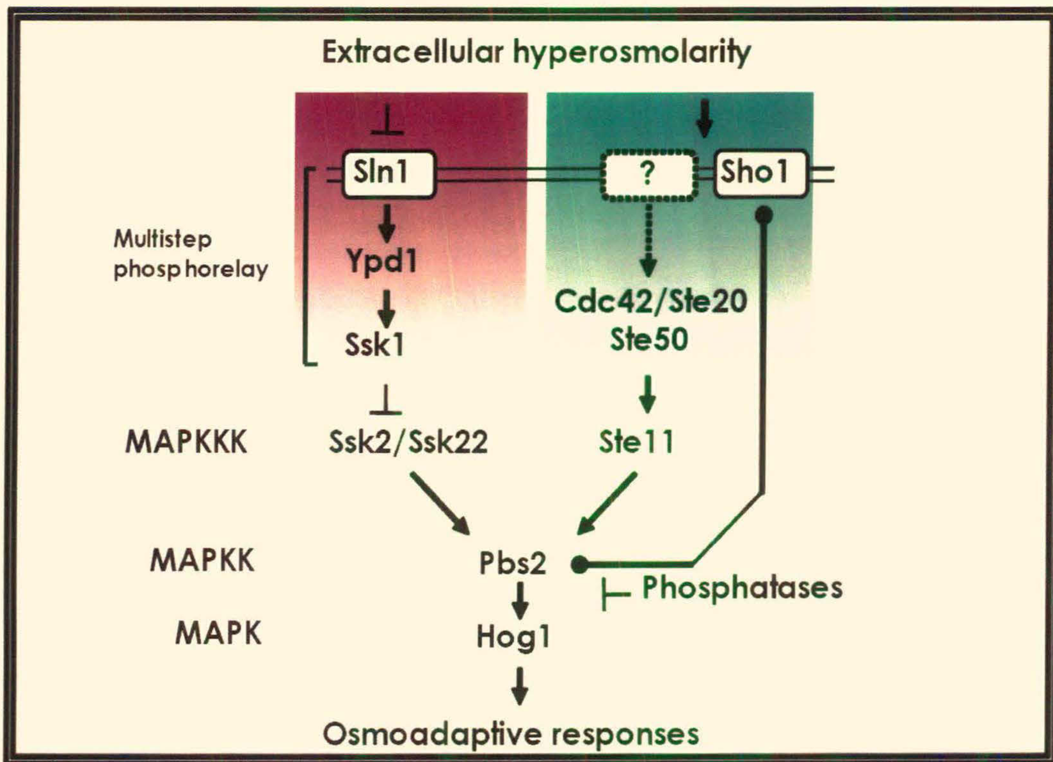


Figure 2.6. The HOG Pathway. The HOG pathway, a MAPK kinase pathway, is constituted by a central core of MAPK kinases and two osmosensing branches Sln1 and Sho1. The signal from two MAPKK converges at the MAPKK Pbs2. Once Hog1p has been activated different osmoadaptive responeses are unleashed.

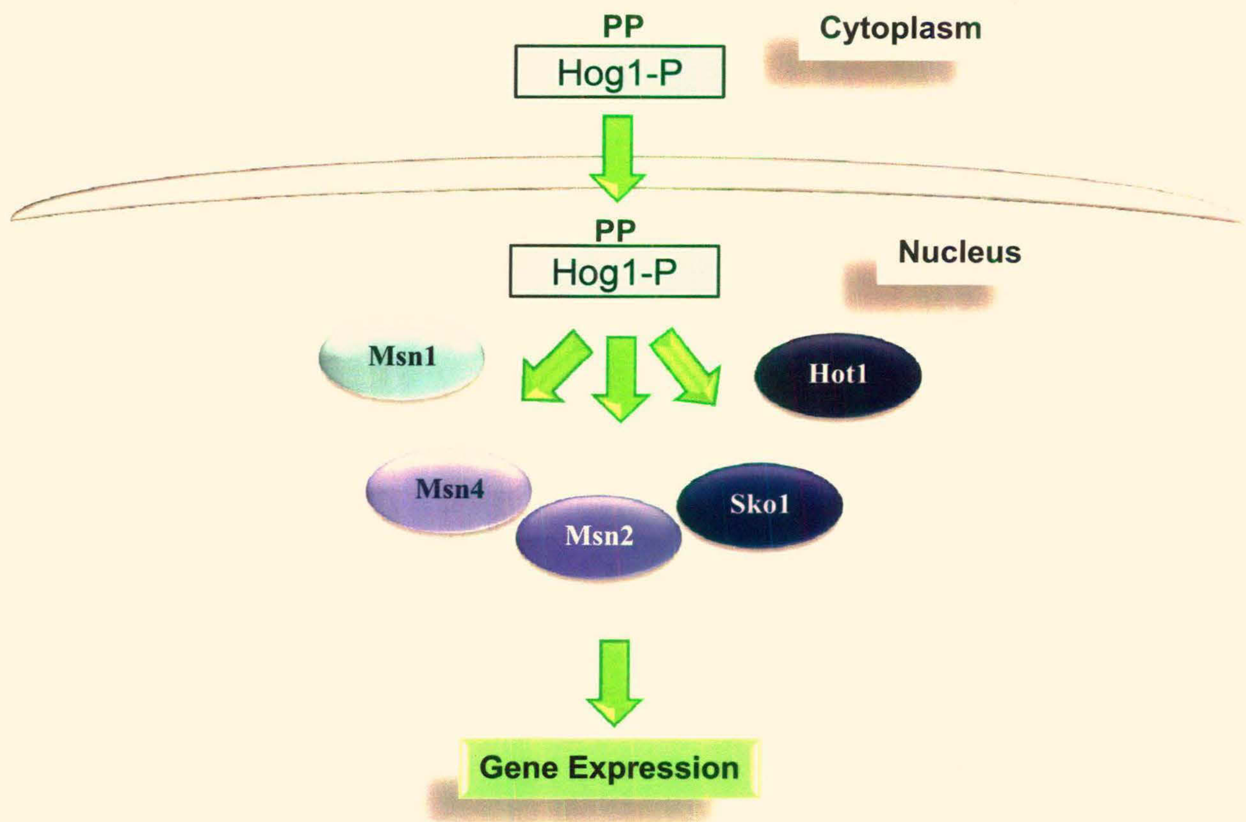


Figure 2.7. Transcription factors under the control of the MAPK Hog1. At least five known transcription factors are under the control of MAPK Hog1p. Msn2p/Msn4p are a pair of apparently redundant transcription factors, Hot1 is one of the best characterized transcription factors under the control of Hog1p and belongs to bZIP class of TFs, Msn1p is distantly related to Hot1p and Sko1p is a protein that belongs to the ATF/CREB family of AP1-related TFs (ATF).

2.3.2.1.2 Calcineurin pathway

In addition to HOG1 MAP kinase pathway, yeast has another signal transduction pathway that is specific for the ionic stress of high NaCl concentrations. This pathway includes calmodulin and Calcineurin (CAN). Calcineurin is a calcium- and calmodulin-dependent serine-threonine protein phosphatase conserved throughout eukaryotic organisms, in which it mediates numerous calcium-dependent signaling events (Aramburu et al., 2000; Rusnak and Mertz, 2000). CAN is a heterodimeric phosphatase 2B with two catalytic subunits, CNA1 and CNA2, and a regulatory subunit CNB. To study CAN function, it can be inactivated by deleting either both *CNA1* and *CNA2* or *CNB1* or by the addition of the anti-inflammatory drug FK506 (Liu, 1993). In *S. cerevisiae*, CAN-dependent signaling is activated by a rise in intracellular Ca^{2+}, which in turn is stimulated by a shift to high temperature (Zhao et al., 1998), hypo-osmotic shock (Batiza et al., 1996), sustained exposure to mating pheromone (Foor et al., 1992; Cyert, 2003), or an increase in the level of extracellular ions such as Na^+, Li^+, and Ca^{2+} (Nakamura et al., 1993; Mendoza et al., 1994; Wieland et al., 1995). Typical phenotypes caused by inactivation of CAN are sensitivity to Na^+, Li^+, and other ions (Mendoza et al., 1994). The CAN phosphatase exerts its effect by dephosphorylating and thereby activating nuclear translocation of the Crz1p zinc finger-containing transcription factor (Matheos et al., 1997; Stathopoulos and Cyert, 1997). Crz1p binds to a promoter element called the CAN-dependent response element (Stathopoulos and Cyert, 1997).

The role of CAN in ion homeostasis is effected by controlling *PMC1*, *PMR1*, and *PMR2*, which encode P-type ATPases that mediate transport of Ca^{2+} ions between different cellular compartments (Cunningham and Fink, 1996), *ENA1* (Garciadeblas et al., 1993), and *TRK1* and *TRK2*, which encode K^+ uptake systems. Control of *TRK1* and *-2* appears to be independent of Crz1p (Mendizabal et al., 2001). Only a part of ENA1 expression is CAN-dependent (Stathopoulos and Cyert, 1997) suggesting that other Na^+-stress response pathways also contribute to ENA1 induction (Ganster et al., 1998). Although no potential biological sensor for Na^+ has been identified (Zhu, 2001), external high NaCl stress increases intracellular Ca^{2+} concentration, causing calmodulin to transmit the signal to downstream components such as CAN (Matheos et al., 1997). CAN mutants (i.e., *cna1cna2* and *cnb*) fail to grow in growth medium having high concentration of either Na^+, Li^+, or Mn^{2+} (Nakamura et al., 1993; Breuder et al., 1994; Mendoza et al., 1994) suggest that CAN participates in regulating the intracellular concentration of several ions (Cyert et al., 1991; Garrett-Engele et al., 1995). In addition

to *ENA1*, some other gene(s) are also contributing to salt tolerances that have been regulated by CAN osmopathway (Mendizabal et al., 1998).

2.3.2.2 Study of regulatory factors in yeast

Rapid technical developments and insightful use of yeast as a model organism have greatly facilitated our discovery of these regulator genes (Serrano, 1996). The art of heterologous protein expression in *S. cerevisiae* has been refined over many years. One advantage of the yeast system is the availability of strong and constitutive promoters that drive the expression of proteins which may belong to cross species. Not only individual genes but also members of an entire gene family, as well as the regulators that control their expression, may be systematically deleted with relative ease in yeast. This allows heterologous expression in a well-controlled system devoid of background contamination from endogenous proteins of similar function. Multiple gene deletion in yeast mostly relies on the efficiency of homologous recombination to target chromosomal loci and on the subsequent crossing of various mutants to produce the desired combination. As a result, it is possible to create an entirely new "designer strain" that serves almost any experimental need.

Automated sequencing lead to an explosion of genome sequencing projects, resulting in the discovery of novel genes of unknown function. This is where the *S. cerevisiae* system demonstrates its most powerful feature, functional complementation: essentially the restoration to wild-type phenotypes by *YFG* (your favorite gene) in a yeast strain lacking the putative homolog. One of the striking advantages of functional complementation is that mutations in *YFG* may be rapidly screened for loss-of-function phenotypes in structure-function analysis. When a genome sequencing or chromosomal mapping project yields a novel gene *YFG*, the first step is to search for putative homologs whose functions have been studied and/or documented in databases. Once a putative homolog is identified on the basis of sequence similarity, *YFG* can be cloned into a yeast expression vector and transformed into the strain with a deletion in the corresponding yeast gene (*Δscyfg*). Alternatively, if *ScYFG* is essential, it can be replaced with the heterologous gene.

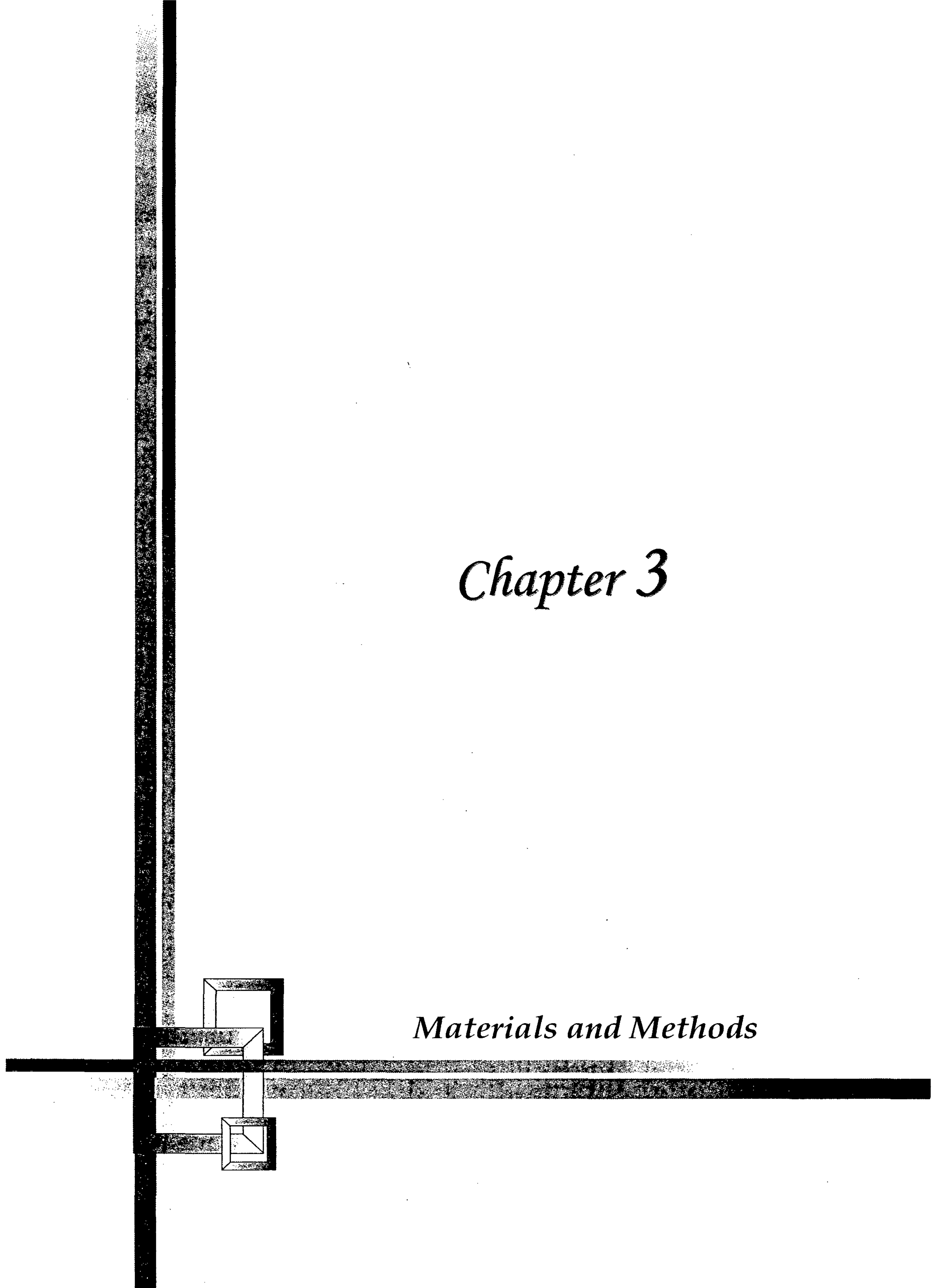
Functional complementation yields the first experimental clue to the biological role of a novel gene, which can be subsequently confirmed by biochemical characterization. It is important to note that there need not be sequence similarity between *YFG* and its yeast counterpart for functional complementation to be applied successfully. For example, the K^+ uptake defect of the yeast *trk1trk2* deletion mutant prevents growth in K^+-limited

medium. Functional complementation of this growth limitation has been successfully exploited for expression cloning, mutagenesis, and trafficking studies of a variety of unrelated K^+ channels from plants (Anderson et al., 1992; Bertl et al., 1995). Since similar signal transduction pathways of yeast and plants have been proposed (Pardo et al., 1998; Lee et al., 1999), determinants of plant stress tolerance could be identified by functional complementation of osmotic, sensitive yeast mutants. A putative MAPK has been identified from *Pisum sativum* (PsMAPK), with 47% primary sequence identity to Hog1p, functionally suppressed salt-induced cell growth inhibition of *hog1* (Popping et al., 1996). Combinations of *Arabidopsis* proteins ATMEKK1 (MAPKKK) and MEK1 (MAPKK), or ATMEKK1 and ATMKK2 (MAPKK) suppressed growth defects of *pbs2Δ* (encodes the MAPKK of the HOG pathway), implicating these as functional components of an osmotic stress MAP kinase cascade (Ichimura et al., 1998). AtDBF2 kinase of *Arabidopsis* mediated functional sufficiency of yeast cells for LiCl tolerance (Lee et al., 1999). *Arabidopsis SAL1* was isolated by complementation of a mutant defective for ENA ATPase activity (Quintero et al., 1996).

Two *Arabidopsis* transcription factor-encoding cDNAs *STO* (salt tolerance) and *STZ* (salt tolerance zinc finger) suppressed the Na^+/Li^+-sensitive phenotype of a CAN-deficient (*cna1-2Δ* or *cnbΔ*) mutant (Lippuner et al., 1996). STO is similar to *Arabidopsis* CONSTANS. *STZ*-mediated salt tolerance is at least partially dependent on *ENA1*, implying a function that involves regulation of the Na^+-ATPase, whereas STO function is independent of *ENA1*. NtSLT1 is a tobacco protein that suppresses the Na^+/Li^+-sensitive phenotype of a CAN-deficient (*cnbΔ*) mutant (Matsumoto et al., 2001). Since yeast has an ion transport systems at the membranes similar to that in plants (Liang et al., 1997), it was possible to clone i. e. an Arabidopsis Ca^{2+}-ATPase (Liang et al., 1997) by functional complementation of a yeast mutant. Furthermore, an *Arabidopsis* cDNA of *AtGSK1*, a gene induced by NaCl and ABA treatments in the plant, can rescue the NaCl stress-sensitive phenotype of a yeast CAN mutant (Piao et al., 1999). The functionality of a yeast gene in the plant has also been demonstrated. When the yeast *HAL1* gene is overexpressed in yeast, the tolerance of *S. cerevisiae* to NaCl was enhanced (Rios et al., 1997). The intracellular K^+ concentration is increased and the Na^+ concentration decreased. Overexpression of the *HAL1* gene in tomato also improved the salt tolerance of the transgenic lines, which were also able to retain more K^+ (Gisbert et al., 2000).

In tobacco and *Arabidopsis* NACK-PQR pathway, similar to HOG pathway, have been reported (Soyano et al., 2003). Tobacco MAPK kinase NQK1 can functionally

complement Pbs2p (Soyano et al., 2003). A number of examples are available where salt-tolerance determinant plant genes are screened using calcineurin mutants (Anderson et al., 1992; Peng and Verma, 1995; Lippuner et al., 1996; Quintero et al., 1996; Piao et al., 1999), however, example of plant genes; particularly transcription factors suppressing osmosensitivity of *hog1* mutant is rare. ASR1 from tomato though provides osmotolerance to *hog1* mutant and binds to DNA; its sequence shows similarity to late embryogenesis protein family rather than to any transcription factor.



Chapter 3

Materials and Methods

3.1 Materials

3.1.1 Plant materials

Material	Source
Cicer arietinum L.	PusaBGD72 (Dept. of Genetics, IARI, New Delhi, INDIA) and ICCV2 (ICRISAT, Hyderabad, INDIA)
Nicotiana tabacum var. xanthi	Wild type plants were procured from SLS, JNU, New Delhi, INDIA

3.1.2 Yeast strains

Strain	Genotype	Source
BCY123	*Mat a, pep4::HIS3 prb1::LEU2 bar1::HISG lys2::GAL1/10-GAL4 can1 ade2 trp1 ura3 his3 leu2-3, 112 Δlys2cir* +GAL +RAF +SUC	Dr. Nilanjan
BCY123a	Same as BCY213, except *cna1*::HIS3 *cna2*::TRP1	This Study
BCY123b	Same as BCY213, except *cnb*::TRP1	This Study
BCY123c	Same as BCY213, except *hog1*::TRP1	This Study
BCY123d	Same as BCY213, except *msn2*::HIS3 *msn4*::TRP1	This Study
BCY123e	Same as BCY213, except *msn1*::HIS3 *hot1*::TRP1	This Study
BCY123f	Same as BCY213, except *hog1*::TRP1 *cnb*::HIS3	This Study
BCY123g	Same as BCY213, except *hog1*::TRP1 *crz1*::HIS3	This Study
BCY123h	Same as BCY213, except *hog1*::TRP1 *ena1*::HIS3	This Study
BY4742	MAT α *his3Δ leu2Δ lys2Δ ura3Δ* YJL059W::kanMX4	This Study
PJ694A	*MATa trp1-901 leu2-3,112 ura3-52 his3-200 gal4Δ gal80Δ LYS2::GAL-HIS3 GAL2-ADE2 met2::GAL7-lacZ*	Dr. Natrajan

3.1.3 Bacterial strains

Strain	Genotype
Escherichia coli DH5α	Φ8dlacZ Δ M15, recA1, endA1, gyr A96, thi-1, hsd17 (r_k^-, m_k^-) supE44, relA1,deoR, (LacZYA-argF)U19
Agrobacterium tumefaciens (GV3101)	Carry pMP90 Ti-plasmid with gentamicin selection and rifampicin chromosomal selection

3.1.4 Plasmid vectors

Strain	Source	Purpose
pGEM-T Easy vector	Promega	PCR product cloning.
pBI101.2 vector	Clontech	Binary vector with GUS for promoter activity studies.
pBI121 vector	Clontech	Binary vector with GUS for overexpression studies.
pCambia 1302	Clontech	Binary vector with GUS and GFP for subcellular localization
$pGBKT_7$	Clontech	Yeast one hybrid
pYES-2.1-V5 His-TOPO	Invitrogen	Yeast expression vector
pYES-2.1-V5 His-*lacZ*	Invitrogen	Promoter studies in yeast
pGEX4T-2	Amersham	Bacterial expression vactor

3.1.5 Chemicals and materials

Type	Material	Source
Molecular weight markers	100 bp DNA ladder, 1 Kb DNA ladder	Fermentas
X-ray film	Hyperfilm™ MP	Kodak
Nylon Membrane	Hybond N^+	Amersham
Antibiotics	Ampicillin, Kanamycin, Cefatoxime, Rifampicin	Sigma
Radioisotopes	$\alpha^{32}P$ dCTP, $[\gamma P^{32}]$ATP	Perkin Elmer
Disposable filters	PVDF 0.45 µm filter unit	Millipore
Enzymes	Commonly used restriction enzymes	NEB
	Taq DNA Polymerase	Clontech
	T4 DNA Ligase	NEB
	RNase	Amersham
Dyes	Ethidium Bromide, Xylene cyanol, Methylene Blue, Coomasie Brilliant Blue	Amersham
Culture media components	Tryptone, Yeast Extract, Agar, MS salts, BAP, NAA, PDA, Beef Extract, Peptone	Difco, Himedia, Sigma
Locally available chemicals	Isopropanol, iso-amyl alcohol, $CaCl_2$, NaCl, NaOH, Glucose, Methanol, $MgCl_2$, KOH, Potassium acetate, Chloroform, Glycerol, Acetic acid, NaH_2PO_4, Na_2HPO_4, $MgSO_4$, HCl, H_2SO_4, Glycine, KCl, Sucrose, Pot. Dichromate, Sodium hypochlorite, Mercuric chloride, tri-Sodium citrate, Formaldehyde.	Qualigens, HiMedia
Foreign chemicals	RNaseOut, DEPC, HEPES, IPTG, MOPS, Sephadex G-50, EDTA, CTAB, Acrylamide, Bis-Acrylamide, TEMED, Triton-X-100, X-gal, X-gluc, MUG, 4-MU	Amersham, Sigma, USB
Kits	Matchmaker library construction and screening kit	Clontech
	polyA Tract mRNA isolation kit	Roche
	PCR-selected cDNA subtractive kit	Clontech
	5' RACE system for rapid amplification of cDNA ends	Invitrogen
	Advantage 2 polymerase mix	Clontech

3.1.6 Oligonucleotides used in the present study

Primer	Sequence (5'- 3')	Comment (s)
FCNA1	CTGCCATAAAACACTCTCAACGCCAATGACAGAGCAGAAAGCCCTAAG	CNA1 knockout
RCNA1	ATAAAAAAGAATAAAATGAGATTTACTACATAAGAACACCTTTGGTG	CNA1 knockout
FCNA2	CCAGTACTTCTTCTTGAACCCGCAATGTCTGTTATTAATTTCACAGG	CNA2 knockout
RCNA2	ATGTACAGTGGAAATAGGAGCTTTCTCTATTTCTTAGCATTTTTGACGA	CNA2 knockout
FCNB	CATAGAAGCATTTTTATTTCTTAAAATGTCTGTTATTAATTTCACAGG	CNB knockout
RCNB	TAAATGAATGAAGTGTCCCCTAGTCCTATTTCTTAGCATTTTTGACGA	CNB knockout
FHOG	AAAACTACAACTATCGTATATAATAATGTCTGTTATTAATTTCACAGG	HOG1 knockout
RHOG	GGTTAGGGACATTAAAAAAACACGTCTATTTCTTAGCATTTTTGACGA	HOG1 knockout
FMSN2	TGCTCATAGAAGAACTAGATCTAAAATGTCTGTTATTAATTTCACAGG	MSN2 knockout
RMSN2	TATCGAATTAAAAAAATGGGGTCTACTATTTCTTAGCATTTTTGACGA	MSN2 knockout
FMSN4	TTTTCTTCTTATTAAAAACAATATAATGACAGAGCAGAAAGCCCTAGT	MSN4 knockout
RMSN4	TTATTTGCTTTTGACCTTATTTTTTCTACATAAGAACACCTTTGGTGG	MSN4 knockout
FMSN1	TGCTCATAGAAGAACTAGATCTAAAATGGCAAGTAACCAGCACATAGG	MSN1 knockout
RMSN1	TATCGAATTAAAAAAATGGGGTCTATCACTTCAAAGTCTCTGGAATAT	MSN1 knockout

FHOT1	TTTTCTTCTTATTAAAAACAATATAATGTCTGGAATGGGTATTGCGAT	HOT1 knockout
RHOT1	TTATTTGCTTTTGACCTTATTTTTTCTATATTCCAGCAAGGCTCTCTT	HOT1 knockout
FCRZ1	AGTTTCGTCAGACAGTACAAGGAAGATGACAGAGCAGAAAGCCCTAAG	CRZ1 knockout
RCRZ1	TATTCAAAGCTTAAAAAAACAAAAACTACATAAGAACACCTTTGGTG	CRZ1 knockout
FENA1	TCGTACACAGAATTGAAAATTTTCGATGACAGAGCAGAAAGCCCTAAG	ENA1 knockout
RENA1	ATAGGGAGCACTTAATAGGCCCTGCCTACATAAGAACACCTTTGGTG	ENA1 knockout
RHIS3	AGTTCGACAACTGCGTACGG	Diagnostic PCR
RTRP1	AGT AGTATGTTGCAGTCTTTTG	Diagnostic PCR
FCTT1	TACTCTCTACAAAACGGTTTTCCGTAC	CTT1 probe
RCTT1	ATAATCCCAGTATATGGTAGTGTCCTG	CTT1 probe
FHSP12	ATGTCTGACGCAGGTAGAAAAGGATTC	HSP12 probe
RHSP12	ACTTCTTGGTTGGGTCTTCTTCACCGT	HSP12 probe
FGPD1	ATGTCTGCTGCTGCTGATAGATTAAAC	GPD1 probe
RGPD1	AAGAGGATAGCAATTGGACACCTTTAG	GPD1 probe
FGPP2	GGATTGACTACTAAACCTCTATCTTTG	GPP2 probe
RGPP2	GCCATTCCTGCCCTTCAGATATGGTTC	GPP2 probe
FENA1	ACAAGTCTAACTGAAGGTTTGACCCAAG	ENA1 probe
RENA1	TAACGGCACCTAAAGCTTCCAGAGAATC	ENA1 probe
FSTL1	ATAAGCAGAACCAGTCACTGGGGACTTACG	STL1 probe
RSTL1	AGTAGATAAGGCGTAGATTGTTGCGAAGAC	STL1 probe
FPYFL	CCCTCGAGATGGCTTTAGAGTTAGAAGCT	CaZF Cloning
RPYFL	GCTCTAGACTACACCGTTTCATCATCATG	CaZF Cloning
F(-Asn)	CCCTCGAGATGGCTTTAGAGTTAGAAGCT	CaZF Cloning
R(-Asn)	GCTCTAGACTATTCGTAGTGGCATCGTTTGT	CaZF Cloning
F(-DLNL)	CCCTCGAGATGGCTTTAGAGTTAGAAGCT	CaZF Cloning
R(-DLNL)	CGGGATCCGCCCTCAGATATTGTGATTCCACTGCT	CaZF Cloning
F(-Asn)	CCCTCGAGATGGCTTTAGAGTTAGAAGCT	CaZF Cloning
R(-Asn)	CGGGATCCAGGCGCAGGTAGATTGAGGTCAAATCC	CaZF Cloning
F(-KRPR)	CCCTCGAGATGGCTTTAGAGTTAGAAGCT	CaZF Cloning
R(-KRPR)	CGGGATCCGCTTTCCACCTCCTGTTCACCGATTCC	CaZF Cloning
F(-DDE)	CCCTCGAGATGGCTTTAGAGTTAGAAGCT	CaZF Cloning
R(-DDE)	CGGGATCCCCAAAAACACACGTGGCCTTTTGGCGGT	CaZF Cloning
F(-TD)	(NdeI/XhoI)ATGGTGAAGCTGAATCACCGCTGC	CaZF Cloning
R(1/179)	CGGGATCCGATACCTCCTTCGTAGTGGCA	CaZF Cloning
F(198/280)	CGGGATCCAGCAGTGGAATCACAATATCTGAG	CaZF Cloning
FGFP	CATGCCATGGCTTTAGAGTTAGAAGCT	CaZF Cloning
RGFP	GAAGATCTTGCACCGTTTCATCATC	CaZF Cloning
M13F	CGCCAGGGTTTTCCCAGTCACGAC	Sequencing
M13R	AGCGGATAACAATTTCACACAGGA	Sequencing
AP1	GTAATACGACTCACTATAGGGC	Genome walking
AP2	ACTATAGGGCACGCGTGGT	Genome walking
CaZF-pGEX/F	CGGGATTCATGGCTTTAGAGTTAGAAGCT	Protein expression
CaZF-pGEX/R	CGGGATTCCTACACCGTTTCATCATCATG	Protein expression
CaZF-pBI/F	GCTCTAGAATGGCTTTAGAGTTAGAAGCT	*In Planta* expression
CaZF-pBI/R	GCTCTAGACTACACCGTTTCATCATCATG	*In Planta* expression
CaZF-pGB/F	CCCTCGAGATGGCTTTAGAGTTAGAAGCT	Yeast one hybrid
CaZF-pGB/R	CCCTCGAGCTACACCGTTTCATCATCATG	Yeast one hybrid
FActin	CCACGAGACAACATTTAACTC	DNA-array
RActin	TATTCTGCCTTTGCAATCCAC	DNA-array
FNPTII	TTTTCTCCCAATCAGGCTTG	DNA-array
RNPTII	TCAGGCTCTTTCACTCCATC	DNA-array

3.1.7 Concentrations of various antibodies used in the present study

Antibiotic	Abbreviation	Concentration
Ampicillin	Amp	100 µg/ml
Kanamycin	Kan	50 µg/ml
Rifampicin	Rif	30 µg/ml
Hygromycin	Hyg	10 µg/ml

3.2 Methods

3.2.1 General Sterilization Procedures

All the glassware, tissue culture tools and culture media were sterilized by autoclaving at 121.6°C under 15 lb psi pressures for 15 minutes. The antibiotics and other heat labile components were filter sterilized with dispensable syringe driven PVDF filter unit of 0.22µm pore size (Millex™, Millipore, USA).

3.2.2 Nutrient media

Media	Composition
LB	10 g/l Tryptone, 5 g/l Yeast Extract, 10 g/l NaCl. Adjust pH to 7.0 with NaOH. Add 1.5% agar for plates
YEB	10 g/l Beef Extract, 2 g/l Yeast Extract, 5 g/l Peptone, 5 g/l Sucrose, 2 mM $MgSO_4$ Adjust pH to 7.0 with NaOH. Add 1.5% agar for plates
YP	20 g/l Peptone, 10 g/l Yeast extract, 50 mg/l Adenine hemi sulphate (Selectively). Adjust pH to 6.5 with HCl. 20 g/l Glucose and/or 20 g/l Galactose and 20 g/l Raffinose. Add 2% agar for plates
SD	6.7 g/l Yeast nitrogen base without amino acids. Adjust pH to 5.8 with KOH, Autoclave. 100 ml of 10x Drop-out Solution
SD (for *BCY123*)	6.7 g/l Yeast nitrogen base without amino acids, 6.7 g/l NaOH, 10 g/l Succinic acid, 20 g/l dextrose. Adjust pH to 5.8 with KOH, Autoclave. 100 ml of 10x Drop-out Solution
MS	Potassium nitrate 1900 mg/l, Ammonium nitrate 1650 mg/l, Calcium chloride.$2H_2O_2$ 440 mg/l, Potassium dihydrogen phosphate 170 mg/l, Manganese sulphate.H_2O 16.89 mg/l, Boric acid 6.20 mg/l, Potassium iodide 0.83 mg/l, Sodium molybdate anhydrous 0.21 mg/l, Zinc sulphate.$7H_2O$ 8.60 mg/l, Copper sulphate.$5H_2O$ 0.025 mg/l, Cobalt chloride. $6H_2O$ 0.025 mg/l, Ferrous sulphate.$7H_2O$ 27.80 mg/l, EDTA disodium salt.$2H_2O$ 37.30 mg/l, Myo-inositol 100 mg/l, Thiamine hydrochloride 10 mg/l, Pyridoxine hydrochloride 1 mg/l, Nicotinic acid 1 mg/l, Sucrose 3%. Adjusted pH to 5.8 with 2M NaOH, 0.8-1% Difco Bacto Agar for plates
MSBN	MS + BA (1µg/ml) + NAA (0.1 µg/ml), Sucrose 3%. Adjusted pH to 5.8 with 2M NaOH, 0.8-1% Difco Bacto Agar for plates
MSBNCK	MSBN + Cefatoxin (40 µg/ml) + Kanamycin (50 µg/ml), Sucrose 3%. Adjusted pH to 5.8 with 2M NaOH, 0.8-1% Difco Bacto Agar for plates
SOB	20 g/l Tryptone, 5 g/l Yeast Extract, 0.5 g/l NaCl. Add 950 ml of water + 10 ml of 250 mM KCl. Adjust pH to 7.0 with 5 M KOH. After autoclaving add 5 ml of 2 M $MgCl_2$
ITB	10.88 g/l $MnCl_2.4H_2O$, 2.20 g/l $CaCl_2.2H_2O$, 18.65 g/l KCl, 20 ml PIPES (0.5 M, pH=6.7. H_2O to 1 l. Sterilize by filteration
Z-Buffer	16.1 g/l Na2HPO4.7H2O, 5.5 g/l NaH2PO4.H2O, 0.75 g/l KCl, 0.246 g/l MgSO4.7H2O. Adjust pH to 7.0 and autoclave

3.2.3 Plant growth conditions and maintenance

All the chickpea (*Cicer arietinum* L.) varieties used were grown under similar conditions. Seeds were soaked overnight in tap water and sown in soil (3-4 seeds/ pot) in green house with 16/8 h light/dark cycle at 22-25°C, 50-60% relative humidity (RH) and watered regularly during cultivation.

3.2.4 Recombinant DNA techniques for cloning and DNA analysis

3.2.4.1 Polymerase Chain Reaction

Specific DNA fragments were amplified using the polymerase chain reaction (PCR) (Mullis and Faloona, 1987). Rapid amplification of DNA was carried out using the Taq polymerase and a set of convergent primers. Repeating the steps (denaturation, annealing and elongation) for x cycles (usually from 25 to 35) will exponentially enrich the reaction with the primer-flanked DNA sequence. In some cases a suitable synthetic restriction sites were incorporated to the 5′-end of the primer for cloning purposes. The PCR reaction was carried out in a 20 µl reaction volume with the following constituents: 10-50 ng template DNA, 1 µl of 10 pmole each sense and antisense primers, 2 µl of 10 mM dNTPs, 2 µl of 10x Taq-buffer, 2 U Taq-polymerase and H_2O up to 20 µl. The amplification reaction was done in a PCR thermocycler using the following program:

Initial denaturation	2 min 94°C
Denaturation	1 min 94°C
Annealing	1 min 50-65°C
Elongation	2 min 72°C
Repeat steps 2-4 for 24-35 cycles	
Final elongation	10 min 72°C

3.2.4.2 Reverse Transcription PCR (RT-PCR)

The reverse transcription PCR (RT-PCR) is a technique used for mRNA detection and quantification. The RT-PCR reaction was conducted using the Superscript reverse transcriptase (Invitrogen) by following the manufacturer's instructions. In brief, a reaction mixture containing 500 ng isolated total RNA, 0.2 µg/µl random primers/OligodT and RNase free H_2O up to 11 µl was prepared. The reaction was incubated for 10 minutes at 70°C then chilled on ice. To this mixture, 4 µl of 5x Reaction Buffer, 2 µl of 0.1 M DTT, 20 units of RNase inhibitor, 2 µl of 10 mM dNTPs, 1 µl of Reverse Transcriptase enzyme (200u/µl) were added and the reaction was incubated for for 60 minutes at 42°C. Finally, the reaction was heated at 70°C for 10 minutes. Further amplification of synthesized cDNA reaction was carried out by a PCR reaction using the gene specific primers.

3.2.4.3 Separation of DNA on Agarose Gels

DNA samples were mixed with 1/10 volume of 10x DNA loading buffer and then separated on horizontal agarose gels (10 x 7 x 0.3 cm) containing 1x TAE buffer. The gel was prepared by dissolving Agarose in 1x TAE and the concentration of the gel ranged between 1-2% depending on the size of the expected DNA fragment, shorter the fragment higher agarose concentration. Electric current of 3 V/cm was used for 1-2 h to run the gel, and the gel was ended depending on the distance between the migrated bands of the dyes present in the DNA loading buffer. Ethidium bromide solution (0.1% w/v) was used to satin the DNA fragments. The DNA detection was done under UV light. Before exposure to the UV light, the gel was rinsed briefly in H_2O to reduce background staining. In a gel-documentation station, gels were visualized on a UV-transilluminator and documented. The sizes and amount of the DNA fragments were determined using DNA standards.

3.2.4.4 Elution of DNA from agarose gel

The PCR product was fractionated on 1% agarose/EtBr gel. The band was cut by using sterile blade and collected in a 1.5 ml sterile micro-centrifuge tube. The gel elution was performed by using QIAEK II gel extraction kit (Qiagen, Germany). The elution was done according to the manufacturers instructions with minor modifications. 600 µl of the QX1 buffer and 10µl of silica beads provided in the kit was added to the eppendorf containing the gel slice and incubated at 60°C for 10 min to dissolve the agarose. After the complete dissolution of agarose, a brief spin of 13,000 rpm for 1 min was given to pellet down the beads to which DNA has bound. These beads were then washed with 500µl of QX1 buffer and twice with 500µl of PE buffer. Then the beads were dried at 37°C and finally dissolved in 20µl of autoclaved water.

3.2.4.5 Purification of PCR products

PCR products were purified by alcohol precipitation method. To one volume of PCR product, 3M sodium acetate (one tenth vol.) and 2.5 times absolute cold ethanol was added, centrifuged at 13,000 rpm for 20 min at 4°C. Pellet was washed twice with 500 µl of 70% ethanol, air dried and dissolved in 20 µl of sterile water.

3.2.4.6 T/A cloning of PCR Products

The DNA molecule amplified using the Taq- Polymerase is characterized by the presence of additional deoxyadenosine nucleotides (dA) at the 3′-end of the PCR product, which is due to the terminal deoxy-nucleotidiltransferase activity nature of the Taq-polymerase enzyme. PCR product with the 3′-dA overhangs can be used to clone in

a vector having a complementary 3′-deoxytimidine (dT). For this purpose the pGEM®-TEasy vector system kit (Promega) was used. The ligation reaction was performed following the manufacturer's instructions.

3.2.4.7 Restriction Digestion of DNA Molecules

The restriction enzymes of endonucleases type II were used to digest a double stranded DNA molecule for analytical and cloning purposes. The enzymes cut the DNA either as 5′ or 3′ 'sticky' overhangs or as blunt ends. The digestion reactions were incubated in a buffer system optimized for the used enzyme and in the case of double digestion a universal buffer system was used. The activity of the restriction enzymes was estimated in units (U), where 1 U stands for the amount of enzyme cutting completely at optimal conditions 1 μg of λ DNA for 60 minutes.

3.2.4.8 Ligation of DNA Fragments

The conventional cloning of a DNA fragment into a selected plasmid was performed using the T4-DNA ligase (NEB) enzyme, which is able to catalyze the formation of a phosphodiesther chemical bond between free 5′-phosphate and 3′-OH groups of double-stranded DNA fragments and vectors. The donor DNA fragment (10x accesses to the vector) was incubated with the vector DNA, 2 μl of ligation buffer and 1 μl of T4-DNA ligase for 16 h at 16°C.

3.2.4.9 Preparation of Competent Bacterial Cells

For cloning purpose, *E. coli* DH5α bacterial strain were made competent by the below given methods and used for transformation.

Calcium Chloride Method

The $CaCl_2$ method was adopted from Sambrook et.al., (2001) with some minor modifications. From the overnight grown pre-culture of bacterial cells, 1 ml of inoculum was used to inoculate 100 ml LB medium in a culture flask. This culture was grown at 37°C with vigorous shaking (200-250 rpm) to an A_{600} of 0.3-0.4. The culture was chilled on ice for 15-20 min, transferred to 50 ml Oak-ridge tubes and centrifuged at 5000 rpm for 5 min at 4°C in Sorvall® RC5C plus centrifuge (Kendro Lab., USA) with SA-600 rotor. The pellet in each tube was gently suspended in 0.5 volumes (of original culture) of ice-cold 100mM $CaCl_2$ by gently swirling the tubes and incubated on ice for 30 min. The cells were collected by centrifugation as above and resuspended in 0.1 volumes ice-cold 100mM $CaCl_2$ by gently swirling the tube.

Preparation of ultra-competent bacterial cells

The competent cells were prepared as described by Inoue et.al., (1990) with few modifications. Inoculate 10 ml of SOB media with freshly streaked DH5α bacterial cells and were grown overnight at 37°C. 800 μl of this overnite grown culture was inoculated in 100 ml SOB media and grown at 22°C with vigorous shaking at 200-250 rpm till the OD_{600} reaches to 0.55. The culture flask was then incubated on ice for 10 min. The culture was transferred to sterile Oakridge centrifuge tubes, 25 ml each, and centrifuged at 2500x g (4,000 rpm) for 10 min at 4°C. The pellet obtained was resuspended in 32 ml of ice-cold ITB (Inoue Transformation Buffer), incubated on ice for 10 min and centrifuged at 2500x g for 10 min at 4°C. The pellet obtained was gently resuspended in 8 ml of HTB and 600 μl of DMSO was added. Cells were kept on ice bath for 10 min. 100 μl of the cell suspension was dispensed in 1.5 ml micro-centrifuge tubes and snap-frozen in liquid nitrogen. The frozen competent cells were stored at - 80°C for future use.

3.2.4.10. Transformation

Competent *E. coli* cells were transformed according to the standard protocol given by Hanahan (1983). A vial of competent cells, stored at - 80°C was carefully thawed on ice avoiding any temperature shock. The ligated product or plasmid was directly added to 100 μl competent cell suspension, mixed by gentle tapping and subsequently kept on ice for 30 min. All the steps of transformation were carried out in laminar hood under sterile conditions. The cells were then given a heat shock at 42°C for 90 sec and quick chilled on ice for 5 min. This is followed by addition of 0.9 ml of LB and the cells were allowed to grow at 37°C for 45 min with gentle shaking. The transformed competent cells were plated on LB plate containing appropriate antibiotic. Blue-white selection if needed was carried out by plating the cells on X-gal/IPTG plate. The plates were then incubated at 37°C overnight.

3.2.4.11. Confirmation for the presence of insert

The presence of the insert in the clone was confirmed by the colony PCR by using either gene specific primers or primers compatible with cloning vector. Individual colonies were picked from overnight grown plate and mixed in 20 μl sterile water in a 0.2 ml micro-centrifuge tubes. The cells were lysed by boiling for 2 min and centrifuged at 13,000 rpm for 30 sec. 10 μl of the supernatant was taken as template for PCR. The master mix was prepared according to the number of the PCR reactions and distributed in thin-walled PCR tubes as discussed in the previous paragraphs. Number of PCR cycles

and cycling conditions were adjusted according to the Tm of primers used for amplification.

3.2.4.12. Alkaline lysis midiprep of plasmid DNA

A single colony of bacterial cell containing the desired clone was inoculated to the 100 ml of LB medium containing the appropriate antibiotic and allowed to grow overnight at 37°C. The bacterial cells were harvested by centrifugation at 5000 rpm for 10 min at 4°C. The pellet was resuspended in 5ml of ice cold solution I (50mM glucose, 10mM EDTA, 25mM TrisCl). Then 5 ml of freshly prepared solution II (0.2N NaOH,1% SDS) was added and mixed gently by inversion, and incubated for 5 min at room temperature followed by addition of 5 ml of ice cold solution III (3M potassium acetate, pH 4.8) and the mixture was incubated on ice for 15 min. This mixture was then centrifuged at 14,000 rpm for 30 min at 4°C and the supernatant was transferred to a fresh Oakridge tube. The supernatant was subjected to RNase treatment 20 μg/ml at 37°C for 45 min. The supernatant was extracted twice with phenol: chloroform: isoamyl alcohol (25:24:1) and followed by separation of upper aqueous phase containing the plasmid in a fresh Oakridge tube. Equal volume of isopropanol was added to precipitate the DNA by centrifugation at 13,000 rpm for 20 min at room temperature. This was followed by washing with 70% alcohol. The pellet was dried at 37°C and dissolved in 100 μl of sterile water.

3.2.4.13 Purification of Plasmid by PEG Precipitation for Sequencing

Eight microliter of 4M NaCl and 40 μl of 13% polyethylene glycol (PEG 8000) was added to the plasmid dissolved in 32 μl sterile water and the mixture was incubated on ice for 30 min. DNA was pelleted by spinning at 13,000 rpm for 20 min at 4°C. The pellet obtained was washed twice with 70% alcohol, dried and dissolved in sterile water. Visual quantification of DNA was done and 150 ng of plasmid in 2 μl was used for automated sequencing with 96 capillary based DNA analyzer (Hitachi and ABI PRISM, Applied Biosystems).

3.2.4.14 Spectrophotometeric estimation of nucleic acid

The quality and quantity of nucleic acid was determined by measuring the absorbance at 260 nm and 280 nm. The amount was calculated using 1.0 A_{260} = 50 μg/ml for DNA. The purity of nucleic acid was determined by calculating the ratio A_{260}/A_{280} for each sample.

3.2.5 Molecular analysis of gene expression

Before starting RNA work, mortar, pestle, glassware, spatula and other required materials were baked at 180°C for 5-6 h. Gel electrophoresis assembly and other plastic wares were treated with 3% H_2O_2 overnight. All the reagents are made in DEPC treated sterile Milli-Q (or equivalent) water.

3.2.5.1 Treatments for RNA isolation

For chickpea seedling -The chickpea seedlings were given different stress and hormone treatment for the mentioned period of time. After treatment the seedlings were harvested and frozen in liquid nitrogen for RNA extraction.

For yeast cells -Cells were grown in YPGalRaf at 30 °C to late log/stationary phase. Cultures were diluted to an OD_{600} of ca. 0.1 in YPGalRaf medium, and then further grown at 30°C till OD_{600} reached to 0.5. After this, cells were subjected to saline stress for different time points and different concentrations of NaCl as mentioned in figures. The saline stress was given by suspending pelleted cells in salt-containing medium. After saline stress, cells were centrifuged for 3 min at 7,000 rpm, and total RNA was extracted from untreated cells or cells treated with NaCl.

3.2.5.2 Isolation of RNA from chickpea seedlings

Total RNA was isolated from chickpea with TRIZOL Reagent according to the protocol provided by the manufacturer (Invitrogen, USA) with few modifications. About 0.8g plants tissue was crushed to fine powder with mortar and pestle in liquid nitrogen without letting it to thaw. The powdered material was transferred to a 2 ml eppendorf tube, immediately 1 ml TRIZOL Reagent was added to the tube and it was vigorously shaked in order to homogenize the sample quickly. The homogenized samples were incubated for 15 min at room temperature for complete dissociation of nucleoprotein complexes. 0.6 volume of chloroform was added per ml of TRIZOL reagent used and tube was vigorously shaked for 30 sec with tube capped tightly, incubated at RT for 10 min and centrifuged at 9,000 rpm for 15 min at 4°C. Following centrifugation, the upper aqueous phase was collected in separate tube (kept on ice) without disturbing the lower whitish layer. The RNA from the aqueous layer was precipitated by mixing with 0.7 volumes of isopropyl alcohol, incubated for 10 min at RT and centrifuged at 9,000 rpm for 10 min at 4°C. The supernatant was discarded by inverting the tubes on tissue paper and RNA pellet was washed two times with 75% ethanol by dislodging the pellet from the surface of tube with vigorous shaking and centrifuging at 9,000 rpm for 5 min at 4°C.

At the end of the procedure, RNA pellet was briefly dried for 10 min and dissolved in adequate volume of DEPC-treated water or for long term storage, the ethanol washed pellet was left in 75% ethanol and kept at -80°C.

3.2.5.3 Isolation of RNA from yeast cells

Total RNA was extracted from untreated cells or cells treated with NaCl by using hot phenol method as described by Schmitt *et.al.,* (1990). In brief, 10 ml of culture was harvested by centrifugation at 13,000 rpm for 3 min at 4°C. Pellet was resuspended thoroughly in 0.4 ml AE-buffer (50 mM CH_3COONa, pH=5.3; 10 mM EDTA). 40 µl of 10% SDS was added and vortexed. Equal volume (0.440 ml) of phenol was added and again vortexed. Reaction was incubated at 65°C for 4 min, chilled rapidly in a dry ice/ethanol bath until the phenol crystals appeared. Mix was centrifuged at 13,000 rpm for 5 min at RT. Upper aqueous layer was saved and extracted with chloroform at RT for 5 min. RNA was precipitated by adding 3 M CH_3COONa (pH=0.3 M) to a final concentration of 0.3 M (i. e., 40 µl) and 2.5 volume of absolute cold ethanol, centrifuged at 13,000 rpm for 20 min at 4°C. Pellet was washed with cold 70% ethanol, dried and dissolved in DEPC-treated water or suspended in 70% ethanol for long term storage in -70°C.

3.2.5.4 RNA quantification

RNA was dissolved in DEPC treated water and incubated at 55°C for 10 min and quickly chilled on ice. After brief centrifugation, 2 µl of the RNA was diluted 500 times by adding 1 ml of DEPC-treated water and mixed thoroughly. The OD of this diluted RNA was taken at 260 nm spectrophotometer (U-2010, HITACHI) against DEPC-treated water as blank. Concentration of the RNA was calculated according to the following formula:

$$\text{RNA conc. } (\mu g/\mu l) = O.D_{260} \times 40 \times \text{Dilution factor} / 1000$$

Purity of the RNA was checked by taking OD at 230, 260, and 280 nm wavelengths. The RNA was indicated as pure if the ratio of $O.D_{(260/280)} \approx 1.7\text{-}2.0$.

3.2.5.5 Denaturing formaldehyde gel for RNA electrophoresis

Total RNA was run in 1.2 % denaturing formaldehyde gel. For preparation of gel, 1.2g agarose was added to 72 ml DEPC treated water and boiled for 1.5 min. Once the temperature comes down to 60°C, 18 ml formaldehyde and 10 ml 10X MOPS buffer was added. The contents were mixed by swirling. The molten gel was poured in casting tray with combs already fitted into it. Meanwhile, RNA samples were prepared by mixing 20

μg of total RNA, 2 μl of 10X MOPS buffer, 4 μl of formaldehyde, 1 μl of 10 mg/ml EtBr and 10 μl of formamide. Samples were heat denatured at 85°C for 10 min and immediately chilled on ice for 2 min. RNA loading dye (1 ml contains 500 μl formamide, 166 μl formaldehyde, 200 μl 5X MOPS and 134 μl DEPC water) was added to each RNA sample in 1:3 (v/v) ratio. The samples were run at 60-70 V for 5-6 h in 1X MOPS buffer.

3.2.5.6 Transfer of total RNA on Nylon Membrane

The gel was rinsed with DEPC treated water for 30 min to remove formaldehyde and it was equilibrated with 20X SSC for 30 min. The RNA was transferred to Hybond–N^+ Nylon membrane (Amersham, UK) by vertical capillary action using 20X SSC for 16 h. After transfer the RNA was cross-linked to the nylon membrane in UV crosslinker (Stratagene, USA) at 1200kJ/cm^2 and this RNA cross-linked membrane was treated with 5% glacial acetic acid for 15 min. To check the RNA transfer on the membrane, it was stained with 0.04% methylene blue (solution prepared in 0.5 M Na-acetate, pH 5.2). Excess of the stain on the membrane was removed by washing with sterile MQ water. Image of ribosomal RNA was captured on Fluor-S™ MultiImager (Bio-Rad, USA) at highest resolution available to show equal loading of RNA. The cross-linked nylon membrane was wrapped in a saran wrap to avoid it from drying.

3.2.5.7 Radioactive probe preparation, purification and hybridization

For probe preparation radiolabel was used, hence all steps were performed in radioactive room taking adequate safety measures. In a hybridization incubator, the RNA cross-linked nylon membranes were incubated at 60°C with 10 ml of pre-hybridization solution (0.5M Phosphate buffer, pH 7.2, 7% SDS, and 1mM EDTA, pH 8.0) in hybridization bottles for 2-4 h. In the meantime the probe was prepared using Megaprime DNA labeling kit (Amersham Biosc, UK). Transcript expression analysis in chickpea and tobacco was done with full length *CaZF* cDNA. In yeast, DNA fragments containing the ORF of the following genes were used as probes: *CTT1* (YGR088W) from position +1 to +540 (0.54 kb), *HSP12* (YFL014W) from position +1 to +330 (0.33 kb), *GPD1*(YDL022W from position +1 to +540 (0.54 kb), *GPP2/HOR2* (YER062C) from position +1 to +480 (0.48 kb) , *ENA1* from position +90 to +1000 (0.91 kb) and *STL1* (YDR536W) from position +40 to +1032 (0.99 kb). For probe preparation, 10-20 ng of DNA, 2.5 μl of primer was used and the volume was adjusted to 25 μl by adding water. This reaction mix was heat denatured at 95°C for 5 min, quick chilled on ice for 1 min

and briefly spinned. To this premix 2 µl of each of the dNTPs (without dCTP), 2.5 µl of reaction buffer, 1.5 µl of $\alpha\,^{32}P$ dCTP (20 µC/µl), 1 µl of Klenow enzyme was added. The content was mixed by giving a brief spin and then the reaction mixture was incubated at 37°C for 30 min. The labelled probe was purified by passing through the sephadex G-50 column. Purified probe was denatured for 5 min in boiling water bath and quick chilled for 5 min. After a brief spin, the probe was added directly to the pre-hybridization solution kept in hybridization bottle. The probe was left for hybridization for 14-16 h at 60°C in hybridization incubator.

3.2.5.8 Washing and Autoradiography

After 16-18 h of hybridization the membrane was washed with 2X SSC, 0.1% SDS at room temperature for 15 min, twice with 0.5XSSC, 0.1% SDS at 60°C for 15 min. Then the membrane was exposed to X-ray film in the Hypercassette™ (Amersham Pharmacia biotech, U.K) for the time period depending upon the signal intensity for autoradiography. Subsequently, the X-ray film was developed using Developer and Fixer solutions (Kodak Affiliate Products, India). The autoradiograms obtained were scanned in Fluor-S™ MultiImager (Bio-Rad, USA).

3.2.6 Southern hybridization

3.2.6.1 Isolation of plant genomic DNA

Genomic DNA was isolated as mentioned by Murray and Thompson (1980) with some modifications. Five gram tissue was crushed in liquid nitrogen, transferred to fresh oakridge tube and 5-8 ml extraction buffer (2% CTAB, 1.4M NaCl, 20mM EDTA, pH 8.0, 100mM Tris-HCl, pH 8.0, 100mM β-ME) was added to the ground tissue. Subsequently, the tubes were incubated at 60°C for 1 h. To this, 5-8 ml of chloroform: isoamyl alcohol (24:1) was added and mixed gently for 2-3 h followed by centrifugation at 10,000x g for 10 min at room temperature. The upper aqueous phase was transferred to another vial, and once again DNA was extracted with 5-8 ml of chloroform: isoamyl alcohol (24:1). To the final aqueous phase 0.6 volume of isopropanol was added for precipitating the genomic DNA which was then spooled out. The genomic DNA was then washed thrice with 70% ethanol, dried in vacuum, dissolved in TE containing 10 mg/ml RNase and incubated at 37°C for 30 min. This was followed by extraction with phenol: chloroform: isoamyl alcohol (25:24:1) and the aqueous phase was transferred to a fresh tube. Thereafter the genomic DNA was precipitated by adding equal volume of

isopropanol. The pellet was obtained by centrifugation at 10,000xg for 20 min at 4°C and washed with 70% ethanol, air dried and dissolved in TE.

3.2.6.2 Digestion of genomic DNA

Digestion of 20 μg of genomic DNA was performed overnight with selected restriction enzymes. The digested DNA was precipitated by adding $1/10^{th}$ of its volume of 3 M sodium acetate, pH 5.2 and twice the volume of ice cold absolute alcohol. The sample was mixed thoroughly and left at 4°C O/N. Subsequently, they were centrifuged at 10,000 rpm for 10 min. The pelleted DNA was washed with 70% alcohol twice, air dried and dissolved in requisite volume of sterile water.

3.2.6.3 Southern blotting

Genomic DNA was electrophoresed overnight on 0.8% agarose gel. Following electrophoresis, DNA was visualized by ethidium bromide staining. DNA from the gel was transferred to nylon membrane using the Capillary method as described in Sambrook et al., (2001). Briefly, the gel was treated first with depurination solution (0.2 N HCl) for 20 min if the DNA fragments were more than 10 Kb in size followed by 10 min treatment in denaturation solution (1.5 M NaCl, 0.5 N NaOH) and then 2-3 washes of 10 min each in neutralizing solution (1 M ammonium acetate). The DNA was transferred to nylon membrane (Hybond-N, Amersham, England) by capillary transfer in 10X SSC (1.5 M NaCl, 0.15 M sodium citrate, pH 7.0) for 16-18 h and then the blot was cross-linked as previously described. The membrane was pre-hybridized and hybridized as described under northern hybridization.

3.2.7 Construction of subtractive cDNA library

Subtracted cDNA library was constructed by using CLONTECH PCR-Select cDNA subtraction kit (CLONTECH Laboratories, Palo Alto, CA) according to established protocols (Figure 3.1). Four subtracted cDNA libraries were constructed with poly (A^+) RNA isolated from C (control), 3d, 6d and 12d DH stressed seedlings of PUSABGD72 and ICCV2.

3.2.7.1 Plant materials and stress treatments

C. arietinum L. cv PUSABGD72 and ICCV2), were grown in 3 L pot in composite soil (peat compost to vermiculite, 1:1), for 12 days (d) after germination at 22 ± 2^0C/50±5% relative humidity with a photoperiod of 12 h with appropriate watering. Both the cultivars were grown in same pot to have same soil moisture content. The pots were irrigated with 200 ml water everyday. For drought treatment, soil-grown plants

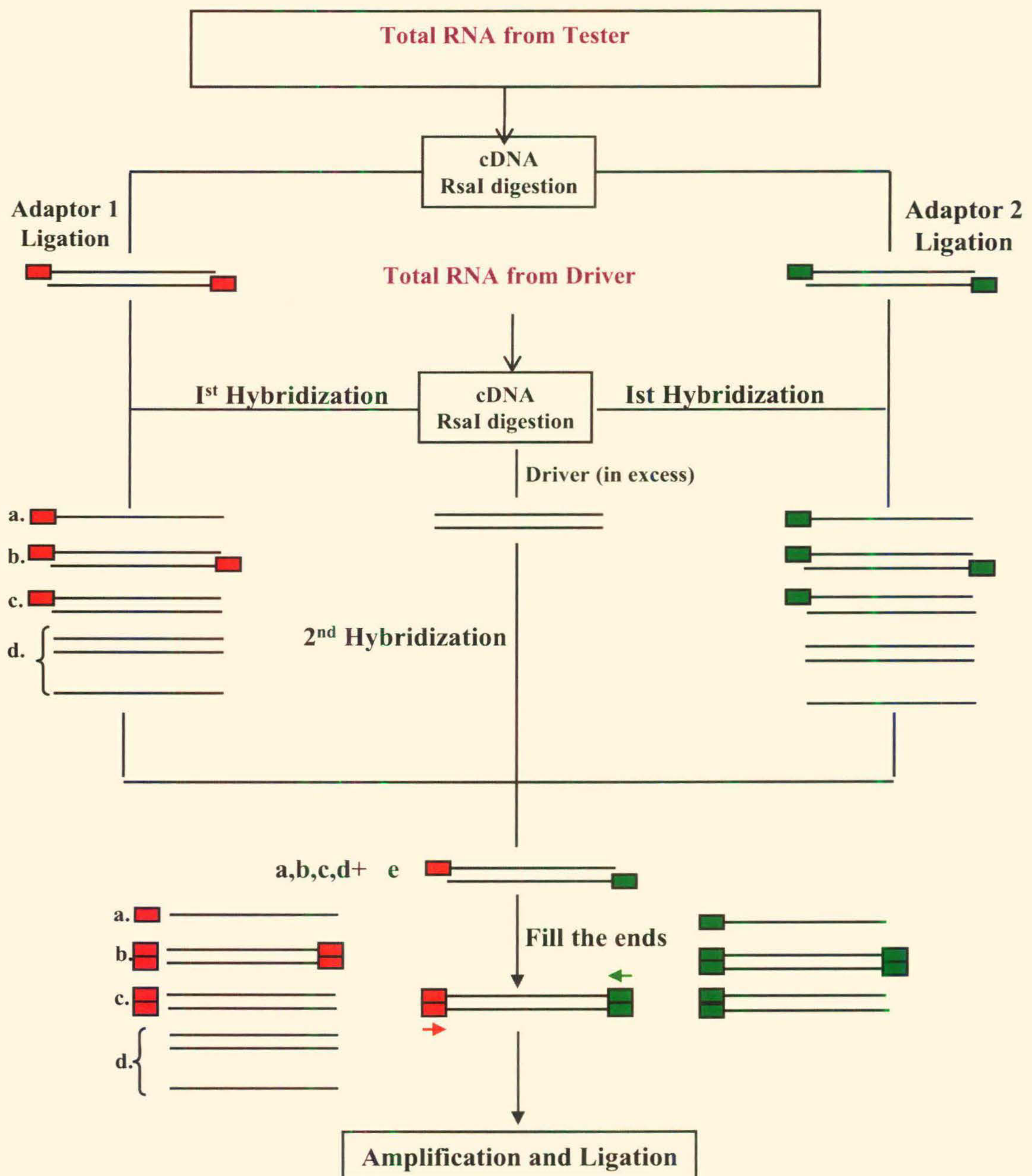


Figure 3.1: Diagrammatic representation of the strategy employed for construction of subtracted library by suppression PCR method. The two different adaptor ligated "Tester cDNAs" were separately subjected to "First Hybridization" with excess of "Driver cDNA" resulting into type a, b, c and d molecules. "Second hybridization" was done by mixing the two different "First hybridization" components in the presence of excess of driver cDNA. Apart from a, b, c and d type molecules "e" type molecules were also formed. The "e" type molecules represented differential genes, which amplified in the presence of adaptor specific 1 and 2R primers.

were subjected to progressive drought by withholding water for 3, 6, and 12 d respectively. In this period the soil moisture content decreased from approximately 50% to approximately 15% at the end of 12 d. For control, plants were kept under the same condition for the same period. Drought stressed plants were harvested at the same time of the day to avoid diurnal changes; immediately frozen in liquid nitrogen and stored at -70°C.

3.2.7.2 Isolation of high quality total RNA and mRNA

Total RNA from C (no stress)/drought treated chickpea cultivars was isolated using Trizol method. Spectrophotometric estimation quantity and quality of total RNA was done as described above. mRNA was isolated using oligo-(dT) tagged magnetic beads (Roche Diagnostics GmbH, Germany). Spectrophotometric estimation of isolated mRNA at OD_{260} was done. The mRNA isolated from C/DH treated PUSABGD72 and ICCV2 plants were referred as tester and driver mRNA, respectively.

3.2.7.3 First strand cDNA synthesis

First strand cDNA synthesis was performed using 2.0 µg of mRNA each from tester and driver population for each library preparation. 1 µl of first strand cDNA synthesis primer was added to 2.0 µg of mRNA in microcentrifuge tube and total volume was made to 5.0 µl. The contents were mixed and spun briefly. After incubation in thermal cycler at 70°C for 2 min the tubes were cooled on ice and briefly centrifuged. To this, following components were added to; 2.0 µl 5X First-Strand Buffer, 1 µl dNTP mix (10mM each), 1 µl sterile H_2O and 1 µl AMV Reverse Transcriptase (20 units/µl). After mixing and brief spin, the tubes were incubated at 42°C in air incubator for 1.5 h. Tubes were then placed on ice to terminate the reaction.

3.2.7.4 Second-strand synthesis

The following components were added to the first-strand synthesis reaction tubes (containing 10 µl); 48.4 µl sterile water, 16.0 µl 5X Second-Strand Buffer, 1.6 µl dNTP mix (10mM) and 4.0 µl 20X Second-Strand Enzyme Cocktail. The total volume of the reaction was made upto 80 µl. The contents were mixed properly and incubated at 16°C for 2 h. 2.0 µl of T4 DNA Polymerase was added and incubated at 16°C for 30 min. 4.0 µl of 20X EDTA/Glycogen mix was added to terminate the second-strand synthesis reaction. Further, 100 µl of phenol: chloroform: isoamyl alcohol (25:24:1) mix was added. The contents were vortexed thoroughly, and centrifuged at 14,000 rpm for 10 min at room temperature. The top aqueous layer was transferred to a sterile 0.5 ml

microcentrifuge tube. 40 µl of 4M NH_4OAc and 300 µl of 95% ethanol was added and immediately proceed with precipitation. Pellet was washed with 500 µl of 80% ethanol. The pellet was air dried and dissolved in 43.5 µl of sterile H_2O.

3.2.7.5 *Rsa* I Digestion

*Rsa*I digestion was performed with each experimental double stranded tester and driver cDNAs to generate shorter, blunt-ended double stranded fragments which are optimal for subtraction and necessary for adaptor ligation. Following reagents were added; 43.5 µl double stranded cDNA, 5.0 µl 10X *Rsa* I restriction buffer and 1.5 µl *Rsa* I (10 U/µl). Contents were mixed thoroughly. The reaction was carried out at 37°C for 1.5 h. To terminate the reaction, 2.5 µl of 20X EDTA/Glycogen mix was added. Then 50 µl of phenol: chloroform: Isoamyl alcohol mix in 25:24:1 ratio was added, vortexed thoroughly and centrifuged at 14,000 rpm for 10 min to separate the aqueous phase. The extraction was repeated twice and the top aqueous layer was transferred to fresh 0.5 ml tube. To this aqueous layer 25 µl of 4M NH_4OAc and 187.5 µl of 95% ethanol was added and immediately proceeded with precipitation. Pellet was washed gently with 200 µl of 80% ethanol. Pellets were air dried and dissolved in 5.5 µl of H_2O and stored at -20^0C.

3.2.7.6 Adaptor Ligation to tester cDNA

One microliter of each *Rsa*I digested tester cDNA was diluted with 5 µl of sterile H_2O. Ligation mix was prepared by combining the following reagents in two separate 0.5 ml micro-centrifuge tubes; 3 µl sterile H_2O, 2 µl 5X Ligation Buffer, 1 µl T4 DNA ligase (400 units/µl) and 2 µl of diluted tester cDNA. To one of the two tubes Adaptor1 and to the other Adaptor 2R were added. Contents were pipetted in & out to mix them thoroughly. To prepare unsubtracted tester control 2 µl from each tube was taken in a fresh tube and mixed. This, after ligation, would act as unsubtracted tester control. Tubes were centrifuged briefly and incubated at 16°C overnight. Ligation reaction was terminated by adding 1.0 µl of EDTA/Glycogen mix. Samples were incubated at 72°C for 5 min and briefly spun to inactivate ligase. One microlitre unsubtracted tester control was diluted with 1 ml of sterile H_2O. These samples were later used for PCR. Till then, the samples were stored at -20°C.

3.2.7.7 First Hybridization

The 4X Hybridization buffer was kept at 37°C for 10 min to ensure that no precipitate remains. First hybridization reactions were then set. The first reaction mixture contained 1.0 µl 4X Hybridization Buffer, 1.5 µl Adaptor 2R-ligated Tester cDNA and 1.5 µl *Rsa* I

digested driver cDNA. The second reaction mixture contained 1.0 µl 4X Hybridization Buffer, 1.5 µl Adaptor 1-ligated Tester cDNA and 1.5 µl *Rsa* I digested driver cDNA. Samples were overlaid with mineral oil and centrifuged briefly and subsequently, incubated in a thermal cycler at 98°C for 1.5 min and then at 68°C for 8 h. These two hybridized samples were designated as first hybridization sample 1 and 2R. Immediately, the second hybridization was performed.

3.2.7.8 Second Hybridization

The two samples 1 & 2R from the first hybridization were mixed together, and fresh denatured driver cDNA was added to further enrich for differentially expressed sequences. The primary hybridization samples were not denatured at this stage and the entire procedure was performed while the samples were still in thermal cycler at 68°C. Following components were added for second hybridization; 1.0 µl of driver cDNA, 1.0 µl 4X Hybridization Buffer and 2.0 µl Sterile H_2O in 0.5 ml PCR tube. From this sample mixture 1.0 µl was transferred to a microcentrifuge tube, overlaid with 1 drop of mineral oil and this tube was incubated in thermal cycler at 98°C for 1.5 min. The freshly denatured driver cDNA was removed from the thermal cycler. Strictly following this step the driver was mixed with the first hybridization samples 1 and 2R simultaneously, which ensured that the two hybridization samples mix together only in the presence of freshly denatured driver. For this, following protocol was followed. The Micropipette was set at 15 µl. Pipette tip was gently touched to the mineral oil and sample interface of the tube containing hybridization sample 2R. The entire sample was carefully drawn halfway into the pipette tip without caring if a small amount of mineral oil is transferred with the sample. Pipette tip was removed from the tube, and a small amount of air was drawn into the tip, creating a slight air space below the droplet of sample. The pipette tip then contained hybridization sample 2R and the driver cDNA separated by a small pocket of air. The entire mixture was transferred to the tube containing hybridization sample 1. Samples were mixed by pipetting up and down, briefly centrifuged if necessary and incubated at 68°C overnight. 200 µl of dilution buffer was added to the tube and mixed by pipetting. Heated in a thermal cycler at 68°C for 7 min and stored at –20°C.

3.2.7.9 PCR Amplification

Differentially expressed cDNAs were selectively amplified using the reactions described in this section. Prior to thermal cycling, missing strands of the adaptors were filled in by a brief incubation at 75°C. This created the binding site for PCR Primer 1. In

the first amplification, only ds cDNAs with different adaptor sequences on each end were exponentially amplified. The second, nested PCR was performed to further reduce the background and to enrich for differentially expressed sequences. Two PCR reactions, one with subtracted tester 1 cDNA and the other with unsubtracted tester control were performed. All PCRs were done using a PTC-200 Thermal cycler (MJ Research). PCR reaction was prepared as follows

Sterile H_2O	19.5 µl
10X PCR reaction buffer	2.5 µl
dNTP mix (10 mM)	0.5 µl
PCR Primer 1 (10 µM)	1.0 µl
50X Advantage cDNA Polymerase Mix	0.5 µl
Total volume	24.0 µl

PCR conditions:

Denaturation	94°C for 30 sec	27 cycles
Annealing of primers	66°C for 30 sec	
Primer extension	72°C for 1.5 min	

3.2.7.10 Secondary PCR

Three microliter of each primary PCR mixture was diluted with 27 µl of H_2O. One microlitre of this diluted primary PCR product was used for secondary PCR.

Sterile H_2O	18.5 µl
10X PCR reaction buffer	2.5 µl
Nested PCR primer 1 (10 µM)	1.0 µl
Nested PCR primer 2R (10 µM)	1.0 µl
dNTP mix (10 mM)	0.5 µl
50X Advantage cDNA Polymerase Mix	0.5 µl
Total volume	24.0 µl

PCR conditions:

Denaturation	94°C for 30 sec	10-12 cycles
Annealing of primers	68°C for 30 sec	
Primer extension	72°C for 1.5 min	

Eight microliter of PCR product was analyzed on a 2.0% agarose/EtBr gel and the remaining PCR product was stored at -20^{0}C. This PCR product was enriched for differentially expressed cDNAs.

3.2.7.11 Cloning of amplified differential cDNA

These cDNAs were directly ligated to pGEMTEasy T/A cloning vector (Promega), transformed to *E.coli* DH5α competent cells and plated on IPTG+X-Gal+Amp LB agar

plates. White colonies were patched and insert size was verified by colony PCR using M13 F/R primers. Positive clones were subsequently sequenced and analyzed.

3.2.8 DNA array/Macroarray

3.2.8.1 DNA array preparation

Individual clones of the subtracted cDNA library were amplified, purified, and denatured by adding an equal volume of 0.6 M sodium hydroxide. Equal volumes of each denatured PCR product (about 100 ng) were spotted on Hybond™ N membranes (Amersham Pharmacia Biotech, NJ, USA) using a 96 well dot-blot apparatus (BIO-RAD Laboratories, CA, USA). In addition, PCR products of chickpea actin cDNA (Accession No. AJ012685) using primers (5'-GGTAACATTGTCTTGAGTGG-3'and 5'-CCAGATCCGTAACAATACAC-3') and neomycin phosphotransferase (*NPTII*) gene from the binary vector pBI121 (Accession No. AF485783.1) using primers (5'-TGCTCGACGTTGTCACTGAAG-3' and 5'-GTCAAGAAGGCGATAGAAGGC-3') were respectively spotted as an internal control and negative control. The membranes were neutralized with neutralization buffer (0.5 M Tris-HCl, pH 7.4, 1.5 M NaCl) for 3 min, washed with 2X SSC, and immobilized with UV cross-linker (Stratagene, La Jolla, CA, USA).

3.2.8.2 DNA array hybridization

Probes were prepared by first-strand reverse transcription (Powerscript™ RT, BD Biosciences, CA, USA) with 1μg mRNAs isolated from different samples and labeled with α^{32}P-dCTP (20 μCi/μl). Radio-labeled cDNAs were purified by Sephadex G-50 (Amersham Pharmacia Biotech, NJ, USA), suspended in pre-hybridization buffer (7% SDS, 0.3 M Sodium phosphate pH 7.4, 1 mM EDTA) and hybridized at 60°C for overnight. The membranes were then washed three times with washing buffer (1X SSC, 1% SDS, 20 min each at 60°C). Autoradiographs were scanned employing a FSMI (Fluor-S-Multiimager, CA, Bio-Rad, USA) to acquire images and signal intensities analyzed by subtracting background noise.

3.2.8.3 Data analysis for DNA-array

Data analysis was performed using ImageQuant software (Molecular Dynamics, Sunnyvale, CA). The radioactive intensity of each spot was quantified as volume values and the local background values were subtracted, resulting in subtracted volume values (sVol). Actin cDNA was used as the internal control whose subtracted volume value was designated as sRef. Normalization among all images was performed by dividing sVol of

each spot by the sRef value within the same image, resulting in a normalized volume value (nVol) for each spot. nVol values were comparable between all images. To access the reproducibility of the macroarray analysis, three independent experiments were conducted. Data for the expression profile analysis were averages of the three independent experiments. The ratios for the signal intensities of each EST between BGD72 and/or ICCV2 were measured as changes in the differential expression of genes represented by cDNA spots on the macroarray. Expression profiles of stress inducible cDNAs were also analyzed by the hierarchical SOTA (Self-organizing tree algorithm) clustering on the log-transformed-fold induction expression values across four time points by using MultiExperiment Viewer (MEV) Software (The Institute for Genome Research; http://rana.lbl.gov/EisenSoftware.htm) (Eisen et al., 1998).

3.2.9 Full-length gene isolation by 5′-RACE

Subtractive cDNA libraries constructed with drought-tolerant (PUSABGD72) and drought-sensitive (ICCV2) cultivar of chickpea (*Cicer arietinum*) at different stages of drought resulted in some EST clones that express higher in the drought-tolerant cultivar in response to drought. One such EST encoding zinc finger protein was used in this study. Using the 5′ RACE System Version 2.0 (Invitrogen, USA) cDNA end of the genes were amplified according to the manufacturer's instructions with minor modifications according to the experimental need.

3.2.9.1 First Strand cDNA Synthesis

In a thin walled 0.2 ml PCR tube 2.5 pmoles of GSP1, 2.5 μg of RNA (preferably from the time period of the sample where the clone has high mRNA transcript level) were added and volume was made up to 15.5 μl with DEPC-treated sterile water. The mixture was incubated for 10 min at 70°C in thermal cycler to denature the RNA and immediately chilled on ice for 1 min. After brief centrifugation, following components were added in the order given- 2.5 μl of 10X PCR buffer, 2.5 μl of 25mM $MgCl_2$, 1 μl of 10mM dNTP mix, 2.5 μl of 0.1M DTT and mixed gently. After quick spin, this mixture was incubated at 42°C for 1 min then 1 μl of SUPERSCRIPT™ II Reverse Transcriptase was added and incubated at 42°C for 50 min. This reaction was terminated by incubating at 70°C for 15 min and after a brief spin 1 μl of RNase H was added and incubated for 30 min at 37°C to remove the RNA. The mixture was collected by brief centrifugation and kept on ice. This reaction mixture can be stored at -20°C.

3.2.9.2 Purification of cDNA

The binding solution (6M NaI) was equilibrated to RT. To the first strand reaction, 120 µl of binding solution was mixed and this mixture was transferred to a GLASSMAX spin cartridge. After centrifuging column at 13,000xg for 20 sec the flow through (saved until recovery of the cDNA was confirmed) was transferred to a microcentrifuge tube and the column was put back into the collection tube. This column was then washed with 0.4 ml of cold (4°C) 1X wash buffer four times by centrifuging at 13,000xg for 20 sec. Then the column was washed two times with 400 µl of cold (4°C) 70% ethanol. After removing the final 70% ethanol wash from the tube, the column was centrifuged at 13,000xg for 1 min. In a fresh tube the single stranded cDNA was recovered by adding 50 µl of preheated water at 65°C and centrifuging at 13,000xg for 20 sec.

3.2.9.3 *TdT* tailing of cDNA

In a 0.2 ml tube, following components were added and mixed gently; 6.5 µl of DEPC-treated water, 5.0 µl of 5X tailing buffer, 2.5 µl of 2mM dCTP and 10 µl of earlier GLASSMAX purified cDNA (The cDNA can be used in variable amounts according to the relative amount of RNA transcript of the desired gene). This mixture was incubated at 94°C for 2-3 min and quickly chilled on ice. After collecting the mixture by brief centrifugation, 1 µl of *TdT* was added gently and the tube was incubated for 10 min at 37°C. To heat inactivate the *TdT*, reaction mixture was heated at 65°C for 10 min. After brief centrifugation the tube was kept at 4°C.

3.2.9.4 PCR of dC-tailed cDNA

In a 0.2 ml thin walled PCR tube, following components were added; 34 µl of sterilized distilled water, 5 µl of 10X PCR buffer, 1 µl of 10mM dNTP mix, 2 µl of 10 µM Nested GSP2, 2 µl of 10 µM Abridged Anchor Primer, 5 µl of dC-tailed cDNA and 1 µl of 50X Titanium *Taq* DNA Polymerase. PCR of 30-35 cycles was performed with following conditions.

Step	Condition	
Pre-amplification denaturation	94°C for 2 min	
Denaturation	94°C for 0.5-1 min	30-35 cycles
Annealing of primers	55°C for 0.5-1 min	
Primer extension	72°C for 1-2 min	
Final extension	72°C for 5-7 min	
Indefinite hold	4°C, until samples were removed	

Eight microlitres of 5′ RACE product was analyzed on 1% EtBr /agarose gel. To confirm the validity of specific amplification nested amplification was done.

3.2.9.5 Nested Amplification

After diluting the primary PCR product 100 times, nested amplification of the primary PCR product was performed using the nested primer and AUAP (Abridged Universal amplification primer) or UAP (Universal Amplification Primer) primers and keeping the PCR conditions same as in the primary PCR. The PCR products were eluted from the 0.8% agarose gel after electrophoresis and cloned into pGEMTEasy T/A cloning vector and sequenced with M13 sequencing primers.

3.2.10 Promoter isolation by Genome Walking

The promoter isolation was done by using the method described in Universal Genomewalker^{TM} Kit (Clontech, USA). From this kit a pool of uncloned, adaptor-ligated genomic DNA fragments were obtained, which were later used for isolation of gene specific promoter. In order to find out cis-acting element organization of *CaZF* a pair of primers was designed and the PCR was performed. The PCR product was cloned in pGEMT and has been given for sequencing. The sequence was analyzed on PLACE signal scan search and PLANTCARE search program.

3.2.10.1 Determination of quantity and purity of genomic DNA

High quality genomic DNA was isolated from chickpea using the protocol discussed above. The quality and quantity of genomic DNA was checked.

3.2.10.2 Digestion of genomic DNA

In four different 1.5 ml sterile tubes, four digestion reactions were set up using the enzymes *Dra*I, *Eco*RV, *Pvu*II and *Stu*I. All these enzymes produce blunt ends. In each reaction following components were combined:

Genomic DNA (0.1 μg/μl)	25 μl
Restriction enzyme	8 μl
Restriction enzyme buffer	10 μl
Deionized H_2O	57 μl
Total volume	100 μl

Mixed gently and incubated at 37°C for 2 h. The tubes were tapped gently and again kept for 16-18 h. From each tube 5 μl reaction mix was checked for digestion on 0.5% agarose/EtBr.

3.2.10.3 Purification of DNA

To each of the reaction tube, an equal amount (95 µl) of phenol was added and slowly vortexed for 10 sec. After brief spin aqueous layer was transferred to a new tube and again the above step was repeated to remove protein contamination. After second extraction, 2 volumes (190 µl) of ice cold 95% ethanol, 1/10 volumes (9.5 µl) of 3M NaOAc, and 20 µg of glycogen was added and vortexed slowly for 10 sec. To pellet the digested DNA, the tubes were centrifuged at 15,000 rpm for 10 min and the supernatant was decanted. The pellets obtained were washed with in 100 µl of ice cold 80% ethanol and centrifuged at 15,000 rpm for 5 min. The supernatant was decanted, pellet was air dried and dissolved in 20 µl of TE (10/0.1, pH 7.5). After a slow speed vortex for 5 sec, 1 µl of the digested DNA quality & quantity was checked on a 0.5% agarose/ EtBr gel.

3.2.10.4 Ligation of Genomic DNA to Genome-walker adaptors

For ligation, 4.0 µl of each digested and purified DNA was taken in 0.5 ml tubes and to each of the four tubes following was added; 1.9 µl Genomewalker adaptor (25µM), 1.6 µl ligation buffer and 0.5 T4 DNA ligase (6 units/µl).

3.2.11 DNA -Binding Assay (EMSA)

3.2.11.1 Preparation of recombinant protein

To generate a GST-fusion protein, the corresponding ORF of *CaZF* was amplified by PCR with primers flanked with restriction site for *EcoRI* and inserted into pGEX4T2 expression vector and introduced into *Escherichia coli* BL21 (DE3).

3.2.11.2 Over expression and purification of GST-CaZF

For the over expression of GST-CaZF, a single colony of BL21 (DE3) containing the construct pGEX-4T-2-GSTCaZF, was inoculated in 10 ml of LB with 50µg/ml of ampicillin. This was allowed to grow at 37°C overnight and next day 3 L of LB was reinoculated with the overnight grown culture so that starting O.D is 0.2.The culture was allowed to grow at 30°C till the O.D. reached to 1.0-1.5 (3-4 h) and the protein was induced by adding 1mM IPTG . Then it was again allowed to grow for another 3 h at 30°C .The cells were harvested at 4°C and washed with ice cold 10mM phosphate buffer pH 7.0 (10 mM phosphate buffer pH7.0, 140 mM NaCl, 2.7 mM KCl). The cells were resuspended in 6-8 ml of phosphate buffer pH 7.0 and 1 mM PMSF was added which acts as a protease inhibitor. This cell suspension was sonicated 3 times with 20 sec pulse and kept in ice. Then it was centrifuged at 13,000 rpm at 4°C for 15 min. The supernatant was transferred to fresh 15 ml falcon tube and 1 ml of washed glutathione beads were

added. 0.01% Triton-X-100 was added and kept for rocking for 2 h at 4°C. These beads with protein bound were centrifuged at high speed to remove supernatant. Beads were washed with 20 ml of phosphate buffer (pH 7.0) and then protein was eluted with elution buffer (10mM reduced Glutathione, 50mM Tris-HCl pH 8.0) and collected in different fractions. These fractions were run on SDS-PAGE to check the fraction with the highest concentration of GST-CaZF and dialyzed. After dialysis the protein concentration was estimated by Bradford assay, then protein was stored in small aliquots.

3.2.11.3 SDS-PAGE

SDS-PAGE was performed as described by Laemmli (1970). The protein samples were denatured by adding 1/5 volume of 5X Laemmli buffer (50 mM Tris HCl pH 6.8, 100 mM DTT, 2% SDS, 0.1% bromophenol blue, 10% glycerol) and boiling for 5min in water bath and then run on the 8% gel. Initially the proteins were run at low voltage so that the proteins get stacked and when the proteins enter the separating gel then the voltage was increased. Gels were stained with Coomassie brilliant blue R-250 (CBB-0.2%, 50% methanol, 10% acetic acid) and excess stain was removed by destainer.

Components	8% resolving gel (10 ml)	5% stacking gel (4 ml)
Water	4.6 ml	2.7 ml
30% Acrylamide (29:1)	2.7 ml	0.67 ml
1.5 M Tris pH 8.8	2.5 ml	-
1.0 M Tris pH 6.8	-	0.5 ml
10% SDS	0.1 ml	0.1 ml
10% Ammonium persulfate	0.1 ml	0.04 ml
TEMED	0.006 ml	0.004 ml

3.2.11.4 EMSA

The binding reactions were performed in 25 μl binding buffer (17 mM HEPES pH 7.4, 60 mM KCl, 1.2 mM DTT, 0.12 mM EDTA, 7.5 mM $MgCl_2$, 17% glycerol) and 1 μg of poly dI:dC. Gel-shift assays were performed with 10,000 c.p.m. of ^{32}P-end-labeled probe A, a tetramer of TTGAC<u>AGT</u>GTCACGCG TTGAC<u>AGT</u>GTCACGCG (core nucleotides are underlined) or mutated probe M1, a tetramer of TT**c**A**g**<u>AGT</u>GTCAC**c**C**g**TTGAC<u>AGT</u>GTCACGCG (mutated bases are in bold lower case letters). For competition experiments unlabeled double stranded self and nonspecific or mutated oligos were included in the reaction mixture. After incubation for 20 min at room temperature, the mixtures were subjected to electrophoresis in 6% polyacrylamide

gel in 0.5XTBE buffer, dried and auto-radiographed as described previously (Urao *et al.*, 1993).

Component	6% PAGE (40 ml)
30% Acrylamide (29:1)	8 ml
5XTBE	8 ml
50% Glycerol	1.6 ml
10% APS	0.4 ml
TEMED	0.032 ml
H_2O	21.97 ml

3.2.12 Yeast One-Hybrid Assay

3.2.12.1 Preparation of recombinant protein

CaZF protein coding sequence or the truncated forms were PCR amplified by using the gene specific primers with NdeI and EcoRI restriction sites as 5' overhangs such that it should be in frame with GAL4-DNA binding domain. The PCR amplified fragment was 0.840 kb in size and it was gel purified. The 0.84 kb fragment and pGBKT7 vector was digested with NdeI and EcoRI restriction enzymes, purified and ligated. The resulting construct was transformed into *E.coli.* Insert-containing plasmid was identified by PCR and restriction analysis. The constructs were transformed into an auxotropic yeast strain PJ69-4A (Cagney et al., 2000) that contains three reporter genes, *HIS3, ADE2, and β-GAL,* under the control of GAL4 promoter, and plated on synthetic medium lacking histidine and adenine. β-Galactosidase assay of the transformed colonies was done with ortho-nitrophenyl-β-D-galactoside (ONPG). Presence of different form of CaZF in the transformed colonies were confirmed by PCR and sequencing.

3.2.12.2 Yeast Transformation

Single yeast colony was inoculated in 5 ml YPD and grown overnight at 30°C. Next day OD_{600} was taken and culture was diluted to 0.2 OD in 30 ml YPD and allowed to grow for approx 3 h till the OD reached 0.6-0.8. The cells were pelleted at 4,000 rpm for 3 min at RT, supernatant discarded and cells thoroughly resuspended in 30 ml sterile water. Cells were again pelleted at 4,000 rpm for 3 min at room temperature and supernatant discarded. The pellet was resuspended in 1 ml of hood open sterile water. In a fresh 1.5 ml eppendorf tube add the components in the given order; 100 μl competent cells, 240 μl of 50% glycerol, 36 μl of 1 M lithium acetate, 25 μl of ss-DNA (2.0 mg/ml), 1-5 μg of plasmid DNA to be transformed. Vortex the tubes vigorously until the cells are completely mixed and then incubated at 30°C for 30-60 min with shaking at 200 rpm,

followed by heat shock for 15 min at 42°C water bath. Cells were chilled on ice for 1-2 min and then pelleted at 13,000 rpm for 20 sec. Pellet was washed twice with 500 µl of sterile water. Appropriate volume of this transformed mix was plated on correct selective medium.

3.2.12.3 ONPG assay

Single yeast colony was inoculated in 5 ml YPD and grown overnight at 30°C. 2.0 ml of this culture was re-inoculated in 8.0 ml of YPD and incubated further at 30°C, 200 rpm for 3-5 h until the OD_{600} became 0.6-0.8. Exact OD_{600} was recorded for future calculations. 1.5 ml of culture was pelleted at 13,000 rpm for 30 sec in triplicates. The pellet was washed with 1.5 ml of Z-buffer and again pelleted. Each pellet was then resuspended in 300 µl of Z-buffer (now, the concentration factor is 1.5/0.3=5-fold). In a fresh 1.5 ml eppendorf tube 100 µl of the cell suspension was taken for the assay. Cells were freezed in liquid N_2 for 0.5-1.0 min. Frozen cells were placed in 37°C water bath for 0.5-1.0 min till the cells were completely thawed. The freeze/thaw cycle was repeated at least 5-times so that the cells had broken-open. A blank tube was set with 100 µl of Z-buffer. For each reaction 0.7 ml of (Z-buffer+β-mercaptoethanol) and 160 µl of ONPG (4 mg/ml in Z-buffer) was added, including the blank tube. Tubes were incubated at 30°C, 200 rpm and a timer was set. 0.4 ml of 1 M Na_2CO_3 was added to each reaction after the yellow color developed. The elapsed time in min was noted down. Cells were pelleted at 13,000 rpm for 3 min at RT. The supernatant was carefully taken in clean cuvettes and OD_{420} was measured in a calibrated spectrophotometer against the blank at A_{420}. β-galactosidase units [1 U of β-galactosidase is defined as the amount which hydrolyze 1 µmole of ONPG to o-nitrophenol and D-galactose per min per cell (Miller, 1972) were calculated according to the following formula;

$$\beta\text{-galactosidase units} = 1{,}000 \times OD_{420} / (T \times V \times OD_{600})$$

Where, T = elapsed time (min) of incubation

V = 0.1 ml × concentration factor (here, 5)

$OD_{600} = A_{600}$ of 1 ml of culture

3.2.13 Gene disruption and complementation of yeast (*Saccharomyces cerevisiae*)

3.2.13.1 Deletion of complete open reading frames in yeast

The construction of deletions or disruptions of specific sequences from the genome and the subsequent analysis of the mutant phenotypes is one of the most powerful genetic tools in baker's yeast *Saccharomyces cerevisiae*. Advanced methods to construct such

deletions will become even more important in the near future when the sequence of the entire yeast genome has been determined (Dujon et al., 1994) and the function of the newly identified open reading frames (ORFs) will be studied. A gene inactivation requires the in vitro creation of a construction in which a selectable marker is sandwiched by the 5' and the 3' flanking sequences of the target ORF. Direct gene deletion of the target genes with the marker module was done by PCR-based gene deletion strategy (Baudin *et al.*, 1993; Wach *et al.*, 1994). This strategy generates such a construction by one step PCR amplification. The deletion 'cassette' used to replace each yeast gene was constructed in a sequential two-step PCR reaction using two pairs of primers. Each primer used contains two distinct regions, one which allows homologous recombination at the target locus and will be named the deleting sequence, the second part which permits the PCR amplification of a selectable marker. The deleting sequences, which are respectively the 5' (oligopro) and 3' (oligoterm) flanking sequences of the ORF, range from 35 to 51 nucleotides in length and are followed by a short stretch of 17 nucleotides homologous to the selectable marker.

3.2.13.2 Deletion Strain Confirmation

The correct replacement of the gene with selective marker was verified in the deletion mutants by diagnostic PCR using whole yeast cells from isolated colonies. The appearance of PCR products of the expected size using primers that span the left and right junctions of the deletion module within the genome confirmed the correct deletion.

3.2.13.3 Deletion Mutant Construction

After confirmation of the fidelity of the constructs by sequencing and diagnostic PCR amplifications, the gene disruptions were constructed by direct transformation of crude PCR mix into MATa and MATα haploids using a standard lithium acetate transformation protocol followed by selection of colonies on agar plates with appropriate selection.

3.2.13.4 Complementation of deletion mutants

For complementation studies selective deletion muatants' strains were transformed with constructed plasmids or with empty pYES-2.1 by the Lithium-acetate/PEG method (Gietz *et al.*, 1995). Transformants were selected for uracil prototrophy by plating on synthetic media lacking uracil (SC-Ura$^-$). Ura$^+$ colonies were selected thereupon.

3.2.14 Yeast Spot Assay

For drop tests, overnight YPGal grown yeast cells were diluted to OD_{600}=0.4 in 2% Gal, 50 mM MES pH 5.5 and incubated for 3h and then further serially diluted with YP to obtain 10, 10^2, 10^3 and 10^4 cells. Three microliters of each dilution was then spotted onto

YPGalRaf with/without NaCl, LiCl, KCl or sorbitol as mentioned or onto complete synthetic uracil$^-$ medium supplemented with 2% Gal, 0.2% sucrose and $MnCl_2$ as indicated in the figures. Plates were incubated at 30°C and unless otherwise indicated, colony growth was inspected after 2-4 days.

3.2.15 Functional analysis of *CaZF* in tobacco

3.2.15.1 Cloning of *CaZF* in a plant binary vector pBI121

To establish the functional significance of the *CaZF* gene, the complete ORF was cloned in the plant expression binary vector pBI121 using a set of primers with *XbaI* and *BamHI* restriction sites as overhangs. The amplified PCR product digested along with vector with *XbaI* and *BamHI* and ligated.

3.2.15.2 Cloning of *CaZF* in a plant binary vector pCAMBIA1302

The *CaZF* coding region without the translation stop codon was cloned in pCAMBIA1302 to produce the protein fused to GFP using PCR primers having *NcoI* and *BglII* restriction sites as their 5' overhangs. The PCR amplified fragments were digested with *NcoI* and *BglII* and cloned in pCAMBIA1302 vector.

3.2.15.3 Transformation of Agrobacterium tumefaciens

Recombinant plasmids constructed in pBI101.2 and pBI121 were transferred into *Agrobacterium* by the freeze-thaw method. For the preparation of competent cells, *Agrobacterium tumefacians* (GV3101) was grown in 50 ml YEM medium (0.04% yeast extract, 1% mannitol, 0.01% NaCl, 0.02% $MgSO_4.7H_2O$ and 0.05% K_2HPO_4) at 28°C with vigorous shaking until the OD_{600} reached 0.5. The culture was chilled on ice and centrifuged at 5,000 rpm for 5 min at 4°C. The pellet was resuspended in 1 ml of ice cold $CaCl_2$ (20 mM) and 0.1 ml aliquots were dispensed in pre- chilled eppendorf tubes and stored at -80°C. Transformation of *Agrobacterium* with various plasmid constructs was done by mixing 1 μg of DNA with competent cells followed by immediate freezing in liquid nitrogen. Subsequently cells were thawed by incubating the eppendorf tube at 37°C for 5 min. Thereafter 1 ml of YEM medium was added to the tube and incubated at 28°C for 1 h. Cells were spread on a YEM agar plate supplemented with 50 μg/ml kanamycin and 25 μg/ml rifampicin and incubated at 28°C. Transformed colonies that appeared after 1-2 d were analyzed either by PCR or by colony hybridization and the positive colonies were confirmed by restriction digestion of the purified recombinant plasmid.

3.2.15.4 Tobacco Plant Transformation

One positive colony of *Agrobacterium tumefacians* from each construct was used to transform tobacco by leaf disc method (Horsch et al., 1985). A single colony was inoculated into 30 ml of liquid YEB medium with kanamycin plus rifampicin and grown for one day 28°C. One ml of this bacterial culture was inoculated into fresh medium and grown overnight. Uniformed sized, healthy, young tobacco leaves were harvested and leaf squares (1 cm^2) were prepared. The leaf squares were then immersed in *Agrobacterium* culture containing various constructs (1:10 dilution of overnight grown culture in liquid MSO medium for 5 min. Leaf squares were taken out, blot dried and placed upside down on the MSO (MS supplemented with 1 mg NAA, 1 mg BAP) culture plates. The leaf explants were cultured for 2-3 days on medium without antibiotics.

3.2.15.5 Selection and Plant Regeneration

Immediately after co-cultivation, explants were transferred to MS medium with BAP and NAA containing 50 μg/ml kanamycin and 250 μg/ml cefataxime. After 3-4 weeks, shoots with a defined stem were removed from explants and placed on MS rooting medium. As soon as the roots started appearing, the plantlets were removed from culture jars and rinsed in water and planted in pots containing vermiculite. After 7-10 d of hardening the plants were transferred to soil and grown under green house condition to allow production of seeds.

3.2.15.6 Seed Plating in Culture Medium

Seeds of wild type and transformed tobacco were surface sterilized with 2 ml of sterilization solution [30% v/v bleach (100% bleach contain 5% hypochlorite) with 1 μl/ml of 20% Triton X- 100] and gently mixed by inversion for 20 min. The seeds were allowed to settle for 1 or 2 min before decanting the bleach. The tubes with seeds was filled with sterile water and mixed. Washing of seeds was repeated several times till the bleach smell disappeared. The seeds were finally resuspended in required volume of sterile water and then plated on GM medium containing kanamycin. Sterile water was allowed to evaporate in laminar flow hood and plates transferred to the culture room. The seedlings, which survived on kanamycin were transferred to soil pots.

3.2.16 Analyses of *CaZF*OX tobacco transgenic plants

3.2.16.1 Abiotic stress tolerance evaluation

T1 seeds of vector control and *CaZF*OX transgenic tobacco lines were surface sterilized, germinated and grown essentially as described by Mukhopadhyay et al.,

(2004). For salt stress, the seedlings were grown on half strength of Moorashige and Skoog medium containing 300 mM NaCl solution. For dehydration stress treatments, the seeds were geminated on 1/2MS medium containing 0.3 M mannitol and grown for 20 d. For heat stress, *CaZF*OX and vector control seeds were allowed to germinate on 0.5MS medium at 39°C.

3.2.16.2 Cellular localization of CaZF protein

For cellular localization of CaZF protein leaf peels from transgenic tobacco plants (from T1 seeds) expressing CaZF protein fused to GFP were used. Histochemical localization of GFP activity was analyzed after incubating the samples in DAPI (1μg/μl) for 10 min. Samples were observed under fluorescent microscope with FITC filter. The same sample was stained with Orcein to confirm the position of nucleus.

3.2.16.3 Morphological analysis of CaZF overexpressing lines

For the study of morphological phenotype, transgenic seeds along with vector control were plated on ½ MS in square plate and were allowed to grow vertically. Seeds were germinated in synthetic soil (3agropete:1vermiculite) in green house for phenotypic analysis of soil grown seedlings.

3.2.16.4 Confocal imaging

For confocal imaging 6d-old T1 tobacco seedlings of *CaZF*OX and vector control were incubated in water containing 2.5μM FM4-64 for 5 min and imaged using Confocal Scanning Microscope (Leica Microsystems, Exton, PA) at 543 nm excitation and 590 nm emissions.

3.2.16.5 Auxin Transport Assay

Auxin transport was measured essentially as previously described (Shin et al., 2005). Six day old vertically grown seedling was used for the experiment. Briefly, agar blocks of 1mm in diameter containing 7.7×10^{-8} M ^{3}H-IAA (Amersham) were placed at root tips in three independent experiments. After incubation for 3 h, a 0.5 mm section of the root close to the agar block was dissected and discarded. Two consecutive 2-mm segments below the incision line were then collected separately and 15 roots were pooled and placed into glass scintillation vials containing 5ml scintillation fluid. Radio-activities in these two pools of root segments were measured using a Beckman Coulter LS6500 Scintillation counter (Fullerton, CA, USA). The amount of the auxin transport was the average of three separate experiments with appropriate standard deviation.

3.2.17 qReal-Time PCR

Total RNA (*CaZF*OX/Vec) was prepared from 200 mg of seedlings using the RNeasy plant mini kit (Qiagen). cDNA was prepared with 2 μg total RNA using High- Capacaity cDNA Reverse Trinscription kit (Applied Biosystemes, Foster City, CA, USA). For the prepration of cDNA 2 μl of 10X RT buffer, 0.8 μl of 25XdNTP mix (100 mM each), 1 μl of 10X random primer, 1 μl of multiscribe RT, 1 μl of RNAs inhibitor and 3.2 μl of MQ was added in 0.2 ml PCR tube and then 10 μl of total RNA was added (final conc.2 μg) and the tubes were incubated for reverse transcription at 25°C for 10 min, 37°C for 120 min, 85°C for 5 sec and 4°C forever.

For qRT-PCR, PCR reactions were performed in an optical 48-well plate with Applied Biosystems StepOne™ (Applied Biosystems USA), using SYBR Green to monitor dsDNA synthesis. Reactions were Performed in a 20 μl volume contained 10 μl 26 SYBR Green Master Mix (Applied Biosystems), 1.0 ng cDNA and 5 μM of each gene-specific primer. PCR cycles were performed as: 95°C for 10 min; 40 cycles of 95°C for 15 sec and 60°C for 1 min. Primer titration and dissociation experiments were performed to ensure that no primer dimmers is formed.

3.2.18 Functional analysis of 5' upstream region of *CaZF* in tobacco

3.2.18.1 Cloning of 5' upstream region of *CaZF* in pBI101.2 vector

To characterize the promoter further and to determine the regulatory properties of *CaZF* promoter, it has been amplified by genome walking and subsequently cloned into the binary vector pBI101.2 (Jefferson, 1987) as a transcriptional fusion in front of a promoterless β-glucuronidase (GUS) gene.

3.2.18.2 Transformation of Agrobacterium tumefaciens

Recombinant plasmids constructed in pBI101.2 were transferred into *Agrobacterium* by the freeze-thaw method as mentioned above. Transformation of *Agrobacterium* with various plasmid constructs was done by mixing 1 μg of DNA with competent cells followed by immediate freezing in liquid nitrogen. Subsequently cells were thawed by incubating the eppendorf tube at 37°C for 5 min. Thereafter 1 ml of YEM medium was added to the tube and incubated at 28°C for 1 h. Cells were spread on a YEM agar plate supplemented with 50 μg/ml kanamycin and 25 μg/ml rifampicin and incubated at 28°C. Transformed colonies that appeared after 1-2 days were analyzed either by PCR or by colony hybridization and the positive colonies were confirmed by restriction digestion of the purified recombinant plasmid.

3.2.18.3 Tobacco Plant Transformation

One positive colony of *Agrobacterium tumefacians* from each construct was used to transform tobacco by leaf disc method (Horsch et al., 1985).

3.2.18.4 Selection and Plant Regeneration

Immediately after co-cultivation, explants were transferred to MS medium with BAP and NAA containing 50 µg/ml kanamycin and 250 µg/ml cefatoxime. After 3-4 weeks, shoots with a defined stem were removed from explants and placed on MS rooting medium. As soon as the roots started appearing, the plantlets were removed from culture jars and rinsed in water and planted in pots containing vermiculite. After 7-10 d of hardening the plants were transferred to soil and grown under green house condition to allow production of seeds.

3.2.19 GUS assay

3.2.19.1 GUS histochemical assay

GUS histochemical assay was carried out in intact tissues (organ or whole seedlings or free hand cut sections). The tissue from the control and transgenic plants were submerged in a fixative buffer in microtiter plates kept on ice (2% formaldehyde, 50 mM sodium phosphate buffer pH 7.0, 0.05% Triton X-100), and vacuum infilterated for 4-5 min. The fixation buffer was removed and and tissue was washed twice with 50 mM phosphate buffer pH 7.0.Then the tissue was stained by adding 600 µl of X-gluc buffer (1.5 mM X-gluc, 50 mM phosphate buffer pH 7.0, 0.1% Triton X-100) and vacuum infilterated for 10 min. The sample tissue with X-gluc buffer was kept at 37°C overnight in darkness. Then X-gluc buffer was removed and the tissue was kept in 70% ethanol to remove the chlorophyll.

3.2.19.2 GUS spectrophotometric assay

The fresh frozen in liquid nitrogen was ground in 1 ml of extraction buffer (50 mM phosphate buffer pH 7.0, 5 mM DTT, 1 mM EDTA, 0.1% sarcosyl, 0.1% Triton X-100) at 4°C. The suspension was transferred to fresh eppendorf tube and centrifuged at 10,000 g for 5 min at 4°C. The supernatant was transferred to fresh eppendorf tube and 50 µl of the supernatant was added to the tube containing 450 µl of assay buffer (1 mM MUG in extraction buffer) and incubated at 37°C for 30 min. GUS activity was determined by fluorometric assay as described by Jefferson (1987) in which MUG was used as a substrate. Total protein was estimated by Bradford assay (Bradford, 1976). The GUS

specific activity was recorded as nanomoles of 4-MU formed per microgram of protein per 1min. from the initial velocity of the reaction (Jefferson *et al.*, 1987).

3.2.20 Proline Assay

Proline estimation was done as described by Magne and Larher (1992). Briefly, approximately 0.25 g of tissue was homogenized in 5 ml of 3% sulphosalicylic acid and filtered. The suspension (1 ml) was mixed with equal volumes of glacial acetic acid and Ninhydrin solution. The mixture was heated in a boiling water bath for one hour and reaction terminated by incubation on ice. Toluene (2 ml) was added and the mixture stirred for 20-30 sec. The toluene layer was separated and absorbance measured at 520 nM.

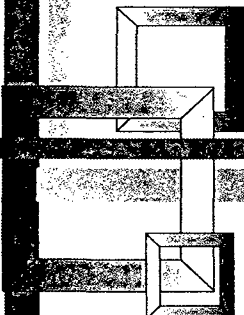
3.2.21 Chlorophyll estimation

Leaf discs of 1.0 cm diameter were excised from healthy and fully expanded tobacco leaves of same age (30 days post germination) from *CaZF*-expressing and vector-control plants. The discs were floated in a solution of NaCl (150 mM or 300 mM) or water (experimental control) for 72 h (Fan et al., 1997). The discs were then used for measuring chlorophyll spectrophotometrically after extraction in 80% cold acetone. The salinity and water treatments were carried out in continous white light at 25±2°C. The experiments were done with three experimental repeats of each vector-control and transgenic lines.

3.2.22 Glycerol estimation

Overnight grown yeast cells in YPGal medium were diluted to OD_{600}=0.3 and grown for 4 h at 30°C. Then they were subjected to increased osmolarity (500 mM NaCl). For glycerol measurement, at different time points of stress, 2 ml samples were taken, boiled for 15 min and then centrifuged to remove cellular debris. The supernatant was used for glycerol measurement by using Free Glycerol Reagent (Sigma, USA) according to the manufacturers' instructions. Glycerol accumulation was expressed in mg/ml (equivalent to triloen content).

Chapter 4



Comparative Transcriptional Profiling of Drought tolerant and Susceptible Chickpea Cultivars

4.1 Introduction

Drought continues to be one of the most significant environmental stresses with continuing decrease in soil moisture content and increase in global temperature (Graham and Vance, 2003). Rapid expansion of water-stressed areas necessitates improvement of crop traits conferring stress tolerance and adaptation through conventional breeding or genetic manipulation. For cultivated crops, like chickpea, where crop improvement through conventional breeding is difficult because of narrow genetic base (Udupa et al., 1999), comparative gene expression profiling is an alternative to identify pathways and genes regulating stress response. In general, comparison of gene expression profiles between contrasting genotypes provides much information in understanding the spatial and temporal patterns of gene expression required for abiotic stress tolerance. If this hypothesis is true, it will be possible to identify key regulatory genes in drought response pathways in further studies, increasing our knowledge of stress physiology, which will lead to the development of more drought tolerant crops. Plants induce expression of a number of genes in response to water limitation. At the cellular level part of the early response results from cell damage and meant for the damage repair. The other part of the response corresponds to adaptive processes that bring about changes in the metabolic process and structure of the cell that allows cell to continue metabolism under low water potential (Ingram and Bartels, 1996). A wide variety of techniques and strategies allow the identification of genes involved in molecular stress response (Cushman and Bohnert, 2000). With the advent of microarrays and protein profiling a lot of information on gene expression have been gathered. Although conventional gene-by-gene analysis is needed to validate the implication of a particular gene in stress response, gene expression analyses in response to various stresses in a number of plants have established the basic functional framework of plant response to abiotic stresses.

Most of the data on gene expression in response to drought and other abiotic stresses are generated using Arabidopsis (Thomashow, 1999; Seki et al., 2001; Cheong et al., 2002). However, it is not an ideal model to study abiotic stress response, as it does not experience variety of environmental changes causing physiological re-adjustments. In view of the wide genetic diversity exists in the plant kingdom, individual crop type should be studied to understand crop-specific response to a particular stress. At least ten different publications are available in literature on rice gene expression analysis in response to salinity only (Kawasaki et al., 2001; Ueda et al., 2002; Rabbani et al., 2003;

Sahi et al., 2003; Chao et al., 2005; Shiozaki et al., 2005; Walia et al., 2005; Walia et al., 2007; Zhou et al., 2007; Kumari et al., 2008). A comparative gene expression study between a salt-tolerant and a salt-sensitive rice cultivar showed gene expression, particularly those related to protein synthesis and turn over, is delayed in the sensitive variety and perhaps responsible for differential response (Yamane et al., 2003). On the other hand, a recent report showed that constitutive overexpression of some stress responsive genes in the salt-tolerant variety, which is otherwise inducible in the sensitive one, seems to be the mechanism for salt-tolerance. A transcriptional profiling of developing maize kernels in response to water deficit indicated that two classes of stress-responsive genes exist; one being specific to concurrent application of stress and another remains affected after transient stress (Yu and Setter, 2003). A previous study from this laboratory also indicated that some genes remained expressed even after removal of dehydration stress and may lead to adaptation (Boominathan et al., 2004). All these data supports a hypothesis that the plant that is well adapted to stress has two basic mechanisms of stress-tolerance; *i.e.* constitutive expression of genes required for adaptation or physiological reprogramming and quick expression of genes required to repair cellular damage in adverse condition. Comparative expression study with cultivars of contrasting features has become useful to identify these two classes of genes. However, there is a paucity of information concerning the number and types of genes involved in drought tolerance and how they interact to produce effective tolerance. In contrast to Arabidopsis and cereals, much less effort is observed in genomic studies of legume crops, in spite of their importance in nitrogen fixation and as a source of protein to a wide population. In this study chickpea (*Cicer arietinum*), a popular food legume that ranks third in global production was used to study gene expression analysis. Although chickpea is generally grown in relatively less irrigated lands and some cultivars adapt well with water-limited environment (Ma et al., 2001), drought poses a serious threat to chickpea production causing 40-50% reduction from its yield potential (Ahmad, 2005). Lack of enough resource impedes the progress of genomic study in chickpea. Recently a pulse microarray made of about 750 cDNAs from chickpea, grasspea and lentil was used to compare gene expression analysis in response to drought, cold and salinity in chickpea cultivars with contrasting stress-tolerance features (Mantri et al., 2007). An EST database has been generated from a library constructed by subtractive suppressive hybridization (SSH) of root tissue of two chickpea cultivars (Jayashree, 2005). Comparative proteome

maps of chickpea nucleus and cell wall revealed differentially expressed proteins during dehydration stress (Bhushan et al., 2007; Pandey et al., 2008).

To identify differentially expressing genes construction of SSH library is a useful tool, where genomic resource is limiting. Therefore, to identify genes differentially expressing in a relatively drought-tolerant and a sensitive chickpea cultivar in response to progressive depletion of water we have constructed SSH libraries from whole seedlings of two cultivars at different stages of stress. This chapter has demonstrated that the SSH and macroarray could be effectively used for gene expression profiling of chickpea responses to drought stress.

Hence, the aims of the experiments detailed in the current chapter were to:

1. Analyse the physiological changes in chickpea cultivars under progressive water depletion.
2. Construction of subtracted EST libraries from the chickpea seedling at different drought conditions and study the expression of ESTs in chickpea seedlings in response to drought.
3. Interpret the results from transcriptional profiling in the context of putative gene functions and genotypes in which they were expressed to try and uncover the mechanism and pathways involved in drought tolerance in chickpeas.

4.2 Results

4.2.1 Differential drought tolerance in two chickpea cultivars

A comparison of drought tolerance between two cultivated chickpea (*Cicer arietinum,* cv. PUSABGD72 and ICCV2) varieties was conducted by measuring the changes in leaf relative water content (RWC), chlorophyll content, Abscisic acid and proline after discontinued irrigation (DH stress) to establish their contrasting characters. The seedlings were grown for 12d after germination before stopping irrigation. RWC of the leaves were measured at different time points as a measure of stress-adaptation as it (RWC) accounts for osmotic adjustment, which is considered as one of the most important mechanisms of plants for adaptation in water-limited environment. During the treatment both the cultivars showed a little increase in RWC after 3d. This could be due to more transport of water from the other compartments of the plant to leaf to maintain turgor or resulted from osmotic adjustments due to more synthesis of osmolytes. After this initial increase, leaf RWC of both the cultivars showed steep decrease up to 12d (Figure 4.1A), with PUSABGD72 showing about 10% higher RWC than ICCV2.

Although role of proline in stress tolerance is debatable, its accumulation is considered as one of the indicators of adaptive response (Delauney et al., 1993; Yoshiba et al., 1997). Both the cultivars showed increase in proline content within 3d of treatment and maintained the increase up to 12d. However, proline accumulation in PUSABGD72 was more than two fold of ICCV2 (Figure 4.1B). Chlorophyll content is considered as the indicator of rate of photosynthesis. Chlorophyll content started decreasing with the treatment in both the cultivars. Throughout the course of treatment better maintenance in the rate of photosynthesis was exhibited by PUSABGD72 (Figure 4.1C). ABA acts as a key regulator of dehydration response (Skriver and Mundy, 1990). Pattern of ABA accumulation in both the cultivars followed the same course. Initial ABA content of PUSABGD72 was higher than ICCV2. There was a sharp increase in the accumulation of ABA within 3d indicating its involvement in early response to stress. However, in the later period of treatment ABA content was re-adjusted and maintained. Overall, ABA accumulation in PUSABGD72 was 3 fold higher than ICCV2 throughout the treatment (Figure 4.1D). Morphological differences were also observed after 12d DH treatment given to both cultivars at the same time (Figure 4.1E). Altogether, with respect to the above assays PUSABGD72 clearly displayed a better tolerance to drought stress than ICCV2.

4.2.2 Construction of subtracted cDNA library and dfferential screening

To characterize the molecular nature of drought tolerance in PUSABGD72, a PCR-based method Suppression Subtractive Hybridization (SSH) (Diatchenko et al., 1996) approach was chosen. SSH is a powerful technique that generates differentially expressed ESTs under drought stress. Subtraction step at mRNA level not only normalizes the sequence abundance but also enriches the transcripts that are differentially expressed in response to drought stress. Four subtracted cDNA libraries were constructed with poly (A^+) RNA isolated from control C (ctrl), 3d, 6d and 12d DH stressed seedlings of PUSABGD72 and ICCV2 (Figure 4.2). Earlier some ESTs resulted from a subtracted cDNA library constituted with only PUSABGD72 at control and 5h dehydration stress condition was reported (Boominathan et al, 2004). Those ESTs were also included in this study. All libraries appeared to be of reasonable quality having inserts ranged in size from 350 to 1500bp (Figure 4.3A and B). 2700 randomly selected clones from all the four libraries were single-pass sequenced. After screening out the redundant sequences 319 high-quality unique EST sequences were generated. These sequences were analyzed by the current GenBank database using the BLASTX algorithm for their putative functional

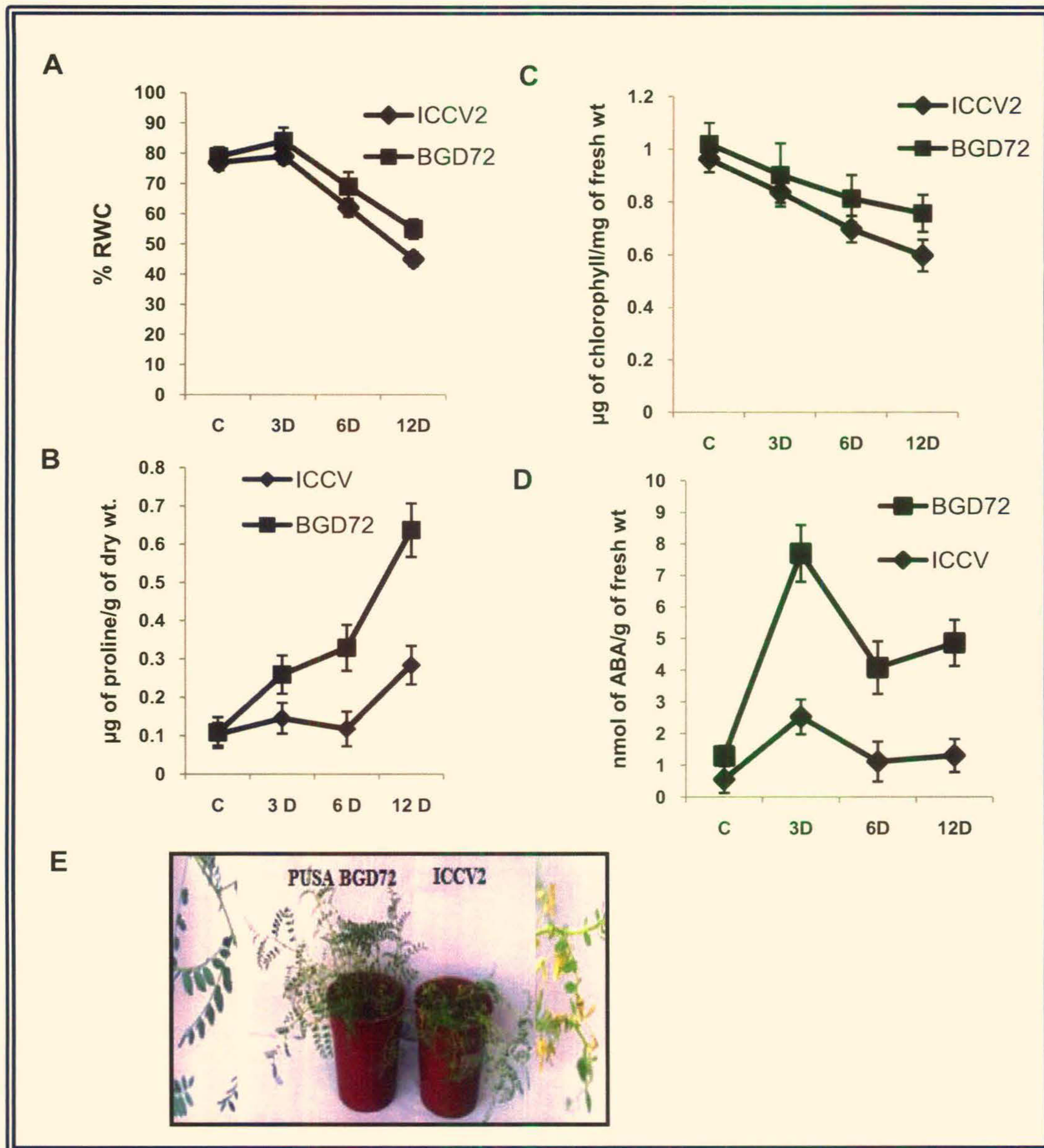


Figure 4.1. Effect of drought stress on chickpea seedlings. Comparative analysis of leaf relative water content (RWC) **(A),** proline **(B)**, chlorophyll content **(C)**, and ABA **(D)** accumulation between two (BGD72 , ICCV2) varieties of chickpea in a time dependent manner under drought stress. Phenotypic differences were also recorded after 12d DH treatment **(E)**. All experiments were done in triplicates, and average mean values were plotted against drought stress duration.

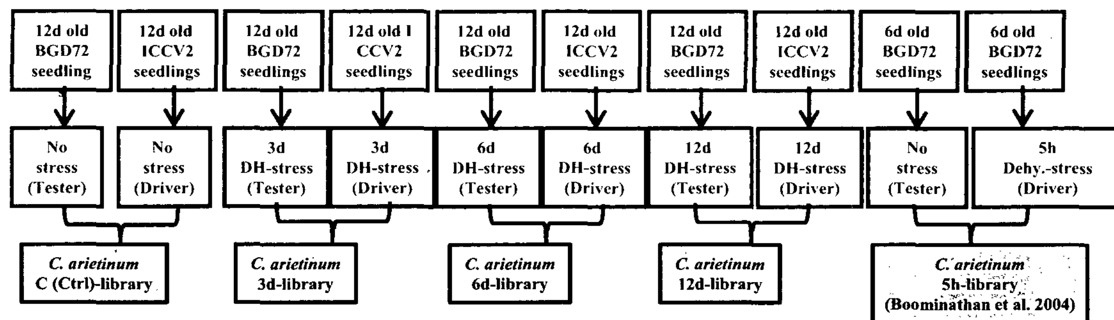


Figure 4.2. Schematic representation of four (this study) and one (Boominathan et al. 2004) subtractive libraries made with chickpea seedlings.

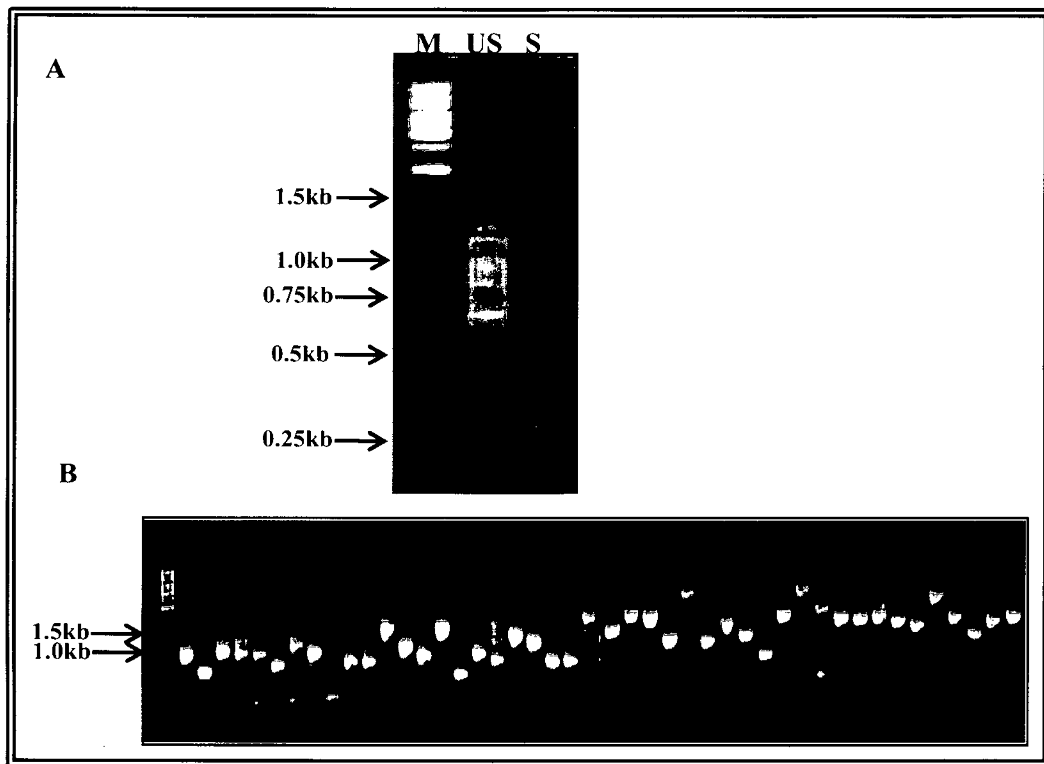


Fig. 4.3 Validation of subtractive libraries made between PUSA BGD72 and in ICCV2. (A) 1% EtBr-agarose gel showing the smear of amplified subtracted cDNAs. (M, 1 Kb ladder; US, "unsubtracted cDNA"; S, "subtracted cDNA" obtained after primary and secondary PCR amplifications). **(B)**1% EtBr stained gel showing PCR amplification of the positive clones.

classification. The ESTs generated were deposited into GenBank for accession numbers. A total of 312 out of 319 ESTs showed significant similarity to known sequences in the databases. The remaining 7 ESTs are not homologous to known sequences and were deemed novel (Table 4.1). Of these 312 ESTs, 277 can be functionally categorized according to their BLASTX match whilst the rest 35 ESTs along with 7 novel ESTs were kept under 'unclassified' category.

Table 4.1: Functional categorization of ESTs generated by subtracted cDNA libraries.

GenBank Match	Putative fuction	E-value	Accession No.
Cell Defense			
AAC49797.1	MRP like ABC transporter	8E-77	FL512349
CAA10134.1	Basic blue Cu protein	7E-21	FL512383
CAA31760.1	Disease resistance response protein	1E-43	FL512398
CAD59565.1	PDR like ABC transporter	3E-77	FL512404
BAC66711.1	Put. Cold shock protein	0.021	FL512475
AAQ93011.1	Put chloroplast FtsH proteinase	8E-26	FL518918
BAE71245.1	FtsH like protein PftF precursor	2E-88	FL518951
ABO61512.1	Leu rich recepter like protein	4.E-05	FL512357
AAB32504.1	Class 10 PR protein	1E-23	FL512394
BAF00814.1	MRP like AB ransporter	7E-43	FL518997
ABG34276.1	Polygalacturonase like protein	8E-52	FL518998
BAA29056.1	Polygalacturonase inhibiting protein	7E-47	CD051271
AAK59442.1	Chitinase family 19	1E-34	CD051291
BAB01963.1	Polygalacturonase inhibiting protein	3E-10	CD051270
BAB16429.1	Elicitor inducible gene	8E-11	FL512456
Cell Transport			
CAB61749.1	Aquaporin	7E-29	FL512407
CAD56222.1	Aquaporin like Water channel protein	4E-51	FL512354
AAB60858.1	Vacuolor assembly protein	2E-43	CD051281
C85065	Kinesin like protein	2E-52	CD051276
Cellular Organisation			
CAA05771.1	Lipid transfer protein precursor	4E-53	CD051321
CAA05771	Lipid transfer protein	1E-53	FL512385
AAF26451	Non specific lipid transfer	9E-14	FL512469
CAA78515.1	Dehydrin	6E-12	FL512347
AAO65979.1	Seed protein	3E-18	FL512360
CAB71135.1	Put. Imbibition protein	1E-99	FL512365
AAX86047.1	α -Tubulin	7E-77	FL512378
CAA10131.1	Chalcone synthetase	1E-24	FL512402
AAF18411.1	Integral mem. Protein	8E-107	FL512408
ABD32895.1	HSP70	9E-105	FL512415
BAE71209.1	Importin	3E-82	FL512417
CAA04767.1	Ripening induced protein	5E-32	FL512428
BAD38204.1	HVA-22 homolog	3.E-06	CD051272
AAA33671.1	Probable HSP	4E-15	FL518913
BAB09641.1	Ceramidase family protein	1E-26	FL518915
AAN31874.1	Anion exchange protein	3E-92	FL518920
ABD32352.1	HSP	3E-56	FL518941
AAM61627.1	Put ER lumen protein retaining receptor	5E-83	FL518946
AAR23312.1	Cellulose synthase	1E-108	FL518949
ABO47740.1	α- tubuline	8E-55	FL518958
CAB75430.1	Put 16KD mem protein	3E-40	FL518959
ABD32895.1	70KD HSP	2E-93	FL518962
CAB45653.1	Put tonoplast intrinsic protein	1E-26	FL518966
CAA05979.1	Adenine ntd translocator	4E-80	FL518967
AAL38353.1	HSP	3E-48	FL518986
ABD32895.1	HSP 70 cognate	6E-77	FL518996
CAA10192.1	Glycine rich protein	2E-09	FL512344
CAB71135.1	Imbibition protein	2E-38	FL519000

AAG15412.1	Seed maturation protein	5E-29	FL519001
CAB53509.1	Histone 2A	2E-22	CD051290
AAN77521.1	Dehydrin	3E-25	CD051297
CAA12027	LEA protein 2	6E-37	CD051326
CAA12026.1	LEA-1	6E-16	CD051271
AAM61711.1	Prolyl-4-hydroxylase	e-100	CD051295
AAC33276.1	Fiber protein 1	5E-33	CD051339
AAN77521.1	Dehydrin1	4E-38	FL512434
AAM61107.1	Probable nitrate transporter	3E-12	FL512436
BAB16458.1	Nitrate transporter	5E-10	FL512438
CAA48210	Triose Phos translocator	4E-52	FL512441
AAB71830.1	Annexin	1E-15	FL512442
AAK53759.1	Probable K+ transporter	1E-25	FL512444
AAK76516.1	Aconitase family	4E-70	FL512448
AAF63170.1	Endomembrane protein 70	3E-71	FL512454
CAA10127.1	Nucleolar protein	2E-25	FL512465
AAM08004.1	Put mem protein	1E-20	FL512450
AAD30230.1	Membrane protein (CDC50 family protein)	1E-54	FL512474
CAA66038.1	Proline rich protein	3E-15	FL512352
CAB81548.2	Put. Proline rich protein	4E-79	FL512405
Energy metabolism			
CAA65008.1	Metallothionein-like protein 1 (MT-1)	2E-26	FL512338
AAF04584.1	Type 1 metallothionein	1E-44	FL512409
CAA10132.1	Superoxide dismutase	3E-82	FL512362
CAA39819.1	Cu/Zn superoxide dismutase II	4E-62	FL512366
CAB71128.2	Cationic peroxidase	1E-82	FL512384
AAY56795.1	Vacuolor ATP synthetase	8E-79	FL512392
ABO20848.1	Monooxygenase	9E-48	FL512410
CAA06156.1	Cyt. P450 monooxygenase	1E-31	FL518911
CAA59444	Catalase	3.E-09	FL512416
BAA10929.1	Unsp. Monooxygenase	2E-42	FL512427
AABO1223	Put dehydrogenase E1 beta	8E-45	FL518914
ABN08957.1	H+-transporting two-sector ATPase	2E-58	FL518930
ABA01324.1	Epoxide hydrolase	7E-16	FL518928
AAW51769.1	Catalase fragment	5E-14	FL518979
BAD26579.1	Cyt P450 like_TBP	1E-45	FL518994
CAA08855.1	Copper amine oxidase	2E-84	FL512335
CAC29436.1	P type H+ATPase	1E-11	CD051280
CAA45098	Thioredoxin F type, chloroplast precursor	0.007	CD051307
AAM61418.1	Mitochondrial uncoupling protein	2E-18	CD051283
AAG14962.1	Cyt P450 dependent monooxygenase	8E-62	CD051325
BAB33250.1	NADH dehydrogenase ND1	4E-27	CD051327
CAB78385.1	Put-3-isopropylmalate dehydrogenase	1.E+00	FL512446
Hormone biosynthesis			
CAA53730.1	Lipoxygenase	2E-44	FL512369
ABO77438.1	Methionine adenosyl transferase	3E-46	FL518953
AAT40304.1	S-adenosylmethionine synthetase (*Medicago*)	3E-62	FL518971
AAA81377.1	SAM synthetase (*Arabidopsis*)	2E-22	CD051262
CAC43237.1	Lipoxygenase	2E-57	CD051273
BAC10549.1	Nine cis epoxycarotenoid dioxygenase	4E-52	CD051315
Metabolism			
AAA33642	Fructose-bisphosphate aldolase 1	3E-97	FL512350
AAR29343.1	Allantoinase	4E-31	FL512353
CAB10455.1	Lipase	9E-25	FL512358
ABM01871.1	Chloroplast rubisco activase	7E-56	FL512364
AAB81011.1	Asn synthetase	6E-69	FL512372
AAD27878.1	Chlorophyll a/b binding protein	1E-58	FL512373
ABI94075.1	Chloroplast rubisco activase small protein isoform	8E-30	FL512377
CAB10455.1	Triacylglycerol lipase like protein	9E-24	FL512391
BAC76729.1	α – amylase	4E-23	FL512401
CAC84547.1	Dicarboxylate/tricarboxylate carrier	1E-99	FL512403
AAD01737.1	GDP mannose pyrophosphorylase	2E-17	FL512412
ABP49577.1	Microsomal omega-6-desaturase	2E-103	FL512413
AAN75219.1	Chloroplast translocon	2E-68	FL512420
ABQ88337.1|	β – cobalamine synthase	2E-44	FL512422

BAE07181.1	Met. Synthase	2E-60	FL512423
AAK65960.1	Sucrose synthase	5E-23	FL512424
AAB06756.2	1-L-myoinositol-1-P-synthetase	6E-12	CD051303
AAM61665.1	Leuco-anthocynidine dioxygenase	5E-36	CD051278
CAH59405.1	Light harvesting protein	1E-72	FL518912
ABW21688.1	Enolase, isoform 1	1E-96	FL518916
ACC59198.1	1-acyl-sn-glycerol-3-Phos acyltransferase	4E-70	FL518917
AAQ84168.1	1-deoxy-o-xylulose-5-Phos reductoisomerase	6E-89	FL518921
AAM28620.1	adenosine monophosphate binding protein	7E-63	FL518923
CAA04512.1	Second sucrose synthase	7E-79	FL518924
CAA36396.1	Glyceraldehydes-3-Phos dehydrogenase	1E-56	FL518927
ACC63885.1	Caffeic acid-o-methyltransferase	4E-67	FL518929
CAA76854.1	Ketol acid reductoisomerase	7E-37	FL518933
AAK64167.1	Methionin synthase	6E-83	FL518940
CAA84494.1	α- 1,4-glucan phosphorylase	1E-42	FL518943
CAA42443.1	P-protein	9E-59	FL518944
AAN15627.1	Nucleotide sugar epimerase like protein	2E-92	FL518945
AAP83930.1	Rubisco activase b form precursor	6E-17	FL518947
AAA33652.1	Carbonic anhydrase	3E-77	FL518948
AAB01223.1	Pyruvate dehydrogenase E1 b	6E-63	FL518950
AAM63830.1	Put PS-I reaction	2E-23	FL518955
AAT85058.1	Put C type cyt synthase	2E-42	FL518957
AAG52429.1	Put amino peptidase	1E-55	FL518960
CAB50768.1	Cyt P450	9E-37	FL518968
AAB46611.1	Asparate aminotransferase	5E-61	FL518970
AAB99755.1	Malate dehydrogenase	4.E-06	FL518972
AAF98217.1	Mannose – 6 – Phos isomerase	3E-51	FL518974
AAN17423.1	P-protein like protein	3E-21	FL518978
ABB20913.1	Rubisco activase	7E-78	FL518984
AAN75219.1	Rubisco activase (small isoform)	4E-53	FL518985
AAB00860.1	Microsomal omega-6-desaturase	7E-92	FL518987
ABB29955.1	Hydroxyacyl glutathione hydrolase	2E-28	FL518990
BAA33879.1	α-amylase	6E-68	FL518991
CAA10290.1	Rubisco small subunit	4E-25	FL518995
AAM65487.1	Chl a/b bp	2E-25	FL512336
CAA63482.1	Glycolate oxidase	1E-74	FL512340
CAA06819.1	Cysteine synthase	1E-51	FL512341
BAB10198.1	Alcohol dehydrogenase	4E-12	CD051265
AAL67089.1	β-amylase	1E-89	CD051266
AAK27718.1	ADP glucose pyrophosphorylase	1E-33	CD051279
BAB08397.1	Phosphoribosylanthranilate transferase like protein	2E-17	CD051285
CAC07424.1	Cinnamoyl CoA reductase	2E-58	CD051301
AAK15160.1	Put apyrase	8E-48	CD051304
CAB78780.1	Trehalose-6-Phos synthase homolog	2E-25	CD051305
AAN15946.1	Rubisco activase(small isoform)	2E-12	CD051311
AAD25783.1	Aldehyde dehydrogenase family	1E-22	FL519005
AAM91301.1	Phosphoglucomutase	1E-34	CD051347
AAB99632.1	Phophonopyruvate decarboxylase like protein	2.E-04	FL519008
AAG09205.1	Trans-cinnamate-4-monooxygenase	e-125	CD051342
CAA11857.1	Delta-8-sphingolipid desaturase	4.E-08	CD051350
AAF79428.1	Long chain fatty acid condensing enzyme	e-111	CD051352
CAA89019.1	Cobalamine independent methionine synthase	4E-12	CD051358
AAL67089.1	Put β- amylase	2E-90	FL519010
CAB78780.1	Trehalose-6-phosphate synthase like protein	2E-25	CD051305
AAL37169.1	Put chloroplast targeted b amylase	2E-26	FL512429
AAD01804.1	Lipase (class 3) family	3E-14	FL512437
CAA06339.1	UDP galactose-4-epimerase	1E-34	FL512443
AAD02832.1	Raffinose synthase	7E-38	FL512447
AAF26084.1	Put alkaline neutral invertase	1E-66	FL512451
BAC01214.1	Fructose-1,6-bisphosphatase	6E-65	FL512453
AAO38524.1	Asparagines synthetase	5E-66	FL512455
AAL37169.1	Glycosyl hydrolase family 14	3E-89	FL512460
BAB40340.1	Probable 12 oxophytodienoate reductase	7E-26	FL512470
AAC28107.1	Nodule enhanced sucrose synthase	4E-32	FL518926

Protein degradation			
ABH08753.1	Ubiquitin	3E-63	FL512379
CAA08906.1	Cysteine proteinase	5E-49	FL512381
CAB88363.1	Prolyl-peptidyl isomerase	1E-47	FL512388
ABH08753.1	Polyubiquitin	3E-52	FL512426
ABR25718.1	Monoubiquitin	4E-28	FL518932
EAA21327.1	Putative senescence associated protein	2E-13	FL518988
ABD32628.1	Thioprotease	2E-72	FL512333
ABF18679.1	Early leaf senescence abundant cysteine proteinase	1E-72	FL512339
CAA08906.1	Cysteine proteinase	2E-86	FL512342
BAA88898.1	Cysteine proteinase type protein	1E-52	CD051336
BAB08738.1	ATP dependent clp protease	8E-73	CD051341
CAA51821.1	Ubiquitin conjugating protein	9E-56	CD051293
AAO43306.1	Putative polyubiquitin	2E-29	FL512431
AAA34123.1	Hexameric polyubiquitin	4E-27	FL512462
AAM64530.1	Ubiquitin homolog	3E-57	FL512466
Signal Transduction			
ABQ95992.1	14-3-3 brain protein homolog	3E-56	FL512351
CAC43238.1	Calcium binding protein	1E-20	FL512355
AAM62466.1	Stress related protein	4.E-04	FL512361
AAM63746.1	ADP ribosylation factor like protein	7E-70	FL512368
CAA88841.1	Phosphoglycerate kinase	5E-86	FL512374
AAS65786.1	Protein kinase family (arabi)	3E-36	FL512375
BAB10839.1	Receptor like protein kinase	5E-48	FL512376
AAD10151.2	Put WD40 repeat protein	E-118	FL512386
CAL25342.1	Putative Zinc binding protein	2E-84	FL512395
AAL47552.1	IAA-AA hydrolase	7E-78	FL512418
CAA48210.1	Phosphate translocator	3E-69	FL512425
AAK50348.1	CBL-interacting protein kinase	6E-23	FL512472
AAG50535.1	Put. Ser-thr protein kinase	8E-26	CD347670
AAS65786.1	Protein kinase family protein	9E-37	FL512375
AAC62851.1	Photolyase	2E-48	FL518952
AAF18411.1	Integral membrane protein	4E-108	FL518989
BAD35220.1	Put nucleolar GTP bp	2E-24	FL512332
AAL47352.1	WD repeat protein like protein	4E-26	CD051264
AAM65034.1	Put protein kinase	3E-34	CD051343
AAD17804.1	Nodule enhanced protein phosphatase	1E-84	FL519004
AAM91135.1	G-protein coupled receptor like protein	5E-63	CD051322
AAM62611.1	ADP ribosylation factor like protein	2.E-09	CD051324
AAF04915.1	Jasmonic acid 2	2E-19	CD051357
CAA67554.1	Protein kinase (CIPK25)	8.E-05	CD051317
CAA49171.1	Omnipotent suppressor protein	1E-30	FL519009
AAD17804.1	Nodule enhanced phophorus protein	1.E-07	FL519011
AAM83095.1	SOS2 like protein (CIPK6)	1E-43	FL512440
AAF69681.1	High affinity Fe^{-2}-Pb^{-2} permease	2E-12	FL512457
CAB90633.1	Protein phosphatase 2C	5E-48	CD051312
CAA49512.1	Glycerol kinase related	2E-56	FL512459
BAA33803.1	Chloroplast phosphoglycerate kinase	7E-58	FL512468
Transcription			
AAQ10954.1	Zn finger protein	1E-23	FL512348
CAD59768.1	Putative reverse transcriptase	2E-09	FL512380
CAE45592.1	Transcription factor BTF3	2E-43	FL512406
AAZ14831.1	AP2 transcription factor	8E-22	FL512414
ABH02865.1	Transcription factor Myb-1	9E-33	FL512419
BAB09451.1	Histone acyl transferase	2E-59	FL518980
AAN31856.1	RNA helicase	1E-21	CD051282
AAM47901.1	RAP2.6	4E-17	CD051355
CAB96991.1	Put Zn finger protein	4E-22	CD051330
AAC49772.1	AP2 domain like protein	4E-17	CF074502
AAC49770.1	Put AP2 domain transcriptional regulator	9.E-09	FL519007
CAC92868.1	Transcriptional repressor of GlcNag	2E-46	FL519012
AAL61938.1	Aspartyl-tRNA synthetase	3E-46	FL512433
AAD26942.1	Zn finger protein	5E-16	FL512439
AAO13360.1	Dehydration responsive element bp3	2E-19	FL512463
AAL66951.1	α-NAC	2E-09	FL518992

AAM66970.1	Put. RNA bp	5E-23	FL512359
ABB87134.1	RNA bp	8E-41	FL512337
BAE71244.1	RNA bp	5E-29	FL518922
AAM66970.1	RNA bp cp29 protein	9E-24	FL512387
Translation			
AAN74635.1	DEAD box RNA helicase	3E-115	FL512356
CAI48073.1	60S ribosomal protein L37a	4E-46	FL512367
AAZ32899.1	Elongation factor 2	1E-75	FL512382
AAF27938.1	Translation initiation factor 5A	1E-14	FL512389
AAN15375.1	Translation initiation factor eIF-2	1E-43	FL512390
AAM63913.1	40s ribosomal protein S7 homolog	1E-17	FL512399
AAZ32899.1	Elongation factor 2	2E-28	FL512400
CAA74893.1	Translation elongation factor Tu	7E-39	FL512421
CAA06245.1	Elongation factor 1 alpha	1E-52	FL518919
BAD09700.1	Put ribosomal protein large subunit	1E-83	FL518935
AAK25759.1	Ribosomal protein L18a	2E-34	FL518931
ABK63942.1	Ribosomal protein L3	3E-42	FL518934
ABK63942.1	60S ribosomal protein L3	2E-48	FL518937
AAL09401.1	Ribosomal protein L17	1E-61	FL518938
AAQ22726.1	40S ribosomal protein S25	8E-29	FL518942
CAA07226.1	Ribosome associated protein p40	4E-83	FL518954
AAB81972.1	Ribosomal protein S14	5E-41	FL518961
CAG47084.1	40S ribosomal protein S9 like	3E-12	FL518963
CAA39950.1	Ribosomal protein L11	5E-46	FL518964
ABF97261.1|	Put 40S ribosomal protein S3	4E-67	FL518973
ACF06499.1	Put 40S ribosomal protein	2E-63	FL518976
AAM65734.1	60s ribosomal protein L13a	6E-71	FL518981
ACF06499.1	40S ribosomal protein	1E-21	FL518982
BAA96366.1	40S ribosomal protein S13	4E-29	FL512445
AAM62795.1	60S ribosomal protein L27A	6E-19	FL512452
CAD56219.1	Ribosomal protein S3a	2E-101	FL512334
AAD28753.1	60S ribosomal protein L37A	1E-46	CD051286
BAA20879.1	Eukaryotic translation initiation factor 5A-1	3E-59	CD051287
AAC14469.1	40S ribosomal protein S11	2E-32	CD051346
AAL79739.1|	Ribosomal protein	4E-16	CD051267
AAN31818.1	Ribosomal protein S15	2E-58	CD051284
CAA11705.1	Elongation factor	1E-25	CD051300
AAM67061.1	Ribosomal protein S2	1E-50	CD051333
AAM63913.1	40S ribosomal protein S7 homolog	4E-17	CD051338
Unclassified			
AAS21370.1	PB-1 domain containing protein	2.E+00	FL518925
ACF06596.1	Callus protein P23	6E-37	FL518939
AAP21292.1	Armadillo b catenin repeat	E-29	FL518965
CAB81288.1	DER2.2	3E-41	FL518969
ABK95544.1	Unknown protein	5E-26	FL518975
CAB79503.1	Put KH domain protein	4E-44	FL518977
ABQ41951.1	SOUL protein	8E-26	FL518983
ABO61376.1	Serine hydroxymethyltransferase	1E-39	FL518993
CAA66108.1	Specific tissue protein	2E-110	FL512343
CAC04249.1	PPF-1 protein	2E-48	CD051344
AAL06916.1	Expressed protein	3E-08	CD051334
ABO61512.1	Put ABA responsive protein	1E-16	FL512397
BAE19944.1	UV opsin	1E+00	FL512363
BAB09624.1	Put. Protein (Arabi.)	4E-33	FL512370
CAN81117.1	Unknown protein (Arabi)	9E-70	FL512371
AAN13040.1	Put CCR-4 associated factor	7E-22	FL512393
AAF33786.1	Cold induced alfalfa gene	1.E+00	FL512473
CAA11429.1	Zwille protein shoot meristem	3.E-05	FL512476
CAF18246.1	Put. Leunig	4E-17	FL512477
AAM67211.1	Serine rich protein	4E-16	CD051340
AAK00390.1	Phi – 1 like protein	1E-51	CD051263
AAL87341.1	LIN 1 protein	2E-30	FL519002
AAF33786.1	Cold induced alfalfa gene	9E-14	CD051294
AAO22640.1	Put protein	6.E-07	FL519003
AAM62421.1	Drm3	5E-17	CD051331

AAK00390.1	Put phi-1 protein	4E-16	FL519006
AAC64888.1	Hyp protein	6E-42	CD051269
BAB62576.1	Put protein	8E-40	CD051275
AAK59423.1	Putative protein	4E-21	CD051298
AAM64945.1	PDI like protein	9.E-07	CD051306
AAL29690.1	Profiling	3E-55	FL512430
AAM34266.1	VTC2	2E-80	FL512432
BAA33810.1	Phi-1	3.E-08	FL512435
AAM14063.1	Put cullin	2E-32	FL512458
AAM12036.1	Anther specific protein	3E-23	FL512461
AAK98750.1	Transposase of Tn10	1E-86	FL512464
BAB96814.1	Zwille protein	E-05	FL512467
ACA04850.1	SAP	2E-40	FL512411
BAB33421.1	Put SAP	7E-27	FL518999
AAF75749.1	Dehydration induced protein	6E-28	FL512471
CAC85245.1	Salt tolerant protein	1E-42	FL512396
CAC85245.1	Salt tolerance protein 4	4E-17	FL518936

4.2.3 Identification of drought responsive transcripts differentially expressed more in PUSABGD72

Drought induced genes identified in this study show homology with genes that are involved in variety of cellular functions. These ESTs were places in eleven functional classifications based on their putative functions. They mostly represent gened involved in metabolism (24%), cellular organization (19%), protein metabolism (degradation and synthesis) (16%) and signal transduction (11%). Among the other classes are cell defense genes (7%), cell transport (2%), energy metabolism (7%), hormone biosynthesis (3%), transcription (5%). The cDNA clones that did not match with protein database accounted for 12% are shown under the category as 'unclassified' (Figure 4.4). There were a number of highly redundant known stresses responsive ESTs in the libraries, indicating their abundance in drought stressed seedlings.

4.2.4 Comparative expression of ESTs in PUSABGD72 with respect to ICCV2

DNA-arrayed Nylon membranes (reverse-northern blots) have become a useful tool for the analysis of gene expression. In the present study, reverse-northern blots were used to compare the expressions of 319 unique ESTs obtained from four (C, 3d, 6d and 12d) subtracted libraries between PUSABGD72 and ICCV2. Differentially expressed ESTs in response to drought were identified by differential hybridization intensities with radio-labeled first strand cDNA probes prepared using poly (A^+) RNA isolated from control/stressed samples of PUSABGD72 or ICCV2 (Figure 4.5). To verify reproducibility, each of the clones was tested at least three times with three independent drought stress experiments. Expression ratio was calculated according to (Seki et al., 2001; Boominathan et al., 2004). Signal intensity of each spot was normalized by subtracting the intensity of the negative control (NPTII). Fold expression was presented

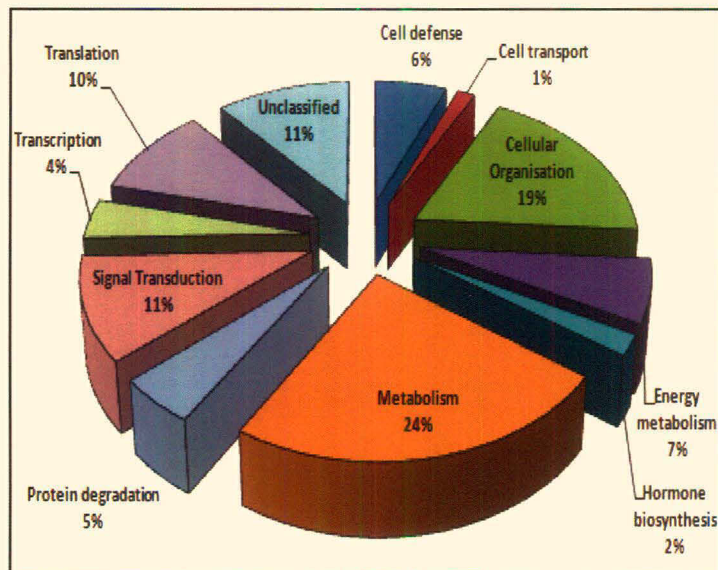


Figure 4.4. Functional categorization of ESTs. The identified ESTs were assigned with a putative function using BLASTX algorithm and clusters of proteins were classified with known or putative functional annotation. Detail information of cluster in given in supplemental table S1.

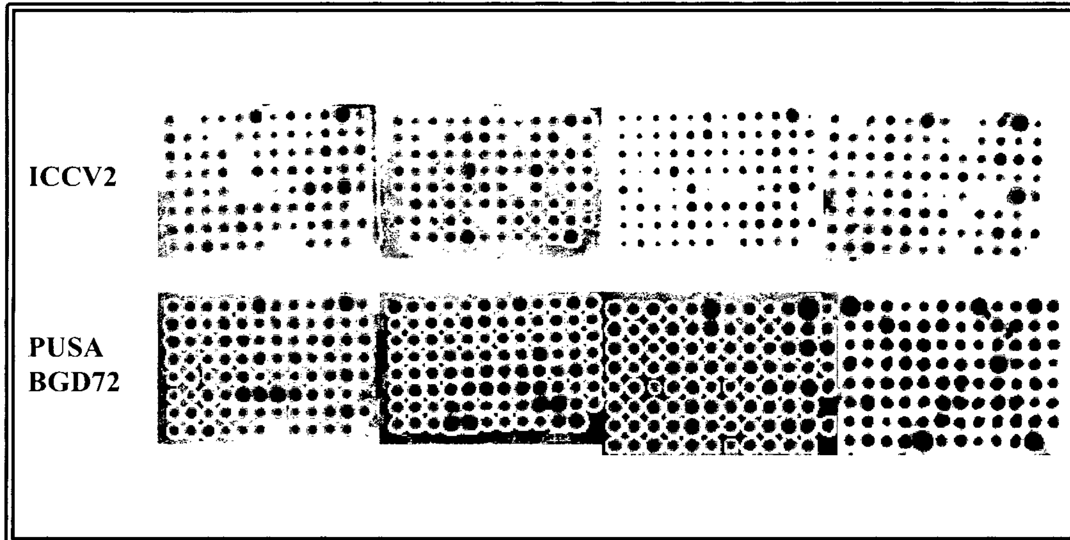


Figure 4.5. Representative macroarrays showing fold induction of the transcripts obtained from subtraction library. Positive clones were PCR amplified and approximately 100 ng of the product was blotted. A replica of the blot was also made. The two blots were respectively hybridized with radiolabelled first strand cDNA probe prepared from 1μg mRNA from PUSABGD72 (tolerant) and ICCV2 (susceptible) chickpea cultivars.

as the expression ratio (control/stressed) of PUSABGD72 to ICCV2 relative to the ratio of intensity of actin. Genes showing ≥2 fold expression at any time point in PUSABGD72 in comparison to ICCV2 were considered as differentially expressed and studied further. Approximately, 23%, 42.5%, 55.62% and 53.5% of the ESTs showed more than two-fold abundance in PUSABGD72 at control and 3d, 6d and 12d DH conditions respectively. Although, the genes expressing more than two fold in PUSABGD72 in comparison to ICCV2 at control condition naturally include drought-responsive and non-responsive genes, relatively higher number of genes differentially expressing in response to drought strongly correlates differential drought-tolerance of the cultivars. 19.5% of all the ESTs were ≥2 fold abundant in PUSABGD72 at all the time points.

To achieve a comprehensive overview of relative expression profiles, 319 ESTs were clustered according to their relative expression patterns in PUSABGD72 in comparison to ICCV2 by the hierarchical clustering method using the correlation coefficient of average linkage of the log-transformed ratio (Eisen et al., 1998). Expression profiling of genes, compared between PUSABGD72 and ICCV2, revealed 11 groups by SOTA clustering, according to the distance of correlation (Figure 4.6). The data was taken in terms of fold expression of control/stressed PUSABGD72 with respect to ICCV2. In addition the data sets were log-transformed to the base 2 to level the scale of expression and to reduce the noise. Only the clusters with n>6 were used to study the co-expression patterns for functionally similar proteins. Detailed information on proteins within each cluster can be found in Table 4.2. Out of the most abundant clusters, the genes belonging to cluster 1, 10 and 11 particularly never expressed less in PUSABGD72 in comparison to ICCV2. The composition of these clusters presents those genes whose expression probably necessitates by the cell for its survival under unfavorable conditions. Cluster 1 genes are mainly classified in cellular organization, metabolism and protein translation category. Cluster 10 genes exhibited higher expression in PUSABGD72 at all the time points monitored in this study. The genes related to cellular organization, metabolism and signal transduction mostly constitute this cluster. Cluster 11 genes represent those, which are expressed comparatively more in PUSABGD72 only at 3d and at 6d DH conditions, but at the later phase of stress expressed at the level of ICCV2. Important genes to mention in this cluster are several defense related genes. Interestingly, all these three clusters include a lot of genes for ribosomal proteins and translation elongation, which corroborates with an earlier study showing a salt-tolerant rice variety expressing protein synthesis genes early in salt stress (Kawasaki et al., 2001). Most of signal transducing genes lay in cluster

10 (16 genes) and they show a steady abundance in PUSABGD72 at all the time points. Interestingly, three ESTs representing a CBL-interacting protein kinase, a receptor-like kinase and a phospho glycerate showed more expression in PUSABGD72 only at 6d DH (cluster 3). The gene clustering based on monitoring of gene expression patterns propose that various pathways in response to drought stress should exist in chickpea and their interplay can lead to differential stress tolerance.

Table 4.2: Detailed cluster information made by SOTA clustering of PUSABGD72 in comparison to ICCV2.

Annotation	Classification	Acc. No.	Log_2(ctrl)	Log_2 (3d)	Log_2 (6d)	Log_2 (12d)
Cluster 1						
Class 10 PR protein	Cell defense	FL512394	0.151	0.227	0.731	0.222
Put. Cold shock protein	Cell defense	FL512475	0.275	0.454	0.084	0.743
Chitinase family 19	Cell defense	CD051291	0.41	0.705	0.934	1.057
Vacuolor assembly protein	Cell transport	CD051281	0.176	0.287	0.299	0.736
Anion exchange protein	Cellular Organisation	FL518920	0.343	-0.134	0.469	1.316
Put 16KD mem protein	Cellular Organisation	FL518959	0.703	1.077	0.619	0.84
70KD HSP	Cellular Organisation	FL518962	0.546	0.696	0.736	1.064
Put tonoplast intrinsic protein	Cellular Organisation	FL518966	1.208	0.807	0.7	0.021
Fiber protein 1	Cellular Organisation	CD051339	0.642	0.485	0.124	1.005
Probable nitrate transporter	Cellular Organisation	FL512436	0.697	0.687	0.848	1.091
Put dehydrogenase E1 beta	Energy metabolism	FL518914	0.935	0.153	0.69	1.329
Epoxide hydrolase	Energy metabolism	FL518928	0.738	0	0.617	1.902
Cu amine oxidase	Energy metabolism	FL512335	0.526	0.766	0.766	0.74
NADH dehydrogenase subunit	Energy metabolism	CD051327	0.163	0.678	0.848	0.361
Nine cis epoxycarotenoid dioxygenase	Hormone biosynthesis	CD051315	0.678	0.084	0.642	0.743
Lipase	Metabolism	FL512358	0.172	0.07	0.189	0.722
Triacylglycerol lipase like protein	Metabolism	FL512391	1.079	0.084	0.163	0.626
α – amylase	Metabolism	FL512401	0.214	1.669	0.189	0.645
Dicarb/tricarboxy carrier	Metabolism	FL512403	0.204	0.251	0.705	1.736
Second sucrose synthase	Metabolism	FL518924	0.394	0.623	0.469	1.702
Put PS-I reaction	Metabolism	FL518955	0.214	0.151	0.972	1.238
α-amylase	Metabolism	FL518991	0.433	1.118	0.017	0.719
Chl a/b bp	Metabolism	FL512336	0.526	0.401	0.74	1.16
Polyubiquitin	Protein degradation	FL512379	0.176	1.131	0.536	0.264
Cys. Proteinase	Protein degradation	FL512381	0.184	1.245	0.506	1.201
Hexameric polyubiquitin	Protein degradation	FL512462	0.184	1.245	0.506	1.201
Phosphoglycerate kinase	Signal Transduction	FL512468	0.214	0.263	0.816	1.238
AP2 transcription factor	Transcription	FL512414	0.252	1.014	0.287	0.7
Put HAT	Transcription	FL518980	0.365	0.575	0.574	1.151
Translation initiation factor e/F-2	Translation	FL512390	0.214	1.084	0.696	1.134
Translation elongation factor	Translation	FL512421	0.163	0.678	0.848	0.361
Put ribosomal protein large subunit	Translation	FL518935	0.234	0.151	0.607	1.16
Put 40S ribosomal protein	Translation	FL518976	0.111	1.084	0.548	1.175
Ribosomal protein S3a	Translation	FL512334	0.379	0.516	0.536	0.623
Ribosomal protein	Translation	CD051267	0.009	0.136	0.367	0.84
Elongation factor	Translation	CD051300	0.642	1.014	0.287	0.7
Specific tissue protein	Unclassified	FL512343	0.172	0.124	0.189	0.722
LIN 1 protein	Unclassified	FL519002	0.697	0.687	0.848	1.091
Put phi-1 protein	Unclassified	FL519006	0.112	0.151	0.888	1.637
PDI like protein	Unclassified	CD051306	0.144	0.029	0.799	1.05
Phi-1	Unclassified	FL512435	0.41	0.705	0.934	1.057
Transposase of Tn10	Unclassified	FL512464	0.1	0.433	0.789	1.475
Cluster 2						
Polygalacturonase like protein	Cell defense	FL518998	0.621	1.239	-1.816	1.221
HSP	Cellular Organisation	FL518986	0.32	1.281	-0.86	1.243
Put SAP	Cellular Organisation	FL518999	1.15	1.124	-0.834	0.956
Put vacuolor ATP synthase	Energy metabolism	FL518930	0.734	-0.105	-0.667	0.642

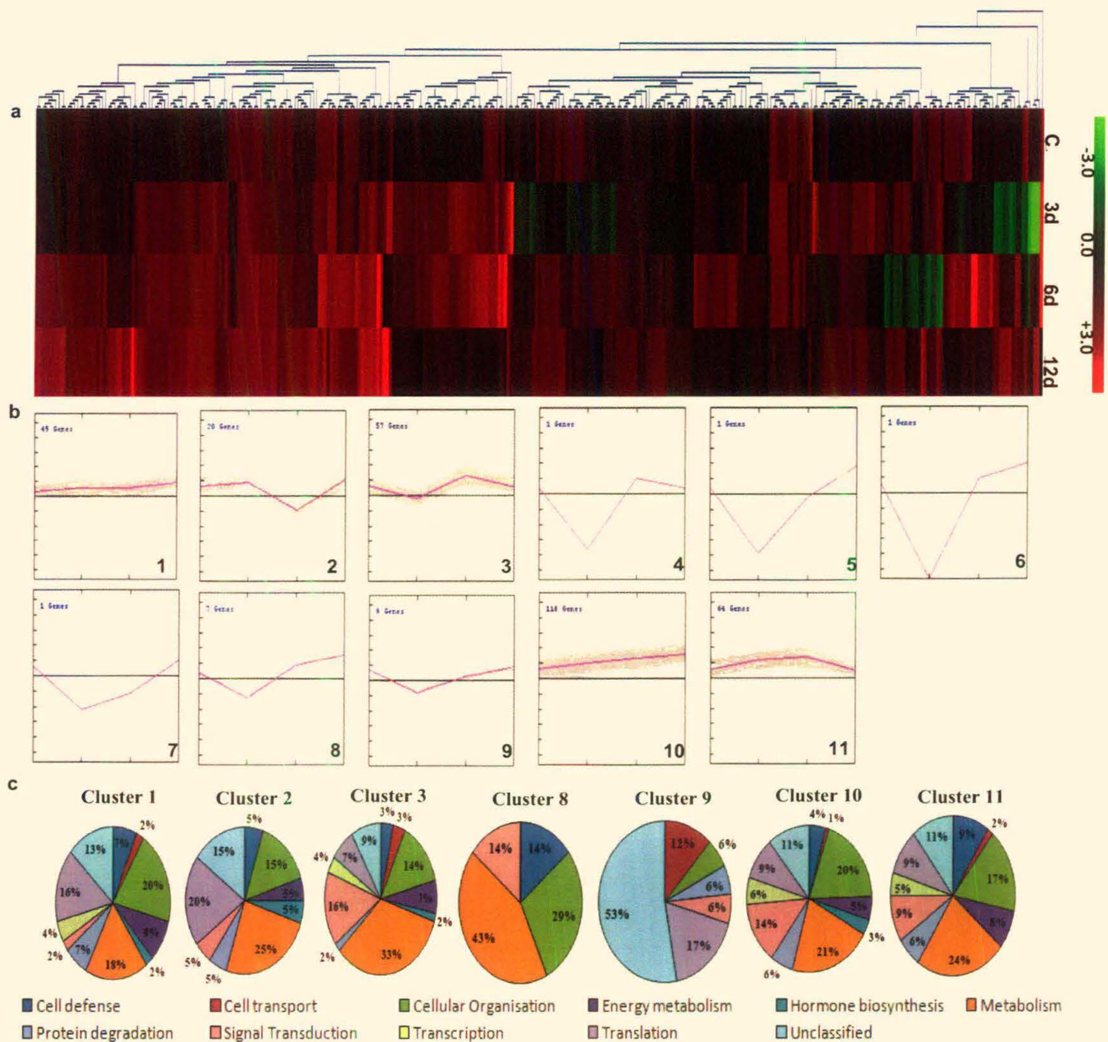


Figure 4.6. Hierarchical clustering analysis of unique genes based on their gene expression patterns in BGD72 in comparison to ICCV2. The 319 differentially expresed genes were clustered into 11 clusters based on their expression profiles. **(a)**, the SOTA clustering tree. **(b)**, expression profiles of SOTA clusters. The expression profile of each individual gene in the cluster is denoted by *grey line* and the mean expression profile is depicted by *pink line* for individual cluster. The number of genes in each cluster is given in the left upper corner and the cluster number is given in the right lower corner. **(c)**, functional characterization of genes of each cluster.

SAM synthetase	Hormone biosynthesis	FL518971	0.313	0.888	-0.865	0.486
Cyt P450	Metabolism	FL518968	1.265	0.098	-1.056	0.745
Asparate aminotransferase	Metabolism	FL518970	0.512	0.401	-0.591	1.223
Mannose – 6 – Phos isomerase	Metabolism	FL518974	1.012	0.934	-0.873	1.048
P-protein like protein	Metabolism	FL518978	0.575	1.007	-1.05	1.81
Rubisco activase (small isoform)	Metabolism	FL518985	1.146	1.566	-1.458	1.682
Senescence	Protein degradation	FL518988	1.084	0.832	-1.498	0.85
Integral membrane protein	Signal transduction	FL518989	0.379	1.251	-0.464	1.226
Elongation factor 2	Translation	FL512400	0.373	1.59	-0.044	0.887
Ribosomal protein L11	Translation	FL518964	0.816	0.748	-0.484	2.108
Put ribosomal protein	Translation	FL518973	0.163	1.014	-1.006	1.193
60s ribosomal protein L13a	Translation	FL518981	0.399	0.614	-1.175	1.042
Armadillo b catenin repeat	Unclassified	FL518965	0.306	1.214	-1.012	0.972
Predicted protein (cicer)	Unclassified	FL518969	0.179	1.208	-0.613	0.377
Hypo protein	Unclassified	FL518993	0.339	0.782	-1.204	0.771
Cluster3						
Put chloroplast FtsH proteinase	Cell defense	FL518918	0.605	0.292	1.55	1.07
Lipid transfer protein precursor	Cell defense	CD051321	0.034	-0.377	1.637	1.098
Aquaporin	Cell transport	FL512407	0.605	-0.234	1.888	0.509
Kinesin like protein	Cell transport	CD051276	0.151	-0.599	1.782	1.345
Put. Imbibition protein	Cellular Organisation	FL512365	1.079	-0.494	0.516	0.851
α-Tubulin	Cellular Organisation	FL512378	0.383	0.227	1.516	0.403
Salt tolerant protein	Cellular Organisation	FL512396	1.078	-0.599	1.782	0.627
Integral mem. Protein	Cellular Organisation	FL512408	0.251	0.151	2.342	0.315
Metallothionein	Cellular Organisation	FL512409	0.321	-0.377	1.832	0.465
SAP	Cellular Organisation	FL512411	0.895	-0.667	1.683	0.239
HSP	Cellular Organisation	FL518941	0.596	0.138	1.022	1.16
Superoxide dismutase	Energy metabolism	FL512362	1.104	0.31	1.214	0.18
Cationic peroxidase	Energy metabolism	FL512384	1.08	-0.494	1.084	0.695
Monooxygenase	Energy metabolism	FL512410	1.546	-0.234	1.604	0.241
Unsp. Monooxygenase	Energy metabolism	FL512427	0.649	-0.286	0.731	0.328
Lipoxygenase	Hormone biosynthesis	FL512369	0.189	-0.12	0.669	0.619
Allantoinase	Metabolism	FL512353	1.102	0.251	1.683	0.287
Asn synthetase	Metabolism	FL512372	1.105	-0.089	1.202	1.269
Chlorophyll. a/b bp	Metabolism	FL512373	0.411	-0.044	1.949	0.403
Rubisco	Metabolism	FL512377	0.697	-0.713	0.848	0.515
Sucrose synthase	Metabolism	FL512424	0.293	-0.415	0.895	0.777
1-L-myoinositol-1-P-synthetase	Metabolism	CD051303	0.282	0.07	1.269	0.283
Enolase, isoform 1	Metabolism	FL518916	0.835	-0.103	0.81	1.357
1-deoxy-o-xylulose-5-Phos reductoisomerase	Metabolism	FL518921	0.707	-0.411	1.158	1.218
Glyceraldehydes-3-Phos dehydrogenase	Metabolism	FL518927	0.322	0.322	1.112	0.446
α - 1,4-glucan phosphorylase	Metabolism	FL518943	0.605	0.111	2.815	0.64
P-protein	Metabolism	FL518944	1.009	0.287	1.144	1.096
Put apyrase	Metabolism	CD051304	0.832	0.014	1.084	1.214
Trans-cinnamate-4-monooxygenase	Metabolism	CD051342	0.3	-0.396	0.978	-0.004
Delta-8-sphingolipid desaturase	Metabolism	CD051350	0.282	0.07	1.269	0.283
Long chain fatty acid condensing enzyme	Metabolism	CD051352	0.122	-0.136	0.748	-0.226
Trehalose-6-phos 4H	Metabolism	CD051305	0.35	-0.044	0.824	0.438
UDP galactose-4-epimerase	Metabolism	FL512443	0.832	0.014	1.084	1.214
Fructose-1,6-bisphosphatase	Metabolism	FL512453	0.731	-0.136	0.807	0.745
Asparagines synthetase	Metabolism	FL512455	0.918	-0.201	0.799	0.661
Polyubiquitin	Protein degradation	FL512426	0.3	-0.396	0.978	-0.004
Ca bp	Signal Transduction	FL512355	0.309	-0.737	1.124	0.759
Stress related protein	Signal Transduction	FL512361	0.433	-0.761	1.144	0.873
Phosphoglycerate kinase	Signal Transduction	FL512374	1.41	-0.136	1.459	0.745
Protein kinase family(arabi)	Signal Transduction	FL512375	1.11	-0.201	0.799	0.661
Receptor like protein kinase	Signal Transduction	FL512376	0.41	-0.667	0.934	0.238
Put Zn bp	Signal Transduction	FL512395	1.145	-0.494	1.687	0.144
IAA-AA hydrolase	Signal Transduction	FL512418	1.134	-0.304	1.982	0.263
CBL-interacting protein kinase	Signal Transduction	FL512472	0.208	0.263	1.551	0.02
Omnipotent suppressor protein	Signal Transduction	FL519009	0.208	0.263	1.551	0.02
Transcription factor BTF3	Transcription	FL512406	0.135	0.202	1.275	0.224
Transcription factor Myb-1	Transcription	FL512419	0.034	-0.377	2.154	0.235
60S ribosomal protein L37a	Translation	FL512367	0.963	-0.434	1.007	0.34

Elongation factor 2	Translation	FL512382	0.556	-0.286	0.895	1.07
Ribosomal protein L18a	Translation	FL518931	0.275	-0.029	3.479	0.729
40S ribosomal protein S7 homolog	Translation	CD051338	0.293	-0.415	0.895	0.777
SOUL protein	Unclassified	FL518983	0.489	0.07	0.84	0.934
PPF-1 protein	Unclassified	CD051344	0.649	-0.286	0.731	0.328
Unknown protein (Arabi)	Unclassified	FL512371	1.081	-0.556	1.257	1.292
Cold induced alfalfa gene	Unclassified	FL512473	0.122	-0.136	0.748	-0.226
Put protein	Unclassified	CD051275	1.132	0.151	1.05	0.315
Cluster4						
40S ribosomal protein S25	Translation	FL518942	0.36	-3.644	1.021	0.363
Cluster5						
Ribosomal protein L3	Translation	FL518934	0.433	-3.837	-0.209	1.808
Cluster6						
Put ER lumen protein retaining receptor	Cellular Organisation	FL518946	0.625	-5.644	0.92	1.962
Cluster7						
Ceramidase family protein	Cellular Organisation	FL518915	0.605	-2.238	-1.162	1.01
Cluster8						
FtsH like protein PftF precursor	Cell defense	FL518951	0.1	-1.515	0.789	1.953
Prolyl-4-hydroxylase	Cellular Organisation	CD051295	0.275	-0.916	0.766	1.804
Nucleolar protein	Cellular Organisation	FL512465	0.614	-1.434	1.503	1.158
GDP mannose pyrophosphorylase	Metabolism	FL512412	0.287	-0.916	0.766	1.804
1-acyl-sn-glycerol-3-Phos acyltransferase	Metabolism	FL518917	0.374	-1.283	0.679	1.202
Pyruvate dehydrogenase E1 b	Metabolism	FL518950	0.44	-1.286	0.547	1.733
Photolyase	Signal transduction	FL518952	0.614	-1.434	1.503	1.158
Cluster9						
Basic blue Cu protein	Cell defense	FL512383	0.275	-0.971	0.651	0.714
HSP70	Cellular Organisation	FL512415	0.229	-0.578	0.322	0.708
Probable HSP	Cellular Organisation	FL518913	1.642	-0.941	-0.407	1.643
Vacuolor ATP synthetase	Energy metabolism	FL512392	0.724	-0.667	0.098	0.732
Phosphate translocator	Signal Transduction	FL512425	1.239	-1.322	0.124	1.005
Put. RT	Transcription	FL512380	0.16	-0.667	0.333	1.256
UV opsin	Unclassified	FL512363	1.079	-0.69	0.623	0.617
Putative protein	Unclassified	CD051298	0.281	-0.713	0.411	0.439
Albumin 2	Unclassified	FL512461	0.724	-0.667	0.098	0.732
Cluster10						
Lipid transfer protein	Cell defense	FL512385	0.212	1.043	1.379	1.228
Lipid transfer protein	Cell defense	FL512385	0.379	1.299	1.195	1.692
Class 10 PR protein	Cell defense	FL512394	1.024	1.379	0.622	1.958
MRP1	Cell defense	FL512449	0.163	1.064	1	1.134
Elicitor inducible gene	Cell defense	FL512456	0.484	0.642	1.104	1.39
Aquaporin like Water channel protein	Cell transport	FL512354	0.135	1.05	2.281	2.106
Dehydrin	Cellular Organisation	FL512347	0.715	1.316	1.091	1.085
Chalcone synthetase	Cellular Organisation	FL512402	-0.112	1	0.888	2.18
RNA bp	Cellular Organisation	FL518922	0.11	0.614	1.277	2.741
Salt tolerance protein 4	Cellular Organisation	FL518936	0.692	1.322	1.584	1.154
Cellulase synthase	Cellular Organisation	FL518949	1.235	0.731	0.85	1.683
Adenine ntd translocator	Cellular Organisation	FL518967	0.299	0.782	0.7	1.764
HSP	Cellular Organisation	FL518996	1.064	2.141	0.308	1.79
RNA bp	Cellular Organisation	FL512337	0.595	0.986	1.521	1.59
Metallothionein	Cellular Organisation	FL512338	0.978	1.151	1.683	1.77
Glycine rich protein	Cellular Organisation	FL512344	0.354	1.642	1.526	1.566
Imbibition protein	Cellular Organisation	FL519000	1.079	1.151	1.475	1.782
Seed maturation protein	Cellular Organisation	FL519001	0.411	0.526	1.516	1.731
Histone 2A	Cellular Organisation	CD051290	0.214	0.903	0.956	1.124
Dehydrin	Cellular Organisation	CD051297	1.322	1.22	1.428	1.828
LEA protein 2	Cellular Organisation	CD051326	1.106	1.379	1.58	1.642
LEA-1	Cellular Organisation	CD051271	1.157	1.104	1.275	1.674
Dehydrin1	Cellular Organisation	FL512434	0.669	1.057	1.556	1.669
Triose Phos translocator	Cellular Organisation	FL512441	0.74	0.864	1.029	1.516
Annexin	Cellular Organisation	FL512442	0.275	0.595	1.077	1.406
Probable K+ transporter	Cellular Organisation	FL512444	0.212	1.077	1.379	1.228
Aconitase family	Cellular Organisation	FL512448	0.356	1.384	0.84	1.281
Dehydration induced protein	Cellular Organisation	FL512471	0.74	1.77	1.996	2.266
Cu/Zn superoxide dismutase II	Energy metabolism	FL512366	0.428	1.293	1.618	1.634
Catalase fragment	Energy metabolism	FL518979	0.287	1.131	1.454	1.2

Unspecific monooxygenase	Energy metabolism	FL518994	0.744	2.101	0.379	3.569
P type H+ATPase	Energy metabolism	CD051280	1.47	1.59	1.872	1.58
Thioredoxin F type, chloroplast precursor	Energy metabolism	CD051307	0.516	1.077	1.356	1.48
Mitochondrial uncoupling protein	Energy metabolism	CD051283	0.993	0.888	1.585	1.124
Methionine adenosyl transferase	Hormone biosynthesis	FL518953	0.316	0.595	1.535	1.984
SAM	Hormone biosynthesis	CD051262	0.401	0.642	1.333	1.214
Lipoxygenase	Hormone biosynthesis	CD051273	0.642	1.245	0.748	1.201
Lipoxygenase	Hormone biosynthesis	CD051273	0.605	0.895	1.888	1.281
Rubisco activase	Metabolism	FL512364	0.275	0.971	0.856	1.314
Light harvesting protein	Metabolism	FL518912	0.623	1.146	1.742	2.144
AMP bp	Metabolism	FL518923	0.503	-0.013	1.299	1.968
Caffeic acid-o-methyltransferase	Metabolism	FL518929	0.27	0.189	0.806	2.121
Rubisco activase b form precursor	Metabolism	FL518947	0.163	0.098	1.327	1.997
Carbonic anhydrase	Metabolism	FL518948	0.231	1.485	1.847	2.104
Put C type cyt synthase	Metabolism	FL518957	0.498	1.138	0.859	1.809
Rubisco activase	Metabolism	FL518984	0.422	0.401	1.007	1.559
w-6-desaturase	Metabolism	FL518987	0.43	0.978	2.762	2.407
Hydroxyacyl glutathione hydrolase	Metabolism	FL518990	0.888	1.043	0.597	1.206
Glycolate oxidase	Metabolism	FL512340	1.163	0.978	1.189	1.58
Cysteine synthase	Metabolism	FL512341	0.212	1.077	1.35	1.22
Alcohol dehydrogenase	Metabolism	CD051265	1.079	0.949	1.157	1.339
β-amylase	Metabolism	CD051266	0.614	1.417	1.687	2.284
ADP glucose pyrophosphorylase	Metabolism	CD051279	0.963	1.05	1.287	1.345
Phosphoribosylanthranilate transferase like protein	Metabolism	CD051285	0.428	1	1.281	1.357
Cinnamoyl CoA reductase	Metabolism	CD051301	0.74	0.864	1.029	1.516
Trehalose-6-Phos synthase homolog	Metabolism	CD051305	0.212	1.077	1.379	1.228
Aldehyde dehydrogenase family	Metabolism	FL519005	0.163	1.064	1	1.134
Phosphoglucomutase	Metabolism	CD051347	0.373	1.411	1.575	1.585
Put β-amylase	Metabolism	FL519010	1.057	1.411	1.58	1.609
Put chloroplast targeted b amylase	Metabolism	FL512429	0.428	1	1.281	1.357
Lipase(class 3)family	Metabolism	FL512437	0.383	0.345	1.516	1.748
Put alkaline neutral invertase	Metabolism	FL512451	0.724	0.807	1.036	1.58
Thioprotease	Protein degradation	FL512333	0.888	1.157	1.59	1.57
Early leaf senescence abundant cysteine proteinase	Protein degradation	FL512339	1.22	1.05	2.281	2.106
Cysteine proteinase	Protein degradation	FL512342	0.411	2.316	1.47	1.531
Cysteine proteinase type protein	Protein degradation	CD051336	1.214	1.293	1.848	1.683
ATP dependent clp protease	Protein degradation	CD051341	1.131	1.144	1.868	1.195
Ubiquitin conjugating protein	Protein degradation	CD051293	0.918	1.163	1.511	1.651
Put ubiquitin	Protein degradation	FL512431	0.731	0.807	1.202	1.345
14-3-3 brain protein homolog	Signal Transduction	FL512351	1.011	0.401	2.151	1.16
Nodule enhanced sucrose synthase	Signal transduction	FL518926	1.9	1.144	0.547	1.09
α-NAC	Signal transduction	FL518992	0.484	1.31	0.048	2.777
Put nucleolar GTP bp	Signal Transduction	FL512332	0.516	1.202	1.263	1.379
WD repeat protein like protein	Signal Transduction	CD051264	1.235	0.993	1.144	1.275
Put protein kinase	Signal Transduction	CD051343	1.233	1.293	1.618	1.687
Nodule enhanced protein phosphatase	Signal Transduction	FL519004	0.356	1.384	0.84	1.281
G-protein coupled receptor like protein	Signal Transduction	CD051322	1.079	1.526	1.475	1.664
ADP ribosylation factor like protein	Signal Transduction	CD051324	0.724	0.705	1.036	1.58
Jasmonic acid 2	Signal Transduction	CD051357	1.111	1.669	1.687	1.811
Protein kinase	Signal Transduction	CD051317	1.134	0.993	1.687	1.189
SOS2 like protein	Signal Transduction	FL512440	0.642	1.245	1.422	1.722
Put mem protein	Signal Transduction	FL512450	1.079	1.526	1.475	1.664
High affinity Fe+2-Pb+2 permease	Signal Transduction	FL512457	0.722	0.731	0.85	1.683
Protein phosphatase 2C	Signal Transduction	CD051312	1.07	1.043	1.876	1.228
Glycerol kinase related	Signal Transduction	FL512459	0.299	0.333	1.293	1.824
Zn finger protein	Transcription	FL512348	0.926	1.091	1.57	1.421
RNA helicase	Transcription	CD051282	0.411	1.233	1.47	1.59
Put Zn finger protein	Transcription	CD051330	0.632	0.623	1.35	1.876
AP2 domain like protein	Transcription	CF074502	1.077	1.35	1.406	1.637
Transcriptional repressor of GlcNag	Transcription	FL519012	1.177	1.281	2.293	1.57
Zn finger protein	Transcription	FL512439	0.642	1.22	1.884	1.828
Dehydration responsive element bp3	Transcription	FL512463	0.731	1.281	1.299	1.733
DEAD box RNA helicase	Translation	FL512356	0.212	1.077	1.35	1.147

Translation initiation factor 5A	Translation	FL512389	0.356	1.384	0.84	1.179
Ribosomal protein L17	Translation	FL518938	0.332	0.526	0.767	1.972
40S ribosomal protein S9 like	Translation	FL518963	0.484	0.642	1.104	1.39
40S ribosomal protein;P6-1	Translation	FL518982	0.662	0.941	0.952	1.535
40S ribosomal protein S13	Translation	FL512445	0.516	1.077	1.356	1.48
60S ribosomal protein L27A	Translation	FL512452	0.202	1.379	1.58	1.642
60S ribosomal protein L37A	Translation	CD051286	0.623	0.748	1.257	1.292
Eukaryotic translation initiation factor 5A-1	Translation	CD051287	0.731	0.536	1.202	1.345
40S ribosomal protein S11	Translation	CD051346	0.506	0.986	1.214	1.47
Ribosomal protein S15	Translation	CD051284	0.895	0.705	1.144	1.31
PB-1 domain containing protein	Unclassified	FL518925	1.115	0.705	0.612	1.41
Callus protein P23	Unclassified	FL518939	0.625	0.696	1.899	1.569
Put KH domain protein	Unclassified	FL518977	0.394	1.384	1.212	1.166
Put. Protein (Arabi.)	Unclassified	FL512370	0.428	1	1.501	1.357
Put. Leunig/B-120	Unclassified	FL512477	1.177	1.281	2.293	1.57
Phi – 1 like protein	Unclassified	CD051263	0.722	0.31	1.214	1.411
Cold induced alfalfa gene	Unclassified	CD051294	0.383	0.345	1.516	1.748
Put protein	Unclassified	FL519003	1.145	0.595	1.077	1.406
Hyp protein	Unclassified	CD051269	0.244	1.22	1.157	1.417
Profiling	Unclassified	FL512430	0.623	0.748	1.257	1.292
VTC2	Unclassified	FL512432	0.411	0.526	1.516	1.731
Put cullin	Unclassified	FL512458	0.379	1.299	1.195	1.692
Zwille protein	Unclassified	FL512467	0.316	0.595	1.535	1.984
Cluster11						
MRP like ABC transporter	Cell defense	FL512349	1.147	1.057	2.242	0.465
Disease resistance response protein	Cell defense	FL512398	1.07	1.144	1.918	0.299
MRP like ABC transporter	Cell defense	FL518997	1.049	1.189	2.371	0.862
Polygalacturonase inhibiting protein	Cell defense	CD051270	0.993	1.521	1.48	0.632
Non specific lipid transfer	Cell defense	FL512469	1.038	1.74	1.899	0.352
Kinesin like protein	Cell transport	CD051276	0.275	1.47	1.832	0.465
Proline rich protein	Cellular Organisation	FL512352	0.197	1.475	1.856	0.643
Put. RNA bp	Cellular Organisation	FL512359	0.354	1.642	1.575	0.328
Seed protein	Cellular Organisation	FL512360	0.507	0.642	1.333	0.225
RNA bp cp29 protein	Cellular Organisation	FL512387	0.261	1.111	1.531	0.37
Put. Proline rich protein APG	Cellular Organisation	FL512405	0.244	1.761	0.585	0.31
Importin	Cellular Organisation	FL512417	1.227	1.098	1.287	0.154
Ripening induced protein	Cellular Organisation	FL512428	0.197	1.163	0.766	0.33
α - tubuline	Cellular Organisation	FL518958	0.463	1.214	0.919	0.358
HVA22 homolog	Cellular Organisation	CD051272	0.209	1.111	1.491	0.521
Nitrate transporter	Cellular Organisation	FL512438	0.993	1.521	1.48	0.632
Endomembrane protein 70	Cellular Organisation	FL512454	0.623	0.714	1.036	0.755
Cyt. P450monooxygenase	Energy metabolism	FL518911	0.918	1.163	0.888	0.188
Cu/Zn superoxide dismutase II	Energy metabolism	FL512366	0.373	0.872	0.809	0.314
Cyt. P450monooxygenase	Energy metabolism	FL518911	0.963	0.696	1.499	0.684
Cyt P450 dependent subunit	Energy metabolism	CD051325	0.222	1.245	2.098	0.308
Put-3-isopropylmalate dehydrogenase	Energy metabolism	FL512446	0.261	1.111	1.531	0.37
Fructose biphos. Aldose	Metabolism	FL512350	0.31	1.05	0.971	-0.076
Chloroplast translocon	Metabolism	FL512420	0.222	1.245	2.098	0.308
β – cobalamine synthase	Metabolism	FL512422	0.19	0.575	1.091	0.457
Met. synthase	Metabolism	FL512423	0.286	1.546	1.157	0.649
Leuco-anthocynidine dioxygenase	Metabolism	CD051278	0.209	1.111	1.491	0.521
Ketol acid reductoisomerase	Metabolism	FL518933	0.197	1.696	1.146	0.604
Methionin synthase	Metabolism	FL518940	0.643	1.07	2.535	0.437
Nucleotide sugar epimerase like protein	Metabolism	FL518945	1.007	1.585	1.331	0.467
Put amino peptidase	Metabolism	FL518960	0.394	1.47	1.542	0.63
Malate dehydrogenase	Metabolism	FL518972	1.276	0.848	0.68	0.731
Rubisco	Metabolism	FL518995	0.165	3.136	1.163	0.677
Rubisco activase(small isoform)	Metabolism	CD051311	0.35	1.021	0.848	0.401
Phophonopyruvate decarboxylase like protein	Metabolism	FL519008	0.19	0.575	1.091	0.457
Raffinose synthase	Metabolism	FL512447	0.35	1.021	0.848	0.401
Glycosyl hydrolase family 14	Metabolism	FL512460	0.322	1.007	1.124	0.476
Prolyl-peptidyl isomerase	Protein degradation	FL512388	0.35	1.021	0.848	0.27
Monoubiquitin	Protein degradation	FL518932	0.219	1.757	1.295	0.46

Cysteine proteinase type protein	Protein degradation	CD051336	0.399	1.202	1.322	1.042
Ubiquitin homolog	Protein degradation	FL512466	0.354	1.642	1.575	0.328
ADP ribosylation factor like protein	Signal Transduction	FL512368	0.322	1.007	1.124	0.476
Put WD40 repeat protein	Signal Transduction	FL512386	1.271	1.077	1.356	0.743
Put ABA responsive protein	Signal Transduction	FL512397	1.214	1.104	1.848	0.281
Membrane protein/B-12	Signal Transduction	FL512474	0.114	0.757	1.138	0.429
Protein kinase family protein	Signal transduction	FL512375	1.346	0.614	1.036	0.755
Nodule enhanced phophorus protein	Signal Transduction	FL519011	0.111	0.546	1.428	0.396
RAP2.6	Transcription	CD051355	0.261	1.111	1.531	0.37
Put AP2 domain transcriptional regulator	Transcription	FL519007	1.227	1.651	1.782	1.131
Aspartyl-tRNA synthetase	Transcription	FL512433	1.41	1.541	1.459	0.745
40s ribosomal protein 57 homolog	Translation	FL512399	0.506	1.428	0.856	0.812
Elongation factor 1 alpha	Translation	FL518919	1.644	2.325	4.373	0.333
60S ribosomal protein L3	Translation	FL518937	0.402	1	1.16	0.017
Ribosome associated protein p40	Translation	FL518954	0.07	1.74	2.95	0.352
Ribosomal protein S14	Translation	FL518961	0.283	1.111	1.194	0.729
Ribosomal protein S2	Translation	CD051333	0.286	1.546	1.157	0.649
Unknown protein	Unclassified	FL518975	0.942	1.333	2.121	0.377
PPF-1 protein	Unclassified	CD051334	0.197	1.163	0.766	0.33
Leu rich recepter like protein	Unclassified	FL512357	0.009	2.316	0.714	0.403
Put CCR-4 associated factor	Unclassified	FL512393	1.106	1.379	0.799	0.649
Zwille protein shoot meristem	Unclassified	FL512476	0.111	0.546	1.428	0.396
Serine rich protein	Unclassified	CD051340	0.24	1.111	0.903	0.202
Drm3	Unclassified	CD051331	0.275	0.774	1.687	0.144

4.2.5 Monitoring expression profiles of high expressing genes

The comparative expression profiles under drought also suggested a different stress-specific gene expression programming in PUSABGD72 in comparison to that in ICCV2. So, in order to delineate the differential basal as well as induced tolerance of PUSABGD72 over ICCV2, we took an attempt to identify the potential candidate genes, which might have caused the differential stress-responses of the contrasting cultivars, on the basis of their expression patterns. As it is mentioned earlier that one of the mechanisms for improved stress-tolerance might be inherent constitutive expression of some genes responsible for tolerance. For this purpose, a comparison between the expression patterns of those genes in two cultivars were carried out which were showing ≥2 fold relative expression at basal level *i.e.* at unstressed condition. A comparative transcript profiling revealed that approximately 23% (77 genes) genes were having ≥2 fold basal expression in PUSABGD72 and about 84% of them showed more expression during the course of stress, which shows that most of the genes in this category are drought-responsive. The genes showing ≥2 fold relative expression at the unstressed condition and ≥3 fold relative expression in PUSABGD72 in comparison to ICCV2 at any point of stress were chosen. This stringent parameter identified 49 genes out of 77 described above (Table 4.3).

Table 4.3: Transcripts expression of selected 53 genes in PUSABGD72 seedlings in response to drought stress at different time points with the fold-expression values.

EST Accession No.	E-value	Putative fuction	C	SD(±)	3d	SD(±)	6d	SD(±)	12d	SD(±)
Cell Defense										
FL512398	1E-43	Disease resistance response protein	2.10	0.09	2.21	0.14	3.78	0.16	1.23	0.10
FL512394	1E-23	Class 10 PR protein	2.03	0.20	2.60	0.27	1.54	0.16	3.88	0.23
FL512357	4.E-05	Leu rich recepter like protein	1.01	0.22	4.98	0.19	1.64	0.13	1.32	0.15
Cell transport										
FL512354	4E-51	Aquaporin like Water channel protein	2.10	0.20	2.07	0.11	0.12	0.12	4.30	0.18
FL512349	8E-77	MRP like ABC transporter	2.21	0.07	2.08	0.15	4.73	0.22	1.38	0.11
FL518997	7E-43	MRP like ABC transporter	2.07	0.21	2.28	0.14	5.17	0.04	1.82	0.16
Cellular Organisation										
FL512450	1E-20	Put mem protein	2.11	0.12	2.88	0.14	2.78	0.14	3.17	0.14
FL512469	9E-14	Non specific lipid transfer	2.05	0.09	3.34	0.14	3.73	0.11	1.28	0.06
FL512352	3E-15	Proline rich protein	2.15	0.27	2.78	0.10	3.62	0.18	1.56	0.29
FL512405	4E-79	Put. Proline rich protein	2.18	0.01	3.39	0.13	1.50	0.14	1.24	0.01
FL518949	1E-108	Cellulase synthase	2.35	0.34	1.66	0.16	1.80	0.14	3.21	0.17
FL518996	6E-77	HSP 70 cognate	2.09	0.08	4.41	0.10	1.24	0.14	3.46	0.21
FL519000	2E-38	Imbibition protein	2.11	0.22	2.22	0.21	2.78	0.17	3.44	0.15
CD051297	3E-25	Dehydrin	2.50	0.29	2.33	0.15	2.69	0.22	3.55	0.20
CD051326	6E-37	LEA protein 2	2.15	0.15	2.60	0.14	2.99	0.04	3.12	0.13
CD051271	6E-16	LEA-1	2.23	0.05	2.15	0.13	2.42	0.16	3.19	0.18
Energy metabolism										
FL512338	2E-26	Metallothionein	2.04	0.17	2.22	0.13	3.21	0.18	3.41	0.14
FL512366	8E-62	Cu/Zn superoxide dismutase II	2.15	0.17	2.45	0.19	3.07	0.14	3.10	0.16
CD051280	1E-11	P type H^+ATPase	2.77	0.27	3.01	0.07	3.66	0.14	2.99	0.10
Metabolism										
FL512353	4E-31	Allantoinase	2.15	0.10	1.19	0.11	3.21	0.03	1.22	0.11
FL518945	2E-92	Nucleotide sugar epimerase like protein	2.01	0.12	3.00	0.18	2.52	0.12	1.38	0.12
CD051266	1E-89	β-amylase	2.00	0.23	2.67	0.08	3.22	0.13	4.87	0.02
FL519010	2E-90	Put β-amylase	2.08	0.24	2.66	0.13	2.99	0.29	3.05	0.16
FL518926	4E-32	Nodule enhanced sucrose synthase	3.73	0.41	2.21	0.09	1.46	0.23	2.13	0.15
Protein degradation										
FL512339	1E-72	Early leaf senescence abundant cysteine proteinase	2.33	0.02	2.07	0.16	4.86	0.22	4.30	0.27
CD051336	1E-52	Cysteine proteinase type protein	2.32	0.13	2.45	0.16	3.60	0.06	3.21	0.08
CD051341	8E-73	ATP dependent clp protease	2.19	0.07	2.21	0.19	3.65	0.14	2.29	0.06
CD051293	9E-56	Ubiquitin conjugating protein	2.19	0.06	2.24	0.17	2.85	0.14	3.14	0.28
Signal Transduction										
FL512351	3E-56	14-3-3 brain protein homolog	2.02	0.06	1.32	0.24	4.44	0.21	2.24	0.07
CD051343	3E-34	Put protein kinase	2.35	0.02	2.45	0.03	3.07	0.07	3.22	0.23
CD051322	5E-63	G-protein coupled receptor like protein	2.11	0.15	2.88	0.18	2.78	0.06	3.17	0.06
CD051357	2E-19	Jasmonic acid 2	2.16	0.02	3.18	0.22	3.22	0.15	3.51	0.06
CD051317	8.E-05	Protein kinase (CIPK25)	2.19	0.06	1.99	0.14	3.22	0.16	2.28	0.19
FL512440	1E-43	SOS2 like protein (CIPK6)	2.19	0.34	2.37	0.18	2.68	0.26	3.30	0.16
CD051312	5E-48	Protein phosphatase 2C	2.10	0.19	2.06	0.19	3.67	0.14	2.34	0.15
FL512472	6E-23	CBL-interacting protein kinase	1.16	0.15	1.20	0.14	2.93	0.03	1.01	0.14
Transcription										
CF074502	4E-17	AP2 domain like protein	2.11	0.07	2.55	0.09	2.65	0.11	3.11	0.07
FL519007	9.E-09	Put AP2 domain transcriptional regulator	2.34	0.04	3.14	0.23	3.44	0.17	2.19	0.07
FL519012	2E-46	Transcriptional repressor of GlcNag	2.26	0.02	2.43	0.12	4.90	0.12	2.97	0.07
FL512439	5E-16	Zn finger protein	2.19	0.21	2.33	0.13	3.69	0.12	3.55	0.13
FL512463	2E-19	Dehydration responsive	2.14	0.23	2.43	0.13	2.46	0.51	3.32	0.18

		element bp3								
FL518992	2.E-09	α-NAC	2.14	0.26	2.48	0.19	1.03	0.20	6.86	0.17
FL512359	5E-23	Put. RNA bp	2.28	0.04	3.12	0.16	2.98	0.18	1.26	0.08
Translation										
FL518919	1E-52	Elongation factor 1 alpha	3.13	0.24	5.01	0.16	20.72	0.18	1.26	0.17
FL512452	6E-19	60S ribosomal protein L27A	2.15	0.23	2.60	0.21	2.99	0.18	3.12	0.15
FL518931	2E-34	Ribosomal protein L18a	1.21	0.13	0.98	0.12	11.15	0.18	1.66	0.15
FL518954	4E-83	Ribosome associated protein p40	1.05	0.17	3.34	0.16	7.73	0.21	1.28	0.18
Unclassified										
FL512477	4E-17	Put. Leunig	2.26	0.25	2.43	0.03	4.90	0.17	2.97	0.25
FL512471	6E-28	Dehydration induced protein	2.67	0.05	3.41	0.23	3.99	0.13	4.81	0.23
FL512397	1E-16	Put ABA responsive protein	2.32	0.05	2.15	0.29	3.60	0.09	1.22	0.06
FL512396	1E-42	Salt tolerant protein	2.11	0.03	1.66	0.12	3.44	0.10	1.54	0.03
FL518936	4E-17	Salt tolerance protein 4	2.03	0.29	2.50	0.14	3.00	0.14	2.23	0.18
FL512411	2E-40	SAP	2.12	0.10	1.63	0.20	3.21	0.18	1.18	0.11

Some ESTs that expressed at equivalent level in both the cultivars under control condition, however, highly induced in PUSABGD72 within 3d in response to stress were also chosen for study. Other than the ESTs mentioned above, four other ESTs were identified in this group. One of them is a CBL-interacting protein kinase (FL512472), two represent ribosomal proteins (FL518931, FL518954) and one is a leucine rich repeat protein (FL512357). Absolute expression of all these ESTs in two contrasting cultivars were compared and presented in Figure 4.7. According to the expression in the tolerant cultivar PUSABGD72 these 53 ESTs can be clustered in four groups (Table 4.4). The mean curves of cluster 1 and 4 showed a steady increase of gene expression from unstressed condition to the end of stress treatment, although, there is a basic difference between these two clusters. Average expression intensity of the cluster 4 genes is much higher than that of cluster 1 and there is a uniformity of expression of these genes. Two genes (FL512394 and FL518992) of cluster 1 displayed a rapid induction at 3d; but at 6d time point their expression went down lower than their basal expression, however, induced again at 12d. We have repeatedly checked their expression to avoid any error. 44 out of 53 high expressing genes belong to these two clusters. Interestingly, most of the ESTs representing signal transduction exhibited a steady increase in stress condition from their basal level. Only two of them, encoding a protein phosphates 2C and a CBL-interacting protein kinase showed sudden high expression at 6d and then reduced at 12d. Similar expression pattern of both these ESTs supports their mutual cooperation in stress signaling. Five genes of cluster 3 that showed sudden high expression at 6d condition mostly represent proteins of unknown function. Another EST of this group encodes a putative RNA binding protein and expressed suddenly 20 fold high at 6d in BGD72. Expression of this EST in ICCV2 also followed a similar pattern, but with a much lower

absolute value. Cluster 2 genes, which showed rapid high fold of expression within 3d and maintained that up to 6d need special attention. All the four genes of this group represent protein metabolism. Three of them code for protein synthesis (elongation factor, ribosomal proteins), and expressed more than 15 fold early in stress. The other represents protein degradation and are expressed about 4-fold higher than its basal expression. Comparatively, fold expression of these genes in the sensitive cultivar ICCV2 at this time period is much lower. Interestingly, two ESTs representing elongation factor 1 alpha and ribosomal protein L18a also showed early induction in ICCV2, but their absolute values of abundance are much lower than those in PUSABGD72. Further, their expression levels are not maintained throughout the stress.

Table 4.4: Detailed 53 genes-cluster information made by SOTA clustering of PUSABGD72.

Annotation	Classification	Acc. No.	Log_2(ctrl)	Log_2 (3d)	Log_2 (6d)	Log_2 (12d)
Cluster 1						
Class 10 PR protein	Cell Defence	FL512394	0.804	2.304	0.114	1.792
MRP like ABC transporter	Cell Transport	FL512349	0.716	2.100	1.670	2.080
MRP like ABC transporter	Cell Transport	FL518997	1.102	2.261	0.901	2.487
Put mem protein	Cellular Organization	FL512450	0.363	1.179	1.838	2.028
Non specific lipid transfer	Cellular Organization	FL512469	0.354	1.445	1.771	0.706
Cellulase synthase	Cellular Organization	FL518949	0.383	0.184	0.706	1.854
HSP 70 cognate	Cellular Organization	FL518996	1.145	2.414	0.142	2.780
Metellothionein	Cellular Organization	FL512338	0.729	0.705	2.220	2.816
LEA-1	Cellular Organization	CD051271	0.724	1.366	2.035	2.434
Cu/Zn superoxide dismutase II	Energy metabolism	FL512366	1.132	1.443	2.222	3.108
P type H^+ATPase	Energy metabolism	CD051280	0.770	1.874	1.251	3.241
Allantoinase	Metabolism	FL512353	0.282	0.536	1.773	2.369
Ubiquitin conjugating protein	Protein Degradation	CD051293	1.111	1.596	1.141	2.392
Put protein kinase	Signal Transduction	CD051343	0.903	1.213	1.993	2.878
Jasmonic acid 2	Signal Transduction	CD051357	0.356	1.275	1.826	2.451
Protein kinase	Signal Transduction	CD051317	0.352	0.974	1.609	2.171
Protein phosphatase 2C	Signal Transduction	CD051312	0.460	1.503	2.684	1.688
CBL-interacting protein kinase	Signal Transduction	FL512472	0.516	0.245	2.573	2.072
AP2 domain like protein	Transcription	CF074502	0.385	1.257	1.738	2.247
Put AP2 domain transcriptional regulator	Transcription	FL519007	0.310	1.059	1.667	2.499
Transcriptional repressor of GlcNag	Transcription	FL519012	0.307	0.484	1.823	2.432
alpha-NAC	Transcription	FL518992	0.623	2.189	0.188	2.273
60S ribosomal protein L27A	Translation	FL512452	0.310	1.059	1.303	1.506
Put. Leunig	Unclassified	FL512477	0.707	0.883	2.223	2.831
Put. Proline rich protein	Unclassified	FL512405	0.427	1.766	2.068	2.340
Salt tolerance protein 4	Unclassified	FL518936	0.410	1.460	1.766	1.781
Cluster 2						
Cysteine proteinase type protein	Protein Degradation	CD051336	1.281	2.637	2.802	1.713
Elongation factor 1 alpha	Translation	FL518919	1.072	5.755	4.492	2.814
Ribosomal protein L18a	Translation	FL518931	0.642	3.659	3.381	2.602
Ribosome associated protein p40	Translation	FL518954	0.714	2.527	3.385	2.013
Cluster 3						
Nodule enhanced sucrose synthase	Metabolism	FL518926	0.459	1.311	3.171	1.874
Put. RNA bp	Transcription	FL512359	0.300	0.514	4.650	2.227
Put ABA responsive protein	Unclassified	FL512397	0.414	1.622	3.188	0.846
Proline rich protein	Unclassified	FL512352	0.900	2.070	4.511	2.667
Salt tolerant protein	Unclassified	FL512396	0.834	1.897	3.106	2.449

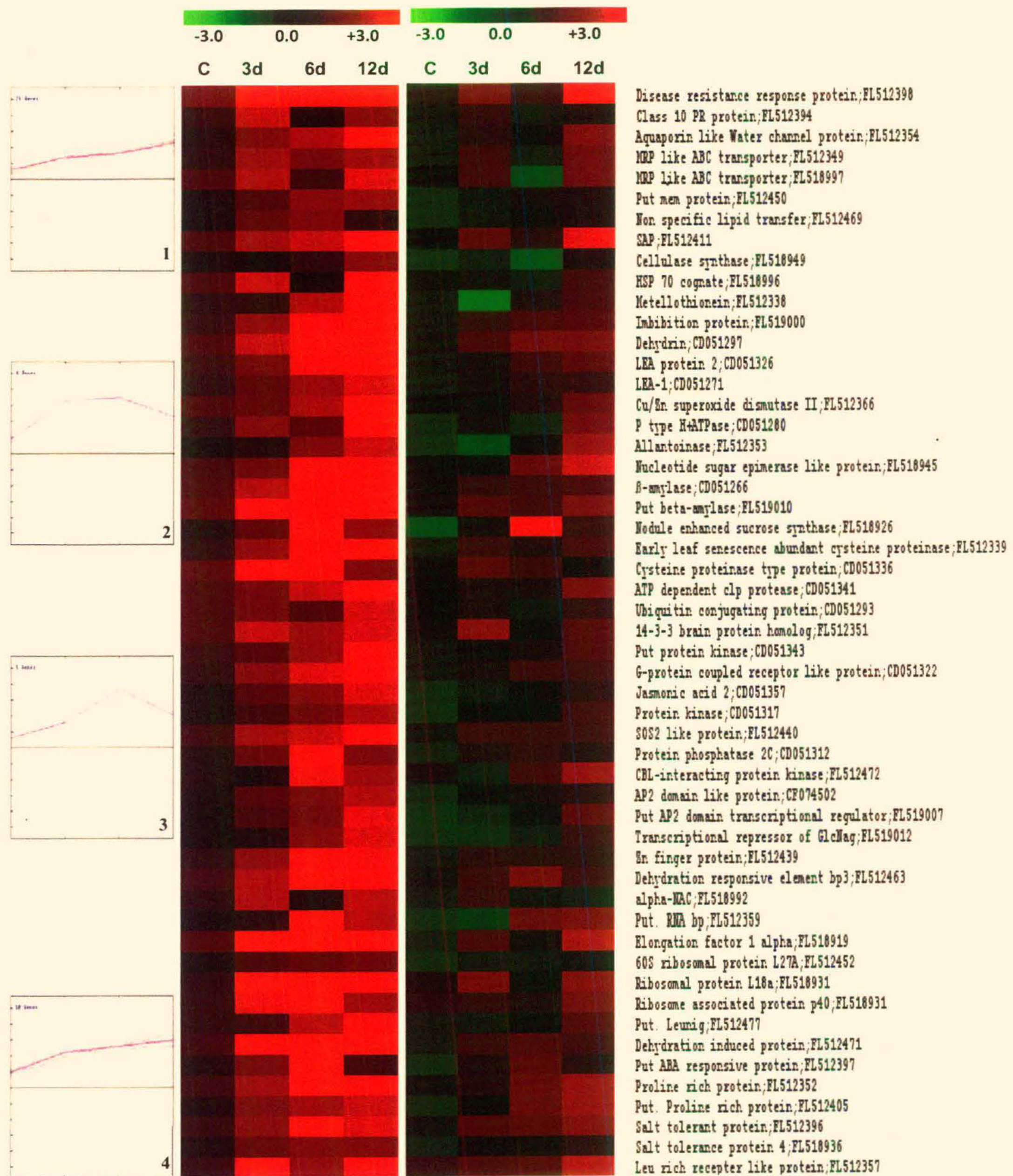


Figure 4.7. Hierarchical clustering analysis of selected genes based on their gene expression patterns (text). The 53 differentially expressed genes were grouped into four clusters based on their expression profiles. **(a),** expression profiles of SOTA clusters. The expression profile of each individual gene in the cluster is denoted by *grey line* and the mean expression profile is depicted by *pink line* for individual cluster. The number of genes in each cluster is given in the left upper corner and the cluster number is given in the right lower corner. **(b),** the comparative expression profiles of selected genes in PUSABGD72 and ICCV2.

Cluster 4						
Disease resistance response protein	Cell Defence	FL512398	1.535	2.792	2.737	3.364
Aquaporin like Water channel protein	Cell Transport	FL512354	0.609	1.798	2.169	3.954
SAP	Cellular Organization	FL512411	1.105	2.175	2.355	2.809
Imbibition protein	Cellular Organization	FL519000	0.847	1.984	2.627	2.991
LEA protein 2	Cellular Organization	CD051326	0.873	1.630	2.560	2.954
Nucleotide sugar epimerase like protein	Metabolism	FL518945	1.050	1.571	2.894	2.873
β-amylase	Metabolism	CD051266	1.111	2.242	2.617	3.365
Put b amylase	Metabolism	FL519010	1.257	2.668	2.590	3.368
Early leaf senescence abundant cysteine proteinase	Protein Degradation	FL512339	0.761	1.950	2.742	3.128
ATP dependent clp protease	Protein Degradation	CD051341	1.111	1.586	2.313	2.940
14-3-3 brain protein homolog	Signal Transduction	FL512351	1.136	2.331	2.078	2.418
G-protein coupled receptor like protein	Signal Transduction	CD051322	0.766	1.891	2.462	2.591
SOS2 like protein	Signal Transduction	FL512440	0.911	2.156	2.333	2.633
Zn finger protein	Transcription	FL512439	0.718	1.938	2.601	2.545
Dehydration responsive element bp3	Transcription	FL512463	0.911	2.192	3.071	2.644
Dehydration induced protein	Unclassified	FL512471	0.925	2.694	3.209	3.252
Leu rich recepter like protein	Unclassified	FL512357	0.824	3.479	1.964	2.657

4.2.6 Macroarray data validation by northern analysis

To validate the results obtained by reverse northern analysis, ten ESTs (FL512354, FL512338, FL512352, CD051280, FL512397, CD051326, CD051266, FL512439, FL512463 and FL518919) were selected for RNA blot and their expressions in both the cultivars were monitored (Figure 4.8). In general, the results of RNA gel blot analysis were consistent with the expression data obtained by reverse-northern analysis. The difference in gene expression is not only related to the stress type and conditions but may also vary within the species and the degree of tolerance of the plant. The differential expression pattern of the ESTs observed might be applicable to the two particular cultivars used in this study. But the genes identified on the basis of differential expression patterns strongly corroborate with similar studies in other plants (Kathiresana et al., 2006) and, therefore, appears to be applicable beyond this species.

4.2.7 Working pathway for drought tolerance mechanism

Based on the comparative gene expression analyses in this study and with the support of the published literature on transgenic expression of different genes leading to stress tolerance in other system we have outlined a working pathway for drought tolerance mechanism (Figure 4.9) in chickpea seedlings and proposed some candidate genes (Figure 4.9 bold letter) that might lead to drought tolerance in overexpressing transgenic system.

4.3 Discussion

Plants perceive and respond to stress. Upon perception of the stress, the signal is communicated to the downstream components ultimately leading to up regulation and down regulation of gene expression that lead to synthesis and repression of proteins required for the initial damage-repair and physiological re-programming for better adaptation. Physiological parameters studied under drought stress conditions indicated that PUSABGD72 is more tolerant compared to ICCV2. It is likely that there might be specific constitutive stress tolerant characters, which are inherent in tolerant species. Alternatively, tolerant species might have more efficient and quicker mechanisms for stress perception and enhanced expression of stress responsive genes, which maintain cellular survival and recovery. It is also possible that at least some of the stress responsive genes expressed in tolerant species are novel, and may have greater adaptive significance. Thus, identification of stress-responsive genes and their expression kinetics in the tolerant crop will be of interest.

A comparative expression patterns of those genes in two cultivars were carried out which were showing ≥2 fold relative expression at basal level *i.e.* at unstressed condition. Approximately 23% (77 genes) genes were having ≥2 fold basal expression in PUSABGD72 and about 84% of them showed more expression during the course of stress, which shows that most of the genes in this category are drought-responsive. Out of these 77 genes, 49 genes showed a striking ≥2 fold relative expression at the unstressed condition and ≥3 fold relative expression in PUSABGD72 in comparison to ICCV2 at any point of stress. Eight of them belong to signal transduction category e.g. CBLinteracting protein kinase (FL512440), putative protein kinases (CD051343, CD051317), protein phosphatase 2C (CD051312), G-protein coupled receptor (CD051322), 14-3-3 protein homolog (FL512351). Implication of SOS2-like protein kinases in providing abiotic stress tolerance by activating the membrane-bound transporters is well documented (Gong et al., 2001; Cheong et al., 2002; Qiu et al., 2004; Batelli et al., 2007). Protein phosphatase 2C is shown to interact with SOS2 and mediated ABA-responsive signals (Gosti et al., 1999). Seven genes of transcription factor category mostly represent AP2-domain proteins having the potential to mediate distinct responses to abiotic stresses such as drought, salt and cold (Yamaguchi-Shinozaki and Shinozaki, 1994; Liu et al., 1998). Another gene in this group putatively encodes α-NAC transcription factor. Overexpression of NAC transcription factor family protein in *Arabidopsis* revealed that several stress-inducible genes were upregulated in the

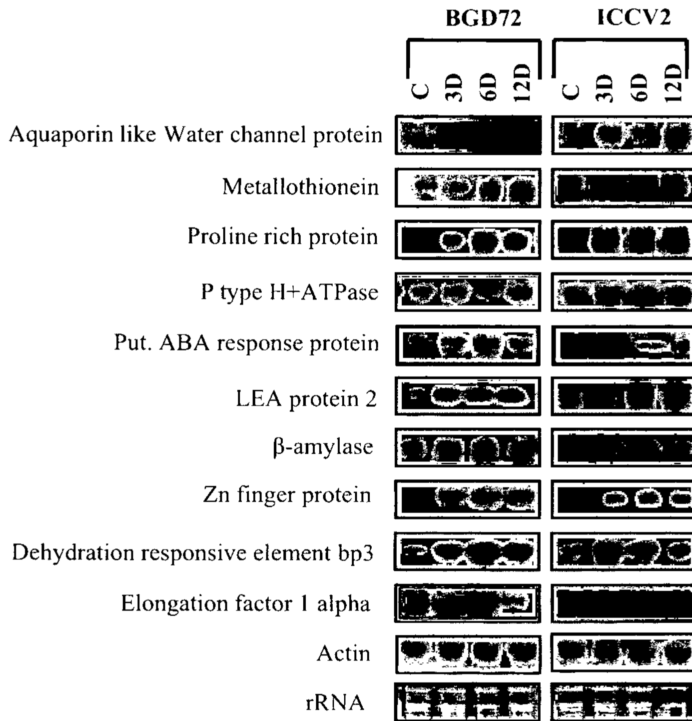


Figure 4.8. Northern analysis showing induced expression of ten selected stress responsive genes :(FL512354, FL512338, FL512352, CD051280, FL512397, CD051326, CD051266, FL512439, FL512463 and FL518919) in BGD72 and ICCV2. 20μg of total RNA isolated from control/stressed seedlings of BGD72/ICCV2 were separated on formaldehyde denaturing gel and transferred to nylon membrane and probed with α32P-dCTP labeled cDNAs corresponding to indicated EST clones. A PCR product of chickpea actin cDNA was used as an internal control and 25S ribosomal RNA was shown as loading control. Time points in days (d) are indicated.

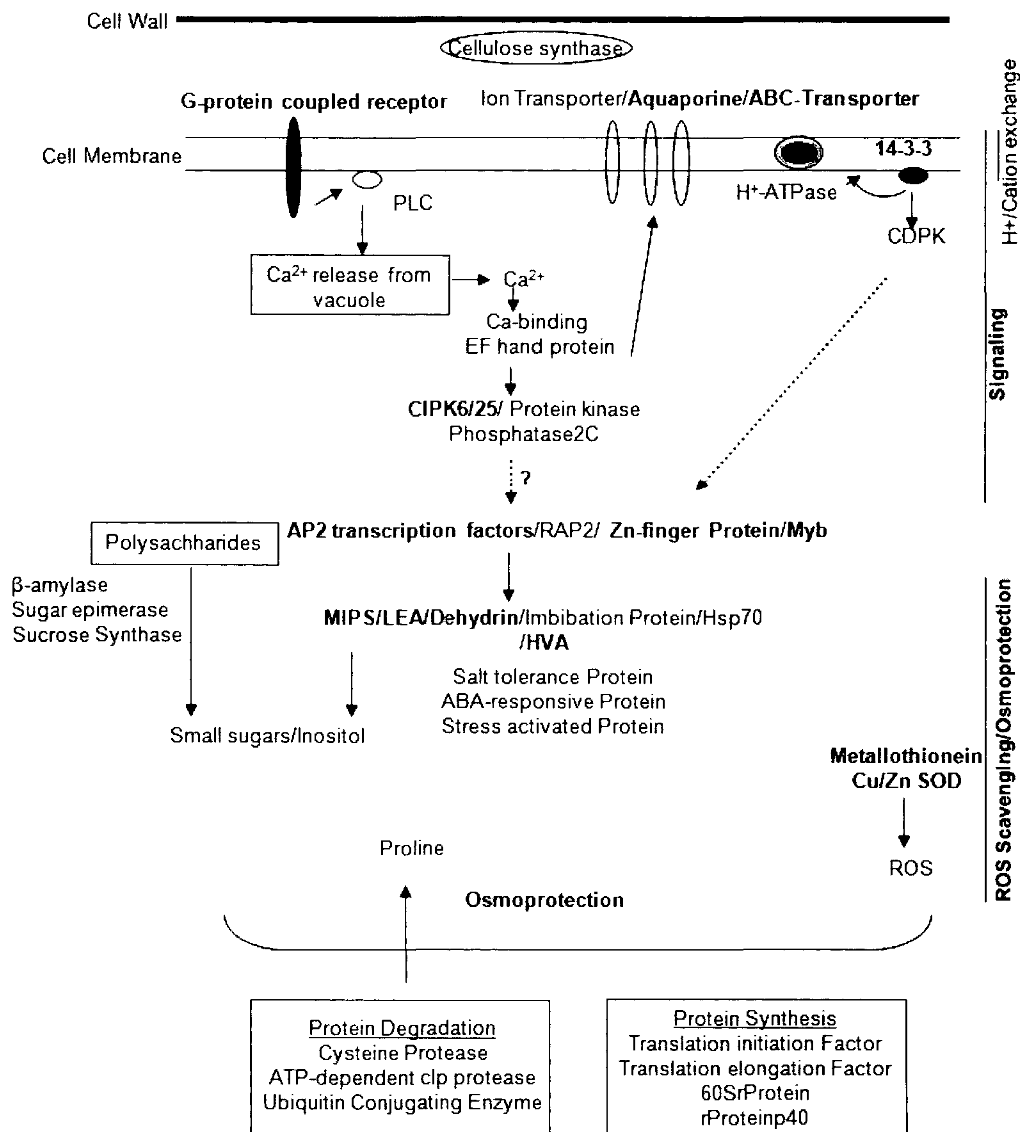


Figure 4.9. An outline of putative pathway for drought-tolerance mechanism in chickpea based on expression data generated in this study. The candidate genes that may provide drought tolerance are predicted on the basis of published literature are shown in bold letters.

transgenic plants, and the plants showed significantly increased drought tolerance (Tran et al., 2004). Zinc finger proteins (FL512439) are ubiquitous; some of them are shown responsive to and provide tolerance to abiotic stresses (Mukhopadhyay et al., 2004; Davletova et al., 2005). Six genes of unknown function represent well-known stress responsive genes encoding e.g. ABAresponsive protein (FL512397), stress activated protein (FL512411), salt tolerance proteins (FL512396, FL518936), dehydration induced protein (FL512471). High expression of ten genes under cellular organization group is well understood as they putatively encode LEAs and dehydrins. Dehydrin, LEA and proline rich proteins are thought to provide stability to other proteins in osmotic stress (Ingram and Bartels, 1996). High relative expression of two genes involved in protein synthesis and four genes involved in protein degradation indicate better protein turnover during the stress condition in the tolerant cultivar as previously reported in case of a salt-tolerant rice variety in salt stress (Seki et al., 2001).

Overexpression of certain enzymes such as superoxide dismutase has been implicated in free radical detoxification and scavenging of free radicals under oxidative stress. Halophytic plants like mangroves have been reported to have a high level of SOD activity, which plays a major role in defending the mangrove species against severe abiotic stresses (Yan and Guizhu, 2007). SOTA clustering of these genes predicted some important speculations. Few important genes of these clusters are hereby mentioned. One of them putatively encodes PR-10 protein. Although, PR-10 proteins are implicated in cellular defense as they express during pathogen attack; abiotic stresses like drought and salinity also induce their expression. The other gene encodes a NAC transcription factor. It appears from their biphasic expression pattern that they are required at early and again at the late phase of DH response. Role of specific glycinerich RNA binding protein in stress responses by stomatal regulation is reported (Kim et al., 2008). High fold of expression of protein metabolism genes strongly indicates a high rate of protein metabolism in the tolerant cultivar early in the stress. This correlative evidence suggests that high rate of protein turn over may be one of the mechanisms for drought tolerance in PUSABGD72.

The focus of this study was on a set of genes, which were highly expressed in a drought-tolerant chickpea cultivar in comparison to a sensitive one early in response to drought stress. Based on the comparative gene expression data and with the supporting evidence from published literature, a preliminary outline of drought-tolerance mechanism in chickpea was sketched. From the list of high expressing ESTs in this study few

candidate genes were predicted that may be helpful in improving the stress tolerance of crops by gene manipulation. Calcium has emerged as a ubiquitous second messenger to a multitude of plant responses including stress. In plants intracellular calcium level is quickly modulated by extracellular stress. G-protein coupled receptor (GPCR) has important role in this respect. Activated membrane-bound G-protein coupled receptors transducer signals through the interacting heterotrimeric G-proteins that ultimately transfer the signal to membranelocalized phopholipase C (Misra et al., 2007).

Study with chemical inhibitor of phospholipase C (PLC) has shown that increased cellular calcium-mediated signaling via PLC is essential for proline accumulation in Arabidopsis in response to ionic hyperosmotic stress (Parre et al., 2007). Therefore, we believe that GPCR might be a good candidate gene and enrichment of GPCR, and thereby the heterotrimeric G-proteins, in the cell membrane will improve the calcium-mediated signaling. 14-3-3 proteins interact with and regulate the activity of P-type H^+-ATPase that creats proton gradient across the membrane (Fuglsang et al., 2007). 14-3-3 proteins also activate calcium dependent protein kinases (Camoni et al., 1998). Overexpression of *Arabidopsis* 14-3-3 protein in cotton improved drought tolerance (Yan et al. 2004) and, therefore, can be considered as a candidate gene. Intracellular calcium activates CBL-interacting protein kinases (CIPK) and the other kinase via calcium-binding proteins. Chickpea homologues of CIPK6 and CIPK25 have been identified in this study. Overexpression of chickpea CIPK6 improved salt and drought tolerance in transgenic tobacco (Tripathi et al., 2009). Apart from that overexpression of rice CIPK3, CIPK12 and CIPK15 improved drought tolerance and accumulation of proline in transgenic plants (Xiang et al., 2007) and supports that the chickpea CIPKs may be considered as candidate genes. CIPK24 (SOS2) and CIPK23 activate Na^+/H^+ antiporter and K^+-transporter respectively (Qiu et al., 2004; Lee et al., 2007).

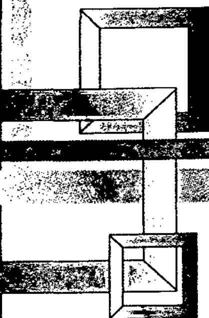
Expression of *Vicia faba* aquaporine in *Arabidopsis* (Xu et al., 2008) and Indian Mustard aquaporine in tobacco (Zhang et al., 2008) improved drought tolerance. Chickpea homologue of *AtMRP4*, an ABC transporter, has been identified in our study. Disruption of *AtMRP4* increased drought sensitivity (Klein et al., 2004). Therefore, Aquaporine and MRP-4 like ABC transporter seems to be essential genes for drought tolerance. Apart from regulating the activity of the transporters kinases and phosphatases may (dotted arrow) modulate activity of transcription factors by post-translational modulation. AP2 family transcription factors were shown to upregulate different down

stream genes to ultimately activate osmoprotective processes. AP2 family transcription factors like DREB1, DREB2CA (Agarwal et al., 2006), had been studied in detail.

Zinc finger proteins like OSISAPI (Mukhopadhyay et al., 2004), STZ (Sakamoto et al., 2004) and MYB-family proteins (Cheong et al., 2002) were shown to enhance drought and salt tolerance to various crops. Myoinositol phosphate synthase (MIPS) catalyzes synthesis of inositol and enhanced salt tolerance in overexpressing transgenic plants (Majee et al., 2004). It is postulated that proteins common referred as late embryogenesis abundant proteins (LEA) protect the structures of other protein during desiccation (Kobayashi et al., 2008). Hsp70 also acts as a general chaperon (Sung et al., 2001). A dehydrin gene from *P. patens* was shown essential for osmotic tolerance (Saavedra et al., 2006). A previous proteomic study has shown that drought increased accumulation of cellulose synthase in chickpea cell wall (Bhushan et al., 2007). Generation of reactive oxygen species (ROS) is a common phenomenon in stress. Two comparatively high expressing ESTs in the tolerant chickpea cultivar encode Copper/Zinc superoxide dismutase (Cu/Zn SOD), the first enzyme in the enzymatic antioxidative pathway, and metallothionein. Constitutive expression of a mangrove Cu/Zn SOD increased drought and salinity tolerance of rice (Prashanth et al., 2008). *CLMT2*, a type-2 metallothionein induction contributes to the survival of wild watermelon under severe drought (Akashi et al., 2004). All these activities leading to metabolic shift in adverse condition require efficient protein turn over mechanism. Among the ESTs putatively involved in protein metabolism the one encoding translation initiation factor eIF1 may be a useful candidate as overexpression of eIF1 of a salt tolerant grass Festuca rubra in rice enhanced salt-adaptation by regulation ion-homeostasis and intracellular redox status (C. J. Diédhiou, 2008).

Chapter 5

Cloning and In-vitro characterization of CaZF, a C2H2 zinc-finger protein from chickpea



5.1 Introduction

The transcription control of stress-inducible genes is crucial to plant responses to environmental stresses. To date, several kinds of transcription factors involved in environmental stresses were functionally characterized. The Cys-2/His-2-type zinc finger, also called the classical or TFIIIA-type finger, is one of the best-characterized DNA-binding motifs found in eukaryotic transcription factors. This motif is represented by the signature CX2-4CX3FX5LX2HX3-5H, consisting of about 30 amino acids and 2 pairs of conserved Cys and His bound tetrahedrally to a zinc ion (Pabo et al., 2001). There are two prominent structural features of plant TFIIIA-type zinc-finger proteins which distinguish them from their counterparts in other eucaryotes. One is the long spacers of diverse lengths between adjacent fingers. These spacers are highly variable in length and sequence from one protein to another. Another structural feature of the plant zinc-finger proteins is the high conservation of a unique six-amino acid stretch, QALGGH (underlined are the conserved Leu and His), within a putative DNA-contacting surface of each finger. The only TFIIIA-type zinc-finger protein without the QALGGH sequence is PCP1 in potato (Kuhn and Frommer, 1995). *In vitro* binding assays with mutagenized proteins have demonstrated that this conserved sequence is critical for DNA-binding activity (Kubo et al., 1998). Interestingly, this sequence has so far not been found in the zinc fingers of any other organisms, suggesting that it may have evolved in association with plant-specific regulatory processes.

In the past decade, many identified C2H2-type zinc finger proteins play various roles in developmental processes or responses to abiotic stresses in plants (Takatsuji, 1999). Because petunia (*Petunia hybrida*) ZPTs were the first reported among this type of protein, we call this zinc-finger motif the ZPT type. Plant ZPT-type proteins have one to four fingers each and can be classified according to the number of fingers. Two-fingered protein genes, here called ZPT2-related genes, constitute the major class of the ZPT-type family genes and include 14 ZPT2 genes in petunia (Takatsuji et al., 1992; Takatsuji et al., 1994; Kubo et al., 1998); *WZF1* in wheat [*Triticum aestivum*; (Sakamoto et al., 1993)]; *STZ* (Lippuner et al., 1996), five ZAT genes (Meissner and Michael, 1997), and *RDH41* (Iida et al., 2000) in *Arabidopsis*; *Pszf1* in pea [*Pisum sativum*; (Michael et al., 1996)]; *Mszpt2-1* in alfalfa [*Medicago sativa*; (Frugier et al., 2000)]; and *SCOF-1* in soybean [*Glycine max*; (Kim et al., 2001)]. To elucidate the functions of plant ZPT2-related proteins, extensive studies had been made with *Arabidopsis* ZPT2-related genes like, *AZF1*, *AZF2*, *AZF3*, and *STZ* (Sakamoto et al., 2000). Role of these AZF and STZ

proteins in transcriptional regulation in plants has been estabilished. All four proteins were localized in nuclei and bound to DNA in a sequence-specific manner. Among the four genes, *AZF2* and *STZ* showed strong induction by various stresses and ABA. The expression of the *STZ* gene increases with salt treatment in plants which suggests the role of STZ in the regulatory processes associated with salt tolerance in plants. A C2H2-type zinc finger protein from soybean, SCOF-1, is ABA- and cold-inducible and can enhance the binding efficiency of another bZIP transcription factor SGBF-1 to ABRE (Kim et al., 2001). RHL41/ZAT12 mediates light acclimatization response in *Arabidopsis* (Iida et al., 2000).

The DNA-binding ability of the Cys-2/His-2-type zinc-finger motif that has been identified in many transcription factors as a DNA-binding motif, has been observed by in vitro gel-shift assay by taking the EP2 sequence as a probe because it had already been reported that some of the petunia ZPT2 proteins bind to this probe in a sequence-specific manner. EP2 is a 26-bp sequence composed of two 13-bp EP1S sequences repeated tandemly. The EP2 sequence contains three A(G/C)T sequences, two in the 13-bp EP1S sequence and the other at the junction of the tandem repeats. EP1S was originally identified as a cis-element within the EPSPS gene promoter in petunia (Takatsuji et al., 1992).

EPF family is a subfamily of TFIIIA-type zinc finger proteins of plants. The proteins of this family are characterized by the long (19-65 amino acids) linkers of various lengths that separate the zinc fingers. The structure of the EPF proteins suggests that they may interact in unique manner with their target DNA. Understanding the mechanism of this protein-DNA interaction would certainly be beneficial to the molecular characterization of proteins. Preliminary characterization of the DNA-binding activities of some EPF proteins of petunia has revealed some unique protein-DNA interactions. ZPT2-2 (renamed from EPF2-5) with a spacer of 44 amino acids, binds to two separate AGT core sites with each finger making contact with one core site (Takatsuji et al., 1994). This interaction is sensitive to the spacing between the two core sites, the optimum being 13bp. Another two-fingered protein, ZPT2-1 (renamed from EPF1), which has a spacer of 61 amino acids, binds to the same core sites but tolerates a spacing of 13 to 16 bp. In petunia, 14 two-fingered proteins have been identified so far and the lengths of their spacers range from 19 to 65 amino acids (Kubo et al., 1998). With this observation it is tempting to speculate that these two-fingered proteins with such diverse lengths of spacers have rather variable preferences for the spacings in the target-DNA sequence.

All of the putative AZF1, AZF2, AZF3, and STZ proteins contained a short stretch of basic amino acids (KRKRSKR) near the N terminus. This region is conserved among most ZPT2-related proteins and might function as a potential nuclear localization signal. In vivo targeting experiments indicate that AZFs and STZ are nuclear-localized proteins (Sakamoto et al., 2004).

With this brief introduction, the aims of the experiments detailed in the current chapter were to:

1. Cloning of full length CaZF from chickpea.
2. Bacterial expression of CaZF protein and in vitro binding assays with EP2S as probe.
3. Heterologous expression of CaZF in yeast and estimating its transactivation ability.
4. Raising CaZF expressing transgenic tobacco for cellular localization.
5. Studying differential CaZF expression under different stress conditions

5.2 Results

5.2.1 Isolation of a partial clone

Subtracted cDNA libraries constructed between two chickpea (*Cicer arietinum*) cultivars at different points of drought-stress resulted in a number of EST clones. Sequence annotation using BLASTx of one of the clones revealed significant homology with putative C2H2 zinc-finger containing protein. This EST expressing more in PUSABGD72 than in ICCV2 at different points of stress (Figure 5.1) was taken for further studies.

5.2.2 CaZF encodes an EPF type C2H2 zinc finger protein

The selected clone was partial and the full-length *CaZF* was isolated by 5' Random Amplification of cDNA ends (RACE) (Figure 5.2). The amplified fragments were cloned and sequenced. Full-length cDNA (*CaZF*) constructed by 5' RACE was 1185bp in length (GenBank accession EU513298).

5.2.3 *In silico* analysis of CaZF encoding protein

Sequence analysis revealed an 843bp open reading frame (ORF) of 280 amino acid, 139bp long 5' and 203bp long 3' untranslated region (UTR). Deduced amino acid sequence shows (Figure 5.3A) CaZF is an EPF type C2H2 zinc finger protein having two canonical TFIIIA-type zinc finger motifs ($CX_2CX_3FX_5LX_2HX_3H$). Both the zinc finger motifs contain conserved QALGGH sequence. A short spacer sequence of 28 amino acids separates two zinc fingers. Detailed comparisons of the amino acid sequences among

plant zinc finger proteins revealed three conserved regions other than the zinc fingers. CaZF contains a short basic region with a consensus of KXKRSKRXR (B-box), near the N-terminus. Another is a region, consisting of three acidic residues followed by hydrophobic residues rich in Leu, with a consensus of EXEXXAXCLXXL (L-box) located between B-box and the first zinc-finger. The other is a short hydrophobic region containing a highly conserved DLNL sequence as a core (DLN-box) close to the C-terminus. CaZF possesses a serine-glutamine rich region at the N-terminus, between L-box and first zinc-finger. CaZF contains two combinations of highly basic region followed by acidic amino acids near the C-terminus (Figure 5.3B).

5.2.4 Phylogenetic analysis of CaZF protein

Comparison of CaZF protein with other known EPF family protein shows that among the studied proteins PIF1 (GB: AAQ54302), a pathogen inducible zinc finger protein from capsicum shows maximum sequence similarity with CaZF of only about 55% homology (expect = 5e-45). No other protein shows any significant stretch of homology outside the conserved domains. Phylogenetic analysis showed that CaZF and one *Arachis* protein (ZFP248) shares the same clad (Figure 5.4).

5.2.5 CaZF binds in vitro to EP sequence repeat

CaZF protein was tested for its ability to bind EP1S sequence. EP1S is a 13 bp sequence with an inverted repeat of TGACA separated by a G (Figure 5.5A). EPF family proteins have spacers of variable lengths between two zinc fingers. Proteins with spacers shorter than 44 amino acids show high specificity of binding to tandemly repeated EP1S with the core G residue separated by 13bp (Takatsuji et al., 1994). Therefore, an EP2S (EP1S dimer) tetramer with 13bp separations between the core G residues was used as a probe for gel shift assay. *CaZF* cDNA was cloned in pGEX4T-2 vector fused to glutathione-S-transferase (GST) and expressed in *E. coli.* Figure 5.5B shows that CaZF protein efficiently bound EP2S tetramer in a sequence specific manner.

5.2.6 CaZF activates transcription in yeast

To determine whether CaZF protein is capable of regulating transcription, *CaZF* ORF was expressed as a fusion to GAL4 DNA-binding domain in a yeast reporter strain carrying *His3, Ade2 and LacZ* reporter genes under *GAL4* promoter. Transformed yeast colonies grew on auxotropic medium lacking histidine and adenine (Figure 5.6A) suggesting that CaZF can function as a transcription activator.

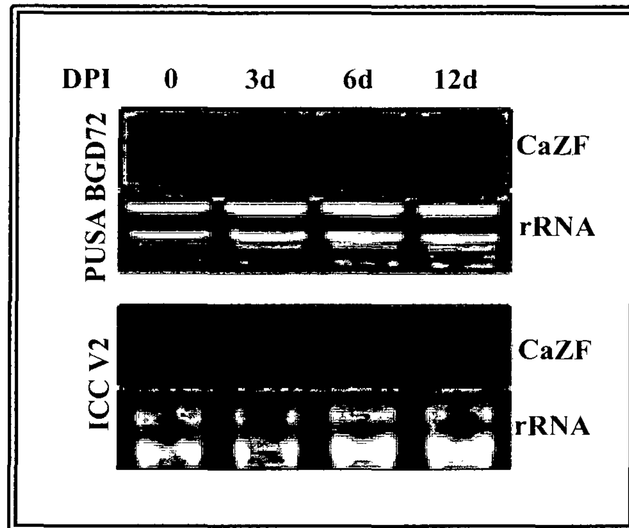


Figure 5.1. Expression of CaZF in chickpea var. PUSABGD72 and ICCV2 under different drought conditions. Samples harvested at day post-irrigation (DPI) is mentioned. Total RNA (20 μg/lane) from chickpea seedlings were hybridized with probe prepared from CaZF cDNA as described under "Experimental Procedures". Ribosomal RNAs are shown as loading control.

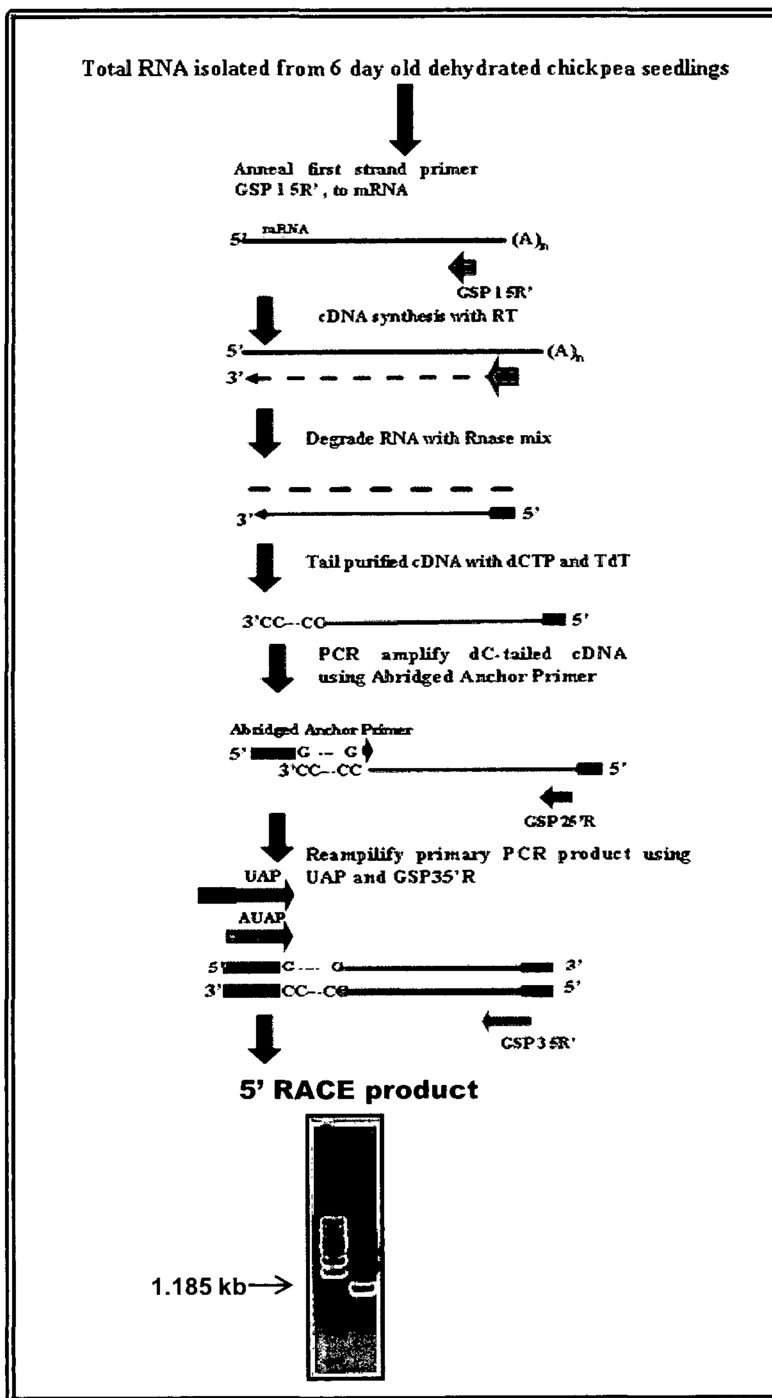


Figure 5.2. Schematic representation of 5'-RACE. Full length cDNA of *CaZF* was isolated using a 5'RACE-PCR based strategy.

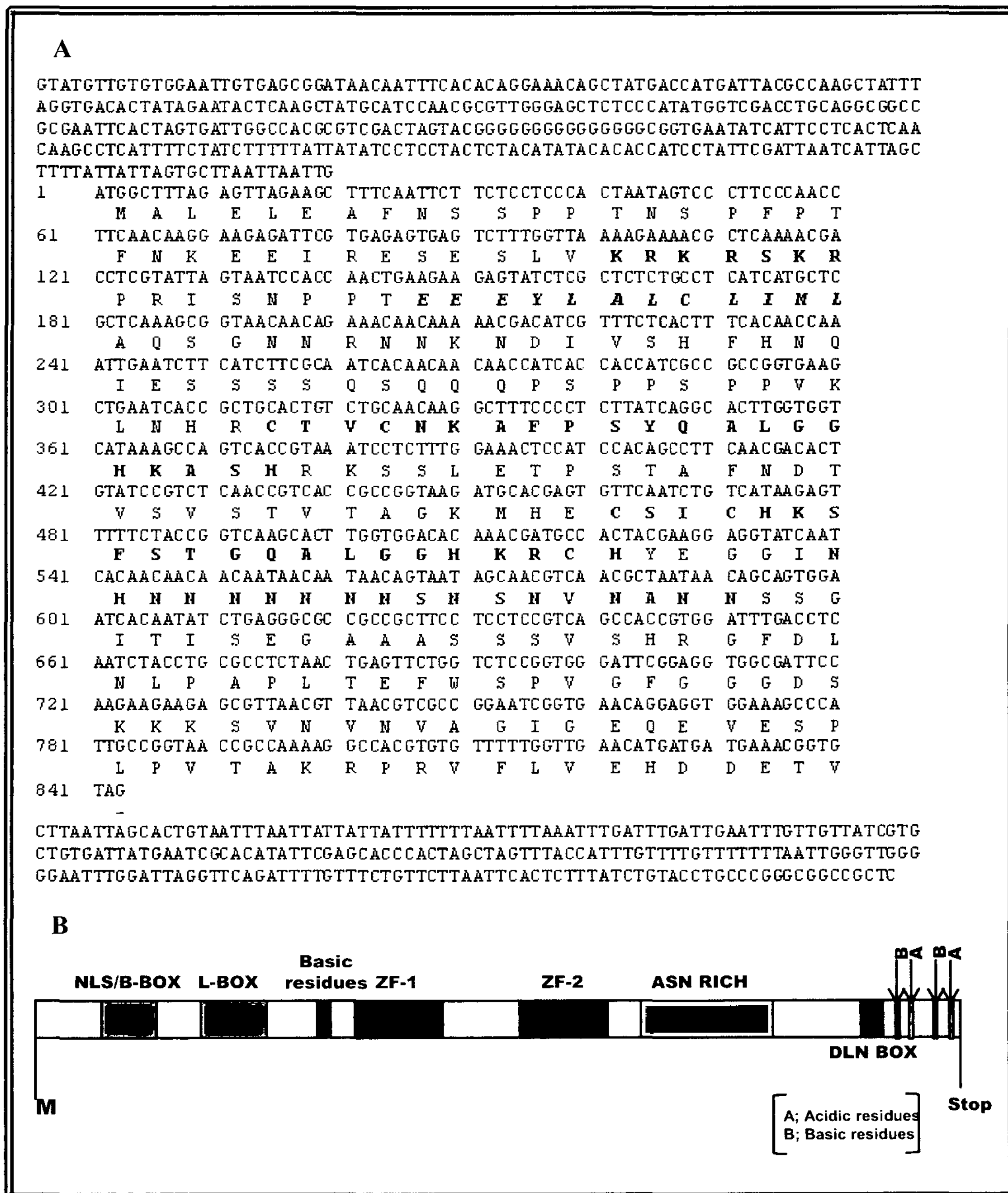


Figure 5.3. In silico analysis of amino acid sequence of CaZF. (*A*) Deduced amino acid sequence of CaZF protein. The basic B-box in CaZF is indicated by *bold letters*, L-box by *bold italic letters*, zinc finger motifs by *red letters* and Asn-rich region by *green letters*. (***B***) Schematic representation of different domains present in CaZF protein.

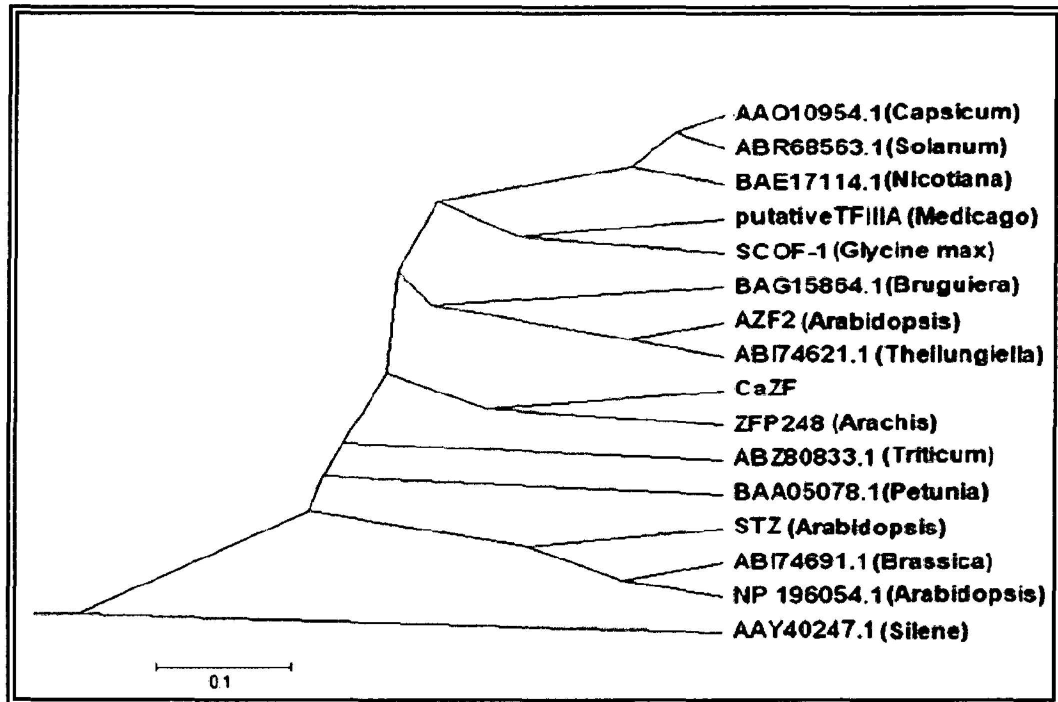


Figure 5.4. Phylogenetic tree showing relationship between CaZF and other well-studied C2H2 zinc-finger family proteins. The tree was generated using the neighbor-joining algorithm of MEGA 2.0 software, version 2.1. The bar indicates the scale for branch length.

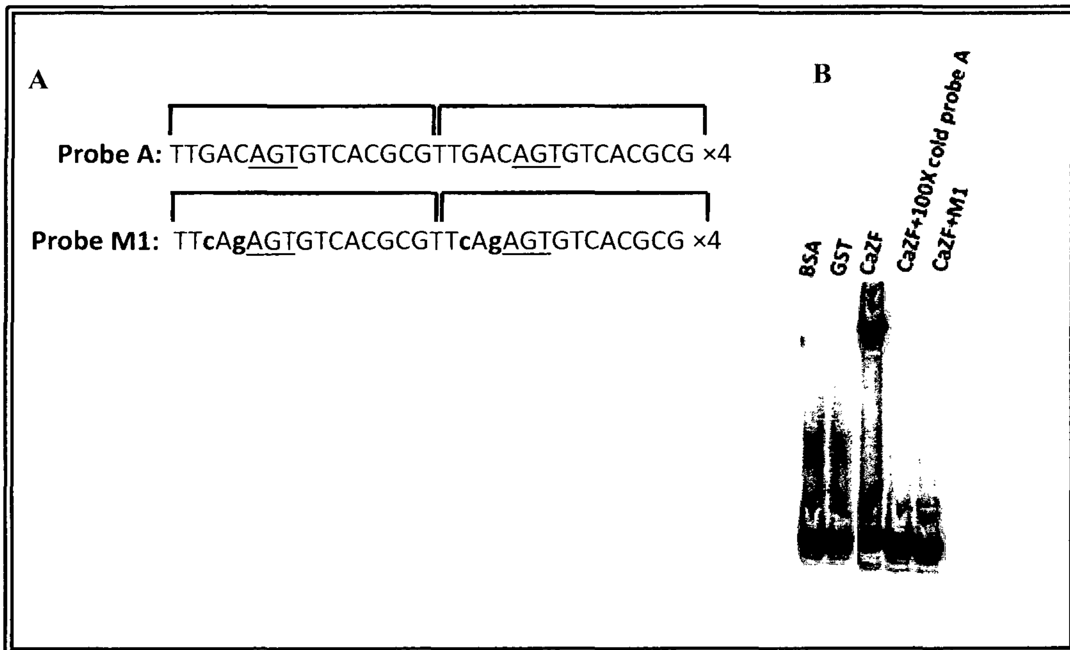


Figure 5.5. CaZF binds to DNA in a sequence specific manner. *A*, The 32bp EP2S sequence tested for gel-shift assay is either wild-type or mutant version M1. Monomers are shown, and tetramers were used in the experiments. Core nucleotides are *underlined* and modified bases are in *bold small case letters*. ***B***, Gel-shift assays demonstrating that CaZF binds to the EP2S probe. The probes (1 ng) used in all reactions were ^{32}P-labeled tetramers of the oligonucleotides shown in ***A***.

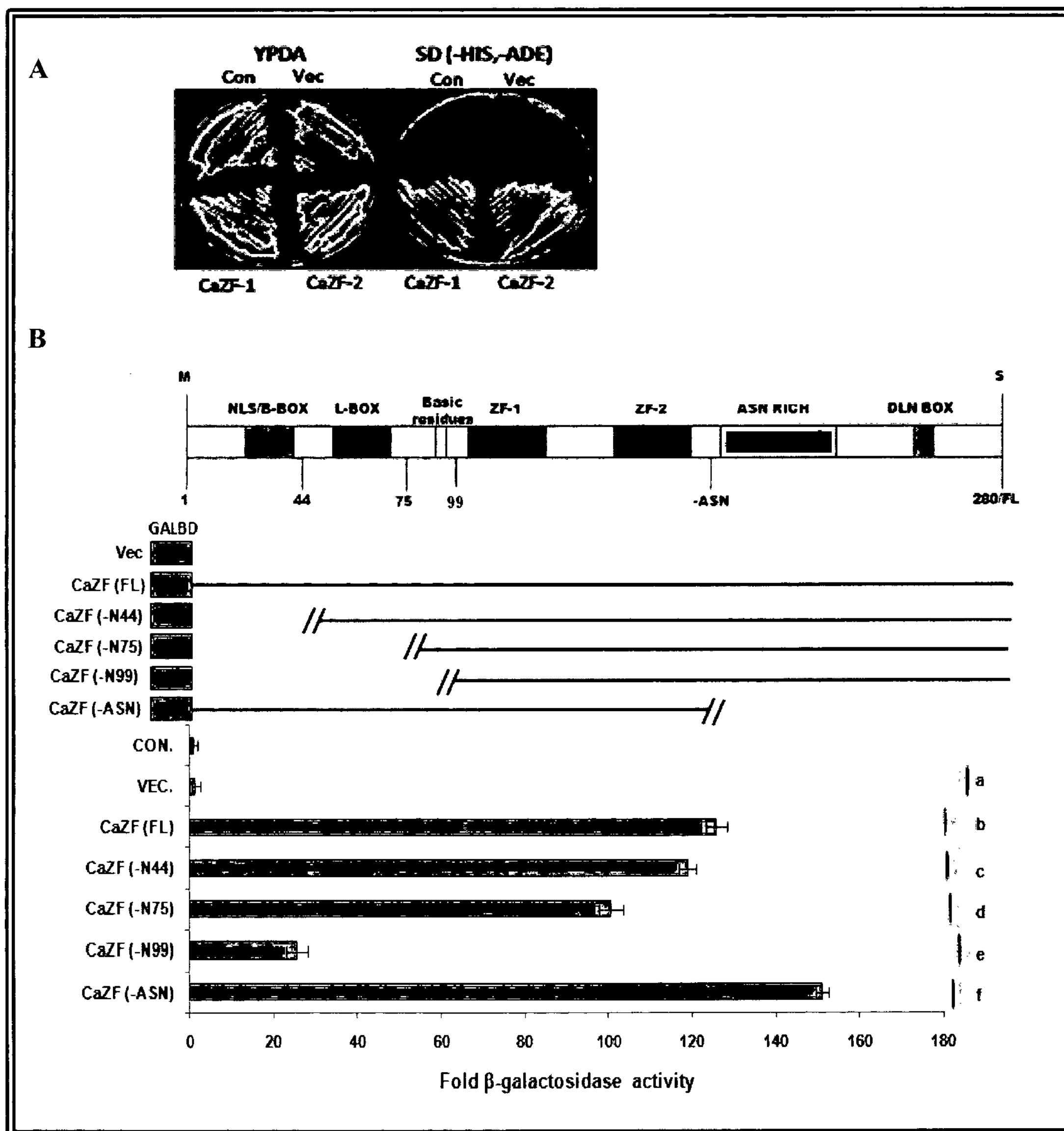


Figure 5.6. Transactivation assay of CaZF in yeast. Full length and truncated *CaZF* cDNA were cloned into pGBKT7 for expression of CaZF protein as a fusion with GAL4-DNA binding domain and introduced into a yeast strain PJ69-4A carrying three reporter genes, *HIS3*, *ADE2*, and *LacZ*, under the control of the *GAL4* promoter. (***A***) Activation of *HIS3* and *ADE2* reporter genes is shown by growth of the transformants growing in SD (-histidine,-adenine) medium against control (con.) and vector (vec.) transformed. (***B***) *LacZ* activation by different deletion constructs of CaZF is shown by β-galactosidase assay of the transformants presented as fold increase in activity.

5.2.7 Transactivation Domain mapping of CaZF in yeast

In order to identify the transactivation domain two CaZF deletion constructs were introduced in the yeast reporter strain and β-galactosidase activity was assayed. Deletion of C-terminal amino acids after the second C2H2 domain (-Asn) produced higher β-galactosidase activity than the full-length protein (Figure 5.6B). Removal of N-terminal amino acids up to the first C2H2 domain (-N99) caused significant reduction of β-galactosidase activity. To further locate the transactivation domain, two more N-terminal deletion mutants, one from 1-44 aminoacids (N44) and other 1-75 aminoacids (N75) were constructed. β-galactosidase assay showed that the aminoacids from 44-75 (L-box) are most important for transactivation property of the protein.

5.2.8 Genomic Organization of *CaZF*

To understand the genome organization (intron number and size) of *CaZF*, preliminary analysis was done using the cDNA and genomic DNA from chickpea as templates. Full length *CaZF* (including 5' and 3' UTR) was amplified from cDNA and genomic clone by using primers designed from 5' and 3' ends of *CaZF* cDNA. The PCR product obtained with genomic DNA as template was same in size as compared to the corresponding cDNA amplification of about ~ 0.850kb (Figure 5.7A, Lanes 2 & 3). These fragments were cloned and sequenced for further validation. The same size and sequence of the genomic fragment of *CaZF* as compared to cDNA show that it is an intron-less gene.

5.2.9 Southern analysis

To know the copy number of *CaZF* in chickpea genome, Southern hybridization was performed. 20μg of the genomic DNA was digested completely with restriction enzymes; *BamHI, EcoRI, MseI, HindIII* and *XbaI* separately. The digested genomic DNA were resolved on 0.8% agarose gel and subsequently transferred to nylon membrane via capillary method as discussed in 'Materials and Methods'. Southern blot was hybridized at high stringency with full length *CaZF* cDNA as a probe. *BamHI, EcoRI,* and *MseI* being the non-cutters of *CaZF*, gave a single band after hybridization whereas the single site cutters (*HindIII, XbaI*) gave two bands after hybridization (Figure 5.7B). A small number of bands were observed in each of the digests, indicating the *CaZF* gene is either single or low copy number.

5.2.10 Cellular localization of CaZF

For cellular localization of *CaZF*, cDNA-encoding CaZF was cloned into binary vector (pCAMBIA1302) in fusion to green fluorescence protein (GFP) at the C-terminal end under *35S CamV* promoter between *NcoI-BglII* (Figure 5.8A). The construct along

with empty pCAMBIA1302 vector were introduced into tobacco (*Nicotiana tabaccum cv.* xanthii) separately by *Agrobacterium* mediated transformation. For the nuclear staining, tobacco leaf peals were incubated for 10 min with DAPI (1μg/μl) before observing under fluorescent microscope with FITC filter. Expression of CaZF protein fused to green fluorescence protein (GFP) in tobacco demonstrated that the protein is localized in nucleus (Figure 5.8B). To confirm the position of the nucleus same samples were stained with DAPI stain which specifically stains nucleus.

5.2.11 Expression analysis of *CaZF* under various stress treatments

CaZF expression pattern was analyzed in response to abiotic stress, plant hormones, signaling molecules and physical factors by using RNA gel-blot analysis. Cold, DH, salt, abscisic acid (ABA), jasmonic acid (JA) and salicylic acid (SA) treatments for five hours alter the *CaZF* expression (Figure 5.9A). Though the expression was found to be much higher in ABA and MeJA treatments than any other, wounding did not alter *CaZF* expression.

The level of the CaZF transcript slowly increases from a basal level and reached at maximum level of approximately 3-4-fold in 3.0 h of cold and salt treatment followed by a decrease in expression till 24.0 h. For the dehydration stress, CaZF mRNA started accumulating within 0.5 h of stress and reached its maximum level (3-fold) within one hour. Though maintaining the expression till 5.0 h of stress, steap decline of the transcript level was observed after 24.0 h (Figure 5.9B). For ABA treatment (100μM) the transcript follows the same pattern as salt, started accumulation within 1.0 h, reaching maximum upto 3-fold at 3.0 h and then gradually decreased in 24.0 h. under JA treatment CaZF transcript level behaved in a similar manner as in cold stress, i.e., reached maximimum level in 3.0 h but decreased upto 24.0 h (Figure 5.9C).

5.2.12 Tissue specific expression of *CaZF*

CaZF has relatively detectable expression in stem in comparison to root and leaf. However, in cold, dehydration and salt stress conditions the shoot specific expression increased several fold in comparison to control condition. *CaZF* has shown a root specific expression under dehydration and salt stress conditions. On the other hand leaf specific *CaZF* expression could be seen in cold and dehydration stress (Figure 5.10A). In case of ABA treatment, *CaZF* transcript accumulation had shown a steep increase in root, shoot and leaf tissues in comparison to control conditions whereas under JA treatment only shoot specific expression could be seen (Figure 5.10B). This result gives an indication

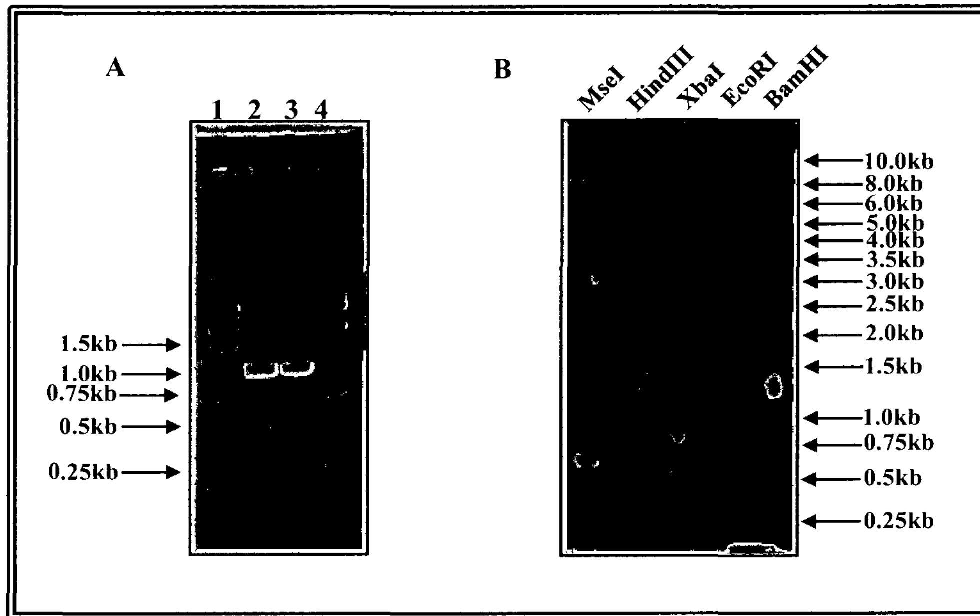


Figure 5.7. Genomic organsation of *CaZF*. (***A***) PCR amplified product of *CaZF* using genomic DNA (lane2) and cDNA (lane 3) as template with ORF flanking primers. (***B***) *CaZF* is a unique or low copy number gene. Chickpea DNA (approximately 10 μg) was digested with indicated restriction endonucleases, and blotted by Southern transfer was hybridized with ^{32}P-labeled probe used for northern hybridization. *CaZF* has single *HindIII* and *XbaI* sites giving two bands.

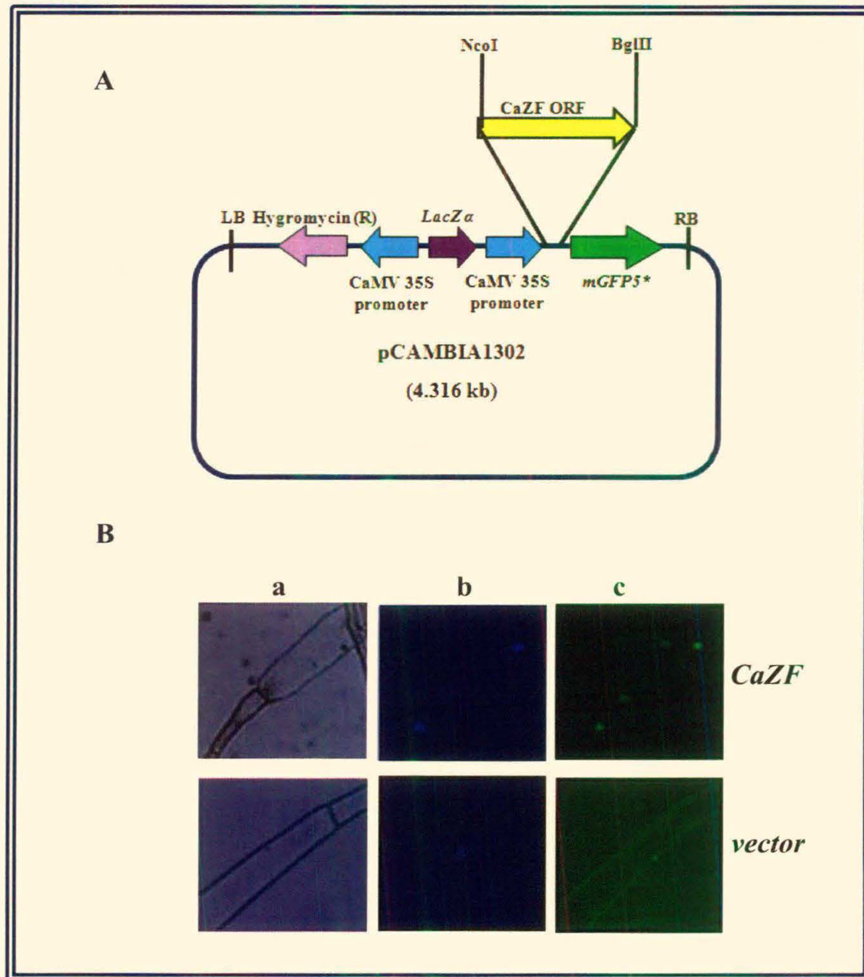


Figure 5.8. CaZF protein localizes in nucleus. (*A*) *CaZF* ORF was cloned in pCAMBIA1302 to make CaZF protein fused with GFP at its C-terminus (***B***) pCAMBIA-*CaZF* construct was introduced in *N. tabacum* leaf explant through *Agrobacterium* mediated transformation. Leaf peels of the transgenic and vector transformed plants were analyzed under microscope for phase contrast (a) for GFP activity (c). The sample was restained with DAPI to confirm the nucleus position (b).

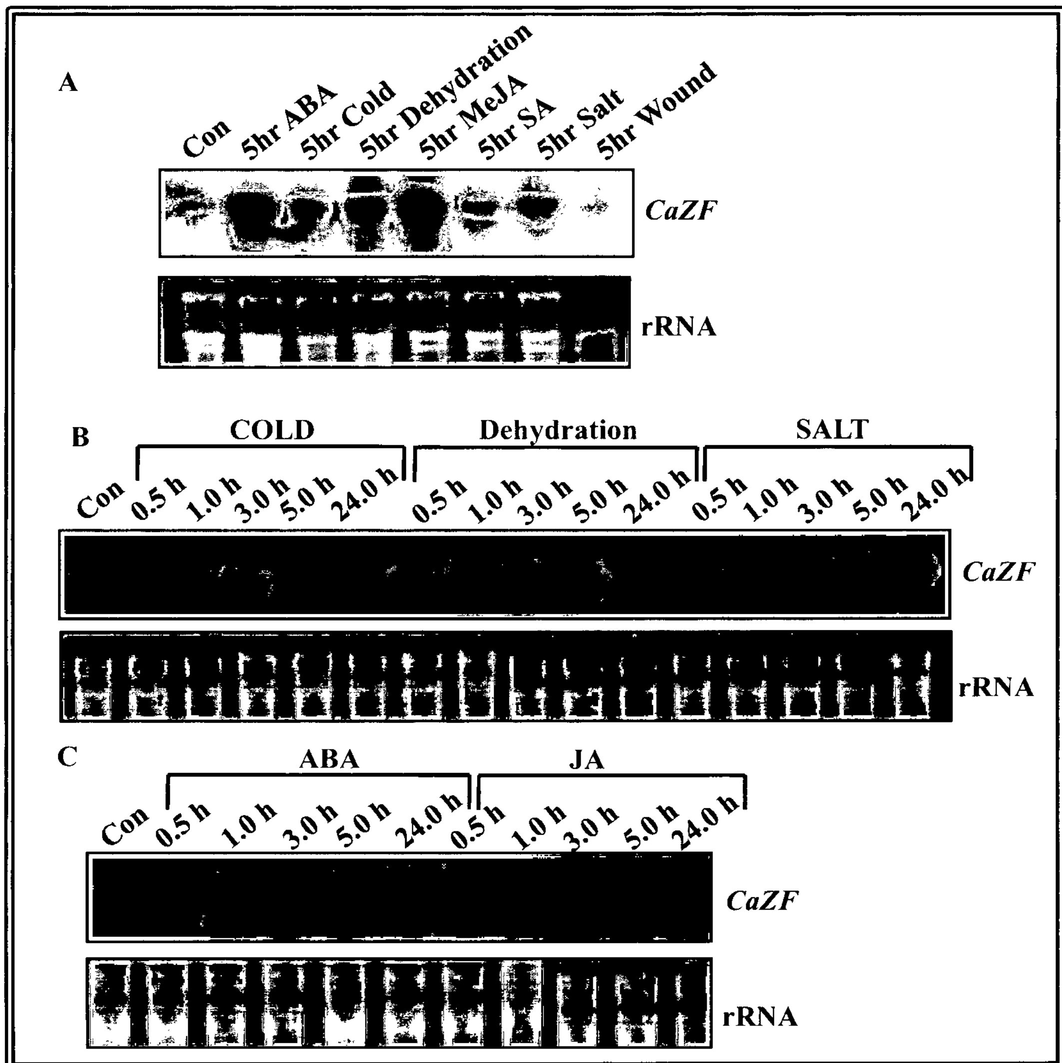


Figure 5.9. Expression pattern of *CaZF* under different stress conditions in chickpea seedlings. 20 μg of total RNA was blotted onto a northern membrane and hybridized with radiolabelled *CaZF*. (***A***) Different abiotic and hormonal stresses given for 5h. (***B***) Cold, dehydration (withdrawal from soil) and salt (150mM). (***C***) ABA (100μM) and JA (100μM). Ethidium bromide-stained rRNA was taken as loading control.

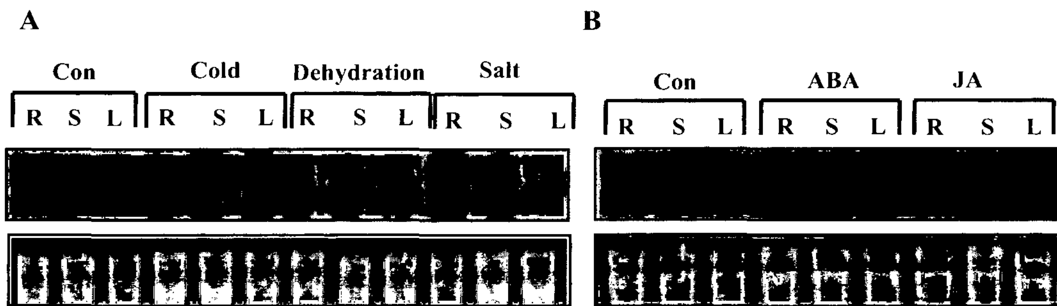


Figure 5.10. Expression pattern of CaZF in plant organs [root (R), shoot (S) and leaf (L)] under different stress conditions. (***A***) Cold, dehydration and salt. (***B***) ABA and JA.

that *CaZF* promoter contains cis-acting elements involved in stress-responsive gene expression.

5.3 Discussion

In this chapter we have characterized *CaZF*, encoding plant-specific transcription factor with two Cys-2/His-2 zinc finger motifs. It showed high expression ratio in PUSABGD72 than in ICCV2 which made us curious to know the possible role of this gene in drought tolerance mechanisms. A number of stress-induced plant proteins of this family have been studied. They are involved in a variety of physiological processes and encode diverse functions as transcription activators or repressors without showing much structural variability. In silico analysis show that CaZF is a 280 amino acid protein in comparison to other EPF family members which are relatively longer than this. Among the studied proteins PIF1 (GB: AAQ54302), a pathogen inducible zinc finger protein from capsicum shows maximum sequence similarity with CaZF of only about 55% homology (expect = 5e-45). Notably, PIF1 is also highly expressed in a pathogen tolerant variety compared to a sensitive one in response to infection (Ohkawa et al., 2005). CaZF contains B-box near the N-terminus, which may function as a potential nuclear localization signal (NLS) and/or may participate in DNA binding. L-box and DLN-box close to the C-terminus, both may play a role in protein-protein interactions or in maintaining the folded structure (Sakamoto et al., 2000). Serine-glutamine rich region at the N-terminus might function as a transactivation domain as suggested for ZPT2-1 and Pszfl (Takatsuji et al., 1992; Michael et al., 1996) or might be a phophorylation site for post-translational modification; and an asparagine rich stretch after the second zinc finger at the C-terminus. Similar asparagine-rich domains are also present in some stress-inducible zinc finger proteins such as SCOF-1, EPF2-5, and STZ (Kim et al., 2001). Like *STO* and *STZ*, the *Arabidopsis* cDNAs, which increase salt tolerance in yeast in a Calcineurin independent manner, *SCOF-1* and *EPF2-5*, CaZF contains highly basic region followed by acidic amino acids near the C-terminus. But, CaZF has two such combinations of basic and acidic amino acid stretches.

CaZF possesses EPF type C2H2 zinc finger motifs that has been identified in some transcription factors from petunia by their ability to bind a target sequence EP1S core sequence (TGACAGTGTCA) present in the promoter of their target gene *EPSPS* (5-enolpyruvylshikimate-3-phosphate synthase) (Takatsuji et al., 1992; Takatsuji et al., 1994). Therefore, the DNA binding ability of CaZF was tested by taking EP2S as a probe.

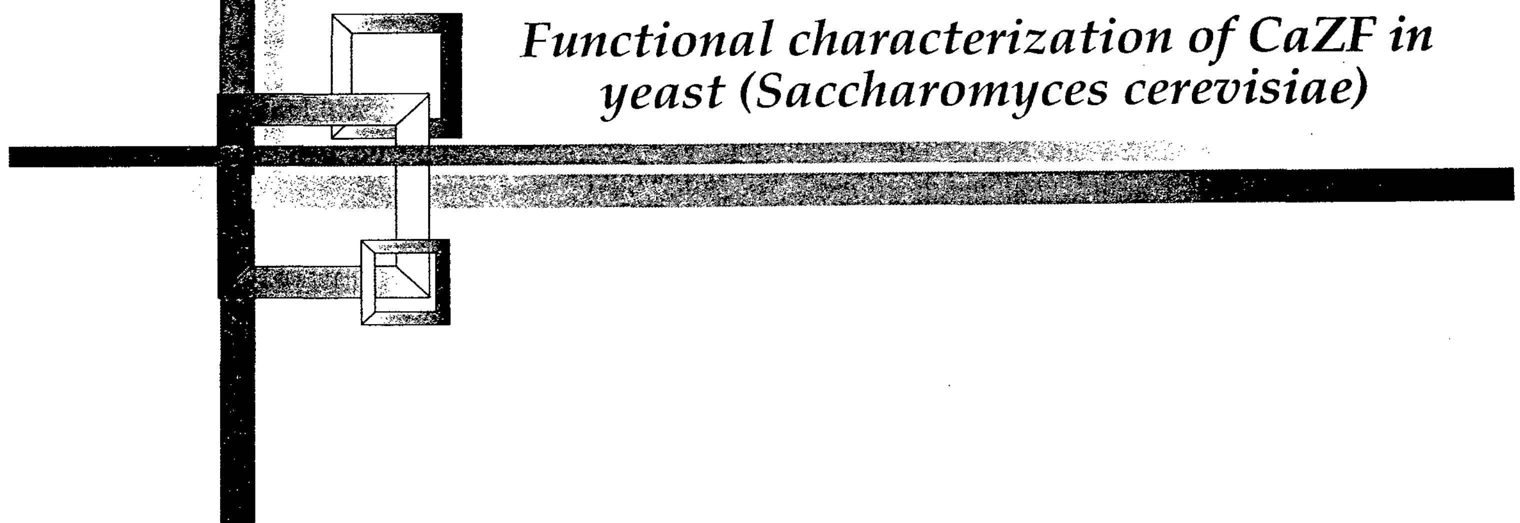
The gel mobility shift assay revealed that CaZF protein bound to the EP2S core sequence specifically and does not shows any gel shift after mutating the core sequence.

Since CaZF is a transcription factor so to determine its capability to regulate transcription became necessary. The activity was quantitated by β-galactosidase assay. (-Asn) truncated form of protein produced higher β-galactosidase activity than the full-length protein so increase in transcription activity after C-terminal deletion of CaZF is most likely due to removal of DLN-box mediated repression. Transcription repressor proteins e.g. ERF, STZ and AZF have a conserved DLN-box motif ($^{L}/_{F}$DLN$^{L}/_{F}$P) at their C-terminus and that was shown to be essential for repressor activity (Ohta et al., 2001; Sakamoto et al., 2004). Removal of N-terminal amino acids (-N99) caused significant reduction of β-galactosidase activity demonstrating essentiality of this domain for transactivation. Transactivation domain mapping showed that the amino acids from 44-75 (L-box) are most important for transactivation property of the protein.

In order to adapt, and then survive in adverse environment, plants must modulate biochemical activities and development based on stress sensing and stress responsive signal transduction pathway. Evidence has emerged indicating the involvement of several signal-transduction pathways, based on the detection of ABA-dependent and ABA-independent pathways with osmotic, ionic and oxidative stress dependent sensing and response components (Liu et al., 1998; Kasuga et al., 1999; Zhu, 2001). Using RNA gel-blot analysis, we showed that *CaZF* clearly induced at the transcription level by abiotic stresses such as drought, cold, and high salt. *CaZF* was also induced by ABA. These results indicate that CaZF protein function under abiotic stress conditions. The expression of *CaZF* was induced by SA and MeJA which are one of the several key regulators that mediate a plant's response to biotic and abiotic stresses, such as pathogen infection, wounding, and UV irradiation. This indicates that there is a cross-talk between ABA and SA/MeJA-signaling pathways under abiotic and biotic stress conditions. From above observations we can speculate that CaZF protein may play an important role under abiotic and biotic stress conditions, but the different manner of response to each stress suggested that this protein function in different signal transduction pathways under stress conditions.

Chapter 6

Functional characterization of CaZF in yeast (*Saccharomyces cerevisiae*)



6.1 Introduction

Cells constantly evaluate and respond to sudden and adverse changes in environment by certain mechanisms that not only initiate the repair of macromolecular damage but also establish a tolerant state, which helps to prevent further damage. Salinity is one of the major abiotic stresses that adversely affect crop productivity and quality. The maladies caused by salt stress arise from the disruption of cellular aqueous and ionic equilibria, so tolerance determinants include effectors that function to restore cellular homeostasis. At the cellular level, these responses involve both modulation of enzymatic activities and changes in gene expression, and require the participation of sensor systems and signal transduction pathways (Serrano, 1996). The studies on the mechanisms of salt tolerance in model organisms like the budding yeast, *Saccharomyces cerevisiae* are leading to the understanding of basic principles of osmotic regulation and ion homeostasis in the cell (Ferrando et al., 1995). This knowledge may provide the tools for engineering salt tolerance in cultivated plants (Serrano and Gaxiola, 1994; Serrano, 1996).

In high osmotic condition, *S. cerevisiae,* initiates an efficient adaptive response, which maintains cellular Na^{+}/K^{+} balance, retains turgor and repairs cellular damages. Principally, two interconnected pathways regulate this adaptive response. The PP2B phosphatase calcineurin (CaN) is a focal component of a Ca^{+2}-dependent signal transduction pathway that mediates Na^{+}, Li^{+} and Mn^{+2} tolerances of *S. cerevisiae.* A functional CaN is a heterodimer composed of two catalytic subunits, CNA1 and CNA2, and a regulatory subunit CNB. Elevated cytosolic Ca^{2+} activates CaN due to extracellular hyperionic stress. It then dephosphorylates a C2H2 zinc finger transcription factor CRZ1/TCN1 (Matheos et al., 1997; Stathopoulos and Cyert, 1997) causing its transport to nucleus to activate expression of a P-type ATPase ENA1/PMR2A for Na^{+} and Li^{+} efflux (Garciadeblas et al., 1993), but only a part of *ENA1* expression is CaN-dependent (Stathopoulos and Cyert, 1997) suggesting that other Na^{+}-stress response pathways also contribute to *ENA1* induction (Ganster et al., 1998). Calcineurin mutants (i.e., *cna1cna2* and *cnb*) fail to grow in growth medium having high concentration of either Na^{+}, Li^{+}, or Mn^{2+} (Nakamura et al., 1993; Breuder et al., 1994; Mendoza et al., 1994) suggest that CAN participates in regulating the intracellular concentration of several ions (Cyert et al., 1991; Garrett-Engele et al., 1995). In addition to *ENA1*, some other gene (s) are also contributing to salt tolerances that have been regulated by CaN osmopathway (Mendizabal et al., 1998).

The high osmolarity glycerol (HOG) pathway is regulated by a mitogen activated protein kinase (MAPK) Hog1p (Brewster et al., 1993; Albertyn et al., 1994). Drastic reduction of osmotolerance in the *hog1* mutants demonstrates the essentiality of this module in hyperosmotic stress. Triggering of HOG pathway results in the rapid phosphorylation and nuclear translocation of Hog1p. Nuclear Hog1p mediates regulation, both repression and activation, of gene expression. For instance, Hog1p phosphorylates and hence inactivates the transcriptional repressor Sko1p, thereby inducing transcription of, among others, the sodium pump-encoding *ENA1* gene (Proft and Serrano, 1999). In addition, at least two different osmosignalling branches, through a series of downstream components, activate MAPK kinase Pbs2p, which in turn phosphorylates and activates MAPK, Hog1p (Maeda et al., 1994; Maeda et al., 1995; Gustin et al., 1998). Activated Hog1p after moving to nucleus further induces downstream osmoresponsive genes through at least five transcription regulators. Msn2p, Msn4p (Martinez-Pastor et al., 1996; Schmitt and McEntee, 1996; Rep et al., 1999) are two functionally redundant C2H2 zinc finger proteins and activate STRE (Stress responsive upstream activator element) mediated induction of several general stress responsive genes CTT1, HSP12, DDR2, TPS2 etc, required possibly for damage repair (Estruch, 2000; Rep et al., 2000; Mager and Siderius, 2002). Two other Hog1p-regulated transcription activators, Msn1p and Hot1p regulate GPD1, GPP2, genes for glycerol biosynthesis enzymes (Rep et al., 1999). Under osmotic stress Hog1p regulated transcription factors recruit activated Hog1p directly to osmoresponsive promoters (Alepuz et al., 2001; Proft and Struhl, 2002) that further stimulate recruitment of RNA Pol II (Alepuz et al., 2003) and Rpd3 histone deacetylase to promote transcription initiation (De Nadal et al., 2004). Sko1p (Proft and Serrano, 1999), related to bZIP/ATF family of transcriptional regulators (Wilkinson et al., 1996), represses *ENA1* expression through CRE (Cyclic AMP Responsive Element) in unstressed condition. Under hypertonic stress Sko1p is phosphorylated by Hog1p and converted into a transcription activator by recruiting SAGA histone deacetylase and SWI/SNF complex to promote chromatin remodeling (Proft and Struhl, 2002) and induce *ENA1* expression in conjunction with Calcineurin/Crz1p mediated pathway (Proft and Serrano, 1999).

Research over the past decade has identified several cellular mechanisms of salt tolerance in yeast that are conserved in plant cells; and isolation, and characterization of a number of plant salt tolerance determinants was based on homologous function (Haro et al., 1993; Serrano and Gaxiola, 1994; Frommer and Ninnemann, 1995; Niu et al., 1995;

Bressan et al., 1998; Hasegawa et al., 2000) in yeast. Calcium sensor-regulated stress response pathways seem to be structurally and functionally conserved in plants (Luan et al., 1993; Liu and Zhu, 1998; Covic et al., 1999) and some abiotic stress-related proteins are often found to functionally complement yeast calcineurin knockouts. In tobacco and *Arabidopsis* NACK-PQR pathway, similar to HOG pathway, have been reported (Soyano et al., 2003). Tobacco MAPK kinase NQK1 can functionally complement Pbs2p (Soyano et al., 2003).

Hence, main aims of the following chapter are:

1. To investigate if CaZF can provide osmotolerance to yeast cells against ionic and non-ionic stresses.
2. To know whether CaZF can suppress the osmosensitive phenotypes of *hog1*, *cnb* and *hog1cnb* mutants.
3. To estimate the effect on glycerol accumulation.
4. To investigate the effect of CaZF expression on stress-responsive genes regulated by Hog1p and CAN in different mutant backgrounds

6.2 Results

6.2.1 *CaZF* expression in yeast is galactose inducible

Full length *CaZF* cDNA was cloned in *XhoI-BamHI* restriction sites of pYES2.1 vector (Figure 6.1). *CaZF* cDNA was expressed under a galactose-inducible promoter in a protease deficient *Saccharomyces cerevisiae* strain BCY123 that reduces degradation of the heterologous protein (Zhao et al., 1994). In contrast to vector transformed control the *CaZF*-transformed colonies were able to grow in galactose-containing medium supplemented with 250mM LiCl or 500mM NaCl. Li^+ and Na^+ are transported through the plasma membrane by same system. The maximum concentration of each salt tolerated by the transformed colonies is 300mM LiCl and 700mM NaCl at 10^{-6} dilution when incubated at 30°C for 4d. Introduction of *CaZF* cDNA in two other yeast strains, BY4742 and PJ69-4A (data not shown) with different genetic background allowed the transformed colonies to grow on a medium supplemented with 250mM LiCl or 500mM NaCl, demonstrated that CaZF could function in a broad genetic spectrum. Salt tolerance of the transformed colonies was galactose inducible, as they did not grow LiCl-supplemented medium when galactose was replaced with glucose showing expression of the cDNA was necessary for salt tolerance (Figure 6.2A).

6.2.2 *CaZF* provides osmotolerance to yeast

BCY123 harboring *CaZF* cDNA also exhibited tolerance against other ionic and non-ionic osmolytes such as $MnCl_2$, KCl and sorbitol (Figure 6.2B) demonstrating *CaZF* can provide tolerance against general osmotic stress. In liquid medium (YPGalRaf) BCY123 harboring *CaZF* grew almost two fold (doubling time 4.8±0.2 h) faster than the vector control strain (doubling time 8.9±0.2 h) in presence of 500mM NaCl. In absence of salt no difference was observed between the growth rates of yeast strains with or without *CaZF* (doubling time 3.1±0.1h) as shown in the solid medium indicating CaZF requires osmotic stress for its function and provides growth advantages only in osmotic stress.

6.2.3 CaZF suppresses salt sensitivity of Calcineurin (CAN) and HOG pathway mutants

Inter-connected pathways regulated by Hog1p MAP kinase and CaN protein phosphatase determine most of the responses to hyperosmotic stress. A number of salt tolerance determinants in plants have been isolated by their ability to suppress salt sensitivity of the yeast mutants (Dreyer et al., 1999). Therefore, CaZF was tested for its ability to provide osmotic tolerance to some of the well-studied yeast mutants. CaZF suppressed the salt sensitivity when expressed in *cna1cna2* double mutant, lacking both the redundant catalytic subunits and *cnb* mutant lacking the regulatory subunit of calcineurin on 400mM NaCl (Figure 6.3A). However, CaZF could not protect the *cnb* mutant against the ionic osmolytes KCl (data not shown) and NaCl to the extent as it did for the wild type cells. This was also reflected when growth rates were measured in liquid medium. *cnb* mutant cells expressing CaZF grew faster (doubling time 7.2±0.16h) than that carrying only vector (doubling time 11.8±0.3h) but grew at a much slower rate than the wild type cells expressing *CaZF* (doubling time 4.8±0.2h). These data indicate that CaZF functions through a pathway, which is additive to but independent of Calcineurin pathway. Alternatively, CaZF can complement salt sensitivity of the *can* mutants independently but requires the CaN pathway for its full function. Interestingly, growth rates of the *cnb* mutant and the wild type cells harboring CaZF in non-ionic osmolyte sorbitol containing medium were comparable (Figure 6.3B). The probable explanation is Calcineurin pathway protects the cells against toxicity of only ionic osmolytes while HOG pathway protects against hypertonic stress due to both ionic and nonionic osmolytes (Zakrzewska et al., 2007).

As CaZF enhanced growth of wild type yeast in presence of nonionic osmolyte, we wanted to test whether it can function in the background of HOG pathway mutants.

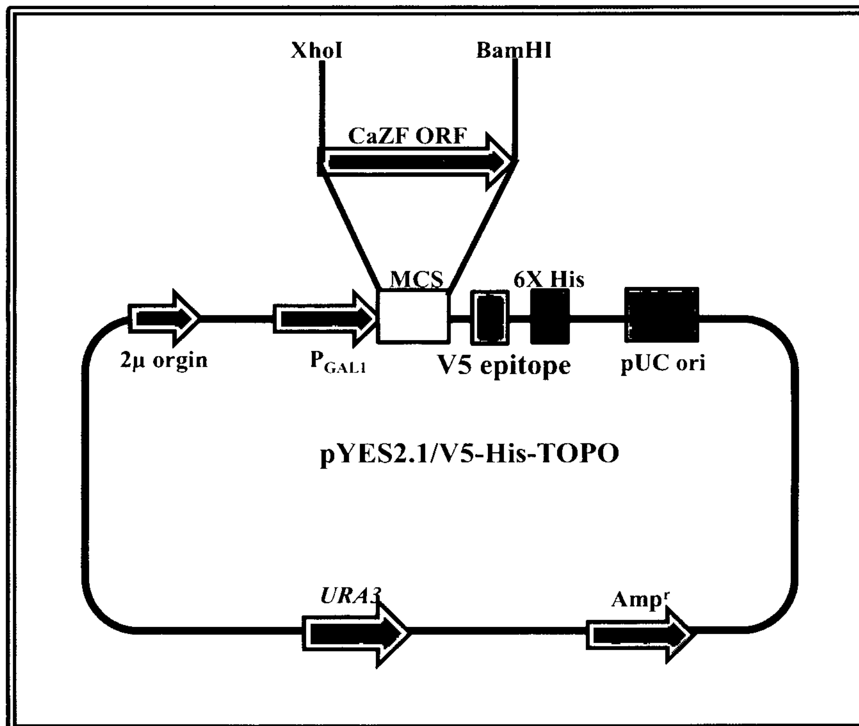


Figure 6.1. Schemetic representation of strategy used for *CaZF*-ORF cloning in plant transformation vector pYES2.1/V5-His-TOPO. The *CaZF* ORF was amplified with a pair of primers having *XhoI* and *BamHI* restriction sites in their 5'-flanking regions. The vector and PCR product were subsequently digested and ligated.

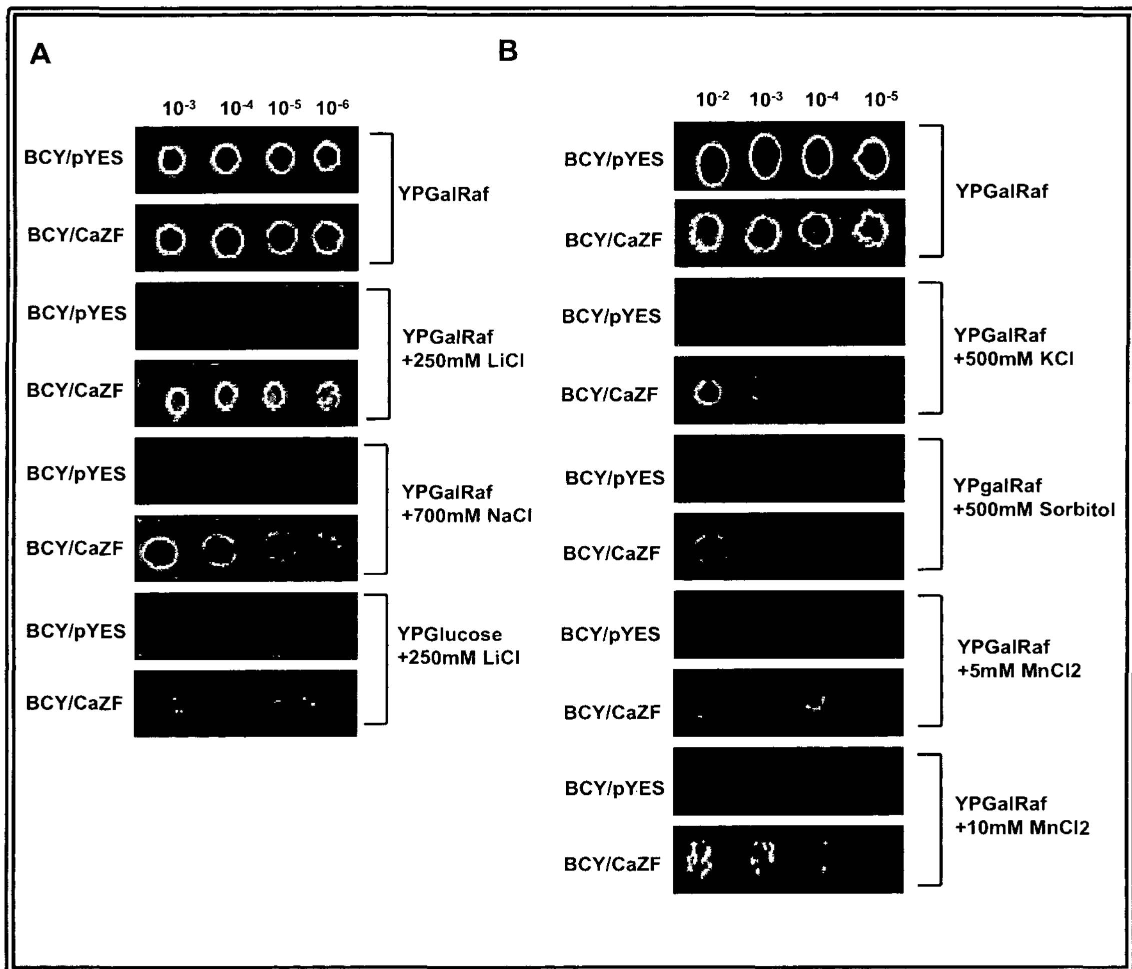


Figure 6.2. *CaZF* provides tolerance to yeast cells against osmotic stress. (*A*) Yeast strain BCY123 harboring only vector (pYES2.1) or CaZF was spotted onto YPGalRaf medium supplemented with 250mM LiCl or 700mM NaCl, or onto YPGlu medium containing 250mM LiCl. Plates without or with salt were shown after incubation at 30^0C for 2d or 4d respectively. **(*B*)** The same strains, as in ***A***, were spotted onto YPGalRaf medium containing either 500mM KCl, 500mM sorbitol or $MnCl_2$ and incubated at 30^0C for 4d.

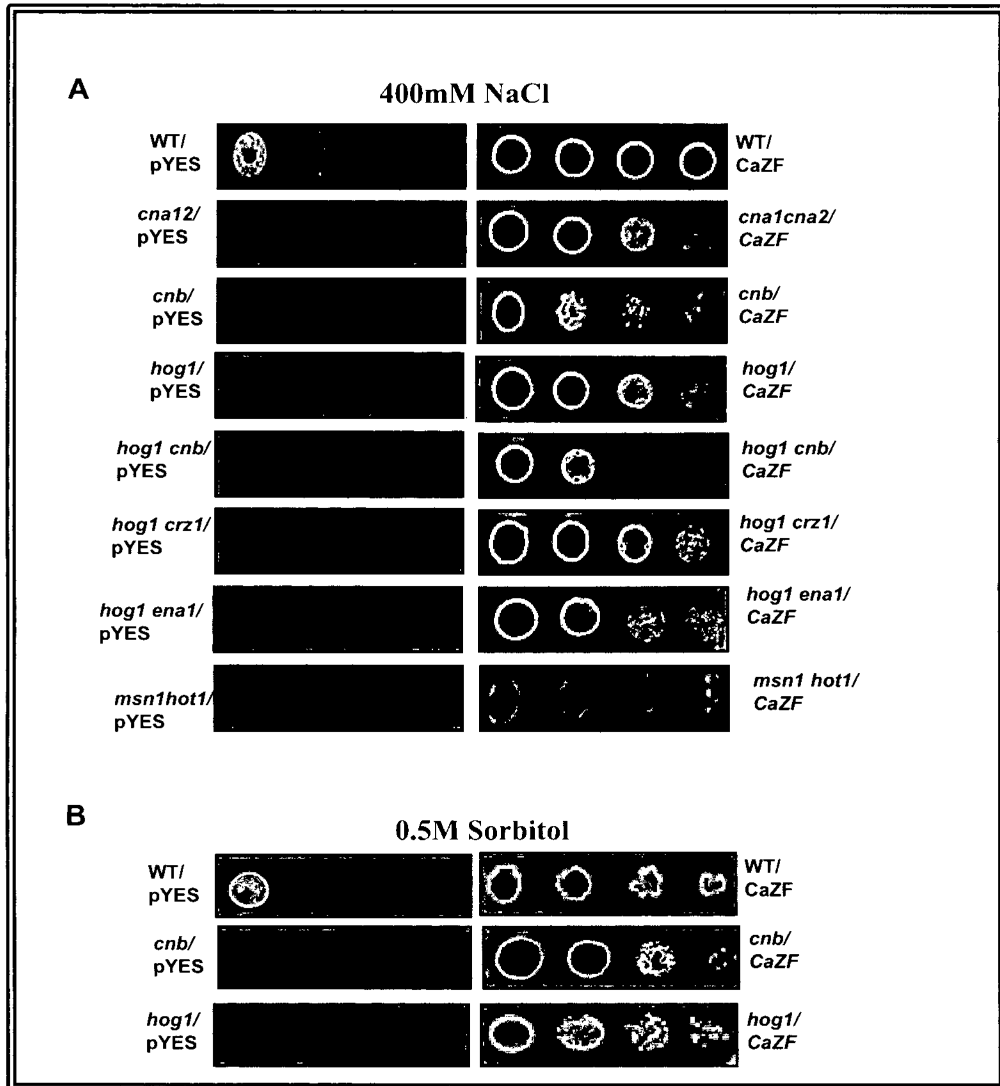


Figure 6.3. CaZF suppresses salt sensitive phenotype of Hog and Calcineurin mutants. ***(A)*** Wild type and mutant BCY123 cells harbouring either only vector (pYES2.1) or CaZF were spotted onto YPGalRaf plates, containing 0.4M NaCl. ***(B)*** Wild type BCY123 and *hog1* or *cnb* mutants expressing CaZF were spotted onto YPGalRaf medium containing 0.5M sorbitol. All spotting experiments were performed, as described under "Materials and Methods".

Expression of *CaZF* suppressed salt sensitivity of *hog1* mutant. As in case of the calcineurin mutants, CaZF provided much less tolerance to the *hog1* strain than it provided for the wild type strain carrying only vector against the ionic and nonionic osmolytes. The result in the solid medium was also supported by the growth rates in liquid medium with 500mM NaCl; as doubling time of *hog1* strain carrying pYES was 12.0±0.15h as opposed to 7.8±0.2h for the *hog1* cells expressing CaZF that was much higher than that required for the wild type cells expressing CaZF (4.8±0.2h). The osmosensitive phenotype of yeast mutant lacking both *Hot1* and *Msn1*, transcriptional activators of the Hog pathway, can also be suppressed by *CaZF* expression (Figure 6.3A) in solid and liquid hypertonic medium as well. Under hypertonic stress Hog1p, through Sko1p, activates expression of *Ena1* (Proft and Serrano, 1999; Garcia-Gimeno and Struhl, 2000; Proft et al., 2001), which is also regulated by Calcineurin through a transcription activator Crz1p, a C2H2 zinc finger protein (Matheos et al., 1997; Stathopoulos and Cyert, 1997; Mendizabal et al., 1998).

As CaZF could suppress osmosensitivity of *hog1* and *cnb* mutants separately, a double mutant *hog1cnb* was constructed to test the functional ability of *CaZF* in absence of both Hog1p and CaN. Surprisingly, *CaZF* expression suppressed salt sensitivity of *hog1cnb*. Analysis of the effect of CaZF expression in these mutants of HOG and CaN pathways mutants suggests that CaZF mediated suppression of osmosensitivity involves a pathway(s) that is independent of but additive to Hog1p and CaN regulated pathways. Simultaneously, reduced growth rate of CaZF expressing mutant cells in comparison to the wild type cells expressing the plant gene also evokes a possibility that *CaZF* may require both the pathways to function in its full strength. Nevertheless, the data presented above suggests that hyperosmotic-adaptation pathway (s) independent of Hog1p and calcineurin exists in yeast.

6.2.4 CaZF induces expression of HOG and Calcineurin regulated genes

We have shown that CaZF can enhance hyperosmotic stress tolerance in budding yeast and it can suppress the osmosensitive phenotype of the mutants lacking Hog1p and/or CaN activities. We further investigated whether CaZF expression has any influence on expressions of the genes those are regulated by HOG pathway and involved in glycerol production and damage repair. We analyzed in wild type and *hog1* background, expressions of two genes, *GPD1* and *GPP2*, involved in glycerol synthesis and two general stress response genes *CTT1* and *HSP12*, predominantly controlled through STRE. Expressions of all the four genes, with different expression kinetics, in the vector control

wild type are enhanced quickly after the salt stress and remained expressed even after 3h (Figure 6.4A). Expression of CaZF did not show any significant effect on the expression of these genes under control condition except an increase in *CTT1* expression. However, under salt stress, expression of *CTT1* and of *HSP12* throughout the course of experiment were higher in CaZF expressing cells, suggesting CaZF function in yeast is not constitutive and it requires some stress-induced pathway (s) for its function. As expected *hog1* mutation caused significant reduction in expression of all these genes. Surprisingly, *hog1* mutant harboring CaZF induced expression of all these genes almost at the level of CaZF expressing wild type strain in response to salt stress. HOG pathway regulated genes were shown to recruit Hog1p at their promoter for the osmotic stress-mediated expression. Salt induced expression of these genes in absence of Hog1p suggests that either CaZF along with some hog1-independent factors is directly activating the expression of these genes or there exist a Hog1p-independent salt inducible pathway in yeast, which is activated by CaZF under salt stress and ultimately causing expression of these genes. CaZF-regulated Hog1p-independent expression of these genes though is not sufficient for providing osmotolerance to the extent as with the wild type background indicating that Hog1p is indispensable for a part of the osmoadaptation mechanism.

Expressions of the genes mentioned so far are also regulated by complex mechanisms, which are not related with HOG pathway and they are also involved in other stress responses (Hohmann, 2002). Therefore, we have analyzed a comparatively simpler expression system that is exclusively controlled by Hog1p. As reported earlier (Zhao et al., 1994), *STL1* (a gene encoding a putative hexose transporter) expression is completely abolished in *hog1* and *hot1* mutants in response to salt stress. Hog1p and Hot1p are recruited in an interdependent manner on *STL1* promoter during acute salt stress as supported by chromatin immunoprecipitation (Proft et al., 2006). In this study, expression of *STL1* was undetectable in absence of salt stress in wild type cells with or without CaZF expression, again supporting the data that the plant protein is not constitutively active in yeast. In presence of 500mM NaCl, *STL1* expression was quickly increased with a peak at 20min at the experimental condition (Figure 6.4B). Expression of CaZF had no significant effect on *STL1* expression in wild type cells in presence of salt. As expected *STL1* expression was undetectable in *hog1* mutant, however, under salt stress CaZF in *hog1* mutant not only induced *STL1* expression to the extent as it did in wild type background, the expression peak was shifted to 10min; the shifting of peak of *STL1* expression was also evident in *msn1hot1* double mutant, where the expression at 10min

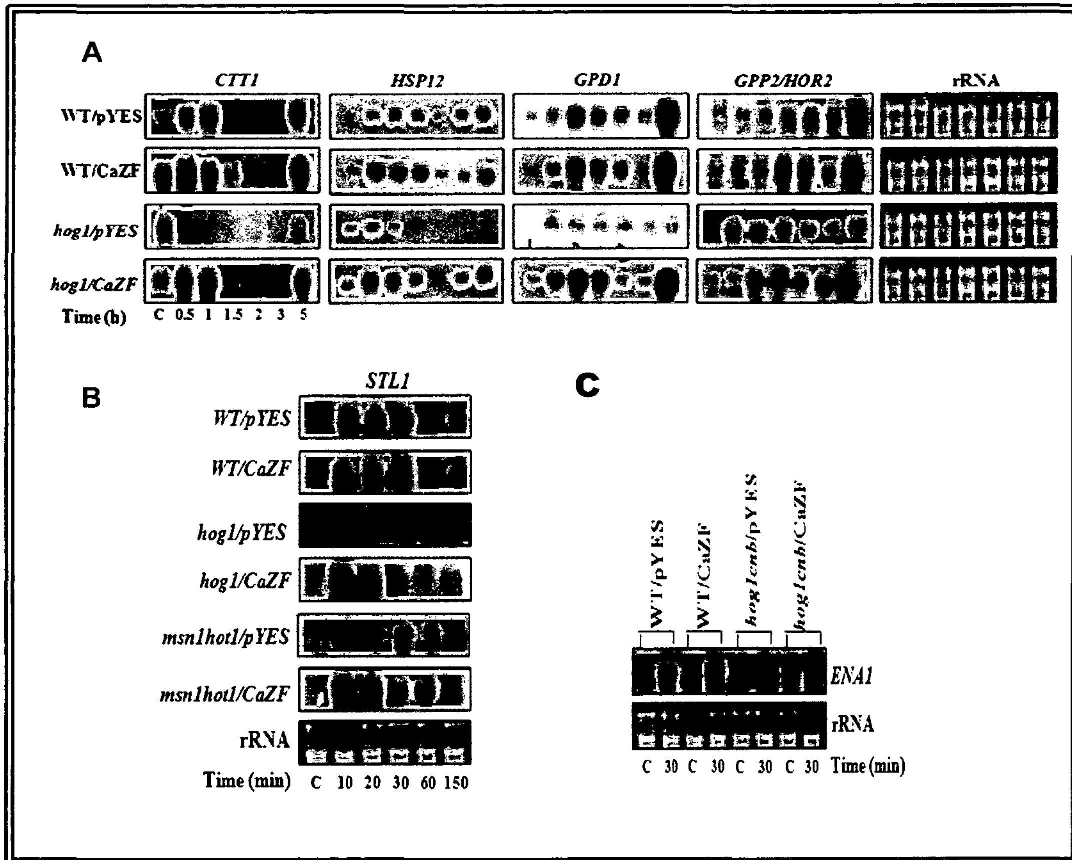


Figure 6.4. Effect of CaZF on stress-responsive gene expression. Wild type or mutant cells as mentioned harboring either only vector (pYES2.1) or CaZF were grown in YPGalRaf medium and treated with 500mM NaCl for mentioned period of time. Northern analysis was performed with probes (*A; CTT1, HSP12, GPD1, GPP2, **B**; STL1, C; ENA1*) representing genes mentioned in the figure. Probe preparation and hybridization was done as described under "Materials and Methods".

was comparable to that at 20min. Interestingly, CaZF-mediated *STL1* expression persisted for relatively shorter time period in *hog1*. Induction of *STL1* expression in response to salt stress in *hog1* and *msn1hot1* mutants confirmed that CaZF functions in a salt stress-dependent but Hog1p-independent manner to activate the expression of Hog1p-regulated genes.

As CaZF could suppress salt sensitivity of *hog1cnb* double mutant we intended to analyze *ENA1* expression, which is regulated independently by both Calcineurin and Hog1p, in CaZF expressing cells (Figure 6.4C). In wild type and mutant background CaZF did not alter steady state low expression level of ENA1 transcript in control condition. Expression of *ENA1* was increased rapidly after exposure to salt in wild type cells and that is further enhanced by more than 1.5 fold in cells harboring CaZF. *ENA1* expression was hardly detectable in *hog1cnb* cells and that is slightly enhanced in response to salt. However, in CaZF expressing mutant cells *ENA1* transcript was accumulated at an equivalent level of wild type cells. To test whether *ENA1* gene product is essential for CaZF function, it was expressed in *hog1ena1* double mutant. Figure 6.3A shows that CaZF expression suppressed salt stress sensitivity not only of *hog1cnb* but also of *hog1crz1* and *hog1ena1* double mutant indicating ENA1 is not essential for CaZF function.

6.2.5 CaZF induces glycerol accumulation

To further clarify whether the effects of CaZF on HOG and CAN-pathway gene expression described above are relevant for a functional osmotic stress response; we estimated glycerol production during or after stress exposure. Figure 6.5 shows that in control condition mutation of the HOG pathway genes and CaZF expression had no influence on constitutive level of total glycerol. Mutant lacking *Hog1* did not stimulate glycerol production in response to stress even at the later stage, while *msn1hot1* double mutant started accumulating glycerol later in stress. However, irrespective of genetic background CaZF enhanced production of glycerol in response to stress though comparatively less in the mutant strains than in the wild type cells corroborating the comparative osmotic tolerance levels of the wild type and the mutant strains expressing CaZF. This result shows that CaZF not only increases the expression of HOG-pathway genes, but also activates the functional osmotic stress response to salt stress in absence of *Hog1*.

6.2.6 CaZF directly binds to STRE and activates CTT1 promoter

CTT1, HSP12, GPD1 and *GPP2,* the well-studied general stress response genes regulated by HOG pathway possess stress tolerance responsive elements in their upstream activating sequence. Msn2p and Msn4p, two C2H2 zinc finger proteins bind to STRE of *CTT1* and *HSP12* to activate their expressions. To determine the mechanism of action of CaZF in yeast we, therefore, tested the ability of CaZF to bind STRE. CaZF protein fused to glutathione-S-transferase (GST) was purified from bacteria and used for gel mobility shift assay using a radiolabeled probe derived from *CTT1* promoter having tetramer of STRE core element (AAGGGG). Figure 6.6A clearly shows that CaZF bound to STRE in a sequence specific manner as replacement of a 'G' residue with 'A' residue in the core element of the probe (M1) totally abolished the binding while another replacement outside the core element (M2) maintained the binding efficiency.

We have shown that expression of CaZF was able to induce expression of *CTT1* (and three other STRE-containing genes) in *hog1* background. Therefore, we tested the ability of CaZF to activate *CTT1* promoter in wild type and *hog1* mutant. 800bp (-137 to -937) upstream activating sequence including the translation start codon of *CTT1* was amplified and inserted before *LacZ* reporter gene to regulate its expression. Wild type and *hog1* yeast strains were co-transformed with the reporter construct and CaZF-expressing plasmid and activity of LacZ was assayed. Figure 6.6B shows that CaZF induced CTT1 expression by more than 50 fold in absence of salt and almost 150 fold of the basal level in presence of salt in the wild type strain. In *hog1* background the inductions were 10 and 60 folds in absence and in presence of salt stress respectively. A CaZF deletion construct lacking its transactivation domain could not activate the *CTT1* promoter and a *CTT1* promoter construct with mutated STRE was not activated by expression of CaZF. This result demonstrated that CaZF was able to activate yeast stress tolerance responsive element in Hog1-independent manner by directly binding to it.

6.2.7 Requirement of CaZF C-terminal domain for salt tolerance

Transactivation assay in yeast determined that the N-terminal domain of CaZF is responsible for its transactivation property and accordingly removal of that domain made CaZF unable to provide salt-tolerance. To investigate whether the C-terminal domain has any role in its activity, we made serial deletion constructs from the C-terminal end of the protein. Removal of last 22 amino acids did not make any difference in the activity of CaZF. However, further removal of 33 amino acids totally abolished the capability of

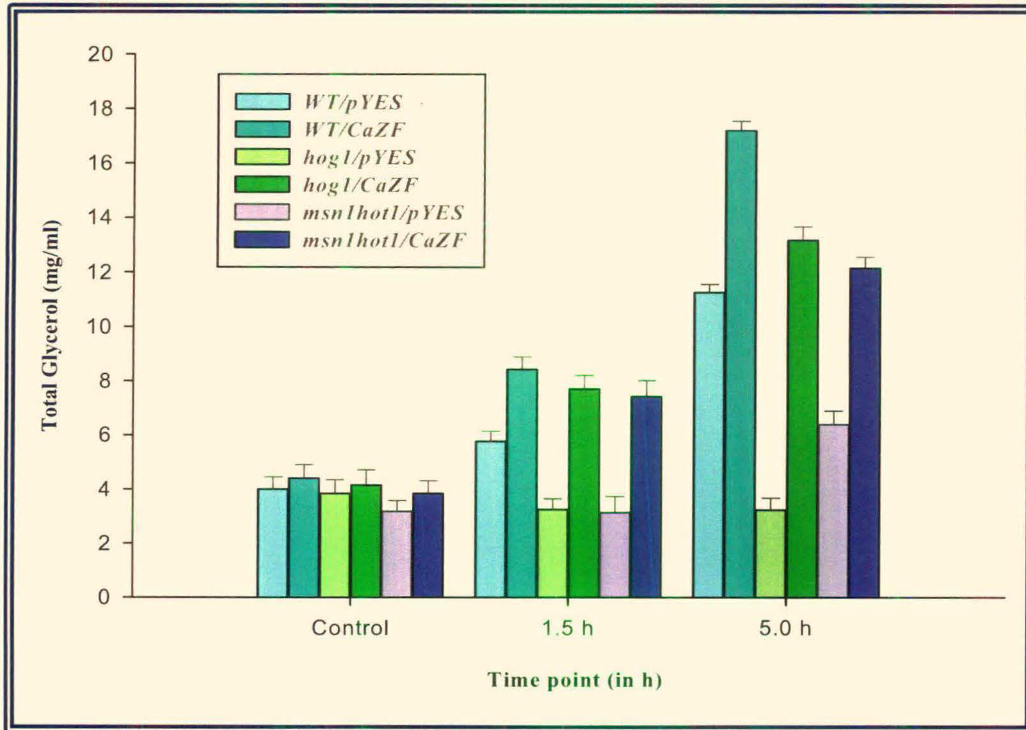
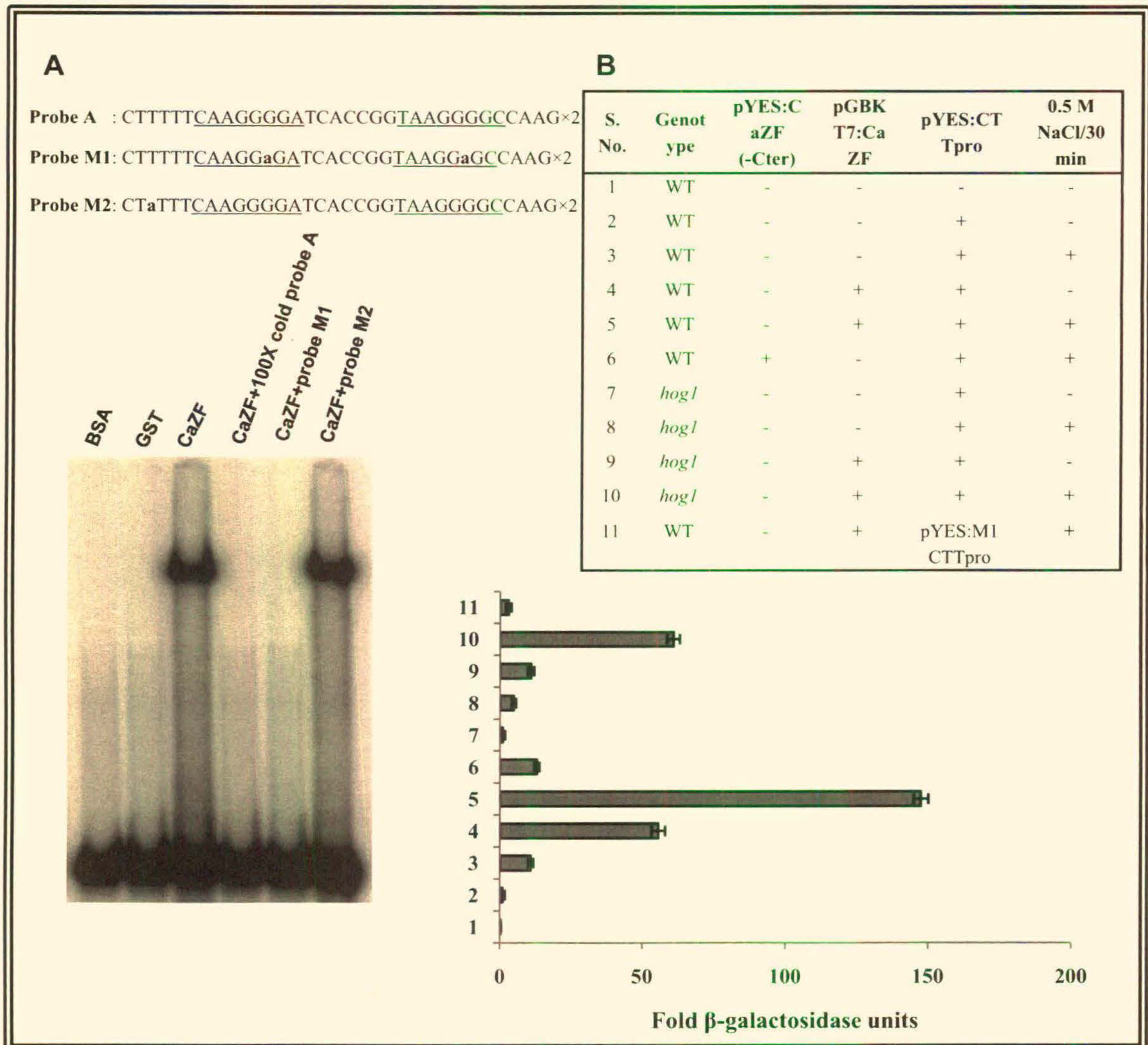


Figure 6.5. Effect of CaZF expression on accumulation of glycerol in wild type and mutant yeast cells in response to salt treatment. Total glycerol [(mg/ml); equivalent to Triloen content] was estimated in wild type and mutant cells carrying either only vector (pYES2.1) or expressing CaZF after treatment with sodium chloride for mentioned time period in the figure.



S. No.	Genotype	pYES:CaZF (-Cter)	pGBKT7:CaZF	pYES:CTTpro	0.5 M NaCl/30 min
1	WT	-	-	-	-
2	WT	-	-	+	-
3	WT	-	-	+	+
4	WT	-	+	+	-
5	WT	-	+	+	+
6	WT	+	-	+	+
7	*hog1*	-	-	+	-
8	*hog1*	-	-	+	+
9	*hog1*	-	+	+	-
10	*hog1*	-	+	+	+
11	WT	-	+	pYES:M1 CTTpro	+

Figure 6.6 *CaZF* directly binds to STRE and activates *CTT1* promoter. ***(A)*** Gel-shift assay demonstrating that CaZF binds to the STRE sequence containing probe. Either wild-type or mutant versions (M1 and M2) of STRE are used. STRE sequences are *underlined* and modified bases are in *bold small case letters*. Recombinant CaZF protein was expressed in *E. coli* DH5α as GST-fused proteins and purified by GST-agarose columns. The probes (1 ng) used in all reactions were ^{32}P-labeled dimers of the oligonucleotides shown in *top panel*. ***(B)*** Transactivation assay of CTT1pro-*LacZ* construct by CaZF. Full length or truncated CaZF proteins and LacZ reporter gene fused to CTT1 promoter fragment or its mutant were cointroduced in wild type (WT) or *hog1* BCY123 yeast strains. The transformed yeast strains were treated with/without 0.5M NaCl for 30min. Activity of β-galactosidase of each sample (average of three independent transformants) as mentioned in table (*lower panel*) was determined and presented in the form of fold induction in activity (*right panel*).

CaZF to provide any salt-tolerance (Figure 6.7). Altogether, these results suggest importance of the C-terminal domain with acidic and basic stretches in CaZF function.

6.3 Discussion

The gene of this study encodes a C2H2 zinc finger protein that is ubiquitous in eukaryotes. A number of stress-induced plant proteins of this family have been studied. They are involved in a variety of physiological processes and encode diverse functions as transcription activators or repressors without showing much structural variability. Several reports describe use of yeast mutants to screen and characterize plant salt tolerance determinants (Sakamoto et al., 2004; Moretti et al., 2006). As yeast and plant stress tolerance systems share quite similar pathways we intended to use yeast salt-sensitive mutants for the characterization of *CaZF*.

In order to understand the functional mechanism we have expressed the *CaZF* cDNA in budding yeast, as this model system (Lippuner et al., 1996) is being deployed because of easy manipulation genes and close functional similarity of osmotic stress response pathways (Serrano and Gaxiola, 1994; Zhu et al., 1997; Hasegawa et al., 2000). A number of examples are available where salt-tolerance determinant plant genes are screened using CaN mutants (Anderson et al., 1992; Peng and Verma, 1995; Lippuner et al., 1996; Quintero et al., 1996; Piao et al., 1999), however, example of plant genes; particularly transcription factors suppressing osmosensitivity of *hog1* mutant is rare. *ASR1* from tomato though provides osmotolerance to *hog1* mutant and binds to DNA; its sequence shows similarity to late embryogenesis protein family rather than to any transcription factor. *ASR1* expression is growth inhibitory in wild type background. In any case, *CaZF* presents a unique example of a plant gene complementing salt-sensitivity of *hog1cnb* double mutant. Moreover, *CaZF* also enhanced growth in presence of sorbitol, showing its ability as a general osmoprotectant and its function is not restricted to a genetic background.

Both HOG and CaN pathways function through a number of transcription activators of zinc finger family of proteins e.g. Msn2p/Msn4p, Sgd1p and Crz1p/Tcn1p. Among them Msn2p/Msn4p and Crz1p belongs to C2H2 zinc finger family (Matheos et al., 1997; Stathopoulos and Cyert, 1997). Despite Msn2p/Msn4p and Crz1p are much larger proteins and have no overall sequence similarity with CaZF, the plant protein most likely is able to functionally substitute both these transcription activators. This is quite evident in case of *CTT1*, *HSP12* and *ENA1* expression in CaZF expressing wild type cells in stress. Expressions of *CTT1* and *HSP12*, predominantly regulated by Msn2p/Msn4p

through STRE (Schuller et al., 1994; Rep et al., 2000) and that of *GPD1* and *GPP2*, regulated by Hot1p and Msn1p (Albertyn et al., 1994; Rep et al., 1999) and not dependent on functional STREs, reveals a striking difference when compared in CaZF-expressing wild type cells in presence of salt. In wild type cells CaZF further induced expressions of *CTT1* and *HSP12* but not of *GPD1* and *GPP2* in salt stress. Thus, CaZF function is not redundant rather additive to Hog1p-mediated expression of these C2H2 zinc finger and STRE-regulated genes. In *hog1* background, under salt stress CaZF is able to induce expression of these genes to a similar extent as Hog1p does in the wild type cells. This also seems to be the mechanism for Crz1p-regulated gene *ENA1*. *ENA1* expression is partially regulated by Crz1p (Stathopoulos and Cyert, 1997; Proft and Serrano, 1999). Accordingly, CaZF further enhanced *ENA1* expression by only about 1.5 fold in wild type background under salt stress. In *hog1cnb* background it was found that CaZF was able to activate expression of *ENA1* independent of Hog1p and CaN to the same level as those regulatory enzymes do in wild type cells. While Crz1p requires CaN-mediated post-translational modification to be active (Matheos et al., 1997), CaZF does not require that. However, CaZF seems to require Hog1p and Calcineurin-independent but stress-dependent post-translational modifications and/or protein-protein interaction for its full functional ability.

Whereas, *GPD1*, *GPP2* and *STL1* genes regulated predominantly by Hot1p, which is not a C2H2 zinc finger protein; influence of CaZF on their expression was evident only in absence of *Hog1* and *Hot1*. *GPD1* and *GPP2* expressions are also regulated by other proteins (e.g. Rap1p for *GPD1*) (Eriksson et al., 2000) and irrespective of combination of gene knockouts involving Hog1p and Hot1p; *GPD1* and *GPP2* remains salt inducible. But CaZF seems to induce expression of these genes by similar mechanism used by Hog1p and Hot1p. The reason being it induces expression of *STL1*, which is exclusively regulated by Hog1p and Hot1p (Proft et al., 2006). There is possibility that CaZF utilizes other proteins and/or other salt inducible pathways to mimic Hog1p-regulated activation, but cannot totally replace Hog1p as *STL1* expression kinetics differs in presence and in absence of Hog1p. CaZF seems to require these genes for salt-tolerance because their end product, the glycerol synthesis in the mutant cells harboring the plant gene nicely correlates their growth in presence of salt.

ENA1, a P-type ATPase, is the first member of cluster of four to five genes encoding very similar proteins and plays a major role in detoxification of sodium and lithium cations. A complex mechanism involving HOG and CaN pathways regulates *ENA1*

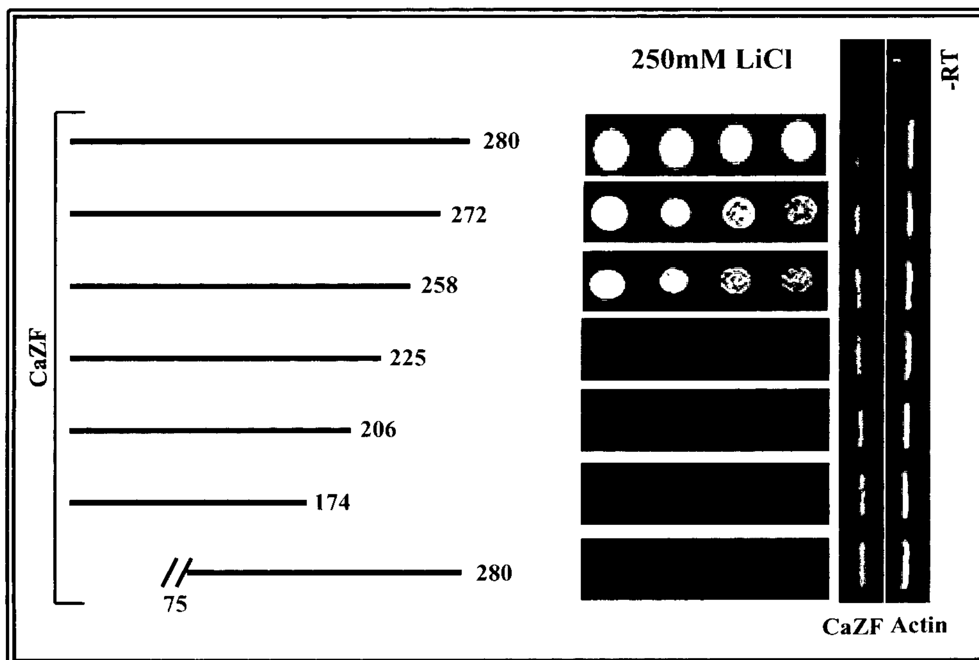


Figure 6.7. Determination of salt tolerance ability of CaZF deletion constructs. BCY123 cells transformed with C-terminal deletion constructs of CaZF as shown were tested for their ability of providing salt tolerance against 250mM LiCl. All spotting experiments were performed as described under "Materials and Methods".

induction in response to salt. In this study *ENA1* expression found to be increased marginally in *hog1cnb* cells in response to salt suggesting these two enzymes (Hog1p and CaN) are the major regulators of salt-responsive *ENA1* expression. Expression of CaZF confers salt tolerance and induces *ENA1* expression in *hog1cnb* suggests that CaZF functions by activating Na^+/Li^+ extrusion system and at the same time also by mechanisms not involving Ena1p as it enhanced salt tolerance of *hog1ena1* mutants. *ENA1*-independent mechanism may involve other cation efflux systems, such as *NHA1* and *SNQ2* or K^+-influx systems like *TRK1* (Mendoza et al., 1994; Mulet et al., 1999; Obata et al., 2007). Similar *ENA1*-independent salt tolerance was also provided by other plant proteins e.g. STO and SLT1 (Lippuner et al., 1996; Matsumoto et al., 2001). Most likely CaZF does not function at the level of Hog1p; rather it works downstream to it. The reasons being, CaZF structurally resembles a transcription factor and expression of CaZF is not toxic like expression of ASR1. ASR1 expression in control condition was growth-inhibitory like a constitutively active Hog1p mutant (Moretti et al., 2006; Maskin et al., 2007).

In conclusion, expression of CaZF in yeast provides evidence that at least some of the crucial stress tolerance determining genes, which are regulated by Hog1p MAP kinase, CaN protein phosphatase and their target transcription factors during osmotic stress, can also be activated to the same extent in absence of their regulatory enzymes/transcription activators. Activation of those genes by a heterologous gene leads to production of the HOG pathway end product i.e. glycerol. In at least one previous instance it was shown that a plant gene (*ASR1*) can induce synthesis of glycerol in salt stress in absence of Hog1p (Moretti et al., 2006; Maskin et al., 2007). The level of dependence of yeast cells on *Hog1* differs with intensity and extent of stress conditions (Posas et al., 2000). Therefore, influence of Hog1p on expression of genes is relative to the experimental condition. We have analyzed gene expression and glycerol estimation at 0.5M NaCl and so there may be a possibility that in this experimental condition influence of Hog1p is relatively less in providing salt-adaptation. Since, CaZF is salt inducible and may work in conjunction or in parallel to Hog1p and CaN, it can thus be strongly predicted that CaZF can be used a substrate for post-translational modification and/or target for protein-protein interaction. The possible explanations of the above finding are; firstly it can induce gene expression and consequently provide growth advantage only in presence of stress; and secondly it requires its C-terminal domain, which is not required for its transcription activation property, for its function.

A close *in silico* analysis of the amino acid sequence (Figure 6.8) reveals that this domain contains apart from alternate stretches of basic and acidic amino acids, a potential site for protein kinase C phosphorylation (SKK) and a potential site for cAMP/cGMP-dependent protein kinase phosphorylation (KKKS). In yeast cAMP-dependent protein kinase A (PKA) is an essential component of general stress response pathway. In normal growth condition PKA phosphorylates C2H2 zinc finger proteins Crz1p, Msn2p and Msn4p to prevent their nuclear localization. Upon inactivation of PKA or activation of the phosphatase calcineurin during stress those proteins get dephosphorylated and are accumulated in the nucleus to activate their target genes (Smith et al., 1998; Cyert, 2003; Kafadar and Cyert, 2004). CaZF, being a C2H2 zinc finger protein may be regulated by the same mechanism and, thereby, stress-mediated activation of CaZF-function can be explained.

Pattern-ID: **ASN_GLYCOSYLATION**
Pattern-DE: N-glycosylation site
Pattern: N[^P][ST][^P]
138 NDTV
187 NNSN
196 NNSS

Pattern-ID: **CAMP_PHOSPHO_SITE**
Pattern-DE: cAMP- and cGMP-dependent protein kinase phosphorylation site
Pattern: [RK]{2}.[ST]
35 RKRS
126 RKSS
241 KKKS

Pattern-ID: **PKC_PHOSPHO_SITE**
Pattern-DE: Protein kinase C phosphorylation site
Pattern: [ST].[RK]
38 SKR
124 SHR
214 SHR
240 SKK
264 TAK

Pattern-ID: **CK2_PHOSPHO_SITE**
Pattern-DE: Casein kinase II phosphorylation site
Pattern: [ST].{2}[DE]
48 TEEE
128 SSLE
202 TISE

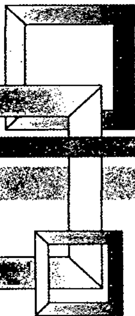
Pattern-ID: **MYRISTYL**
Pattern-DE: N-myristoylation site
Pattern: G[^EDRKHPFYW].{2}[STAGCN][^P]
64 GNNRNN
119 GGHKAS
164 GQALGG
178 GINHNN
200 GITISE
206 GAAASS
236 GGGDSK

Pattern-ID: **ZINC_FINGER_C2H2**
Pattern-DE: Zinc finger, C2H2 type, domain
Pattern: C.{2.4}C.{3}[LIVMFYWC].{8}H.{3.5}H
105 CTVCNKAFPSYQALGGHKASH
154 CSICHKSFSTGQALGGHKRCH

Pattern-ID: **CYTOCHROME_C**
Pattern-DE: Cytochrome c family heme-binding site signature
Pattern: C[^CPWHF][^CPWR]CH[^CFYW]
154 CSICHK

Figure. 6.8. Prosite Motif Search. *In silico* analysis of CaZF amino acid sequence reveals important domains of the protein as mentioned.

Chapter 7



Functional characterization of CaZF in constitutively expressing transgenic tobacco plants

7.1 Introduction

Environmental stresses such as cold, salinity and drought are the major obstacles affecting plant growth and crop productivity. To cope with these stresses, plants undergo from physiological and developmental adaptations to cellular metabolism and gene expression changes. Changes in gene expression during stress responses have been extensively studied (Bray, 1993; Thomashow, 1998; Shinozaki and Yamaguchi-Shinozaki, 2000; Zhu, 2001). Many genes have been demonstrated to respond to abiotic (drought and salinity) stress in various plant species. Among these, some stress-inducible genes have been overexpressed in transgenic plants, producing a stress-tolerant phenotype of the plant, confirming that the gene products function in stress tolerance. So far, researches were focused on isolation and functional analysis of stress-inducible genes, and these genes have been classified into two major groups: the effector genes and the regulator genes. The products encoded by the former directly protect plant cells against stresses, but those encoded by the latter function in regulating gene expression and signal transduction (Shinozaki and Yamaguchi-Shinozaki, 2000). In addition, the existence of several drought- and cold-regulatory pathways that are controlled by multiple transcription factors (TFs) has been reported (Seki et al., 2001; Seki et al., 2002).

The transcription control of stress-inducible genes is crucial to plant responses to environmental stresses. To date, several kinds of transcription factors involved in environmental stresses were functionally characterized. The importance of TFs in stress tolerance has been demonstrated by manipulating the expression of a gene encoding a TF in transgenic plants. For example, constitutive overexpression of the TF CBF1/DREB1A resulted in *Arabidopsis* plants with enhanced tolerance to cold stress (Nakashima et al., 2009); inducible expression of *Arabidopsis* CBF3/DREB1B, DREB1C also improved tolerance to drought, salt and cold stresses (Kasuga et al., 1999). Overexpression of *DREB1A, DREB1B, and DREB1C* genes results in multiple biochemical changes that are associated with cold acclimation (Gilmour et al., 2000; Gilmour et al., 2004). Examples of such changes in plants overexpressing DREB1A included elevated levels of proline and total soluble sugars such as sucrose, raffinose, glucose, and fructose. A side effect of the overexpression of *DREB1A, DREB1B, or DREB1C* in transgenic *Arabidopsis* is dwarfism (Liu et al., 1998; Kasuga et al., 1999). Similarly, the development of dwarf phenotypes was also found in transgenic tomato overexpressing *Arabidopsis DREB1B* (Hsieh et al., 2002). Overexpression of CAP2, an AP2/ERF family protein of chickpea also enables plant to be more tolerant to drought and salinity (Shukla et al., 2006).

Furthermore, many other TFs were identified to be involved in stress signal transduction, such as drought-inducible bZIP transcription factors AREB/ABF (Uno et al., 2000); AtMYC2/AtMYB2 from *Arabidopsis* (Abe et al., 2003) and cold induced basic Helix-Loop-Helix protein OsbHLH from rice (Wang et al., 2003). The Cys-2/His-2-type zinc finger, also called the classical or TFIIIA-type finger, is one of the best-characterized DNA-binding motifs found in eukaryotic transcription factors. To date, many identified C2H2-type zinc finger proteins have been implicated in developmental processes or responses to abiotic stresses in plants (Takatsuji, 1999; Shoji Sugano, 2003; Chinnusamy et al., 2004; Baier et al., 2006; Chinnusamy et al., 2007; Udvardi et al., 2007). Best known among them is *Arabidopsis* SUPERMAN (SUP), which is involved in the control of cell division in flower whorls (Sakai et al., 1995). Other *SUP*-like genes of *Arabidopsis*, *AtZFP10* and *AtZFP11*, cause dwarfed growth and abnormal leaf phenotypes when overexpressed in tobacco (Dinkins et al., 2002; Dinkins et al., 2003). Petunia *LIF* overexpression also resulted in a dramatic increase in lateral shoots and reduced plant height. Functional study of zinc finger protein, MIF1 (encoding putative small zinc finger proteins that are highly conserved in plants) by constitutive overexpression using the 35S promoter revealed pleiotropic defects of plant development, including reductions in size of all organs, morphological alterations of the cotyledons and flowers, and ectopic root hairs on the hypocotyl and cotyledons. Both cell division and elongation were inhibited by overexpressed MIF1. Other example includes a C2H2-type zinc finger protein from soybean. SCOF-1 is ABA- and cold-inducible and can enhance the binding efficiency of another bZIP transcription factor SGBF-1 to ABRE (Kim et al., 2001). Constitutive overexpression of SCOF-1 in *Arabidopsis* plants induced COR gene expression and enhanced cold tolerance of transgenic tobacco and *Arabidopsis* plants. A cold, salt-inducible zinc finger STZ/ZAT10 from *Arabidopsis* can complement yeast calcineurin mutants and increase salt tolerance of transgenic *Arabidopsis* (Lippuner et al., 1996; Sakamoto et al., 2000). Transgenic *Arabidopsis* plants overexpressing *STZ* showed dwarf phenotype and growth inhibition (Sakamoto et al., 2004). Overexpression of ZPT2-3, a cold-induced C2H2-type zinc finger protein gene from petunia can improve drought tolerance in transgenic plants (Shoji Sugano, 2003). RHL41/ZAT12 mediates light acclimatization response in *Arabidopsis* (Iida et al., 2000). *Medicago sativa* Mszpt2-1 is required for nodule differentiation in nitrogen-fixing roots (Frugier et al., 2000). Recently, a *Medicago truncatula* zinc finger protein Mt-ZIP1 was shown to be regulated by cytokinin, ABA and methyl jasmonate but not by cold (Xu and Ma, 2004).

A change in mineral nutrient availability and heterogeneous distribution of ground water induces a various adaptive mechanisms in plants, among which plasticity of root development is a crucial factor (Gregory, 1998). In several species, root proliferation in nutrient-rich regions leads to an increased ratio of root surface to exposed soil volume, which facilitates the uptake of nutrients (Ludlow and Muchow, 1990). The development of shoot and root tissue in plants is highly coordinated process. Leaf development occurs in conjunction with continuous development of lateral roots to extract more water and nutrient from the soil. In order to achieve maximum extraction of soil moisture the requirements are (a) deep proliferation of root (b) optimum root density through the soil profile and (c) adequate longitudinal conductance in main root (Fisher et al., 1982). With this brief introduction the major objectives of the current chapter are:

1. Raising transgenic tobacco overexpressing *CaZF*.
2. *CaZF* expression analysis of putative transgenic lines.
3. Phenotypic characterization of *CaZF*OX transgenic lines.
4. Cloning of *CaZF* upstream activating sequence.
5. *In vitro* and *in vivo* interaction studies of *CaZF*-UAS with CAP2.

7.2 Results

7.2.1 Overexpression of CaZF in transgenic tobacco

7.2.1.1 Cloning of *CaZF* cDNA in plant binary vector pBI121

The complete ORF of *CaZF* was cloned in *BamHI-SmaI* restriction site of plant expression vector pBI121 (Figure 7.1). The colonies grown on the kanamycin selected plates were first checked by colony PCR using gene specific primers and then by restriction digestion.

7.2.1.2 Agrobacterium mediated transformation of Nicotiana tabaccum

25 d-old tobacco leaf disc were used as explants for transformation. Leaf discs from axenically grown tobacco plants were used as explants for transformation. Leaf disc were infiltrated with *Agrobacterium* strain GV3101 harbouring the pBI121::*CaZF* plasmid. Following co-cultivation, explants were selected on MS medium containing BAP 1μg and NAA 0.1 μg containing kanamycin (50μg/L) and cefatoxim (250mg/L). Regeneration was initiated three weeks later and small sized shootlets originated from explants were transferred to fresh selection medium (Figure 7.2A). The fully-grown plantlets were transferred on to rooting medium with a basal Murashige-Skoog (MS) medium (Figure 7.2B and C). The plants were subsequently transferred to pots containing agropite:vermiculite for hardening in green house. Representative of each, vector control

and *CaZF* transgenic plant is shown in Figure 7.2D. For control experiments, the pBI121 vector was also used to transform tobacco. In both the cases, expression was driven by *CaMV35S* promoter.

7.2.1.3 Confirmations of putative transgenic lines

Integration of the transgene in T_0 plants was tested by PCR-amplification using gene specific primers and genomic DNA isolated from the leaves of the putative transgenic plants. Six-plants were found PCR positive and showed a PCR product of 0.800kb (Figure 7.2E). Further the transgene expression was checked by northern analysis in PCR positive putative transgenic tobacco lines. Two lines which were showing highest and two with lowest *CaZF* mRNA abundance were selected for further study (Figure 7.2F). The plants were grown to maturity and seed setting. Seeds obtained from these lines were further used for the characterization of the transgenic plants.

7.2.2 Phenotypic analysis of putative transgenic lines

6d old T_1 seedlings from two independent lines each of high- and low-expressing transgenic tobacco were compared with the vector transformed tobacco (taken as control) seedlings for morphological phenotype. Seeds of vector control and both the constructs were plated on half strength MS medium and allowed to grow vertically in square plates for six days. No difference was observed between the transgenic lines and the control seeds in the rate and the period taken for germination. However, the T_1 seedling shows number of morphological differences in comparison to vector control plants. Roots of *CaZF* overexpressing (*CaZF*OX) lines exhibited more than two fold (211%) higher rate of root growth than that of control seedlings (Figure 7.3A). The difference in rate of root growth is graphically represented in the Figure 7.3B. To investigate whether longer root phenotype caused by *CaZF* overexpression has altered cellular organization at the root tip, cell positions were visualized by confocal imaging after staining the root tips with a fluorescent dye FM4-64. *CaZF*OX root tips showed no gross deformation in cellular arrangement in comparison to those of vector control. However, *CaZF* roots showed significantly higher number of cells in the apical meristem region. Increased cell number is evident in the epidermal, cortical and stellar layers and that caused extra linings of cell layers in *CaZF*OX roots indicating frequent cell division. In conjunction with enhanced cell division, zone of cell division is much longer in these seedlings (Figure 7.3C). There was more number of lateral roots in the transgenic lines as compared to the vector control plants. 16d-old transgenic seedlings show an average of 2.5 numbers of lateral roots whereas the vector control was having no visibly developed lateral roots (Figure 7.4A).

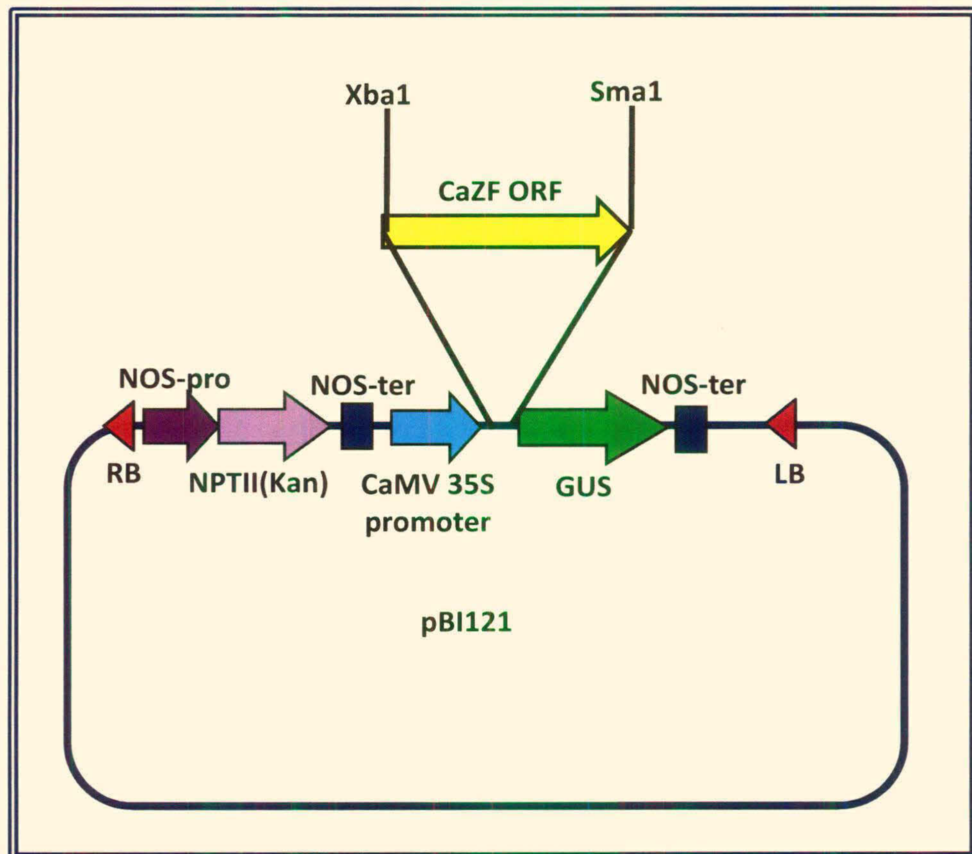


Figure 7.1. Schemetic representation of strategy used for *CaZF*-ORF cloning in plant transformation vector pBI121. The *CaZF* ORF was amplified with a pair of primers having *XbaI* and *SmaI* restriction sites in their 5'-flanking regions. The vector and PCR product were subsequently digested and ligated.

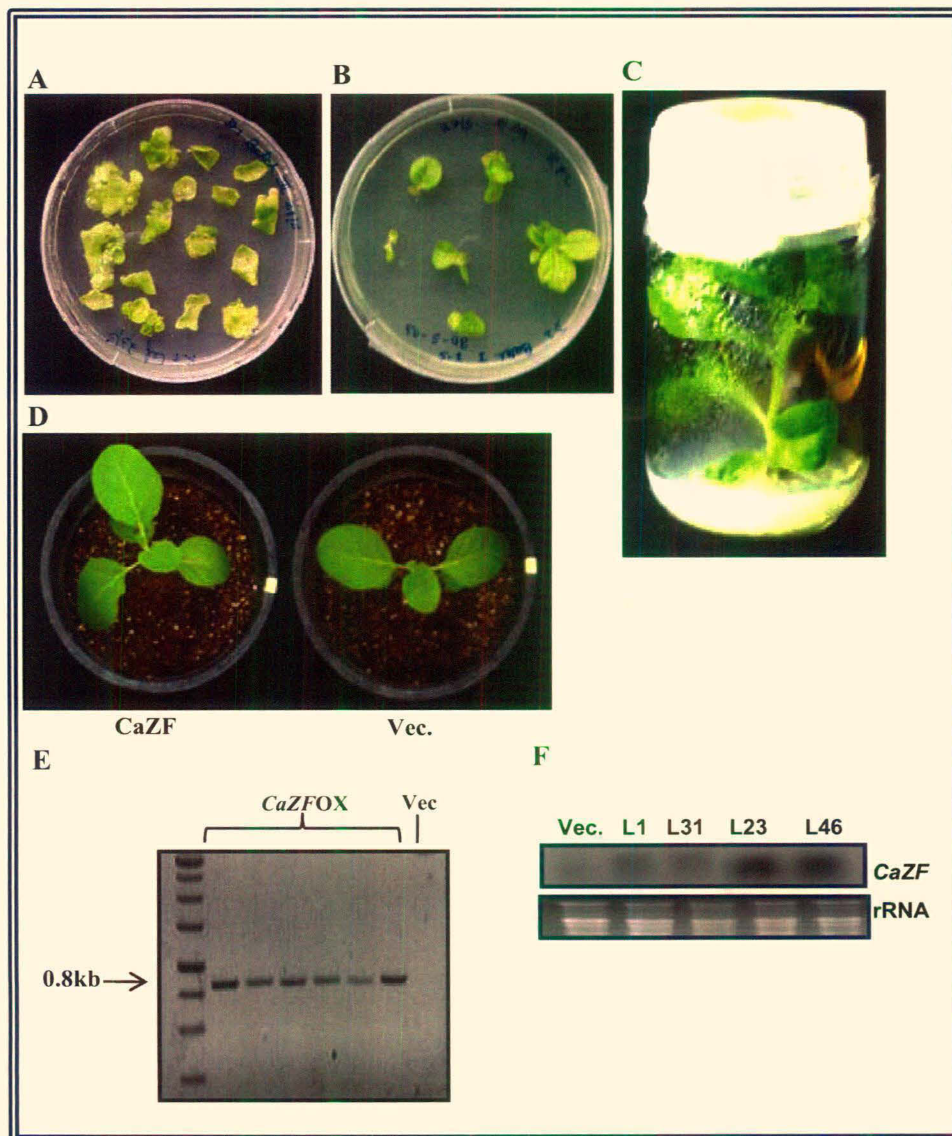


Figure 7.2. Regeneration of *CaZF* overexpressing transgenic tobacco plants. Leaf discs of tobacco were co-cultivated with *Agrobacterium* containing pBI121 (vector control, Vec) or pBI121-*CaZF* , and transferred to selection medium containing kanamycin (50 μg/μl) and cefatoxime (250 μg/μl). (***A-C***) Different stages of regeneration in tissue culture. (***D***) For hardening in green house. (***E***) Genomic DNA PCR for transgene insertion in vector control and transgenic plants. (***F***) Expression analysis of different *CaZF*OX lines by northern blotting. rRNA was taken as loading control.

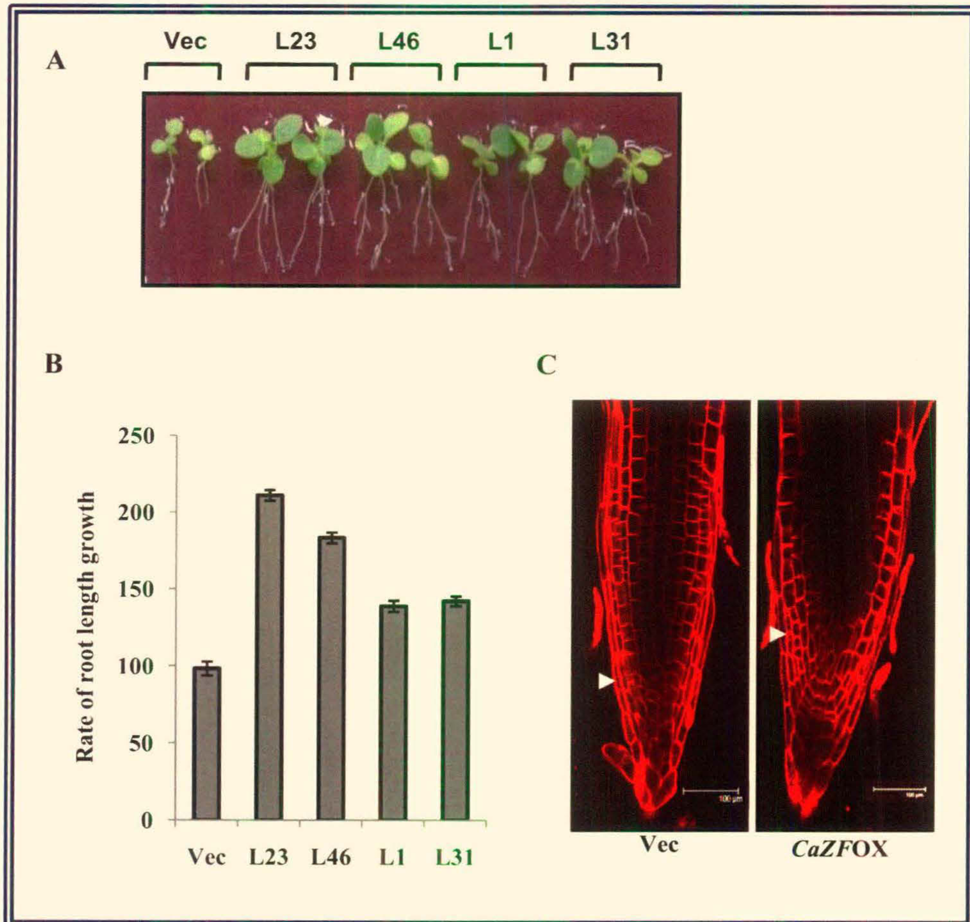


Figure 7.3. Root morphology of *CaZF* overexpressing transgenic lines. (*A*) Root morphology of vertically grown 6-d old tobacco T1 seedlings transformed with pBI121 (vector control) or PBI121:*CaZF*. Two representative lines each of high expressing and low expressing *CaZF*-expressing plants are shown. (***B***) Comparison of rate of root length growth of the vector control and *CaZF*-expressing plants. Averages of three measurements of thirty seedlings each are shown. (***C***) Confocal imaging of root apices from vector control and *CaZF*-expressing tobacco seedlings respectively at equal magnification stained with fluorescent dye FM4-64. Arrow marks the start of elongation zone. Scale Bar, 100μm.

Graphical representation of number of lateral roots in vector control and four transgenic lines are presented in Figure 7.4B. *CaZF*OX lines also showed longer and robust root hair in comparison with the vector control tobacco (Figure 7.4C). Leaf surface area of the transgenic lines was found much bigger than that of the vector control plants. Leaf surface area of vector control and *CaZF*OX transgenic lines were measured using the standard graphical method. Average surface area of the third and fourth leaves (from the bottom) of 10 seedlings (16d after germination in soil) from each transgenic line were measured and found to be about almost three fold of that of vector control (Figure 7.5A&B). Further exploring the reason behind the increase in the leaf area in the transgenic plants, ventral epidermal peel from both side of mid-rib of the leaf base, middle lamina and leaf tip of the wild type and transgenic lines were compared for cell size. Six samples from each leaf and leaves from four plants of each line were taken. Average cell size (from 24 samples of each line) of the ventral epidermal surface of the fourth leaf from the bottom of 16d old transgenic plants was approximately three times of the average cell size from the same area of the vector control leaf; though these results do not exclude effects of transgene expression on the cell division (Figure 7.5C). Similarly average fresh weight of the transgenic seedlings of the same age was almost three fold that of wild type seedling (Figure 7.5D). Increase in the root length, number of lateral roots and leaf size can be correlated with the expression of transgene in the tobacco plant as expression of the transgene in *CaZF*OX lines L1 and L31 is much less compared to that in other two lines, L23 and L46.

7.2.3 Auxin transport in *CaZF* overexpressing lines

Plant hormone auxin regulates a number of cellular and developmental processes of different tissues including root. Root length and lateral root formation are severely compromised in plants defective in auxin transport. Therefore, to check whether the altered root phenotype of the *CaZF*OX lines shows the difference in the polar auxin transportation, basipetal auxin transport at the root tips of the *CaZF*OX lines were compared with that of the vector control seedlings. For the experiment seedlings were grown vertically on half strength MS plates for 6d. To check the transportation, agar block containing radioactive Indole Acetic Acid (IAA); 3-[5(n)-^{3}H] IndolylAceticAcid was placed at the root tips of the seedlings for two hours and then basipetal auxin transport was observed in the root tissue of vector control and *CaZF*OX lines 2mm above the agar block using liquid scintillation counter (LSC). Significant increase in the auxin transport was observed in the *CaZF*OX lines when compared with Vec (Figure 7.6A).

7.2.4 Analysis of early auxin inducible genes

To analyze the activation of auxin response pathway in *CaZF*OX lines, the expression of early auxin inducible genes *IAA4.2* and *IAA2.5* was analyzed in the vector control and transgenic plants. Real-Time-PCR of tobacco *IAA2.5* and *IAA4.2* clearly demonstrated that the constitutive expression of these genes in the transgenic lines is 2-4 folds higher than in vector control. *Actin* has been taken as an internal control for normalization (Figure 7.6B).

7.2.5 Effect of dehydration, salt and heat stress on tissue culture grown CaZF overexpressing lines

To evaluate the effect of dehydration stress on *CaZF*OX and vector control seedlings, seeds were allowed to germinate on half strength MS medium containing 0.3M mannitol. Germination of seeds in mannitol medium was delayed in comparison to the control medium [0.5MS (without mannitol)]. In addition, seed germination of vector control was further delayed in comparison to the *CaZF*OX lines in mannitol medium. After 20d of germination in mannitol medium, though any difference in leaf color was not observed; however, growth rates of *CaZF*OX lines were found to be much higher than vector control seedlings (Figure 7.7A). The response of transgenic tobacco seed germination to mannitol suggests involvement of *CaZF* in osmotic stress tolerance. To assess the effect of high salt on the seed germination/growth of the vector control and T_1 plants overexpressing *CaZF*, surface-sterilized seeds were plated on 0.5MS supplemented with 0.3M NaCl. In the presence of high salt, vector transformed seeds showed almost no germination (only one out of thirty-two seeds in one repeat) until 20d, while on average 85-95% *CaZF* overexpressing T_1 seeds showed germination within 15d. Seeds of vector transformed and *CaZF* overexpressing transgenic plants showed no remarkable difference in germination and growth in 0.5MS (without salt, taken as control plate) (Figure 7.7B).

For studying the effect of heat stress on seed germination/growth of the vector control and *CaZF*OX transgenic lines, surface-sterilized seeds were plated on 0.5MS and incubated at 39°C. Under heat stress, vector transformed seeds showed almost no germination until 10d, while on average 80-90% *CaZF*OX T_1 seeds showed germination within 7d. Vector control and *CaZF* transgenic seeds showed almost same germination/growth behavior when allowed to germinate under normal conditions (Figure 7.7C).

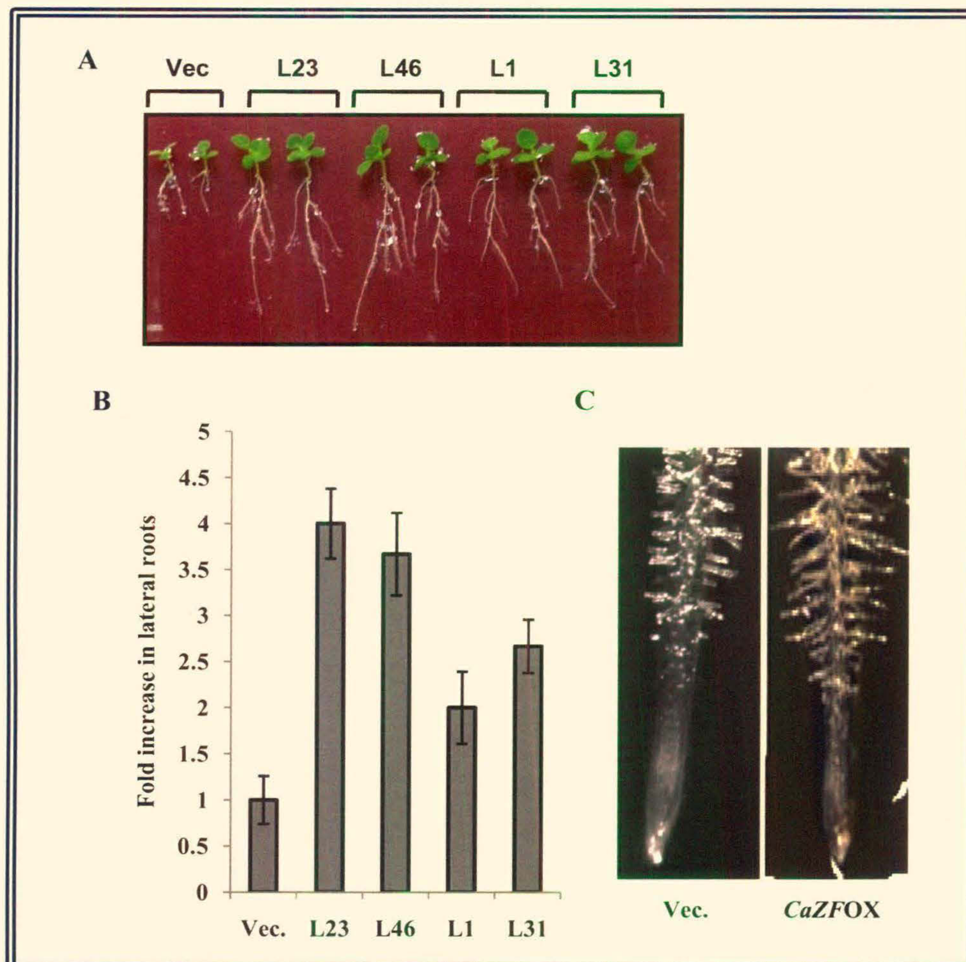


Figure 7.4. Phenotypic analysis of CaZF-overexpressing transgenic lines. (***A***) Morphology of 20-d old soil grown vector control and *CaZF*OX T1 tobacco plants. (***B***) Graphical representation of fold increase in lateral roots of vector control and *CaZF*OX lines (***C***) Stereomicroscope imaging of root apices of vertically grown T1 seedlings of vector control and *CaZF*OX tobacco seedlings.

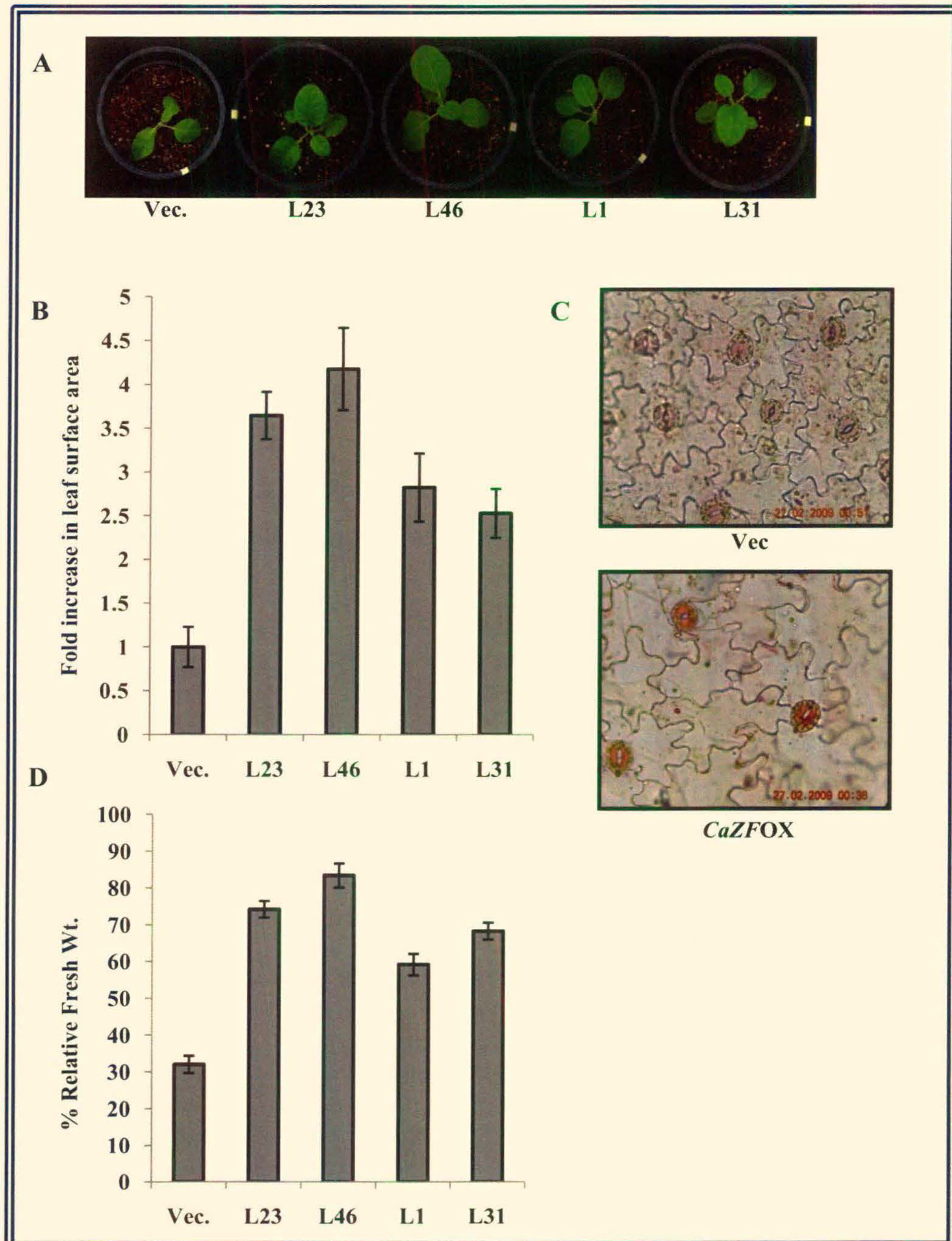


Figure 7.5. Phenotypic analysis of soil-grown representative vector control (Vec) and *CaZF*OX transgenic plants. (***A***) Three weeks-old soil grown vector control, and *CaZF*OX transgenic plants showing expanded leaves. (***B***) Fold increase in expanded leaf surface area in vector control and transgenic plants. (***C***) Epidermal peels from the ventral surface of the middle lamina of the vector control and transgenic (*CaZF*L46) leaves showing cell size. (***D***) %Relative fresh weight of two-week old vector control and transgenic plants under normal growth conditions.

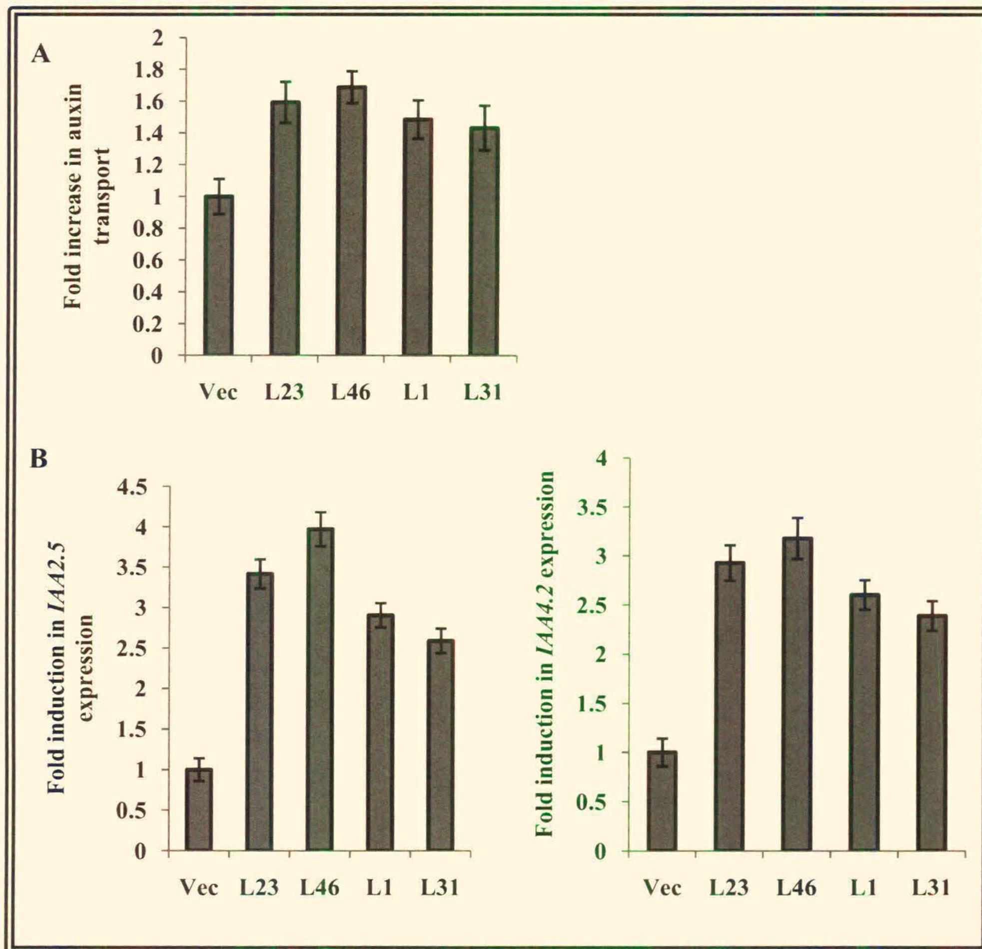


Figure 7.6. Auxin response in transgenic *CaZFOX* plants. (*A*) Comparison of basipetal root auxin transport in 6-d old vector control and *CaZFOX* seedlings in a root segment 2 mm above the site of auxin application at the root tip. Data are presented as fold increase of auxin transport, relative to vector control tobacco. (***B***) qRealTime-PCR of *IAA4.2 and IAA2.5* in vector control and transgenic seedlings (L23, L46, L1 and L31). Readings presented was taken from an average of three independent experiments and normalized against *Actin.*

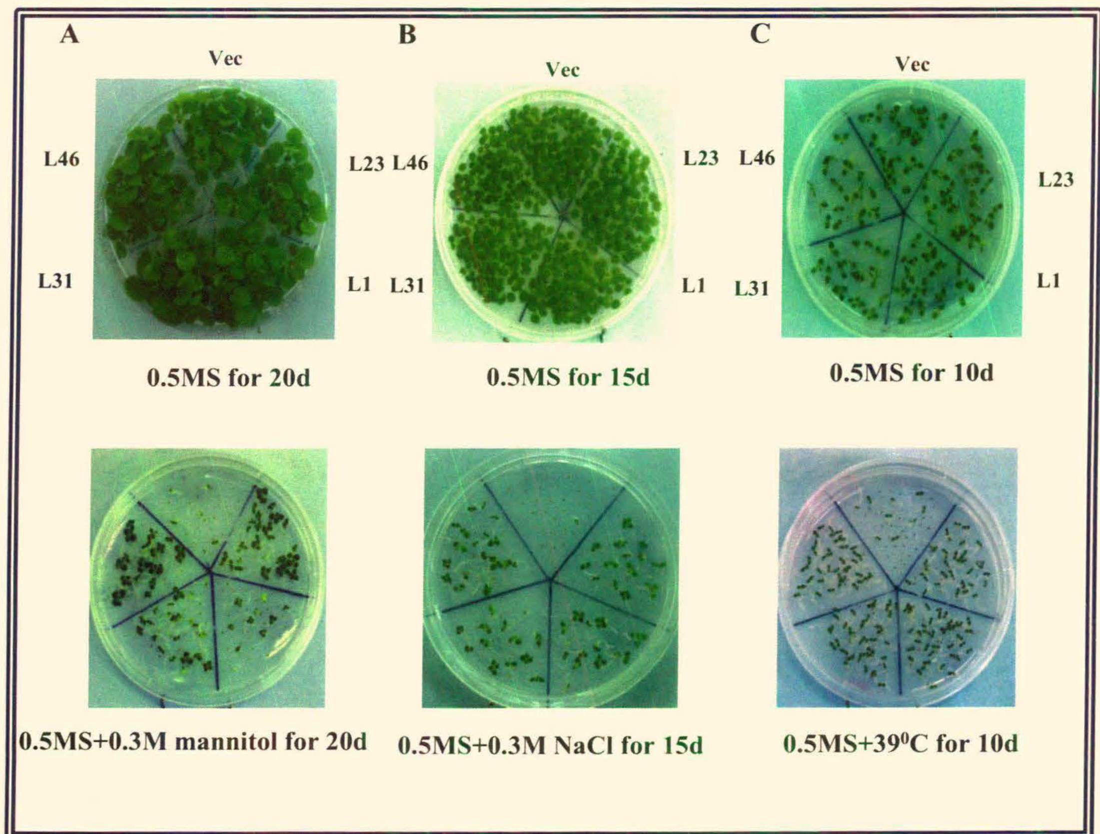


Figure 7.7. Effect of dehydration, salt and heat stress on seedlings. (*A*) Effect of dehydration stress on vector control and T1 progenies of *CaZF*OX transgenic lines (L23, L46, L1 and L31). Seeds were germinated and grown on medium containing 0.3M mannitol for 20d (*Lower panel*). (***B***) Effect of salt stress on vector control and T1 transgenic tobacco lines. Seeds were germinated and grown on medium containing 0.3M NaCl for 15d (*Lower panel*). (***C***) Effect of heat stress on vector control and T1 transgenic tobacco lines. Seeds were plated on 0.5MS and incubated at 39^0C for 10d (*Lower panel*). Seedlings grown under control condition are presented in *upper panel* of each stress condition.

7.2.6 Effect of salt and drought stress on green house grown CaZFOX transgenic lines

Two weeks old vector control and *CaZF*OX transgenic lines were assessed for the salt stress tolerance through irrigation of 0.2M NaCl solution for 2-weeks and then with water for 1-week for recovery. The transgenic lines showed much better recovery than wild type plants (Figure 7.8A). To examine whether overexpression of *CaZF* affects the tolerance to drought stress, vector control and *CaZF* transgenic plants were grown in pots for 2-weeks and then left them unwatered for 2 weeks. For recovery they were watered for 1-week. Vector control plants showed more bleaching and loss of turgor in comparison to the transgenic plants (Figure 7.8B). The same experiment was performed with the mature plants. The 50d-old vector control and transgenic plants were not irrigated for 50d. From the figure it is evident that the *CaZF*OX plants are more tolerant to drought stress in comparison to the control plants (Figure 7.8C).

7.2.7. Transcript analysis of the stress inducible marker genes in *Nicotiana tabacum*

The earlier experiments showed that the *CaZF* expressing transgenic lines were tolerant to salt and drought stress in comparison to vector control plants. The expression of tobacco stress inducible marker genes, *NtERD10B* and *NTERD10C* was analyzed under control condition. qReal-Time PCR analysis showed that the transcript level of both *NTRED10B* and *NTERD10C* are constitutively expressed under control condition in the transgenic lines (Figure 7.8D).

7.2.8 Biochemical response of *CaZF*OX transgenic tobacco plants

7.2.8.1 Determination of proline content

To evaluate the physiological changes of transgenic plants, free proline content as osmolyte in the vector control and *CaZF*OX transgenic tobacco was determined under control condition as well as under drought treatment. After 5h of dehydration, free proline content in the control and *CaZF*OX transgenic plants were measured. The vector control seedlings contains 0.038μg of free proline in comparison to *CaZF*OX transgenic lines L23, L46, L1 and L31 which contains 0.089, 0.112, 0.054 and 0.067μg respectively. However, even under control condition *CaZF*OX transgenic lines accumulate higher levels of free proline as compared with the control seedlings. The basal level of free proline content in vector control is 0.030μg where as *CaZF*OX lines L23, L46, L1 and L31 contains 0.052, 0.062, 0.041, 0.049μg of free proline respectively (Figure 7.9A).

7.2.8.2 Determination of chlorophyll content

To gain the better understanding of physiological changes in *CaZF*OX transgenic plants as compared to vector control tobacco plants, leaf disks from all four lines of T_1 transgenic plants and vector transformed plants were floated separately on water, 150mM or 300mM NaCl for 72h and subsequently total chlorophyll content was quantitated. Chlorophyll content of the vector-transformed and *CaZF*-expressing plants was comparable in presence of water. However, salinity-induced loss of chlorophyll was much lower in *CaZF*OX lines (average 13.2% and 23.1% for L1/L21, and 27.8% and 51.2% for L17/L46 at 150mM and 300mM NaCl respectively) compared with that in the vector control (average 62.3% and 76.4% at 150mM and 300mM NaCl respectively) (Figure 7.9B). From the damage caused by salt stress it was evident that *CaZF*OX transgenic tobacco plants have a better ability to tolerate salinity stress as compared to vector control plants.

7.2.9 Analysis of altered gene expression due to CaZF overexpression in transgenic N. tabacum

Physiological parameters studied under different stress conditions indicated that *CaZF*OX transgenic seedlings are more tolerant compared to vector control seedlings. It is likely that *CaZF* expression has altered the expression of some genes related to abiotic stress and auxin signaling and help in maintaining cellular survival and recovery. To gain insight into the identification of *CaZF*-responsive genes and their expression subtractive cDNA hybridization approach was perceived, generating differentially expressed ESTs. A subtractive cDNA library was constructed with poly (A^+) RNA isolated from C (vector control) and *CaZF*OX transgenic (*CaZF*L46) two week old seedlings as described under "Materials and Methods". Approximately 500 randomly selected clones from the library were single-pass sequenced. After screening out the redundant sequences, 222 high-quality unique EST sequences were generated. These sequences were analyzed by the current GenBank database using the BLASTX algorithm for their putative functional classification. The ESTs generated were deposited into GenBank for accession numbers. A total of 207 out of 222 ESTs showed significant similarity to known sequences in the databases. The remaining 15 ESTs are not homologous to known sequences and were deemed novel (Table 7.1). Of these 222 ESTs, 189 can be functionally categorized according to their BLASTX match whilst the rest 18 ESTs along with 15 novel ESTs were kept under 'unclassified' category. These ESTs were placed in ten functional classifications based on their putative functions. They mostly represent genes involved in

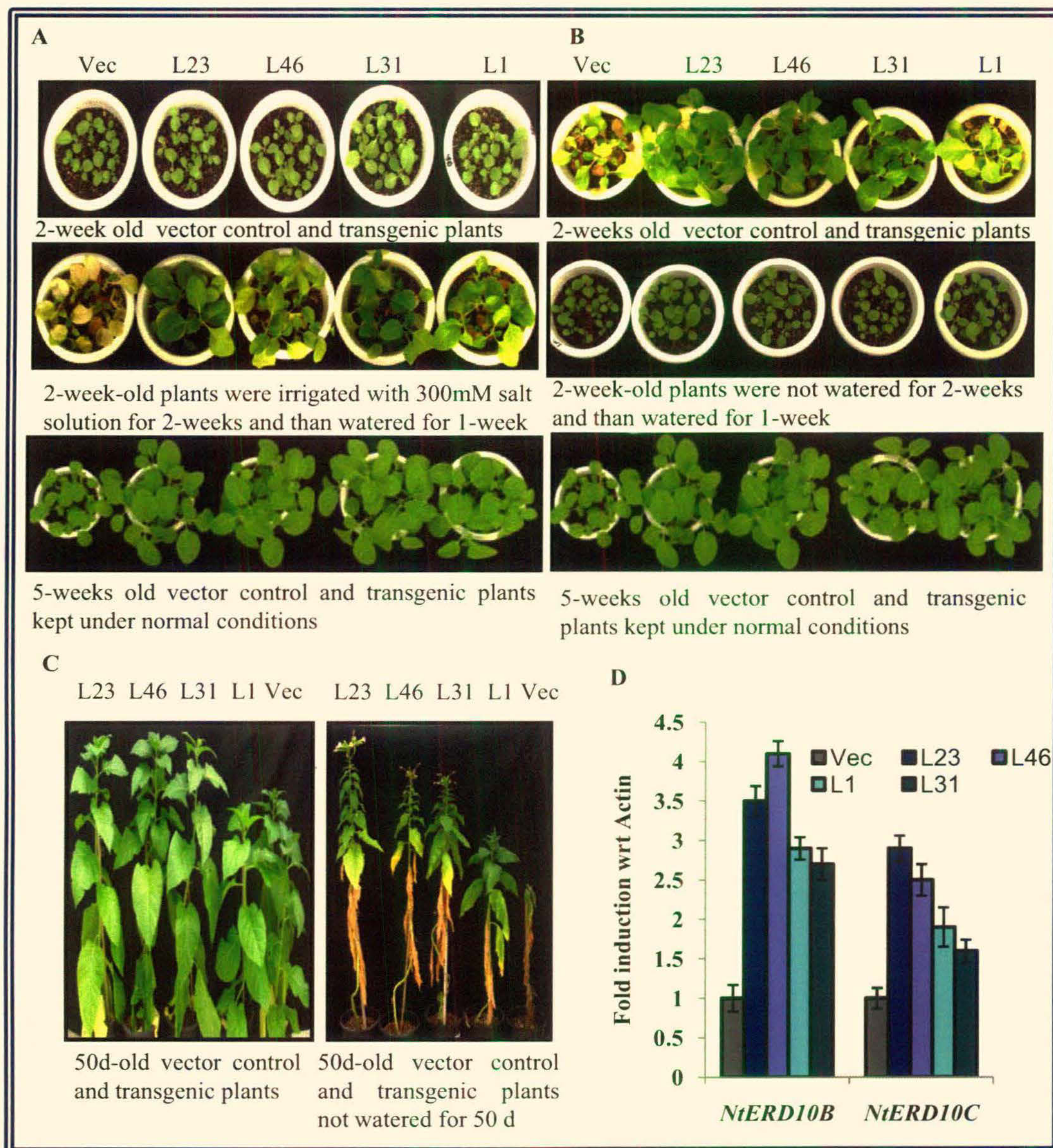


Figure 7.8. Effect of salt and dehydration stress on soil grown vector control and transgenic plants .(*A*) Two-weeks-old vector control (Vec) and transgenic T1 seedlings were irrigated with 200mM salt solution for 2-weeks and then with water for 1-week. Seedlings of vector control and transgenic lines under control condition are presented in *upper* and *lower panels*. (***B***) Two-weeks old vector control and transgenic T1 seedlings were not watered for 2-weeks and then watered for 1-week. Seedlings of vector control and transgenic lines under control condition are presented in *upper* and *lower panels*. (***C***) 50d-old vector control and transgenic T1 plants were not watered for 50d. Representatives of 10 plants of each line under an experiment are shown. ***(D)*** Expression of abiotic stress marker genes *NtERD10B* and *NtERD10C* in vector control (Vec) and transgenic tobacco lines in normal growth condition.

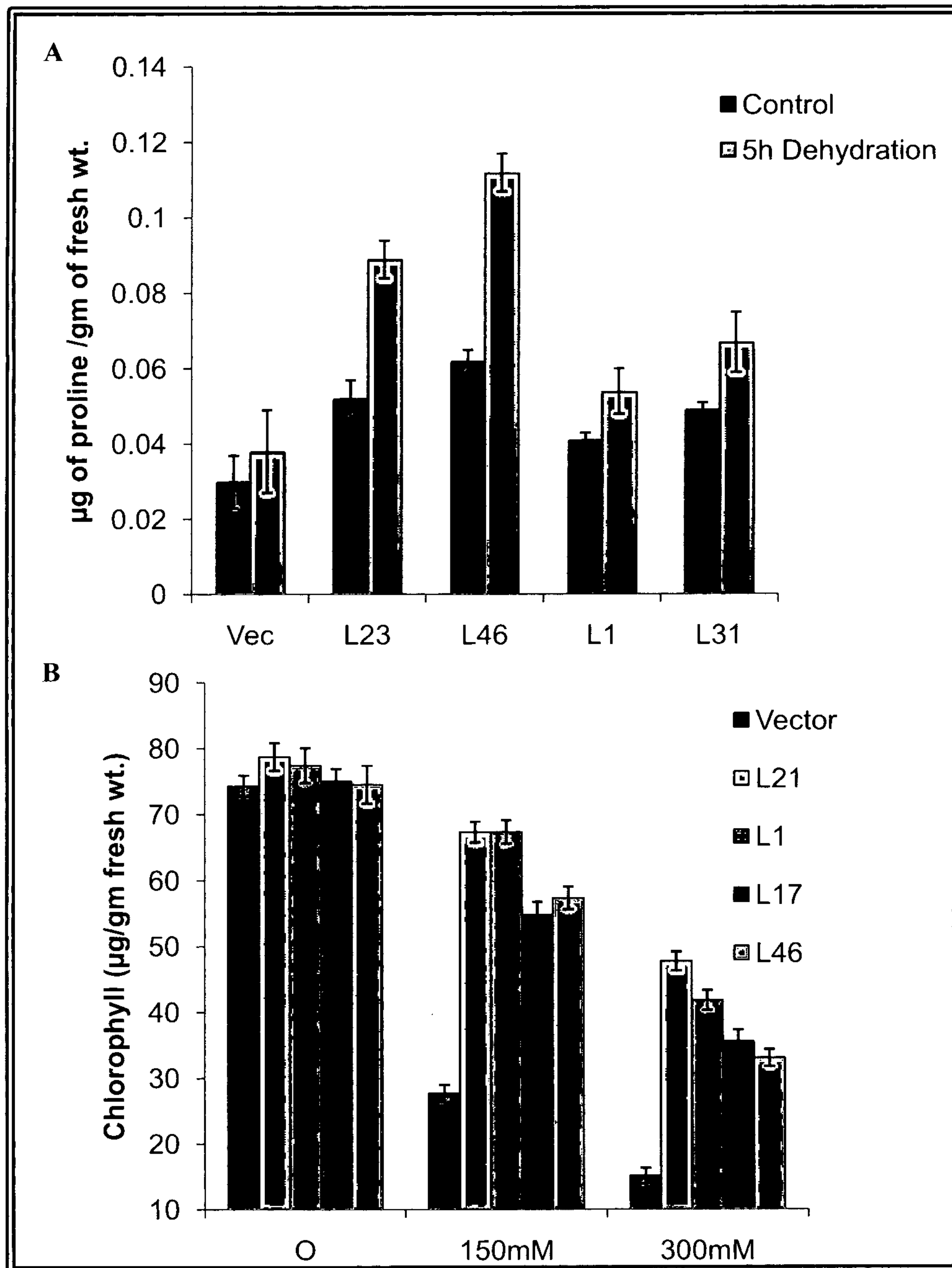


Figure 7.9. Biochemical response of *CaZF*OX transgenic tobacco plants. Comparative analysis of proline (*A*) and chlorophyll content (*B*) between Vec and *CaZF*OX transgenic lines under control or stress conditions. All experiments were done in triplicates, and average mean values were plotted.

metabolism (17.12%), transcription (13.96%), cell defense genes (11.7%), energy metabolism (11.26%), and signal transduction (14.41%). Other categories are hormone biosynthesis (0.9%), cellular organization (8.56%), protein metabolism (2.7%) and translation (4.5%). The 'unclassified' cDNA clones as described above accounted for 14.86% (Figure 7.10). Among the whole ESTs, a number of ESTs related to abiotic stress response and auxin response were identified. To further validate the library twelve ESTs [GO308103, GO308041, GO308056, GO308100, GO308155, GO308064, GO308113, GO308149, GO308183, GO308200, GO308212 and GO308225] were selected for qReal-Time PCR expression analysis in both, vector and *CaZF*OX seedlings. A few of them are graphically presented in Figure 7.11.

Table 7.1: Functional categorization of ESTs generated by subtracted cDNA libraries.

Gene bank match	Annotation	E-value	Accession No.
Cell Defence			
BAB09414.1	ABC transporter, ATP-binding protein-like	8E-56	GO308234
ABD28700.1	ATP binding , related	1E-24	GO308122
CAH10191.1	avirulence-like protein 1	5E-62	GO308065
CAA06925.1	Avr9 elicitor response protein	3E-62	GO308172
AAG43549.1	Avr9/Cf-9 rapidly elicited protein 1	7E-62	GO308066
AAG43549.1	Avr9/Cf-9 rapidly elicited protein 111B	1E-41	GO308198
AAG43557.1	Avr9/Cf-9 rapidly elicited protein 65	2E-44	GO308135
AAB23374.1	basic chitinase	4E-86	GO308216
AAL26909.1	dehydration-responsive protein RD22	1E-27	GO308113
ABB47791.2	dehydration-responsive protein, putative, expr	8E-45	GO308112
ACA24495.1	gamma reponse I-like protein	3E-66	GO308034
AAB48305.1	Hs1pro-1	4E-77	GO308211
AAN17462.1	hypersensitive-induced reaction protein 1	2E-79	GO308047
AAT45202.1	lipid transfer protein 1 precursor	1E-55	GO308050
AAS13435.1	lipid-transfer protein	1E-47	GO308200
BAE98764.1	MRP-like ABC transporter	4E-71	GO308103
BAD07484.1	PDR-type ABC transporter 2	2E-88	GO308105
ABD33460.1	putative NBS-LRR resistance disease protein rsp22	2E-45	GO308159
CAF33484.1	putative pathogenesis-related protein	5E-15	GO308060
ACG50004.1	salt responsive protein 2	3E-88	GO308149
ABJ89813.1	wound-induced protein kinase	7E-65	GO308130
AAX20033.1	GDSL-lipase protein	4E-87	GO308055
AAG34872.1	In2-1 protein	5E-62	GO308169
BAG68298.1	serine palmitoyltransferase	2E-82	GO308185
ABE98329.1	SRC2-like protein	3E-6	GO308136
CAA47374.1	prb-1b	9E-65	GO308054
Cellular Organization			
CAD13178.1	alpha-tubulin	3E-64	GO308121
AAR37366.1	beta-tubulin	1E-151	GO308028
AAO03579.1	cellulose synthase-like protein D4	1E-141	GO308219
CAA18105.1	glycine-rich protein	4E-5	GO308131
BAA83710.1	heat shock factor	3E-37	GO308183
AAR17080.1	heat shock protein 70-3	3E-110	GO308064
ACD45076.1	heat-shock protein 70	3E-69	GO308058

CAB01913.1	Histone H4 homologue	7E-38	GO308072
AAR12194.1	molecular chaperone Hsp90-2	5E-118	GO308096
AAM65650.1	pectinesterase, putative	2E-90	GO308217
CAB75430.1	putative 16kDa membrane protein	3E-64	GO308043
CAI53895.2	putative receptor associated protein	2E-107	GO308162
AAC79095.1	putative ribonucleoprotein	1E-69	GO308046
AAZ80876.1	putative sorbitol transporter	2E-60	GO308038
AAS46241.1	xyloglucan endotransglucosylase-hydrolase XTH3	4E-125	GO308192
AAS46244.1	xyloglucan endotransglucosylase-hydrolase XTH9	3E-48	GO308108
AAG43444.1	xyloglucan endotransglycosylase	1E-118	GO308107
CAA58003.1	xyloglucan endo-transglycosylase	2E-14	GO308093
AAQ55288.2	phytocalpain	1E-69	GO308218
Energy Metabolism			
CAA86468.1	1-aminocyclopropane-1-carboxylate deaminase	8E-104	GO308070
BAA07828.1	4-coumarate:coenzyme A ligase	9E-93	GO308138
CAA70968.2	amino acid transporter	6E-89	GO308030
BAA25685.1	arginine decarboxylase	4E-13	GO308119
AAK13318.1	ATP:citrate lyase	2E-45	GO308049
CAA65063.1	c subunit of V-type ATPase	4E-22	GO308171
AAA57551.1	catalase	7E-62	GO308238
AAF81310.1	Contains similarity to a dehydrogenase	9E-17	GO308083
AAT84461.1	cytochrome b5 isoform Cb5-D	4E-7	GO308061
BAA10929.1	cytochrome P450 like_TBP	1E-39	GO308073
AAB58728.1	cytosolic NADP-malic enzyme [*L. esculentum*]	1E-39	GO308062
ABI98681.2	cytosolic NADP-malic enzyme [*N. tabacum*]	1E-82	GO308068
AAB02006.1	epoxide hydrolase	2E-100	GO308227
CAA54045.1	H(+)-transporting ATPase	3E-100	GO308158
ABC01898.1	mitochondrial carrier-like protein	2E-102	GO308205
ABR67418.1	mitochondrial FAD carrier	1E-30	GO308129
BAB18781.1	mitochondrial protein-like protein	9E-75	GO308199
CAC19856.1	mitochondrial succinate dehydrogenase iron-sulphur subunit	1E-72	GO308123
CAA69601.2	NADH glutamate dehydrogenase [*N. tabacum*]	5E-139	GO308127
CAA69601.2	NADH glutamate dehydrogenase	4E-139	GO308098
CAD33241.1	putative mitochondrial NAD-dependent malate dehydrogenase	2E-126	GO308228
ACF17669.1	putative pyruvate dehydrogenase E1 alpha subunit	5E-143	GO308128
AAC48918.1	salicylic acid binding catalase	2E-141	GO308039
AAD33072.1	secretory peroxidase	3E-126	GO308032
BAG80556.1	UDP-glucose:glucosyltransferase	3E-40	GO308053
Hormone Biosynthesis			
AAP83138.1	lipoxygenase	2E-111	GO308156
ABY55855.1	S-adenosylmethionine decarboxylase 2	2E-44	GO308120
Metabolism			
CAA45700.1	23 kDa polypeptide of water-oxidizing complex of photosystem II	1E-74	GO308026
BAD80839.1	2-Hydroxyisoflavanone dehydratase	1E-23	GO308029
ABW17197.1	alanine aminotransferase 2	7E-92	GO308195
AAM75140.1	alkaline alpha galactosidase II	8E-93	GO308091
BAE98988.1	beta-1,4-N-acetylglucosaminyltransferase like protein	4E-91	GO308174
AAK30294.1	beta-amylase	4E-58	GO308150
CAA84525.1	chlorophyll a,b binding protein type I	7E-89	GO308036
AAO62942.1	chlorophyll a/b binding protein [*N. tabacum*]	2E-93	GO308201
CAK24966.1	chlorophyll a/b binding protein [Solanum]	9E-65	GO308057
AAB61236.1	chlorophyll a/b-binding protein	4E-38	GO308193
ABB55370.1	chlorophyll a-b binding protein 3C-like	1E-118	GO308104
ABC59516.1	chloroplast photosystem II 22 kDa component	4E-64	GO308180

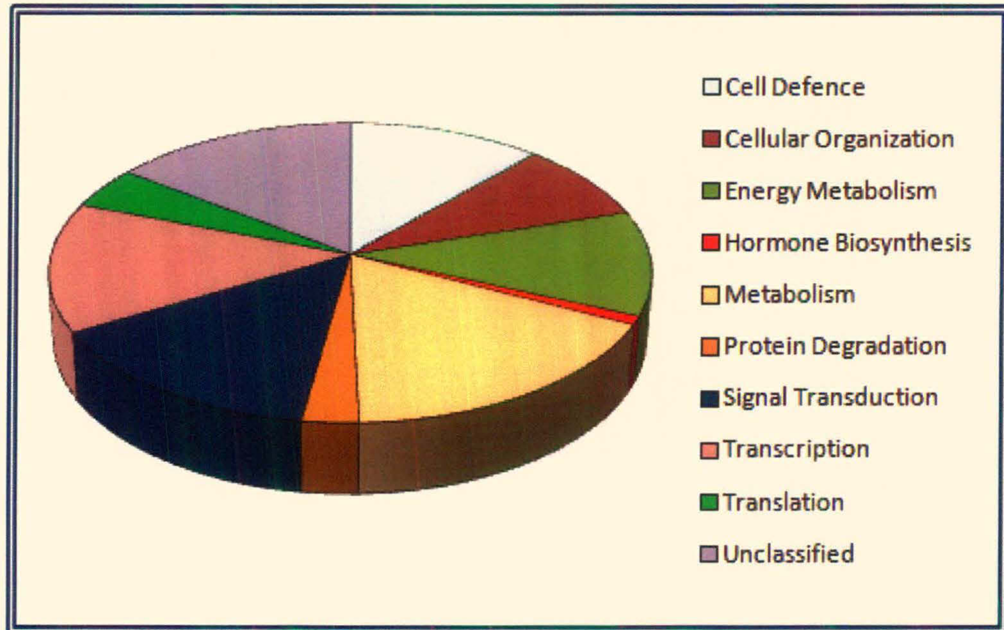


Figure 7.10. Functional categorization of ESTs. The identified ESTs were assigned with a putative function using BLASTX algorithm and clusters of proteins were classified with known or putative functional annotation. Detail information of cluster in given in Table 7.1.

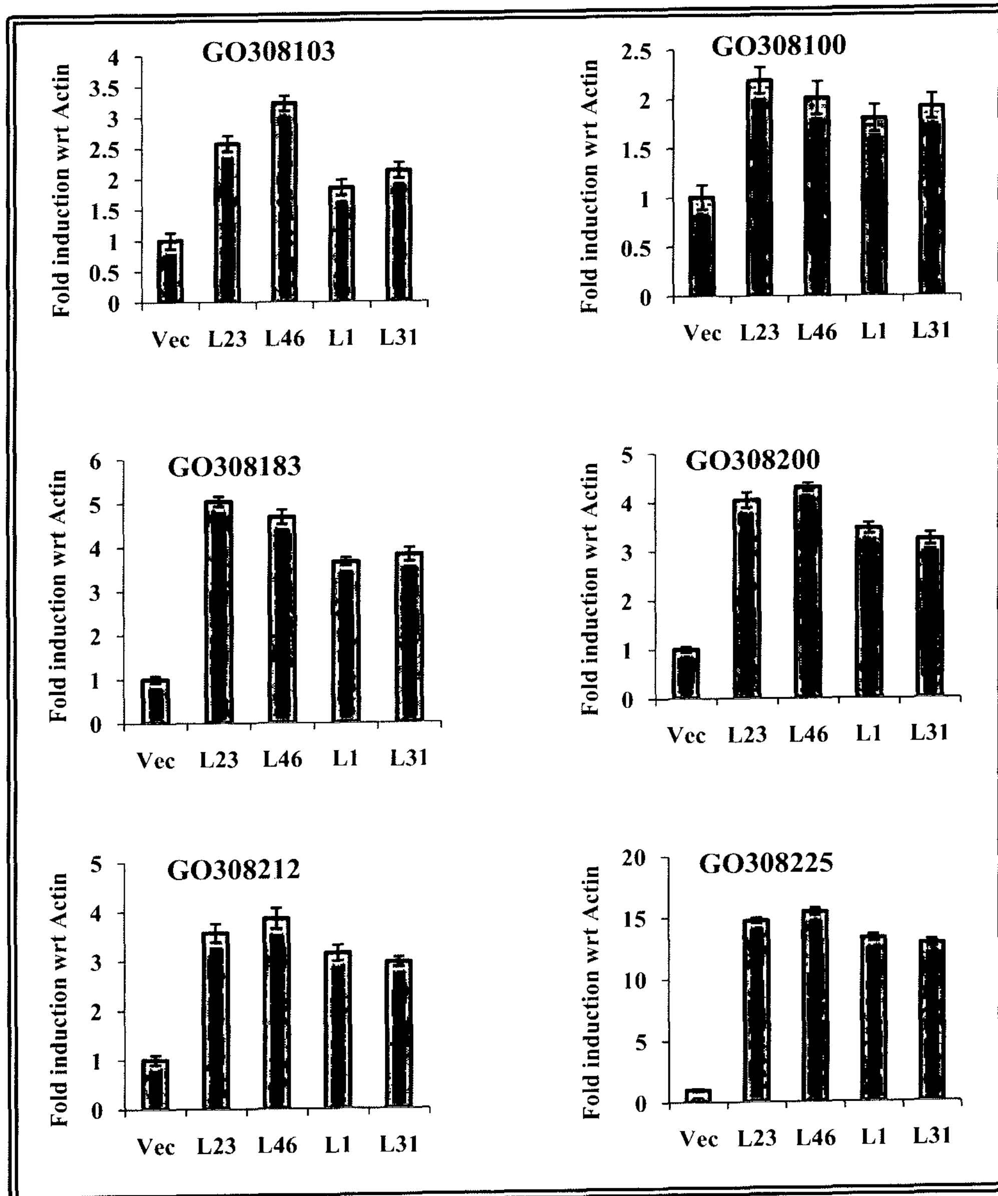


Figure 7.11. qReal-Time PCR of selected library genes. mRNA abundance of some of the library genes was calculated by qReal-Time PCR. Actin was used as an internal control. GO308103, MRP-like ABC transporter; GO308100, Auxin efflux carrier family protein-like protein; GO308183, Heat-shock factor; GO308200, Lipid transfer protein; GO308212, CIPK16 and GO308225, Elongation-factor 1α.

ACB05667.1	chloroplast rubisco activase	3E-43	GO308080
AAF19345.1	diacylglycerol acylCoA acyltransferase	6E-76	GO308052
BAB03027.1	glutamine-fructose-6-phosphate transaminase 2	2E-89	GO308077
CAC80374.1	glyceraldehyde-3-phosphate dehydrogenase	3E-46	GO308090
ABY20971.1	glyceraldehyde-3-phosphate dehydrogenase A subunit	8E-66	GO308075
AAC33509.1	glycolate oxidase	1E-17	GO308067
BAA25393.1	light harvesting chlorophyll a/b-binding protein	8E-117	GO308242
BAA25395.1	light harvesting chlorophyll a/b-binding protein2	2E-119	GO308044
CAC12826.1	malate dehydrogenase [*N. tabacum*]	2E-42	GO308194
CAH60894.1	malate dehydrogenase [*S. lycopersicum*]	5E-78	GO308097
ABI95860.1	methionine synthase	6E-105	GO308025
ABC55421.1	myo-inositol-1-phosphate synthase	8E-115	GO308175
BAG09382.1	peroxisomal glycolate oxidase	3E-112	GO308081
BAG09382.1	peroxisomal glycolate oxidase [*G. max*]	3E-114	GO308031
BAB89366.1	phosphoenolpyruvate carboxylase	2E-83	GO308243
CAJ19272.1	plastocyanin precursor	2E-48	GO308222
BAA04634.1	PSI-H precursor	3E-08	GO308173
AAK72885.1	putative lipid acyl hydrolase	5E-57	GO308146
AAK26130.1	putative thiamin biosynthesis protein	8E-119	GO308115
CAB59430.1	quinolinate phosphoribosyltransferase	9E-62	GO308187
AAA34116.1	ribulose-1, 5-bisphosphate carboxylase small subunit	2E-24	GO308230
AAM62603.1	rubisco expression protein, putative	5E-9	GO308085
AAF24126.1	soluble starch synthase	1E-22	GO308178
ABV25893.1	starch synthase isoform I	2E-22	GO308186
ABJ99591.1	type III chlorophyll a/b-binding protein	8E-91	GO308118
AAB97152.1	Mg protoporphyrin IX chelatase	9E-70	GO308182
Protein degradation			
AAF62403.1	harpin inducing protein	3E-38	GO308206
BAD07806.1	putative HECT ubiquitin-protein ligase 3	2E-74	GO308240
ACH87168.1	senescence-related protein	1E-56	GO308224
AAC00572.1	similar to zinc metalloproteinases	1E-52	GO308207
ABD65144.1	ubiquitin carboxyl-terminal hydrolase, putative	2E-49	GO308037
ABY19385.1	pheophorbide A oxygenase 2	2E-36	GO308168
Signal Transduction			
AAA86052.1	abscisic stress ripening protein	2E-26	GO308190
AAU14832.1	adenosine kinase isoform 1S	7E-44	GO308059
BAD73344.1	auxin efflux carrier family protein-like	9E-12	GO308100
ABK41009.1	auxin/indole-3-acetic acid	4E-31	GO308155
ABK06394.1	Ca^{2+}-binding protein	6E-29	GO308099
CAC43238.1	calcium binding protein [*S. rostrata*]	6E-39	GO308076
AAF31152.1	calcium-binding protein [*O. europaea*]	6E-22	GO308163
AAR99412.1	calmodulin	1E-48	GO308164
AAQ63461.1	calmodulin 4	2E-79	GO308063
ABM55247.1	calmodulin-binding protein	5E-79	GO308134
BAA95793.1	carbonic anhydrase	9E-54	GO308221
ABJ91223.1	CBL-interacting protein kinase 16	5E-79	GO308212
AAF33670.1	cyclic nucleotide-gated calmodulin-binding ion channel	1E-110	GO308125
BAD00043.1	MAP kinase phosphatase	2E-71	GO308232
BAC57589.1	membrane located receptor-like protein	3E-06	GO308191
ABY85198.2	mitogen activated protein kinase 1	2E-15	GO308229
CAA58594.1	Petunia Shaggy kinase 4	1E-70	GO308109
CAA52979.1	phosphate translocator	4E-137	GO308086
ABP57375.1	phosphoinositide-specific phospholipase C	4E-77	GO308027
BAD95059.1	potassium transport protein-like	3E-59	GO308048

AAM63486.1	protein kinase-like protein	6E-36	GO308071
AAQ67229.1	protein phosphatase 2A catalytic subunit	9E-55	GO308196
AAD11598.1	putative calcium channel	9E-93	GO308110
CAC37356.1	putative membrane protein	3E-35	GO308235
AAC33204.1	Putative protein kinase	1E-30	GO308210
BAE99831.1	putative receptor-like protein kinase	1E-85	GO308116
BAF02199.1	putative serine/threonine protein kinase	9E-97	GO308040
BAD09759.1	putative signal recognition particle receptor	2E-76	GO308239
AAM44081.1	type IIB calcium ATPase MCA5	5E-81	GO308151
ABC01915.1	vacuolar sorting receptor protein PV72-like protein	2E-62	GO308142
ABD32569.1	V-ATPase subunit C	4E-111	GO308203
ABG66292.1	VHS domain-containing protein, putative, expressed	5E-33	GO308231
Transcription			
CAB45908.1	Beta-COP-like protein	3E-78	GO308244
AAO13360.1	DREB3 [*L. esculentum*]	1E-31	GO308209
ACE73695.1	DREB3 [*N. tabacum*]	1E-86	GO308139
ACE73696.1	DREB4	7E-110	GO308056
ABV89652.1	early-responsive to dehydration 4	8E-94	GO308189
AAX20034.1	ethylene responsive element binding protein C1	9E-25	GO308226
AAG51287.1	helicase, putative	7E-46	GO308153
CAF74711.1	MYC transcription factor	7E-30	GO308106
AAF66823.1	poly(A)-binding protein	2E-149	GO308111
AAL87345.1	putative chloroplast nucleoid DNA-binding protein	7E-38	GO308167
CAD59768.1	putative reverse transcriptase	8E-12	GO308095
BAD10335.1	putative transcriptional regulator	2E-43	GO308042
ACF74549.1	RAV transcription factor	2E-62	GO308051
CAB10245.1	RNA polymerase II fifth largest subunit like protein	6E-62	GO308082
BAA77383.1	transcription factor NtWRKY2	7E-137	GO308041
BAF48804.1	wound-responsive AP2 like factor 2	2E-72	GO308088
ACF04195.1	WRKY	4E-12	GO308147
BAB61055.1	WRKY DNA-binding protein	1E-137	GO308154
ACJ04728.1	WRKY transcription factor-30	1E-37	GO308213
AAX95671.1	Zn-finger in Ran binding protein and others, putative	4E-18	GO308045
ABE01085.1	BTF3	5E-54	GO308177
ACD49740.1	BURP domain-containing protein	2E-55	GO308181
ABE02823.1	GRAS1	6E-114	GO308233
ABD72959.1	GRAS2	1E-112	GO308157
AAM47025.1	nam-like protein 1	1E-65	GO308102
AAM34773.1	nam-like protein 10	1E-59	GO308141
AAM34770.1	nam-like protein 7	3E-88	GO308170
BAA33810.1	phi-1	7E-140	GO308069
CAF18246.1	STY-L protein	2E-104	GO308245
CAA88492.1	TAF-2	2E-76	GO308140
BAA87058.1	WIZZ	4E-42	GO308236
Translation			
AAM63791.1	40S ribosomal protein S19-like	8E-10	GO308223
CAA09042.1	40S ribosomal protein S6	3E-43	GO308133
ABA40437.1	40S ribosomal protein S7-like protein	1E-67	GO308084
ABR25618.1	60S ribosomal protein l2	3E-21	GO308035
ABA40469.1	60S ribosomal protein L21-like protein	4E-39	GO308184
ABA40469.1	60S ribosomal protein L21-like protein [*Solanum*]	4E-49	GO308074
ABX71676.1	60S ribosomal protein L6-like protein [*Panax*]	2E-25	GO308144
BAA09709.1	elongation factor-1 alpha	7E-09	GO308225
CAB10520.1	ribosomal protein	2E-22	GO308214

ABB72816.1	ribosomal protein L24-like protein	5E-28	GO308237
Unclassified			
CAD29735.1	allene oxide synthase	7E-107	GO308148
AAW02789.1	aluminum-induced protein	2E-36	GO308152
AAM98103.1	At1g02660/T14P4_9	2E-20	GO308092
AAL24253.1	AT4g20170/F1C12_90	1E-57	GO308176
AAO42869.1	At5g42860	4E-15	GO308126
BAD88358.1	CBS domain containing protein-like	8E-34	GO308137
AAK69757.1	chromomethylase CMT2	1E-82	GO308220
ABX09988.1	cullin 4	3E-113	GO308166
EEB19994.1	Cylicin-1, putative	5.7	GO308078
CAC05495.1	DNA gyrase subunit B-like protein	4E-115	GO308087
AAG00249.1	F1N21.14	1E-83	GO308160
AAG00256.1	F1N21.7	3E-32	GO308114
CAD37200.1	GDA2 protein	1E-50	GO308132
ACF74342.1	gonadotropin beta chain	6E-15	GO308188
CAB61744.1	hypothetical protein [*C. arietinum*]	5E-34	GO308145
CAI84656.1	hypothetical protein [*N. tabacum*]	8E-08	GO308208
CAN76368.1	hypothetical protein [*V. vinifera*]	3E-83	GO308124
EEA59336.1	hypothetical protein BRAFLDRAFT_83344	3.8	GO308079
EAZ28165.1	hypothetical protein OsJ_011648	1E-17	GO308204
ABA46779.1	meloidogyne-induced giant cell protein-like protein	6E-43	GO308143
BAA06151.1	pit2	7E-44	GO308161
CAB79691.1	putative protein	1E-52	GO308246
BAD45511.1	putative RAD26	2E-5	GO308033
BAD45605.1	putative t-complex protein 1 theta chain	3E-103	GO308101
AAK11255.1	regulator of gene silencing	6E-75	GO308197
BAF47120.1	translationally controlled tumor protein like protein	1E-43	GO308179
ABA59556.1	U-box protein	2E-63	GO308117
ABK92936.1	unknown [*P. trichocarpa*]	8E-119	GO308165
ABB87113.1	unknown [*S. tuberosum*]	2E-62	GO308241
ACG30009.1	unknown [*Z. mays*]	2E-78	GO308089
BAD43828.1	unknown protein [*A. thaliana*]	5E-13	GO308215
BAA89236.1	unnamed protein product [*N. tabacum*]	8E-70	GO308094
CAO45813.1	unnamed protein product [*V. vinifera*]	4E-122	GO308202

7.2.10 Cloning of upstream activating sequences (UAS)

In order to dissect the cis-acting elements responsible for *CaZF* regulation its promoter (UAS) was cloned, sequenced and analyzed. For isolation of the 5'-upstream region, a genomic library from chickpea was constructed using Genome Walker™ Universal Kit (Clontech) following the manual instruction. Nested primers were designed according to the manual of the kit. The amplified product of ≈1.8kb was cloned and sequenced (Figure 7.12). *In silico* analysis of *CaZF* promoter has been done by PLACE signal scan search (http://www.dna.affrc.go.jp/PLACE/signalscan.html) and PLANTCARE (http://bioinformatics.psb.ugent.be/webtools/plantcare/html) search programs, which revealed the presence of a number of cis-acting elements like, ABRE and LTRECOREATCOR15 (known for ABA response), MBS (Myb-binding site

involved in drought inducibility), P-box (GA-responsive element, TCA (SA-responsive element), DRE (Dehydration responsive element binding, DREB-binding site), GCCCORE (defense and JA-responsive element), TGA-element and CATATGGMSAUR (invoved in auxin-response). Besides these elements many light responsive elements are also present (Figure 7.13).

To determine the regulation of *CaZF* gene expression, ≈1.8kb 5'-upstream region was cloned into the binary vector pBI101.2 (Jefferosn *et al.,* 1987) as a transcriptional fusion in front of a promoterless β-glucuronidase (GUS) gene (Figure 7.14). The resulting construct has been used to transform tobacco (*N. tabacum*) by agrobacterium mediated plant transformation (Gelvin *et al.,* 1987). Transformed shootlets were confirmed by selecting on kanamycin supplemented medium and were analyzed. To confirm the insertion of the constructs, genomic PCR was performed using gene specific forward primers and vector specific GUSR primer taking genomic DNA from different transgenic lines as a template.

7.2.11 Interaction studies of CaZF with CAP2

CAP2, an AP2/ERF containing transcription factor binds to DRE (dehydration responsive element) present in the promoter region of stress responsive genes and activate their expression. Since *CaZF*OX plants exhibited developmental and stress tolerance phenotypes as the *CAP2*OX plants (Shukla et al., 2006) so it was speculated that CAP2 might have a role in *CaZF*-UAS activation.

7.2.11.1 CAP2 binds to DRE in *CaZF*-UAS in gel shift assay

CaZF possess many stress responsive cis-acting elements in their upstream activating sequence including one of the most important elements, DRE. To determine the mechanism of activation of *CaZF*-UAS, the ability of CAP2 to bind DRE in the *CaZF* promoter was tested. A gel-shift assay demonstrates that CAP2 binds to the DRE in the *CaZF* upstream sequences. Recombinant CAP2 protein was expressed in *E. coli* DH5α as glutathione-S-transferase (GST)-fused proteins and purified by GST-agarose columns. The probes (1ng) used in all reactions were ^{32}P-labeled dimer of oligonucleotide having DRE as core element (ACCGAC). Figure 7.15A clearly shows that CAP2 bound to DRE in a sequence specific manner as replacement of a 'G' residue with 'A' residue in the core element of the probe (M1) totally abolished the binding while another replacement outside the core element (M2) maintained the binding efficiency.

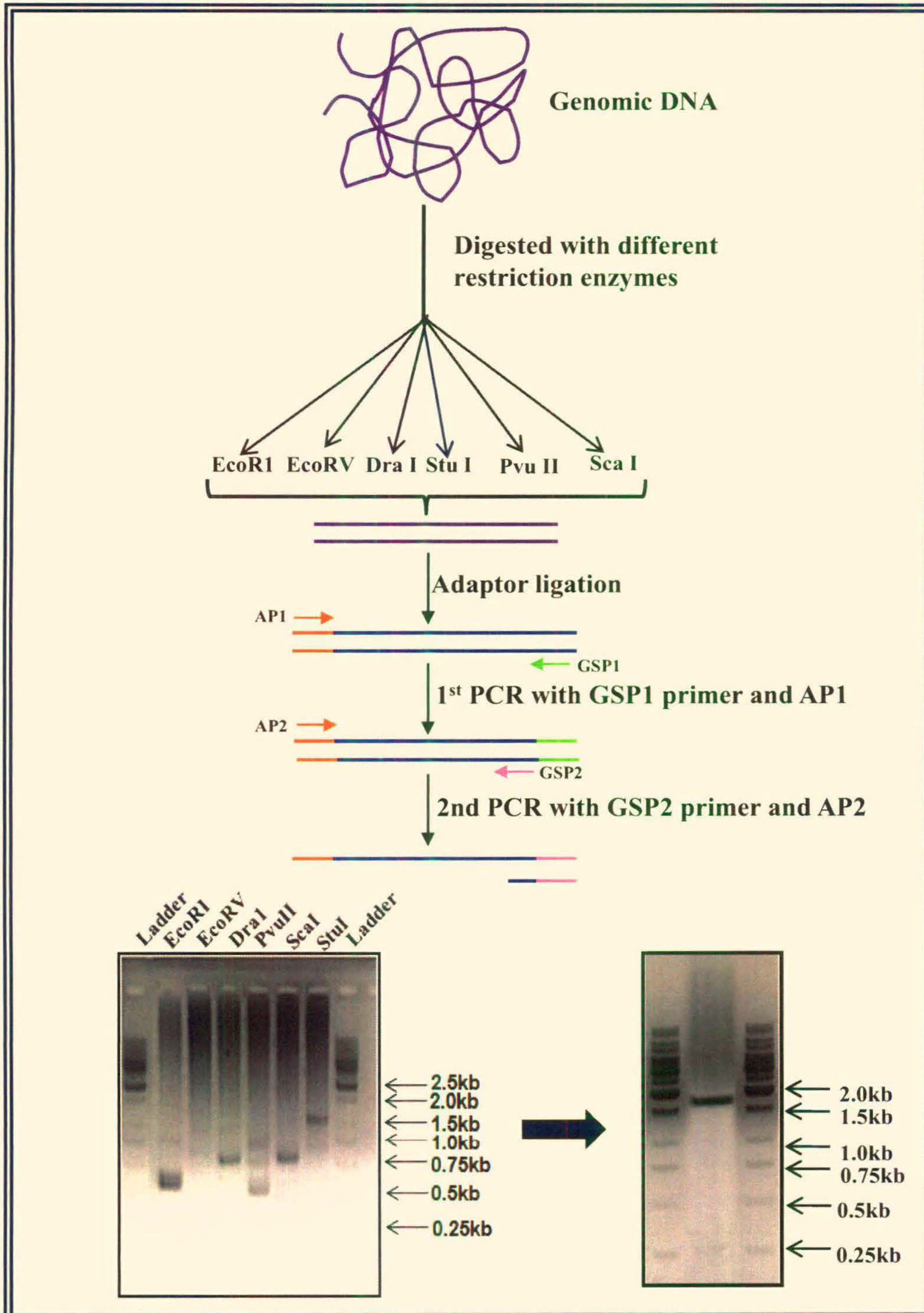


Figure 7.12. Isolation of 5'-upstream sequence of *CaZF* gene. The flow chart for Genome walking and the secondary PCR of genome walking with *EcoRI, EcoRV, DraI, PvuII, ScaI* and *StuI* chickpea library using AP2 & GSP1, GSP2 (gene specific) primers.

-1800

GGATTCACCGACTGTGGCCGGCTGGGTGTGGCGGACCGCTATCAGGACATAGCGTTGG
CTACCCGTGATATTGCTGAAGAGCTTGGCGGCGAATGGGCTGACCGCTTCCTCGTGCTT
TACGGTATCGCCGCTCCCGATTCGCAGCGCATCGCCTTCTATCGCCTTCTTGACGAGTTC
TTCTGAGCGGGACTCTGGGGTTCGAAATGACCGACCAAGCGACGCCCAACCTGCCATC
ACGAGATTTCGATTCCACCGCCGCCTTCTATGAAAGGTTGGGCTTCGGAATCGTTTTCCG
GGACGCCGGCTGGATGATCCTCCAGCGCGGGGATCTCATGCTGGAGCTCTTCGCCCACC
CTAGGGGGAGGCTAACTGAAACACGGAAGGAGACAATACCGGAAGGAACCCGCGCTA
TGACGGCAATAAAAAGACAGAATAAAACGCACGGTGTTGGGTCGTTTGTTCATAAACG
CGGGGTTCGGTCCCAGGGCTGGCACTCTGTCGATACCCCACCGAGACCCCATTGGGGCC
AATACGCCCGCGTTTCTTCCTTTTCCCCACCCCACCCCCCAAGTTCGGGTGAAG**GCCGA**
CGGCTCGCAGCCAACGTCGGGGCGGCAGGCCCTGCCATAGCCTCAGGTTACTCATATG
T**ACTTTA**GATTGATTTAAAACTTCATTTTTAATTTAAAAGGATCTAGGTGAAGATCCTTT
TTGATAATCTCATGACCAAATCCCTTA**ACGTG**AGTTTTCGTTCCACTGAGCGTCAGACC
CCGTAGAAAAGATCAAAGGATCTTCTTGAGATCCTTTTTTTCTGCGCGTAATCTGCTGCT
TGCAAACAAAAAAACCACCGCTACCAGCGGTGGTTTAAACGCCGGATCAAGAGCTACCA
A**CCATCTTTTT**CCGAAGGTAACTGGCTTCAGCAGAGCGCAGATACCAAATACTGTTCTT
CTAGTGTAGCCGTAGTTAGGCCACCACTTCAAGAACTCTGTAGCACCGCCTACATACCT
CGCTCTGCTAATCCTGTTACCAGTGGCTGCTGCCAGTGGCGATAAGTCG**TGTCTC**ACCG
GGTTGGACTCAAGACGATAGTTACCGGATAAGGCGCAGCGGTCGGGCTG**AACGG**GGGG
TTCGTGCACACAGCCCAGCTTGGAGCG**AACGAC**CTACACCGAACTGAGATACCTACAG
CGTGAGCTATGAG**AAAG**CGCCACGCTTCCCGAAGGGAGAAAGGCGGACAGGTATCCGG
TAAGCGGCAGGGTCGGAACAGGAGAGCGCACGAGGGAGCTTCCAGGGGGAAACGCCT
GGTATCTTTATAGTCCTGTCGGGTTTCGCCACCTCTGACTTGAGCGTCGATTTTTGTGAT
GCTCGTCAGGGGGGCGGAGCCTATGGAAAAACGCCAGCAACGCGGCCTTTTTACGGTTC
CTGGCCTTTTGCTGGCCTTTTGCTCACATGTTCTTTCCTGCGTTATCCCCTGATTCTGTGG
ATAACCGTATTACCGCCTTTGAGTGAGCTGATACCGCTCGCCGCAGCCGAACGACCGAG
CGCAGCGAGTCAGTGAGCGAGGAAGCGGAAGAGCGCCCAATACGCAAACCGCCTCTCC
CCGCGCGTTGGCCGATTCATTAATGCAGCTGGCACGACAGGTTTCCCGACTGGAAAGCG
GGCAGTGAGCGCAACG**CAATTG**ATGTGAGTTAGCTCACTCATTAGGCACCCCAGGCTTT
ACACTTTATGCTTCCGGCTCGTATGTTGTGTGGAATTGTGAGCGGATAACAATTTCACAC
AGGAAACAGCTATGACCATGATTACGCCAAGCTATTT

-1

Stress Responsive Element

Element	Sequence
ABRELATERD1 site	ACGTG
LTRECOREATCOR15	CCGAC
MYCCONSENSUSAT site	CAATTG
MYBCOREATCYCB1 site	AACGG
P-box	CCTTTTG
TCA Element	CCATCTTTTT
DRECRTCOREAT site	GCCGAC

Auxin responsive elements

Element	Sequence
NTBBF1ARROLB site	ACTTTA
ARFAT site	TGTCTC
DOFCOREZM site	AAAG
CATATGGMSAUR site	CATATG
TGA Element	AACGAC

Figure 7.13. ***In silico a*nalysis of the *CaZF* – UAS.** Study of *CaZF*-UAS sequence with PLACE and PLANTcare tools shows the presence of many important cis-acting elements involved in stress and auxin response (highlighted with different colors).

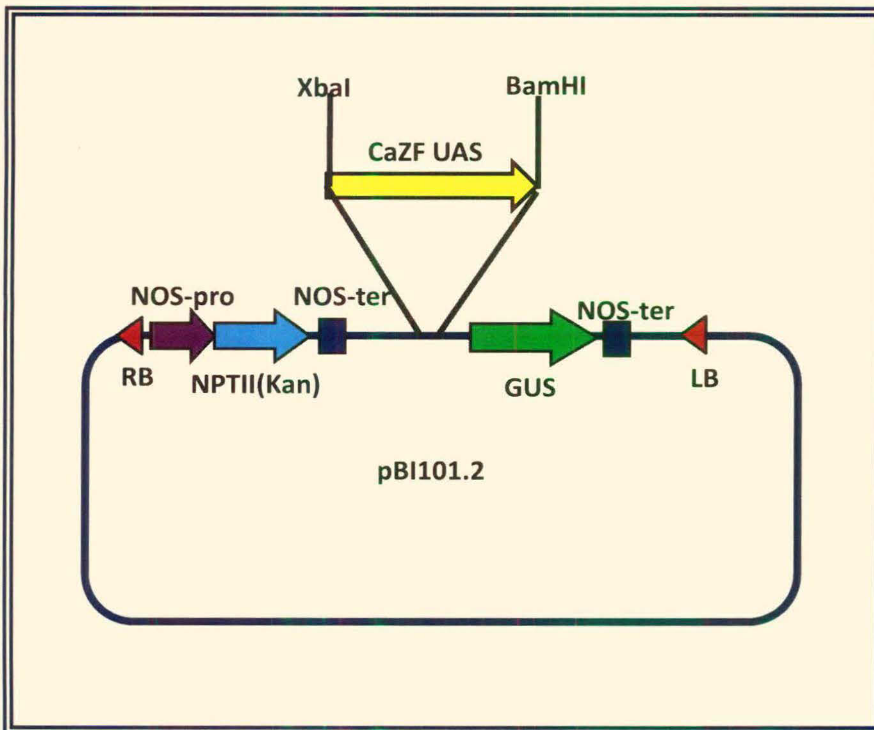


Figure 7.14. Regulation of CaZF expression. The *CaZF* UAS was amplified with a pair of primers having *XbaI* and *BamHI* restriction sites in their 5'-flanking regions. The vector and PCR product were subsequently digested and ligated.

7.2.11.2 CAP2 activates *CaZF* promoter expression in yeast

As most of the cellular mechanisms controlling the growth and stress responses are being conserved in yeast and plants so yeast was used as the model system for speculating the role of *CaZF* gene *in planta*. For this purpose the ability of CAP2 to activate *CaZF* promoter in yeast was tested. 1800bp upstream activating sequence including the DRE was amplified and inserted before *LacZ* reporter gene in pYES2.1-*LacZ* vector to regulate its expression. Yeast cells were co-transformed with the *CaZF*-UAS-*LacZ* reporter construct and CAP2-expressing construct (pGBKT7-*CAP2*) and activity of *LacZ* was assayed. Figure 7.15B shows that CAP2 induced *CaZF* expression by more than 80-fold. A *CaZF* promoter construct with mutated DRE (M1, mutation in the DRE core sequence) was not activated by expression of CAP2. This result together with the result of gel-shift assay clearly demonstrated that CAP2 was able to activate *CaZF*-UAS by directly binding to it.

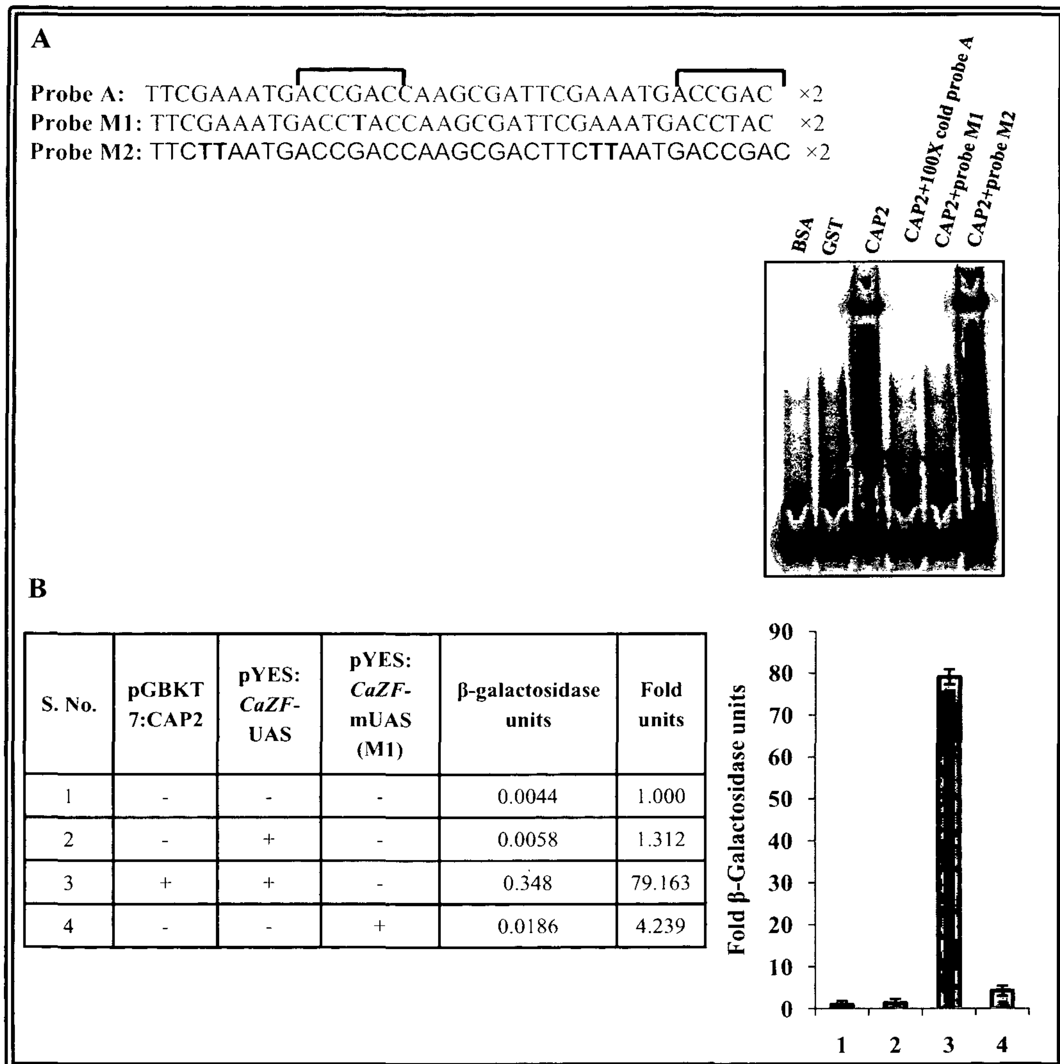
7.2.11.3 CAP2 activates *CaZF* promoter expression *in planta*

To establish the functional significance of CAP2-*CaZF*UAS interaction further studies were made *in planta*. The complete ORF of *CAP2* gene was cloned in pCAMBIA (pCAMBIA:*CAP2*) and 1.8kb CaZF-UAS was cloned in pBI101.2 (PBI101:*CaZF*-UAS). These two constructs were co-introduced into *Agrobacterium tumefaciens*. The transformed colonies were selected on antibiotic containing medium. The positively transformed colonies harboring both the constructs were checked by colony PCR using specific primers for both constructs. *Agrobacterium* colony having both constructs was introduced into tobacco plants using *Agrobacterium*-mediated transformation. The transgenic shootlets were selected on medium containing both hygromycin (pCAMBIA selection marker) and kanamycin (pBI101 selection marker). Transgenic shootlets harboring both *CAP2* and *CaZF*-UAS were verified by genomic DNA PCR and positive ones were further chosen for transient expression analysis. The shootlets harboring *CaZF*-UAS (PBI101:CaZF-UAS) with or without pCAMBIA vector were taken as experimental controls. To assess the effect of CAP2 on *CaZF* expression MUG-assay was performed with transformed shootlets and experimental controls. Activity of *GUS* gene in *CAP2*+*CaZF*-UAS containing shootlets was assayed and found to be approximately three-fold stronger in comparison to only *CaZF*-UAS or *CaZF*-UAS+pCAMBIA vector containing shootlets (Figure 7.16).

7.3 Discussion

The physiological and biochemical changes in plants under particular stress conditions are related to altered gene expression. Onset of a stress triggers some initial sensors, which then activate cytoplasmic Ca^{2+} and protein signalling pathways, leading to stress-responsive gene expression and physiological changes (Bressan et al., 1998; Xiong et al., 2002). Also, accumulation of abscisic acid (ABA) plays an important role in abiotic stress signaling and transduction pathways, mediating many responses. It is well documented that abiotic stresses in general alter the abundance of many transcripts and proteins through regulation of both gene expression and protein turnover (Seki et al., 2002), indicating that transcriptional and post-transcriptional regulation play an essential role in the adaptation of cellular functions to the environmental changes. In the past few years, TFs have been identified as regulatory proteins that play important roles in the plant response to various environmental stresses (Liu and Zhu, 1998; Liu et al., 1998). One of the most important classes of TFs involved in regulation of stress responses is C2H2 zinc finger proteins (ZFPs). ZFPs constitute an abundant family of nucleic acid binding proteins in the genomes of higher and lower eukaryotes. Several ZFPs in plants, e.g. *Arabidopsis* (AZFPs) and *Petunia* (ZPTs), have already been functionally characterized. They are involved in a variety of processes such as the regulation of floral organogenesis, leaf initiation, lateral shoot initiation, gametogenesis and stress response. ZPT2-related proteins have been previously described as candidates to show how a subset of transcription factors might be involved in the water stress response at the level of transcriptional regulation (Sakamoto et al., 2000).

Here, a chickpea (*C. arietinum*) gene, *CaZF*, encoding a plant-specific transcription factor with two Cys-2/His-2-type zinc finger motifs (ZPT2-related proteins) is characterized. The ZPT2-related proteins consist of a relatively large family of transcription factors in plants (Takatsuji, 1999). Using northern blot analysis, we have shown that *CaZF* gene was clearly induced at the transcription level by abiotic stresses like drought, cold and salt. This gene was also induced by ABA and JA suggesting that the regulatory function of CaZF protein may be involved in plant responses to abiotic stress conditions. For a further understanding of the *CaZF* function under abiotic stress conditions *in planta* a hetrologous system was used as chickpea is a recalcitrant crop and tough for raising stable transformants. We chose to generate transgenic tobacco overexpressing *CaZF* under the control of the constitutive CaMV 35S promoter. We obtained two independent transgenic lines each of high- and low-overexpressing *CaZF*. In



S. No.	pGBKT 7:CAP2	pYES: *CaZF*-UAS	pYES: *CaZF*-mUAS (M1)	β-galactosidase units	Fold units
1	-	-	-	0.0044	1.000
2	-	+	-	0.0058	1.312
3	+	+	-	0.348	79.163
4	-	-	+	0.0186	4.239

Figure 7.15. CAP2 binds with *CaZF*-UAS in a sequence specific manner. (*A*) The DRE sequence in *CaZF*-UAS tested for gel-shift assay is either wild-type or mutant version M1/M2. Monomers are shown, and dimers were used in the experiments. Core nucleotides are *in bracket* and modified bases are in *bold letters (Left panel)*. Gel-shift assays demonstrating that CAP2 binds to the DRE probe. (***B***) *LacZ* activation of *CaZF*-UAS by CAP2 is shown by β-galactosidase assay of the transformants presented as fold increase in activity. Assay was done by taking three independent transformed colonies in triplets.

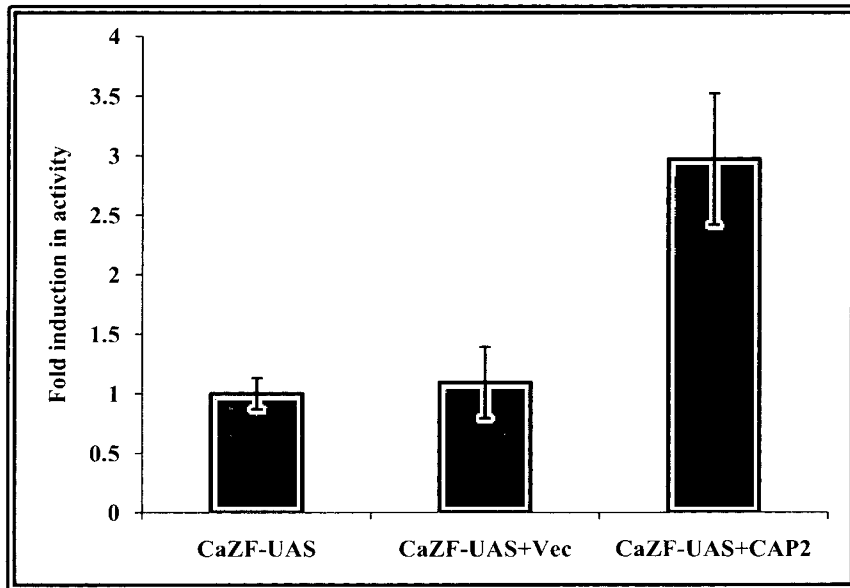


Figure 7.16. *Inplanta* validation of CAP2 interaction with *CaZF*-UAS. *GUS* gene expression in *CAP2+CaZF*-UAS containing shootlets and experimental controls was quantified by MUG activity. Shootlets having only *CaZF*-UAS with or without pCAMBIA vector were taken as controls.

all independent lines, overexpression of *CaZF* enhanced the growth and developement of seedlings under normal growth conditions as well as under drought stress, salinity and heat stress signifies that *CaZF* could work as a stress tolerant determinant.

Biogenesis of lateral root is affected by the interplay of various environmental and endogenous factors, including biosynthesis and distribution of phytohormones like auxin, ABA and ethylene. Lateral root formation is also promoted by salt stress. Mutation in genes *ABI3* and *ARF8* compromise with lateral root formation and growth (Ruegger et al., 1998; Signora et al., 2001; Brady et al., 2003; He et al., 2004). On the other hand, expression of tobacco ethylene receptors NTHK1 and NTHK2 showed more sensitivity to salt stress (Zhang et al., 2001; Xie et al., 2002). So it is evident that signaling pathways of ABA, ethylene, and auxin have several common points of crosstalk. Expression of *Arabidopsis* gene *AtNAC2* is induced by salt stress, auxin, ABA and ethylene; however, the induction under salt stress is suppressed in the mutant plants insensitive to ethylene and resistant to auxin. Interestingly overexpression of *AtNAC2*, similar to *CaZF*-overexpression, promoted the lateral root formation led to a hypothesis that salt induced expression of *AtNAC2* is dependent on ethylene and auxin signaling pathway (He et al., 2005). Presence of auxin and salt responsive *cis*-acting elements in the upstream activating sequence of *CaZF* prompted us to think a similar regulation of *CaZF*. There is strong evidence that plant hormones, such as auxin play a very important role in plant growth and developmental processes. Auxin is essential for cell division and expansion so *in vitro* auxin has been used widely in cell culture and plant regeneration. Root phenotype, enhanced cell division in root and auxin transport assays of *CaZF*OX tobacco plants suggested that this factor could have a role in auxin transport and/or signaling. This hypothesis was further supported by the fact that *CaZFOX* tobacco seedlings showed increased expression of *IAA2.5* and *IAA4.2* genes, which mediate auxin signal transduction. The marked induction of their expression in 35S::*CaZF* seedlings suggests that either auxin biosynthesis is induced or some early steps of auxin perception are fastened. Alternatively, one possibility is that auxin distribution is altered as supported by increased basipetal auxin transport in roots of *CaZF*OX seedlings. Furthermore, the lateral root growth of 35S::*CaZF* seedlings might also be indicative of a change in polar auxin transport (PAT), as PAT is crucial for lateral root initiation and emergence (Reed et al., 1998; Bhalerao et al., 2002). Leaf development occurs in conjunction with continuous development of lateral roots to extract more water and nutrient from soil. In reverse, lateral roots are believed to develop in response to shoot-derived auxin. Therefore, there

is a possibility that production of auxin was increased due to bigger leaf surface area in the *CaZF*OX transgenic plants.

The transcriptional profiling by subtractive cDNA approach suggests that *CaZF* regulates a collection of transcripts involved in metabolism (17.12%), transcription (13.96%), cell defense genes (11.7%), energy metabolism (11.26%), signal transduction (14.41%), hormone biosynthesis (0.9%), cellular organization (8.56%), protein metabolism (2.7%) and translation (4.5%). Many signature genes for abiotic stress and auxin response have been found in the library. mRNA abundance of these genes was quantified by qReal-Time PCR showed the increased expression level in *CaZF*OX lines. Results of expression analysis are also in good agreement with the functional characterization of gain-of-function lines for *CaZF*. The enhancement of salt, dehydration and heat stress tolerance by *CaZF* overexpression can thus be explained by increased expression of stress responsive ESTs like *AtMRP4* homologue, CIPK16, Ca^{2+} binding proteins, dehydrin, HSPs, HSFs, DREB, etc. Another possibility of transgenic plants' tolerance to drought and salt is due to the increase in the proline content in the *CaZF*OX lines. Among the genes whose expression is controlled by the environmental cues are those that encode chlorophyll a/b-binding (CAB) proteins, PSI and PSII subunits, oxygen-evolving complex and Rubisco subunits. Most genes associated with photosynthesis are under the control of a transcriptional regulatory network evolved to control plant response to external stimuli. Those genes may be the target for TFs such as *CaZF*. The expression of these genes is induced in *CaZF*OX lines supporting the hypothesis that it may play a role in regulating genes involved in photosynthesis and carbohydrate metabolism, thus accounting for increased growth.

In silico analysis of the *CaZF*-promoter showed presence of some putative cis-acting elements reported to be involved in gene expression associated with drought, high-salt, and cold stresses. A few important cis-acting elements in *CaZF*-UAS to be mentioned here are DRE (CCGAC), ABRE (RACGTGGC), MYCRS (CANNTG), and MYBRS (RAACYR). Cis-acting elements, like DRE, ABRE, are involved in gene expression associated with drought, high-salt, and cold stresses. ABRE functions as a cis-acting element for the expression of ABA-inducible genes. Expression of *CaZF* was strongly induced by ABA treatment, which may regulate its expression under drought and high-salt stress conditions. It has been found that a number of genes downstream to DREB1 does not posses DRE/CRT in their promoter region. A hypothesis is therefore proposed that DREB1 regulates a number of other TFs, which in turn regulate the expression of a

number of DREB1-downstream genes. Altogether these proposed DREB1-regulated TFs are called DREB-regulon (Fowler and Thomashow, 2002). DREB regulon is mainly involved in cold, dehydration and salt stress response and is probably the one that has attracted most attention. Recently, *Arabidopsis* STZ/ZAT10 has been identified as a downstream target of DREB1A/CBF3 transcription factor using microarray systems, suggesting that STZ/ZAT10 is involved in response to stresses through DREB1A/CBF3 signaling pathway (Maruyama et al., 2004). Over-expression of *STZ* in *Arabidopsis* has resulted in growth retardation and repression of several genes involved in photosynthesis and related metabolism. This means that STZ factor, like DREB1 may be involved in growth retardation through repression of photosynthesis and carbohydrate metabolism genes. Similarly, the enhanced stress tolerance and developmental phenotype, in addition to altered auxin transport associated with *CaZF* overexpression, suggested that it may play a role in *CAP2* (AP2/ERF transcription factor) signaling cascade. There are several lines of evidences supporting this speculation. The *CAP2* overexpression and *CaZF* overexpression phenotypes and behavioral responses match in a number of ways. Transcripts level of both the genes increased by dehydration, cold, salt and abscisic acid. Overexpression of both the molecules in tobacco caused drastic increase in the leaf cell size, and, thereby, in leaf surface area and number of lateral roots. Transgenic plants demonstrated more tolerance to dehydration and salt stress than the wild-type plants. Transgenic plants expressed higher steady-state transcript levels of abiotic stress-response genes *NtERD10B* and *NtERD10C* and auxin-response genes *IAA4.2* and *IAA2.5*. In addition, CAP2 binds to DRE present in the *CaZF*-UAS and activates it in yeast system. *In planta* expression analysis was done which further supported this hypothesis. Taken together, the binding and activation assays, stress-responsive expression patterns and phenotypic characterization revealed that *CaZF* might function as a downstream component of CAP2 protein and help in regulating the expression of CAP2-target genes in response to drought and salinity stress.

In summary, transgenic tobacco lines overexpressing *CaZF* showed higher constitutive expression of abiotic stress-responsive genes, improved tolerance to high salinity and osmotic stresses related to ABA-signaling. Overexpressing lines had morphological features characteristic of auxin sensitivity/transport, demonstrating interaction of two signal transduction pathways and a potential involvement of a transcription factor in both the pathways. In addition, this study suggests a signaling relation between CaZF and CAP2 in grain legume chickpea.

Summary

Plants are sessile organisms that are exposed to a constant barrage of environmental stresses which impact on growth, development and reproduction. Multiple signalling pathways regulate plant stress responses and a significant overlap between the patterns of gene expression in plants and response to different stresses exists. Recent progresses have been made to understand the complex cascade of gene expression in abiotic stress responses especially in identifying specificity and cross talk in stress signalling pathways.

The main objective of this work was to identify drought-related genes by following SSH approach. To understand stress tolerance conferred by multiple genes, individual genetic factors determining stress tolerance need to be elucidated. We took an attempt to profile the transcriptome of drought-tolerant and susceptible chickpea cultivars. Using this strategy we enriched those genes that may contribute to constitutive or inducible tolerance mechanism by screening for genes showing differential expression levels between resistant and susceptible lines. We also identified a set of genes that show significant induction in drought-tolerant variety upon drought stress. These genes may also serve as 'candidate genes' for gene-manipulation and crop improvement in future. Once identified, the next objective was to functionally characterize one of the selected genes. In the present work, an attempt has been made to functionally characterize one of the C2H2 zinc-finger family protein (CaZF) from *Cicer arietinum* under abiotic stress environment. The important results obtained during this study are summarized below;

1. A comparison of drought tolerance between two cultivated chickpea (*Cicer arietinum,* cv. PUSABGD72 and ICCV2) varieties was conducted by measuring the changes in leaf relative water content (RWC), chlorophyll content, Abscisic acid and proline after discontinued irrigation (DH stress) to establish their contrasting characters.
2. Four subtracted cDNA libraries were constructed with poly (A^+) RNA isolated from control C (ctrl), 3d, 6d and 12d DH stressed seedlings of PUSABGD72 (drought-tolerant) and ICCV2 (drought-susceptible).
3. Selected clones from all the four libraries were single-pass sequenced and high-quality unique EST sequences were generated. ESTs were annotated and placed in eleven functional classifications based on their putative functions.
4. Expression profiling of all ESTs was done by reverse-northern blot analysis between PUSABGD72 and ICCV2.
5. 19.5% of all the ESTs were $\geq$2 fold abundant in PUSABGD72 at all the time points.

6. 319 ESTs were clustered according to their relative expression patterns in PUSABGD72 in comparison to ICCV2 by the hierarchical clustering method to achieve a comprehensive overview of relative expression profiles.
7. The comparative expression profiles under drought suggested a different stress-specific gene expression programming in PUSABGD72 in comparison to that in ICCV2.
8. Based on the comparative gene expression analyses in this study and with the support of the published literature we have outlined a working pathway for drought tolerance mechanism in chickpea seedlings and proposed some candidate genes that might lead to drought tolerance in overexpressing transgenic system.
9. A chickpea EST (GenBank accession EU513298) revealed significant homology with putative C2H2 zinc-finger containing protein by sequence annotation using BLASTx of all clones was taken for further study.
10. The selected partial clone was made full-length by 5'RACE. Full-length cDNA (*CaZF*) was 1185bp in length.
11. *In silico* sequence analysis revealed an 843bp ORF of 280 amino acid, 139bp long 5' and 203bp long 3'UTR. Deduced amino acid sequence shows CaZF have two canonical TFIIIA-type zinc finger motifs. Three conserved regions other than the zinc fingers are B-box (near the N-terminus), L-box (between B-box and the first zinc-finger) and DLN-box (close to the C-terminus). CaZF possesses a serine-glutamine rich region at the N-terminus, between L-box and first zinc-finger and Asn-rich region just after second zinc finger. CaZF contains two combinations of highly basic region followed by acidic amino acids near the C-terminus.
12. Phylogenetic analysis shows that PIF1 (GB: AAQ54302), a pathogen inducible zinc finger protein from capsicum shows maximum sequence similarity with CaZF. CaZF and one *Arachis* protein (ZFP248) shares the same clad.
13. The gel mobility shift assay revealed that CaZF proteins bind to the EP2S core sequence in a sequence specific manner as no binding was observed with altered core sequence.
14. CaZF can transactivate the reporter genes in yeast. Further, transactivation domain mapping showed that the aminoacids from 44-75 (L-box) are most important for transactivation property of the protein.
15. *CaZF* is single or low copy number, intron less gene.
16. CaZF protein is nuclear localized.

17. Cold, DH, salt, abscisic acid (ABA), jasmonic acid (JA) and salicylic acid (SA) treatments for five hours alter the *CaZF* expression but wounding did not alter *CaZF* expression.
18. CaZF has relatively more detectable expression in stem in comparison to root and leaf under control conditions. However, in cold, dehydration and salt stress conditions the shoot specific expression increased several fold.
19. Yeast strain (BCY123) harboring *CaZF* cDNA exhibited tolerance against ionic and non-ionic osmolytes such as NaCl, $MnCl_2$, KCl and sorbitol demonstrating that it can provide tolerance against general osmotic stress.
20. CaZF mediated suppression of osmosensitivity involves a pathway(s) that is through or parallel to Hog1p and CaN regulated pathways.
21. Heterologous expression of CaZF provides osmotolerance in *S. cerevisiae* through Hog1p and Calcineurin dependent as well as independent pathways. CaZF partially suppresses salt-hypersensitive phenotypes of *hog1*, *can* and *hog1can* mutants and in conjunction, stimulates HOG and CAN pathway genes with subsequent accumulation of glycerol in absence of Hog1p and CAN.
22. CaZF expression suppressed salt stress sensitivity not only of *hog1cnb* but also of *hog1crz1* and *hog1ena1* double mutant indicating ENA1 is not essential for CaZF function.
23. CaZF was able to activate yeast stress tolerance responsive element in Hog1-independent manner by directly binding to it.
24. For functional characterization of CaZF, transgenic tobacco overexpressing CaZF were raised. Two each of relatively high- and low-expressing transgenic lines were studied.
25. Roots of *CaZF*OX lines exhibited more than two fold higher rate of root growth than that of control seedlings. *CaZF* roots showed significantly higher number of cells in the apical meristem region. Increased cell number is evident in the epidermal, cortical and steller layers and that caused extra linings of cell layers in *CaZF*OX roots indicating frequent cell division. There was more number of lateral roots in the transgenic lines as compared to the vector control plants. Leaf surface area of the transgenic lines was found much bigger than that of the wild type plants. Average cell size of *CaZF*OX lines was found to be higher that in the vector control.

26. Significant increase in the auxin transport was observed in the *CaZF*OX lines when compared with vector control.
27. *CaZF*OX lines exhibited increased tolerance to dehydration, salt and heat stress.
28. Free proline and chlorophyll content of *CaZF*OX transgenic lines was found to be higher in control and as well as in stress conditions.
29. To identify the downstream genes of CaZF, a subtractive cDNA library was constructed with C (vector control) and *CaZF*OX transgenic two week old seedlings and 222 high-quality unique EST sequences were generated. Many genes related to abiotic stress and auxin response are reported.
30. To identify the cis acting elements present in *CaZF* upstream activating sequence, 1.8kb *CaZF*-UAS was cloned. In silico analysis of the *CaZF*-promoter showed some putative cis-acting elements reported to be involved in gene expression associated with drought, high-salt, and cold stresses. Few important cis-acting elements are DRE (CCGAC), ABRE (RACGTGGC), MYCRS (CANNTG), and MYBRS (RAACYR).
31. CAP2, an AP2/ERF containing transcription factor binds to DRE (dehydration responsive element) present in the promoter region of stress responsive genes and activate their expression. The binding and activation assays, stress-responsive expression patterns and phenotypic characterization revealed that *CaZF* might function as a downstream component of CAP2 proteins through regulating the expression of signaling genes in response to drought and salinity stress.

Bibliography

Abe H, Urao T, Ito T, Seki M, Shinozaki K, Yamaguchi-Shinozaki K (2003) Arabidopsis AtMYC2 (bHLH) and AtMYB2 (MYB) function as transcriptional activators in abscisic acid signaling. Plant Cell **15:** 63-78

Agarwal P, Agarwal P, Reddy M, Sopory S (2006) Role of DREB transcription factors in abiotic and biotic stress tolerance in plants. Plant Cell Reports **25:** 1263-1274

Ahmad F, Gaur P., Crosser J. (2005) Chickpea (*Cicer arietinum* L.). *Genetic Resources, Chromosome Engineering and Crop Improvement - Grain Legumes* **1:** 185-214

Akashi K, Miyake C, Yokota A (2001) Citrulline, a novel compatible solute in drought-tolerant wild watermelon leaves, is an efficient hydroxyl radical scavenger. FEBS Lett **508: 438-**442

Akashi K, Nishimura N, Ishida Y, Yokota A (2004) Potent hydroxyl radical-scavenging activity of drought-induced type-2 metallothionein in wild watermelon. Biochem Biophys Res Commun **323:** 72-78

Akhtar N, Pahlman AK, Larsson K, Corbett AH, Adler L (2000) SGD1 encodes an essential nuclear protein of Saccharomyces cerevisiae that affects expression of the GPD1 gene for glycerol 3-phosphate dehydrogenase. FEBS Lett **483:** 87-92

Albertyn J, Hohmann S, Thevelein JM, Prior BA (1994) GPD1, which encodes glycerol-3-phosphate dehydrogenase, is essential for growth under osmotic stress in Saccharomyces cerevisiae, and its expression is regulated by the high-osmolarity glycerol response pathway. Mol Cell Biol **14:** 4135-4144

Albrecht V, Weinl S, Blazevic D, D'Angelo C, Batistic O, Kolukisaoglu U, Bock R, Schulz B, Harter K, Kudla J (2003) The calcium sensor CBL1 integrates plant responses to abiotic stresses. Plant J **36:** 457-470

Alepuz PM, de Nadal E, Zapater M, Ammerer G, Posas F (2003) Osmostress-induced transcription by Hot1 depends on a Hog1-mediated recruitment of the RNA Pol II. Embo J **22:** 2433-2442

Alepuz PM, Jovanovic A, Reiser V, Ammerer G (2001) Stress-induced map kinase Hog1 is part of transcription activation complexes. Mol Cell **7:** 767-777

Anderson JA, Huprikar SS, Kochian LV, Lucas WJ, Gaber RF (1992) Functional Expression of a Probable Arabidopsis thaliana Potassium Channel in Saccharomyces cerevisiae. Proceedings of the National Academy of Sciences **89:** 3736-3740

Aramburu J, Rao A, Klee CB (2000) Calcineurin: from structure to function. Curr Top Cell Regul **36:** 237-295

Arumuganathan K, Earle E (1991) Nuclear DNA content of some important plant species. Plant Molecular Biology Reporter **9:** 208-218

Asai T, Tena G, Plotnikova J, Willmann MR, Chiu WL, Gomez-Gomez L, Boller T, Ausubel FM, Sheen J (2002) MAP kinase signalling cascade in Arabidopsis innate immunity. Nature **415:** 977-983

Aukerman MJ, Schmidt RJ, Burr B, Burr FA (1991) An arginine to lysine substitution in the bZIP domain of an opaque-2 mutant in maize abolishes specific DNA binding. Genes Dev **5:** 310-320

Baier M, Kandlbinder A, Dietz K-J, Golldack D (2006) New insights into abiotic stress signalling in plants. *In* Progress in Botany, pp 248-274

Bartels D, Salamini F (2001) Desiccation tolerance in the resurrection plant Craterostigma plantagineum. A contribution to the study of drought tolerance at the molecular level. Plant Physiol **127:** 1346-1353

Bartels D, Sunkar R (2005) Drought and salt tolerance in plants. CRC Critical Reviews in Plant Sciences **24:** 23-58

Batelli G, Verslues PE, Agius F, Qiu Q, Fujii H, Pan S, Schumaker KS, Grillo S, Zhu JK (2007) SOS2 promotes salt tolerance in part by interacting with the vacuolar H+-ATPase and upregulating its transport activity. Mol Cell Biol **27:** 7781-7790

Batiza AF, Schulz T, Masson PH (1996) Yeast respond to hypotonic shock with a calcium pulse. J Biol Chem **271:** 23357-23362

Baudin A, Ozier-Kalogeropoulos O, Denouel A, Lacroute F, Cullin C (1993) A simple and efficient method for direct gene deletion in Saccharomyces cerevisiae. Nucleic Acids Res **21:** 3329-3330

Berbel A, Navarro C, Ferrandiz C, Canas LA, Madueno F, Beltran JP (2001) Analysis of PEAM4, the pea AP1 functional homologue, supports a model for AP1-like genes controlling both floral meristem and floral organ identity in different plant species. Plant J **25: 441-451**

Berrocal-Lobo M, Molina A, Solano R (2002) Constitutive expression of ETHYLENE-RESPONSE-FACTOR1 in Arabidopsis confers resistance to several necrotrophic fungi. Plant J **29:** 23-32

Bertl A, Anderson JA, Slayman CL, Gaber RF (1995) Use of Saccharomyces cerevisiae for patch-clamp analysis of heterologous membrane proteins: characterization of Kat1, an inward-rectifying K+ channel from Arabidopsis thaliana, and comparison with endogeneous yeast channels and carriers. Proc Natl Acad Sci U S A **92:** 2701-2705

Bhalerao RP, Eklof J, Ljung K, Marchant A, Bennett M, Sandberg G (2002) Shoot-derived auxin is essential for early lateral root emergence in Arabidopsis seedlings. Plant J **29:** 325-332

Bhushan D, Pandey A, Choudhary MK, Datta A, Chakraborty S, Chakraborty N (2007) Comparative proteomics analysis of differentially expressed proteins in chickpea extracellular matrix during dehydration stress. Mol Cell Proteomics **6:** 1868-1884

Blokhina O, Virolainen E, Fagerstedt KV (2003) Antioxidants, oxidative damage and oxygen deprivation stress: a review. Ann Bot (Lond) **91 Spec No:** 179-194

Boggon TJ, Shan WS, Santagata S, Myers SC, Shapiro L (1999) Implication of tubby proteins as transcription factors by structure-based functional analysis. Science **286:** 2119-2125

Bohnert HJ, Jensen RG (1996) Strategies for engineering water stress tolerance in plants. Trends in Biotechnology **14** 89-97

Bohnert HJ, Nelson DE, Jensen RG (1995) Adaptations to Environmental Stresses. Plant Cell **7:** 1099-1111

Boominathan P, Shukla R, Kumar A, Manna D, Negi D, Verma PK, Chattopadhyay D (2004) Long term transcript accumulation during the development of dehydration adaptation in Cicer arietinum. Plant Physiol **135:** 1608-1620

Borden KL, Boddy MN, Lally J, O'Reilly NJ, Martin S, Howe K, Solomon E, Freemont PS (1995) The solution structure of the RING finger domain from the acute promyelocytic leukaemia proto-oncoprotein PML. EMBO J **14:** 1532-1541

Bradford MM (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem **72:** 248-254

Brady SM, Sarkar SF, Bonetta D, McCourt P (2003) The ABSCISIC ACID INSENSITIVE 3 (ABI3) gene is modulated by farnesylation and is involved in auxin signaling and lateral root development in Arabidopsis. Plant J **34:** 67-75

Bray EA (1993) Molecular Responses to Water Deficit. Plant Physiol **103:** 1035-1040

Bray EA (1997) Plant responses to water deficit. Trends in Plant Science **2:** 48-54

Bray EA (2002) Classification of genes differentially expressed during water-deficit stress in Arabidopsis thaliana: an analysis using microarray and differential expression data. Ann Bot (Lond) **89 Spec No:** 803-811

Bressan RA, Hasegawa PM, Pardo JM (1998) Plants use calcium to resolve salt stress. Trends Plant Sci. **3:** 411-412

Breuder T, Hemenway CS, Movva NR, Cardenas ME, Heitman J (1994) Calcineurin is essential in cyclosporin A- and FK506-sensitive yeast strains. Proc Natl Acad Sci U S A **91:** 5372-5376

Brewster JL, de Valoir T, Dwyer ND, Winter E, Gustin MC (1993) An osmosensing signal transduction pathway in yeast. Science **259:** 1760-1763

C. J. Diédhiou OVPKJDDG (2008) The SUI-homologous translation initiation factor <i>eIF-1</i> is involved in regulation of ion homeostasis in rice. Plant Biology **10:** 298-309

Cagney G, Uetz P, Fields S (2000) High-throughput screening for protein-protein interactions using two-hybrid assay. Methods Enzymol **328:** 3-14

Camoni L, Harper JF, Palmgren MG (1998) 14-3-3 proteins activate a plant calcium-dependent protein kinase (CDPK). FEBS Letters **430:** 381-384

Chao DY, Luo YH, Shi M, Luo D, Lin HX (2005) Salt-responsive genes in rice revealed by cDNA microarray analysis. Cell Res **15:** 796-810

Chehab EW, Patharkar OR, Hegeman AD, Taybi T, Cushman JC (2004) Autophosphorylation and subcellular localization dynamics of a salt- and water deficit-induced calcium-dependent protein kinase from ice plant. Plant Physiol **135:** 1430-1446

Chen TH, Murata N (2002) Enhancement of tolerance of abiotic stress by metabolic engineering of betaines and other compatible solutes. Curr Opin Plant Biol **5:** 250-257

Chen W, Provart NJ, Glazebrook J, Katagiri F, Chang HS, Eulgem T, Mauch F, Luan S, Zou G, Whitham SA, Budworth PR, Tao Y, Xie Z, Chen X, Lam S, Kreps JA, Harper JF, Si-Ammour A, Mauch-Mani B, Heinlein M, Kobayashi K, Hohn T, Dangl JL, Wang X, Zhu T (2002) Expression profile matrix of Arabidopsis transcription factor genes suggests their putative functions in response to environmental stresses. Plant Cell **14:** 559-574

Chen Z, Malamy J, Henning J, Conrath U, Sanchez-Casas P, Silva H, Ricigliano J, Klessig DK (1995) Induction, modification, and transduction of the salicylic acid signal in plant defense responses. Proc Natl Acad Sci U S A **92:** 4134-4137

Cheng NH, Pittman JK, Zhu JK, Hirschi KD (2004) The protein kinase SOS2 activates the Arabidopsis H(+)/Ca(2+) antiporter CAX1 to integrate calcium transport and salt tolerance. J Biol Chem **279:** 2922-2926

Cheong YH, Chang HS, Gupta R, Wang X, Zhu T, Luan S (2002) Transcriptional profiling reveals novel interactions between wounding, pathogen, abiotic stress, and hormonal responses in Arabidopsis. Plant Physiol **129:** 661-677

Cheong YH, Kim KN, Pandey GK, Gupta R, Grant JJ, Luan S (2003) CBL1, a calcium sensor that differentially regulates salt, drought, and cold responses in Arabidopsis. Plant Cell **15:** 1833-1845

Chinnusamy V, Schumaker K, Zhu JK (2004) Molecular genetic perspectives on cross-talk and specificity in abiotic stress signalling in plants. J Exp Bot **55:** 225-236

Chinnusamy V, Zhu J, Zhu JK (2007) Cold stress regulation of gene expression in plants. Trends Plant Sci **12:** 444-451

Choi H, Hong J, Ha J, Kang J, Kim SY (2000) ABFs, a family of ABA-responsive element binding factors. J Biol Chem **275:** 1723-1730

Covic L, Silva NF, Lew RR (1999) Functional characterization of ARAKIN (ATMEKK1): a possible mediator in an osmotic stress response pathway in higher plants. Biochim Biophys Acta **1451:** 242-254

Cunningham KW, Fink GR (1996) Calcineurin inhibits VCX1-dependent H+/Ca2+ exchange and induces Ca2+ ATPases in Saccharomyces cerevisiae. Mol Cell Biol **16:** 2226-2237

Cushman JC, Bohnert HJ (2000) Genomic approaches to plant stress tolerance. Curr Opin Plant Biol **3:** 117-124

Cyert MS (2003) Calcineurin signaling in Saccharomyces cerevisiae: how yeast go crazy in response to stress. Biochem Biophys Res Commun **311:** 1143-1150

Cyert MS, Kunisawa R, Kaim D, Thorner J (1991) Yeast has homologs (CNA1 and CNA2 gene products) of mammalian calcineurin, a calmodulin-regulated phosphoprotein phosphatase. Proc Natl Acad Sci U S A **88:** 7376-7380

Davletova S, Schlauch K, Coutu J, Mittler R (2005) The Zinc-Finger Protein Zat12 Plays a Central Role in Reactive Oxygen and Abiotic Stress Signaling in Arabidopsis. Plant Physiol. **139:** 847-856

De Nadal E, Zapater M, Alepuz PM, Sumoy L, Mas G, Posas F (2004) The MAPK Hogl recruits Rpd3 histone deacetylase to activate osmoresponsive genes. Nature **427:** 370-374

Delauney AJ, Hu CA, Kishor PB, Verma DP (1993) Cloning of ornithine delta-aminotransferase cDNA from Vigna aconitifolia by trans-complementation in Escherichia coli and regulation of proline biosynthesis. J Biol Chem **268:** 18673-18678

Deng X, Phillips J, Meijer AH, Salamini F, Bartels D (2002) Characterization of five novel dehydration-responsive homeodomain leucine zipper genes from the resurrection plant Craterostigma plantagineum. Plant Mol Biol **49:** 601-610

Diatchenko L, Lau YF, Campbell AP, Chenchik A, Moqadam F, Huang B, Lukyanov S, Lukyanov K, Gurskaya N, Sverdlov ED, Siebert PD (1996) Suppression subtractive hybridization: a method for generating differentially regulated or tissue-specific cDNA probes and libraries. Proc Natl Acad Sci U S A **93:** 6025-6030

Dietrich RA, Richberg MH, Schmidt R, Dean C, Dangl JL (1997) A novel zinc finger protein is encoded by the Arabidopsis LSD1 gene and functions as a negative regulator of plant cell death. Cell **88:** 685-694

Dimova DK, Stevaux O, Frolov MV, Dyson NJ (2003) Cell cycle-dependent and cell cycle-independent control of transcription by the Drosophila E2F/RB pathway. Genes Dev **17:** 2308-2320

Dinkins R, Pflipsen C, Thompson A, Collins GB (2002) Ectopic Expression of an Arabidopsis Single Zinc Finger Gene in Tobacco Results in Dwarf Plants. Plant Cell Physiol. **43:** 743-750

Dinkins RD, Pflipsen C, Collins GB (2003) Expression and deletion analysis of an Arabidopsis SUPERMAN-like zinc finger gene.. Plant Science **165:** 33-41

Dreyer I, Horeau C, Lemaillet G, Zimmermann S, Bush D, Rodriguez-Navarro A, Schachtman D, Spalding E, Sentenac H, Gaber R (1999) Identification and characterization of plant transporters using heterologous expression systems. J. Exp. Bot. **50:** 1073-1087

Dubouzet JG, Sakuma Y, Ito Y, Kasuga M, Dubouzet EG, Miura S, Seki M, Shinozaki K, Yamaguchi-Shinozaki K (2003) OsDREB genes in rice, Oryza sativa L., encode transcription activators that function in drought-, high-salt- and cold-responsive gene expression. Plant J **33:** 751-763

Dujon B, Alexandraki D, Andre B, Ansorge W, Baladron V, Ballesta JP, Banrevi A, Bolle PA, Bolotin-Fukuhara M, Bossier P, et al. (1994) Complete DNA sequence of yeast chromosome XI. Nature **369:** 371-378

Durner J, Klessig DF (1996) Salicylic acid is a modulator of tobacco and mammalian catalases. J Biol Chem **271:** 28492-28501

Eisen MB, Spellman PT, Brown PO, Botstein D (1998) Cluster analysis and display of genome-wide expression patterns. Proc Natl Acad Sci U S A **95:** 14863-14868

Eriksson P, Alipour H, Adler L, Blomberg A (2000) Rap1p-binding sites in the saccharomyces cerevisiae GPD1 promoter are involved in its response to NaCl. J Biol Chem **275:** 29368-29376

Estruch F (2000) Stress-controlled transcription factors, stress-induced genes and stress tolerance in budding yeast. FEMS Microbiol Rev **24:** 469-486

Eulgem T, Rushton PJ, Robatzek S, Somssich IE (2000) The WRKY superfamily of plant transcription factors. Trends Plant Sci **5:** 199-206

Fan L, Zheng S, Wang X (1997) Antisense Suppression of Phospholipase D[alpha] Retards Abscisic Acid[mdash] and Ethylene-Promoted Senescence of Postharvest Arabidopsis Leaves. Plant Cell **9:** 2183-2196

Fan W, Dong X (2002) In vivo interaction between NPR1 and transcription factor TGA2 leads to salicylic acid-mediated gene activation in Arabidopsis. Plant Cell **14:** 1377-1389

Ferrando A, Kron SJ, Rios G, Fink GR, Serrano R (1995) Regulation of cation transport in Saccharomyces cerevisiae by the salt tolerance gene HAL3. Mol Cell Biol **15:** 5470-5481

Feuerstein R, Wang X, Song D, Cooke NE, Liebhaber SA (1994) The LIM/double zinc-finger motif functions as a protein dimerization domain. Proc Natl Acad Sci U S A **91:** 10655-10659

Finkelstein RR, Somerville CR (1990) Three Classes of Abscisic Acid (ABA)-Insensitive Mutations of Arabidopsis Define Genes that Control Overlapping Subsets of ABA Responses. Plant Physiol. **94:** 1172-1179

Fisher KS, Jhonson EC, Edameades GO (1982) Breeding and selection for Drought Resistance in Crops with Emphasis on Rice,. *In*. IARI, Philippines, pp 377-399

Foor F, Parent SA, Morin N, Dahl AM, Ramadan N, Chrebet G, Bostian KA, Nielsen JB (1992) Calcineurin mediates inhibition by FK506 and cyclosporin of recovery from alpha-factor arrest in yeast. Nature **360:** 682-684

Fowler S, Thomashow MF (2002) Arabidopsis Transcriptome Profiling Indicates That Multiple Regulatory Pathways Are Activated during Cold Acclimation in Addition to the CBF Cold Response Pathway. Plant Cell **14:** 1675-1690

Frommer WB, Ninnemann O (1995) Heterologous Expression of Genes in Bacterial, Fungal, Animal, and Plant Cells. Annual Review of Plant Physiology and Plant Molecular Biology **46:** 419-444

Frugier F, Poirier S, Satiat-Jeunemaitre B, Kondorosi A, Crespi M (2000) A Kruppel-like zinc finger protein is involved in nitrogen-fixing root nodule organogenesis. Genes Dev **14:** 475-482

Fuglsang AT, Guo Y, Cuin TA, Qiu Q, Song C, Kristiansen KA, Bych K, Schulz A, Shabala S, Schumaker KS, Palmgren MG, Zhu JK (2007) Arabidopsis protein kinase PKS5 inhibits the plasma membrane H+ -ATPase by preventing interaction with 14-3-3 protein. Plant Cell **19:** 1617-1634

Fujita M, Fujita Y, Maruyama K, Seki M, Hiratsu K, Ohme-Takagi M, Tran LS, Yamaguchi-Shinozaki K, Shinozaki K (2004) A dehydration-induced NAC protein, RD26, is involved in a novel ABA-dependent stress-signaling pathway. Plant J **39:** 863-876

Fujita Y, Fujita M, Satoh R, Maruyama K, Parvez MM, Seki M, Hiratsu K, Ohme-Takagi M, Shinozaki K, Yamaguchi-Shinozaki K (2005) AREB1 Is a transcription activator of novel ABRE-dependent ABA signaling that enhances drought stress tolerance in Arabidopsis. Plant Cell **17:** 3470-3488

Furihata T, Maruyama K, Fujita Y, Umezawa T, Yoshida R, Shinozaki K, Yamaguchi-Shinozaki K (2006) Abscisic acid-dependent multisite phosphorylation regulates the activity of a transcription activator AREB1. Proc Natl Acad Sci U S A **103:** 1988-1993

Ganster RW, McCartney RR, Schmidt MC (1998) Identification of a calcineurin-independent pathway required for sodium ion stress response in Saccharomyces cerevisiae. Genetics **150:** 31-42

Garcia-Gimeno MA, Struhl K (2000) Aca1 and Aca2, ATF/CREB Activators in Saccharomyces cerevisiae, Are Important for Carbon Source Utilization but Not the Response to Stress. Mol. Cell. Biol. **20:** 4340-4349

Garciadeblas B, Rubio F, Quintero FJ, Banuelos MA, Haro R, Rodriguez-Navarro A (1993) Differential expression of two genes encoding isoforms of the ATPase involved in sodium efflux in Saccharomyces cerevisiae. Mol Gen Genet **236:** 363-368

Garrett-Engele P, Moilanen B, Cyert MS (1995) Calcineurin, the Ca2+/calmodulin-dependent protein phosphatase, is essential in yeast mutants with cell integrity defects and in mutants that lack a functional vacuolar H(+)-ATPase. Mol Cell Biol **15:** 4103-4114

Gietz RD, Schiestl RH, Willems AR, Woods RA (1995) Studies on the transformation of intact yeast cells by the LiAc/SS-DNA/PEG procedure. Yeast **11:** 355-360

Gilmour SJ, Fowler SG, Thomashow MF (2004) Arabidopsis transcriptional activators CBF1, CBF2, and CBF3 have matching functional activities. Plant Mol Biol **54:** 767-781

Gilmour SJ, Sebolt AM, Salazar MP, Everard JD, Thomashow MF (2000) Overexpression of the Arabidopsis CBF3 transcriptional activator mimics multiple biochemical changes associated with cold acclimation. Plant Physiol **124:** 1854-1865

Gisbert C, Rus AM, Bolarin MC, Lopez-Coronado JM, Arrillaga I, Montesinos C, Caro M, Serrano R, Moreno V (2000) The yeast HAL1 gene improves salt tolerance of transgenic tomato. Plant Physiol **123:** 393-402

Goff SA, Cone KC, Chandler VL (1992) Functional analysis of the transcriptional activator encoded by the maize B gene: evidence for a direct functional interaction between two classes of regulatory proteins. Genes Dev **6:** 864-875

Gong D, Guo Y, Schumaker KS, Zhu JK (2004) The SOS3 family of calcium sensors and SOS2 family of protein kinases in Arabidopsis. Plant Physiol **134:** 919-926

Gong Z, Koiwa H, Cushman MA, Ray A, Bufford D, Kore-eda S, Matsumoto TK, Zhu J, Cushman JC, Bressan RA, Hasegawa PM (2001) Genes that are uniquely stress regulated in salt overly sensitive (sos) mutants. Plant Physiol **126:** 363-375

Gosti F, Beaudoin N, Serizet C, Webb AA, Vartanian N, Giraudat J (1999) ABI1 protein phosphatase 2C is a negative regulator of abscisic acid signaling. Plant Cell **11:** 1897-1910

Goyal K, Walton LJ, Tunnacliffe A (2005) LEA proteins prevent protein aggregation due to water stress. Biochem J **388:** 151-157

Graham PH, Vance CP (2003) Legumes: importance and constraints to greater use. Plant Physiol **131:** 872-877

Grandori C, Cowley SM, James LP, Eisenman RN (2000) The Myc/Max/Mad network and the transcriptional control of cell behavior. Annu Rev Cell Dev Biol **16:** 653-699

Gregory P (1998) Root growth of chickpea, febabean, lentil and pea and effect of water and salt stress. Kluwer Academic Publishers, London, U.K

Grover A, Sahi C, Sanan N, Grover A (1999) Taming abiotic stresses in plants through genetic engineering: current strategies and perspective. Plant Sci **143:** 101-111

Gu YQ, Wildermuth MC, Chakravarthy S, Loh YT, Yang C, He X, Han Y, Martin GB (2002) Tomato transcription factors pti4, pti5, and pti6 activate defense responses when expressed in Arabidopsis. Plant Cell **14:** 817-831

Guilfoyle TJ, Ulmasov T, Hagen G (1998) The ARF family of transcription factors and their role in plant hormone-responsive transcription. Cell Mol Life Sci **54:** 619-627

Guiltinan MJ, Miller L (1994) Molecular characterization of the DNA-binding and dimerization domains of the bZIP transcription factor, EmBP-1. Plant Mol Biol **26:** 1041-1053

Guo Y, Gan S (2006) AtNAP, a NAC family transcription factor, has an important role in leaf senescence. Plant J **46:** 601-612

Gurley WB (2000) HSP101: a key component for the acquisition of thermotolerance in plants. Plant Cell **12:** 457-460

Gustin MC, Albertyn J, Alexander M, Davenport K (1998) MAP kinase pathways in the yeast Saccharomyces cerevisiae. Microbiol Mol Biol Rev **62:** 1264-1300

Hanahan D (1983) Studies on transformation of Escherichia coli with plasmids. J Mol Biol **166:** 557-580

Harmon AC, Gribskov M, Harper JF (2000) CDPKs - a kinase for every Ca2+ signal? Trends Plant Sci **5:** 154-159

Haro R, Baneulos MA, Quintero FJ, Rubio F, Rodriguez-Navarro A (1993) Genetic basis of sodium exclusion and sodium tolerance in yeast: A model for plants. Physiol. Plant. **89:** 868-874

Hasegawa PM, Bressan RA, Zhu JK, Bohnert HJ (2000) Plant Cellular and Molecular Responses to High Salinity. Annu Rev Plant Physiol Plant Mol Biol **51:** 463-499

He P, Warren RF, Zhao T, Shan L, Zhu L, Tang X, Zhou JM (2001) Overexpression of Pti5 in tomato potentiates pathogen-induced defense gene expression and enhances disease

resistance to Pseudomonas syringae pv. tomato. Mol Plant Microbe Interact **14:** 1453-1457

He XJ, Mu RL, Cao WH, Zhang ZG, Zhang JS, Chen SY (2005) AtNAC2, a transcription factor downstream of ethylene and auxin signaling pathways, is involved in salt stress response and lateral root development. Plant J **44:** 903-916

He XJ, Zhang ZG, Yan DQ, Zhang JS, Chen SY (2004) A salt-responsive receptor-like kinase gene regulated by the ethylene signaling pathway encodes a plasma membrane serine/threonine kinase. Theor Appl Genet **109:** 377-383

Heath MC (2000) Hypersensitive response-related death. Plant Mol Biol **44:** 321-334

Hohmann S (2002) Osmotic stress signaling and osmoadaptation in yeasts. Microbiol Mol Biol Rev **66:** 300-372

Hong SW, Vierling E (2001) Hsp101 is necessary for heat tolerance but dispensable for development and germination in the absence of stress. Plant J **27:** 25-35

Horsch RB, Rogers SG, Fraley RT (1985) Transgenic plants. Cold Spring Harb Symp Quant Biol **50:** 433-437

Hoyos ME, Zhang S (2000) Calcium-independent activation of salicylic acid-induced protein kinase and a 40-kilodalton protein kinase by hyperosmotic stress. Plant Physiol **122:** 1355-1363

Hsieh TH, Lee JT, Charng YY, Chan MT (2002) Tomato plants ectopically expressing Arabidopsis CBF1 show enhanced resistance to water deficit stress. Plant Physiol **130:** 618-626

Ichimura K, Mizoguchi T, Irie K, Morris P, Giraudat J, Matsumoto K, Shinozaki K (1998) Isolation of ATMEKK1 (a MAP kinase kinase kinase)-interacting proteins and analysis of a MAP kinase cascade in Arabidopsis. Biochem Biophys Res Commun **253:** 532-543

Iida A, Kazuoka T, Torikai S, Kikuchi H, Oeda K (2000) A zinc finger protein RHL41 mediates the light acclimatization response in Arabidopsis. Plant J **24:** 191-203

Ingram J, Bartels D (1996) The Molecular Basis of Dehydration Tolerance in Plants. Annu Rev Plant Physiol Plant Mol Biol **47:** 377-403

Inoue H, Nojima H, Okayama H (1990) High efficiency transformation of Escherichia coli with plasmids. Gene **96:** 23-28

Ito Y, Katsura K, Maruyama K, Taji T, Kobayashi M, Seki M, Shinozaki K, Yamaguchi-Shinozaki K (2006) Functional analysis of rice DREB1/CBF-type transcription factors involved in cold-responsive gene expression in transgenic rice. Plant Cell Physiol **47:** 141-153

Iuchi S, Kobayashi M, Taji T, Naramoto M, Seki M, Kato T, Tabata S, Kakubari Y, Yamaguchi-Shinozaki K, Shinozaki K (2001) Regulation of drought tolerance by gene manipulation of 9-cis-epoxycarotenoid dioxygenase, a key enzyme in abscisic acid biosynthesis in Arabidopsis. Plant J **27:** 325-333

Jaglo KR, Kleff S, Amundsen KL, Zhang X, Haake V, Zhang JZ, Deits T, Thomashow MF (2001) Components of the Arabidopsis C-repeat/dehydration-responsive element binding factor cold-response pathway are conserved in Brassica napus and other plant species. Plant Physiol **127:** 910-917

Jakoby M, Weisshaar B, Droge-Laser W, Vicente-Carbajosa J, Tiedemann J, Kroj T, Parcy F (2002) bZIP transcription factors in Arabidopsis. Trends Plant Sci **7:** 106-111

Jayashree B (2005) A legume genomics resource: The Chickpea Root Expressed Sequence Tag Database. *In* Electronic Journal of Biotechnology, Vol 8

Jefferson R (1987) Assaying chimeric genes in plants: The GUS gene fusion system. Plant Molecular Biology Reporter **5:** 387-405

Jofuku KD, den Boer BG, Van Montagu M, Okamuro JK (1994) Control of Arabidopsis flower and seed development by the homeotic gene APETALA2. Plant Cell **6:** 1211-1225

Johnson JD, Zhang W, Rudnick A, Rutter WJ, German MS (1997) Transcriptional synergy between LIM-homeodomain proteins and basic helix-loop-helix proteins: the LIM2 domain determines specificity. Mol Cell Biol **17:** 3488-3496

Jonak C, Kiegerl S, Ligterink W, Barker PJ, Huskisson NS, Hirt H (1996) Stress signaling in plants: a mitogen-activated protein kinase pathway is activated by cold and drought. Proc Natl Acad Sci U S A **93:** 11274-11279

Jonak C, Ligterink W, Hirt H (1999) MAP kinases in plant signal transduction. Cell Mol Life Sci **55:** 204-213

Kafadar KA, Cyert MS (2004) Integration of stress responses: modulation of calcineurin signaling in Saccharomyces cerevisiae by protein kinase A. Eukaryot Cell **3:** 1147-1153

Kang JY, Choi HI, Im MY, Kim SY (2002) Arabidopsis basic leucine zipper proteins that mediate stress-responsive abscisic acid signaling. Plant Cell **14:** 343-357

Kasuga M, Liu Q, Miura S, Yamaguchi-Shinozaki K, Shinozaki K (1999) Improving plant drought, salt, and freezing tolerance by gene transfer of a single stress-inducible transcription factor. Nat Biotechnol **17:** 287-291

Katagiri F, Chua NH (1992) Plant transcription factors: present knowledge and future challenges. Trends Genet **8:** 22-27

Kathiresana A, Lafittea HR, Chena J, Mansuetoa L, Bruskiewich R, Bennett J (2006) Gene expression microarrays and their application in drought stress research. Field Crops Research **97:** 101-110

Kawasaki S, Borchert C, Deyholos M, Wang H, Brazille S, Kawai K, Galbraith D, Bohnert HJ (2001) Gene expression profiles during the initial phase of salt stress in rice. Plant Cell **13:** 889-905

Kim JC, Lee SH, Cheong YH, Yoo CM, Lee SI, Chun HJ, Yun DJ, Hong JC, Lee SY, Lim CO, Cho MJ (2001) A novel cold-inducible zinc finger protein from soybean, SCOF-1, enhances cold tolerance in transgenic plants. Plant J **25:** 247-259

Kim KN, Cheong YH, Grant JJ, Pandey GK, Luan S (2003) CIPK3, a calcium sensor-associated protein kinase that regulates abscisic acid and cold signal transduction in Arabidopsis. Plant Cell **15:** 411-423

Kim KN, Cheong YH, Gupta R, Luan S (2000) Interaction specificity of Arabidopsis calcineurin B-like calcium sensors and their target kinases. Plant Physiol **124:** 1844-1853

Kim MI, Park SW, Yu SH, Cho HS, Ha HJ, Hwang I, Pai HS (2001) Molecular characterization of the NeIF2Bbeta gene encoding a putative eIF2B beta-subunit in Nicotiana tabacum. Mol Cells **11:** 110-114

Kim YH, Song HO, Ko KM, Singaravelu G, Jee C, Kang J, Ahnn J (2008) A Novel Calcineurin-interacting Protein, CNP-3, Modulates Calcineurin Deficient Phenotypes in Caenorhabditis elegans. Mol Cells **25**

Klein M, Geisler M, Suh SJ, Kolukisaoglu H, Uuml, Azevedo L, Plaza S, Curtis MD, Richter A, Weder B, Schulz B, Martinoia E (2004) Disruption of AtMRP4, a guard cell plasma membrane ABCC-type ABC transporter, leads to deregulation of stomatal opening and increased drought susceptibility. The Plant Journal **39:** 219-236

Knight H, Brandt S, Knight MR (1998) A history of stress alters drought calcium signalling pathways in Arabidopsis. Plant J **16:** 681-687

Knight H, Trewavas AJ, Knight MR (1996) Cold calcium signaling in Arabidopsis involves two cellular pools and a change in calcium signature after acclimation. Plant Cell **8:** 489-503

Knight MR, Campbell AK, Smith SM, Trewavas AJ (1991) Transgenic plant aequorin reports the effects of touch and cold-shock and elicitors on cytoplasmic calcium. Nature **352:** 524-526

Kobayashi F, Ishibashi M, Takumi S (2008) Transcriptional activation of Cor / Lea genes and increase in abiotic stress tolerance through expression of a wheat DREB2 homolog in transgenic tobacco. Transgenic Research **17:** 755-767

Kohler C, Hennig L, Spillane C, Pien S, Gruissem W, Grossniklaus U (2003) The Polycomb-group protein MEDEA regulates seed development by controlling expression of the MADS-box gene PHERES1. Genes Dev **17:** 1540-1553

Kong Y, Flick MJ, Kudla AJ, Konieczny SF (1997) Muscle LIM protein promotes myogenesis by enhancing the activity of MyoD. Mol Cell Biol **17:** 4750-4760

Koornneef M, Alonso-Blanco C, Vreugdenhil D (2004) Naturally occurring genetic variation in Arabidopsis thaliana. Annu Rev Plant Biol **55:** 141-172

Kovtun Y, Chiu WL, Tena G, Sheen J (2000) Functional analysis of oxidative stress-activated mitogen-activated protein kinase cascade in plants. Proc Natl Acad Sci U S A **97:** 2940-2945

Kreps JA, Wu Y, Chang HS, Zhu T, Wang X, Harper JF (2002) Transcriptome changes for Arabidopsis in response to salt, osmotic, and cold stress. Plant Physiol **130:** 2129-2141

Kubo K, Sakamoto A, Kobayashi A, Rybka Z, Kanno Y, Nakagawa H, Takatsuji H (1998) Cys2/His2 zinc-finger protein family of petunia: evolution and general mechanism of target-sequence recognition. Nucleic Acids Res **26:** 608-615

Kudla J, Xu Q, Harter K, Gruissem W, Luan S (1999) Genes for calcineurin B-like proteins in Arabidopsis are differentially regulated by stress signals. Proc Natl Acad Sci U S A **96:** 4718-4723

Kuhn C, Frommer WB (1995) A novel zinc finger protein encoded by a couch potato homologue from Solanum tuberosum enables a sucrose transport-deficient yeast strain to grow on sucrose. Mol Gen Genet **247:** 759-763

Kumari S, Panjabi Nee Sabharwal V, Kushwaha HR, Sopory SK, Singla-Pareek SL, Pareek A (2008) Transcriptome map for seedling stage specific salinity stress response indicates a specific set of genes as candidate for saline tolerance in Oryza sativa L. Funct Integr Genomics

Kupicha FK (1981) Vicieae. *In* RM Polhill, PH Raven, eds, Advances in legume systematics, Vol 1, Kew Royal Botanic Gardens, pp 377–381

Kuroda S, Tokunaga C, Kiyohara Y, Higuchi O, Konishi H, Mizuno K, Gill GN, Kikkawa U (1996) Protein-protein interaction of zinc finger LIM domains with protein kinase C. J Biol Chem **271:** 31029-31032

Kwak JM, Mori IC, Pei ZM, Leonhardt N, Torres MA, Dangl JL, Bloom RE, Bodde S, Jones JD, Schroeder JI (2003) NADPH oxidase AtrbohD and AtrbohF genes function in ROS-dependent ABA signaling in Arabidopsis. EMBO J **22:** 2623-2633

Laemmli UK (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature **227:** 680-685

Lang V, Mantyla E, Welin B, Sundberg B, Palva ET (1994) Alterations in Water Status, Endogenous Abscisic Acid Content, and Expression of rab18 Gene during the Development of Freezing Tolerance in Arabidopsis thaliana. Plant Physiol. **104:** 1341-1349

Lee JH, Hubel A, Schoffl F (1995) Derepression of the activity of genetically engineered heat shock factor causes constitutive synthesis of heat shock proteins and increased thermotolerance in transgenic Arabidopsis. Plant J **8:** 603-612

Lee JH, Van Montagu M, Verbruggen N (1999) A highly conserved kinase is an essential component for stress tolerance in yeast and plant cells. Proc Natl Acad Sci U S A **96:** 5873-5877

Lee SC, Lan WZ, Kim BG, Li L, Cheong YH, Pandey GK, Lu G, Buchanan BB, Luan S (2007) A protein phosphorylation/dephosphorylation network regulates a plant potassium channel. Proc Natl Acad Sci U S A **104:** 15959-15964

Leung J, Giraudat J (1998) Abscisic Acid Signal Transduction. Annu Rev Plant Physiol Plant Mol Biol **49:** 199-222

Liang F, Cunningham KW, Harper JF, Sze H (1997) ECA1 complements yeast mutants defective in Ca2+ pumps and encodes an endoplasmic reticulum-type Ca2+-ATPase in Arabidopsis thaliana. Proc Natl Acad Sci U S A **94:** 8579-8584

Liotenberg S, North H, Marion-Poll A (1999) Molecular biology and regulation of abscisic acid biosynthesis in plants. Plant Physiol. Biochem. **37:** 341-350

Lippuner V, Cyert MS, Gasser CS (1996) Two classes of plant cDNA clones differentially complement yeast calcineurin mutants and increase salt tolerance of wild-type yeast. J Biol Chem **271:** 12859-12866

Liu J (1993) FK506 and ciclosporin: molecular probes for studying intracellular signal transduction. Trends Pharmacol Sci **14:** 182-188

Liu J, Zhu JK (1997) An Arabidopsis mutant that requires increased calcium for potassium nutrition and salt tolerance. Proc Natl Acad Sci U S A **94:** 14960-14964

Liu J, Zhu JK (1998) A calcium sensor homolog required for plant salt tolerance. Science **280:** 1943-1945

Liu L, White MJ, MacRae TH (1999) Transcription factors and their genes in higher plants functional domains, evolution and regulation. Eur J Biochem **262:** 247-257

Liu Q, Kasuga M, Sakuma Y, Abe H, Miura S, Yamaguchi-Shinozaki K, Shinozaki K (1998) Two transcription factors, DREB1 and DREB2, with an EREBP/AP2 DNA binding domain separate two cellular signal transduction pathways in drought- and low-temperature-responsive gene expression, respectively, in Arabidopsis. Plant Cell **10:** 1391-1406

Luan S, Kudla J, Rodriguez-Concepcion M, Yalovsky S, Gruissem W (2002) Calmodulins and calcineurin B-like proteins: calcium sensors for specific signal response coupling in plants. Plant Cell **14 Suppl:** S389-400

Luan S, Li W, Rusnak F, Assmann SM, Schreiber SL (1993) Immunosuppressants implicate protein phosphatase regulation of K+ channels in guard cells. Proc Natl Acad Sci U S A **90:** 2202-2206

Ludlow MM, Muchow RC (1990) A critical evaluation of traits for improving crops yields in water-limited environments. Advances Agronomy **43:** 107-153

Ma Q, Behboudian MH, Turner NC, Palta JA (2001) Gas exchange by pods and subtending leaves and internal recycling of CO(2) by pods of chickpea (Cicer arietinum L.) subjected to water deficits. J Exp Bot **52:** 123-131

Maeda T, Takekawa M, Saito H (1995) Activation of yeast PBS2 MAPKK by MAPKKKs or by binding of an SH3-containing osmosensor. Science **269:** 554-558

Maeda T, Wurgler-Murphy SM, Saito H (1994) A two-component system that regulates an osmosensing MAP kinase cascade in yeast. Nature **369:** 242-245

Mager WH, Siderius M (2002) Novel insights into the osmotic stress response of yeast. FEMS Yeast Res **2:** 251-257

Magne C, Larher F (1992) High sugar content of extracts interferes with colorimetric determination of amino acids and free proline. Anal Biochem **200:** 115-118

Majee M, Maitra S, Dastidar KG, Pattnaik S, Chatterjee A, Hait NC, Das KP, Majumder AL (2004) A novel salt-tolerant L-myo-inositol-1-phosphate synthase from Porteresia coarctata (Roxb.) Tateoka, a halophytic wild rice: molecular cloning, bacterial overexpression, characterization, and functional introgression into tobacco-conferring salt tolerance phenotype. J Biol Chem **279:** 28539-28552

Maleck K, Levine A, Eulgem T, Morgan A, Schmid J, Lawton KA, Dangl JL, Dietrich RA (2000) The transcriptome of Arabidopsis thaliana during systemic acquired resistance. Nat Genet **26:** 403-410

Mantri NL, Ford R, Coram TE, Pang EC (2007) Transcriptional profiling of chickpea genes differentially regulated in response to high-salinity, cold and drought. BMC Genomics **8:** 303

Mantyla E, Lang V, Palva ET (1995) Role of Abscisic Acid in Drought-Induced Freezing Tolerance, Cold Acclimation, and Accumulation of LT178 and RAB18 Proteins in Arabidopsis thaliana. Plant Physiol. **107:** 141-148

Martin C, Paz-Ares J (1997) MYB transcription factors in plants. Trends Genet **13:** 67-73

Martinez-Pastor MT, Marchler G, Schuller C, Marchler-Bauer A, Ruis H, Estruch F (1996) The Saccharomyces cerevisiae zinc finger proteins Msn2p and Msn4p are required for transcriptional induction through the stress response element (STRE). Embo J **15:** 2227-2235

Maruyama K, Sakuma Y, Kasuga M, Ito Y, Seki M, Goda H, Shimada Y, Yoshida S, Shinozaki K, Yamaguchi-Shinozaki K (2004) Identification of cold-inducible downstream genes of the Arabidopsis DREB1A/CBF3 transcriptional factor using two microarray systems. Plant J **38:** 982-993

Maskin L, Frankel N, Gudesblat G, Demergasso MJ, Pietrasanta LI, Iusem ND (2007) Dimerization and DNA-binding of ASR1, a small hydrophilic protein abundant in plant tissues suffering from water loss. Biochem Biophys Res Commun **352:** 831-835

Matheos DP, Kingsbury TJ, Ahsan US, Cunningham KW (1997) Tcn1p/Crz1p, a calcineurin-dependent transcription factor that differentially regulates gene expression in Saccharomyces cerevisiae. Genes Dev **11:** 3445-3458

Matsumoto TK, Pardo JM, Takeda S, Bressan RA, Hasegawa PM (2001) Tobacco and Arabidiopsis SLT1 mediate salt tolerance of yeast. Plant Mol Biol **45:** 489-500

Meissner R, Michael AJ (1997) Isolation and characterisation of a diverse family of Arabidopsis two and three-fingered C2H2 zinc finger protein genes and cDNAs. Plant Mol Biol **33:** 615-624

Mendizabal I, Pascual-Ahuir A, Serrano R, de Larrinoa IF (2001) Promoter sequences regulated by the calcineurin-activated transcription factor Crz1 in the yeast ENA1 gene. Mol Genet Genomics **265:** 801-811

Mendizabal I, Rios G, Mulet JM, Serrano R, de Larrinoa IF (1998) Yeast putative transcription factors involved in salt tolerance. FEBS Lett **425:** 323-328

Mendoza I, Rubio F, Rodriguez-Navarro A, Pardo JM (1994) The protein phosphatase calcineurin is essential for NaCl tolerance of Saccharomyces cerevisiae. J Biol Chem **269:** 8792-8796

Meskiene I, Bogre L, Glaser W, Balog J, Brandstotter M, Zwerger K, Ammerer G, Hirt H (1998) MP2C, a plant protein phosphatase 2C, functions as a negative regulator of mitogen-activated protein kinase pathways in yeast and plants. Proc Natl Acad Sci U S A **95:** 1938-1943

Michael AJ, Hofer JM, Ellis TH (1996) Isolation by PCR of a cDNA clone from pea petals with similarity to petunia and wheat zinc finger proteins. Plant Mol Biol **30:** 1051-1058

Miller J, McLachlan AD, Klug A (1985) Repetitive zinc-binding domains in the protein transcription factor IIIA from Xenopus oocytes. EMBO J **4:** 1609-1614

Miller JH (1972) Experiments in Molecular Genetics Cold Spring Harbor Lab. Press, Plainview, NY

Misra S, Wu Y, Venkataraman G, Sopory SK, Tuteja N (2007) Heterotrimeric G-protein complex and G-protein-coupled receptor from a legume (Pisum sativum): role in salinity and heat stress and cross-talk with phospholipase C. Plant J **51:** 656-669

Mittler R (2002) Oxidative stress, antioxidants and stress tolerance. Trends Plant Sci **7:** 405-410

Mohapatra SS, Poole RJ, Dhindsa RS (1988) Abscisic Acid-Regulated Gene Expression in Relation to Freezing Tolerance in Alfalfa. Plant Physiol **87:** 468-473

Moretti MB, Maskin L, Gudesblat G, Garcia SC, Iusem ND (2006) ASR1, a stress-induced tomato protein, protects yeast from osmotic stress. Physiologia Plantarum **127:** 111-118

Mukhopadhyay A, Vij S, Tyagi AK (2004) Overexpression of a zinc-finger protein gene from rice confers tolerance to cold, dehydration, and salt stress in transgenic tobacco. Proc Natl Acad Sci U S A **101:** 6309-6314

Mulet JM, Leube MP, Kron SJ, Rios G, Fink GR, Serrano R (1999) A novel mechanism of ion homeostasis and salt tolerance in yeast: the Hal4 and Hal5 protein kinases modulate the Trk1-Trk2 potassium transporter. Mol Cell Biol **19:** 3328-3337

Mullis KB, Faloona FA (1987) Specific synthesis of DNA in vitro via a polymerase-catalyzed chain reaction. Methods Enzymol **155:** 335-350

Munns R, James RA, Lauchli A (2006) Approaches to increasing the salt tolerance of wheat and other cereals. J Exp Bot **57:** 1025-1043

Murray MG, Thompson WF (1980) Rapid isolation of high molecular weight plant DNA. Nucl. Acids Res. **8:** 4321-4326

Nakamura T, Liu Y, Hirata D, Namba H, Harada S, Hirokawa T, Miyakawa T (1993) Protein phosphatase type 2B (calcineurin)-mediated, FK506-sensitive regulation of intracellular ions in yeast is an important determinant for adaptation to high salt stress conditions. Embo J **12:** 4063-4071

Nakashima K, Ito Y, Yamaguchi-Shinozaki K (2009) Transcriptional Regulatory Networks in Response to Abiotic Stresses in Arabidopsis and Grasses. Plant Physiol. **149:** 88-95

Niu X, Bressan RA, Hasegawa PM, Pardo JM (1995) Ion Homeostasis in NaCl Stress Environments. Plant Physiol **109:** 735-742

Novillo F, Alonso JM, Ecker JR, Salinas J (2004) CBF2/DREB1C is a negative regulator of CBF1/DREB1B and CBF3/DREB1A expression and plays a central role in stress tolerance in Arabidopsis. Proc Natl Acad Sci U S A **101:** 3985-3990

Nuccio ML, Rhodes D, McNeil SD, Hanson AD (1999) Metabolic engineering of plants for osmotic stress resistance. Curr Opin Plant Biol **2:** 128-134

Obata T, Kitamoto HK, Nakamura A, Fukuda A, Tanaka Y (2007) Rice shaker potassium channel OsKAT1 confers tolerance to salinity stress on yeast and rice cells. Plant Physiol **144:** 1978-1985

Ohkawa T, Washburn JO, Sitapara R, Sid E, Volkman LE (2005) Specific binding of Autographa californica M nucleopolyhedrovirus occlusion-derived virus to midgut cells of Heliothis virescens larvae is mediated by products of pif genes Ac119 and Ac022 but not by Ac115. J Virol **79:** 15258-15264

Ohta M, Matsui K, Hiratsu K, Shinshi H, Ohme-Takagi M (2001) Repression domains of class II ERF transcriptional repressors share an essential motif for active repression. Plant Cell **13:** 1959-1968

Ohtake Y, Takahashi T, Komeda Y (2000) Salicylic acid induces the expression of a number of receptor-like kinase genes in Arabidopsis thaliana. Plant Cell Physiol **41:** 1038-1044

Ota IM, Varshavsky A (1993) A yeast protein similar to bacterial two-component regulators. Science **262:** 566-569

Oztur ZN, Talame V, Deyholos M, Michalowski CB, Galbraith DW, Gozukirmizi N, Tuberosa R, Bohnert HJ (2002) Monitoring large-scale changes in transcript abundance in drought- and salt-stressed barley. Plant Mol Biol **48:** 551-573

Pabo CO, Peisach E, Grant RA (2001) Design and selection of novel Cys2His2 zinc finger proteins. Annu Rev Biochem **70:** 313-340

Pandey A, Chakraborty S, Datta A, Chakraborty N (2008) Proteomics approach to identify dehydration responsive nuclear proteins from chickpea (Cicer arietinum L.). Mol Cell Proteomics **7:** 88-107

Pandey G (2008) Emergence of a novel calcium signaling pathway in plants: CBL-CIPK signaling network. Physiology and Molecular Biology of Plants **14:** 51-68

Pardo JM, Reddy MP, Yang S, Maggio A, Huh GH, Matsumoto T, Coca MA, Paino-D'Urzo M, Koiwa H, Yun DJ, Watad AA, Bressan RA, Hasegawa PM (1998) Stress signaling through Ca2+/calmodulin-dependent protein phosphatase calcineurin mediates salt adaptation in plants. Proc Natl Acad Sci U S A **95:** 9681-9686

Parre E, Ghars MA, Leprince A-S, Thiery L, Lefebvre D, Bordenave M, Richard L, Mazars C, Abdelly C, Savoure A (2007) Calcium Signaling via Phospholipase C Is Essential for Proline Accumulation upon Ionic But Not Nonionic Hyperosmotic Stresses in Arabidopsis. Plant Physiol. **144:** 503-512

Pascual-Ahuir A, Serrano R, Proft M (2001) The Skolp repressor and Gcn4p activator antagonistically modulate stress-regulated transcription in Saccharomyces cerevisiae. Mol Cell Biol **21:** 16-25

Patel S, Caplan J, Dinesh-Kumar SP (2006) Autophagy in the control of programmed cell death. Curr Opin Plant Biol **9:** 391-396

Pavletich NP, Pabo CO (1991) Zinc finger-DNA recognition: crystal structure of a Zif268-DNA complex at 2.1 A. Science **252:** 809-817

Peng Z, Verma DP (1995) A rice HAL2-like gene encodes a Ca(2+)-sensitive 3'(2'),5'-diphosphonucleoside 3'(2')-phosphohydrolase and complements yeast met22 and Escherichia coli cysQ mutations. J Biol Chem **270:** 29105-29110

Piao HL, Pih KT, Lim JH, Kang SG, Jin JB, Kim SH, Hwang I (1999) An Arabidopsis GSK3/shaggy-like gene that complements yeast salt stress-sensitive mutants is induced by NaCl and abscisic acid. Plant Physiol **119:** 1527-1534

Pontier D, Miao ZH, Lam E (2001) Trans-dominant suppression of plant TGA factors reveals their negative and positive roles in plant defense responses. Plant J **27:** 529-538

Popping B, Gibbons T, Watson MD (1996) The Pisum sativum MAP kinase homologue (PsMAPK) rescues the Saccharomyces cerevisiae hog1 deletion mutant under conditions of high osmotic stress. Plant Mol Biol **31:** 355-363

Posas F, Chambers JR, Heyman JA, Hoeffler JP, de Nadal E, Arino J (2000) The transcriptional response of yeast to saline stress. J Biol Chem **275:** 17249-17255

Posas F, Wurgler-Murphy SM, Maeda T, Witten EA, Thai TC, Saito H (1996) Yeast HOG1 MAP kinase cascade is regulated by a multistep phosphorelay mechanism in the SLN1-YPD1-SSK1 "two-component" osmosensor. Cell **86:** 865-875

Prashanth SR, Sadhasivam V, Parida A (2008) Over expression of cytosolic copper/zinc superoxide dismutase from a mangrove plant Avicennia marina in indica rice var Pusa Basmati-1 confers abiotic stress tolerance. Transgenic Res **17:** 281-291

Proft M, Mas G, de Nadal E, Vendrell A, Noriega N, Struhl K, Posas F (2006) The stress-activated Hog1 kinase is a selective transcriptional elongation factor for genes responding to osmotic stress. Mol Cell **23:** 241-250

Proft M, Pascual-Ahuir A, de Nadal E, Arino J, Serrano R, Posas F (2001) Regulation of the Sko1 transcriptional repressor by the Hog1 MAP kinase in response to osmotic stress. EMBO J **20:** 1123-1133

Proft M, Serrano R (1999) Repressors and upstream repressing sequences of the stress-regulated ENA1 gene in Saccharomyces cerevisiae: bZIP protein Sko1p confers HOG-dependent osmotic regulation. Mol Cell Biol **19:** 537-546

Proft M, Struhl K (2002) Hog1 kinase converts the Sko1-Cyc8-Tup1 repressor complex into an activator that recruits SAGA and SWI/SNF in response to osmotic stress. Mol Cell **9:** 1307-1317

Qiu QS, Guo Y, Quintero FJ, Pardo JM, Schumaker KS, Zhu JK (2004) Regulation of vacuolar Na+/H+ exchange in Arabidopsis thaliana by the salt-overly-sensitive (SOS) pathway. J Biol Chem **279:** 207-215

Queitsch C, Hong SW, Vierling E, Lindquist S (2000) Heat shock protein 101 plays a crucial role in thermotolerance in Arabidopsis. Plant Cell **12:** 479-492

Quintero FJ, Garciadeblas B, Rodriguez-Navarro A (1996) The SAL1 gene of Arabidopsis, encoding an enzyme with 3'(2'),5'-bisphosphate nucleotidase and inositol polyphosphate 1-phosphatase activities, increases salt tolerance in yeast. Plant Cell **8:** 529-537

Rabbani MA, Maruyama K, Abe H, Khan MA, Katsura K, Ito Y, Yoshiwara K, Seki M, Shinozaki K, Yamaguchi-Shinozaki K (2003) Monitoring expression profiles of rice genes under cold, drought, and high-salinity stresses and abscisic acid application using cDNA microarray and RNA gel-blot analyses. Plant Physiol **133:** 1755-1767

Reed RC, Brady SR, Muday GK (1998) Inhibition of auxin movement from the shoot into the root inhibits lateral root development in Arabidopsis. Plant Physiol **118:** 1369-1378

Rep M, Krantz M, Thevelein JM, Hohmann S (2000) The transcriptional response of Saccharomyces cerevisiae to osmotic shock. Hotlp and Msn2p/Msn4p are required for the induction of subsets of high osmolarity glycerol pathway-dependent genes. J Biol Chem **275:** 8290-8300

Rep M, Reiser V, Gartner U, Thevelein JM, Hohmann S, Ammerer G, Ruis H (1999) Osmotic stress-induced gene expression in Saccharomyces cerevisiae requires Msnlp and the novel nuclear factor Hotlp. Mol Cell Biol **19:** 5474-5485

Riechmann JL, Heard J, Martin G, Reuber L, Jiang C, Keddie J, Adam L, Pineda O, Ratcliffe OJ, Samaha RR, Creelman R, Pilgrim M, Broun P, Zhang JZ, Ghandehari D, Sherman BK, Yu G (2000) Arabidopsis transcription factors: genome-wide comparative analysis among eukaryotes. Science **290:** 2105-2110

Riechmann JL, Meyerowitz EM (1998) The AP2/EREBP family of plant transcription factors. Biol Chem **379:** 633-646

Rios G, Ferrando A, Serrano R (1997) Mechanisms of salt tolerance conferred by overexpression of the HAL1 gene in Saccharomyces cerevisiae. Yeast **13:** 515-528

Robinson MJ, Cobb MH (1997) Mitogen-activated protein kinase pathways. Curr Opin Cell Biol **9:** 180-186

Ruegger M, Dewey E, Gray WM, Hobbie L, Turner J, Estelle M (1998) The TIR1 protein of Arabidopsis functions in auxin response and is related to human SKP2 and yeast grrlp. Genes Dev **12:** 198-207

Rusnak F, Mertz P (2000) Calcineurin: form and function. Physiol Rev **80:** 1483-1521

Ryals JA, Neuenschwander UH, Willits MG, Molina A, Steiner HY, Hunt MD (1996) Systemic Acquired Resistance. Plant Cell **8:** 1809-1819

Saavedra L, Svensson J, Carballo V, Izmendi D, Welin B, Vidal S (2006) A dehydrin gene in Physcomitrella patens is required for salt and osmotic stress tolerance. Plant J **45:** 237-249

Sahi C, Agarwal M, Reddy MK, Sopory SK, Grover A (2003) Isolation and expression analysis of salt stress-associated ESTs from contrasting rice cultivars using a PCR-based subtraction method. Theor Appl Genet **106:** 620-628

Saijo Y, Hata S, Kyozuka J, Shimamoto K, Izui K (2000) Over-expression of a single Ca2+-dependent protein kinase confers both cold and salt/drought tolerance on rice plants. Plant J **23:** 319-327

Sainz MB, Grotewold E, Chandler VL (1997) Evidence for direct activation of an anthocyanin promoter by the maize C1 protein and comparison of DNA binding by related Myb domain proteins. Plant Cell **9:** 611-625

Sakai H, Medrano LJ, Meyerowitz EM (1995) Role of SUPERMAN in maintaining Arabidopsis floral whorl boundaries. Nature **378:** 199-203

Sakamoto A, Minami M, Huh GH, Iwabuchi M (1993) The putative zinc-finger protein WZF1 interacts with a cis-acting element of wheat histone genes. Eur J Biochem **217:** 1049-1056

Sakamoto H, Araki T, Meshi T, Iwabuchi M (2000) Expression of a subset of the Arabidopsis Cys(2)/His(2)-type zinc-finger protein gene family under water stress. Gene **248:** 23-32

Sakamoto H, Maruyama K, Sakuma Y, Meshi T, Iwabuchi M, Shinozaki K, Yamaguchi-Shinozaki K (2004) Arabidopsis Cys2/His2-type zinc-finger proteins function as transcription repressors under drought, cold, and high-salinity stress conditions. Plant Physiol **136:** 2734-2746

Sambrook J, Russell D, Sambrook J (2001) Molecular Cloning: A Laboratory Manual (3-Volume Set). {Cold Spring Harbor Laboratory Press}

Sanchez-Garcia I, Rabbitts TH (1994) The LIM domain: a new structural motif found in zinc-finger-like proteins. Trends Genet **10:** 315-320

Sanders D, Pelloux J, Brownlee C, Harper JF (2002) Calcium at the crossroads of signaling. Plant Cell **14 Suppl:** S401-417

Schmitt AP, McEntee K (1996) Msn2p, a zinc finger DNA-binding protein, is the transcriptional activator of the multistress response in Saccharomyces cerevisiae. Proc Natl Acad Sci U S A **93:** 5777-5782

Schmitt ME, Brown TA, Trumpower BL (1990) A rapid and simple method for preparation of RNA from Saccharomyces cerevisiae. Nucleic Acids Res **18:** 3091-3092

Schuller C, Brewster JL, Alexander MR, Gustin MC, Ruis H (1994) The HOG pathway controls osmotic regulation of transcription via the stress response element (STRE) of the Saccharomyces cerevisiae CTT1 gene. Embo J **13:** 4382-4389

Schweighofer A, Hirt H, Meskiene I (2004) Plant PP2C phosphatases: emerging functions in stress signaling. Trends Plant Sci **9:** 236-243

Seki M, Narusaka M, Abe H, Kasuga M, Yamaguchi-Shinozaki K, Carninci P, Hayashizaki Y, Shinozaki K (2001) Monitoring the expression pattern of 1300 Arabidopsis genes under drought and cold stresses by using a full-length cDNA microarray. Plant Cell **13:** 61-72

Seki M, Narusaka M, Ishida J, Nanjo T, Fujita M, Oono Y, Kamiya A, Nakajima M, Enju A, Sakurai T, Satou M, Akiyama K, Taji T, Yamaguchi-Shinozaki K, Carninci P, Kawai J, Hayashizaki Y, Shinozaki K (2002) Monitoring the expression profiles of 7000 Arabidopsis genes under drought, cold and high-salinity stresses using a full-length cDNA microarray. Plant J **31:** 279-292

Serrano R (1996) Salt tolerance in plants and microorganisms: toxicity targets and defense responses. Int Rev Cytol **165:** 1-52

Serrano R, Gaxiola R (1994) Microbial models and salt stress tolerance in plants. Crit. Rev. Plant Sci. **13:** 121-138

Serrano R, Montesinos C (2003) Molecular Bases of Desiccation Tolerance in Plant Cells and Potential Applications in Food Dehydration. Food Science and Technology International **9:** 157-161

Sheen J (1996) Ca2+-dependent protein kinases and stress signal transduction in plants. Science **274:** 1900-1902

Shi J, Kim K-N, Ritz O, Albrecht V, Gupta R, Harter K, Luan S, Kudla J (1999) Novel Protein Kinases Associated with Calcineurin B-like Calcium Sensors in Arabidopsis. Plant Cell **11:** 2393-2406

Shin H, Shin HS, Guo Z, Blancaflor EB, Masson PH, Chen R (2005) Complex regulation of Arabidopsis AGR1/PIN2-mediated root gravitropic response and basipetal auxin transport by cantharidin-sensitive protein phosphatases. Plant J **42:** 188-200

Shinozaki K, Yamaguchi-Shinozaki K (1996) Molecular responses to drought and cold stress. Curr Opin Biotechnol **7:** 161-167

Shinozaki K, Yamaguchi-Shinozaki K (2000) Molecular responses to dehydration and low temperature: differences and cross-talk between two stress signaling pathways. Curr Opin Plant Biol **3:** 217-223

Shinozaki K, Yamaguchi-Shinozaki K (2007) Gene networks involved in drought stress response and tolerance. J Exp Bot **58:** 221-227

Shiozaki N, Yamada M, Yoshiba Y (2005) Analysis of salt-stress-inducible ESTs isolated by PCR-subtraction in salt-tolerant rice. Theor Appl Genet **110:** 1177-1186

Shoji Sugano HKZRRCJSKMMO-THT (2003) Stress-responsive zinc finger gene <i>ZPT2-3</i> plays a role in drought tolerance in petunia. The Plant Journal **36:** 830-841

Shou H, Bordallo P, Wang K (2004) Expression of the Nicotiana protein kinase (NPK1) enhanced drought tolerance in transgenic maize. J Exp Bot **55:** 1013-1019

Shukla RK, Raha S, Tripathi V, Chattopadhyay D (2006) Expression of CAP2, an APETALA2-Family Transcription Factor from Chickpea, Enhances Growth and Tolerance to Dehydration and Salt Stress in Transgenic Tobacco. Plant Physiol. **142:** 113-123

Signora L, De Smet I, Foyer CH, Zhang H (2001) ABA plays a central role in mediating the regulatory effects of nitrate on root branching in Arabidopsis. Plant J **28:** 655-662

Siminovitch D, Cloutier Y (1982) Twenty-Four-Hour Induction of Freezing and Drought Tolerance in Plumules of Winter Rye Seedlings by Desiccation Stress at Room Temperature in the Dark. Plant Physiol. **69:** 250-255

Skriver K, Mundy J (1990) Gene expression in response to abscisic acid and osmotic stress. Plant Cell **2:** 503-512

Smallwood M, Bowles DJ (2002) Plants in a cold climate. Philos Trans R Soc Lond B Biol Sci **357:** 831-847

Smith A, Ward MP, Garrett S (1998) Yeast PKA represses Msn2p/Msn4p-dependent gene expression to regulate growth, stress response and glycogen accumulation. EMBO J **17:** 3556-3564

Snedden WA, Fromm H (1998) Calmodulin, calmodulin-related proteins and plant responses to the environment. **3:** 299-304

Soyano T, Nishihama R, Morikiyo K, Ishikawa M, Machida Y (2003) NQK1/NtMEK1 is a MAPKK that acts in the NPK1 MAPKKK-mediated MAPK cascade and is required for plant cytokinesis. Genes Dev **17:** 1055-1067

Sprague GF, Jr. (1998) Control of MAP kinase signaling specificity or how not to go HOG wild. Genes Dev **12:** 2817-2820

Stathopoulos AM, Cyert MS (1997) Calcineurin acts through the CRZ1/TCN1-encoded transcription factor to regulate gene expression in yeast. Genes Dev **11:** 3432-3444

Steponkus PL, Uemura M, Joseph RA, Gilmour SJ, Thomashow MF (1998) Mode of action of the COR15a gene on the freezing tolerance of Arabidopsis thaliana. Proc Natl Acad Sci U S A **95:** 14570-14575

Su H, Balderas E, Vera-Estrella R, Golldack D, Quigley F, Zhao C, Pantoja O, Bohnert HJ (2003) Expression of the cation transporter McHKT1 in a halophyte. Plant Mol Biol **52:** 967-980

Sung DY, Vierling E, Guy CL (2001) Comprehensive expression profile analysis of the Arabidopsis Hsp70 gene family. Plant Physiol **126:** 789-800

Takatsuji H (1998) Zinc-finger transcription factors in plants. Cell Mol Life Sci **54:** 582-596

Takatsuji H (1999) Zinc-finger proteins: the classical zinc finger emerges in contemporary plant science. Plant Mol Biol **39:** 1073-1078

Takatsuji H, Mori M, Benfey PN, Ren L, Chua NH (1992) Characterization of a zinc finger DNA-binding protein expressed specifically in Petunia petals and seedlings. Embo J **11:** 241-249

Takatsuji H, Nakamura N, Katsumoto Y (1994) A new family of zinc finger proteins in petunia: structure, DNA sequence recognition, and floral organ-specific expression. Plant Cell **6:** 947-958

Tamura T, Hara K, Yamaguchi Y, Koizumi N, Sano H (2003) Osmotic stress tolerance of transgenic tobacco expressing a gene encoding a membrane-located receptor-like protein from tobacco plants. Plant Physiol **131:** 454-462

Taylor PWJ, Ford R (2007) Chickpea. *In* C Kole, ed, Pulses, Sugar and Tuber Crops: Genome Mapping and Molecular Breeding in Plants., Vol 3. Springer-Verlag, Berlin-Heidelberg, pp 109-121

Teige M, Scheikl E, Eulgem T, Doczi R, Ichimura K, Shinozaki K, Dangl JL, Hirt H (2004) The MKK2 pathway mediates cold and salt stress signaling in Arabidopsis. Mol Cell **15:** 141-152

Tester M, Davenport R (2003) Na+ tolerance and Na+ transport in higher plants. Ann Bot (Lond) **91:** 503-527

Thomashow MF (1998) Role of cold-responsive genes in plant freezing tolerance. Plant Physiol **118:** 1-8

Thomashow MF (1999) PLANT COLD ACCLIMATION: Freezing Tolerance Genes and Regulatory Mechanisms. Annu Rev Plant Physiol Plant Mol Biol **50:** 571-599

Tran LS, Nakashima K, Sakuma Y, Simpson SD, Fujita Y, Maruyama K, Fujita M, Seki M, Shinozaki K, Yamaguchi-Shinozaki K (2004) Isolation and functional analysis of Arabidopsis stress-inducible NAC transcription factors that bind to a drought-responsive cis-element in the early responsive to dehydration stress 1 promoter. Plant Cell **16:** 2481-2498

Tripathi V, Parasuraman B, Laxmi A, Chattopadhyay D (2009) CIPK6, a CBL-interacting protein kinase is required for development and salt tolerance in plant. Plant J

Udupa SM, Robertson LD, Weigand F, Baum M, Kahl G (1999) Allelic variation at (TAA)n microsatellite loci in a world collection of chickpea (Cicer arietinum L.) germplasm. Mol Gen Genet **261:** 354-363

Udupa SM, Sharma A, Sharma RP, Pai RA (1993) Narrow genetic variability in *Cicer arietinum L.* as revealed by RFLP Analysis Journal of Biotechnology and Biochemistry **2:** 83-86

Udvardi MK, Kakar K, Wandrey M, Montanari O, Murray J, Andriankaja A, Zhang J-Y, Benedito V, Hofer JMI, Chueng F, Town CD (2007) Legume Transcription Factors: Global Regulators of Plant Development and Response to the Environment. Plant Physiol. **144:** 538-549

Ueda A, Shi W, Nakamura T, Takabe T (2002) Analysis of salt-inducible genes in barley roots by differential display. J Plant Res **115:** 119-130

Uemura M, Joseph RA, Steponkus PL (1995) Cold Acclimation of Arabidopsis thaliana (Effect on Plasma Membrane Lipid Composition and Freeze-Induced Lesions). Plant Physiol **109:** 15-30

Umezawa T, Fujita M, Fujita Y, Yamaguchi-Shinozaki K, Shinozaki K (2006) Engineering drought tolerance in plants: discovering and tailoring genes to unlock the future. Curr Opin Biotechnol **17:** 113-122

Umezawa T, Yoshida R, Maruyama K, Yamaguchi-Shinozaki K, Shinozaki K (2004) SRK2C, a SNF1-related protein kinase 2, improves drought tolerance by controlling stress-responsive gene expression in Arabidopsis thaliana. Proc Natl Acad Sci U S A **101: 17306-**17311

Uno Y, Furihata T, Abe H, Yoshida R, Shinozaki K, Yamaguchi-Shinozaki K (2000) Arabidopsis basic leucine zipper transcription factors involved in an abscisic acid-dependent signal transduction pathway under drought and high-salinity conditions. Proc Natl Acad Sci U S A **97:** 11632-11637

Urao T, Yakubov B, Satoh R, Yamaguchi-Shinozaki K, Seki M, Hirayama T, Shinozaki K (1999) A transmembrane hybrid-type histidine kinase in Arabidopsis functions as an osmosensor. Plant Cell **11:** 1743-1754

Urao T, Yamaguchi-Shinozaki K, Urao S, Shinozaki K (1993) An Arabidopsis myb homolog is induced by dehydration stress and its gene product binds to the conserved MYB recognition sequence. Plant Cell **5:** 1529-1539

Valliyodan B, Nguyen HT (2006) Understanding regulatory networks and engineering for enhanced drought tolerance in plants. Curr Opin Plant Biol **9:** 189-195

VandenBosch K, Stacey G (2003) Advances in Legume Biology. Plant Physiol. **131**

Vera-Estrella R, Barkla BJ, Bohnert HJ, Pantoja O (2004) Novel regulation of aquaporins during osmotic stress. Plant Physiol **135:** 2318-2329

Vij S, Tyagi AK (2007) Emerging trends in the functional genomics of the abiotic stress response in crop plants. Plant Biotechnol J **5:** 361-380

Vinocur B, Altman A (2005) Recent advances in engineering plant tolerance to abiotic stress: achievements and limitations. Curr Opin Biotechnol **16:** 123-132

Wach A, Brachat A, Pohlmann R, Philippsen P (1994) New heterologous modules for classical or PCR-based gene disruptions in Saccharomyces cerevisiae. Yeast **10:** 1793-1808

Walia H, Wilson C, Condamine P, Liu X, Ismail AM, Zeng L, Wanamaker SI, Mandal J, Xu J, Cui X, Close TJ (2005) Comparative transcriptional profiling of two contrasting rice genotypes under salinity stress during the vegetative growth stage. Plant Physiol **139:** 822-835

Walia H, Wilson C, Zeng L, Ismail AM, Condamine P, Close TJ (2007) Genome-wide transcriptional analysis of salinity stressed japonica and indica rice genotypes during panicle initiation stage. Plant Mol Biol **63:** 609-623

Wang W, Vinocur B, Altman A (2003) Plant responses to drought, salinity and extreme temperatures: towards genetic engineering for stress tolerance. Planta **218:** 1-14

Wang YJ, Zhang ZG, He XJ, Zhou HL, Wen YX, Dai JX, Zhang JS, Chen SY (2003) A rice transcription factor OsbHLH1 is involved in cold stress response. Theor Appl Genet **107:** 1402-1409

Washburn KB, Davis EA, Ackerman S (1997) Coactivators and TAFs of transcription activation in wheat. Plant Mol Biol **35:** 1037-1043

Wieland J, Nitsche AM, Strayle J, Steiner H, Rudolph HK (1995) The PMR2 gene cluster encodes functionally distinct isoforms of a putative Na+ pump in the yeast plasma membrane. EMBO J **14:** 3870-3882

Wilkinson MG, Samuels M, Takeda T, Toone WM, Shieh JC, Toda T, Millar JB, Jones N (1996) The Atf1 transcription factor is a target for the Sty1 stress-activated MAP kinase pathway in fission yeast. Genes Dev **10:** 2289-2301

Winicov I, Bastola D (1997) Salt tolerance in crop plants: new approaches through tissue culture and gene regulation. Acta Physiologiae Plantarum **19:** 435-449

Winicov II, Bastola DR (1999) Transgenic overexpression of the transcription factor alfin1 enhances expression of the endogenous MsPRP2 gene in alfalfa and improves salinity tolerance of the plants. Plant Physiol **120:** 473-480

Wu K, Tian L, Hollingworth J, Brown DC, Miki B (2002) Functional analysis of tomato Pti4 in Arabidopsis. Plant Physiol **128:** 30-37

Xiang Y, Huang Y, Xiong L (2007) Characterization of Stress-Responsive CIPK Genes in Rice for Stress Tolerance Improvement. Plant Physiol. **144:** 1416-1428

Xie C, Zhang ZG, Zhang JS, He XJ, Cao WH, He SJ, Chen SY (2002) Spatial expression and characterization of a putative ethylene receptor protein NTHK1 in tobacco. Plant Cell Physiol **43:** 810-815

Xiong L, Schumaker KS, Zhu JK (2002) Cell signaling during cold, drought, and salt stress. Plant Cell **14 Suppl:** S165-183

Xiong L, Wang RG, Mao G, Koczan JM (2006) Identification of drought tolerance determinants by genetic analysis of root response to drought stress and abscisic Acid. Plant Physiol **142:** 1065-1074

Xiong L, Yang Y (2003) Disease resistance and abiotic stress tolerance in rice are inversely modulated by an abscisic acid-inducible mitogen-activated protein kinase. Plant Cell **15:** 745-759

Xu H, Jiang X, Zhan K, Cheng X, Chen X, Pardo JM, Cui D (2008) Functional characterization of a wheat plasma membrane Na+/H+ antiporter in yeast. Arch Biochem Biophys **473:** 8-15

Xu Y, Ma QH (2004) Medicago truncatula Mt-ZFP1 encoding a root enhanced zinc finger protein is regulated by cytokinin, abscisic acid and jasmonate, but not cold. DNA Seq **15:** 104-109

Yamaguchi-Shinozaki K, Kasuga M, Liu Q, Nakashima K, Sakuma Y, Abe H, Shinwari ZK, Seki M, Shinozaki K (2002) Biological mechanisms of drought stress response. JIRCAS Working Report **23:** 1-8

Yamaguchi-Shinozaki K, Shinozaki K (1994) A novel cis-acting element in an Arabidopsis gene is involved in responsiveness to drought, low-temperature, or high-salt stress. Plant Cell **6:** 251-264

Yamaguchi-Shinozaki K, Shinozaki K (2006) Transcriptional regulatory networks in cellular responses and tolerance to dehydration and cold stresses. Annu Rev Plant Biol **57:** 781-803

Yamane K, Kawasaki M, Taniguchi M, Miyake H (2003) Differential effect of NaCl and polyethylene glycol on the ultrastructure of chloroplasts in rice seedlings. J Plant Physiol **160:** 573-575

Yan L, Guizhu C (2007) Physiological adaptability of three mangrove species to salt stress. Acta Ecologica Sinica **27:** 2208-2214

Yanagisawa S (1996) A novel multigene family that the gene for a maize DNA-binding protein, MNB1a belongs to: isolation of genomic clones from this family and some aspects of its molecular evolution. Biochem Mol Biol Int **38:** 665-673

Yoshiba Y, Kiyosue T, Nakashima K, Yamaguchi-Shinozaki K, Shinozaki K (1997) Regulation of levels of proline as an osmolyte in plants under water stress. Plant Cell Physiol **38:** 1095-1102

Yu LX, Setter TL (2003) Comparative transcriptional profiling of placenta and endosperm in developing maize kernels in response to water deficit. Plant Physiol **131:** 568-582

Zakrzewska A, Boorsma A, Delneri D, Brul S, Oliver SG, Klis FM (2007) Cellular processes and pathways that protect Saccharomyces cerevisiae cells against the plasma membrane-perturbing compound chitosan. Eukaryot Cell **6:** 600-608

Zhang J, Klueva N, Wang Z, Wu R, Tuan, Nguyen H (2000) Genetic Engineering for Abiotic Stress Resistance in Crop Plants. In Vitro Cellular & Developmental Biology. Plant **36:** 108-114

Zhang JS, Xie C, Shen YG, Chen SY (2001) A two-component gene (NTHK1) encoding a putative ethylene-receptor homolog is both developmentally and stress regulated in tobacco. Theor Appl Genet **102:** 815-824

Zhang JZ, Creelman RA, Zhu JK (2004) From laboratory to field. Using information from Arabidopsis to engineer salt, cold, and drought tolerance in crops. Plant Physiol **135:** 615-621

Zhang W, Gruszewski HA, Chevone BI, Nessler CL (2008) An Arabidopsis purple acid phosphatase with phytase activity increases foliar ascorbate. Plant Physiol **146:** 431-440

Zhao C, Jung US, Garrett-Engele P, Roe T, Cyert MS, Levin DE (1998) Temperature-induced expression of yeast FKS2 is under the dual control of protein kinase C and calcineurin. Mol Cell Biol **18:** 1013-1022

Zhao S, Douglas NW, Heine MJ, Williams GM, Winther-Larsen HC, Meaden PG (1994) The STL1 gene of Saccharomyces cerevisiae is predicted to encode a sugar transporter-like protein. Gene **146:** 215-219

Zhou J, Wang X, Jiao Y, Qin Y, Liu X, He K, Chen C, Ma L, Wang J, Xiong L, Zhang Q, Fan L, Deng XW (2007) Global genome expression analysis of rice in response to drought and high-salinity stresses in shoot, flag leaf, and panicle. Plant Mol Biol **63:** 591-608

Zhu JK (2001) Cell signaling under salt, water and cold stresses. Curr Opin Plant Biol **4:** 401-406

Zhu JK (2002) Salt and drought stress signal transduction in plants. Annu Rev Plant Biol **53:** 247-273

Zhu JK, Hasegawa PM, Bressan RA (1997) Molecular aspects of osmotic stress in plants. Critical reviews in Plant Sciences **16:** 253-277

Zielinski RE (1998) Calmodulin and Calmodulin-Binding Proteins in Plants. Annu Rev Plant Physiol Plant Mol Biol **49:** 697-725

Publication

CaZF, a Plant Transcription Factor Functions through and Parallel to HOG and Calcineurin Pathways in *Saccharomyces cerevisiae* to Provide Osmotolerance

Deepti Jain[1], Nilanjan Roy[2], Debasis Chattopadhyay[1]*

1 National Institute of Plant Genome Research, Aruna Asaf Ali Marg, New Delhi, India, 2 National Institute for Pharmaceutical Education and Research, SAS Nagar, Punjab, India

[illegible] mutants were deployed to characterize a gene encoding a C2H2 zinc finger protein (CaZF) that is [illegible] in a drought-tolerant variety of chickpea (*Cicer arietinum*) and provides salinity tolerance in [illegible] *Saccharomyces cerevisiae* most of the cellular responses to hyperosmotic stress [illegible] by two [illegible] involving high osmolarity glycerol mitogen-activated protein kinase (Hog1p) [illegible] Calcineurin [illegible] regulated protein phosphatase 2B. In this study we report that heterologous expression of CaZF [illegible] in *S. cerevisiae* through Hog1p and Calcineurin dependent as well as independent pathways. CaZF [illegible] hypersensitive phenotypes of *hog1*, *can* and *hog1can* mutants and in conjunction stimulates HOG [illegible] genes with subsequent accumulation of glycerol in absence of Hog1p and CAN. CaZF directly binds to [illegible] (STRE) to activate STRE-containing promoter in yeast. Transactivation and salt tolerance assays of [illegible] showed that other than the transactivation domain a C-terminal domain composed of acidic and [illegible] required for its function. Altogether, results from this study suggest that CaZF is a potential plant [illegible] and also provide evidence that in budding yeast expression of HOG and CAN pathway genes can [illegible] absence of their regulatory enzymes to provide osmotolerance.

Citation: Jain D, Roy N, Chattopadhyay D (2009) CaZF, a Plant Transcription Factor Functions through and Parallel to HOG and Calcineurin Pathways in *Saccharomyces cerevisiae* to Provide Osmotolerance. PLoS ONE 4(4): e5154. doi:10.1371/journal.pone.0005154

Editor: Samuel P. Hazen, University of Massachusetts Amherst, United States of America

Received January 2, 2009; **Accepted** March 13, 2009; **Published** April 13, 2009

Copyright: © 2009 Jain et al. This is an open-access article distributed under the terms of the Creative Commons Attribution License, which permits unrestricted use, distribution, and reproduction in any medium, provided the original author and source are credited.

Funding: The project was funded by Council for Scientific and Industrial Research (CSIR), INDIA and a seed grant from National Institute for Plant Genome Research. DJ acknowledges CSIR for research fellowship. The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Competing Interests: The authors have declared that no competing interests exist.

* E-mail: debasis_chattopadhyay@nipgr.res.in

Introduction

Plants have to cope with several types of environmental stress conditions. Water deficit, salty soils and cold are the common stress conditions affecting plant development [1]. Among them, high salinity is one of the most serious limiting factors in plant growth and productivity [1]. Cells constantly evaluate and respond to sudden and adverse changes in environment by certain mechanisms that not only initiate the repair of macromolecular damage but also establish a tolerant state, which helps to prevent further damage. Budding yeast (*Saccharomyces cerevisiae*) has been considered an excellent model for the study of the mechanisms underlying tolerance, particularly to saline stress [2], because of the high degree of evolutionary conservation of stress pathways between higher eukaryotes and *S. cerevisiae* and the ease with which yeast genes encoding components of the pathways can be manipulated.

In high osmotic condition, *S. cerevisiae*, initiates an efficient adaptive response, which maintains cellular Na^+/K^+ balance, retains turgor and repairs cellular damages. Principally, two interconnected pathways regulate this adaptive response. Elevated cytosolic Ca^{2+} activates Calcineurin (CAN) due to extracellular hyperionic stress, a heterodimeric phosphatase 2B with two catalytic subunits, CNA1 and CNA2, and a regulatory subunit CNB. It then dephosphorylate a C2H2 zinc finger transcription factor CRZ1/TCN1 [3,4] causing its transport to nucleus to activate expression of a P-type ATPase ENA1/PMR2A for Na^+ and Li^+ efflux [5], but only a part of ENA1 expression is CAN-dependent [4] suggesting that other Na^+-stress response pathways also contribute to ENA1 induction [6]. Calcineurin mutants (i.e., *cna1cna2* and *cnb*) fail to grow in growth medium having high concentration of either Na^+, Li^+, or Mn^{2+} [7–9] suggest that CAN participates in regulating the intracellular concentration of several ions [10,11]. In addition to ENA1, some other gene(s) are also contributing to salt tolerances that have been regulated by calcineurin osmopathway [12].

The high osmolarity glycerol (HOG) pathway is regulated by a mitogen activated protein kinase (MAPK) Hog1p [13,14]. Drastic reduction of osmotolerance in the *hog1* mutants demonstrates the essentiality of this module in hyperosmotic stress. At least two osmosignalling branches, through a series of downstream components, activate MAPK kinase Pbs2p, which in turn phosphorylate and activates MAPK, Hog1p [15–17]. Activated Hog1p after moving to nucleus further induces downstream osmoresponsive genes through at least five transcription regulators. Msn2p, Msn4p [18–20] are two functionally redundant C2H2 zinc finger proteins and activate STRE (Stress responsive upstream activator element) mediated induction of several general stress responsive genes

CTT1, HSP12, DDR2, TPS2 etc, required possibly for damage repair [21–23]. Two other Hog1p-regulated transcription activators, Msn1p and Hot1p regulate GPD1, GPP2, genes for glycerol biosynthesis enzymes [19]. Under osmotic stress Hog1p regulated transcription factors recruit activated Hog1p directly to osmoresponsive promoters [24,25] that further stimulate recruitment of RNA Pol II [26] and Rpd3 histone deacetylase to promote transcription initiation [27]. Sko1p [28], related to bZIP/ATF family of transcriptional regulators [29], represses *ENA1* expression through CRE (Cyclic AMP Responsive Element) in unstressed condition. Under hypertonic stress Sko1p is phosphorylated by Hog1p and converted into a transcription activator by recruiting SAGA histone deacetylase and SWI/SNF complex to promote chromatin remodeling [25] and induce *ENA1* expression in conjunction with Calcineurin/Crz1p mediated pathway [28].

Research over the past decade has identified several cellular mechanisms of salt tolerance in yeast that are conserved in plant cells; and isolation, and characterization of a number of plant salt tolerance determinants was based on homologous function [30–35] in yeast. Calcium sensor-regulated stress response pathways seem to be structurally and functionally conserved in plants [36–38] and some abiotic stress-related proteins are often found to functionally complement yeast calcineurin knockouts. In tobacco and *Arabidopsis* NACK-PQR pathway, similar to HOG pathway, have been reported [39]. Tobacco MAPK kinase NQK1 can functionally complement Pbs2p [39].

As drought and high salinity are amongst the major challenges for plant survival, our interest is focused on one chickpea (*Cicer arietinum*) gene highly expressed in a drought tolerant cultivar in comparison to a drought sensitive cultivar in response to drought and provided tolerance to high salt when expressed in tobacco. The gene, *CaZF* encodes a C2H2 zinc finger protein. As zinc finger proteins are ubiquitous; and drought and salt stress share some common signaling pathways we decided to investigate if there is any osmoregulatory response mediated by CaZF in *S. cerevisiae*, in an attempt to outline the *in vivo* function of chickpea CaZF. Overexpression of *CaZF* cDNA in a galactose-inducible manner in yeast demonstrated that CaZF is able to rapidly improve salt tolerance of yeast cells under saline stress. Moreover, CaZF is able to complement osmotolerance deficiencies in *hog1*, *cnb1*, and *hog1cnb1* double mutants concomitantly with an increased accumulation of osmolyte glycerol and stress-responsive genes regulated by Hog1p and CAN.

Results and Discussion

Differential expression of *CaZF*, a gene for C2H2 zinc finger protein from chickpea

Subtracted cDNA libraries constructed between two chickpea (*Cicer arietinum*) cultivars at different points of drought-stress resulted in a number of EST clones expressing higher in the drought tolerant BGD72 than in the sensitive ICCV2 in response to drought. An EST encoding a putative zinc finger protein expressing more in BGD72 than in ICCV2 at different points of stress (Figure 1) was taken for further studies. Full-length cDNA (*CaZF*) constructed by 5′ RACE was 1185 bp in length (GenBank accession EU513298). Sequence analysis revealed an 843 bp open reading frame (ORF) of 280 amino acid, 139 bp long 5′ and 203 bp long 3′ untranslated region. Deduced amino acid sequence shows (Figure 2A) CaZF is an EPF type C2H2 zinc finger protein having two canonical TFIIIA-type zinc finger motifs ($CX_2CX_3FX_5LX_2HX_3H$). Both the zinc finger motifs contain conserved QALGGH sequence. A short spacer sequence of 28 amino acids separates two zinc fingers. Among the studied proteins PIF1 (GB: AAQ54302), a pathogen inducible zinc finger protein from capsicum shows maximum sequence similarity with CaZF of only about 55% homology (expect = 5e-45). Notably, PIF1 is also highly expressed in a pathogen tolerant variety compared to a sensitive one in response to infection [40]. Detailed comparisons of the amino acid sequences among plant zinc finger proteins revealed three conserved regions other than the zinc fingers. CaZF contains a short basic region with a consensus of KXKRSKRXR (B-box), near the N-terminus, which may function as a potential nuclear localization signal (NLS) and/or may participate in DNA binding. Another is a region, consisting of three acidic residues followed by hydrophobic residues rich in leucine, with a consensus of EXEXXAXCLXXL (L-box) located between B-box and the first zinc-finger. The other is a short hydrophobic region containing a highly conserved DLNL sequence as a core (DLN-box) close to the C-terminus. The latter two may play a role in protein-protein interactions or in maintaining the folded structure. CaZF possesses a serine-glutamine rich region at the N-terminus, between L-box and first zinc-finger, which might function as a transactivation domain as suggested for ZPT2-1 and Pszf1 [41,42] or might be a phosphorylation site for post-translational modification; and an asparagine rich stretch after the second zinc finger at the C-terminus. Similar asparagine-rich domains are also present in some stress-inducible zinc finger proteins such as SCOF-1, EPF2-5, and STZ [43]. Like *STO* and *STZ*, the Arabidopsis cDNAs, which increase salt tolerance in yeast in a Calcineurin independent manner, SCOF-1 and EPF2-5, CaZF contains highly basic region followed by acidic amino acids near the C-terminus. But, CaZF has two such combinations of basic and acidic amino acid stretches. Phylogenetic analysis showed that CaZF and one Arachis protein (ZFP248) shares the same clad (Figure 2B).

CaZF binds in vitro to EP sequence repeat and activates transcription in yeast

CaZF possesses EPF type C2H2 zinc finger motifs that has been identified in some transcription factors from petunia by their ability to bind a target sequence EP1S core sequence (TGA-

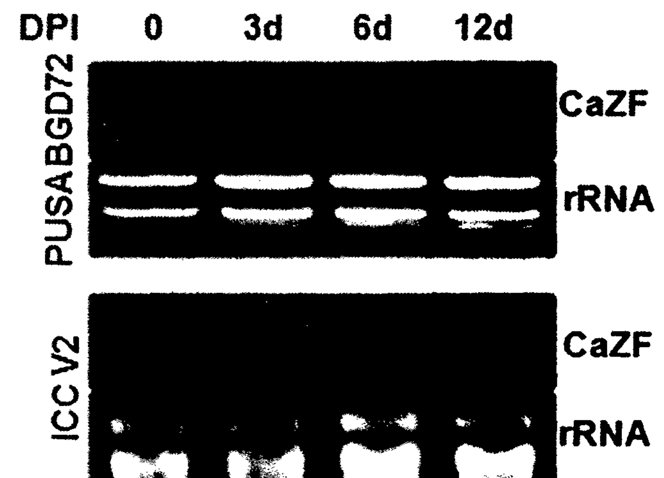


Figure 1. Expression of CaZF in chickpea varieties BGD72 and ICCV2 under different drought conditions. Samples harvested at day post-irrigation (DPI) is mentioned. Total RNA (20 µg/lane) from chickpea seedlings were hybridized with probe prepared from *CaZF* cDNA as described under "Experimental Procedures". Ribosomal RNAs (rRNA) are shown as loading control.
doi:10.1371/journal.pone.0005154.g001

A

MALELEAFNSSPPTNSPFPTFNKEEIRESESLVKRKRSKR
PRISNPPTEEEYLALCLIMLAQSGNNRNNKNDIVSHFHN
QIESSSSQSQQQPSPPSPPVKLNHRCTVCNKAFPSYQALG
GHKASHRKSSLETPSTAFNDTVSVSTVTAGKMHECSICH
KSFSTGQALGGHKRCHYEGGINHNNNNNNNSNSNVNAN
NSSGITISEGAAASSSVSHRGFDLNLPAPLTEFWSPVGFG
GGDSKKKSVNVNVAGIGEQEVESPLPVTAKRPRVFLVE
HDDETV

B

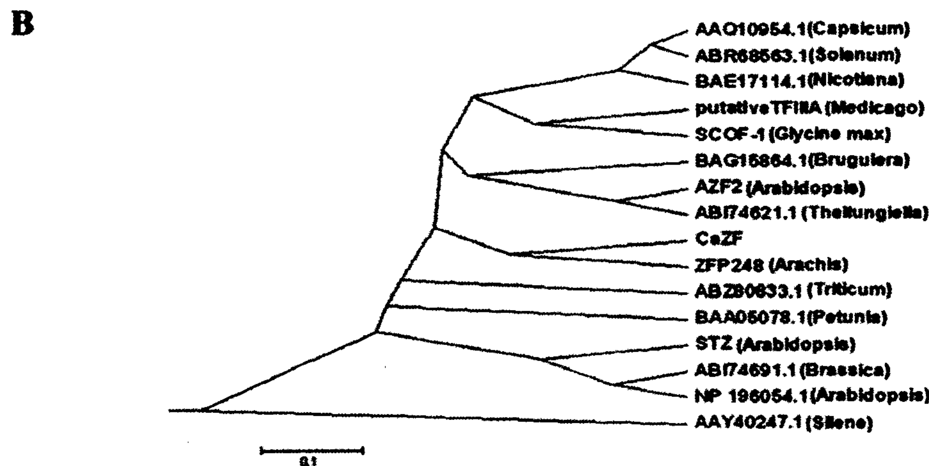


Figure 2. In silico analysis of CaZF protein. *A*, Deduced amino acid sequence of CaZF. The basic B-box in CaZF is indicated by *bold letters*, L-box by an *underline*, zinc finger motifs by *underlined grey letters* and Asn-rich region by *underlined bold letters*. *B*, Phylogenetic tree showing relationship between CaZF and other well-studied C2H2 zinc-finger family proteins. The tree was generated using the neighbor-joining algorithm of MEGA 2.0 software, version 2.1. The bar indicates the scale for branch length.
doi:10.1371/journal.pone.0005154.g002

CAGTGTCA) present in the promoter of their target gene *EPSPS* (5-enolpyruvylshikimate-3-phosphate synthase) [41,44]. Therefore, CaZF protein was tested for its ability to bind EP1S sequence. EP1S is a 13 bp sequence with an inverted repeat of TGACA separated by a G (Figure 3A). EPF family proteins have spacers of variable lengths between two zinc fingers. Proteins with spacers shorter than 44 amino acids show high specificity of binding to tandemly repeated EP1S with the core G residue separated by 13 bp [44]. Therefore, an EP2S (EP1S dimer) tetramer with 13 bp separations between the core G residues was used as a probe for gel shift assay. Figure 3B shows that glutathion-S-transferase (GST) fused CaZF protein expressed in *E. coli* efficiently bound EP2S tetramer. Expression of CaZF protein fused to green fluorescence protein (GFP) at the C-terminal end under *35S CamV* promoter in tobacco demonstrated that the protein is localized in nucleus (Figure 3C). To determine whether CaZF protein is capable of regulating transcription, *CaZF* ORF was expressed as a fusion to GAL4 DNA-binding domain in a yeast reporter strain carrying *His3, Ade2 and LacZ* reporter genes under *GAL4* promoter. Transformed yeast colonies grew on auxotropic medium lacking histidine and adenine (Figure 3D) suggesting that CaZF can function as a transcriptional activator. In order to identify the transactivation domain, two CaZF deletion constructs were introduced into the yeast reporter strain and β-galactosidase activity was assayed. Deletion of C-terminal amino acids after the second C2H2 domain (-Asn) produced higher β-galactosidase activity than the full-length protein (Figure 3E). Increase in transactivation activity after C-terminal deletion of CaZF is most likely due to removal of DLN-box mediated repression. Transcription repressor proteins e.g. ERF, STZ and AZF have a conserved DLN-box motif ($^{L}/_{F}$DLN$^{L}/_{F}$P) at their C-terminus and that was shown to be essential for repressor activity [45,46]. Removal of N-terminal amino acids up to the first C2H2 domain (-N99) caused significant reduction of β-galactosidase activity demonstrating essentiality of this domain for transactivation. To further locate the transactivation domain, two more N-terminal deletion mutants, one from 1–44 aminoacids (N44) and other 1–75 aminoacids (N75) were constructed. β-galactosidase assay showed that the aminoacids from 44–75 (L-box) are most important for transactivation property of the protein.

CaZF-expressing transgenic tobacco plants show salt-tolerance

To establish the functional significance of CaZF *in planta* the complete ORF of CaZF gene was introduced into tobacco plants using *Agrobacterium*-mediated transformation. Out of twelve transgenic lines harboring single copy of the transgene, two relatively high expressing and two relatively low expressing lines

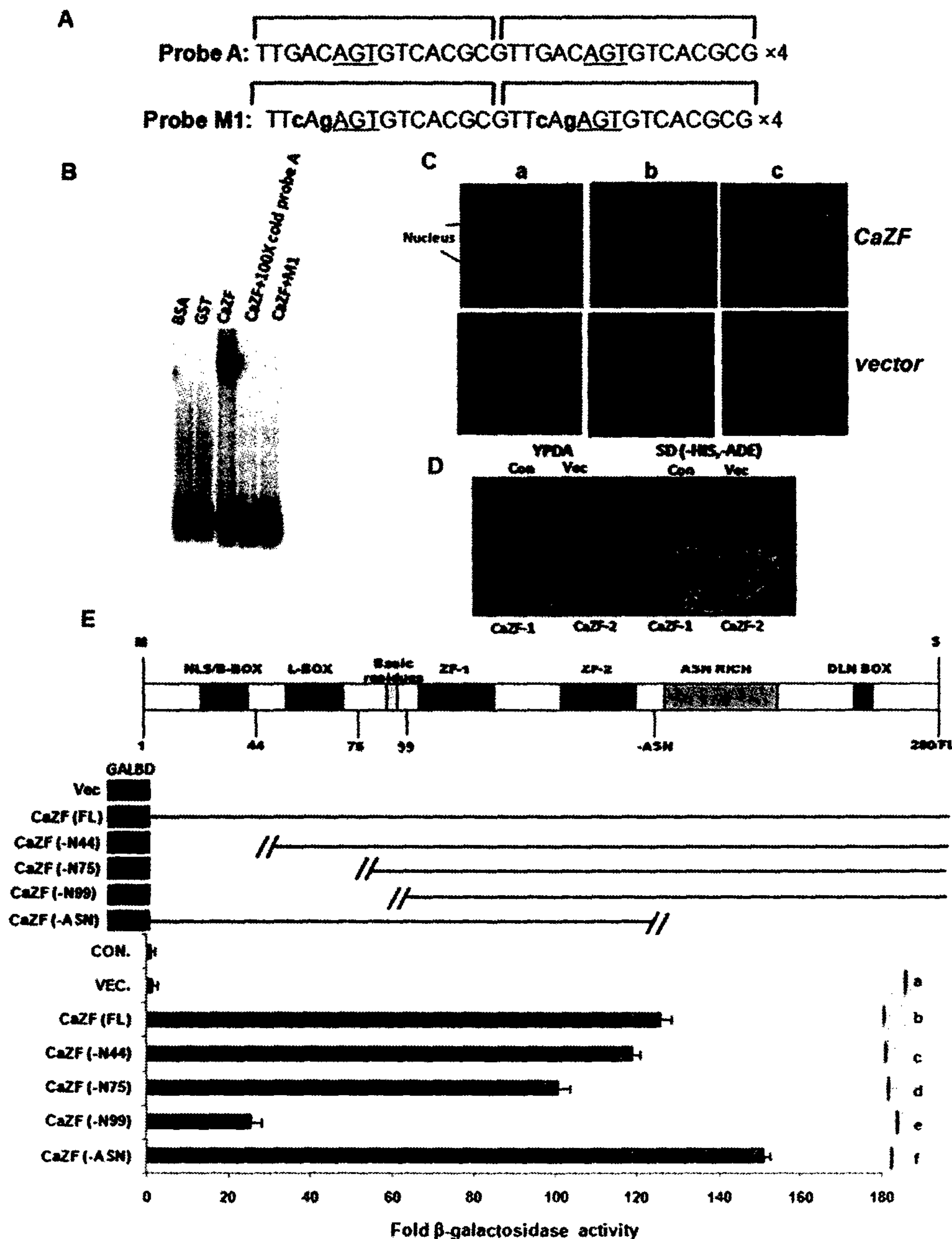


Figure 3. DNA binding, cellular localization and transactivation assay of CaZF. *A*, The 32 bp EP2S sequence tested for gel-shift assay is either wild-type or mutant version M1. Monomers are shown, and tetramers were used in the experiments. Core nucleotides are *underlined* and modified bases are in *bold small case letters*. *B*, Gel-shift assays demonstrating that CaZF binds to the EP2S probe. *C*, CaZF protein localizes in nucleus. Leaf peels of the CaZF overexpressing transgenic and vector transformed plant were analyzed under microscope for phase contrast (a) for GFP activity (c). The sample was restained with DAPI to confirm the nucleus position (b) as indicated. *D, E*, Transactivation assay of CaZF in yeast. Full length and truncated CaZF cDNA were cloned into pGBKT7 for expression of CaZF protein as a fusion with GAL4-DNA binding domain. Activation of *HIS3* and *ADE2* reporter genes is shown by growth of the transformants growing in SD (-histidine,-adenine) medium against control (con.) and vector (vec.) transformed (*D*). *LacZ* activation by different deletion constructs of CaZF is shown by β-galactosidase assay (taken as an average of three independent experiments) of the transformants presented as fold increase in activity (*E*).
doi:10.1371/journal.pone.0005154.g003

(Figure 4A) were chosen for salt-tolerance analysis. The vector transformed and the CaZF-expressing T_1 transgenic lines were germinated simultaneously and grew normally in 0.5 MS (*Murashige* and *Skoog*) medium (Figure 4B). To assess the effect of high salt on the seed germination/growth of the vector control and T_1 plants overexpressing CaZF, surface-sterilized seeds were plated on 0.5 MS supplemented with 200 mM NaCl. In the presence of high salt, vector transformed seeds showed almost no germination (only one out of thirty-two seeds in one repeat) until 20 d, while on average 85–95% CaZF overexpressing T_1 seeds showed germination within 15 d (Figure 4C). To test for salinity tolerance, leaf disks from all four lines of T_1 transgenic plants and vector transformed plants were floated separately on water, 150 mM or 300 mM NaCl for 72 h and subsequently total

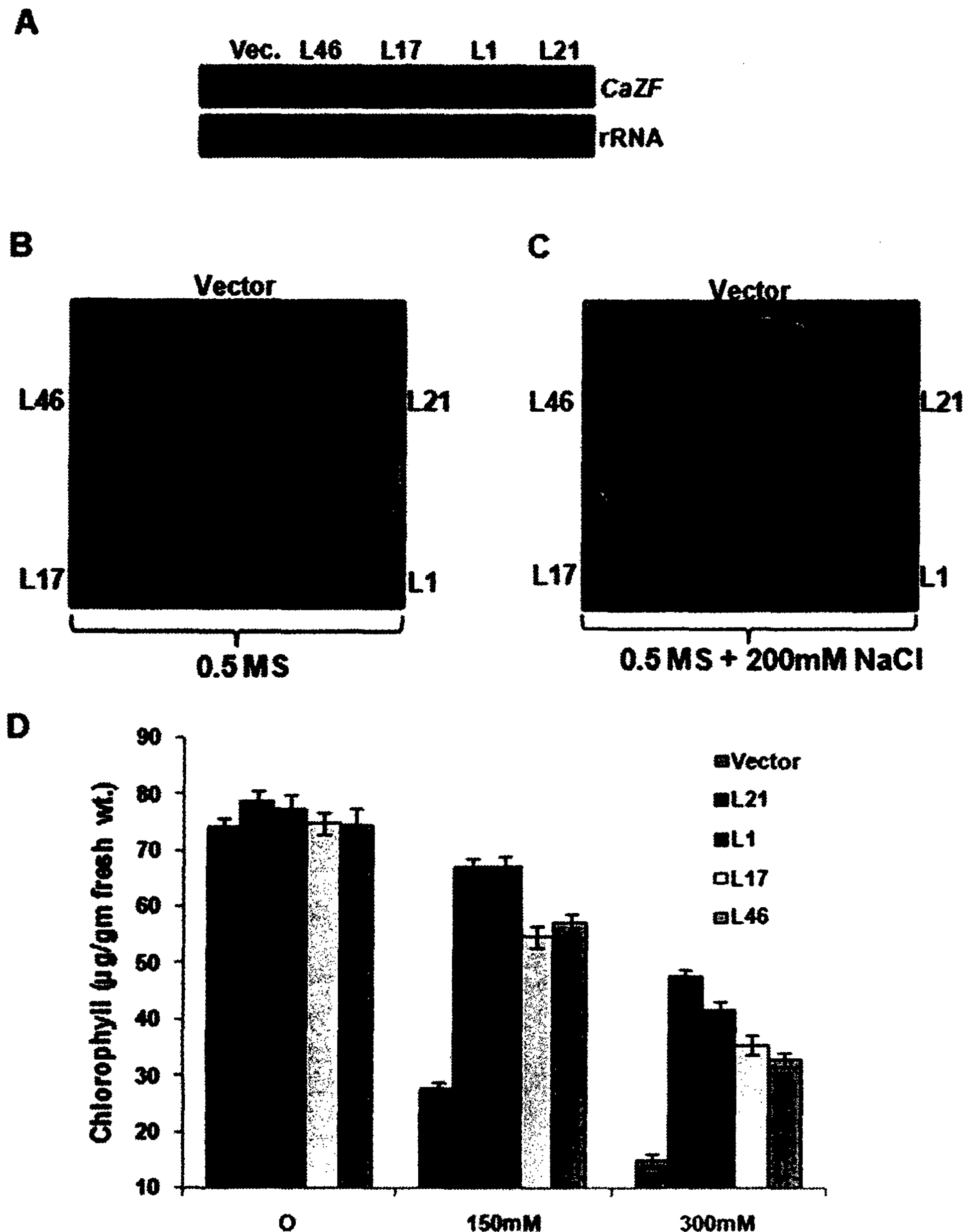


Figure 4. Expression of *CaZF* and salt tolerance of transgenic tobacco. *A*, Northern blot showing expression of *CaZF* in T_1 transgenic tobacco lines transformed with pBI121 without (vector) or with *CaZF* (L21, L1, L17 and L46). Full length *CaZF* cDNA was used as probe. *B*, Vector control and T_1 transgenic progenies were grown on 0.5 MS (*Murashige* and *Skoog*) medium for 10 d. *C*, Effect of salt stress on tobacco seedlings from vector control and T_1 transgenic progenies (CaZFL21, CaZFL1, CaZFL17 and CaZFL46) was demonstrated by germinating seeds on 0.5 MS medium supplemented with 200 mM NaCl for 20 d. Representative figure of three independent experiments are shown. *D*, Chlorophyll content determined by leaf disc senescence assay for salinity tolerance of 30d-old vector control and transgenic tobacco lines overexpressing CaZF, after incubation in water, 150 mM and 300 mM NaCl solutions for 72 hr under continuous white light at 25±2°C. Results of three independent experiments are shown.
doi:10.1371/journal.pone.0005154.g004

chlorophyll content was quantitated. Chlorophyll content of the vector-transformed and CaZF-expressing plants was comparable in presence of water. However, salinity-induced loss of chlorophyll was much lower in CaZF overexpressing lines (average 13.2% and 23.1% for L1/L21, and 27.8% and 51.2% for L17/L46 at 150 mM and 300 mM NaCl respectively) compared with that in the vector control (average 62.3% and 76.4% at 150 mM and 300 mM NaCl respectively) (Figure 4D). From the damage caused by salt stress it was evident that CaZF overexpressing transgenic tobacco plants have a better ability to tolerate salinity stress as compared to vector control plants. The degree of bleaching (yellow color) observed in leaf disks after 72 h can reflect the extent of damage caused by stress. CaZF-expressing transgenic tobacco seedlings also exhibited improved drought tolerance (data not shown).

CaZF enhances osmotolerance in yeast

Several reports describe use of yeast mutants to screen and characterize plant salt tolerance determinants [45,47]. As *CaZF* encodes a ubiquitous C2H2 zinc finger protein, and as yeast and

plant stress tolerance systems share quite similar pathways we intended to use yeast salt-sensitive mutants for the characterization of *CaZF*. *CaZF* cDNA was expressed under a galactose-inducible promoter in a protease deficient *S. cerevisiae* strain BCY123 that reduces degradation of the heterologous protein [48]. In contrast to vector transformed control the *CaZF*-transformed colonies were able to grow in galactose-containing medium supplemented with 250 mM lithium chloride (LiCl) or 500 mM NaCl. Li^+ and Na^+ are transported through the plasma membrane by same system. The maximum concentration of each salt tolerated by the transformed colonies is 300 mM LiCl and 700 mM NaCl at 10^{-6} dilution when incubated at 30°C for 4 days. Introduction of *CaZF* cDNA in two other yeast strains, BY4742 and PJ69-4A (data not shown) with different genetic background allowed the transformed colonies to grow on a medium supplemented with 250 mM LiCl or 500 mM NaCl, demonstrated that CaZF could function in a broad genetic spectrum. Salt tolerance of the transformed colonies was galactose inducible, as they did not grow LiCl-supplemented medium when galactose was replaced with glucose showing expression of the cDNA was necessary for salt tolerance (Figure 5A). BCY123 harboring *CaZF* cDNA also exhibited tolerance against other ionic and non-ionic osmolytes such as $MnCl_2$, KCl and sorbitol (Figure 5B) demonstrating *CaZF* can provide tolerance against general osmotic stress. In liquid medium (YPGalRaf) BCY123 harboring *CaZF* grew almost two fold (doubling time 4.8±0.2 hrs) faster than the vector control strain (doubling time 8.9±0.2 hrs) in presence of 500 mM NaCl. In absence of salt no difference was observed between the growth rates of yeast strains with or without CaZF (doubling time 3.1±0.1 hr) as shown in the solid medium indicating CaZF requires osmotic stress for its function and provides growth advantages only in osmotic stress.

CaZF partially suppresses salt sensitivity of Calcineurin and HOG pathway mutants

Exposure to high salinity causes hypercationic and hyperosmotic stresses to eukaryotic cells [31]. Inter-connected pathways regulated by Hog1p MAP kinase and CAN protein phosphatase determine most of the responses to hyperosmotic stress. A number of salt tolerance determinants in plants have been isolated by their ability to suppress salt sensitivity of the yeast mutants [49]. Therefore, CaZF was tested for its ability to provide osmotic tolerance to some of the well-studied yeast mutants. CaZF suppressed the salt sensitivity when expressed in *cna1cna2* double mutant, lacking both the redundant catalytic subunits and *cnb* mutant lacking the regulatory subunit of Calcineurin on 400 mM NaCl (Figure 6A). However, CaZF could not protect the *cnb* mutant against the ionic osmolytes KCl (data not shown) and

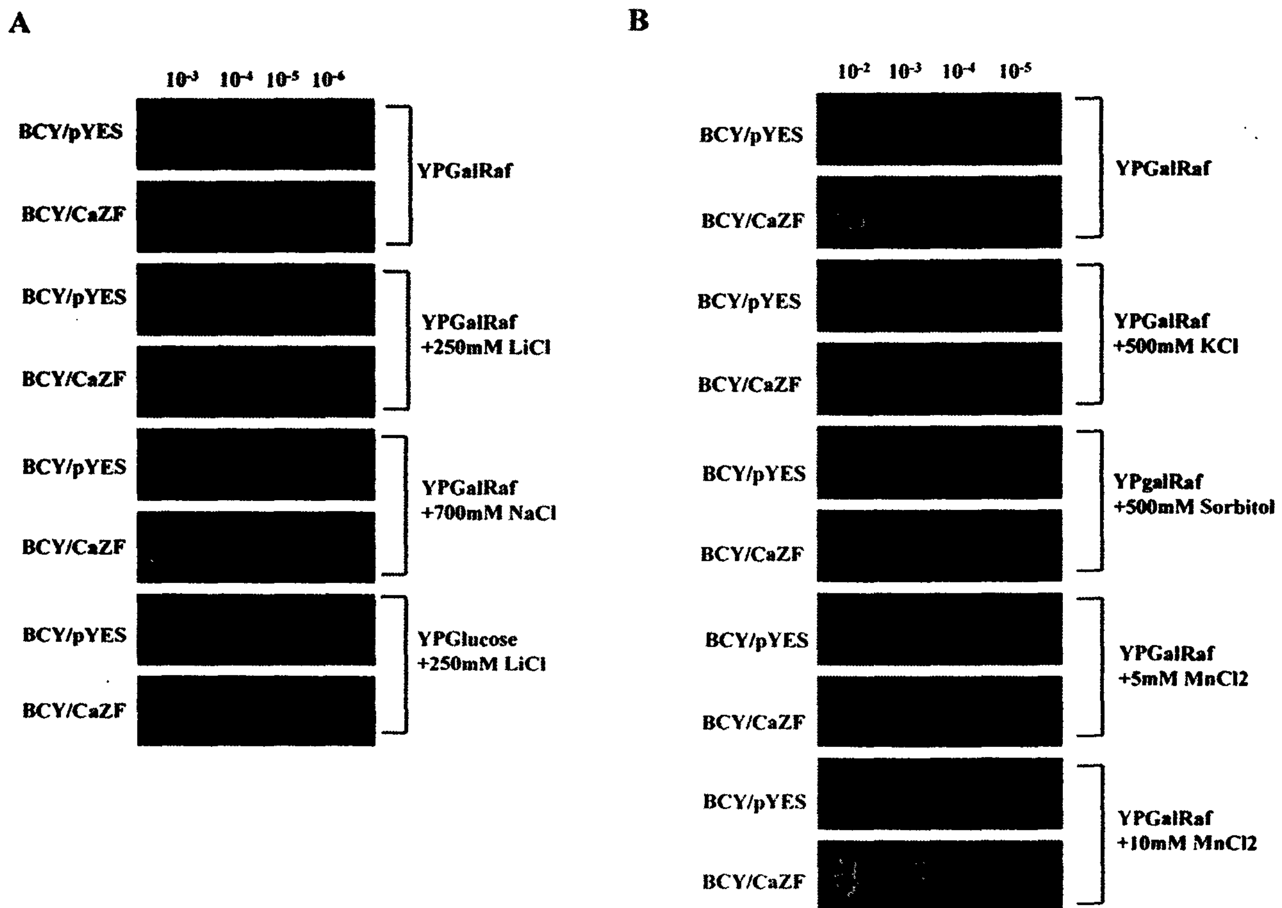


Figure 5. *CaZF* provides tolerance to yeast cells against osmotic stress. *A*, Yeast strain BCY123 harboring only vector (pYES2.1) or CaZF was spotted onto YPGalRaf medium supplemented with 250 mM LiCl or 700 mM NaCl, or onto YPGlu medium containing 250 mM LiCl. Plates without or with salt were shown after incubation at 30°C for 2 d or 4 d respectively. *B*, The same strains, as in *A*, were spotted onto YPGalRaf medium containing either 500 mM KCl, 500 mM sorbitol or $MnCl_2$ and incubated at 30°C for 4 d. Representative figures from three independent experiments are shown.
doi:10.1371/journal.pone.0005154.g005

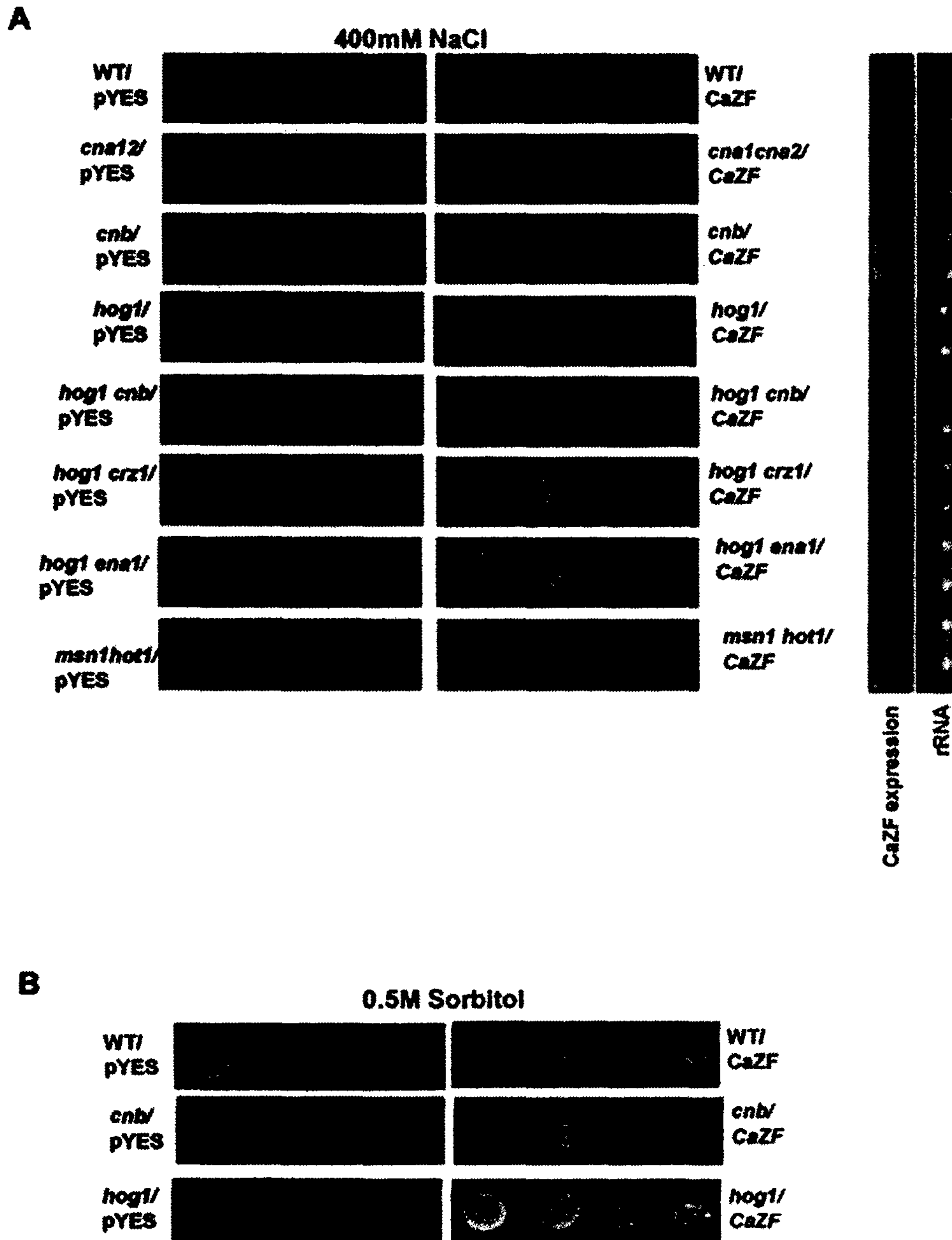


Figure 6. CaZF suppresses salt sensitive phenotype of Hog and Calcineurin mutants. *A*, Wild type and mutant BCY123 cells harbouring either only vector (pYES2.1) or CaZF were spotted onto YPGalRaf plates, containing 0.4 M NaCl. Right panel demonstrated the CaZF expression level in WT and mutant BCY123 yeast strain. rRNA was taken as loading control. *B*, Wild type BCY123 and *hog1* or *cnb* mutants expressing CaZF were spotted onto YPGalRaf medium containing 0.5 M sorbitol. All spotting experiments were performed, as described in Figure. 4. Representative figures from three independent experiments are shown.
doi:10.1371/journal.pone.0005154.g006

NaCl to the extent as it did for the wild type cells. This was also reflected when growth rates were measured in liquid medium. *cnb* mutant cells expressing CaZF grew faster (doubling time 7.2±0.16 h) than that carrying only vector (doubling time 11.8±0.3 h) but grew at a much slower rate than the wild type cells expressing CaZF (doubling time 4.8±0.2 h). These data indicate that CaZF functions through a pathway, which is additive to but independent of Calcineurin pathway. Alternatively, CaZF can partially complement salt sensitivity of the *can* mutants but requires the CAN pathway for its full function. Interestingly, growth rates of the *cnb* mutant and the wild type cells harboring CaZF in non-ionic osmolyte sorbitol containing medium were comparable (Figure 6B). The probable explanation is Calcineurin pathway protects the cells against toxicity of only ionic osmolytes while HOG pathway protects against hypertonic stress due to both ionic and nonionic osmolytes [50].

As CaZF enhanced growth of wild type yeast in presence of nonionic osmolyte, we wanted to test whether it can function in the background of HOG pathway mutants. Expression of *CaZF* suppressed salt sensitivity of *hog1* mutant. As in case of the calcineurin mutants, CaZF provided much less tolerance to the *hog1* strain than it provided for the wild type strain carrying only

vector against the ionic and nonionic osmolytes. The result in the solid medium was also supported by the growth rates in liquid medium with 500 mM NaCl; as doubling time of *hog1* strain carrying pYES was 12.0±0.15 h as opposed to 7.8±0.2 h for the *hog1* cells expressing CaZF that was much higher than that required for the wild type cells expressing CaZF (4.8±0.2 h). The osmosensitive phenotype of yeast mutant lacking both *Hot1* and *Msn1*, transcriptional activators of the Hog pathway, can also be suppressed by *CaZF* expression (Figure 6A) in solid and liquid hypertonic medium as well. Under hypertonic stress Hog1p, through Sko1p, activates expression of *Ena1* [28,51,52], which is also regulated by Calcineurin independent of Hog1p through a transcription activator Crz1p, a C2H2 zinc finger protein [3,4,12]. As CaZF could suppress osmosensitivity of *hog1* and *cnb* mutants separately, we constructed a double mutant *hog1cnb* to test the functional ability of *CaZF* in absence of both Hog1p and Calcineurin. Surprisingly, *CaZF* expression partially suppressed salt sensitivity of *hog1cnb*. Analysis of the effect of CaZF expression in these mutants of HOG and CAN pathways mutants suggests that CaZF mediated suppression of osmosensitivity involves a pathway(s) that is independent of but additive to Hog1p and Calcineurin regulated pathways. Simultaneously, reduced growth rate of CaZF expressing mutant cells in comparison to the wild type cells expressing the plant gene also evokes a possibility that CaZF may require both the pathways to function in its full strength. The data presented above suggests that hyperosmotic-adaptation pathway (s) independent of Hog1p and Calcineurin can be created in yeast.

CaZF induces expression of HOG and Calcineurin regulated genes

We have shown that CaZF can enhance hyperosmotic stress tolerance in budding yeast and it can partially suppress the osmosensitive phenotype of the mutants lacking Hog1p and/or Calcineurin activities. We further investigated whether *CaZF* expression has any influence on expressions of the genes those are regulated by HOG1p and involved in glycerol production and damage repair. We analyzed in wild type and *hog1* background, expressions of two genes, *GPD1* and *GPP2*, involved in glycerol synthesis and two general stress response genes *CTT1* and *HSP12*, predominantly controlled through STRE. Expressions of all the four genes, with different expression kinetics, in the vector control wild type are enhanced quickly after the salt stress and remained expressed even after 3 h (Figure 7A). Expression of CaZF did not show any significant effect on the expression of these genes under control condition except an increase in *CTT1* expression. However, under salt stress, expression of *CTT1* and of *HSP12* throughout the course of experiment was higher in CaZF expressing cells, suggesting that CaZF function in yeast is not constitutive and it requires some stress-induced pathway(s) for its function. As expected, *hog1* mutation caused significant reduction in expression of all these genes. Surprisingly, *hog1* mutant harboring CaZF induced expression of all these genes almost at the level of CaZF expressing wild type strain in response to salt stress. HOG pathway regulated genes were shown to recruit Hog1p at their promoter for the osmotic stress-mediated expression. Salt induced expression of these genes in absence of Hog1p suggests that either CaZF along with some Hog1p-independent factors is directly activating the expression of these genes or a Hog1p-independent salt-inducible pathway in yeast is activated by CaZF under salt stress and ultimately causing expression of these genes. CaZF-regulated Hog1p-independent expression of these genes though is not sufficient for providing osmotolerance to the extent as with the wild type background indicating that Hog1p is indispensable for a part of the osmoadaptation mechanism.

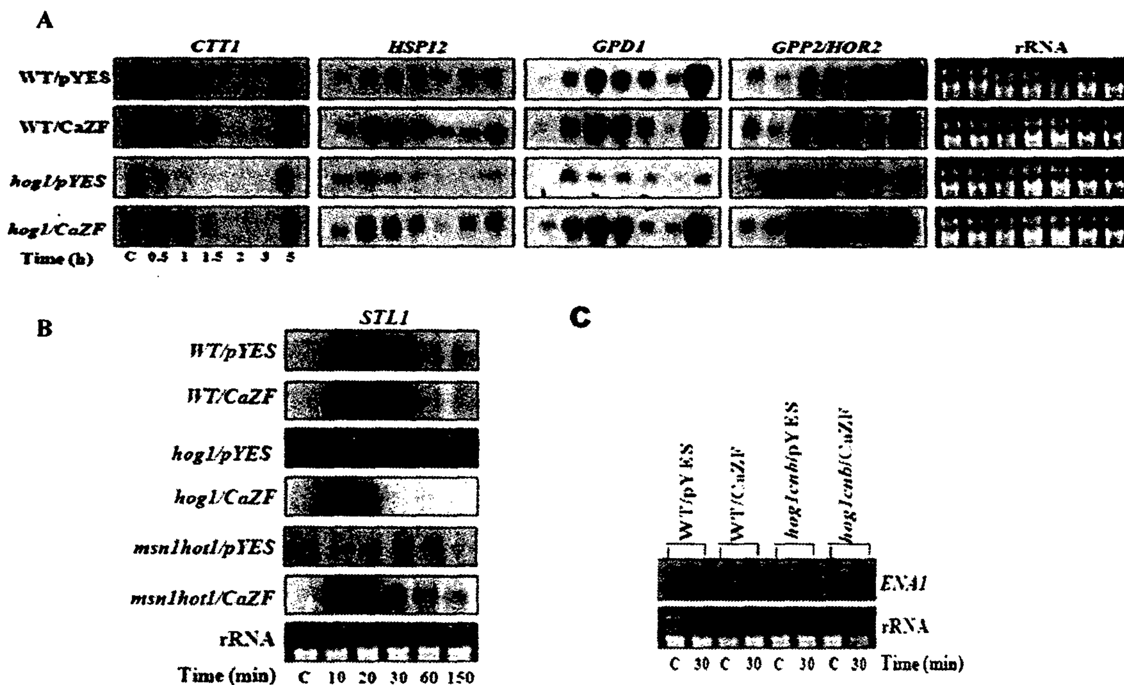


Figure 7. Effect of CaZF on stress-responsive gene expression. Wild type or mutant cells as mentioned harboring either only vector (pYES2.1) or CaZF were grown in YPGalRaf medium and treated with 500 mM NaCl for mentioned period of time. Northern analysis was performed with probes representing genes mentioned in the figure subpanels *A*, *B* and *C*. Representative figures from three independent experiments are shown.
doi:10.1371/journal.pone.0005154.g007

Expressions of the genes mentioned so far are also regulated by complex mechanisms, which are not related with HOG pathway and are also involved in other stress responses [53]. Therefore, we have analyzed a comparatively simpler expression system that is exclusively controlled by Hog1p. As reported earlier [48], *STL1* (a gene encoding a putative hexose transporter) expression is completely abolished in *hog1* and *hot1* mutants in response to salt stress. Hog1p and Hot1p are recruited in an interdependent manner on *STL1* promoter during acute salt stress as supported by chromatin immunoprecipitation [54]. In this study, expression of *STL1* was undetectable in absence of salt stress in wild type cells with or without CaZF expression, again supporting the data that the plant protein is not constitutively active in yeast. In presence of 500 mM NaCl, *STL1* expression was quickly increased with a peak at 20 min at the experimental condition (Figure 7B). Expression of CaZF had no significant effect on *STL1* expression in wild type cells in presence of salt. As expected *STL1* expression was undetectable in *hog1* mutant, however, under salt stress CaZF in *hog1* mutant not only induced *STL1* expression to the extent as it did in wild type background, the expression peak was shifted to 10 min; the shifting of peak of *STL1* expression was also evident in *msn1hot1* double mutant, where the expression at 10 min was comparable to that at 20 min. Interestingly, CaZF-mediated *STL1* expression persisted for relatively shorter time period in *hog1*. Induction of *STL1* expression in response to salt stress in *hog1* and *msn11hot1* mutants confirmed that CaZF functions in a salt stress-dependent manner in yeast and indicates that CaZF also possesses a Hog1p-independent function to activate the expression of some Hog1p-regulated genes.

As CaZF could partially suppress salt sensitivity of *hog1cnb* double mutant we intended to analyze *ENA1* expression, which is regulated independently by both Calcineurin and Hog1p, in CaZF expressing cells (Figure 7C). In wild type and mutant background, CaZF did not alter steady state low expression level of ENA1 transcript in control condition. Expression of *ENA1* was increased rapidly after exposure to salt in wild type cells and that is further enhanced by more than 1.5 fold in cells harboring CaZF. *ENA1* expression was hardly detectable in *hog1cnb* cells and that is slightly enhanced in response to salt. However, in CaZF expressing mutant cells *ENA1* transcript was accumulated at an equivalent level of wild type cells. To test whether *ENA1* gene product is essential for CaZF function, it was expressed in *hog1ena1* double mutant. Figure 6A shows that CaZF expression suppressed salt stress sensitivity not only of *hog1cnb* but also of *hog1crz1* and *hog1ena1* double mutant indicating ENA1 is not essential for CaZF function.

Both HOG and CAN pathways function through a number of transcriptional activators of zinc finger family of proteins e.g. Msn2p/Msn4p, Sgd1p and Crz1p/Tcn1p. Among them Msn2p/Msn4p and Crz1p belongs to C2H2 zinc finger family [3,4]. Despite of the fact that Msn2p/Msn4p and Crz1p are much larger proteins and have no overall sequence similarity with CaZF, the plant protein most likely is able to functionally substitute both these transcription activators. This is quite evident in case of *CTT1, HSP12* and *ENA1* expression in CaZF expressing wild type cells in stress. Expressions of *CTT1* and *HSP12*, predominantly regulated by Msn2p/Msn4p through STRE [21,55] and that of *GPD1* and *GPP2*, regulated by Hot1p and Msn1p [14,19] and not dependent on functional STREs, reveals a striking difference when compared in CaZF-expressing wild type cells in presence of salt. In wild type cells CaZF further induced expressions of *CTT1* and *HSP12* but not of *GPD1* and *GPP2* in salt stress. Thus, CaZF function is not redundant rather additive to Hog1p-mediated expression of these C2H2 zinc finger and STRE-regulated genes. In *hog1* background, under salt stress CaZF is able to induce expression of these genes to a similar extent as Hog1p does in the wild type cells. This also seems to be the mechanism for Crz1p-regulated gene *ENA1*. *ENA1* expression is partially regulated by Crz1p [4,28]. Accordingly, CaZF further enhanced *ENA1* expression by only about 1.5 fold in wild type background under salt stress. In *hog1cnb* background it was found that CaZF was able to activate expression of *ENA1* independent of Hog1p and Calcineurin to the same level as those regulatory enzymes do in wild type cells. While Crz1p requires Calcineurin-mediated post-translational modification to be active [3], CaZF does not require that. However, CaZF seems to require Hog1p and Calcineurin-independent but stress-dependent post-translational modifications and/or protein-protein interaction for its full functional ability.

For the genes (*GPD1, GPP2* and *STL1*) that are regulated predominantly by Hot1p, which is not a C2H2 zinc finger protein, influence of CaZF on their expression was evident only in absence of *Hog1* and *Hot1*. *GPD1* and *GPP2* expressions are also regulated by other proteins (e.g. Rap1p for *GPD1*) [56] and irrespective of combination of gene knockouts involving Hog1p and Hot1p; *GPD1* and *GPP2* remains salt inducible. But CaZF seems to induce expression of these genes by similar mechanism used by Hog1p and Hot1p. The reason being it induces expression of *STL1*, which is exclusively regulated by Hog1p and Hot1p [54]. There is possibility that CaZF utilizes other proteins and/or other salt inducible pathways to mimic Hog1p-regulated activation, but cannot totally replace Hog1p as *STL1* expression kinetics differs in presence and in absence of Hog1p. CaZF seems to require these genes for salt-tolerance because their end product, the glycerol synthesis in the mutant cells harboring the plant gene nicely correlates their growth in presence of salt.

ENA1, a P-type ATPase, is the first member of cluster of four to five genes encoding very similar proteins and plays a major role in detoxification of sodium and lithium cations. A complex mechanism involving different pathways regulates *ENA1* induction in response to salt. Involvements of Hog1p and calcineurin are already discussed. At least two other pathways namely TOR and Hal3/Ppz also regulate *ENA1* expression in response to salt [for review [57,58]]. However, in our experimental system *ENA1* expression increased marginally in *hog1cnb* cells in response to salt suggesting these two enzymes (Hog1p and Calcineurin) are the major regulators of salt-responsive *ENA1* expression. Expression of CaZF confers salt tolerance and induces ENA1 expression in *hog1cnb* suggests that CaZF functions by activating Na^+/Li^+ extrusion system and at the same time also by mechanisms not involving ENA1p as it enhanced salt tolerance of *hog1ena1* mutants. *ENA1*-independent mechanism may involve other cation efflux systems, such as *NHA1* and *SNQ2* or K^+-influx systems like *TRK1* [7,59,60]. Similar *ENA1*-independent salt tolerance was also provided by other plant proteins e.g. STO and SLT1 [61,62].

CaZF induces glycerol accumulation

To further clarify, whether the effects of CaZF on HOG and CAN-pathway gene expression described above are relevant for a functional osmotic stress response; we estimated glycerol production during or after stress exposure. Figure 8 shows that in control condition mutation of the HOG pathway genes and *CaZF* expression had no influence on constitutive level of total glycerol. Mutant lacking *Hog1* did not stimulate glycerol production in response to stress even at the later stage, while *msn1hot1* double mutant started accumulating glycerol later in stress. However, irrespective of genetic background CaZF enhanced production of glycerol in response to stress though comparatively less in the mutant strains than in the wild type cells corroborating the

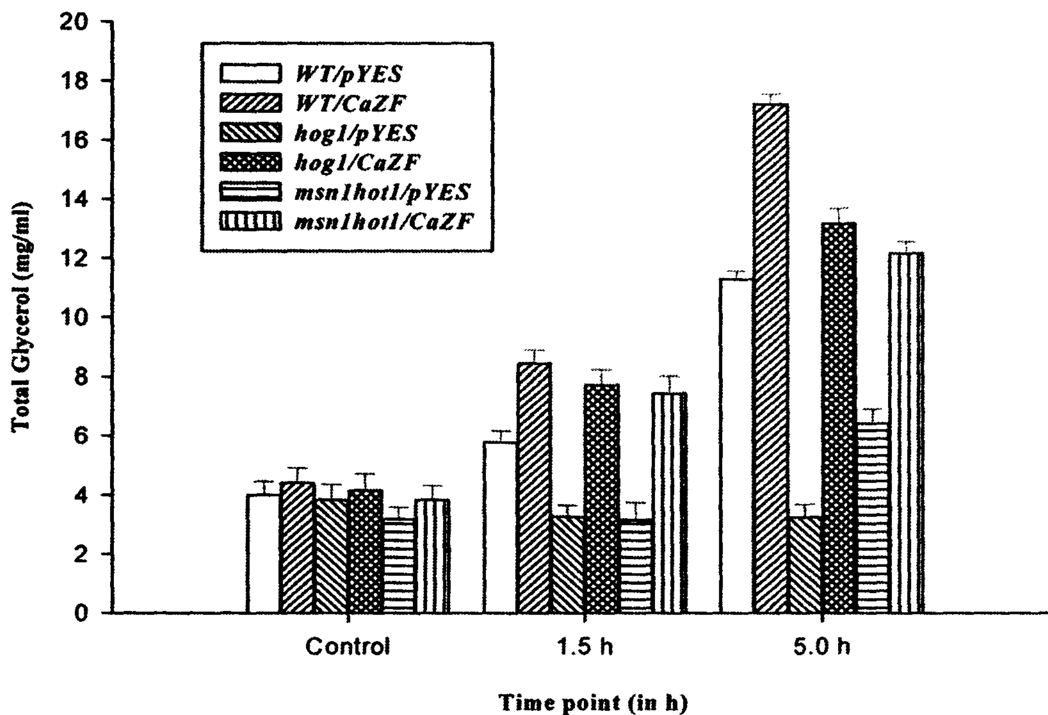


Figure 8. Effect of CaZF expression on accumulation of glycerol in response to salt treatment. Total glycerol [(mg/ml); equivalent to Triloen content] was estimated in wild type and mutant cells carrying either only vector (pYES2.1) or expressing CaZF after treatment with sodium chloride for mentioned time period in the figure. Glycerol assay was done with three experimental repeats and the average value was considered. doi:10.1371/journal.pone.0005154.g008

comparative osmotic tolerance levels of the wild type and the mutant strains expressing CaZF. This result shows that CaZF not only increases the expression of HOG-pathway genes, but also activates the functional osmotic stress response to salt stress in absence of *Hogl*.

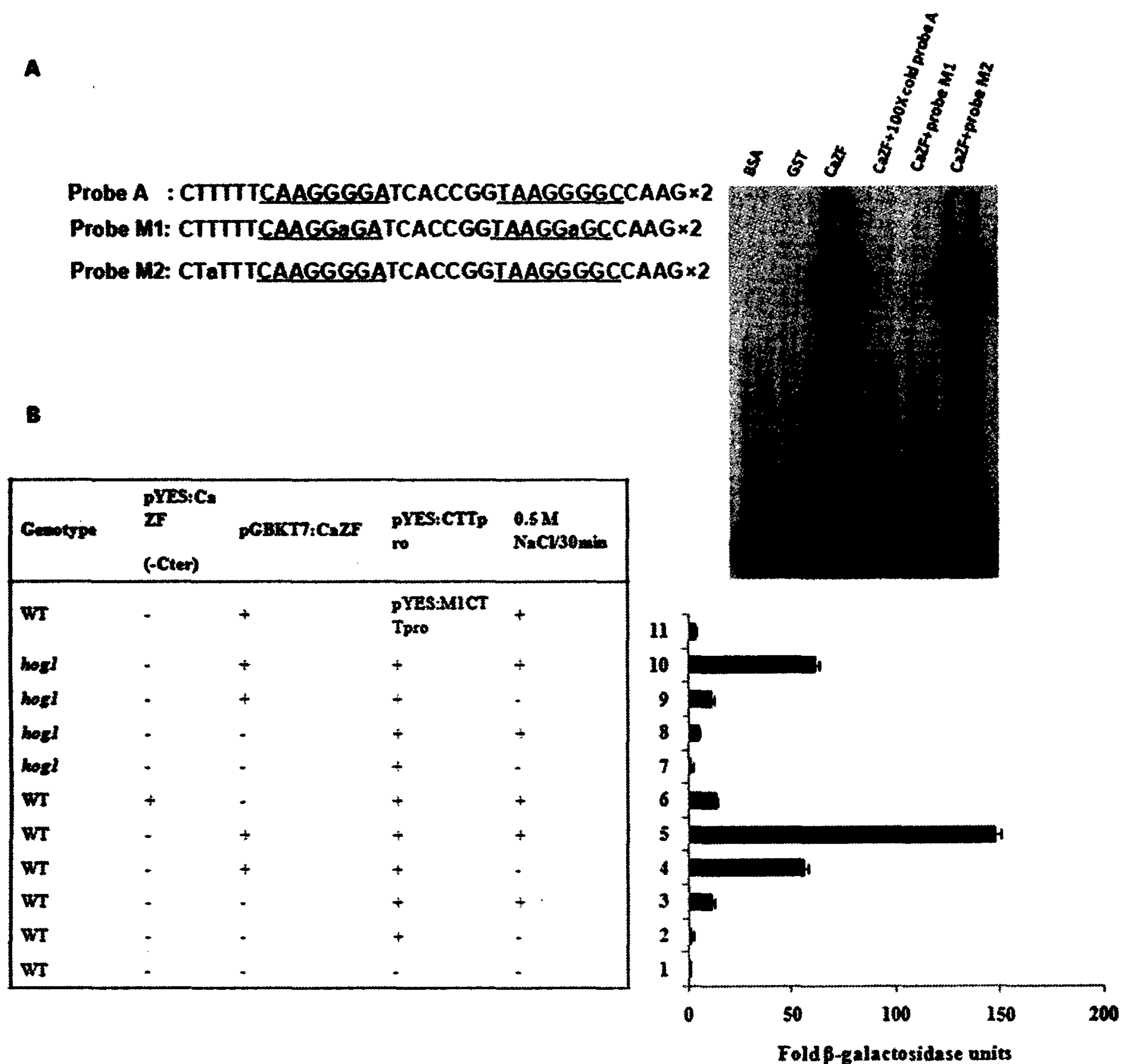
CaZF directly binds to STRE and activates CTT1 promoter

CTT1, *HSP12*, *GPD1* and *GPP2*, the well-studied general stress response genes regulated by HOG pathway possess stress tolerance responsive elements in their upstream activating sequence. Msn2p and Msn4p, two C2H2 zinc finger proteins bind to STRE of *CTT1* and *HSP12* to activate their expressions. To determine the mechanism of action of *CaZF* in yeast we, therefore, tested the ability of CaZF to bind STRE. CaZF protein fused to glutathione-S-transferase (GST) was purified from bacteria and used for gel mobility shift assay using a radiolabeled probe derived from *CTT1* promoter having tetramer of STRE core element (AAGGGG). Figure 9A clearly shows that CaZF bound to STRE in a sequence specific manner as replacement of a 'G' residue with 'A' residue in the core element of the probe (M1) totally abolished the binding while another replacement outside the core element (M2) maintained the binding efficiency.

We have shown that expression of CaZF was able to induce expression of *CTT1* (and three other STRE-containing genes) in *hogl* background. Therefore, we tested the ability of CaZF to activate *CTT1* promoter in wild type and *hogl* mutant. 800 bp (−137 to −937) upstream activating sequence including the translation start codon of *CTT1* was amplified and inserted before *LacZ* reporter gene to regulate its expression. Wild type and *hogl* yeast strains were co-transformed with the reporter construct and CaZF-expressing plasmid and activity of LacZ was assayed. Figure 9B shows that CaZF induced *CTT1* expression by more than 50 fold in absence of salt and almost 150 fold of the basal level in presence of salt in the wild type strain. In *hogl* background the inductions were 10 and 60 folds in absence and in presence of salt stress respectively. A CaZF deletion construct lacking its transactivation domain could not activate the *CTT1* promoter and a *CTT1* promoter construct with mutated STRE was not activated by CaZF. This result demonstrated that CaZF was able to activate yeast stress tolerance responsive element in Hogl-independent manner by directly binding to it.

Requirement of CaZF C-terminal domain for salt tolerance

Transactivation assay in yeast determined that the N-terminal domain of CaZF is responsible for its transactivation property and accordingly removal of that domain made CaZF unable to provide salt-tolerance. To investigate whether the C-terminal domain has any role in its activity, we made serial deletion constructs from the C-terminal end of the protein. Removal of last 22 amino acids did not make any difference in the activity of CaZF. However, further removal of 33 amino acids totally abolished the capability of CaZF to provide any salt-tolerance (Figure 10). A close analysis of the amino acid sequence reveals that this domain contains apart from alternate stretches of basic and acidic amino acids, a potential site for protein kinase C phosphorylation (SKK) and a potential site for cAMP/cGMP-dependent protein kinase phosphorylation (KKKS). In yeast, cAMP-dependent protein kinase A (PKA) is an essential component of general stress response pathway. In normal growth condition PKA phosphorylates C2H2 zinc finger proteins Crz1p, Msn2p and Msn4p to prevent their nuclear localization. Upon inactivation of PKA or activation of the phosphatase calcineurin during stress those proteins get dephosphorylated and are accumulated in the nucleus to activate their



Genotype	pYES:CaZF (-Cter)	pGBKT7:CaZF	pYES:CTTpro	0.5M NaCl/30min
WT	-	+	pYES:M1CTTpro	+
hog1	-	+	+	+
hog1	-	+	+	-
hog1	-	-	+	+
hog1	-	-	+	-
WT	+	-	+	+
WT	-	+	+	+
WT	-	+	+	-
WT	-	-	+	+
WT	-	-	+	-
WT	-	-	-	-

Figure 9. CaZF directly binds to STRE and activates CTT1 promoter. *A*, Gel-shift assay demonstrating that CaZF binds to the STRE sequence containing probe. Either wild-type or mutant versions (M1 and M2) are used. STRE sequences are *underlined* and modified bases are in ***bold small case letters*** (left panel). *B*, Transactivation assay of CTT1-*LacZ* construct by CaZF. Full length or truncated CaZF protein and LacZ reporter fused to CTT1 promoter fragment or its mutant were cointroduced in wild type (WT) or *hog1* BCY123 yeast strains. The transformed yeast strains were treated with/ without 500 mM NaCl for 30 min. Activity of β-galactosidase of each sample (average of three independent transformants) as mentioned in table (left panel) was determined and presented in the form of fold induction in activity (right panel).
doi:10.1371/journal.pone.0005154.g009

target genes [63–65]. CaZF, being a C2H2 zinc finger protein may be regulated by the same mechanism and, thereby, stress-mediated activation of CaZF-function can be explained. Altogether, these results suggest importance of the C-terminal domain with acidic and basic stretches in CaZF function.

Conclusion

We have identified a chickpea gene, CaZF encoding a C2H2 zinc finger protein, which is expressed relatively in higher amount in response to drought stress in a drought-tolerant variety in comparison to a sensitive one. We raised tobacco transgenics overexpressing CaZF showing tolerance to high salinity.

Most likely, in yeast, CaZF does not function at the level of Hog1p; rather it works downstream to it. The reasons being, CaZF structurally resembles a transcription factor and expression of *CaZF* is not toxic like expression of ASR1. ASR1 expression in control condition was growth-inhibitory like a constitutively active Hog1p mutant [47,66]. In spite of inducing gene expression and glycerol production, expression of CaZF could not provide equivalent salt-tolerance to *hog1* and *cnb* mutants as it did in wild type cells. There might be several reasons for that. We have analyzed expressions of only a few genes while Hog1p and Calcineurin together regulate expression of a wide number of genes involved in several pathways. Secondly, Hog1p-mediated stress relief mechanism begins much before of Hog1p-regulated transcriptional induction of downstream genes. Osmotic stress-activated Hog1p phosphorylates Nha1 Na^{+}/H^{+} antiporter, which is crucial for rapid reassociation of those proteins, which were

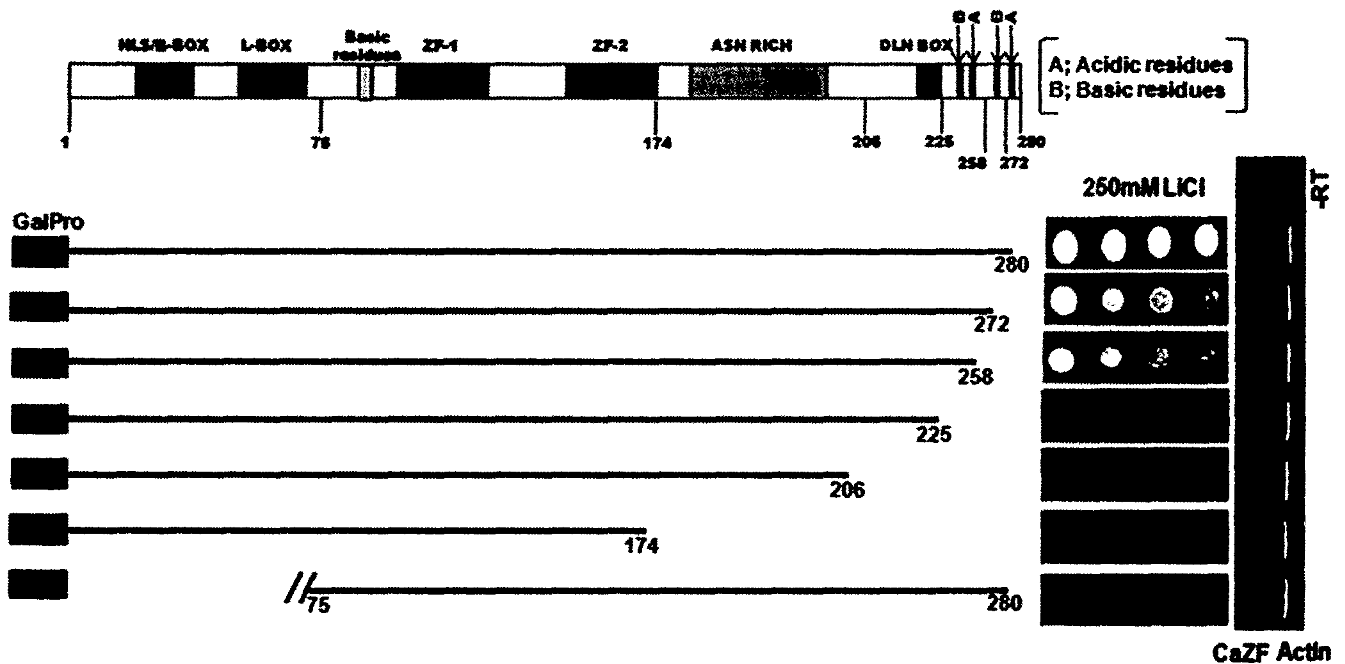


Figure 10. Determination of salt tolerance ability of *CaZF* deletion constructs. BCY123 cells transformed with C-terminal deletion constructs of CaZF as shown in the schematic representation were tested for their ability of providing salt tolerance against 250 mM LiCl. Representative figures from three independent experiments are shown.
doi:10.1371/journal.pone.0005154.g010

dissociated from the chromatin due to stress, with their target sites [24,67]. Apart from that, HOG pathway shares kinases and phosphatases with a lot of interconnecting pathways [for review [53]], which cannot be replaced totally by heterologous expression of one transcription factor.

In conclusion, expression of *CaZF* in yeast provides evidence that at least some of the crucial stress tolerance determining genes, which are regulated by Hog1p MAP kinase, Calcineurin protein phosphatase and their target transcription factors during osmotic stress, can also be activated to the same extent in absence of their regulatory enzymes/transcriptional activators. Activation of those genes by a heterologous gene leads to production of the HOG pathway end product i.e. glycerol. In at least one previous instance it was shown that a plant gene (*ASR1*) could induce synthesis of glycerol in salt stress in absence of Hog1p [47,66]. The level of dependence of yeast cells on *Hog1* differs with intensity and extent of stress conditions. After 20 min of exposure at 800 mM NaCl, 75% of salt stress-responsive genes are strongly dependent on *Hog1* while only 32% of them are strongly dependent on *Hog1* after 10 min exposure at 400 mM NaCl exposure. At 400 mM NaCl 36% of salt-induced genes are independent of *Hog1* in contrast to only 3% at 800 mM [2]. Therefore, influence of Hog1p on expression of genes is relative to the experimental condition. We have analyzed gene expression and glycerol estimation at 500 mM NaCl and so there may be a possibility that in this experimental condition influence of Hog1p is relatively less in providing salt-adaptation. A salt-inducible but Hog1p and Calcineurin-independent pathway definitely uses CaZF as a substrate for post-translational modification and/or target for protein-protein interaction, because it can induce gene expression and consequently provide growth advantage only in presence of stress; and secondly it requires its C-terminal domain, which is not required for its transcription activation property, for its function. CaZF might be a potential target for cyclic AMP-dependent protein Kinase such as protein kinase A. Altogether our experiments in tobacco and in yeast demonstrate that CaZF, a C2H2 zinc finger protein from chickpea is a potential salt tolerant determinant in plant. Unlike several other zinc finger proteins having 'DLNL' motif and acting as transcription repressor, CaZF acts as transcription activator. Other than its transactivation domain, which resides at its N-terminus, the C-terminal aminoacids also play a major role in its activity. CaZF requires post-translational modification and/or interaction with other stress-inducible proteins for its full activity. Our results in yeast model suggest that CaZF can act as a general osmotolerance-determinant by inducing the production of osmolytes by directly activating their promoters.

Materials and Methods

Yeast Strains and Culture Conditions

Yeast strains used in this study are BCY123, BY4742 and PJ69-4A (Table 1). Different mutants used in this study are the derivatives of *Saccharomyces cerevisiae* BCY123 (wild type) [48]. To culture cells, standard yeast media and growth conditions were used. Yeast cells were grown in either YP containing 2% bacto-peptone, 1% Difco yeast extract, 50 μg/mL adenine sulphate supplemented with either 2% dextrose (YPD) or 2% galactose and 2% raffinose (YPGalRaf) or synthetic media containing 0.7% (w/v) yeast nitrogen base supplemented with the required amino acids at 30 μg/mL, 2% (w/v) Glucose, 50 mM succinic acid/Tris (pH 5.5) at 30°C. *Escherichia coli* strain DH5α, used in this study, was grown in Luria broth (LB) medium containing 1% peptone, 0.5% yeast extract and 0.5% NaCl supplemented with ampicillin (50 mg/L) at 37°C. Antibiotics were filter sterilized and added to autoclaved medium.

Table 1. *S. cerevisiae* strains used in this study.

S. NO.	STRAIN	GENOTYPE	SOURCE OF REFERENCE
[illegible]	[illegible]	[illegible] pcb1::LEU2 bar1::HISG lys2::GAL1/10-GAL4 can1 ade2 trp1 ura3 his3 leu2-3, 112 [illegible]	[illegible]
2	BCY123a	Same as BCY213, except *cna1*::HIS3 *cna2*::TRP1	This Study
[illegible]	[illegible]	[illegible] 213, except *cnb*::TRP1	[illegible]
4	BCY123c	Same as BCY213, except *hog1*::TRP1	This Study
[illegible]	[illegible]	[illegible] except *msn2*::HIS3 *msn4*::TRP1	[illegible]
6	BCY123e	Same as BCY213, except *msn1*::HIS3 *hot1*::TRP1	This Study
[illegible]	[illegible]	[illegible] except *hog1*::TRP1 *cnb*::HIS3	[illegible]
8	BCY123g	Same as BCY213, except *hog1*::TRP1 *crz1*::HIS3	This Study
[illegible]	[illegible]	[illegible] except *hog1*::TRP1 *ena1*::HIS3	[illegible]
10	BY4742	MAT α *his3Δ leu2Δ lys2Δ ura3Δ* YJL059W::kanMX4	[76]
[illegible]	[illegible]	[illegible] ura3-52 his3-200 gal4Δ gal80Δ LYS2::GAL-HIS3 GAL2-ADE2 met2::GAL7-lacZ	[illegible]

doi:10.1371/journal.pone.0005154.t001

Plant material used and Identification of cDNA coding for *CaZF*

A drought-tolerant (BGD72) and a drought-sensitive (ICCV2) cultivar of chickpea (*Cicer arietinum*) were used in this study. The cultivars were grown in same pot containing soilrite:vermiculite (1:1). The seedlings were grown in same pot to keep same soil moisture content for both the cultivars. Drought treatment was applied by stopping irrigation to 10 d old chickpea seedlings. Samples were harvested after 0, 3, 6 and 12 d post-irrigation. Subtractive cDNA libraries constructed with these cultivars at different stages of drought resulted in some EST clones that express higher in the drought-tolerant cultivar in response to drought. One such EST encoding C2H2 zinc finger protein was used in this study. 5′RACE System (Life Technologies, Rockville, MD) was used to construct the full length cDNA of *CaZF*. For functional study in yeast the complete and truncated ORF of CaZF was directionally cloned into pYES-2.1-V5 His-TOPO flanked by *XhoI* and *XbaI* restriction sites under galactose-inducible GAL1 promoter.

Subcellular Localization Analysis of Transiently Expressed Fusion Proteins

The CaZF coding region without the translation stop codon was cloned in pCAMBIA1302 to produce the protein fused to GFP using following PCR primers (5′CATGCCATGGCTTTA-GAGTTAGAAGCT3′) and (5′GAAGATCTTGCACCGTTT-CATCATC3′). The PCR amplified fragments were digested with NcoI and BglII and cloned in pCAMBIA1302 vector. The construct was introduced into tobacco (*Nicotiana tabaccum cv. xanthii*) by *Agrobacterium* mediated transformation. For the nuclear staining, tobacco leaf peals were incubated for 10 min with DAPI (1 μg/μl) before observing under fluorescent microscope with FITC filter.

Raising of *CaZF* Overexpressing Transgenic Tobacco Plants

The complete ORF of *CaZF* gene was cloned into the *XbaI-SmaI* site of the pBI-121 vector (Clontech) in the sense orientation. pBI121 without (vector-control) and with *CaZF* were chemically mobilized in to *Agrobacterium tumefaciens* strain GV3101. Tobacco (*Nicotiana tabaccum cv. xanthii*) leaf discs were transformed following standard protocol [68]. Putative T_0 transgenic plants were regenerated from the callus in the presence of kanamycin and integration of the transgene was further confirmed by PCR amplification. The seeds from these plants, i.e. T_0 seeds, were germinated on kanamycin-containing medium and on the basis of segregation analysis and genomic Southern blot; transgenic lines with single transgene insertion were selected for further analyses.

Leaf Disc Assay of CaZF Transgenic Plants

Leaf discs of 1.0 cm diameter were excised from healthy and fully expanded tobacco leaves of same age (30 d post germination) from *CaZF*-expressing and vector-control plants. The discs were floated in a solution of NaCl (150 mM or 300 mM) or water (experimental control) for 72 h [69]. The discs were then used for measuring chlorophyll spectrophotometrically after extraction in 80% cold acetone. The salinity and water treatments were carried out in continous white light at 25±2°C. The experiments were done with three experimental repeats of each vector-control and transgenic lines.

Preparation of Recombinant Proteins and Gel Mobility Shift Assay

To generate a GST-fusion protein, the corresponding ORF of CaZF was amplified by PCR with primers flanked with restriction site for *EcoRI* and inserted into pGEX4T2 expression vector and introduced into *Escherichia coli* BL21 (DE3). Protein expression was induced by 0.5 mM IPTG for 3 h at 30°C. The recombinant proteins were purified from bacterial lysates with Glutathione-Sepharose beads (GE-Amersham) and subsequently monitored by 10% SDS-PAGE. All DNA binding reactions were carried out in 25 mM HEPES-KOH; pH 7.6, 40 mM KCl, 0.1% Nonidet P-40, 0.01 mM $ZnCl_2$, 10 μg/ml poly (dI-dC), and 0.1 mM dithiothreitol. Gel-shift assays were performed with 10,000 c.p.m. of ^{32}P-end-labeled probe A, a tetramer of TTGACAGTGTCACGCG TTGACAGTGTCACGCG (core nucleotides are underlined) or mutated probe M1, a tetramer of TTcAgAGTGTCACcCgTT-GACAGTGTCACGCG (mutated bases are in bold lower case letters. After incubation for 20 min at room temperature, the mixtures were subjected to electrophoresis in 8% polyacrylamide gel as described previously [70].

Yeast One-Hybrid Assay

CaZF protein coding sequence or the truncated forms were cloned in yeast (*S. cerevisiae*) expression vector pGBKT7 (Clone-

tech) at *NdeI-EcoRI* site to express CaZF proteins fused to GAL4 DNA-binding domain. The constructs were transformed into an auxotropic yeast strain PJ69-4A [71] that contains three reporter genes, *HIS3*, *ADE2*, *and β-GAL*, under the control of GAL4 promoter, and plated on synthetic medium lacking histidine and adenine. β-Galactosidase assay of three independent transformed colonies was done in triplicates with ortho-nitrophenyl-β-D-galactoside (ONPG). Presence of different form of CaZF in the transformed colonies were confirmed by PCR and sequencing.

Gene disruption, Complementation and Transformation of Yeast

Direct gene deletion of the target genes with the marker module was done by PCR-based gene deletion strategy [72,73] using the primers mentioned in Supplemental Table S1. *HIS3* and *TRP1* markers were amplified from plasmids pRS413 and pGBKT7 respectively. Correct deletion of the target genes was detected by diagnostic PCR using whole yeast cells from isolated colonies and a set of oligonucleotides designed to bind outside or inside of the replaced target sequence and within the marker module. Disruption of Hog1 gene was confirmed by Western Blot analysis with Hog1p-specific antibody (Supplemental Figure S1). After confirmation of the fidelity of the constructs by sequencing, different yeast strains were transformed with constructed plasmids or with empty pYES-2.1 by the Lithium-acetate/PEG method [74]. Transformants were selected for uracil prototrophy by plating on synthetic media lacking uracil (SC-Ura$^-$). Ura$^+$ colonies were selected thereupon. For osmotolerance experiments and to monitor the growth of mutant yeast strains complemented by CaZF and/or truncated forms, drop tests were performed.

Yeast Spot Assay

For drop tests, overnight YPGal grown yeast cells were diluted to $OD_{600} = 0.4$ in 2% Gal, 50 mM MES pH 5.5 and incubated for 3 h and then further serially diluted with YP to obtain 10, 10^2, 10^3 and 10^4 cells. Three microliters of each dilution was then spotted onto YPGalRaf with/without NaCl, LiCl, KCl or sorbitol as mentioned or onto complete synthetic uracil$^-$ medium supplemented with 2% Gal, 0.2% sucrose and $MnCl_2$ as indicated in the figures. Plates were incubated at 30°C and unless otherwise indicated, colony growth was inspected after 2–4 d.

RNA Isolation

Cells were grown in YPGalRaf at 30°C to late log/stationary phase. Cultures were diluted to an OD_{600} of ca. 0.1 in YPGalRaf medium, and then further grown at 30°C till OD_{600} reached to 0.5. Then the, cells were subjected to saline stress for different time points as mentioned in figures. The saline stress was given by suspending pelleted cells in salt-containing medium. After saline stress, cells were centrifuged for 3 min at 7,000 rpm, and total RNA was extracted from untreated cells or cells treated with NaCl by using hot phenol method as described [75].

Northern and Western Blot Analyses

For Northern blot analysis, total RNA (20 µg/lane) was electrophoresed on 1.2% agarose-formaldehyde gels and transferred to positively charged nylon membranes (GE-Amersham, UK). Membranes were hybridized at 60°C in the presence of hybridization buffer (700 mM NaCl, 40 mM NaH_2PO_4;pH 7.6, 4 mM EDTA, 0.2% polyvinylpyrrolidone, 0.2% Ficoll, 0.1% SDS, 0.2 mg/ml salmon sperm DNA) and 10^6 cpm/ml appropriate ^{32}P-labeled DNA fragment. DNA fragments containing the ORF of the following genes were used as probes: *CTT1* (YGR088W) from position +1 to +540 (0.54 kb), *HSP12* (YFL014W) from position +1 to +330 (0.33 kb), *GPD1*(YDL022W from position +1 to +540 (0.54 kb), *GPP2/HOR2* (YER062C) from position +1 to +480 (0.48 kb), *ENA1* from position +90 to +1000 (0.91 kb) and *STL1* (YDR536W) from position +40 to +1032 (0.99 kb). Probes were labeled using the random-primed DNA labeling kit (GE-Amersham, UK). Filters were washed in 0.1× SSC (1× SSC is 150 mM NaCl, 15 mM sodium citrate, pH 7.0), 0.1% SDS at 55°C. Blots were exposed on Kodak X- Ray films. For Western blot analysis, 20 µg of total cell lysate was analyzed by 12% SDS-PAGE, subsequently transferred to Hybond-C membrane. Specific proteins were detected using antibodies from Santacruz Biotechnology and electrochemiluminiscence (ECL) kit from GE Healthcare. c-Myc (9E-11) and Hog1p (yC-20) antibodies were used to detect myc-tagged GalBD-CaZF and *S. cerevisae* Hog1p respectively. Ponceau-S stained membranes were checked for equal protein loading. For semiquantitative RT-PCR, 1 µg RNA was converted to cDNA using Superscript Reverse Transcriptase (Invitogen). One-tenth of the cDNA product was used for PCR amplification. Amplified product was visible after 22 cycles. Primers used for CaZF are (5′ATGGCTTTAGAGTTAGAAGCTTTCAATTCTTC3′; 5′AGACGGATACAGTGTCGTTGAAGGCTGTGGATG 3′) and for actin are (5′ATGGATTCTGAGGTTGCTGCTTTGGT-TATT3′; 5′AAAGAGTAACCACGTTCACTCAAGATCTTC3′).

Glycerol estimation

Overnight grown yeast cells in YPGal medium were diluted to $OD_{600} = 0.3$ and grown for 4 h at 30°C. Then they were subjected to increased osmolarity (500 mM NaCl). For glycerol measurement, at time points indicated in the Figure. 7, 2 ml samples were taken, boiled for 15 min and then centrifuged to remove cellular debris. The supernatant was used for glycerol measurement by using Free Glycerol Reagent (Sigma, USA) according to the manufacturers' instructions. Assay was done with three independent experimental repeats. Glycerol accumulation was expressed in mg/ml (equivalent to triloen content).

CTT1-*LacZ* β-galactosidase assay

Wild type BCY123 or *hog1* yeast cells were cotransformed with pYES-CTT1-*LacZ* construct containing a 800 bp (−137 to −937) upstream region of *CTT1* fused to *LacZ* and pGBKT7-*CaZF* construct expressing full length or truncated form of *CaZF* cDNA. Three independent transformants were grown to late log phase in SD medium without uracil and histidine. Cells were collected and re-suspended in YPD medium to an OD of 0.2–0.3. Growth was assumed until A_{600} of 0.5–0.7. Cells were harvested and resuspended in YPD with or without salt (500 mM NaCl) for 30 min. Cells from three independent transformants were collected and assayed for β-galactosidase activity as described above.

Electrophoresis mobility shift assays

Recombinant CaZF protein was expressed in *E. coli* DH5α as GST-fused proteins and purified by GST-agarose columns. Gel-shift assays were performed with 10,000 c.p.m. of ^{32}P-end-labeled probe A, a dimer of CTTTTCAAGGGGATCACCGG-TAAGGGGCCAAG (STRE sequences are underlined) or mutated probe M1, a dimer of CTTTTCAAGGaGATCACCGGTAAG-GaGCCAAG or probe M2, a dimer of CTaTTCAAGGGGAT-CACCGGTAAGGGGCCAAG (mutated bases are in bold lower case letters). After incubation for 20 min at room temperature, the mixtures were subjected to electrophoresis in 8% polyacrylamide gel as described previously [70].

Supporting Information

Figure S1 Detection of Hog1p. Hog1p was detected in S. cerevisiae BCY123 (WT) and the corresponding hog1 mutant strain by Western Blot with Hog1p-antibody (yC-20, sc-6815). Blotted membrane stained with Ponceau-S is shown for equivalent loading of protein (20 μg)
Found at: doi:10.1371/journal.pone.0005154.s001 (9.28 MB TIF)

Table S1 Oligonucleotides used in this study
Found at: doi:10.1371/journal.pone.0005154.s002 (0.07 MB DOC)

Acknowledgments

The authors acknowledge Dr. K. Natrajan, School of Life Sciences, Jawaharlal Nehru University, New Delhi, INDIA for providing the yeast strain BY4742.

Author Contributions

Conceived and designed the experiments: DC. Performed the experiments: DJ. Analyzed the data: DJ. Contributed reagents/materials/analysis tools: NR. Wrote the paper: DC.

References

1. Boyer JS (1982) Plant Productivity and Environment. Science 218: 443–448.
2. Posas F, Chambers JR, Heyman JA, Hoeffler JP, de Nadal E, et al. (2000) The transcriptional response of yeast to saline stress. J Biol Chem 275: 17249–17255.
3. Matheos DP, Kingsbury TJ, Ahsan US, Cunningham KW (1997) Tcn1p/Crz1p, a calcineurin-dependent transcription factor that differentially regulates gene expression in Saccharomyces cerevisiae. Genes Dev 11: 3445–3458.
4. Stathopoulos AM, Cyert MS (1997) Calcineurin acts through the CRZ1/TCN1-encoded transcription factor to regulate gene expression in yeast. Genes Dev 11: 3432–3444.
5. Garciadeblas B, Rubio F, Quintero FJ, Banuelos MA, Haro R, et al. (1993) Differential expression of two genes encoding isoforms of the ATPase involved in sodium efflux in Saccharomyces cerevisiae. Mol Gen Genet 236: 363–368.
6. Ganster RW, McCartney RR, Schmidt MC (1998) Identification of a calcineurin-independent pathway required for sodium ion stress response in Saccharomyces cerevisiae. Genetics 150: 31–42.
7. Mendoza I, Rubio F, Rodriguez-Navarro A, Pardo JM (1994) The protein phosphatase calcineurin is essential for NaCl tolerance of Saccharomyces cerevisiae. J Biol Chem 269: 8792–8796.
8. Nakamura T, Liu Y, Hirata D, Namba H, Harada S, et al. (1993) Protein phosphatase type 2B (calcineurin)-mediated, FK506-sensitive regulation of intracellular ions in yeast is an important determinant for adaptation to high salt stress conditions. Embo J 12: 4063–4071.
9. Breuder T, Hemenway CS, Movva NR, Cardenas ME, Heitman J (1994) Calcineurin is essential in cyclosporin A- and FK506-sensitive yeast strains. Proc Natl Acad Sci U S A 91: 5372–5376.
10. Cyert MS, Kunisawa R, Kaim D, Thorner J (1991) Yeast has homologs (CNA1 and CNA2 gene products) of mammalian calcineurin, a calmodulin-regulated phosphoprotein phosphatase. Proc Natl Acad Sci U S A 88: 7376–7380.
11. Garrett-Engele P, Moilanen B, Cyert MS (1995) Calcineurin, the Ca2+/calmodulin-dependent protein phosphatase, is essential in yeast mutants with cell integrity defects and in mutants that lack a functional vacuolar H(+)-ATPase. Mol Cell Biol 15: 4103–4114.
12. Mendizabal I, Rios G, Mulet JM, Serrano R, de Larrinoa IF (1998) Yeast putative transcription factors involved in salt tolerance. FEBS Lett 425: 323–328.
13. Brewster JL, de Valoir T, Dwyer ND, Winter E, Gustin MC (1993) An osmosensing signal transduction pathway in yeast. Science 259: 1760–1763.
14. Albertyn J, Hohmann S, Thevelein JM, Prior BA (1994) GPD1, which encodes glycerol-3-phosphate dehydrogenase, is essential for growth under osmotic stress in Saccharomyces cerevisiae, and its expression is regulated by the high-osmolarity glycerol response pathway. Mol Cell Biol 14: 4135–4144.
15. Gustin MC, Albertyn J, Alexander M, Davenport K (1998) MAP kinase pathways in the yeast Saccharomyces cerevisiae. Microbiol Mol Biol Rev 62: 1264–1300.
16. Maeda T, Takekawa M, Saito H (1995) Activation of yeast PBS2 MAPKK by MAPKKKs or by binding of an SH3-containing osmosensor. Science 269: 554–558.
17. Maeda T, Wurgler-Murphy SM, Saito H (1994) A two-component system that regulates an osmosensing MAP kinase cascade in yeast. Nature 369: 242–245.
18. Martinez-Pastor MT, Marchler G, Schuller C, Marchler-Bauer A, Ruis H, et al. (1996) The Saccharomyces cerevisiae zinc finger proteins Msn2p and Msn4p are required for transcriptional induction through the stress response element (STRE). Embo J 15: 2227–2235.
19. Rep M, Reiser V, Gartner U, Thevelein JM, Hohmann S, et al. (1999) Osmotic stress-induced gene expression in Saccharomyces cerevisiae requires Msn1p and the novel nuclear factor Hot1p. Mol Cell Biol 19: 5474–5485.
20. Schmitt AP, McEntee K (1996) Msn2p, a zinc finger DNA-binding protein, is the transcriptional activator of the multistress response in Saccharomyces cerevisiae. Proc Natl Acad Sci U S A 93: 5777–5782.
21. Rep M, Krantz M, Thevelein JM, Hohmann S (2000) The transcriptional response of Saccharomyces cerevisiae to osmotic shock. Hot1p and Msn2p/Msn4p are required for the induction of subsets of high osmolarity glycerol pathway-dependent genes. J Biol Chem 275: 8290–8300.
22. Estruch F (2000) Stress-controlled transcription factors, stress-induced genes and stress tolerance in budding yeast. FEMS Microbiol Rev 24: 469–486.
23. Mager WH, Siderius M (2002) Novel insights into the osmotic stress response of yeast. FEMS Yeast Res 2: 251–257.
24. Alepuz PM, Jovanovic A, Reiser V, Ammerer G (2001) Stress-induced map kinase Hog1 is part of transcription activation complexes. Mol Cell 7: 767–777.
25. Proft M, Struhl K (2002) Hog1 kinase converts the Sko1-Cyc8-Tup1 repressor complex into an activator that recruits SAGA and SWI/SNF in response to osmotic stress. Mol Cell 9: 1307–1317.
26. Alepuz PM, de Nadal E, Zapater M, Ammerer G, Posas F (2003) Osmostress-induced transcription by Hot1 depends on a Hog1-mediated recruitment of the RNA Pol II. Embo J 22: 2433–2442.
27. De Nadal E, Zapater M, Alepuz PM, Sumoy L, Mas G, et al. (2004) The MAPK Hog1 recruits Rpd3 histone deacetylase to activate osmoresponsive genes. Nature 427: 370–374.
28. Proft M, Serrano R (1999) Repressors and upstream repressing sequences of the stress-regulated ENA1 gene in Saccharomyces cerevisiae: bZIP protein Sko1p confers HOG-dependent osmotic regulation. Mol Cell Biol 19: 537–546.
29. Wilkinson MG, Samuels M, Takeda T, Toone WM, Shieh JC, et al. (1996) The Atf1 transcription factor is a target for the Sty1 stress-activated MAP kinase pathway in fission yeast. Genes Dev 10: 2289–2301.
30. Haro R, Baneulos MA, Quintero FJ, Rubio F, Rodriguez-Navarro A (1993) Genetic basis of sodium exclusion and sodium tolerance in yeast: A model for plants. Physiol Plant 89: 868–874.
31. Serrano R, Gaxiola R (1994) Microbial models and salt stress tolerance in plants. Crit Rev Plant Sci 13: 121–138.
32. Frommer WB, Ninnemann O (1995) Heterologous Expression of Genes in Bacterial, Fungal, Animal, and Plant Cells. Annual Review of Plant Physiology and Plant Molecular Biology 46: 419–444.
33. Niu X, Bressan RA, Hasegawa PM, Pardo JM (1995) Ion Homeostasis in NaCl Stress Environments. Plant Physiol 109: 735–742.
34. Hasegawa PM, Bressan RA, Zhu JK, Bohnert HJ (2000) Plant Cellular and Molecular Responses to High Salinity. Annu Rev Plant Physiol Plant Mol Biol 51: 463–499.
35. Bressan RA, Hasegawa PM, Pardo JM (1998) Plants use calcium to resolve salt stress. Trends Plant Sci 3: 411–412.
36. Luan S, Li W, Rusnak F, Assmann SM, Schreiber SL (1993) Immunosuppressants implicate protein phosphatase regulation of K+ channels in guard cells. Proc Natl Acad Sci U S A 90: 2202–2206.
37. Covic L, Silva NF, Lew RR (1999) Functional characterization of ARAKIN (ATMEKK1): a possible mediator in an osmotic stress response pathway in higher plants. Biochim Biophys Acta 1451: 242–254.
38. Liu J, Zhu JK (1998) A calcium sensor homolog required for plant salt tolerance. Science 280: 1943–1945.
39. Soyano T, Nishihama R, Morikiyo K, Ishikawa M, Machida Y (2003) NQK1/NtMEK1 is a MAPKK that acts in the NPK1 MAPKKK-mediated MAPK cascade and is required for plant cytokinesis. Genes Dev 17: 1055–1067.
40. Ohkawa T, Washburn JO, Sitapara R, Sid E, Volkman LE (2005) Specific binding of Autographa californica M nucleopolyhedrovirus occlusion-derived virus to midgut cells of Heliothis virescens larvae is mediated by products of pif genes Ac119 and Ac022 but not by Ac115. J Virol 79: 15258–15264.
41. Takatsuji H, Mori M, Benfey PN, Ren L, Chua NH (1992) Characterization of a zinc finger DNA-binding protein expressed specifically in Petunia petals and seedlings. Embo J 11: 241–249.
42. Michael AJ, Hofer JM, Ellis TH (1996) Isolation by PCR of a cDNA clone from pea petals with similarity to petunia and wheat zinc finger proteins. Plant Mol Biol 30: 1051–1058.
43. Kim JC, Lee SH, Cheong YH, Yoo CM, Lee SI, et al. (2001) A novel cold-inducible zinc finger protein from soybean, SCOF-1, enhances cold tolerance in transgenic plants. Plant J 25: 247–259.
44. Takatsuji H, Nakamura N, Katsumoto Y (1994) A new family of zinc finger proteins in petunia: structure, DNA sequence recognition, and floral organ-specific expression. Plant Cell 6: 947–958.
45. Sakamoto H, Maruyama K, Sakuma Y, Meshi T, Iwabuchi M, et al. (2004) Arabidopsis Cys2/His2-type zinc-finger proteins function as transcription repressors under drought, cold, and high-salinity stress conditions. Plant Physiol 136: 2734–2746.
46. Ohta M, Matsui K, Hiratsu K, Shinshi H, Ohme-Takagi M (2001) Repression domains of class II ERF transcriptional repressors share an essential motif for active repression. Plant Cell 13: 1959–1968.

47. Moretti MB, Maskin L, Gudesblat G, Garcia SC, Iusem ND (2006) ASR1, a stress-induced tomato protein, protects yeast from osmotic stress. Physiologia Plantarum 127: 111–118.
48. Zhao S, Douglas NW, Heine MJ, Williams GM, Winther-Larsen HC, et al. (1994) The STL1 gene of Saccharomyces cerevisiae is predicted to encode a sugar transporter-like protein. Gene 146: 215–219.
49. Dreyer I, Horeau C, Lemaillet G, Zimmermann S, Bush D, et al. (1999) Identification and characterization of plant transporters using heterologous expression systems. J Exp Bot 50: 1073–1087.
50. Zakrzewska A, Boorsma A, Delneri D, Brul S, Oliver SG, et al. (2007) Cellular processes and pathways that protect Saccharomyces cerevisiae cells against the plasma membrane-perturbing compound chitosan. Eukaryot Cell 6: 600–608.
51. Proft M, Pascual-Ahuir A, de Nadal E, Arino J, Serrano R, et al. (2001) Regulation of the Sko1 transcriptional repressor by the Hog1 MAP kinase in response to osmotic stress. EMBO J 20: 1123–1133.
52. Garcia-Gimeno MA, Struhl K (2000) Aca1 and Aca2, ATF/CREB Activators in Saccharomyces cerevisiae, Are Important for Carbon Source Utilization but Not the Response to Stress. Mol Cell Biol 20: 4340–4349.
53. Hohmann S (2002) Osmotic stress signaling and osmoadaptation in yeasts. Microbiol Mol Biol Rev 66: 300–372.
54. Proft M, Mas G, de Nadal E, Vendrell A, Noriega N, et al. (2006) The stress-activated Hog1 kinase is a selective transcriptional elongation factor for genes responding to osmotic stress. Mol Cell 23: 241–250.
55. Schuller C, Brewster JL, Alexander MR, Gustin MC, Ruis H (1994) The HOG pathway controls osmotic regulation of transcription via the stress response element (STRE) of the Saccharomyces cerevisiae CTT1 gene. Embo J 13: 4382–4389.
56. Eriksson P, Alipour H, Adler L, Blomberg A (2000) Rap1p-binding sites in the saccharomyces cerevisiae GPD1 promoter are involved in its response to NaCl. J Biol Chem 275: 29368–29376.
57. Ruiz A, Arino J (2007) Function and regulation of the Saccharomyces cerevisiae ENA sodium ATPase system. Eukaryot Cell 6: 2175–2183.
58. Platara M, Ruiz A, Serrano R, Palomino A, Moreno F, et al. (2006) The transcriptional response of the yeast Na(+)-ATPase ENA1 gene to alkaline stress involves three main signaling pathways. J Biol Chem 281: 36632–36642.
59. Obata T, Kitamoto HK, Nakamura A, Fukuda A, Tanaka Y (2007) Rice shaker potassium channel OsKAT1 confers tolerance to salinity stress on yeast and rice cells. Plant Physiol 144: 1978–1985.
60. Mulet JM, Leube MP, Kron SJ, Rios G, Fink GR, et al. (1999) A novel mechanism of ion homeostasis and salt tolerance in yeast: the Hal4 and Hal5 protein kinases modulate the Trk1-Trk2 potassium transporter. Mol Cell Biol 19: 3328–3337.
61. Lippuner V, Cyert MS, Gasser CS (1996) Two classes of plant cDNA clones differentially complement yeast calcineurin mutants and increase salt tolerance of wild-type yeast. J Biol Chem 271: 12859–12866.
62. Matsumoto TK, Pardo JM, Takeda S, Bressan RA, Hasegawa PM (2001) Tobacco and Arabidopsis SLT1 mediate salt tolerance of yeast. Plant Mol Biol 45: 489–500.
63. Smith A, Ward MP, Garrett S (1998) Yeast PKA represses Msn2p/Msn4p-dependent gene expression to regulate growth, stress response and glycogen accumulation. EMBO J 17: 3556–3564.
64. Kafadar KA, Cyert MS (2004) Integration of stress responses: modulation of calcineurin signaling in Saccharomyces cerevisiae by protein kinase A. Eukaryot Cell 3: 1147–1153.
65. Cyert MS (2003) Calcineurin signaling in Saccharomyces cerevisiae: how yeast go crazy in response to stress. Biochem Biophys Res Commun 311: 1143–1150.
66. Maskin L, Frankel N, Gudesblat G, Demergasso MJ, Pietrasanta LI, et al. (2007) Dimerization and DNA-binding of ASR1, a small hydrophilic protein abundant in plant tissues suffering from water loss. Biochem Biophys Res Commun 352: 831–835.
67. Proft M, Struhl K (2004) MAP kinase-mediated stress relief that precedes and regulates the timing of transcriptional induction. Cell 118: 351–361.
68. Shukla RK, Raha S, Tripathi V, Chattopadhyay D (2006) Expression of CAP2, an APETALA2-Family Transcription Factor from Chickpea, Enhances Growth and Tolerance to Dehydration and Salt Stress in Transgenic Tobacco. Plant Physiol 142: 113–123.
69. Fan L, Zheng S, Wang X (1997) Antisense Suppression of Phospholipase D[alpha] Retards Abscisic Acid[mdash] and Ethylene-Promoted Senescence of Postharvest Arabidopsis Leaves. Plant Cell 9: 2183–2196.
70. Urao T, Yamaguchi-Shinozaki K, Urao S, Shinozaki K (1993) An Arabidopsis myb homolog is induced by dehydration stress and its gene product binds to the conserved MYB recognition sequence. Plant Cell 5: 1529–1539.
71. Cagney G, Uetz P, Fields S (2000) High-throughput screening for protein-protein interactions using two-hybrid assay. Methods Enzymol 328: 3–14.
72. Baudin A, Ozier-Kalogeropoulos O, Denouel A, Lacroute F, Cullin C (1993) A simple and efficient method for direct gene deletion in Saccharomyces cerevisiae. Nucleic Acids Res 21: 3329–3330.
73. Wach A, Brachat A, Pohlmann R, Philippsen P (1994) New heterologous modules for classical or PCR-based gene disruptions in Saccharomyces cerevisiae. Yeast 10: 1793–1808.
74. Gietz RD, Schiestl RH, Willems AR, Woods RA (1995) Studies on the transformation of intact yeast cells by the LiAc/SS-DNA/PEG procedure. Yeast 11: 355–360.
75. Schmitt ME, Brown TA, Trumpower BL (1990) A rapid and simple method for preparation of RNA from Saccharomyces cerevisiae. Nucleic Acids Res 18: 3091–3092.
76. Pardo JM, Reddy MP, Yang S, Maggio A, Huh GH, et al. (1998) Stress signaling through Ca2+/calmodulin-dependent protein phosphatase calcineurin mediates salt adaptation in plants. Proc Natl Acad Sci U S A 95: 9681–9686.