"CHARACTERIZATION AND VALIDATION OF ZINC FINGER GENE SUPERFAMILY TRANSCRIPTION FACTORS FOR DROUGHT TOLERANCE IN RICE (*Oryza sativa* L.) USING *IN SILICO* AND FUNCTIONAL GENOMICS APPROACHES"

M. Sc. (Ag.) THESIS

by

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INDIRA GANDHI KRISHI VISHWAVIDYALAYA
RAIPUR, (C.G.)

2011

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Thesis

Submitted to the

Indira Gandhi Krishi Vishwavidyalaya, Raipur

by

SANJEET KUMAR

IN PARTIAL FULFILMENT OF THE

REQUIREMENTS FOR THE

DEGREE OF

Master of Science
In

Agriculture

(Plant Molecular Biology and Biotechnology)

Roll. No. 12509 **ID No. 120109032**

JUNE, 2011

CERTIFICATE – I

This is to certify that the thesis entitled **"Characterization and Validation of Zinc Finger Gene Superfamily Transcription Factors for Drought Tolerance in Rice (*Oryza sativa* L.) Using *In Silico* and Functional Genomics Approaches",** submitted in partial fulfillment of the requirements for the degree of **"Master of Science in Agriculture"** of the Indira Gandhi Krishi Vishwavidyalaya, Raipur is a record of the bonafide research work carried out by **Mr. Sanjeet Kumar** under my guidance and supervision. The subject of the thesis has been approved by the Student's Advisory Committee and the Director of Instructions.

No part of the thesis has been submitted for any other degree or diploma (Certificate awarded etc.) or has been published/Published part has been fully acknowledged. All the assistance and help received during the course of the investigations have been duly acknowledged by him.

Date:

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Advisory committee

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Member : Dr. (Smt.) Zenu Jha _____

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Member : Dr. R. R. Saxena _____

CERTIFICATE – II

This is to certify that the thesis entitled **"Characterization and Validation of Zinc Finger Gene Superfamily Transcription Factors for Drought Tolerance in Rice (*Oryza sativa* L.) Using *In Silico* and Functional Genomics Approaches",** submitted by **Mr. Sanjeet Kumar** to the Indira Gandhi Krishi Vishwavidyalaya, Raipur in partial fulfillment of the requirements for the degree of M.Sc. (Ag.) in the Department of Plant Molecular Biology and Biotechnology has been approved by the external examiner and Student's Advisory Committee after oral examination.

EXTERNAL EXAMINER

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DIRECTOR OF INSTRUCTIONS _____

Acknowledgement

Words work as a vector to express our deep emotions and concerns. It is my pride privilege to acknowledge my Major advisor Dr. Girish Chandel, Associate professor, Department of Plant Molecular Biology and Biotechnology, Indira Gandhi Krishi Vishwavidhyalaya, Raipur for providing me a vision for science. His illuminating guidance, crystal suggestions and continuous support through all thick and thin imbued me with the unparallel zeal throughout the whole investigation. Despite his busy schedule and personal engagements he was always there for patient hearing, intelligent advice and has given touch of excellence to this manuscript.

Most humbly and respectfully I wish to express deep sense of gratitude to Dr. D.K. Sharma, Senior Scientist, Head, Department of Plant Molecular Biology and Biotechnology, Indira Gandhi krishi Vishwavidyalaya, Raipur, for his valuable suggestions, believe in me and providing necessary facilities as and when I needed. I would like to extend my sincere thanks to respected members of my advisory committee Dr. D.K. Sharma, Dr. (Smt) Z. Jha, and Dr. R.R. Saxena and Dr. Sanjay Sharma, College of Agriculture, Raipur for their useful suggestions, critical comments and kind help rendered as and when needed. I would like to express my sincere thanks to Dr. A. S. Kotasthane sir for providing Bioinformatics facility throughout my course and research work.

I wish to record my sincere gratitude to Dr. M. P. Pandey, Hon'ble vice Chanceller, Dr. O. P. Kashyap, Dean, Dr. U. K, Mishra, Director of instruction and Dr. S. K, Patil, Director Research Services, I.G.K.V., Raipur for providing me the necessary facilities for Research.

I am exclusively thankful to Dr. U.G. Kulkarni for suggesting me whenever there was need to pour his experience.

I am especially thankful to my seniors and other staff members beside Shiv sir, Lincon sir, Vikrant sir, Neha madam, Mahima madam, Archana madam, Sweta madam, Renu madam, Toshy madam, Manju madam, Rakesh sir, Manoj bhaiya, Jitendra, Lakhan bhaiya, Pushav bhaiya and others for their valuable support, advice and genuine guidance, whenever, I sought throughout my course work.

I owe my gratitude to friends Amit, Ajit, Ravish, Rahul, Onkar, Mahendra, Pallavi, Saroj and my affectionate juniors whose support and help always strengthened me. At the same time equally grateful to friends behind the curtain Dyaneshwar sir, Sada sir, Sandeep sir, Yashwant sir, Mangesh sir, Ravi, Ganesh, Amol, Sushant, Anant, Sagar, Shrikant, Kiran, Suvarna, Varsha, Radha.

I bow to almighty God for blessing me with an embodiment of love and courage so called mother Smt. Seeta Devi and ever inspiring father Late Sudama Prasad, supportive uncle-auntie and wonderful cousins, found them backing me throughout my life whenever it came to a tough time or merry one.

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LIST OF ABBREVIATIONS

Abbreviation	Details
%	- per cent
°C	- degree celsius
µl	- microlitre
ABRE	- abscisic acid responsiveness
ANOVA	-Analysis of variance
bp	- Base pair
dATP	- deoxy adenosine 5’ triphosphate
cDNA	- complementary DNA
dGTP	- deoxy guanosine 5’ triphosphate
DNA	- deoxyribo nucleic acid
dNTPs	- deoxynucleotide triphosphates
DREB	- drought responsive binding site
dTTP	- deoxy thymidine 5’ triphosphate
dCTP	- deoxy cytidine 5’ triphosphate
EDTA	- ethylene diamine tetra acetic acid
ERE	- ethylene-responsive element
et al.	- and others
ESTs	- Expressed sequence tags
gm	- gram
µg	- microgram
ha	- hectare
h.	- hours
i.e.	- that is
LEA	- Late embryogenesis abundant
M	- molar
min	- Minute
mM	- Millie molar
ml	- milliliter

MBS	- MYB binding site
MPSS	- massive parallel signature sequence
μM	- micro molar
ng	- nano gram
nm	- nanometer
PCR	- polymerase chain reaction
QTLs	- Quantitative traits loci
ROS	- Reactive oxygen species
rpm	- rotations per minute
RT-PCR	- Real time Polymerase chain reaction
SNPs	- Single nucleotide polymorphisms
UTR	- Untranslated region
TPM	- transcripts per millions
TFs	- Transcription factors
ZFP	- Zinc finger protein

CHAPTER I

INTRODUCTION

Rice *(Oryza sativa* L.) is the world's most important staple food crop and it is affected by many biotic and abiotic stresses among them drought is a major problem for much of the production area (O'Toole and Chang, 1979; Chang *et al.,* 1982). One-half of the 148 million ha of rice planted annually is in a rainfed environment, but production in this ecosystem accounts for only one quarter of rice grain production. Drought is one of the most common environmental stresses that affect growth and development of plants through alterations in metabolism and gene expression (Ceccarelli *et al*., 1996). However, little progress has been made in characterizing the genetic determinants of drought resistance, because it is a complex phenomenon and comprising of a number of physio-biochemical processes at both cellular and organismic levels at different stages of plant development (Tripathy *et al*., 2000).

The complexity of drought involved the interaction of number of individual component traits that contribute to three main plant strategies, drought escape covers mechanisms permitting the crop to complete the most sensitive stages of its life cycle during the periods of highest water supply, drought avoidance designates mechanisms, which help the plant to maintain a good water status and turgor during a stress period and drought tolerance regroups mechanisms that allow the plant to sustain the least injury under decreasing leaf water potential (Blum *et al*., 1982). To survive under unfavorable conditions, plants have developed a variety of sophisticated strategies (Bohnert *et al*., 1995). Currently, our understanding of processes underlying plant response to drought at the molecular and the whole plant levels has rapidly progressed.

With the recently advancements in the genomic research of rice, several drought related candidate genes have been characterized. *In silico* structural and functional characterization of drought tolerance related gene families such as Zinc finger gene, WRKY, bZIP etc. has paved the path to study and understand the complexity of drought. Functional genomics in rice is thus an important area of research whereby the function of new genes involved in plant development and survival is defined. In this regard, it is relevant to describe the characteristics of a gene encoding a zinc-finger protein that is induced by several stresses. Over expression of the gene, *OSISAP1*(*O. sativa* subspecies *indica* stress-associated protein gene), in tobacco leads to stress tolerance as assessed at the seedling stage. Progress in the mass-scale profiling of the transcriptome, proteome and metabolome has allowed a more holistic approach in investigations of drought tolerance based on the measurement of the concerted expression of thousands of genes and their products.

Recently, a number of stress-inducible genes have been identified using microarray analysis in rice. Rice homologues of CBF/DREB1 and DREB2, 10 OsDREB1s and four OsDREB2s, respectively, have been identified based on rice genome sequence analyses. Similarly, the role of transcriptional factors activating various genes related to drought tolerance in rice is one of major approaches in understanding the molecular mechanism of drought tolerance in rice. TFs are proteins able to specifically bind with short DNA sequences located in the promoter of genes and to interact with the pre-initiation complex of transcription, conducting to activate or to inhibit the RNA polymerase II. Then TFs modulate the transcription rate of their target genes. One TF can modulate the transcription of several genes, including genes encoding

TF_S themselves, and reorients the cell and organism activity for the adaptation to a particular external condition. For this reason, TFs constitute key elements of the adaptation process of plants to their environment and are preferred targets for selection or engineering of complex agronomical traits of interest. Some classes of Zinc-finger motifs (e.g. TFIIIA- and GATA types) among C_2H_2, Gag knuckle, Treble clef, Zinc ribbon and Zn_2/Cys_6, 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.The products of stress-inducible genes function not only in stress tolerance but also in stress response. The transcriptome profiling experiments conducted on drought-stressed plants have highlighted the central role of transcription factors (TFs), and other drought related genes, while unveiling the complex hierarchy of the regulatory network that differentially modulates the expression of dehydration signature genes in a tissue-specific manner.

Recently, isolated three TFIIIA-type zinc finger protein genes *ZFP245* (Huang *et al.,* 2005), *ZFP182* and *ZFP252* (renamed from *RZF71*) from rice and showed the enhanced expression of these genes by various abiotic stresses such as cold, salt and drought (Guo *et al.,* 2007*).* Thus in the present investigation an attempt was made to study the expression of TFIIIA-type zinc finger proteins involved in responses to drought stresses using *in silico* structural, functional and wet lab analysis. The present study was conducted with the following main objectives:

1. *In silico* structural characterization of drought tolerance related zinc finger gene superfamily TFs for putative location, expression, and their role in drought tolerance.

2. Functional characterization and designing of primers for zinc finger gene superfamily TFs related to drought tolerance using genomic tools.

3. Expression analysis of selected drought tolerance related zinc finger gene superfamily TFs using RT-PCR.

CHAPTER II

REVIEW OF LITERATURE

2.1 Rice

"Rice is life"- This slogan of the international year of rice (2004) outlines the importance of rice. Rice, the world's most important cereal crop, is the primary source of food and calories for about half of mankind (Kush, 2005). In Asia, rice provides as much as 80 % of the dietary calories in countries such as Bangladesh and Indonesia and 65% of the Indian population. It is grown globally on 153 million hectares (m ha) and in a wide range of ecosystems under varying temperatures, altitudes and water regimes. About 45% of global rice area is under rainfed ecosystems. In India, of the 44 m ha of total rice area, 33% is in rainfed low lands and 15% in uplands. Of all the abiotic stresses that curtail crop productivity, drought is the most devastating one and the most recalcitrant to 'breeders' efforts. In the past, breeding efforts to improve drought tolerance have been hindered by its quantitative genetic basis and poor understanding of the physiological basis of yield in water-limited conditions (Tuberosa and Salvi, 2006). The major breeding objective in these ecosystems is to improve drought resistance in rice plants but, little progress has been achieved in improving yield under stress due to poor knowledge of the genetic control of drought resistance (Kanagaraj *et al*., 2010).

2.2 Rice and severity of drought

Rice *(Oryza sativa* L.) is the world's most important staple food crop and drought is a major problem for much of the production area (O'Toole and Chang, 1979; Chang *et al.,* 1982). One-half of the 148 million ha of rice planted annually is in a rainfed environment, but production in this ecosystem accounts for only one quarter of rice grain

production. Drought is one of the most common environmental stresses that affect growth and development of plants through alterations in metabolism and gene expression (Ceccarelli *et al*., 1996). However, little progress has been made in characterizing the genetic determinants of drought resistance, because it is a complex phenomenon and comprising of a number of physio-biochemical processes at both cellular and organismic levels at different stages of plant development (Tripathy *et al*., 2000). The country's rice production declined to 89.13 million tonnes in 2009-10 crop years (July-June) from record 99.18 million tonnes in the previous year due to severe drought that affected almost half of the country.

2.3 Drought tolerance mechanism in Rice

Drought is the primary abiotic stress causing not only differences between the mean yield and potential yield but also causing variation from year to year, resulting in yield instability. Phenotype is the result of genotype and environmental interaction. Therefore, assessment of desired genotypes is highly dependent on proper environmental conditions. Abiotic stresses (particularly drought, high temperature, salinity and others) generally reduce crop productivity. These stresses are location-specific, exhibiting variation in frequency, intensity and duration. Stresses can occur at any stage of plant growth and development, thus illustrating the dynamic nature of crop plants and their productivity. Although selection for genotypes with increased productivity in drought-prone environments has been an important aspect of many plant breeding programs, the biological basis for drought tolerance is still poorly understood. Also, drought stress is highly heterogeneous in time, space, degree of stress, growth stage and time of stress exposure (Gupta *et al.,* 1983) and is unpredictable. Due to their secondary mode of life,

plants resort to many adaptive strategies in response to different abiotic stresses such as high salt, dehydration, cold and heat, which ultimately affect the plant growth and productivity (Gill *et al.,* 2003). Against these stresses, plants adapt themselves by different mechanisms including change in morphological and developmental pattern as well as physiological and biochemical responses (Bohnert *et al.,* 1995). Drought combating mechanism comprises drought escape (the ability of a plant to escape periods of drought, especially during the most sensitive periods of its development), drought avoidance (the ability of a plant to withstand a dry period by maintaining a favorable internal water balance under drought) and drought tolerance mechanisms (the ability of a plant to recover from a dry period by producing new leaves from buds that were able to survive the dry spell) (Blum, 1988).

2.4 Physiology, cell biology and biochemistry of drought

Crop plants grown under drought conditions are exposed to a combination of stresses that are attributable to high temperatures, excessive irradiance, soil resistance to root penetration and low water potential. Loss of leaf water causes some passive loss of turgor in guard cells. Abscisic acid production is also induced and leads to a further loss of stomatal turgor. The resulting stomatal closure causes a concomitant decrease in CO_2 availability in the leaves, and hence it assimilates availability to the plant.

Although the photosynthetic machinery has a range of photo protective mechanisms to dissipate excess light energy, the continued exposure of leaves to excessive excitation energy can lead to photoreduction of oxygen and the generation of highly toxic reactive oxygen species (ROS) such as superoxides and peroxides (Niyogi, 1999). These dangerous compounds cause chemical damage to DNA and proteins, and

can therefore have serious or even lethal effects on cellular metabolism. Plants have evolved several strategies to deal with ROS, including the production of chemical antioxidants such as ascorbic acid, glutathione and tocopherol that directly remove potentially damaging electrons from the ROS, and enzymic systems such as peroxidases and superoxide dismutases that scavenge the electrons enzymically (Alscher *et al.*, 1997). The enzymes often use metals such as iron, zinc, copper or manganese as electron acceptors, so the metal ions must be available if enzymic detoxification of ROS is to proceed. In this way, oxidative stress may be linked to mineral deficiencies. In related responses, reactive aldehydes produced through perturbations in redox balances can be removed by the action of aldehyde dehydrogenases and aldose/aldehyde reductases (Kirch *et al.,* 2001*),* and superoxide production in mitochondria can be limited by the alternative oxidase (Purvis, 1997). Another adaptive mechanism for protection against drought is the maintenance of turgor during periods of drought by adjusting the osmotic pressure of cells. There are two main routes whereby this can be achieved. Firstly, the cell can sequester ions into cellular compartments. Secondly, specialized osmolytes such as proline, glycine betaine, mannitol, trehalose, ononitol and ectoine can be synthesized to readjust cellular osmotic potential. These osmolytes are also active in scavenging ROS, especially if they are targeted to the chloroplast (Shen *et al.,* 1997). Other specialized organic molecules can be used to protect cellular membranes against physical damage, and proteins against unfolding. Dehydration induces the partitioning of amphiphilic molecules such as glycosylated flavonols and hydroquinones into membranes; these compounds increase membrane fluidity and depress phase transition temperatures (Hoekstra *et al.,* 2001).

Proline and sugars can coat protein molecules, exclude solute from their surfaces and thereby reduce the rate of unfolding (Hoekstra *et al.,* 2001). During extreme desiccation, tolerant plants synthesize large amounts of nonreducing disaccharides, such as trehalose, which can substitute for water by satisfying hydrogen bonding requirements of polar amino acid residues at protein surfaces, and maintain the folded active states of the proteins. Some late embryogenesis abundant (LEA) proteins and dehydrins might act in a similar fashion (Dure *et al.,* 1989).

2.4.1 LEA proteins

LEA is a major group of proteins that typically accumulate during the late stages of embryogenesis or in response to dehydration, low temperature, salinity or exogenous ABA treatment – indicating their responsiveness to cellular dehydration. LEA proteins are widely distributed among monocot and dicot species, and many different forms have been isolated molecularly. LEA proteins are characterized by a biased amino acid composition, by their high hydrophilicity and solubility in water and often by their solubility after boiling. The homology among different LEA proteins, the presence of conserved protein domains, their ubiquity and the developmental specificity of their expression implies a fundamental role in desiccation tolerance. It has been proposed that these proteins may play a role in protecting cytoplasmic structures during dehydration (Ramanjulu *et al.,* 2002).

LEA proteins have been divided into groups based on predicted biochemical properties and motifs with sequence similarities (Dure *et al*., 1989). A Characteristic feature of group 1 LEA proteins is a 20-amino-acid motif. Dure *et al.,* (1989) predicted that the 11-amino-acid peptide forms an amphipathic α -helix with possibilities for intra-

and inter-molecular interactions. Group 4 LEA proteins are characterized by a conserved N-terminus, predicted to form A -helices and a diverse C-terminal part with a random coil structure. Group 5 LEA proteins contain more hydrophobic residues than the other groups; they are not soluble after boiling and are likely to adapt a globular structure (Cuming, 1999).

2.4.2 Aquaporins

Under non-stress conditions, plants keep their water balance by adjusting the water conductance of their tissues. Vascular tissues and guard cells play an important role in this process. A significant component in cellular water transport is aquaporins (Maurel and Chrispeels, 2001). Aquaporins are a complex family of channel proteins that facilitate the transport of water along transmembrane water potential gradients. Aquaporins can regulate the hydraulic conductivity of membranes and potentiate a 10–20-fold increase in water permeability (Maurel, 1997). Aquaporins have a potential role in plant water relations: the regulation of expression and activity are modulated by dehydration. Several genes that encode aquaporins are up-regulated by dehydration, for example *rd28* from *Arabidopsis* (Yamaguchi-Shinozaki *et al.,* 1992) or the tomato-ripening-associated membrane protein (TRAMP) (Fray *et al.,* 1994). In *C. plantagineum*, several aquaporins were up-regulated by dehydration; some are inducible both by dehydration and ABA, while others are inducible by drought alone, suggesting the involvement of ABA-dependent and -independent signalling pathways (Mariaux *et al.,* 1998).

A consequence of many environmental stresses including dehydration is oxidative stress, i.e. the accumulation of reactive oxygen species (ROS), which damage cellular

structures. Under optimal conditions, leaves are equipped with sufficient antioxidant enzymes and metabolites to cope with ROS. The accumulation of enzymes such as superoxide dismutases, ascorbate peroxidases, catalases, glutathione-S-transferases (GST) and glutathione peroxidases has been observed during stress conditions. The capacity of the antioxidative defence system determines the fate of the cell and whether the cell continues to function or suffers photo-oxidation (Foyer *et al.,* 1994).

2.4.3 Accumulation of osmolytes and soluble sugars

The accumulation of compatible solutes or osmolytes under osmotic stress is well known in many organisms. Osmolytes are synthesized in response to osmotic stress and do not interfere with normal cellular biochemical reactions. They help to maintain an osmotic balance under dehydration conditions. There are several examples of the accumulation of osmolytes contributing to the relatively high water content necessary for growth and cellular metabolism. Osmolytes include sugars, polyols, proline, quaternary ammonium compounds and tertiary sulfonium compounds. The increased synthesis of osmolytes is achieved by modulating genes encoding enzymes of the osmolyte biosynthetic pathway. For instance, simultaneous up-regulation of P-5-C synthase (*P-5-CS*) and down-regulation of the proline dehydrogenase (*ProDH*) gene leads to proline accumulation during water stress (Yoshiba *et al.*, 1997).

2.5 Signal perception of drought

Many of the extracellular signals are perceived by membrane-associated receptor kinases. Changes in protein phosphorylation were observed when plants were exposed to water deficit, suggesting reversible protein phosphorylation as a regulator (Conley *et al.*, 1997). These receptor kinases are composed of an extracellular ligand binding domain, a

transmembrane domain and a cytosolickinase domain. The receptor-like protein kinase gene, *RPK1*, from *Arabidopsis* is induced rapidly by dehydration, high salt and low temperature, suggesting that *RPK1* may function in the transmission of environmental stress signals (Hong *et al*., 1997). Several reports of numerous protein kinases with close sequence similarities to MAPKs and other kinases belonging to the MAPK cascade have been identified in plants in response to dehydration/ABA, suggesting that the MAPK cascade is involved in stress signalling in plants (Mizoguchi *et al*., 1996; Mikolajczyk *et al*., 2000).

A role for calcium signalling in plants during environmental stress a condition has been demonstrated unequivocally (Knight *et al.*, 1997). Isolation of drought and high-salt-induced expression of calcium dependent protein kinases (CDPKs) in plants provides indirect evidence for a role of calcium signalling (Urao *et al*., 1994). Using different Ca^{2+} channel blockers, Knight *et al*., (1997) have demonstrated that the expression of drought responsive genes was inhibited during mannitol treatment in *Arabidopsis*, implicating the importance of calcium signalling in gene expression. Furthermore, a gene (*AtCBL1*; *Arabidopsis thaliana* calcineurin B-like protein, a calcium binding protein) was induced in response to drought, wounding and cold stress (Kudla *et al*., 1999). Calcinuerin B-like proteins are implicated in a variety of signalling pathways in animals (Tong *et al*., 1995) and in adaptation to salt stress in yeast and plants (Nakamura *et al*., 1993; Pardo *et al*., 1998; Liu *et al.*, 1998).

2.6 Modern Approaches for Understanding Drought Tolerance

Drought remains as one of the major constraints limiting rice productivity. Conventional breeding approaches are slow in progress towards developing drought

tolerant rice varieties due to complex nature of drought tolerance mechanism (Ramya *et al*., 2010). Environmental stresses have a great impact on the yield of cereal crops. As detailed earlier, the effect of drought and/or heat stress on yield is highly complex and involves processes as diverse as stem reserve accumulation, gametogenesis, fertilization, embryogenesis, and endosperm and grain development. Our present knowledge on these processes and on their mutual interactions is still scant, especially if the potential impacts of environmental factors also have to be considered. The application of modern research tools to reveal the complex molecular networks behind the observed physiological and developmental responses in higher plants, including cereals, has only recently begun. The overall potential and drawback of modern genetic and genomic approaches in cereal improvement and in deciphering the regulatory mechanisms of abiotic stress tolerance in plants have recently been thoroughly reviewed.

2.6.1 Use of Molecular Markers to Decipher Drought Tolerant Traits

Good genetic maps based on molecular marker technologies are now available for major cereal species (Snape *et al.,* 2005 and Langridge *et al.,* 2006). Many of the traits determining abiotic stress tolerance and the quality and quantity of yield are controlled by a large number of genes, which have only minor individual effects but which act together (quantitative trait loci, QTL). In crop species with large, complex genomes, QTL analysis is an important tool in the identification of genetic markers to assist breeding efforts. This approach is complicated in wheat because of the polyploid nature of the genome and the low levels of polymorphism, but is straightforward in rice, maize and barley (Snape *et al.,* 2005). The strong synteny observed between the genetic maps of cereals, however, may help transfer the knowledge gained for rice or barley to wheat. Studies on the abiotic

stress tolerance of cereals include the extensive analysis of QTLs linked to the field evaluation of stress tolerance (Langridge *et al.,* 2006). Despite extensive efforts, it seems to be very difficult to identify QTLs linked to grain yield and yield components with a high consistency in diverse environments (Bruce *et al.*, 2002). However, Root-ABA1, a major QTL consistently affecting root architecture, leaf ABA concentration, grain yield, and other agronomic traits in maize under both well-watered and water-stressed conditions was recently identified, demonstrating the usefulness of this experimental approach (Giuliani *et al.*, 2005 and Landi *et al.,* 2007). Genomic regions associated with grain yield and its components under drought stress have been identified in rice (Lanceras *et al.,* 2004). In another study, the genetic bases of traits representing source, sink and transport tissues and their relationship to yield have been investigated by QTL analysis in rice (Cui *et al.,* 2003). Correlating genetic information with physiological and morphological traits related to high yield and/or drought tolerance will allow the development of new varieties with improved yield safety under water-limited conditions using molecular marker-assisted breeding. This technology may also be useful to survey drought and heat tolerance in various genotypes, including land races and wild relatives of cereals. In addition, the comparison of QTLs linked to stress tolerance in various cereals may help identify common loci or genes linked, for example, to drought tolerance (Langridge *et al.*, 2006).

2.6.2 Genetic Engineering to Improve Drought Tolerance

Apparently, plants employ multiple mechanisms to ensure dehydration tolerance. At present, our knowledge of the metabolic changes that contribute to dehydration tolerance is incomplete, but information about the biochemical processes contributing to

dehydration tolerance is essential for successful engineering of dehydration tolerance in crop plants. The genetic approaches (Tab. 2.1) offer new ways to evaluate the contribution of individual genes to dehydration tolerance. The past decade has seen the prospect of using genetically modified plants and testing their potential to modulate tolerance through the alteration both of osmolyte levels and enzymes that scavenge reactive oxygen species or transcription factors, and of components involved in signal transduction. Overproduction of proline in transgenic tobacco enhanced root biomass and flower development and helped the cells to maintain water potential, thus enhancing water tolerance (Kavi Kishor *et al*., 1995).

Table: 2.1 List of drought tolerance related candidate genes validated by transgenic approach.

S. No.	Species	Detail of gene	Traits	References
1	Groundnut	*Rd29A:AtDREB1A*	Transcription efficiency, oxidative stress.	Bhatnagar-Mathur, *et al.*, 2007
2	Rice	*CaMV35S:OsSNAC1*	Spikelet fertility, seed yield, stomatal closure, lower transpiration rate.	Hu, *et al.*, 2006
3	Rice	*CaMV35S:OsHARDY*	WUE, PSII rate, lower transpiration rate,*AP2* like transcription factors.	Hu, *et al.*, 2006 and Karaba, *et al.*, 2007
4	Rice	*CaMV35S:OsLEA3-1*	Grain yield and spikelet fertility.	Xiao, *et al.*, 2007
5	Rice	*CaMV35S:OsSKIPa*	Spikelet fertility, grain yield, oxidative stress tolerance.	Hou, *et al.*, 2009
6	Rice	*CaMV35S:OsAP37*	Total grain weight, grain filling rate, number of filled grains.	Oh, *et al.*, 2009
7	Rice	*ZFP252*	Drought and salt tolerance by increasing production of free proline.	Xu, *et al.*, 2008
8	Rice	*OsCIPK12*	Drought tolerance,differentially induced by drought.	Xiang, *et al.*, 2007
9	Maize	*OsRACT:ZmNF-YB2*	Delayed wilting and senescence, stomatal conductance, photosynthesis rate	Nelson, *et al.*, 2007
10	Petunia	*ZPT2-3*	Drought tolerant, regulatory mechanism of petal specific expression.	Bartels, *et al.*, 2005
11	*Arabidopsis*	*CAZFP1*	Drought tolerant, Chl. Content.	Kim, *et al.*, 2004
12	*Arabidopsis*	*STZ*	Drought tolerant, regulatory process in vegetative tissue.	Sakamoto, *et al.*, 2004

2.6.3 Functional genomics to elucidate drought tolerance mechanisms

Functional genomics technologies provide tools for the detection and definition of cellular networks through which stress perception, signal transduction and defensive responses are mediated. Expression profiling may help identify the key molecular events underlying stress tolerance and grain development, as well as their interactions. The number of cereal expressed sequence tag (EST) sequences available in public databases is continuously increasing. The cDNA libraries used to generate these ESTs represent various tissues and growth conditions, but yield- and stress-related libraries dominate. Recently, the digital expression analysis of EST sequences combined with gene annotation (annotation of 29556 different sequences) resulted in the identification of several pathways associated with abiotic stress resistance in wheat (Houde *et al.*, 2006). The number of macro- and micro-array platforms available for cereal research is also increasing. A DNA chip was hybridized containing around 21 000 genes, representing approximately half the rice genome, with cDNA probes characteristic of the successive stages of grain filling (Zhu *et al.*, 2003). They then examined the expression patterns of known genes that could potentially be involved in the process (e.g. genes linked to carbohydrate and fatty acid metabolisms). The expression of 98 of the 491 selected genes exhibited a correlation with grain filling. Based on the expression patterns of these genes, a further 171 genes were found with similar regulation, suggesting that they might also be involved in grain filling. Another methodological refinement led to the identification of a further 28 similar genes (Anderson *et al.*, 2003), which means that at least 297 genes in the rice genome are currently thought to be related to grain filling. The knowledge of the rice genome sequence has allowed the promoter regions that control these genes to be

identified and for common elements in these regions to be discovered. These in turn led to the discovery of nine transcription factors that regulate the expression of the genes (Zhu *et al.,* 2003).

Yamakawa *et al.*, (2007) elucidated the effect of high temperature on grain filling during the milky stage of rice. The number of macro- and micro-array platforms available for cereal research is also increasing. Zhu *et al.*, (2003) hybridized a DNA chip containing around 21 000 genes, representing approximately half the rice genome, with cDNA probes characteristic of the successive stages of grain filling. They then examined the expression patterns of known genes that could potentially be involved in the process (e.g. genes linked to carbohydrate and fatty acid metabolisms). Another methodological refinement led to the identification of a further 28 similar genes (Anderson *et al.* 2003), which means that at least 297 genes in the rice genome are currently thought to be related to grain filling.The knowledge of the rice genome sequence has allowed the promoter regions that control these genes to be identified and for common elements in these regions to be discovered. These in turn led to the discovery of nine transcription factors that regulate the expression of the genes (Zhu *et al.,* 2003).

Zinselmeier *et al.* (2002) analysed the expression of 1502 maize genes in response to moderate or severe water deficiency induced immediately prior to or 2–4 d after pollination, and changes were monitored in the gene expression patterns of the silk and the grain. Over all the treatments, changes were observed in the expression of 179 of the 1502 genes (Fig. 2.1). A large majority of these genes also showed changes in the leaves of water-deficient plants, indicating the general nature of the stress response.

Interestingly, drought caused no decline in starch synthesis genes, in contrast to the response to shading (Zinselmeier *et al*., 2002).

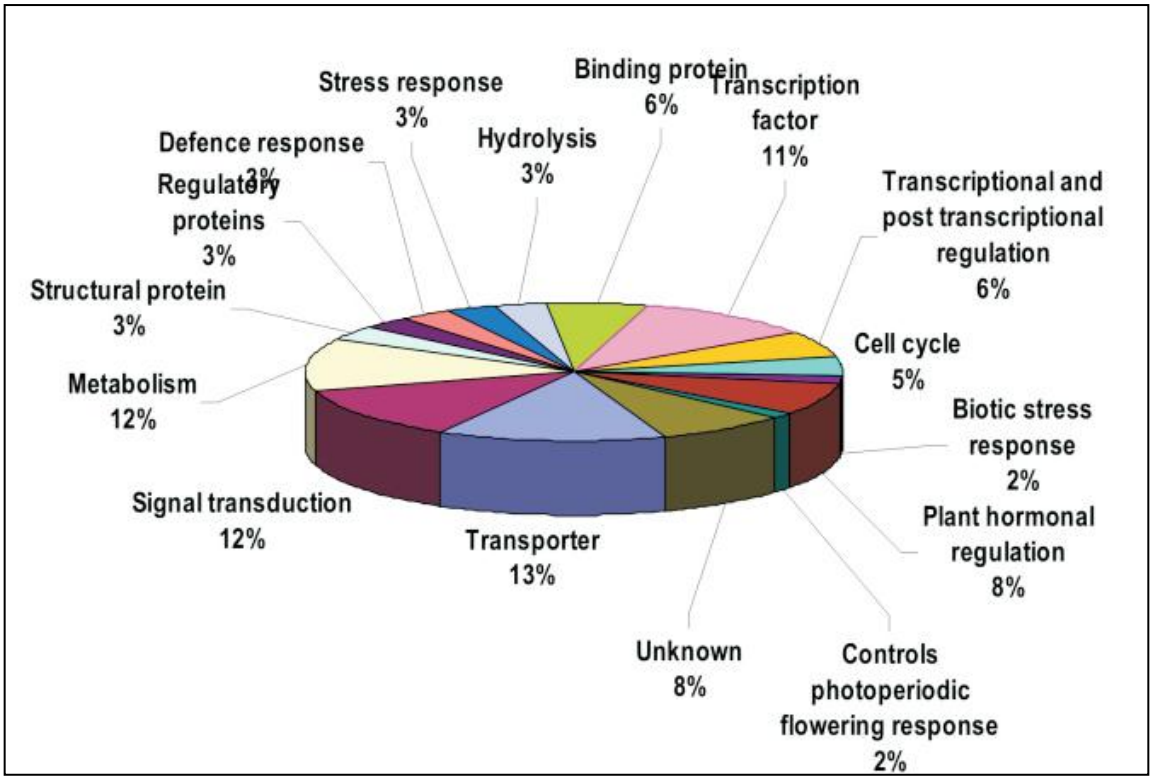

Figure 2.1 Functional classifications of selected drought tolerant genes

2.6.4 Proteomics

Investigating the effect of drought and/or heat stress on protein composition might also be an important step towards understanding the link between environmental factors and plant development.

Proteomic studies in cereals can be based on rice as a model species (Agrawal *et al.*, 2005 and Komatsu *et al*., 2006). A proteomic analysis of drought- and salt-stressed rice plants found that around 3000 proteins could be detected in a single gel and over 1000 could be quantified (Salekdeh *et al.,* 2002). Forty two proteins were found to

change their abundance or position in response to the treatments. The effect of salt stress on young rice panicles has been investigated by the same authors (Dooki *et al.,* 2006). The proteomic analysis of rice leaf sheaths during drought stress identified 10 up-regulated and two down-regulated proteins. One of the drought responsive proteins identified was an actin depolymerizing factor also present at high levels in the leaves of nonstressed drought-resistant cultivars (Ali *et al.,* 2006). Proteomic analysis has been started in all major cereal species in addition to rice. Proteomic reference maps have been compiled for maize (Mechin *et al.,* 2004) and wheat (Vensel *et al.,* 2005) endosperm and for barley grain (Finnie *et al.,* 2002) during the processes of grain filling and maturation. The effect of heat stress on the grain of hexaploid wheat has been thoroughly studied at the protein level (Majoul *et al.,* 2004). The down-regulation of several proteins involved in the starch metabolism and the induction of HSPs were reported (Majoul *et al.,* 2004). As regards the effect of drought on the wheat grain proteome, Hajheidari *et al.* (2007) reported the detection of 121 proteins exhibiting significant changes in response to the stress, of which 57 could be identified. Two-thirds of the identified proteins turned out to be thioredoxin targets, revealing the link between drought and oxidative stresses. In addition, the contrasting protein changes observed in susceptible and tolerant genotypes allowed the identification of potential markers or regulators of drought tolerance.

2.6.5 Metabolomics

The importance of metabolite changes during plant responses to abiotic stress suggests that detailed metabolite profiling may provide valuable insights into stress response mechanisms. A recent report on rice found that 88 main metabolites could be successfully quantified from the extract of rice leaves. The compounds identified covered

pathways of sugar and amino acid metabolism; hence these types of analyses should prove valuable for assessing stress responses. Metabolomic research on cereals has also recently begun (Sato *et al.,* 2004) and may, in the future, provide valuable information, for instance, on the sugar and amino acid metabolism in the vegetative and reproductive organs of cereals under various environmental conditions (Langridge *et al.*, 2006).

2.7 Regulation of drought tolerant genes

What distinguishes dehydration-tolerant plants from nontolerant plants at the molecular level? Characterization of numerous genes induced by dehydration emphasizes that the answer lies not within a single gene. Recent microarray analysis on *Arabidopsis* plants using 1300 cDNAs have revealed that approximately 44 genes were up-regulated in response to dehydration, of which 30 are reported to be novel (Seki *et al*., 2001).

2.7.1 Up-regulation of drought related genes

However, every up-regulated gene does not necessarily have a role in adaptation: some might be induced because of damage caused by stress (Zhu, 2000). Increasing evidence indicates that the genes responding to dehydration can be categorized into two classes, based on their response in terms of time-scale. Some respond immediately within seconds or minutes while others are responding later, in hours, days or even weeks. This allows us to speculate that the early responsive genes may provide initial protection and amplification of signals while the genes that are responding later may be involved in adaptation to stress conditions.

2.7.2 Down-regulation of drought tolerant genes

Although the primary interest is in the identification of the up-regulated genes during water stress, the down-regulation of gene expression also contributes to the

adaptation of plants to stress. For instance, *ProDH* gene expression is down-regulated during water stress, resulting in the accumulation of Proline (Yoshiba *et al*., 1997). In *C. plantagineum*, studies have revealed that transcripts encoding proteins relevant to photosynthesis are down-regulated and have been estimated to represent 36% of the total number of genes altered during the dehydration process (Bockel *et al*., 1998). The exact function of several down-regulated genes is largely unknown. The logical hypothesis is that some of the genes are down-regulated because of the fact that their product might not be suited to the new physiological condition caused by dehydration stress (Chandler and Robertson, 1994).

2.8 Transcription factors of drought tolerant genes

Transcription factors are important components of signal transduction networks conveying diverse signals to specific responses. Genetic modification of plant by altering the expression of individual genes will take long time to make a plant over-expressing a group of genes conferring drought tolerance. Alternatively activation of a set of genes by engineering for their transcriptional activation can confer much greater stress tolerance (Shinozaki *et al.,* 2003 and Yamaguchi-Shinozaki *et al.,* 2005).

2.8.1 Transciptome studies of drought responses

Differences in gene expression during abiotic stress responses such as drought, salinity, cold and high temperature varies to the type and extent of stress. In the model plant *Arabidopsis* deeper insights were gained into functional genomic aspects of multiple stress interactions. Using 1300 full-length clones (Seki *et al.,* 2001) and 7000 full-length clone inserts multi-stress interactions of abiotic stress treatments were studied to overlapping responses as well to identify genes of potential interest to salt, drought and

cold responses. By using 1300 full-length clones, Seki *et al.*, (2001) identified a set of only 44 and 19 genes, which were induced either by drought or cold stress response, respectively. By using 7000 full-length inserts, 299 drought-inducible genes, 213 high salinity-stress-inducible genes, 54 cold-inducible genes and 245 ABA-inducible genes were identified.

2.8.2 *In silico* transcript profiling of a drought-responsive gene

In contrast to digital *in silico* quantification of expression levels based on EST counts, approaches such as SAGE, MPSS, array-based transcript profiling technologies and quantitative real time PCR (qRT-PCR) allow us to perform an assessment of high-throughput expression of thousands of genes in control and stress-treated tissues at various developmental stages. Insights into gene expression patterns and functions coupled with stress tolerance can be explored by EST-based cDNA arrays. Gene expression profiling using cDNA macroarrays (Sreenivasulu *et al.,* 2006) or microarrays are novel approaches to identify higher number of transcripts and pathways related to stress tolerance mechanisms than before. There are several studies reported related to abiotic stress transcriptome profiling in model species such as *Arabidopsis* and rice that have revealed several new stress-related pathways in addition to the previously well described stress-related genes (Desikan *et al*., 2001; Kreps *et al*., 2002 and Oh *et al*., 2005).

2.8.3 Genome-wide transcriptome studies

An important aspect of the current research interest using functional genomic tools is the identification of key regulators based on gene expression patterns related to multi-stress interactions. Genome-wide transcriptome analysis has identified hundreds of

genes encoding transcription factors that are induced or repressed by many environmental stresses (Chen *et al*., 2004). The expression patterns of these transcription factors are highly complex and they suggest that stress tolerance and resistance are controlled at the transcriptional level by an extremely intricate gene regulatory network. Chen *et al*., (2004) identified groups of transcription factors regulated, (a) specifically by abiotic stress (class I) and (b) both by biotic and abiotic stresses (class II) in *Arabidopsis* (Fig. 2.2). Among the class I group, approximately 20 genes were preferentially induced by abiotic stresses such as salinity, osmotic, cold and jasmonic acid treatments.

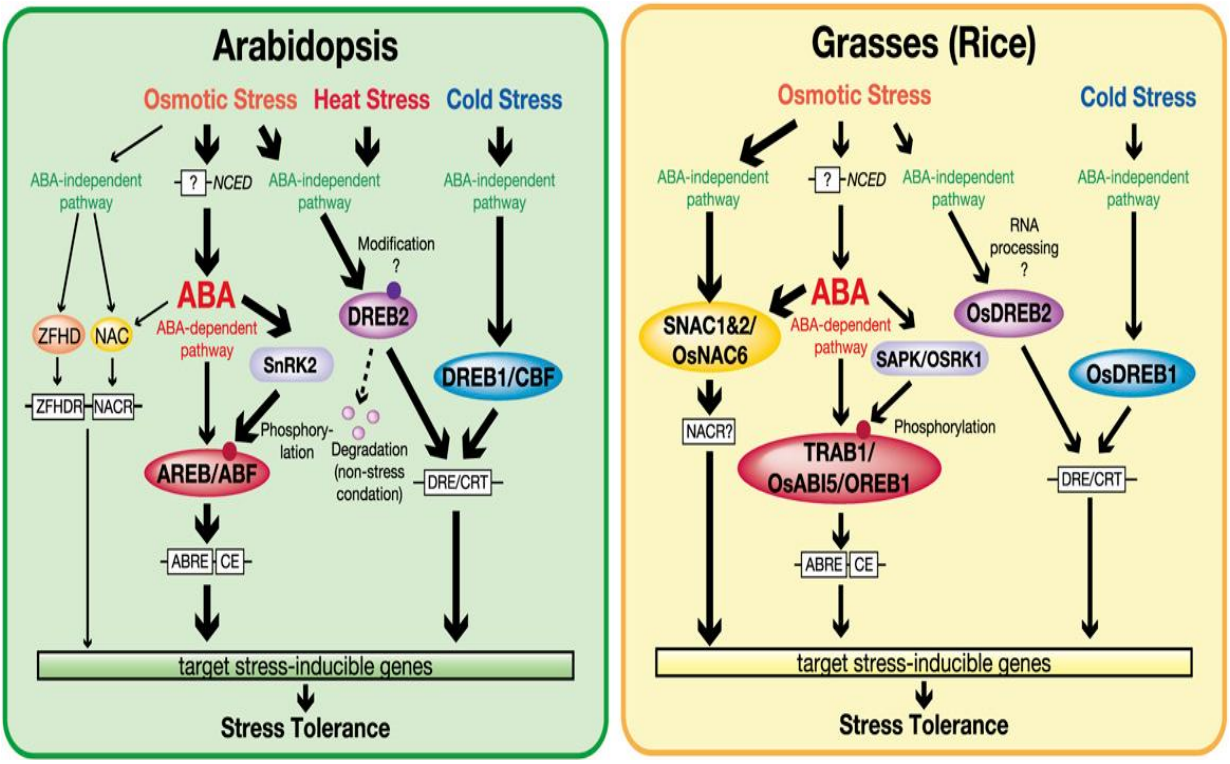

Figure 2.2 Major transcriptional regulatory networks of cis-acting elements and TFs involved in abiotic stress-responsive gene expression in *Arabidopsis* and grasses such as rice.

These transcription factors include DRE/CRT binding factors activated by cold stress, CCA1 and *Athb-8* regulated by hormones, (Baima, 2001), Myb proteins as well as

bZIP/HD-ZIPs and AP2/EREBP domain proteins (Kizis *et al.*, 2001). Further, Seki *et al*., (2002) employed a full-length cDNA microarray containing 7000 independent *Arabidopsis* cDNAs to identify cold, drought and salinity-induced target genes and stress-related transcription factor family members such as DREB, ERF, WRKY, MYB, bZIP, helix-loop-helix and NAC. These results indicate that there is a greater cross-talk between salt and drought stress signaling processes in comparison to salt and cold stresses. Fowler and Thomashow (2002) revealed rapid expression of CBF1 (DREB1b), CBF2 (DREB1c) and CBF3 transcripts (DREB1a) during short-term cold acclimation transcriptome studies of Arabidopsis. Six additional long-term up-regulated genes encode transcription factors: putative zinc finger protein (At4g38960), R2R3-Myb transcription factor AtMYB73, H-protein promoter binding factor 2a (AF079503), the HD-Zip protein AthB-12, and two AP2 domain proteins, RAP2.7 and RAP2.1. Similarly, transcriptome response to dehydration, salinity and ABA has been monitored in sorghum seedlings and identified approximately 22 transcription factors (Buchanan, 2005). These regulators include ABF from bZIP factors, DREB from AP2/EREBP family, HD-ZIP and MYB factors, which are also known to be stress-responsive in other model species such as *Arabidopsis* and rice. Also, there is a greater need to verify the roles that these transcription factors play in the networks for better designing plants that can tolerate a variety of environmental stresses.

2.8.4 Zinc-finger transcription factors unveiling the drought tolerance mechanism

In general, transcription factors have modular structures composed of a few functional domains for binding to target DNAs, for interaction with other proteins including other transcription factors and components of basic transcriptional machinery,

and for other functions. Transcription factors (TFs) regulate genome expression in response to environmental and physiological signals, and some of them switch on plant adaptive developmental and physiological pathways. One TF is encoded by a single gene but regulates the expression of several other genes leading to the activation of complex adaptive mechanisms and hence represents major molecular targets to genetically improve the tolerance of crop plants against different stresses. The term 'zinc finger' represents the sequence motifs in which cysteines and or histidines 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, are present in a number of transcription factors and 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.

Plant growth and crop productivity are largely affected by environmental stresses such as drought, salinity and low temperature. To date, many stress-related genes have been isolated and characterized from various plants. These genes encode products either directly protecting plant cells from abotic stresses or regulating expression of other genes to enhance plant tolerance to the stresses (Xiao. *et al.,* 2007 and Martınez-Atienza *et al.,* 2007). Under the stress conditions, the C-repeat binding factor/dehydration-responsive element binding factor (CBF/DREB) transcription factors induce the expression of downstream genes containing C-repeat/dehydration response elements (CRT/DRE) in their promoters to im- prove plant tolerance (Xiong *et al.,* 2002 and Nakashima *et al.,*

2006). Many other transcription factors such as NAC, MYB, bZIP and zinc finger proteins have been well characterized with their roles in the regulation of stress-responses (Agarwal *et al.,* 2006 and Chinnusamy *et al.,* 2006).

It is valuable to survey the TFIIIA-type zinc finger proteins involved in rice responses to abiotic stresses, not only for our understanding the molecular mechanisms of stress responses in monocots but also for improvement of plant tolerance to abiotic stresses by gene-transfer. Recently, isolated three TFIIIA-type zinc finger protein genes *ZFP245* (Huang *et al.,* 2005), *ZFP182* and *ZFP252* (renamed from *RZF71*) (Guo *et al.,* 2007*),* from rice and found that these genes were induced by various abiotic stresses. As the expression of *ZFP182* in transgenic tobacco or over expression in rice plants increased their tolerance to salt stress, ZFP182 might play a curial role in plant tolerance to salt along with the drought (Huang *et al*., 2007).

CHAPTER-III

MATERIALS AND METHODS

The present investigation entitled **"Characterization and validation of Zinc Finger gene superfamily transcription factors for drought tolerance in Rice (*Oryza sativa* L.) using *in silico* and functional genomics approaches"** was carried out at Department of Plant Molecular Biology and Biotechnology, Indira Gandhi Krishi Vishwavidalaya, Raipur, India. The details of the experiment are explained below.

3.1 Materials

Seven diverse rice genotypes selected based on the genetic structure and drought related phenological characters (Table 3.1) were used as materials for the present study. The plant materials were grown at green house condition (Fig. 3.1) in two treatments stress and control in pots containing equal proportion of sand, silt and clay.

Table 3.1 Rice genotypes with their pedigree and Characteristics

S.No.	Name	Pedigree/Cross combination	Characteristics
1	Indira barani dhan	Swarna X IR 42253	High yielding, deep rooted, drought tolerant.
2	Chapti Gurmatiya	Land race of CG Collection	Long Bold grains, drought tolerant, Tall.
3	Nagina 22	Land races	Up land variety, Known drought and heat tolerant.
4	Swarna	Vaisishtha X Mahsuri	Popular rice cultivar, low Fe and Zn, as local check.
5	Bhataphool	Land race of CG Collection	Long Bold grains, drought tolerant, Tall.
6	Nipponbare	Landrace	Temperate japonica Complete genome sequenced.
7	Wild Rice-6	Selected wild Rice	Small grains, grains shattering while maturity, poor milling quality.

3.2 Methods

3.2.1 Characterization of rice genotypes for drought related traits

Selected rice genotypes were observed for the relative water content and Proline content under controlled as well as stressed conditions. The soil moisture content of the earthen pots was also recorded at the time of the harvest of tissues for the wet lab analysis.

3.2.1.1 Soil moisture content

Soil moisture was measured by collecting soil samples at soil depth of 30 cm. The soil was collected using auger and immediately kept in pre weighed box. The moist soil samples were weighed immediately, thereafter the samples were oven dried at 80^{o}C up to constant weight (for 24 hour) and reweighed after drying to obtain dry weight of soil. The soil moisture content was calculated by using following formula:

$$\text{Soil moisture (\%)} = \frac{\text{Wt. of moist soil} - \text{Wt. of oven dried soil}}{\text{Wt. of oven dried soil}} \times 100$$

3.2.1.2 Relative water content of rice leaves

Relative water content is an appropriate measurement of plant status in terms of the physiological consequence of cellular water deficit. The leaf samples of stressed and unstressed plants were taken after imposing the water stress at 6th days to determine the RWC (%). Each sample was placed in a pre-weighed airtight vial. The samples were placed in a vial, with its basal part to the bottom. Vials were immediately placed in a cooler (around 10°C to 15°C). The samples were taken immediately to the lab as soon as possible. In the lab, vials were weighed to obtain leaf sample fresh weight (W), after

which the samples were hydrated to full turgidity for 4 hours under normal room temperature (Fig. 3.2). After 4 hours the samples were taken out of water and were well dried of any surface moisture quickly and lightly with filter paper and immediately weighed to obtain fully turgid weight (TW). Samples were then oven dried at 80°C for 24 hours and weighed to determine dry weight (DW).

Calculation:

$$\text{RWC}\ (\%) = \left[\frac{(\text{W}-\text{DW})}{(\text{TW}-\text{DW})}\right] \times 100$$

Where, W- sample fresh weight, TW- sample turgid weight and DW- sample dry weight.

3.2.1.3 Estimation of Free Proline in rice leaf tissues

Proline accumulation under various abiotic stresses (heat, cold, drought, moisture and salinity) in important factor for crop plants considered as a tolerance mechanism. It is suggested to act as an osmolyte/compatible as well as a source of nitrogen during recovery from stress.

Leaves were harvested at late vegetative stage and it was immediately transferred to lab for the estimation of proline using the following protocol given by Bates *et al*., 1973.

Procedure:

1. 0.5 g plant tissue was taken and homogenized in 5 ml of 3% Sulphosalycylic acid using pre washed mortar and pestle.
2. The homogenate was filtered through Whatman No. 1 filter paper and collected filtrate was used for the estimation of Proline content.

3. 2 ml of extract was taken in test tube and 2 ml of glacial acetic acid and 2 ml of ninhydrin reagent were added.
4. Reaction mixture was heated in a boiling water bath at 100^{o}C for 1hour. Brick red colour developed
5. After cooling the reaction mixtures, 4 ml of toluene was added and then transferred to a separating funnel
6. After thorough mixing, the chromospheres containing toluene was separated and its absorbance read at 520 nm in spectrophotometer against toluene blank
7. Standard curve of Proline was prepared by taking 5 to 100 µg ml^{-1} concentrations (Fig.: 3.3).
8. Free Proline content in sample was estimated by referring to a standard curve made from known concentrations of Proline by taking following formula:

$$\mu\text{moles per g tissue} = \frac{\mu\text{g proline/ mL x mL toluene}}{115.5} \times \frac{5}{\text{g sample}}$$

Where, 115.5 = Molecular weight of Proline

3.3 *In silico* structural and functional characterization and validation of Zinc Finger gene superfamily transcription factors

The nucleotide sequences of ten known drought tolerance related genes involved in enhancing drought tolerance were obtained from Rice Genome Annotation Project website (http://www.rice.plantbiology.msu.edu/). The corresponding nucleotide sequences were then analyzed for identification of candidate SNPs loci, repetitive sequences and characterization of putative expression pattern.

3.3.1 Structural characterization of Zinc Finger gene superfamily transcription factors for drought tolerance

3.3.1.1 Identification of candidate Single Nucleotide Polymorphism (SNPs)

A single-nucleotide polymorphism (SNP) is a DNA sequence variation occurring when a single nucleotide — A, T, C, or G — in the genome (or other shared sequence) differs between members of a biological species or paired chromosomes in an individual. Oryza SNP database freely available for public use at Oryza SNP consortium (http://www.oryzasnp.org/cgi-bin/gbrowse/osa_snp_tigr) contains Perlgen model based SNPs derived from sequence comparison 10 rice genome including Swarna, Nipponbare, Nagina22 with whole genome sequence of Nipponbare as reference (McNally *et al.*, 2006, Clark *et al.,* 2007). This database was used to identify candidate SNPs within drought tolerance related genes using “Oryza SNP search pages” tool relative to TIGR locus id and chromosome coordinate within one or more cultivars. The search generated cultivar specific SNP identifier reference along with cultivar specific SNP base calls. The SNP base calls were then used to derive obtain information about gene model based classification of each SNPs according to their position in the candidate gene sequences such as intronic, exonic, 5’UTR, 3’UTR etc. The candidate SNPs annotated in 3’ and 5’

untranslated region is particularly useful in developing gene specific markers applicable across Oryza sativa cultivars.

3.3.1.2 Identification of *cis*-acting elements applying PlantCARE for TFs of Zinc Finger genes of drought tolerance in rice

A *cis*-regulatory element is a region of DNA or RNA that regulates the expression of genes located on that same molecule of DNA (often a chromosome). Regulation, which is determined by chromatin structure, the binding of transcription factors and *cis* regulatory DNA sequences, can be inferred computationally by mining transcript profiles and the regional structure and distribution of short response elements in corresponding promoter regions. To analyze the *cis* regulatory elements in the promoter region of the drought responsive genes, the 1 kb upstream sequences of the 10 drought tolerance genes were downloaded using TIGR database. Analyzed for the presence of drought related DRE, ABRE, MYB and MYC conserved motif *cis* sequences, their exact position and their repeated number of occurrence of conserved *cis* elements were also identified using Plant care database (http: //bioinformatics .psb.ugent.be /webtools/ plantcare/ html) and literature search.

3.3.2 Functional characterization of Zinc Finger gene superfamily transcription factors

3.3.2.1 Identification of co-localized Expressed Sequence Tags (ESTs)

An expressed sequence tag or EST is a short sub-sequence of a cDNA sequence used to identify gene transcripts, and is instrumental in gene discovery and gene sequence determination. The locus ID of each Zinc Finger gene superfamily transcription factors for drought tolerance was used as query to search for the ESTs mapped over these

genes and further expression pattern was predicted on the basis of respective tissue expression library using Rice Gene Expression Anatomy Viewer and Digital Northern tools available at TIGR database *(*http://www.tigr.org/tdb/e2k/osa1/dnav/*)*. ESTs corresponding to a tissue library provided information about putative site of expression of the metal related genes in which it was identified.

3.3.2.2 Identification of Massive Parallel Signature Sequence (MPSS) tags

Massive parallel signature sequencing (MPSS) is a sequenced based approach that used to identify and quantify mRNA transcripts present in a sample. MPSS tags corresponding to Zinc Finger gene superfamily transcription factors for drought tolerance genes in rice were identified. The rice MPSS database includes comprehensive set of libraries which can be accessed at http://www.mpss.udel.edu/rice. More than 20 MPSS libraries derived from diverse tissues root, leaves, stem, panicle and germinating seeds abiotically stressed (cold, drought and salt) tissues have been generated for *japonica* cultivar Nipponbare grown under different conditions as light and dark, different developmental stages and several biological replicates. TIGR locus identifier for each gene was used as 'query' to obtain all annotated or non-annotated MPSS tags using 'Query by chromosome position tools' available at TIGR genome browser (http://www.tigr.org/tdb/e2k/osa1/dnav/). The search resulted in 17 and 20 nt long MPSS tags, tag sequence, chromosome coordinate position, tissue library information and transcript abundance values such as TPM (transcripts per million) value, Normalized abundance in different steps and 'p' value. The transcript number under "Norm Abund" category was considered mainly to draw a conclusion about abundance of transcript and henceforth level of expression of gene in the particular tissue type. Nakano *et al.*, (2006),

in a study of MPSS tag based characterization of expression pattern in *Arabidopsis* have reported that TPM<5 corresponds to normalized housekeeping genes and TPM<15 indicated very weak expression hence only those tissue were considered that showed TPM>15.

3.3.2.3 Digital microarray analysis

Digital microarray-based gene expression profiling was used to characterize genes whose expression is changed in response to drought, salinity etc. by comparing gene expression in affected to that in unaffected plants. DNA microarray technology has become the standard tool for the parallel quantification of large numbers of messenger RNA transcripts. The power of this approach has been demonstrated in dissecting plant physiology and development, and in unraveling the underlying cellular signaling pathways. Digital microarray analysis for TFs of Zinc Finger drought tolerance genes was carried out with the help of microarray tools publically available at http://www.ricearray.org/. At this analysis tool, the locus identifier of each gene was used as query and 'Affymetrix GeneChip experiment' platform was selected to download the expression data of all the genes for the reproductive development stage as described by Arora *et al*., (2007). The results were obtained as matching probes from the Affymetrix array. The data were in the form of Log2 transformed signal values generated from the average of three biological replicates. This data is further used to perform the heat map of normalized signal intensity values, for each gene which provides a quantitative measure of the transcript of a particular gene and hence its expression.

3.4 Expression analysis of Zinc Finger gene superfamily transcription factors for drought tolerance

The expression analysis of leaf tissues at late vegetative stage of 7 diverse rice genotypes sown in pot was done.

3.4.1 Collection of leaf tissue samples and isolation of total RNA

Leaf samples for RNA isolation of drought stressed and controlled rice varieties were collected at late vegetative stage. The leaves tissues collected at late vegetative stage were frozen in liquid nitrogen immediately to be used for gene expression studies. For gene expression studies extraction of RNA was done by **RNeasy® Plant Mini Kit.**

1. The harvested leaves were crushed in liquid nitrogen with the help of mortar and pestle.
2. The powder was immediately transferred to an autoclaved 2ml or 1.5 ml micro centrifuge tube.
3. 450 µl of buffer RLT/RLC provided with kit was immediately added, care was taken to avoid thawing of sample.
4. The sample was then vortexed vigorously and incubated at 56 ºC for 3 min.
5. After incubation the micro-centrifuge (mcf) tubes were centrifuged at 13000 rpm for 10 min.
6. The lysate was transferred to QIAshredder spin column (supplied with kit) using large orifice pipette followed by centrifuging at 13000 rpm for 8 min.
7. The supernatant was transferred to new 1.5 ml micro centrifuge tube

8. About 1/2 volume of absolute ethanol was added to the supernatant.

9. It was then mixed immediately by pipetting and whole volume was transferred to RNeasy mini spin column sitting in a 2 ml fresh collection tube.

10. The RNeasy column was centrifuged at 10,000 rpm for few sec.

11. The flow through supernatant was discarded and RNeasy mini spin column was reused by placing it in fresh 2ml collection tube.

12. The column was then washed with 700 µl RW1 buffer by centrifuging at 10000 rpm for 15 sec.

13. 500 µl of RPE buffer was added to RNasy mini spin column followed by centrifuging at 10000 for 5 sec.

14. The flow through was discarded and collection tube was replaced by fresh 2ml collection tube.

15. The step 16 and 17 were repeated twice.

16. The RNeasy spin column was then placed in a new 2ml collection tube.

17. About 30-50 µl (depending on expected yield) of RNase free water was added to the RNeasy spin column and centrifuged at 10000 for 1min.

18. The last step may be repeated with adding about 20 µl RNase free water when yield of RNA is more than 30 µg/ml.

19. The isolated RNA was stored immediately at -20 ºC.

3.4.2 Quantification and estimation of purity of RNA

The isolated RNA was quantified using Nanodrop spectrophotometer (ND1000). Two microliter of isolated RNA was placed over tip of Nanodrop to record absorbance at 230nm. The absorbance ratio (A_{230}/A_{260}) and (A_{230}/A_{280}) was recorded for each sample to estimate the purity of RNA. The acceptable absorbance ratio (A_{230}/A_{260}) for pure RNA was more than 1.8.

3.4.3 cDNA synthesis

RNA once isolated and quantified was used for cDNA synthesis using Thermo Scientific Verso^{TM} cDNA Synthesis Kit as per manufacturer's instructions. The kit consisted of following components and is suitable for 1pg to 1µg of RNA.

a. Verso Enzyme Mix

b. 5X cDNA Synthesis Buffer

c. Anchored Oligo dT (500 ng/µl)

d. Random Hexamer (400 ng/µl)

e. dNTP mix (5mM each)

f. RT Enhancer

The stepwise procedure as described in Thermo Scientific Verso^{TM} cDNA Synthesis Kit was followed. The reaction must be set in ice all the time. The Oligo dT primer of 12nt length and final concentration 1µM was used as primer for cDNA synthesis.

The stepwise protocol for cDNA synthesis was as follow:

1. Template RNA samples were thawed on ice, then required amount of template RNA was dispensed in each tube.

2. Each component as primer solutions, 5x cDNA synthesis buffer, dNTP mix and water (PCR grade) were ice thawed and vortexed briefly to settle the contents.

3. Master Mix (except Verso Enzyme Mix and template RNA) was prepared on ice (Table 3.2).

4. The master mix was mixed by vortexing gently for not more than 5 sec.

5. RNA dependent DNA polymerase (Verso Enzyme Mix) was added to mastermix and mixed by pipetting.

6. Master Mix was distributed in equal amount to each tube. (Master Mix = 20µl – amount of template RNA).

7. The 500µl PCR were then incubated at 42ºC for 30 min. Followed by 95 ºC for 2 min.

8. Stored at -20 ºC till further use.

Table: 3.2 cDNA synthesis (reverse-transcription) Reaction Components

Components	**Volume**	**Final Concentration**
Master mix 5x cDNA synthesis buffer dNTP Mix RNA primer RT Enhancer Verso Enzyme Mix Water (PCR grade)	 4 µl 2 µl 1 µl 1 µl 1 µl Variable	 1x 500 µM each
Template RNA	1-5 µl	1 ng
Total volume	**20 µl**	

3.4.4 Semi quantitative PCR

In semi-quantitative expression profiling cDNA synthesized in previous step were amplified with gene specific primers. The resultant PCR product was then resolved on 0.8% Agarose gel at 100V. The presence of amplicons and their respective intensity were recorded. The relative intensity of amplicons provided basis for quantification of level of expression of gene as high, moderate, low and negligible. The details of PCR components and thermal profile for amplification are given in table 3.5 and table 3.6 respectively.

Table: 3.3 PCR components their quantity used for semi-quantitative PCR.

Components	**Concentration**	**Quantity**
cDNA buffer dNTP mix Primer Forward Primer Reverse Taq polymerase Nanopure water	1,500 ng/µl (10X) (2mM) (25µM) (25µM) (5U/ µl) -	1.5 µl 1.0 µl 2.0 µl 0.5 µl 0.5 µl 0.2 µl 4.5 µl
Total		**10.0 µl**

Table: 3.4 Temperature profiles used for Semi quantitative PCR

Steps	Temperature (^{0}C)	Duration	Cycles	Activity
1	94	4 min	1	Initial Denaturation
2	94	30 sec	↑	Denaturation
3	variable(56-58ºC)	1 min	35	Annealing
4	72	1 min	↓	Extension
5	72	10min	1	Final extension
6	4	For ever	1	Store

3.4.5 Designing of primers for Zinc Finger gene superfamily transcription factors for drought tolerance

Gene specific primers were designed for 10 drought tolerant TF_S genes using batchprimer3 software (http://www.probes.usda.gov/batchprimer3/). Primer designing was done by putting the nucleotide sequences in FASTA format with desired specifications for GC content, annealing temperature, Tm values, primer length and length of amplified fragments with other parameters as default setting (Table 3.5). The details of primer sequences for all 10 genes are given in Table 3.6.

Table: 3.5 Specifications for gene specific primer designing

Criteria	Optimum	Range
Length of target sequence to be amplified	500-700bp	700-1000bp
Tm	60^0C	54-62^0C
GC content	55%	50-60%
Length of primer	20bp	18-20bp

Table: 3.6 Primer sequences of 10 Zinc Finger gene superfamily transcription factors for drought tolerance genes

S.N	Gene	Forward	Reverse
1	LOC_Os01 g19800	GTAGCAGAGGAGCAACCAAT	CAACCTGTGCATTCTAGCTG
2	LOC_Os 02 g 12790	TCACACACATGGCATCCT	GCAGCAGTAGGGGGTAATAA
3	LOC_Os 02 g 45780	GCTTTCCACGACTCTCTTTC	CGACTAGCTAGGAGCTTCTGT
4	LOC_Os 03 g 20870	CGCCCACAAGTTTAATGC	CACAGATGCTCACCTTAACC
5	LOC_Os Os 03 g 22680	CAAGCACTACAGGAGGAGGT	GATGTGACCCATGCAGTTC
6	LOC_Os 04g 49550	CACACTTGTCACCTTCTCTCC	CAAGACATTCGGATCCTCTC
7	LOC_Os02 g 53530	AGTCCCCCTCTGCTCTTACT	GTGGCAACACAAGCTAGGTA
8	LOC_Os 05 g 10670	GGGGCCTCTCTGGTTTAT	TAATATGTCGCCGGATCG
9	LOC_Os 08 g 06280	GATTTGACGAGCAAGGTCAG	CCCCAGACATACAGGATGTAG
10	LOC_Os 09 g 35670	AGGAGGTTTCCTCGTCGT	TGGATAGCTTTCGCTCTAGG

3.4.6 Reagents for Proline estimation

1. Glacial acetic acid (Analytical grade)
2. Sulphosalycylic acid (3%): Three gram of sulphosalycylic acid was dissolved in 100 ml of distilled water.
3. Orthophosphoric acid (6 N): Required volume of orthophosphoric acid (38.1 ml) was taken and volume was made to 100 ml, using distilled water to get 6 N orthophosphoric acid.
4. Acid ninhydrin: Ninhydrin (1.25 g) was dissolved in a blend of 30 ml of glacial acetic acid and 20 ml of 6 N orthophosphoric acids.

3.5 Statistical Analysis

3.5.1 Analysis of variance

The variance is a measure of the variability and is defined as average of square deviation from mean. The records from experiment was analyzed using CRD and as per the procedure given by Cocharan and Cox (1957). ANOVA was performed for the estimation of Proline.

3.5.2 Parameters of Variation

Mean: The mean was calculated by following formula:

$$X = \sum \frac{xi}{n}$$

Where,

$\sum xi$ = summation of all the observation

n = Total number of observation

x = Mean of the character

3.5.3 Critical Difference

Critical difference is the least significant difference, equal to or greater than which all the differences are significant.

Critical Difference (CD) = $\sqrt{2rVe}$ X t 5%

Where,

V_e – Error variance

r – Number of replication

CHAPTER IV

RESULTS AND DISCUSSION

The present study entitled **"Characterization and validation of Zinc Finger gene superfamily transcription factors for drought tolerance in Rice (*Oryza sativa* L.) using *in silico* and functional genomics approaches"** was done to understand and characterise transcription factors genes involved in drought tolerance in rice.

4.1 Quantification of drought stress using physiological and biochemical parameters

Seven different rice genotypes (Indira barani dhan, Chapti Gurmatiya, Nagina 22, Swarna, Bhataphool, Nipponbare and Wild Rice-6) were used in the present study and grown in two treatments i.e. stress and control. The diverse seven rice genotypes were germinated and grown in a mixture of sand and soil (2:1) in a green house at 25 ºC with a 16 h light/dark cycle. Plants were watered at regular interval, to ensure that soil remained moist. Watering was stopped from the 45 days after germination until the soil was dry and leaf rolling becomes visible which typically took 6 days. However, plants grown in control treatment were at 100 percent field capacity. After this dehydration treatment the aerial tissues (leaves) of the control and stressed plants were collected and divided for molecular (frozen in liquid nitrogen for RNA extraction), biochemical (estimation of proline content in leaves) and physiological (estimation of relative water content of leaf) analysis.

4.1.1. Relative water content (RWC) of leaf tissues

The result of relative water content (RWC) among seven known drought tolerant and susceptible rice genotypes at 6^{th} day of stress is shown in Fig 4.2. The RWC in leaves indicates their capability of maintaining turgor (Weatherly, 1950). In the present

investigation, the RWC decreased both in drought tolerant (Indira Barani Dhan, Nagina22, Bhataphool, Chapti Gurmutiya and Nipponbare) and drought susceptible genotypes (Swarna, *Oryza longistimnata*). The decrease was more in susceptible genotype than in the tolerant ones. The relative water content of leaf (RWC) among seven different rice genotypes ranged from 70.83 % to 74.28 % at late vegetative stage under 6th day of stress (50-55 % SMC), where as in control condition, it ranges from 91 to 95 percent (Table 4.1). Under 6th day of water stress, among tolerant genotypes, Indira Barani Dhan maintained maximum RWC (74.54 percent) followed by Nipponbare (74.28 percent) Bhataphool (73.80 percent), and Chapti Gurmatiya (71.79 percent), and of susceptible genotypes, Swarna (71.87 percent) maintained higher RWC than *Oryza longistimnata* (70.83 percent), which showed lowest RWC.

Decrease in RWC in leaves of rice genotypes Nagina22, CR 143-2-2 (drought tolerant), Panidhan, Pusa 169 (drought susceptible) in 45-day old seedlings subjected to water stress using PEG-6000 solution of -10 and -16 bar up to 5 hr was reported by Tyagi *et al.*, 2005. Relative water content is a parameter used to estimate the water potential under stress condition (Kumar *et al*., 2004). The ability of the rice genotypes to maintain high leaf water content under moisture stress conditions is considered as a possible drought resistance mechanisms in rice (Das *et al*. 2005). The presence of higher RWC in tolerant genotypes indicates their ability to retain water, possibly due to better osmo-regulation (Gambao *et al.*, 1991). Similar to our findings, relatively higher RWC have been reported in tolerant cultivars of wheat (Martin *et al*., 1997) and pearl millet (Goyal *et al*., 2001). Based on the results obtained in present investigation, Indira Barani

Dhan found to be more drought tolerant as compared to the other tolerant verities used in the study.

Table: 4.1: Relative water content of seven different rice genotypes

S. No.	Genotypes	Relative water content (%)		Decrease in RWC (%)
		Control	Stress	
1	Indira Barani Dhan	92.10 ± 1.39	74.54 ± 1.75	19.08
2	Chapti Gurmatiya	94.00 ± 0.50	71.79 ± 0.99	23.62
3	Nagina 22	91.66 ± 1.82	72.41 ± 0.38	21.00
4	Swarna	93.75 ± 0.25	71.87 ± 0.91	23.33
5	Bhataphool	95.38 ± 1.88	73.80 ± 1.10	22.62
6	Nipponbare	94.23 ± 0.73	74.28 ± 1.49	21.17
7	Wild Rice 6	93.33 ± 0.62	70.83 ± 1.96	24.10

4.1.2. Variation in Proline Content in tissues of stressed and control rice genotypes

Proline accumulation varies widely, among seven known tolerant and susceptible rice genotypes for drought tolerance used in present investigation. The free proline content in the leaf tissues of different rice varieties were estimated as per the method given by Bates *et al*., (1973). The proline content among seven known tolerant and susceptible rice genotypes at 6^{th} day of stress is shown in Figure 4.3**.** The amount of free proline ranged from 1.01-4.65 µ mole/g of leaf tissue among the tested rice genotypes (Table 4.2). Compared to control, proline content increased significantly in the leaf tissues of all genotypes at 6^{th} day of stress. The increase was higher in Indira Barani dhan (a drought tolerant variety released by IGKV Raipur (4.65 µ mole/g), compared to other

drought tolerant verities viz; Chapti Gurmatiya, Nagina 22 and Bhataphool and among susceptible ones, the proline content of Swarna (1.38 µ mole/g) and wild rice-6 (*Oryza longistiminata*) (1.36 µ mole/g) were more or less same.

Compare to stress vs control tissues of all rice genotypes the Nagina 22 was found with 48.40 fold increase in proline accumulation, followed by Bhataphool (16.75 fold), Nipponbare (14.42 fold), Indira Barani Dhan (7.75 fold) and Chapti Gurmutiya (5.61 fold) at 6^{th} day of stress. Similarly, among susceptible once the fold increase in proline content was higher in Swarna (15.33 fold) as compared to Wild rice 6 (*Oryza longistiminata*) (9.06 fold). Although the fold increase was found to be higher in Nagina 22 (48.40 fold) as compared to Indira Barani dhan (7.75 fold), but the accumulation of proline was higher in Indira Barani Dhan (4.65 µ mole/g) compared to Nagina22 (2.42 µ mole/g) under stress. The results of the present study are consistent with the hypothesis that the synthesis not its level *per se* may be of primary importance in adaptation in stress (Hare and Crees, 1997, 1998). Similarly, Tyagi *et al.*, 2005 have also worked out the variation in free proline accumulation in rice. They reported increase in proline accumulation in the 45 days old rice seedling subjected to water stress using PEG-6000 solution of -10 and -16bar up to 5 hr. Based on the results obtained in present investigation, Indira Barani Dhan found to be more droughts tolerant as compared to the other tolerant verities used in the study.

A significant increase in proline has been observed in response to water stress; favoring osmotic adjustment in rice (Lin *et al.,* 2002; Bohnert and Jensen 1996) suggested that proline accumulation may serve as a means of osmotic adjustment and storing carbon and nitrogen when stress leads to slower growth.

A positive and strong correlation (0.70) was found between RWC and Proline content (Tab. 4.2) and it reveals that with the increase of intensity of drought the Proline content is enhanced (Fig.4.4). Earlier (Mattioni *et al*., 1997), it is reported that the proline accumulation may help to maintain the high RWC for growth and cellular unction.

Table: 4.2 Correlation of drought tolerance indices in two treatments

Variables	Proline (s)	RWC (s)
Proline (s)	**1**	
RWC (s)	0.70	**1**

Values in bold are different from 0 with a significance level alpha =0.05

Table: 4.3 Proline content of seven different rice genotypes

S. No.	**Genotypes**	**Proline (µ mole/g)**		**Increase in proline content (X times)**
		Control	Stress	
1	Indira Barani Dhan	0.60 ± 0.40	4.65 ± 2.52	07.75
2	Chapti Gurmatiya	0.18 ± 0.01	1.01 ± 0.10	05.61
3	Nagina 22	0.05 ± 0.14	2.42 ± 0.29	48.40
4	Swarna	0.09 ± 0.09	1.38 ± 0.74	15.33
5	Bhataphool	0.12 ± 0.07	2.01 ± 0.11	16.75
6	Nipponbare	0.14 ± 0.04	2.02 ± 0.10	14.42
7	Wild Rice 6	0.15 ± 0.03	1.36 ± 0.76	09.06

Table: 4.4 Completely randomize design ANOVA for estimation of proline

Source of variation	Degree of Freedom	Sum of Square	Mean sum of Square	F-calculated	F-Table
Treatment	13	44.21768	3.40136	462.4788*	2.507263
Error	14	0.102965	0.007355		
Total	27				

*significant at 5%

The proline content in seven diverse rice genotypes were analyzed by completely randomize block design. The analysis of variance indicated significant effect between the genotypes at five percent level of significance (Table 4.3).

4.2 *In silico* structural and functional characterization of Zinc Finger gene superfamily transcription factors

The nucleotide sequences of ten known transcription factors (TFs) of Zinc Finger genes involved in enhancing drought tolerance were obtained from Rice Genome Annotation Project website (http://www.rice.plantbiology.msu.edu/). The corresponding nucleotide sequences were then characterized structurally and functionally.

4.2.1 Structural characterization of Zinc Finger gene superfamily transcription factors for drought tolerance

4.2.1.1 Identification of candidate Single Nucleotide Polymorphism (SNPs)

Single Nucleotide Polymorphism (SNPs) is considered as highly polymorphic reproducible DNA markers. SNPs can be identified *in silico* by analysis of sequence under question with a reference sequence. A total of 39 candidate SNPs (Fig. 4.5) were searched in 10 genes of Zinc Finger family out of which it was identified in 9 genes. The number of SNPs per gene varied from 1 in LOC_Os02g45780 to10 in LOC_ Os0510670.

Higher the SNPs greater the polymorphic reproducibility, depicticting the potentiality of genes and it can be applied for studying the traits responsible for drought tolerance.

Feltus *et al.,* (2004) identified SNPs in rice by comparison of *japonica* cultivar Nipponbare and *indica* cultivar 93-11 genome sequences. Putative SNPs in rice have also been identified by sequence alignment of reference sequence (Nipponbare) with partial sequence available for multiple rice genomes (Swarna, Moroberekan and N22) by McNally *et al.,* (2006).

4.2.1.2 Identification of *cis*-acting elements applying PlantCARE for transcription factors (TFs) of Zinc Finger genes of drought tolerance in rice

Transcription factors (TFs) are important components of signal transduction networks conveying diverse signals to specific responses. Several databases for the analysis of plant promoters exist (e.g. http://www.dna.affrc.go.jp/PLACE and http://intra.psb.ugent.be:8080/PlantCARE) and for identification of *cis*-acting elements for TFs of Zinc Finger genes of drought tolerance in rice the used database was http://intra.psb.ugent.be:8080/PlantCARE. The total number of motifs found were 233 among them commonly found motifs were ABRE (cis-acting element involved in the abscisic acid responsiveness), HSE (cis-acting element involved in heat stress responsiveness) and MBS (MYB binding site) involved in drought-inducibility. Motif IIb having sequence CCGCCGCGCT corresponding to LOC_Os02g12790 functioning as abscisic acid responsive element in rice (Tab. 4.5). LOC_Os02g53530 and LOC_Os05g10670 were found with common motif HSE; sequence AAAAAATTTC functioning as cis-acting element involved in heat stress responsiveness. Motif MBS was

found in all the locus id except LOC_Os02g12790, LOC_Os05g10670, and LOC_Os09g35670.

ABA plays crucial role in various signalling processes, it is logical to expect that other stress responsive integration points within ABA responsive promoters also govern gene regulation. In case of rice, genome-wide binding experiments like chromatin-immuno precipitation coupled with microarray (Chip–chip) are lacking. Several databases like PLACE and Plant-CARE among others provide experimental evidences regarding cis-elements and transcription factors in plants (Higo *et al*., 1999 and Lescot *et al*., 2002). Presence of abiotic stress related cis elements in the promoter regions of the genes harbored in the selected QTL region may be responsible for activation of more number of genes in this region during drought (Ramya *et al*., 2010).

Table: 4.5 *Cis*-Acting elements within transcription factors (TFs) of Zinc Finger gene for drought tolerance

LOCUS ID	Motifs Found	Organism	Sequence	Function
LOC_Os 01g19800	ERE	*Dianthus caryophyllus*	ATTTCAAA	ethylene-responsive element
	MBS	*Arabidopsis thaliana*	TAACTG	MYB binding site involved in drought-inducibility
LOC_Os02g 12790	ABRE	*Arabidopsis thaliana*	TACGTG	cis-acting element involved in the abscisic acid responsiveness
	MBS	*Arabidopsis thaliana*	CAACTG	MYB binding site involved in drought-inducibility
	TC-rich repeats	*Nicotiana tabacum*	ATTTTCTCCA	cis-acting element involved in defense and stress responsiveness
	motif IIb	*Oryza sativa*	CCGCCGCGCT	abscisic acid responsive element
LOC_Os 02g45780	MBS	*Arabidopsis thaliana*	CAACTG	MYB binding site involved in drought-inducibility
LOC_Os 02g53530	ABRE	*Arabidopsis thaliana*	CACGTG	cis-acting element involved in the abscisic acid responsiveness
	HSE	*Brassica oleracea*	AAAAAATTTC	cis-acting element involved in heat stress responsiveness
	MBS	*Arabidopsis thaliana*	TAACTG	MYB binding site involved in drought-inducibility
	MBS	*Arabidopsis thaliana*	CAACTG	MYB binding site involved in drought-inducibility

	MBS	*Zea mays*	CGGTCA	MYB binding site involved in drought-inducibility
LOC_Os 03g22680	ABRE	*Triticum aestivum*	GGACACGTGGC	cis-acting element involved in the abscisic acid responsiveness
	MBS	*Arabidopsis thaliana*	TAACTG	MYB binding site involved in drought-inducibility
	TC-rich repeats	*Nicotiana tabacum*	ATTTTCTTCA	cis-acting element involved in defense and stress responsiveness
LOC_Os 04g49550	ABRE	*Arabidopsis thaliana*	CACGTG	cis-acting element involved in the abscisic acid responsiveness
	ABRE	*Hordeum vulgare*	CCTACGTGGC	cis-acting element involved in the abscisic acid responsiveness
	MBS	*Arabidopsis thaliana*	TAACTG	MYB binding site involved in drought-inducibility
LOC_Os 05g10670	HSE	*Brassica oleracea*	AAAAAATTTC	cis-acting element involved in heat stress responsiveness
LOC_Os 08g06280	MBS	*Zea mays*	CGGTCA	MYB binding site involved in drought-inducibility
	TC-rich repeats	*Nicotiana tabacum*	ATTTTCTTCA	cis-acting element involved in defense and stress responsiveness
LOC_Os 09g35670	ERE	*Dianthus caryophyllus*	ATTTCAAA	ethylene-responsive element
	TC-rich repeats	*Nicotiana tabacum*	ATTTTCTCCA	cis-acting element involved in defense and stress responsiveness

4.2.2 Functional characterization of transcription factors (TFs) Zinc Finger gene superfamily transcription factors

4.2.2.1 Identification of co-localized Expressed Sequence Tags (ESTs)

The locus ID of each Zinc Finger gene superfamily transcription factors drought tolerance gene was used as query to search for the ESTs mapped over these genes and further expression pattern was predicted on the basis of respective tissue expression library using Rice Gene Expression Anatomy Viewer and Digital Northern tools available at TIGR database *(http://www.tigr.org/tdb/e2k/osa1/dnav/).* ESTs corresponding to a tissue library provided information about putative site of expression of the drought related genes in which it was identified.

ESTs were identified in all ten drought tolerant genes of Zinc Finger superfamily. A total of 199 ESTs were identified in 10 genes with minimum 6 ESTs in LOC_Os03g22680 and maximum 47 ESTs in LOC_Os05g10670. The ESTs identified in

each gene were then categorised according to their corresponding expression tissue library such as flower, panicle, seed, leaves, roots, stem to understand putative site of expression (Fig.4.6). The ESTs corresponding to LOC_Os09g35670 were found in flower, root and leaf tissue library suggesting expression of the gene in these tissues. The ESTs identified in LOC_Os02g53530 and LOC_Os02g45780 corresponded mostly to whole plant, panicle and leaf tissues which suggested respective phase specific expression of these genes in rice (Tab. 4.6).

ESTs have been most widely used tag based method for transcriptional profiling in plants. ESTs are though potential source for tag based expression profiling but public ESTs databases lack sufficient information about quantitative expression estimation and are less informative about levels of gene expression than recent tag based methods such as Serial Analysis of Gene Expression (SAGE) and Massive Parallel Signature Sequence (MPSS) (Meyer *et al.*, 2004).

Table 4.6 Tissue expression libraries viz. distribution of identified ESTs in Zinc Finger genes

Gene / Library	LOC_Os01g19800	LOC_Os02g53530	LOC_Os02g45780	LOC_Os03g22680	LOC_Os04g49550	LOC_Os05g10670	LOC_Os08g06280	LOC_Os09g35670
Mixed	-	9	6	-	22	8	1	4
Callus	-	-	8	3	-	19	2	5
Whole plant	1	2	3	-	1	2	1	1
Flower	-	-	2		-	1	2	1
Sheath	-	-	-	-	-	-	-	-
Root	4	-	1	-	5	8	1	1
Stem	-	-	1	-	-	-		-
Pistil	-	-	-	-	-	-	2	1
Panicle	-	2	2	1	6	5	6	-
Shoot	2	9	2	2	-	4	9	-
Seed	-	-	6	-	-	-	2	-
Leaf	-	3	-	-	-	-	-	6
Unknown	-	1	-	-	1	-	-	1
Phloem	-	-	1	-	-	-	-	-
Total	7	26	32	6	35	47	26	20

4.2.2.2 Identification of Massive Parallel Signature Sequence (MPSS) tags

Each signature sequence (MPSS tag) in a MPSS dataset was analyzed and compared with all other signatures and all identical signatures were counted. The abundance/ frequency of each tag is expressed in TPM (transcript per million) which is considered as the measure of expression in a corresponding tissue library. Most of the genes were moderately expressed in different tissues while some showed tissue specific expression. The highest number of MPSS tags was found in LOC_Os02g45780 (4011) (Fig. 4.7) where as lowest was obtained in LOC_Os02g12790 (126). Maximum number of mature leaves tissue library was found in LOC_Os09g35670. LOC_Os09g35670 showed highest level of expression in meristematic tissue library (299) while LOC_Os02g53530 showed highest level of expression in leaves (35). *In silico* MPSS analysis revealed that all the ten putative candidate genes showed the presence of corresponding 17 base signature tags. This finding suggests that most of the candidates are expressed genes (Tab. 4.7).

MPSS tag based profiling offers great opportunities for *in silico* applications in functional characterization of genes using web based tools (Dubey and Chandel, 2010). Identifying MPSS signature sequences co-localizing with a gene can yield valuable information about putative spatial or temporal expression of that gene (Banerjee *et al*., 2010). Meyers *et al.,* (2004) and Mikkilineni *et al.,* (2004) also reported use of MPSS tags for quantitative transcriptome profiling of novel and known genes in *Arabidopsis*.

Table 4.7: Tissue expression library viz. distribution of MPSS tags identified within drought tolerance related transcription factors Zinc finger genes.

Locus id / Library	LOC_Os01g19800	LOC_Os02g12790	LOC_Os02g53530	LOC_Os02g45780	LOC_Os03g20870	LOC_Os03g22680	LOC_Os04g49550	LOC_Os05g10670	LOC_Os08g06280	LOC_Os09g35670
Young roots	-	2	-	227	23	18	16	15	24	35
Mature roots	-	4	-	261	56	88	136	13	9	15
Germinating seedlings	8	1	-	-	71	94	-	39	3	122
Stem	1	-	-	219	-	-	34	-	76	7
Young leaves	-	-	11	59	68	-	73	13	-	17
Mature leaves	-	1	21	54	22	-	30	15	65	80
Meristematic tissue	16	3	-	299	2	20	22	46	158	135
Mature pollen	-	2	-	95	-	-	43	1	47	68
Ovary and Mature stigma	-	3	-	116	-	-	182	25	138	51
Immature panical	-	-	-	28	-	71	3	58	82	8
Germinating seed	-	14	5	36	1	35	0	8	47	69
Callus	-	-	-	3	14	48	4	145	18	97
Salt strsd young roots	-	1	-	345	99	19	6	3	58	65
Salt strsd young leaves	-	24	-	63	59	5	16	7	8	62
Water strsd young roots	-	-	4	310	34	41	-	17	6	126
Water strsd young leaves	4	10	-	-	8	2	-	16	27	120
Cold strsd young roots	-	-	-	281	45	59	-	-	13	-
Cold strsd young leaves	3	6	-	306	26	-	90	-	-	21
Unwounded plant	-	11	-	25	0	-	28	-	39	138
Mock treated plant	-	-	-	87	30	8	1	-	62	167
X.oryzae	-	15	12	25	22	2	22	-	30	81
M. grisea	-	12	18	36	69	61	10	-	22	64
Mock treatment	-	1	21	-	76	23	1	-	66	44
Roots	-	-	0	336	2	56	18	29	5	52
Leaves	-	-	35	12	17	4	34	6	1	30
Meristematic tissue	3	-	-	210	2	98	41	-	9	64
F1 Hybrid Mat Root	93	-	-	299	27	152	4	-	1	285
F1 Hyb Mature leaves	-	-	16	61	38	1	25	36	6	94
F1 Hyb Meristematic tissue	104	-	-	210	-	10	35	28	14	140
Developing seeds	-	14	-	-	-	-	26	1	7	150
Beet armyworm dam. leaves	-	-	9	-	-	-	-	-	-	41
Mechanical dam. leaves	-	-	9	6	2	-	-	2	-	83
Total	232	126	162	4011	842	918	914	581	987	2648

TPM: Transcript per million, TPM ≤ 4low expression, TPM≤ 4-5: weakly expressed, TPM ≤ 15-400 moderately expressed, TPM≤500-1000: high level of expression, TPM > 1000: strongly expressed (http://mpss.udel.edu/at/query/php).

4.2.2.3 Digital microarray analysis

Digital microarray expression profiling of transcription factors (TFs) of Zinc Finger drought tolerant genes were carried out by using microarray data from Rice Array database analysis tool site (www.ricearray.org). The results were obtained as matching probes from the Affymetrix array. The data were in the form of Log2 transformed signal values generated from the average of three biological replicates. The analysis revealed that all the genes except LOC_Os02g12790 and LOC_Os08g02680, showed moderate to higher levels of expression based on their signal intensity values. Among the different plant organs, it was observed that majority of the genes expressed more in various temporal stages of development, young and mature leaves. LOC_Os05g10670 showed high level of expression in Nagina-22 and IR64 under both experiments viz normal and drought conditions whereas LOC_Os05g10670 and LOC_Os09g35670 showed higher expression than LOC_Os02g12790 and LOC_Os04g49550 in drought experiment (Fig. 4.8) for transcription factors of Zinc Finger genes. Microarray represents a high throughput means to analyze the expression of a gene and to identify genes involved in a particular biological process.

Based on the spatio- temporal expression data generated from the MPSS analysis, the microarray analysis was carried out particularly under the series accession number GSE6893 comprising expression data for reproductive development in rice. At this analysis tool, the locus identifier of each gene was used as query and 'Affymetrix GeneChip experiment' platform was selected to download the expression data of all the genes for the reproductive development stage as described by Arora *et al*., (2007).

4.3 Expression analysis of drought responsive Zinc Finger gene superfamily transcription factors gene using semi-quantitative RT-PCR

A set of seven diverse rice genotypes including known resistance and susceptible varieties were used for expression analysis using RT-PCR. Based on *in-silico* structural and functional characterization and results obtained from digital microarray expression profiling of TFs genes, one out of ten genes was used for semi-quantitative RT-PCR based expression profiling.

4.3.1. Standardization of RT-PCR parameters for expression profiling of TFs gene related to drought responsiveness

The sequences corresponding to the genes were obtained from the rice genome sequences database (TIGR). The sequences of cDNA from genes were used to design the RT-PCR primers using the BatchPrimer3 software (http://www.probes.usda.gov/batchprimer3/). An Alpha tubulin gene was used as internal control. The melting temperature, template DNA and other parameters related to RT-PCR, of selected genes were standardized for expression analysis. The first-strand cDNA was obtained from 1 µg of total RNA in a 20 µl reaction mixture, and 20 ng/µl of synthesized cDNA was used as template for PCR reaction (94°C for 4 min and then 35 cycles of 30 s at 94°C, 30 s at 55-60°C, and 30 s at 72°C, followed by 72°C for 5 min). After gel electrophoresis, the intensity of each band was measured, and normalized the data using the Alpha tubulin bands of each well, respectively (Fig. 4.9).

4.3.2. Semi-quantitative expression profiling of TFs gene

The semi-quantitative RT-PCR analysis of one drought responsive genes belonging to zinc finger gene superfamily was performed in leaf trascriptome at three time points (6^{th} day, 12^{th} day of stress and 48 hours after re-watering) in vegetative stage. A comparisons in expression level of a gene, belonging to zinc finger gene superfamily namely Os05g10670 were made among seven tested rice genotypes for drought tolerance. Fig 4.10 indicates differential expression of Os05g10670 among seven tested rice genotypes. As shown in Table 4.8**,** the expression of Os05g10670 gene get initiated at 6^{th} day of stress in Nagina22, Nipponbare, Swrana, Wild Rice-6 (*Oryza longistiminata*) and Bhataphool compare to that of control. Based on the expression analysis of Os05g10670 gene, the seven rice genotypes were categorized into three groups' viz. early responsive genotypes with high expression level, early responsive genotypes with low expression level and late responsive genotypes.

4.3.2.1. Early responsive genotypes with high expression level

The expression of Os05g10670 gene slightly higher in the stress samples as compared to the corresponding control for the genotype Nagina22 at 6^{th} and 12^{th} day of drought stress. Similar pattern of expression was exhibited by Swarna at 6^{th} day of stress. In contrast to this, comparatively higher level of expression was observed in the control samples for the four genotypes Bhataphool, Nipponbare and Wildrice-6 at 6^{th} day stress. However, low level expression was observed in Indira Brani Dhan and Chapti gurmutiya at 6^{th} day of stress and got lower expression on stressed samples compare to that of control ones at 12^{th} day treatment. The results obtained through the expression analysis of Os05g10670 gene, revealed that Nagina22 seems to be an early responsive variety towards drought stress as compared to other seven known susceptible and tolerant rice genotypes. The results of semi-quntitative expression

analysis of Os05g10670 gene were found to be in accordance with digital microarray analysis performed in present investigation. In both studies the expression of Os05g10670 gene was higher in Nagina 22 and Swarna. The findings of the in-silico characterization have been validated using RT-PCR.

4.3.2.2. Early responsive genotypes with low expression level

As shown in Fig 4.10, the expression of Os05g10670 gene was found to be low in Wild Rice-6 (*Oryza longistiminata*) and Bhataphool at 6^{th} day of stress. The expression level was high in control leaf tissues as compared to expression in stress leaf tissue for Wild Rice-6 (*Oryza longistiminata*) and Bhataphool. However no expression was observed in both the genotypes at 12^{th} day of stress and after re-watering (Table 4.8). It revealed that Os05g10670 gene was down regulated in Wild Rice-6 (*Oryza longistiminata*) and Bhataphool.

4.3.2.3. Late responsive genotypes

Two rice genotyeps Indira barani Dhan and Chapti gurmutiya shows higher expression of Os05g10670 gene in control samples than the stresses ones. Further when compare to 6^{th} days of water stressed samples, lower expression of Os05g10670 gene clearly indicates the down regulations due to stress. In general expression level was high in control leaf tissues as compared to expression of stress leaf tissues. The expression of Os05g10670 gene was observed almost equal in both stresses as well as in control leaf tissue in Indira Barani Dhan after forty eight hours of re-watering while in Chapti Gurmutiya Os05g10670 gene showed moderate expression in control leaf tissue as compared to stress leaf tissue after forty eight hours of re-watering.

The expression profile of Os05g10670 gene reveled that, although the expression level was high at 6^{th} day of stress in Nagina22 and Swarna but it decreases at 12^{th} day of stress. Our finding indicates that Os05g10670 gene has delayed

expression due to water stress for the two drought tolerant rice varieties. Similarly finding of down regulation of OSbZIP genes using microarray analysis of the known drought tolerant rice genotypes Dagardeshi was also recorded by Khurana *et al*., (personal commutation). Under stress conditions, the C-repeat binding factor/dehydration-responsive element binding factor (CBF/DREB) transcription factors induce the expression of downstream genes containing C-repeat/dehydration response elements (CRT/DRE) in their promoters to improve plant tolerance (Xiong *et al.,* 2002 and Nakashima *et al.,* 2006, Guo *et al.,* 2007*),*). Many other transcription factors such as NAC, MYB, bZIP and zinc finger proteins have been well characterized with their roles in the regulation of stress-responses (Agarwal *et al.,* 2006 and Chinnusamy *et al.,* 2006).

Transcription factors (TFs) regulate genome expression in response to environmental and physiological signals, and some of them switch on plant adaptive developmental and physiological pathways. Plant growth and crop productivity are largely affected by environmental stresses such as drought, salinity and low temperature. To date, many stress-related genes have been isolated and characterized from various plants. These genes encode products either directly protecting plant cells from abotic stresses or regulating expression of other genes to enhance plant tolerance to the stresses (Xiao. *et al.,* 2007 and Martınez-Atienza *et al.,* 2007). The role of ZFP182 gene in increased salt stress tolerance in plants along with the drought tolerance has been reported (Huang *et al*., 2007).

Table 4.8: Differential expression of Os05g10670 among seven rice genotypes.

S. N.	Name of Genotypes	Duration of stress					
		6th day		12th day		Re-watering	
		Stress	Control	Stress	Control	Stress	Control
1	Indira Barani Dhan	√ Low	√ Low	√ Low	√ High	√ High	√ High
2	Chapti Gurmatiya	×	√ Low	√ Negligible	√ Moderate	√ Negligible	√ Moderate
3	Nagina 22	√ High	√ Moderate	√ High	√ Moderate	√ Negligible	√ Moderate
4	Swarna	√ High	√ Moderate	x	×	×	×
5	Bhataphool	×	√ High	×	×	×	×
6	Nipponbare	√ Low	√ High	√ Negligible	√ Negligible	√ Negligible	×
7	Wild Rice 6	√ Moderate	√ Very High	×	√ Negligible	√ Negligible	×

CHAPTER V

SUMMARY, CONCLUSION AND SUGGESTIONS FOR FUTURE RESEARCH WORK

Rice is the world's most important staple food crop and drought is the major constrain to its production. Drought is a complex phenomenon, involving the interaction of number of individual traits by activating several transcription factors in turn expressing the genes responsible for these traits. Characterization of such transcription factors and studying the regulatory mechanism at genomic level with the help of functional genomics tools provide a platform for effective engineering strategies leading to better drought tolerance. The transcriptome profiling experiments conducted on drought-stressed plants have highlighted the central role of transcription factors (TFs), and other drought related genes, while unveiling the complex hierarchy of the regulatory network that differentially modulates the expression of dehydration signature genes in a tissue-specific manner. In order to study the effect of dehydration stress in rice various experiments were conducted with major objectives (i) *In silico* structural characterization of drought tolerance related zinc finger gene superfamily TFs for putative location, expression, and their role in drought tolerance. (ii) Functional characterization and designing of primers for zinc finger gene superfamily TFs related to drought tolerance using genomic tools. (iii) Expression analysis of selected drought tolerance related zinc finger gene superfamily TFs using RT-PCR.

Drought and salinity are among the worst scourges of agriculture. One effective mechanism to reduce damage from these stresses is the accumulation of high intracellular levels of osmoprotectant compounds. These compounds include proline, ectoine, betaines, polyols, and trehalose, and have evolved in many different

organisms. Since some crop plants have low levels of these osmoprotectants or none at all, engineering in osmoprotectant biosynthesis pathways is a potential way to improve stress tolerance. Water loss from plant tissues under drought conditions results in growth inhibition and in a number of other metabolic and physiological changes. The relative water content of leaves under water stress stressed condition after imposing drought for 6 days ranged from 70.83% to 74.54% as compared to regularly watered plants which ranged 92.10% to 95.38%. A significant increase in proline was observed in response to water stress. Proline content in Nagina 22 was found as high as 48 fold more under stress as compared to control. A positive and strong correlation was found between RWC and Proline content.

Many genes that respond to dehydration and that gene product may be involved in dehydration tolerance. In which Transcription factors (TFs) regulate genome expression in response to environmental and physiological signals, and some of them switch on plant adaptive developmental and physiological pathways. Single Nucleotide Polymorphism (SNPs) are highly polymorphic reproducible markers, which was fond in 9 genes out of 10 ranging from 1 (LOC_Os02g45780) to 9 (LOC_ Os0510670).

Transcription factors are key components of signal transduction networks, conveying diverse signals to specific responses for drought stress and *cis*-acting elements are important in this regard. Total number of motifs found were 233 and among them, motif MBS was found in all the locus ids except LOC_Os02g12790, LOC_Os05g10670, and LOC_Os09g35670.

Sequencing tools and sensitive transcriptome analysis techniques together have enabled qualitative and quantitative computational transcriptome characterization using expression tag sequences (ESTs, MPSS and digital

microarray). Ten drought tolerance related transcription factors gene sequences were analyzed for presence of known co-localized ESTs and MPSS tags using genome browsers (Rice Genome Annotation Project at http://rice.plantbiology.msu.edu/), expression tags search tools (PASA- Program to Assemble Spliced Alignments and http://mpss.udel.edu/rice) and digital microarray (http://www.ricearray.org/). A total of 199 ESTs were identified in 10 genes with minimum 6 ESTs in LOC_Os03g22680 and maximum 47 ESTs in LOC_Os05g10670. The ESTs identified in each gene were then categorized according to their corresponding expression tissue library such as flower, panicle, seed, leaves, roots, stem to understand putative site of expression.

The highest number of MPSS tags was found in LOC_Os02g45780 (4011) where as lowest was obtained in LOC_Os02g12790 (126). Maximum number of mature leaves tissue library was found in LOC_Os09g35670.

Among the different plant organs, it was observed that majority of the genes expressed more in various temporal stages of development, young and mature leaves. LOC_Os05g10670 showed high level of expression in experiment Nagina 22 and IR64 under normal and drought conditions whereas LOC_Os05g10670 and LOC_Os09g35670 showed higher expression than LOC_Os02g12790 and LOC_Os04g49550 in experiment Expression data from field droughty rice. Expression tag sequence based computational transcriptome profiling analysis provided a discrete pattern of expression of drought tolerance related transcription factors genes.

The semi-quantitative RT-PCR analysis of one drought responsive genes belonging to zinc finger gene superfamily was performed in leaf trascriptome at three time points (6^{th} day, 12^{th} day of stress and 48 hours after re-watering) in vegetative

stage. Expression profiling of one gene (Os05g10670) indicates differential expression among seven rice genotypes.

Conclusions

- The RWC of leaves of all rice genotypes reduced remarkably when subjected to water stress and significant increase in proline content was observed, revealing that with the increase in intensity of drought, proline content is enhanced and both the parameters were found positively and strongly correlated.
- Higher the SNPs, greater the polymorphic reproducibility, depicting the potentiality of genes and can be applied for understanding the traits responsible for drought. The SNPs which were identified in 9 out of 10 genes, ranging from 1 (LOC_Os02g45780) to 9 (LOC_ Os0510670), in reference to Swarna, Moroberekan, N22 and Nipponbare genomes identified using Rice Genome Annotation project and *Oryza* SNP Consortium Database
- ESTs have been most widely used tag based method for transcriptional profiling in rice. The ESTs identified in LOC_Os02g53530 and LOC_Os02g45780 corresponded mostly to whole plant, panicle and leaf tissues which suggested respective phase specific expression of these genes in rice. A total of 199 ESTs were identified in 10 genes with minimum 6 ESTs in LOC_Os03g22680 and maximum 47 ESTs in LOC_Os05g10670.
- The abundance/ frequency of each tag is expressed in TPM (transcript per million) which is considered as the measure of expression in a corresponding tissue library. MPSS tag based profiling offered great opportunities for *in silico* applications in functional characterization of genes using web based tools. The highest number of MPSS tags was found in LOC_Os02g45780

(4011) where as lowest was obtained in LOC_Os02g12790 (126). Maximum number of mature leaves tissue library was found in LOC_Os09g35670.

- Digital microarray-based gene expression profiling was used to characterize genes whose expression is changed in response to drought, salinity etc. by comparing gene expression in affected to that in unaffected plants. Digital microarray expression profiling of transcription factors (TFs) of Zinc Finger drought tolerant genes were carried out by using microarray data from Rice Array database analysis tool site (*www.ricearray.org*). The results were obtained as matching probes from the Affymetrix array.
- There are several transcription factors imparting drought tolerance to rice. Transcription factors of Zinc Finger gene (LOC_Os05g10670) contributing to drought tolerance gets up regulated.
- The semi-quantitative RT-PCR analysis of one drought responsive gene belonging to zinc finger gene superfamily was performed in leaf trascriptome at three time points (6^{th} day, 12^{th} day of stress and 48 hours after re-watering) in vegetative stage. Expression profiling of one gene (Os05g10670) indicates differential expression among seven rice genotypes. The expression of Os05g10670 gene was high at 6^{th} day of stress in Nagina 22 and Swarna followed by Nipponbare, Bhataphool and Wild rice (*Oryza longistiminata*).

Suggestions for future research work

- Drought stress is among the most serious challenges to crop production. Upon exposure to drought condition. Upon exposure of plants to drought condition many stress related genes are induced. There is need to examine and analyze stress related genes and their products in multiple environments.

- There is need to examine and understand functions of large number of stress related genes in order to improve stress tolerance towards drought in rice.
- Hundreds of genes and their products respond to drought stresses at transcriptional and translational level. Understanding the functions of these stress inducible genes help to unravel the possible mechanism of drought stress tolerance.
- Genes that are significantly up or down regulated in drought tolerant cultivar when compared to a susceptible cultivar can serve as a “transcriptional candidates” for drought tolerance.

"Characterization and validation of zinc finger gene superfamily transcription factors for drought tolerance in rice (*oryza sativa* l.) using *in silico* and functional genomics approaches"

BY

SANJEET KUMAR

ABSTRACT

Rice (*Oryza sativa* L.) is an important cereal food crop and model crop for plant genome analysis. Drought remains as one of the major constraints limiting rice productivity. The discovery of novel genes, determination of their expression patterns in response to abiotic stress, and an improved understanding of their roles in stress adaptation obtained by the use of functional genomics will provide the basis of effective engineering strategies leading to greater stress tolerance. Progress is now anticipated through characterization of various transcription factors involved in imparting drought tolerance to rice. Zinc finger protein, *ZFP252* has been found effective against drought tolerance by increasing production of free Proline.

Seven diverse rice genotypes (Indira barani dhan, Chapti Gurmatiya, Nagina 22, Swarna, Bhataphool, Nipponbare and Wild Rice-6) were grown in green house conditions to characterize the transcription factors of Zinc finger genes for drought tolerance. The gene expression was monitored by RT-PCR. Selected stress responsive genes responsible for transcription related activities were studied for samples subjected to drought stress for a period of six days (RWC 70.83–74.54%). A significant increase in Proline was observed in response to water stress. Proline content in Nagina 22 was found as high as 48 fold more under stress as compared to control. A positive and strong correlation was found between RWC and Proline content.

Single Nucleotide Polymorphism (SNPs) are highly polymorphic reproducible markers, which was identified in 9 genes out of 10, ranging from 1 (LOC_Os02g45780) to 9 (LOC_ Os0510670), in reference to Swarna, Moroberekan, N22 and Nipponbare genomes identified using Rice Genome Annotation project and *Oryza* SNP Consortium Database. Total number of motifs found were 233 and among them, motif MBS was found in all the locus ids except LOC_Os02g12790, LOC_Os05g10670, and LOC_Os09g35670.

A total of 199 ESTs were identified in 10 genes with minimum 6 ESTs in LOC_Os03g22680 and maximum 47 ESTs in LOC_Os05g10670. The highest number of MPSS tags was found in LOC_Os02g45780 (4011) where as lowest was obtained in LOC_Os02g12790 (126). Maximum number of mature leaves tissue library was found in LOC_Os09g35670.

Digital microarray expression profiling of transcription factors (TFs) of Zinc Finger drought tolerant genes were carried out by using microarray data from Rice Array database analysis tool site (www.ricearray.org). LOC_Os05g10670 showed high level of expression in experiment Nagina-22 and IR64 under normal and drought conditions whereas LOC_Os05g10670 and LOC_Os09g35670 showed higher expression than LOC_Os02g12790 and LOC_Os04g49550 in experiment Expression data from field droughted rice under digital microarray analysis.

The semi-quantitative RT-PCR analysis of one drought responsive gene belonging to zinc finger gene superfamily was performed in leaf trascriptome at three time points (6^{th} day, 12^{th} day of stress and 48 hours after re-watering) in vegetative stage. Expression profiling of one gene (Os05g10670) indicates differential expression among seven rice genotypes. The expression of Os05g10670 gene was high at 6^{th} day of stress in Nagina 22 and Swarna followed by Nipponbare, Bhataphool and Wild rice (*Oryza longistiminata*).

Towards developing plants against drought and salt stress tolerance, transcription factors are being widely used to genetically engineering crop plants.

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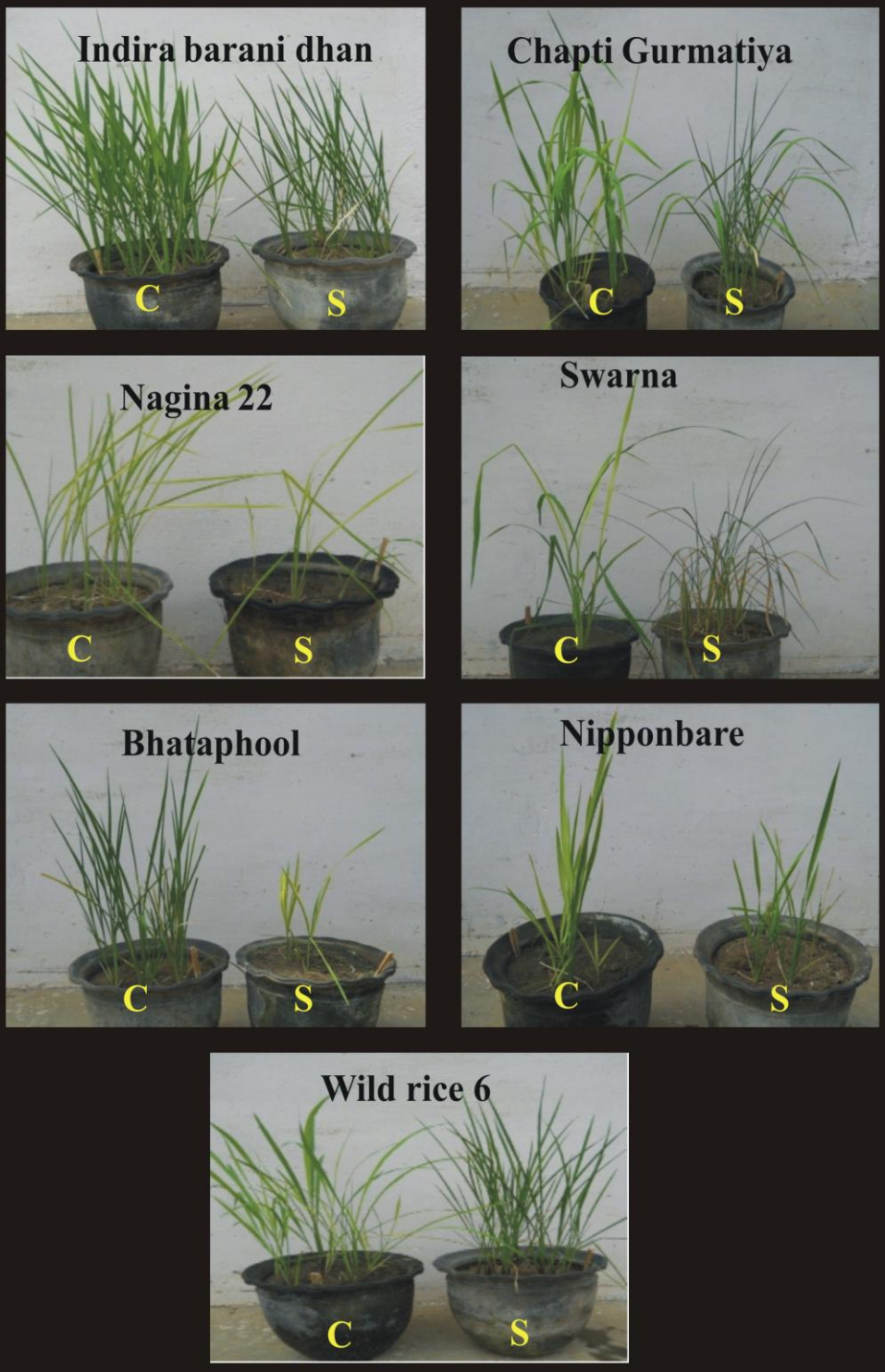


Fig. 4.1: Comparison of seven genotypes with control (C) and 6 days stress(S) condition

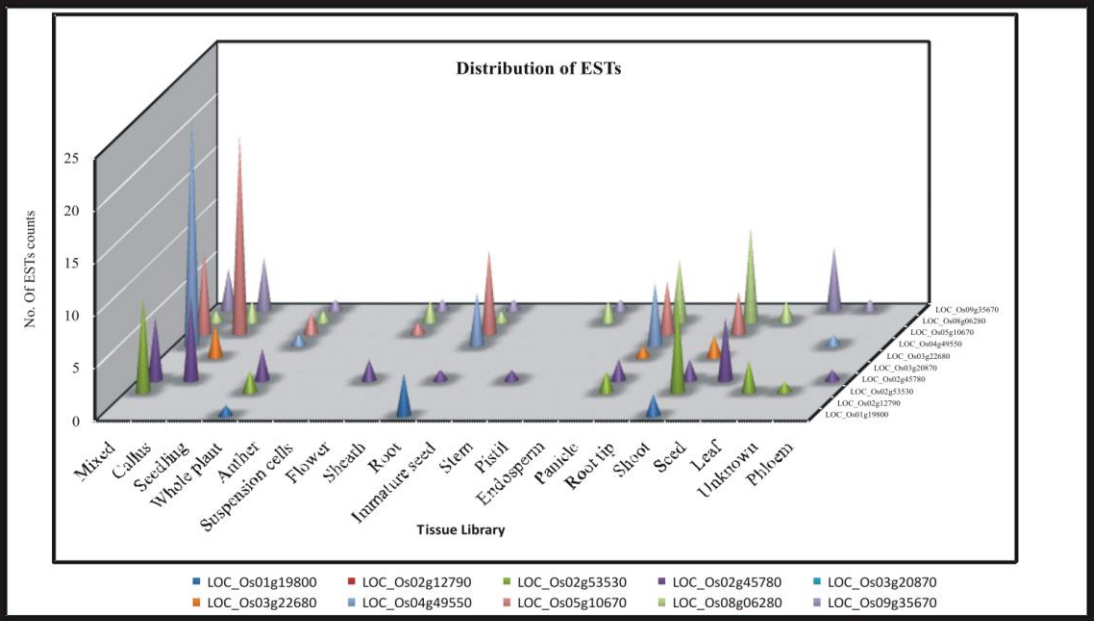
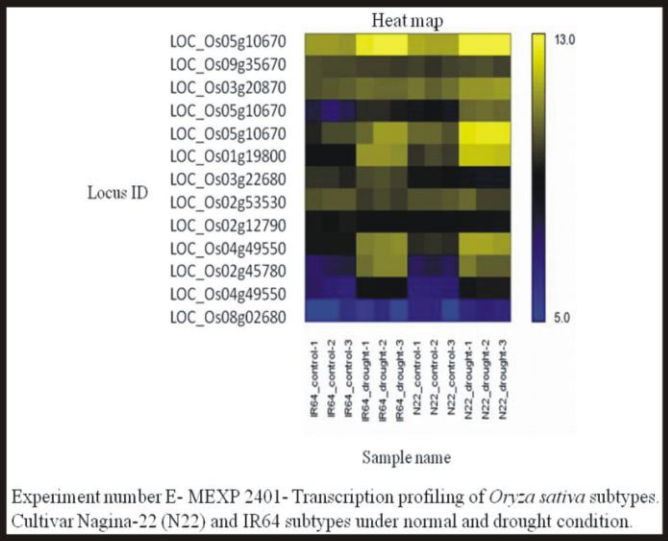
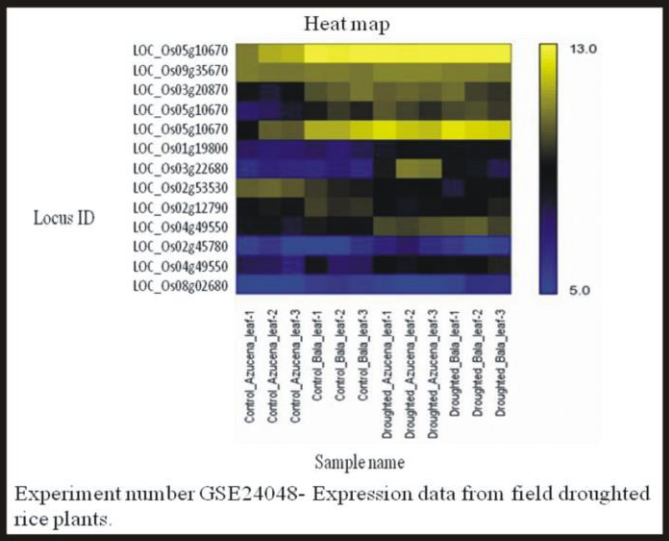


Fig. 4.6: Distribution diagram of the tissue specific expression of transcription factors genes. The x axis indicates different tissue library . The y axis represents no. of ESTs found in different tissue library. The Z axis represents locus IDs of different TFs genes



Experiment number E- MEXP 2401- Transcription profiling of *Oryza sativa* subtypes. Cultivar Nagina-22 (N22) and IR64 subtypes under normal and drought condition.



Experiment number GSE24048- Expression data from field droughted rice plants.

Fig. 4.8: Spatio-temporal expression profiles of drought tolerance related transcription factors genes for Transcription profiling of *Oryza sativa* subtypes Cultivar Nagina-22 (N22) and IR64 subtypes under normal and drought conditions and Expression data from field drought rice plants generated at rice array database tool site (www.ricearray.org). The different control and drought rice plants have been marked at the bottom in both experiments. Color bar at the right represents log2 signal values, blue representing low-level expression, black medium and yellow signifies high-level expression.

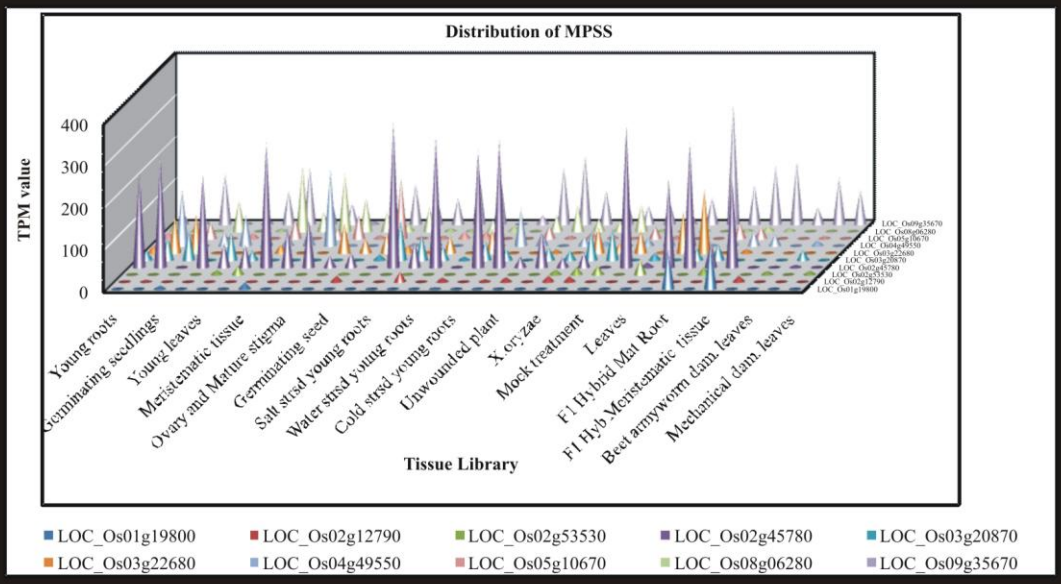


Fig. 4.7: Distribution diagram of the tissue specific expression of transcription factors genes. The x axis indicates different tissue library. The y axis represents no. Of MPSS found in different tissue library. The Z axis represents locus IDs of different TFs genes.

Fig. 3.2: Estimation of Relative water content with control and 6 days stress condition

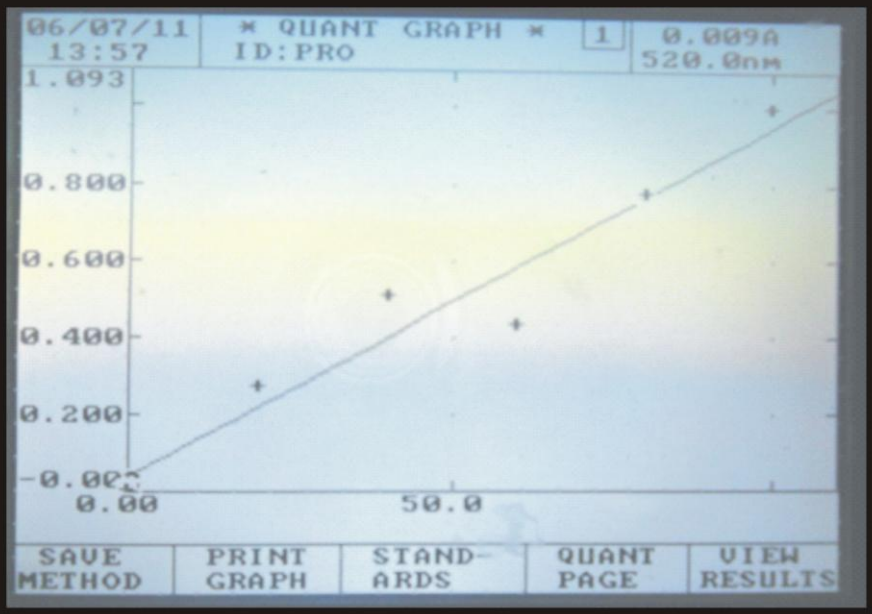


Fig. 3.3: Standard curve prepared for Proline estimation

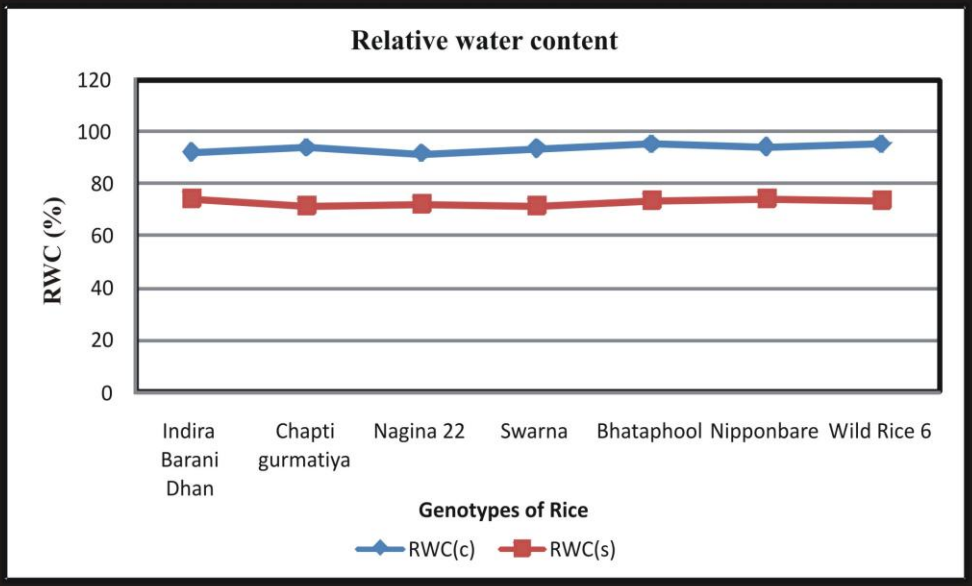


Fig. 4.2: Relative water content in leaves of 45-days old plants of seven rice genotypes at 6th day of stress under stress and control conditions.

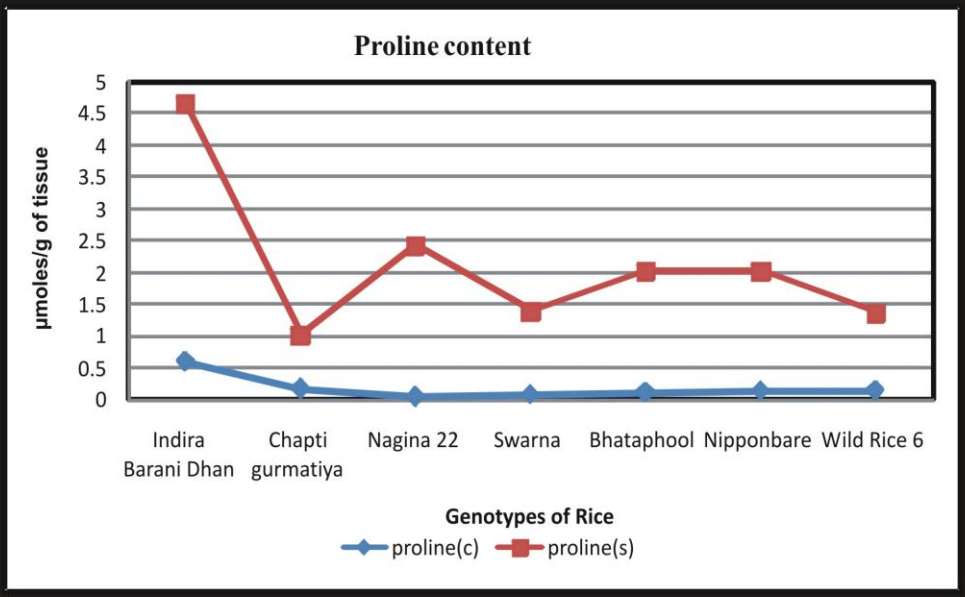


Fig. 4.3: Proline content in leaves of 45-days old plants of seven rice genotypes at 6th day of stress under stress and control conditions.

Fig. 3.1: Seven rice genotypes grown in green house

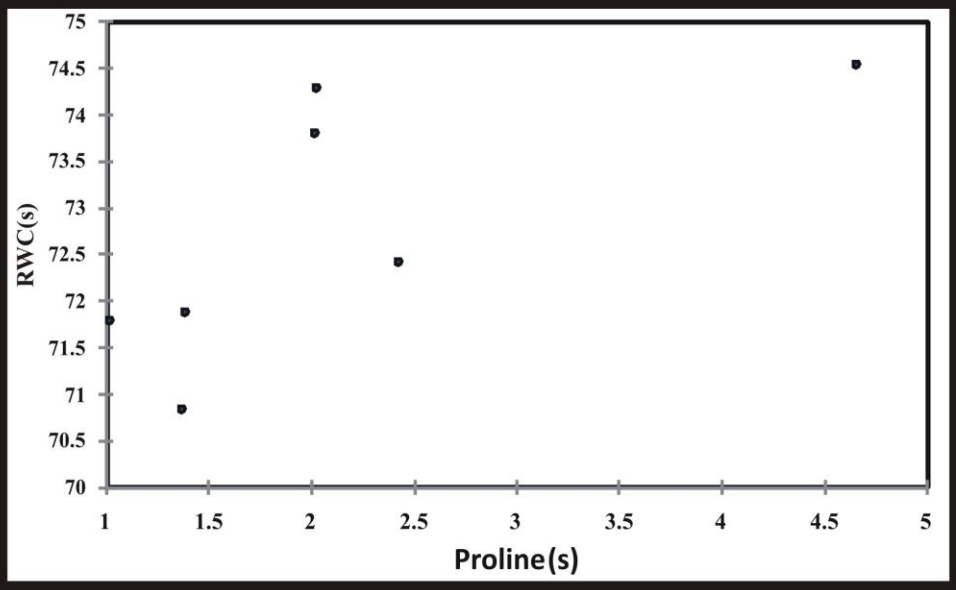


Fig. 4.4: Scatter plots of drought resistance indices (Proline content and RWC)

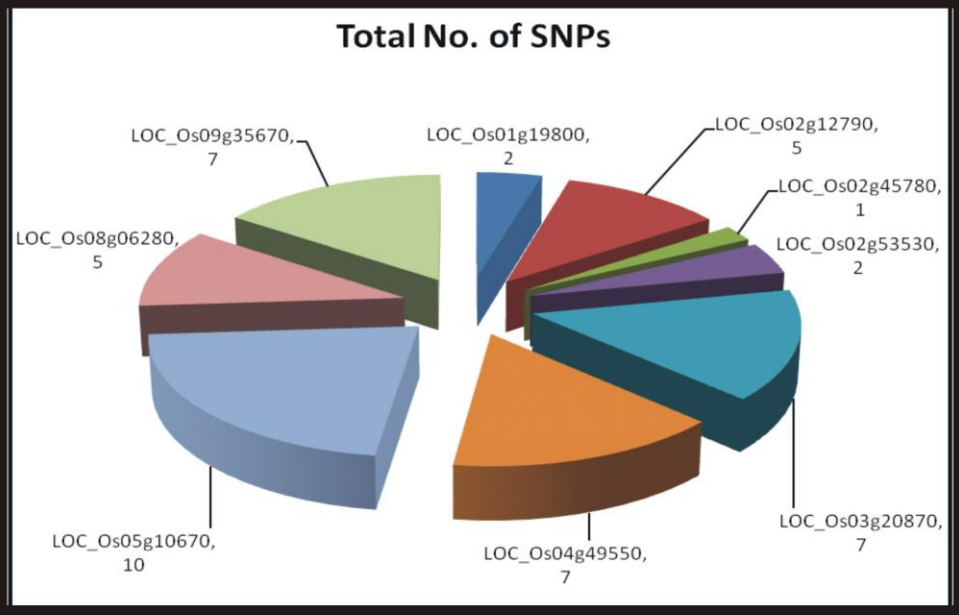


Fig. 4.5: Number of SNPs identified within transcription factors of Zinc Finger gene of drought tolerance in rice