# VALIDATION OF IDENTIFIED GENES FOR WATER STRESS
# IN RICE (*Oryza sativa* L.) MEDIATED BY *Pseudomonas fluorescens*

By
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**(2014-11-105)**

## THESIS

*Submitted in partial fulfillment of the*
*requirement for the degree of*

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**VELLANIKKARA, THRISSUR – 680656**
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**2016**

# DECLARATION

I, hereby declare that the thesis entitled **"Validation of identified genes for water stress in rice (*Oryza sativa* L.) mediated by *Pseudomonas fluorescens"*** is a bonafide record of research work done by me during the course of research and the thesis has not previously formed the basis for the award to me any degree, diploma, fellowship or other similar title of any other University or Society.
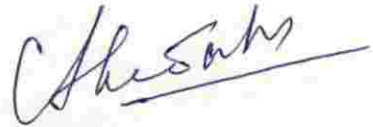
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Certified that the thesis entitled **"Validation of identified genes for water stress in rice (*Oryza sativa* L.) mediated by *Pseudomonas fluorescens* "** is a bonafide record of research work done independently by **Mr. Saakre Manjesh (2014-11-105)** under my guidance and supervision and that it has not previously formed the basis for the award of any degree, diploma, associateship or fellowship to him.

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**Saakre Manjesh**

To my loving
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my guide

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# ABBREVIATIONS

| | |
|---|---|
| ABA | Abscisic acid |
| ABI | Applied Biosystems |
| ACC | 1-Aminocyclopropane-1-Carboxylate |
| Act | Actin |
| AGI | Arabidopsis Genome Initiative |
| AP2-EREBP | APETALA2 and Ethylene-Responsive Element Binding Proteins |
| AthaMap | Arabidopsis thaliana Map |
| BLAST | Basic Local Alignment Search Tool |
| bZIP | Basic Leucine Zipper |
| $^{0}C$ | Degree Celsius |
| cDNA | Complementary Deoxyribo Nucleic Acid |
| CDS | Coding Sequence |
| CFU | Colony Forming Unit |
| CP | Crossing point |
| cm | Centimeter |
| CO I | Cytochrome Oxidase Subunit I |
| CPBMB | Centre for Plant Biotechnology and Molecular Biology |
| Ct | Threshold cycle |
| DBTF | DNA binding transcription factor |
| DIC | Distributed Information Centre |
| DD-RT-PCR | Differentially Display Reverse Transcriptase PCR |
| DEPC | Diethylpyrocarbonate |
| DNA | Deoxyribo Nucleic Acid |
| dNTP | Dehydroxy Nucleotide Tri Phosphate |
| ds | Double stranded |
| EDTA | Ethylene Diamine Tetra Acetic acid |

| | |
|---|---|
| Etbr | Ethidium bromide |
| F | Forward |
| g | Gram |
| GAPDH | Glyceraldehyde-3-phosphate dehydrogenase |
| h | Hour |
| Ha | Hectare |
| HCL | Hydro Chloric Acid |
| HSP | Heat   shock protein |
| IAA | Indole Acetic Acid |
| IDT | Integrated DNA Technologies |
| IRRI | International Rice Research Institute |
| JA | Jasmonic Acid |
| KAU | Kerala Agricultural University |
| Kb | Kilo base |
| kDa | kilo  Dalton |
| LEA | Late Embryogenesis Abundant |
| M | Molar |
| mg | Milligram |
| min | Minute |
| ml | Millilitre |
| mM | Millimolar |
| M-MuLV | Molony murine leukemia virus |
| MOPS | 3-(N-morpholino)propanesulfonic acid |
| mRNA | Messenger RNA |
| MYB | Myeloblastosis B |
| µg | Microgram |
| µl | Microliter |
| µM | Micromole |

| | |
|---|---|
| NCBI | National Center for Biotechnology Information |
| ng | Nano Gram |
| OD | Optical Density |
| OdT | Oligo Dehydroxy Thymine |
| Os | *Oryza sativa* |
| PCR | Polymerase Chain Reaction |
| Pf | Pseudomonas fluorescens |
| PGPR | Plant Growth Promoting Rhizobacteria |
| pH | Hydrogen ion concentration |
| PKDP | Protein Kinase Domain Protein |
| PTB | Pattambi |
| % | Per cent |
| R | Reverse |
| RGAP | Rice Genome Annotation Project |
| RNAse | Ribo Nuclease |
| Rn | Normalized reporter signal |
| RNA | Ribo Nucleic Acid |
| ROS | Reactive Oxygen Species |
| rpm | Revolutions per minute |
| rRNA | Ribosomal Ribo Nucleic Acid |
| RT | Reverse Transcriptase |
| S | Svedberg unit |
| SA | Salicylic acid |
| Sec | Second |
| SES | Standard Evaluation System |
| STIFDB2 | Stress Responsive Transcription Factor Data Base Version 2.0 |
| TAE | Tris Acetate EDTA |

| | |
|---|---|
| TAIR | The Arabidopsis Information Resource |
| TDF | Transcript derived fragment |
| TIGR | The Institute of Genome Research |
| TF | Transcription Factor |
| TFBS | Transcription Factor Binding Site |
| tRNA | Transfer Ribo Nucleic Acid |
| Tris | (hydroxymethyl)aminomethane |
| TSS | Transcription Start Site |
| TUB | Tubulin |
| UBQ | Ubiquitin |
| UTR | Un Translated Region |
| UV | Ultra violet |
| V | Volts |
| Wt | Weight |
| Δ | Delta |

# Introduction

# 1. INTRODUCTION

Rice (*Oryza sativa* L.) is one of the major food crops for about 65 percent of the world's population. It is the staple food for an expansive part of the world, particularly in Asia (Ghadirnezhad and Fallah, 2014). Rice is a typical monocot with long and slender leaves. Its inflorescence bears the edible seed or the grain which is rich in carbohydrates. It has been estimated that a large portion of the world's population depends wholly or partially on rice for its calorie intake.

Climatic factors play a major role in the growth and development of any crop. Among the various factors, water availability is of great significance with regard to rice cultivation (Singh *et al.,* 2008). Rice is predominantly a kharif season crop. However, it is also grown as rabi/summer season crop with assured irrigation wherever winter is not severe. Indian rice production largely depends on monsoon rains and only 59 per cent of area under rice cultivation has assured irrigation (Ramakrishna, 2015).

Plants are sessile and therefore they cannot escape the adverse environmental factors such as biotic and abiotic stresses. Generally, plants do not always grow under optimum growth conditions. They suffer from many adverse situations that cause different types of stresses, and prevent them from reaching maximum development (Singh *et al.,* 2008).

Biotic stress occurs as a result of various effects caused by different living entities such as microbes, beneficial and harmful insects and also weeds (Flynn, 2013), whereas abiotic stresses are manifested as the negative effect of non-living entities on living entities during various environmental conditions. According to Vinebrooke *et al.* (2004), an adverse environmental condition to be called as an abiotic stress, it must have an adverse impact on the individual physiology or the p

opulation performance beyond its normal range of variation in a significant way (Vinebrooke *et al.*, 2004).

Drought is a problem of worldwide importance, affecting the crop production and quality on a large scale and this problem is becoming more serious with respect to the global climate change (Halliwell, 2006). Furthermore, drought is also related to salinity, chilling stress, high temperature stress, acid-alkaline stress, biotic stress, senescence, growth and development, UV-B damage, wounding, embryogenesis, blossoming, signal transduction and many other factors (Castle *et al.*, 2004). Therefore, drought is associated with all parts of plant biology. As of now, research on drought stress has been one of the principle headings in the global plant biology and biological breeding.

In the global agricultural scenario water stress or drought is an unavoidable and chronic feature. Water shortage has been reported in about one third of world's arable land (Kramer, 1980) which reduces the crop production to a very minimum. Water, being an integral part of plant development, plays an important role in the initiation of growth, activation of enzymes, and further maintenance of the plant's developmental process and ultimately affects the economy of a country.

*Pseudomonas fluorescens* encloses a group of saprophytes that are very common in soil, water and other plant surfaces but are nonpathogenic. It is an obligate aerobe, gram negative bacillus which belongs to Plant Growth Promoting Rhizobacteria (PGPR). This group of bacteria plays a major role in promoting plant growth, induction of systemic tolerance and also biological control of pathogens.

The knowledge about the mechanisms of plant adaptation to stress conditions such as biotic as well as abiotic conditions can be exploited in different ways to improve the crop species and to increase the productivity in adverse environmental conditions (Kumar *et al.*, 2015). A major advance in the knowledge and

understanding of plant drought tolerance was the discovery of the ABA signaling pathway. The mechanisms by which plants respond to stress include both ABA-dependent and ABA-independent processes (Tuteja, 2007). One of the major components in the abscisic acid (ABA) signaling pathway that is activated during any abiotic stress in plants is the bZIP transcription factor family (Liu *et al.*, 2014).
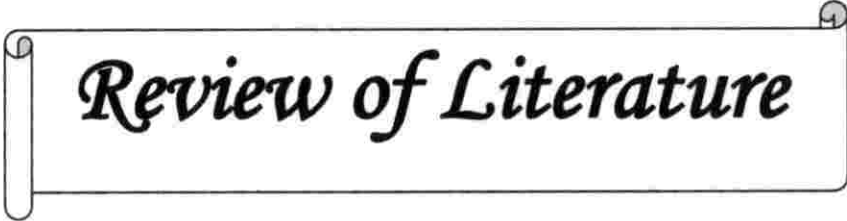
Expression of a variety of genes is induced by various types of abiotic stresses such as drought, high salinity, low temperature *etc.* in various plants. The products of expressed genes are proteins which may include enzymes, transcription activators or membrane proteins which will give tolerance to a particular stress. Quantitative real time PCR (qrt-PCR) technique is highly sensitive, accurate and practically easy to use and hence it has become as a routine bioinstrumentation for gene level measurement (Provenzano and Mocellin, 2007).

Transcriptional control of stress-responsive genes is a vital part of the plant response to a large variety of stresses in which combinatorial control plays a major role. A transcription factor is a protein that binds to specific DNA sequences, thereby controlling the rate of transcription of genetic information from DNA to messenger RNA. A defining feature of transcription factors is that they contain one or more DNA-binding domains (DBDs), which attach to specific sequences of DNA (*cis-*regulatory elements) adjacent to the genes that they regulate. Combinatorial control is defined as the involvement of a distinct number of transcription factors in different but specific combinations so as to produce a wide range of gene expression patterns (Singh, 2005). Combinatorial transcription factors are a pair of regulatory proteins bound to *cis*-regulatory elements present on promoter region of a gene, which can interact and also can regulate the transcription of that particular gene (Bhattacharjee *et al.*, 2013).

Biochemical identification as well as validation studies of individual genes that participate in a combinatorial controlled trait are highly difficult. Bioinformatics

resources shall provide a robust platform to identify the major molecular players involved in combinatorial control.

With this background, the present study was undertaken to analyze quantitative expression of identified genes for water stress in rice, *in-silico* identification of stress inducible Transcription Factor Binding Sites (TFBSs) and combinatorial *cis*-regulatory elements.

# Review of Literature

# 2. REVIEW OF LITERATURE

Rice, botanically known as *Oryza sativa* is a monocotyledonous semi aquatic angiosperm. The genus consists of more than 20 species out of which only two are cultivated. *Oryza sativa* and *Oryza glaberrima* are the cultivated species in Asian and West African countries respectively (Watanabe, 1997).

As the staple food of a majority of the Asian population, the morphology, physiology, agronomy, genetics and biochemistry of rice have been studied extensively. Researches and crop improvement programmes on rice had been started more than a century back (Xu *et al.*, 2009). Till date, more than 40,000 varieties have been reported in rice worldwide. Extensive adoptions of high yielding rice varieties have led many of the Asian countries to become self-sufficient in food production (MoEF, 2011).

In India, rice is grown mainly under four ecosystems namely irrigated ecosystems, rainfed lowlands, rainfed uplands and deep water lowlands. More than 50 % of the area under rice cultivation (55 %) is under rainfed conditions out of which about 80 % is in the eastern part of India, where the rice cultivation is vulnerable to the adverse monsoons (Gupta, 2014).

In India rice is mainly grown as a transplanted crop. Young seedlings of appropriate age are transplanted manually to the main fields from the seedbeds. The percentage of area under direct seed sown crop accounts to about 28% most of which is confined to the eastern India (Pandey and Velasco, 1999).

## 2.1.1. Rice genotype Matta Triveni (PTB 45) used for the present study

Matta Triveni (PTB 45) is a popular rice variety of Kerala, developed at the Regional Agricultural Research Station, Pattambi, Kerala in 1990. It is a reselection of a white rice variety Triveni (PTB 38) developed from Annapoorna (PTB 35) and a

land race, Kavungin Poothala (PTB 15) (Prabhakaran, 2007). The average plant height is 82 cm. The green leaf sheath is erect and broad with 45-50 cm width. The grains are medium bold with red coloured bran. The variety is resistant to lodging. The recommended fertilizer dosage is 90:45:45 kg NPK/ ha. The test weight of seeds amounts to 24.1g.

Matta Triveni is a short duration variety of 100-105 days and tolerant to brown plant hoppers. However it is susceptible to rice blast and sheath blight (KAU, 2011). The variety is recommended for all the three rice seasons of Kerala namely Virippu, Mundakan and Puncha. A yield of 10, 000 kg/ ha has been reported from the Kole regions (single cropped areas) whereas locally the yield was only 5, 500 kg/ha which throws light on the presence of adaptability genes provided by the land races Kavunginpoothala and Thekken Cheera (Prabhakaran, 2007).

## 2.1.2. Biotic and Abiotic Stress

Stress is an altered physiological condition due to the factors, which disrupt the equilibrium (Gaspar *et al.*, 2002). Plants experience a wide range of stresses including biotic as well as abiotic. Biotic factors include the insects, pests, weeds and pathogens whereas abiotic stress is caused by various environmental factors. Chinnusamy *et al.* (2004) reported that if the stress level is moderate and for a short period of time, the injury is also brief and plant can recuperate when the stress period is over. However a severe stress condition leads to forestall in flowering, seed set and induce senescence leading to the death of the plant.

Adverse effects on the growth and productivity of the plants are observed as a result of abiotic stresses. Such a situation triggers a sequence of various molecular, morphological, biochemical and physiological changes in plants. The most common abiotic stresses experienced by plants are drought, temperature extremities, and saline soils. Presently, the entire plant kingdom is in the danger of rapid environmental

fluctuations. Greenhouse gases such as $CO_2$, methane and nitrous oxides have an enormous impact on global environmental conditions leading to extreme temperature changes and weather patterns in many parts of the world (Hirt and Shinozaki, 2004). As per the reports of FAO (2004) globally, around 22% of the agricultural area is affected by saline soils and the area under water deficit is mounting up and is likely to increase even more (Burke et al., 2006).

At present the challenge for crop scientists is to develop crop varieties that are resistant or tolerant to various abiotic stresses so as to improve the yield. This is important to meet the demand of growing population and this necessitates the need for understanding the varied aspects of the stress conditions and the molecular changes associated with it (Venkateswarlu et al., 2012).

### 2.1.2.1. Drought

Drought is one of the major challenges in crop production. It has been found that drought affects around 26 per cent of the total arable land. Among the various stresses that plants experience drought pose a severe threat to food security as it affect the crop yield particularly that of cereals. In water deficit conditions both plant water potential and turgor pressure are reduced significantly and this affects the regular functions of the crop (Hsiao, 1973).

Drought results in reduction in decline in plant water levels, leaf area, dry matter production and transpiration. Geared toward reducing plant water use rate, plants reduce its leaf area as a drought avoidance mechanism and thus conserve water during drought periods (Jones, 1992). Reduction in stomatal conductance during water deficit conditions result in reduced assimilation of carbon and consequently reduces the biomass production (Delfine et al., 2001; Medrano et al., 2002). Plants under drought conditions have more of its dry matter partitioned into the roots than into the shoots (Wilson, 1988; Arora and Mohan, 2001).

### 2.1.2.1.1. Economic yield reduction by drought stress

In barley drought stress reduced the number of tillers, spikes and grains per plant. The individual grain weight was also reduced as a result of the stress condition. The yield reduction during drought stress varies with the stages of development. Lafitte et al., (2007) reported 53-92 % reduction in crop yield when stress was induced during reproductive stage whereas 60% reduction in yield when the drought was induced during grain filling stage. In case of Maize, the grain yield was reduced by 25-60% when the drought stress was during vegetative stage. However during the reproductive stage drought condition, there was a higher reduction in grain yield which amounted to 32-92% (Atteya et al., 2003).

The germination potential, length of hypocotyl and weights of shoot and root (both fresh and dry) were found to be reduced under water deficit conditions that has been induced by polyethylene glycol, according to a study conducted by Zeid and Shedeed (2006). However they observed an increase in the root lengths. In spite of the stress severity, post-anthesis drought was detrimental to the grain yield (Samarah, 2005). In a study conducted on pea, both germination potential as well as early growth of seedlings was found to be impaired due to drought stress in five cultivars under study (Okcu et al., 2005).

In case of Rice, the susceptibility to drought is more obvious during reproductive stage and when the stress condition coincides with the reproductive period, there is maximum reduction in grain yield (Cruz and Toole, 1984).

### 2.2.1. Plant defense against abiotic stress

Plants respond to various stress conditions differently. Subjecting a plant to an abiotic stress leads to the expression of a large number of genes. The product of these genes may result in the increased levels of certain metabolites and proteins, which in turn confers a certain level of protection against these stress conditions (Pooja et al.,

2015). Plants respond to declining water potential through several mechanisms including osmotic adjustment wherein the solutes are accumulated within the cells (Jones, 1992).

Even in the same plot, each plant may adapt very differently from one another. Mittler (2006) experimented on a group of different plant species. Using different stress signals such as drought or cold, the entire group of plant species was stimulated. It was observed that none of the stress responses were similar, even though the plants had been growing in exactly the same environment. Roots form the first line of defense in plants against many of the abiotic stresses. If the soil in which the plants are growing is healthy and biologically diverse, the plant will have a better chance of surviving many of the stressful conditions (Brussaard *et al.*, 2007). Understanding the changes in the cellular, biochemical and molecular machinery of a cell under stress is a key point in breeding crops for stress resistance.

Cellular level destruction of plants is caused at multiple stages of plant growth which may result in phenotypic lethality as a result of stress conditions either singularly or in combinations. However, plants have certain mechanisms to perceive even the delicate changes in growth conditions. Such mechanisms developed by the plants activate distinct signal transduction cascades, which in turn activate stress-responsive genes. The ultimate effect of such activated cascades is the transcriptional reprogramming of morphology, metabolism, physiology and survival (Xiong *et al.*, 2002).

Changes at the transcriptome, cellular, and physiological levels are brought about as a response to various stress conditions in plants and in 2012, Atkinson and Urwin reported such responses are highly complex. Recent studies have shown that plants responses to multiple stresses as well as individual stress are different from each other. A specific route of gene expression is activated in plants which relate to the exact stress conditions encountered. Such a programme of gene expression

involves the activation of kinase cascades, transcription factors and various reactive oxygen species.

Based on leaf temperatures, the tolerance level of rice varieties to drought were evaluated by Hirayama *et al.* (2006) both in upland and lowland rice varieties. The rate of increase in leaf temperatures of the upland rice varieties was slower than that of the low land rice varieties. A high level of correlation in the leaf temperature and photosynthetic rates of the varieties were observed when measured using infrared radiation thermometer during three consecutive years. It was observed that under upland conditions, when leaf temperatures are low, rice varieties maintain high transpiration rate, photosynthetic rates and also produce higher yield. On the other hand varieties having higher leaf temperatures were susceptible to drought. The study thus provided a lead to the fact that leaf temperature can be considered as a useful indicator to estimate the drought tolerance which would be of great importance in rice breeding.

In 2002, Ozturk *et al.* reported the induction of similar defense responses by plants even though various genes are differentially regulated under different stress conditions. This was confirmed by the gene expression profiles of either drought or salt stressed barley plants.

### 2.2.1.1. Abscisic acid

Evolution of complex signaling pathways in plants is an adaptation to survive abiotic stresses and it includes receptors, secondary messengers, phytohormones, and signal transducers (Hossain *et al.*, 2010). The phytohormone, ABA plays a regulatory role in many physiological processes in plants. Diverse stress conditions such as drought, severe cold, and light and temperature extremities results in increased levels of ABA. Elevated ABA levels can enhance plant adaptation to various abiotic stresses (Tuteja, 2007). ABA is popularly known as the stress hormone. With the

variations in the availability of ABA, the response of plants to various environmental conditions is varied. The adequate development of plants is possible only with the involvement of ABA as it is an endogenous signal for stress response.

Like other plant hormones, concentration of ABA in the tissue as well as its affectability to the hormone will determine its response to the stress. The concentration of ABA in a particular tissue is also driven by the various processes such as biosynthesis, catabolism, compartmentation and transport (Taiz and zeiger, 2010).

The action of ABA involves modification of gene expression. Swamy and Smith (2009) reported that analysis of responsive promoters disclosed several potential *cis-* and *trans*-acting regulatory elements.

The level of ABA increases in plant system when the plant is facing dehydration. This turns on certain genes involved in the stress response against drought and are expressed in the plant system. The effect of increased levels of ABA is manifested as complete closure of stomata thus reducing water loss through transpiration. Alteration of gene expression is also observed as a result of high level of ABA (Hirt and Shinozaki, 2004).

Maize, sorghum, rice, barley, soybean, wheat and various other crops have been reported to have endogenous ABA accumulation during drought stressed conditions (Sah *et al.,* 2016). However it was first observed in drought stressed wheat. Studies have also reported ABA accumulation in response to cold and salt stress. Exogenous application of ABA has been found to improve the adaptive responses in many crops (Wei *et al.,* 2015). Mohapatra *et al.* (2008) reported that ABA treatments improved cold resistance in cucumber and lucerne whereas Etehadnia *et al.* in 2008 reported salt stress was alleviated in common bean and potato after ABA treatments.

The major mechanism of action of ABA is by regulating the stomatal opening to limit water loss from leaves under drought conditions. In addition to this, ABA induces the expression of many genes which are expressed as proteins that are important for stress responses and tolerance such as enzymes for osmo protectant synthesis (Fujita *et al.*, 2011). Transcriptome studies have shown that over 50% of the genes regulated by ABA are also governed by drought or salinity, whereas cold-regulated transcriptome shows less overlap with those induced by other stresses. Seki *et al.* (2002) identified 245 ABA-inducible genes in *Arabidopsis*. Among these ABA-inducible genes, 63% (155 genes) were induced by drought, 54% (133 genes) by high salinity and 10% (25 genes) by cold treatment. Rabbani *et al.* (2003) identified 73 stress-inducible genes in rice, among which, 43 genes were induced by ABA. These results demonstrate significant crosstalk between ABA response and abiotic stress signaling pathways, especially for drought and high-salinity.

### 2.2.1.2. Basic leucine zipper proteins

The transcription factors encoded by basic leucine zipper (bZIP) genes are concerned with directing various biological processes. The bZIP transcription factor family is one of the largest such families in plants. The members of this transcription factor familyis mainly involved in stress responses as well as hormone signal transduction (Rodriguez and Connell, 2006). In rice, bZIP proteins have multiple biological processes which including pathogen defense; responses to abiotic stresses; seed development and germination; senescence; and responses to salicylic, jasmonic, and abscisic acids. The expression of the gene on its downstream is brought about by the bZIP transcription factors as a result of its interaction with ABA-responsive elements (ABREs). These *cis*-acting small DNA regions are present in the promoter region of ABA-inducible genes. Hence, the bZIP transcription factors are assigned as ABRE-binding factors (ABFs) or ABRE-binding proteins (AREBs) (Yamaguchi, 2005).

The bZIP family consists of proteins with a DNA Binding Domain (BD). These domains are rich in basic amino acid residues and are presenting adjacent to a leucine zipper dimerization domain. Jakoby *et al.* (2002) reported 75 bZIPs in *Arabidopsis* whereas Correa *et al.* (2008) reported 92 bZIPs in rice.

A number of bZIP proteins such as OSBZ8 (Mukherjee *et al.*, 2006), OsABI5/OREB1 (Hong *et al.*, 2011), OsAREB1/OsABF2 (Yang *et al.*, 2011) and OsbZIP52/RISBZ5 (Liu *et al.*, 2015) have been reported to bind to ABA-responsive elements (ABRE) and regulate ABA-induced ABRE containing genes at transcriptional level. A transcriptional activator is coded by the OsbZIP52 gene that is strongly induced during low temperature stress (4°C). The importance of this gene with respect to cold conditions has been demonstrated using the lines having overexpression of the same gene. The induction of this gene is related to the down regulation of various other abiotic stress-related genes, such as OsLEA3, OsTPP1, and Rab25 ultimately leading to a considerable drop in the tolerance to cold and drought stress in rice. The study suggests that OsbZIP52 functions as a negative regulator both in cold as well as drought stress (Liu *et al.*, 2015). Analogously, the negative impact of OsABI5 gene, having two splicing variants (Zou *et al.*, 2007), have also been studied. It is induced under high saline conditions, indicating that OsABI5 also has a negative impact on response to salt stress (Zou *et al.*, 2008).

Certain stress related genes are induced by multiple stress conditions as in case of OsbZIP46, an ABRE-binding protein that is be induced by multiple stresses such as drought, salinity, cold, and oxidative stress. OsbZIP46 exhibits auxin responses, indicating a possible positive effect of OsbZIP46 in mediating the crosstalk between indole-3-acetic acid and ABA (Tang *et al.*, 2012). Similar to OsbZIP46 (Tang *et al.*, 2012), several other bZIP proteins, including OsbZIP23 (Xiang *et al.*, 2008), OsbZIP72 (Lu *et al.*, 2009), OsABF1 (Amir *et al.*, 2010), and

OsbZIP16 (Chen *et al.*, 2012) are also ABRE-binding factors and act as positive *trans*-activators in response to drought tolerance in rice.

### 2.2.1.3. Heat shock proteins (Hsps)

Heat shock proteins or the Hsps are a group of polypeptides produced as a response to high temperatures by almost all organisms (Lindquist and Craig, 1988). Protection against stress damage is the major function of Hsps. They are also involved in the signal transduction chains as well as intracellular distribution and degradation of proteins.

Based on sequence homology, the eukaryotic Hsps are classified into different families such as Hsp100, Hsp90, Hsp70, Hsp60, Hsp20 and small Hsps (shsps). The molecular size of various families ranges from 15 to 30 kDa (Vierling, 1991). In 1996, Waters *et al.* reported that during a heat stress in plants, the small Hsps are the predominant group of Hsps which are induced as a stress response.

Jong *et al.* (1998) stated that in plants, small Hsps have evolved in parallel after the divergence of plants and animals. Based on localization in different cellular compartments and the amino acid sequence homology all the plant small Hsps encoded by nuclear genes are divided into six classes. The classification also takes into account the immunological cross-reactivity (Waters *et al.*, 1996).

Among all of them, the most abundant and complex member in sHsps in higher plants is the Hsp20 family. In eukaryotes, they are encoded by members of a multi-gene family. The members of Hsp20 family possess a terminal carboxyl-terminal domain of approximately 90 amino acid residues called α-crystallin domain (ACD) which is a conserved sequence (Vierling, 1991). The *Hsp20* gene family not only prevents heat shock but also promote resistance to different stress factors. However, only very little is known about this gene family in rice.

It has been observed that besides providing protective responses against high temperature stress, the Hsp20 proteins are also involved in providing positive responses against various other stress conditions. According to Swindell *et al.* (2007) the Hsps are a group of proteins that are induced to protect plants from the damages caused by various stress factors. In addition to response against the stress caused by heat, one subset of small Hsps or Hsp20 proteins, also act against the stress caused by salt shock, alcohol, amino acid analogs, chilling and oxidative injury, drought and heavy metals (Ouyang *et al.*, 2009). It is thought that Hsp20 proteins play an important role during various stress conditions in plants by helping them to adapt to such situations. Cheong *et al.* in 2002 reported a subset of sHsp that was induced by certain stress factors such as salt, drought, oxidative stress and wounding using microarray studies in *Arabidopsis*. Sachin *et al.* (2007) have observed that in seeds vegetative tissues and other storage organs, various members of the small *Hsp* gene families are developmentally regulated.

Ouyang *et al.* in 2009 identified and characterized 39 OsHsp20 (*Oryza sativa* Hsp20) genes in rice. In the study, each member was described by the gene structure, its expression and localization, and also the phylogenetic relationship using real time PCR. They have used real-time PCR to perform the characterization of the normal and heat shock induced expression of selective OsHsp20 genes.

### 2.2.1.4. Salicylic acid

Salicylic acid (SA) is a phenolic compound which influence the plant growth and development by regulating the plant responses during different biotic and abiotic stress conditions (Miura and Tada, 2014). It regulates photosynthesis, nitrogen metabolism, proline (Pro) metabolism, antioxidant defense system, and plant-water relations under stress conditions to protect the plant system during stress (Khan *et al.*, 2014). In an experiment conducted by Habibi (2012), supplementation of SA to drought stressed *H. vulgare* showed an increased net $CO_2$ assimilation rate attributed

to increased stomatal conductance and a higher plant dry mass. Rice leaves contain very high level of salicylic acid content but its exact role is still poorly understood (Pal *et al.*, 2014).

### 2.2.1.5. Jasmonic acids

Wang *et al.* (2013) reported that a class of polyunsaturated fatty acid derived phytohormones called jasmonic acid (JA) plays an important role in plant growth and defense mechanisms. It supports the plants to regulate its development and growth by responding to environmental fluctuations by providing resistance to abiotic as well as biotic stresses. The action of JA has been observed as either synergistic or antagonistic with other hormones such as gibberellins, salicylic acid, brassinosteroid, ethylene, auxin, and abscisic acid during the signaling process. Such signals regulate gene expression and confer physiological and metabolic adjustments in plants. Understanding of JA pathway in rice is gaining importance in the recent years as rice is one of the major nutritional sources for one third of the global population (Zheng *et al.*, 2015).

### 2.2.1.6. Proline

The amino acid proline, required for primary metabolism has a very high conformational rigidity (Yang, 2009). It has been observed that the amount of proline in higher plants increases during stress conditions. Szabados and Savour (2009) reported its accumulation during drought, high salinity, light and UV irradiation, heavy metals and oxidative stresses and also in response to biotic stress factors. During abiotic stress conditions, proline metabolism has been observed in various plant species. This compound also takes part in protecting the integrity of plasma membrane and act as a scavenger of free radicals (Weltmeier *et al.*, 2006).

## 2.3. Plant Growth Promoting Rhizobacteria (PGPR) and *Pseudomonas fluorescens*

Plant growth promoting rhizobacteria are the soil bacteria inhabiting around/on the root surface and are directly or indirectly involved in promoting plant growth and development via production and secretion of various regulatory chemicals in the vicinity of rhizosphere (Ahmad and Kibert, 2013). Several microbes promote plant growth, and many microbial products that stimulate plant growth have been marketed. In this review we restrict ourselves to bacteria that are derived from and exert this effect on the root. Such bacteria are generally designated as PGPR (Lugtenbergc and Kamilova, 2009). The beneficial effects of these rhizobacteria on plant growth can be direct or indirect. PGPRs are effective in a wide range of crops to enhance its growth and to improve the crop yield (Herman *et al.*, 2008; Minorsky, 2008).

*Pseudomonas fluorescens* is a PGPR bacterium. It colonizes a wide range of ecological niches, including the rhizosphere of plants (Jose *et al.,* 2013). By promoting seed germination, accelerating growth in early stages and inducing root initiation. *Pseudomonas fluorescens* act as a plant growth stimulator (Heinonsalo *et al.*, 2004). Marschner and Timonen (2006) reported the production of various phytohormones including auxins (IAA), gibberellins and cytokinins. They are also reported to produce specific amino acids and other growth promoters that improve plant growth. Matthijs *et al.* (2007) observed that *Pseudomonas fluorescens* have a high capacity for solubilizing phosphate and can also effect in siderophore production. Deveau *et al.* in 2007 reported *P. fluorescens* adhere and colonize the surface of some ectomycorrhizas. This colonization improves the symbiotic relationship between the plant and the ectomycorrhiza, and benefit the host plant. Certain strains of *Pseudomonas fluorescens* promotes the ACC deaminase activity and help plants to resist the stress conditions more efficiently (Arshad *et al.,* 2008).

An additional mechanism by which biocontrol agents can reduce plant biotic and abiotic stresses, enhance plant growth and metabolism, in which *Pseudomonas fluorescens* is a plant growth promoting rhizobacteria, a significant group of bacteria which help in promoting plant growth and inducing systemic resistance (Ganeshan and Arthikala, 2005). *Pseudomonas fluorescens* is also involved in controlling pathogens and forms an integral component of organic farming.

PGPRs are effective in a wide range of crops to enhance its growth and to improve the crop yield (Herman *et al.*, 2008; Minorsky, 2008). *Pseudomonas fluorescens* B16 was isolated from the roots of monocotyledonous plants. However they had been identified to colonize wide range of host plants. The same was found to increase the plant height and other flower and fruit characters of tomato plants (Minorsky, 2008). PGPRs isolated from rice fields were shown to improve plant features including height, root length, and dry matter production of both shoot and root of rice seedlings (Ashrafuzzaman *et al.*, 2009).

Currently biological control agents either antagonistic fungi or bacteria are utilized extensively in plant disease control (Aguilar and Barea, 1997). One of such agents is *Pseudomonas fluorescens*. It has a positive effect on both pathosystem and growth components. *Pseudomonas fluorescens* reduces incubation period of the pathogen, decrease disease intensity and reduces the pathogenic population density both in soil as well as inside the plant (Soesanto *et al.*, 2010).

Under salinity conditions, PGPR have shown beneficial effects in plants on parameters such as germination rate, tolerance to drought, weight of shoots and roots, yield and plant growth (Kloepper *et al.*, 2004; Kokalis *et al.*, 2006).

The combined effect of *Pseudomonas fluorescens* and salicylic acid was observed by Sivakamasudari and Usharani (2012). The plants under study were inoculated with *Rhizoctonia solani* and were given *Pseudomonas fluorescens* as seed

treatment and as soil drenching. The plants were further given salicylic acid treatment after 30 days. It was observed that there was an augmented plant defense enzyme system which improved the systemic resistance due to an altered metabolic state.

Three PGPR strains namely PF1, FP7 and PB2 were tested alone as well as in combinations for knocking off rice sheath blight disease and promoting plant growth under greenhouse and field conditions. The incidence of sheath blight was significantly reduced when the PGPRs were applied in combinations than when applied individually. It was applied as either bacterial suspension through seed, root, foliar and soil application in greenhouse conditions, or as powder-based formulation under field conditions. Mixed application of the three different strains improves the plant development with respect to tiller number, plant stature and grain yield. Application of these strains individually increased the yield by 17.7% whereas it was increased by 25.9% when applied as a mixture. The mixture of PF1 with that of FP7 had given the best results in the experiments conducted by Nandakumar *et al.* in 2001.

Baburao, (2012) analyzed biometric parameters in vegetative stage for Matta Triveni (PTB 45), rice variety. It has been seen that maximum shoot length, root length, no. of tillers, fresh weight, dry weight and recovery in *Pf* treated + drought treated plants and was showed significantly superior over other two such as control and water stressed. In the same study differentially expressed genes were identified and showed all genes were showed upregulated under the influence of *P. fluerescens.*

## 2.4.1. Application of real-time PCR technique for gene expression analysis and validation studies

In rice present studies are mainly concentrated in improving its production as well as its quality. These studies are based on the determination of the functions of various genes present in the rice genome. At present, real-time PCR is one of the best

options available for gene expression analysis due to its high level accuracy, sensitivity, and reproducibility. Li *et al.* (2009) observed that for a reliable result, the importance of choosing the right reference genes for normalization is always critical.

Real-time PCR is a molecular technique based on the polymerase chain reaction. It quantifies the amplification of a specific DNA molecule not the end point of the PCR reaction but during the run of PCR reaction, i.e. in real-time. Qualitative, quantitative and semi-quantitative applications are possible with real-time PCR.

### 2.4.2. Basic terms used in real-time PCR

Data analysis is an important step in real time PCR. The raw data is analyzed to set two important factors namely baseline and threshold values before quantifying the nucleic acid targets in real-time PCR. While using different probes in a single experiment, the baseline and threshold settings are adjusted for each probe separately. Similarly, using SYBR Green detection, the baseline and threshold adjustment for each individual assay is required for analysis of different PCR products from a single experiment. A sigmoidal shaped amplification plot is obtained in the data analysis while using a linear scale wherein the fluorescence is plotted against the number of cycles. Basic terminologies used in data analysis of a real time PCR are explained below.

### 2.4.2.1. Baseline

The baseline is generally measured between cycle number 3 and 15. The fluorescence due to the amplified products is usually not detectable during these cycles. If the expression level of the specific gene is high or if the amount of template is high, the number of cycles used to compute the baseline is reduced. The base is set such that, the amplification plot starts at a cycle number that is greater than the highest baseline cycle number. This is done for individual target sequence using the fluorescence data from the linear scale amplification plot. Automatic and optimized

baseline settings are possible for individual samples using recent versions of software for various real-time cyclers.

### 2.4.2.2. Background

The nonspecific fluorescence detected in the PCR reaction is referred to as background. This may happen due to ineffective quenching of the fluorophore or due the presence of high quantities of double-stranded DNA template when using SYBR Green. The software algorithm of the real-time cycler removes the background component of the signal mathematically.

### 2.4.2.3. Reporter signal

The fluorescent signal that is generated during a real-time PCR is called the reporter signal. It may be either by SYBR Green or by any sequence-specific fluorescently labeled probe.

### 2.4.2.4. Normalized reporter signal (Rn)

A fluorescent dye ROX, is used as an internal reference in some real-time cyclers for normalization of the fluorescent signal. Well-to-well variations which may arise due to pipetting inaccuracies, well position, and fluorescence fluctuations can be corrected by this internal reference. As it is not involved in the PCR reaction directly, its presence does not interfere with real-time PCR assays and the emission spectrum of ROX is completely different from that of the commonly used fluorescent dyes as probes.

### 2.4.2.5. Threshold

The value which lies within the linear region of the amplification plot and which is above the background but below amplification plot is called the threshold.

The threshold is set for each target if quite a few targets are used in same the real-time test.

### 2.4.2.6. Threshold cycle (Ct) or crossing point (Cp)

The cycle at which there is significant increase in fluorescence or the cycle number where the amplification plot crosses the threshold is called the threshold cycle. Ct is required to calculate the initial template quantity and can be a fractional number.

### 2.4.2.7. ΔCt value

The Ct value of the target gene as well as the corresponding endogenous reference genes differ with each other. The reference gene is usually a housekeeping gene. The difference between the two Ct values is called ΔCT value. It is used to normalize the quantity of the initial template used.

$$\Delta Ct = Ct \text{ (target gene)} - Ct \text{ (endogenous reference gene)}$$

### 2.4.2.8. ΔΔCt value

When the difference between the ΔCt values of the sample and that of the reference sample is calculated on an average scale, it is reffered to as the ΔΔCt value. The reference sample is also known as calibrator sample. During the relative quantification procedure, all the samples are normalized to the calibrator sample.

$$\Delta\Delta Ct = \text{average } \Delta Ct \text{ (sample of interest)} - \text{average } \Delta Ct \text{ (reference sample)}$$

### 2.4.2.9. Endogenous reference gene

An endogenous reference gene is a gene whose expression level does not vary with different samples. An example for an endogenous reference gene is the housekeeping genes such as actin. Comparison of the Ct value of the target gene and that of the reference gene is done for the normalization of the expression levels of the specific target gene. The use of the endogenous reference gene is helpful in correcting any possible degradation of RNA and also the variations of nucleic acid content. Inhibitors if present in the sample is corrected with the use of such reference genes. Problems concerned with the efficiency of reverse-transcription, nucleic acid recovery and sample handling are also corrected using the endogenous reference gene.

*eIF-4a* and *ACT1* genes were observed to be the best reference genes in rice according to a study by Li *et al.* (2009). This was confirmed by assessing nine frequently used housekeeping genes in rice for their stability of expression using geNorm software at various developmental stages. However, the two common reference genes *18S* and *25S rRNA*s, showed least stability in expression. Moreover, to obtain a reliable result during normalization of temporal and spatial expression analysis, it is necessary to use multiple but appropriate reference genes collectively.

The expression of those genes having most stable expression has to be used for normalizing the results of real-time PCR. Jain *et al.* (2006) conducted the gene expression analysis in a group of 25 rice samples from different stages of plant growth and development. They assessed 10 housekeeping genes, such as *18S rRNA, 25S rRNA, ACT11, UBC, UBQ10, UBQ5, GAPDH, eIF-4a, eEF-1a* and *b-TUB* which are most frequently used in expression studies. The different tissue samples analyzed had different expression levels with *UBQ5* and *eEF-1a* showing most stable expression across all examined tissue samples. On the other hand, for the samples

from those plants grown under various environmental conditions, *18S* and *25S rRNA* exhibited the highest stable expression.

### 2.4.3. Expression analysis of various stress responsive genes using real-time PCR

A multi-parallel qRT-PCR platform used for expression studies was established for more than 2500 rice transcription factor genes. A detection method that is specific and consistent for the expression of rice transcription factor genes is possible by this resource (Caldana *et al*, 2007).

Different OsHSP genes have different roles in plant responses against abiotic stresses as well as in plant growth and development. Zoua *et al.* (2008) on analyzing expression patterns of nine heat shock protein genes (OsHSPs) in rice by semi-quantitative reverse transcriptase polymerase chain reaction (RT-PCR) observed distinctive expressions in different organs. Abiotic stresses and abscisic acid (ABA) treatment influence the expressions of the OsHSP genes. They were induced strongly by heat shock treatment, but not by cold treatments. Salt stress treatment increased the transcripts of OsHSP80.2, OsHSP71.1 and OsHSP23.7 whereas the expressions of OsHSP80.2 and OsHSP24.1 genes were enhanced on treatment with 10% PEG. In the experiment, when OsHSP71.1 gene was induced by ABA, OsHSP24.1 was suppressed by the same.

For validating 27 rice genes that encode different classes of enzymes in the developing seeds and leaves, such as ADP glucose pyrophosphorylase (AGPase), starch branching enzyme, starch synthase, starch phosphorylase and starch debranching enzyme, Ohdan *et al.* (2005) used quantitative real-time PCR. The gene expression which was tissue and developmental stage-specific showed four expression patterns in the seeds. Group 1 genes, concerned with the structuring of fundamental cell machineries, *de novo* synthesis of glucan primers, and beginning of

starch granule formation were expressed very early in grain formation stage. Group 2 genes, were expressed heavily through the entire endosperm growth stage. Group 3 genes, had transcripts that were low at the onset but increased at the commencement of starch synthesis in the endosperm. They are believed to play critical roles in endosperm as well as starch synthesis. Group 4 genes, complexed with the production of starch in the pericarp, were expressed scantly, mainly at the onset of grain development.

Exposure to salt, drought and various temperature conditions differentially regulate the different annexin genes in *Arabidopsis thaliana* (Cantero *et al.*, 2006). A study was conducted using quantitative real time reverse transcription PCR to assess eight different annexins in *A. thaliana*, using samples from different tissues after exposing to different stimuli. The expression levels were assayed in dry seeds, germinating seeds and also in one week old seedlings. All the eight annexins under study were expressed in germinating seeds which increased after a week. The relative amount of annexins varied in roots, cotyledons and hypocotyls. Red and far red light treatments also affect the expression of annexins. On monitoring the annexin expression changes with respect to different of abiotic stress conditions, it was observed that the expression profile of most of the annexin genes in *Arabidopsis* were regulated differentially which indicated its role in stress responses.

Certain genes involved in some of the biological functions such as signal transduction, metabolism, transport and transcription, are synchronized by auxin content. A study conducted by Jain and Khurana (2009), identified and characterized the members of the GH3, Aux /IAA and SAUR gene families in rice. At various stages of vegetative and reproductive development including the panicle and seed formation, the expression patterns of various auxin-responsive genes were recognized in the study and those belonging to the members of the GH3, Aux/IAA, SAUR and ARF gene families were further analyzed utilizing the microarray analysis. The

expression mode was tissue and developmental stage specific and was confirmed by real-time PCR. The differential expression profiles inveterated their role in diverse developmental processes. A crosstalk between auxin and abiotic stress signaling was indicated during various abiotic stress conditions by the differential expression of several auxin-responsive genes.

A study was conducted by Basu and Roychoudhury (2014) in three varieties of *indica* rice including salt sensitive IR-29 and salt tolerant Pokkali, and Nonabokra to understand their expression profiles after different abiotic stress treatments. The treatments were salinity, dehydration, cold and exogenous ABA application. On considering the transcriptional profiles and protein expression of the candidate genes, namely *NHX-1, NAC-1, HKT-1, OSBZ8, SOS-3, SAPK5, SAPK7 , Rab16A, CRT/DREBP, WRKY24, WRKY71* and *DREBP2*, the corresponding candidate proteins OSBZ8, SAMDC, and GST, considerable differences were revealed between the salt-sensitive as well as salt-tolerant rice varieties. In the salt tolerant varieties, the expression level was higher even at constitutive level. However, in case of salt sensitive variety, the expression was induced only by the respective stress signals. Samples were collected from shoot and/or root tissues at different time periods of exposure to stress conditions.

In the same study, it was observed that there was an increase in the level of transcripts with increase in exposure time to the stress. The expression level was at the peak after 24 hr of stress exposure. The existence of conserved sequence motifs that are indispensable for abiotic stress response was confirmed by *in silico* analysis of the upstream regions of all the genes under study. The multiple stress tolerance in *indica* rice varieties have been conferred by the over representation of the transcriptome and proteome with increase in exposure time (Basu and Roychoudhury, 2014).

In rice, ornithine 1-aminotransferase gene (*OsOAT*) is a direct target of the stress-responsive NAC transcription factor SNAC2 and the over expression of *OsOAT* gene improve drought, osmotic and oxidative stress tolerance. This was confirmed by You *et al.* (2012) by the characterization of the gene which is responsible for both multiple stress and phytohormone treatment. Induction of the gene during drought may be ABA-dependent or ABA-independent. Under normal growth conditions, over expression of *OsOAT* gene increases the 1-OAT activity and proline accumulation. Glutathione content and the activity of reactive oxygen species ROS-scavenging enzymes, is also high in *OsOAT* overexpressing plants. Enhanced ROS-scavenging capacity and proline pre-accumulation are the two main mechanisms by which *OsOAT* overexpressing plants confers stress tolerance.

Wang *et al.* (2014) reported the use *RBE4* as a reference gene in rice for selecting homozygous lines in a multiple transgenic stacking experiment using quantitative real-time PCR. It is a fast and accurate method and can be applied to resolve the gene stacking even up to three transgenes within four generations. The selection accuracy was 100 % while considering a single locus whereas for two loci it was about 92.3 %. The method is more accurate, fast and consistent than the current transgenic methodologies and can be used efficiently for the selection of homozygous plants.

The gene *sos101* in *Arabidopsis thaliana* (*Atsos101*) codes for WD40 repeat containing protein which is a salt stress induced gene. Rice genome contains four orthologues for this gene known as *Ossos101*. In three rice genotypes, the quantitative real-time RT-PCR analysis showed salt responsive temporal gene expression of salt tolerance for the orthologues at two salt concentrations namely 50 mM and 100 mM NaCl. Phylogenic and amino acid sequence analysis revealed that the WD40 repeat domain in SOS101 protein and its rice orthologue have highly conserved regions. The orthologues in other crop plants also showed highly

conserved regions in the WD40 repeat domain in SOS101 protein (Kumar *et al.*, 2015).

## 2.5.1. Transcription factors

A transcription factor (TF) is a protein that has the ability to bind to specific sequence of DNA and by this means it controls the movement of genetic information from DNA to mRNA. Transcription factors execute this function either alone or in combination with other proteins. They can act as either an activator and promote the transcription or can act as a repressor and block the transcription of genes at the downstream. Plant stress responses are regulated by multiple signalling pathways that activate gene transcription and its downstream machinery. Plant genomes consist of a large number of transcription factors (TFs). For example, *Arabidopsis* has about 5.9% of its genome dedicated for the coding of for more than 1,500 TFs (Riechmann *et al.*, 2000). Among these, many of the TFs come under a few but large multigene families such as MYB, AP2/EREBP, bZIP and WRKY. The response of each member of the same family maybe different to different stress conditions and alternatively, some stress responsive genes may allocate the same TFs, as indicated by the considerable overlap of the gene-expression patterns produced as a result of different stress signals (Kreps *et al.*, 2002).

The dehydration-responsive transcription factors (DREB) and C-repeat, binding factors (CBF) attach themselves to DRH and CRT *cis*-acting elements containing the conserved motif (CCGAC). CBF1, CBF2 and CBF3 are the individual members of the CBF/DREB1 and are themselves stress-inducible. AP2/EREBP multi gene family encodes the DREB/CBF proteins and regulate the transcription of several genes such as rd29A, rdl7, cor6.6, cor15a, erdl0, kinl, kin2 and others as a cold and water stress response (Thomashow *et al.*, 2001).

Recently (2006), in transgenic *Arabidopsis*, Sakuma *et al*, reported an active form of DREB2 that was capable of *trans*-activating the target stress-inducible genes which further developed the drought tolerance. The expression of the DREB2 protein is observed under normal growth conditions. However it is activated through the post-translational modifications in the early stages of the osmotic stress response as an effect to the osmotic signals.

The NAC proteins are a family of plant specific transcription factors, also known as the No Apical Meristem (NAM) proteins. The NAC protein, *ANAC072/RD26*, may act as a TF in response to ABA and drought stress, Overexpression of this gene results in up-regulation of a large range of stress-inducible genes and hypersensitivity to ABA (Fujita *et al.*, 2004).

Transcription factors also act as repressors of the water stress response. The AP2 domain transcription factor, ABA repressor 1 (ABR1) is up-regulated by ABA and drought stress (Pandey *et al.*, 2005). The *abr 1* null mutant is stress sensitive and has increased expression of stress response genes under stress conditions (Partdey *et al.*, 2005).

Another negative regulator of ABA response is the AP2 transcription factor ethylene response element binding factor 7 (ERF7). Over expression of ERF7 results in increased stomatal aperture and drought sensitivity (Song *et al.*, 2005).

Another well characterized group of TFs involve in the ABA response element binding proteins *(AREB/ABF)*, a sub-family of bZIP transcription factors. The expression of all four ABF transcription factors is induced by ABA treatment (Choi *et al.*, 2000). *ABF1* is also induced by cold stress; ABF2 (AREB1) and ABF 3 are both are induced by drought, cold stress and salt stress (Singh and Laxmi, 2015). All TFs, except ABF1 have a role as positive regulators if growth, stomatal control and seed germination (Kang *et al.*, 2002). The role of ABF1 appears to be limited to

seed germination (Finkelstein and Lynch, 2000).

TFs containing zinc fingers are also involved in drought responses. One such zinc finger protein, ZAT12, is induced by water stress, as well as many other stress conditions (Davletova *et al.*, 2005). This protein appears to be a positive regulator as over expression results in osmotic stress tolerance, however it is not known if it acts in an ABA-dependent or ABA-independent pathway (Davletova *et al.*, 2005). The role of two other zinc finger TFs, AZF2 and ZAT 10, is still being investigated but they appear to be negative regulators of the water stress response (Sakamoto *et al.*, 2004).

## 2.6.1. Combinatorial control of gene expression

Transcriptional control of genes involved in stress responses is a vital part of the plant response to a broad spectrum of stresses in which combinatorial control plays a major role. Combinatorial control can be defined as the involvement of a distinct number of transcription factors in varying combinations to produce a wide spectrum of expression patterns (Singh, 2005). A pair of regulatory proteins called transcription factors bind to *cis*-regulatory elements which are present on promoter region of a gene would regulate the transcription of that particular gene (Bhattacharjee *et al.*, 2013).

Transcription factors binds to specific DNA in a sequence specific manner. While these sites are degenerate, they do have a certain level of consensus. Such sites, having a fixed consensus region and some variable regions are called *cis*-regulatory elements. There are regions on the DNA, where combinations of *cis*-elements occur in clusters. These *cis*-regulatory modules affect transcription, even if they are located far away from the target gene. Which *cis*-regulatory module would influence the transcription of a gene at a particular time is determined by the combination of DNA binding transcription factors (DBTFs) and co-regulators

attached to a *cis*-regulatory module at that time. The activity of *cis*-regulatory modules might also change when different subsets of *cis*-elements of the *cis*-regulatory modules are bound by transcription factors. Therefore, it is the number and organization of the *cis*-elements that determine the activity and specificity of a *cis*-regulatory module (Bhattacharjee *et al.*, 2013).

Furthermore, for identification and characterization of TF-DNA interactions, experimental and computational methods are used. Experimental methods can be subdivided into *in vitro* and *in vivo* based approaches. The most commonly used method for assessing the genome-wide TF binding is based on chromatin immunoprecipitation (ChIP) integrated with either DNA microarray technology or more recently with massive parallel sequencing (Geertz and Maerkl, 2010).

Weltmeier *et al.* (2006) demonstrated the transcriptional control of *ProDH* gene, encoding the enzyme proline dehydrogenase which can degrade L-proline during hypo-osmotic conditions in *Arabidopsis* plants and reported that AtbZIP53 and AtbZIP10 (TFs) regulates the expression of *ProDH* gene by combinatorial interactions using both *in vitro* and *in vivo* experiments.

### 2.6.2. Bioinformatics approach

It is difficult to identify and validate the individual genes involved in combinatorial regulatory events using wet lab experiments. Bioinformatics tools provide a better and robust platform to acquire biological information and to understand the molecular elements defining a biological problem by integrating available whole genome data sets and tools. Assimilation of large quantity of data from numerous experimental and bioinformatics resources is possible through *in silico* analysis.

## 2.6.2.1. Databases for plant stress response

Plants are frequently subjected to both biotic and abiotic stresses which reduce its productivity significantly (Boyer 1982). There are a large number of databases providing biological information related to plant stresses like PHYTOPROT (Zadeh *et al.*, 2004), PESTD (Jayashree *et al.*, 2006), Plant Stress-Responsive Gene Catalogue (Wanchana *et al.*, 2008), PhytAMP (Hammami *et al.*, 2009), QlicRice (Smita *et al.*, 2011), Plant Stress Gene Database (Prabha *et al.*, 2011), Rice SRTFDB (Priya and Jain 2013), STIFDB2 (Naika *et al.*, 2013), PASmiR (Zhang *et al.*, 2013), Arabidopsis Stress Responsive Gene Database (Subhomoi *et al.*, 2013) and PRGdb 2. 0 (Sanseverino *et al.*, 2013).

### 2.6.2.1.1. Stress-Responsive Transcription Factor Database V 2.0 (STIFDB2

Understanding the various plant stress responses necessitates the detailed knowledge about various gene regulation mechanisms operating during the stress conditions. Stress-responsive Transcription factor database (STIFDB) is a platform which catalogue information on genes involved in stress response and their transcription factor binding sites in *A. thaliana* described by Shameer *et al.* (2009). The updated version of STIFDB is STIFDB2 and it includes 15 stress signals, 5984 genes for stress response and 10 specific transcription factor families. These are concerned with the stress responses in *A. thaliana* and six specific transcription factor families in *O. sativa* (Naika *et al.*, 2013).

### 2.6.2.1.2. AthaMap Database

To identify the target genes of specific transcription factors, positional information of its binding site in the whole genome is a useful approach. Such data is also used to generate models on the gene regulation under the study. AthaMap is a database capable of generating such positional maps for TFBS in the *Arabidopsis thaliana* genome. It was developed using the available binding sites which have been

identified by conducting random binding site selection experiments. Alignment matrices are generated using the sites obtained from these experiments. A program called PASTER utilizes these matrices to spot the genomic positions of TFBS within the *A. thaliana* genome. Considering that sequence recognition is not specific for species but maybe similar for the members in the same plant TF family, the matrix-based searches are carried out with transcription factors from many diverse plant species. Positional information is then imported into the AthaMap database which is then displayed online either by giving the gene model number (AGI) that can be obtained from the TAIR database or by entering the specific chromosomal position (Steffens *et al.*, 2004).

Earlier version of AthaMap enclosed information of $7.4 \times 10^6$ putative binding sites for 36 diverse transcription factors. This represented 16 TF families. The combinatorial *cis*-regulatory elements included were more than $1.8 \times 10^5$. Latest version of AthaMap includes a transcription factor binding site map of target genes of *Arabidopsis thaliana* based on both *in vivo* and experimentally verified binding sites. The database is now equipped with more than eighty nine thousand TFBS based and describes experimentally determined sites for 48 *Arabidopsis thaliana* TFs constituting 13 different TF families. Bulow *et al.* (2006) reported that all the genes annotated in the AthaMap database is linked to various other databases which provide useful information on *A. thaliana such as* TAIR, TIGR and MIPS.

### 2.6.2.1.2.1. AthaMap search tool

AthaMap allows the user to opt for a specific quality of the TFBS in the database so that highly conserved TF binding sites can be determined by matrix-based screening. High sequence conservation is denoted by higher scores which allow the exclusion of TFBS with lower conserved sequences. As per the TAIR annotations, sequences, exons, introns, CDS and UTRs are shown in various colours once the search is completed.

The search program determines the individual position, particular site score, threshold and also the maximum score possible score for the site (Hertz and Stormo, 1999). It is possible to compare the score of the site as well as the maximum score. A highly conserved binding site is represented by a high score that is close to the maximum score whereas a binding site with lower conserved regions is represented by a low score nearer to the threshold value. By comparing the particular score of a site with that of the threshold and maximum obtained in PASTER, the quality of a putative binding site can be judged (Bulow *et al.*, 2010).

### 2.6.2.1.3. Identification of combinatorial *cis*-regulatory elements

Expression of different genes is regulated mostly by the transcription factors (TFs) binding to the *cis* -regulatory sequences. The TFs taking part in the gene regulation of the neighbouring genes can be assumed using these regulatory sequences. Steffenes *et al.* (2005) identified about 1500 TFs in *Arabidopsis thaliana*, corresponding to 5% of the total genes. MYB and MYB-related (190 members), AP2/ EREBP, bHLH, NAC, C2H2 (Zn), HD, MADS, bZIP and WRKY are the largest TF families reported. Transcription factor binding sites map of *A. thaliana* is of non-restrictive nature which allows the detection of regulatory sequences within transcribed as well as coding regions (Steffens *et al.*, 2005).

### 2.6.2.1.3.1. A web based tool for the identification of co-localizing TFBS

Identification of combinatorial elements is achieved by the co-localization tool in the AthaMap database. Two similar or dissimilar transcription factors may be obtained from the drop down list for the co-localization analysis. A restriction score is used to restrict to the highly conserved binding sites, for the TFs with their binding sites which have been identified by matrix-based screenings. The score restrictions are not required in case of TFBS obtained from TBP and CBF because the TFBS for TBP (TATA box) and CBF (CAAT box) are positionally definite. Similarly, it is not

being applied to those sites determined by pattern search also. Co-localization can also be done with the Transcription Start Sites (TSS) annotated to AthaMap. Maximum spacer length allowed is between 0 and 100bp (Steffens *et al.*, 2005).

The co-localization analysis result obtained on the same page provides particulars on the co-localizing TFBS, their absolute chromosomal positions and the orientation of the sites. It also provides information about the neighbouring genes and their positions in relation to the start site for translation. In the co-localization result list there are highlighted regions. These regions correspond to the absolute with the respective co-localizing binding sites. A summary of the total number of co-localizations obtained can be viewed using the option "show overview" displayed on the result page. It can be viewed for individual spacer length up to the maximum spacer length selected (http://www.athamap.de/description_colocalization.php).

Baburao (2012) identified differentially expressed genes by DD-RT-PCR and transcript derived fragments (TDFs) found nucleotide sequence similarity (in both BLAST N and BLAST X) with cytochrome oxidase subunit I, protein kinases, ABA responsive element protein, heat shock proteins and MYB transcription factor which are all operating in major signaling pathways.

# Materials and Methods

# 3. MATERIALS AND METHODS

The study on "Validation of identified genes for water stress in rice (*Oryza sativa* L.) mediated by *Pseudomonas fluorescens*" was carried out at the Centre for Plant Biotechnology and Molecular Biology (CPBMB), IT – BT Complex, College of Horticulture, Vellanikkara during period from August, 2014 to July, 2016. Materials used and methodologies adopted for the study is described in this chapter.

### 3.1.1. Plant material and *Pseudomonas fluorescens*

Rice (*Oryza sativa* L.) variety Matta Triveni (PTB-45), a popular upland rice variety of Kerala which is susceptible to drought was used in the present study. The seed material was obtained from the Department of Plant Breeding and Genetics, College of Horticulture, Kerala Agricultural University, Vellanikkara. *Pseudomonas fluorescens* strain pfl (KAU strain) was obtained from the Centre for Plant Biotechnology and Molecular Biology (CPBMB), IT-BT Complex, College of Horticulture, Vellanikkara.

### 3.1.2. Identified gene sequences

Nucleotide sequences of Transcript Derived Fragments (TDFs) of differentially expressed genes were identified by Baburao (2012) was used for similarity search, quantitative expression study and for in-silico identification of TFBSs and combinatorial cis-regulatory elements.

### 3.1.3. Chemicals, glassware and plastic ware

All the chemicals used in the study were of good quality (AR/GR grade) obtained from various firms such as Invitrogen, MERK India Ltd, Sisco Research Laboratories, Himedia and Sigma. Molecular grade enzymes, buffers and DEPC were supplied by Bangalore GeNei Ltd. All the plastic wares were obtained from Tarson India Ltd. and borosilicate glass wares were used. RevertAid first strand cDNA synthesis kit was purchased from Thermo Scientific and SYBR® Premix Ex Taq ™ II (Tli RNaseH Plus) for real time PCR was obtained by Takara Bio India Ltd.

For sterilization of glass wares and supplies hot air oven and autoclave were used. High precision electronic balance (Shimadzu), pH meter (EuTech Instruments PC 510), micropipettes (Eppendorf), Icematic (F100 compact) and high speed refrigerated centrifuge (KUBOTA 6500) were used for total RNA extraction. Extracted RNA samples were stored at -80°C and reagents were stored at 4°C in refrigerator (Samsung). RNase ZAP was obtained from Ambion, Inc, USA. The RNA and cDNA samples were separated by gel electrophoresis (BioRad). The gels were visualized and converted into digital image data using the GS-900™ calibrated densitometer (BioRad). Softwares used for the analysis of the digital image data are Quantity one and PD Quest. First strand cDNA samples were amplified by using ProFlex 3 x 32-well PCR System and gradient PCR was done by using SureCycler 8800 from Agilent technologies. Applied Biosystems 7300 real-time PCR System was used for quantitative gene expression analysis and also used optical 96-well reaction plate with barcode (PCR compatible DNA/RNA/RNase free) and splash free support base by MicroAmp™.

Instruments available at CPBMB were utilized for the work while Bioinformatics softwares and Applied Biosystems 7300 Real-Time PCR System were accessed from Distributed Information Centre (DIC).

## 3.2. Methods

### 3.2.1. Treatments for the experiment

The plants were given three treatments. Plants without any treatment served as absolute control ($T_1$). Water was withheld in second treatment ($T_2$) and in third treatment water was withheld in plants for which *Pseudomonas fluorescens* was applied ($T_3$). Five pots were maintained for each treatment and four plants were maintained in each pot with the spacing of 15 cm each. The design used was completely randomized design (CRD). Plants were given two applications of *Pseudomonas fluorescens* (*Pf*), i.e., seed treatment and foliar spray. Seeds of rice variety Matta Triveni were soaked for 24 hours in *Pf* solution (10 g ($1 \times 10^{10}$CFU/g)/litre/kg of seeds) and sowed in tray containing red soil. Pots were filled with red soil, clay and cow dung in 1:1:1 ratio and then seedlings were transplanted to the pots. Foliar spray of *Pf* (two percent) was given for plants 45 days after sowing. Water was withheld till the leaves got completely rolled, after which leaf samples were taken for expression studies. Plants were harvested 105 days after sowing and biometric analysis was done after harvest.

**Table 1. Details of treatments given to plants**

| Treatments | Type of plants |
|---|---|
| Treatment 1 ($T_1$) | Absolute Control |
| Treatment 2 ($T_2$) | Water stressed |
| Treatment 3 ($T_3$) | Water stressed + *Pseudomonas flurescencens* (*Pf*) treated |

**Table 2. Details of *Pf* treatments given to T₃ plants**

| Sl. No. | Treatment | Days after sowing | Dosage of *Pf* |
|---------|-----------|-------------------|----------------|
| 1. | Seed treatment | - | 10 g ($1\times10^{10}$CFU/g)/liter/kg of seeds |
| 2. | Foliar spray | ~50 days | 2 Percent |

### 3.2.2. Imposition of water stress

Water was withheld continuously for 15 days during reproductive stage (panicle initiation stage) one week after foliar spray. Plants exhibited leaf rolling score as per Standard Evaluation System (IRRI, 1996) was taken for total RNA isolation and biometric parameters were recorded after harvest.

**Table 3. Leaf rolling scores description**

| Scale | Description |
|-------|-------------|
| 0 | Leaves healthy |
| 1 | Leaves starts to fold |
| 3 | Leaves folding (deep V- shaped) |
| 5 | Leaves fully cupped (U- shaped) |
| 7 | Leaves margins touching (O-shaped) |
| 9 | Leaves tightly rolled |

### 3.2.3. Measurement of biometric parameters

The plant observations were taken on shoot length, root length, fresh weight, dry weight, yield and 1000 seeds weight for biometric analysis in control, water stressed and water stressed + *Pf* treated plants after harvest. Statistical analysis was carried out using Duncans multiple range test.

### 3.3.1. Similarity search of identified TDFs

The nucleotide sequence of all the sequences were compared with DNA, EST, and protein sequences from rice genome databases such as TIGR (The Institute of Genome Research) rice (http://blast.jcvi.org/eukblast/index.cgi?project=osa1), Plant Transcription Factor Database V3.0 (http://planttfdb.cbi.pku.edu.cn/), DRTF (Dtabase of Rice Transcription Factor; http://drtf.cbi.pku.edu.cn/), RGAP (Rice Genome Annotation Project; http://rice.plantbiology.msu.edu/index.shtm) database using BLASTX (nucleotide translated query vs. protein database) search for its homology with the sequence of a gene already recorded in the rice genome databases. Proteins with BLASTX scores above 50 bits and significantly low expected value (E-value) were designated as known functions.

### 3.4.1. Total RNA extraction

#### A. General precautions

Precautions were strictly followed to avoid contamination and degradation during RNA isolation to get intact and prime quality RNA. All the glassware, mortar and pestle, microtips and microcentrifuge tubes were treated with 0.1 % DEPC treated water overnight and then double autoclaved. Solutions like 70 per cent ethanol and MOPS buffer were prepared with autoclaved DEPC treated water. Gloves were worn throughout the experiment.

## B. Sample collection

Young tender leaves from all the three different treatments i.e, control plants ($T_1$), water stressed ($T_2$) and water stressed + *Pseudomonas fluorescens* treated plants ($T_3$) were collected early in the morning for RNA isolation. Leaves were then washed quickly with DEPC treated water.

## C. Isolation of total RNA

Isolation of good quality and quantity of RNA is a pre requisite for synthesis of first strand cDNA. Total RNA was extracted from all the three treatments using TRI reagent method.

### Reagents

TRI reagent (Sigma-Aldrich)

Chloroform

Ice cold isopropanol

70 per cent ethanol (v/v)

Autoclaved DEPC treated water

### Procedure

1. 0.1 g of leaf tissue was weighed and ground well in liquid nitrogen using mortar and pestle.
2. One ml of TRIzol per 0.1 g of leaf tissue was added and the homogenate was incubated till the solution became brown.
3. The homogenate was transferred to a 2 ml microcentrifuge tube.
4. The contents were then centrifuged at 12,000 g at $4^0$C for 10 min.

5. The supernatant was transferred to a 2 ml microcentrifuge tube.

6. 200 µl of chloroform was added to the supernatant and the contents were mixed by shaking. It was then kept at room temperature for 10 min.

7. The contents were then centrifuged at 12,000 g at $4^0C$ for 15 min.

8. The supernatant was transferred to a fresh 1.5 ml microcentrifuge tube.

9. 500 µl of isopropanol was added to the supernatant. The contents were mixed by slight inversion then incubated for 10 min. and centrifuged at 12,000 g at $4^0C$ for 10 min.

10. The supernatant was discarded and the pellet was washed with one ml of 75 per cent ethanol by centrifugation at 7500 g at $4^0C$ for 10 min.

11. The supernatant was discarded and the pellet was air dried under laminar air flow for 15 min.

12. The pellet was dissolved in 15 µl autoclaved DEPC treated water.

### 3.4.2. Quality and quantity analysis of total RNA

### 3.4.2.1. Quality analysis by formaldehyde-agarose gel electrophoresis

One per cent denaturing gel was prepared and electrophoresis was performed to check the quality of RNA, following the method as described by Sambrook *et al.* (1989).

### Materials for formaldehyde-agarose gel electrophoresis

1. Agarose (GeNei, Low EEO)
2. 10X MOPS buffer (pH 7.0)
3. 6X Loading/Traking dye
4. Electrophoresis unit (Biorad), power pack, casting tray, comb

5. Ethidium bromide solution (0.3µg/ml)

6. Gel documentation and analysis system (Biorad)

Chemical composition of buffer and dyes used in gel electrophoresis are given in Appendix II.

**Procedure**

1. 800 ml of 1X MOPS buffer was prepared by diluting 10X stock solution with autoclaved DEPC treated water. This buffer was used to fill the electrophoresis tank and to prepare the gel.

2. The open ends of gel casting tray were sealed with cello tape placed on a horizontal platform. The comb was placed at one end properly.

3. 0.8 per cent agarose was dissolved properly in 1X MOPS buffer by boiling in a microwave oven.

4. The solution was poured into the gel casting tray and allowed to solidify.

5. After the gel was completely set (20 to 30 min. at room temperature), the comb and cello tape were removed.

6. The gel placed in the electrophoresis tank containing 1X MOPS buffer (with the wells near the cathode) such that it is submerged to a depth of 1 cm.

7. 1 µl of 6X gel loading dye was mixed with 4 µl (20 µg/lane) of RNA and then loaded in the wells. λ DNA/*Eco* RI + *Hind* III double digest (Bangalore GeNei) marker was loaded in the first well.

8. The cathode and anode were connected to the power pack and the gel was run at a constant voltage of 25 volts.

9. The power was switched off when the tracking dye moved more than half of gel from the well.

10. The gel placed in a gel documentation system, bands were visualized under UV light and the gel image was documented.

### 3.4.2.2. Spectrophotometric analysis of total RNA

The samples which gave three discrete bands specific to RNA on an agarose gel electrophoresis were further analyzed and quantity and quality of RNA was estimated using NanoDrop® ND-1000 spectrophotometer (NanoDrop Technologies Inc., USA). Before taking sample readings, the instrument was set to zero by taking 1 µl double autoclaved DEPC treated distilled water as blank. One microlitre of RNA from each sample was used for quantification. The absorbance of nucleic acid samples was measured at a wavelength of 230/280, $OD_{230}/OD_{280}$ and $OD_{260}/OD_{230}$ ratios were recorded to assess the purity of RNA. A ratio of 1.8 to 2.0 for $OD_{260}/OD_{280}$ and above 1.0 for $OD_{260}/OD_{230}$ indicated good quality RNA. Good quality total RNA from all three treatments was extracted separately.

### 3.4.3. Reverse Transcription Polymerase Chain Reaction

Reverse transcription polymerase chain reaction is one of many variants of polymerase chain reaction. It involves an initial incubation of RNA sample with a reverse transcriptase enzyme and oligodT primer. This PCR is widely used in expression profiling, to determine the expression of a gene and for diagnosis of RNA viruses.

### 3.4.3.1. First strand cDNA synthesis

The isolated total RNA predominantly consists of rRNA, tRNA and mRNA. The isolation of non-degraded mRNA is very essential for successful first strand synthesis and subsequent PCR amplification. Mature mRNA molecules carry poly (A) at their 3' termini, to which oligo dT primers anneal. Reverse Transcriptase enzyme can synthesize cDNA from isolated RNA under favorable conditions. The single stranded cDNA was used for quantitative real time PCR without further purification.

Total RNA was used to synthesize first strand cDNA using Thermo Scientific RevertAid H Minus First Strand cDNA kit (Thermo Scientific) as per the manufacturer's guildlines.

### Materials

1. RNA sample

2. Oligo (dT)$_{18}$ primer(500µg/ml)

3. Nuclease free water

4. First strand buffer (5X)

5. 10 mM dNTP mix (2.5 mM each)

6. RiboLock RNase Inhibitor (20 units/µl)

7. RevertAid H Minus M-MuLV Reverse Transcriptase (200 units/µl)

**Procedure**

1. 3µl of total RNA (~1 µg) was added to a 0.2 ml micro centrifuge tube.

2. Added 1 µl of oligo $(dT)_{18}$ primer.

3. Added 12 µl nuclease free water.

4. The reaction mix was centrifuged briefly and incubated at 65°C for 5 min in a thermocycler and then quick chilled on ice.

5. The reaction mix was spanned briefly to collect the content at the bottom of the tube and the reagents were added one by one as follows

   ➢ 4 µl First strand buffer (5X)

   ➢ 1µl RiboLock RNase Inhibitor (20 units/µl)

   ➢ 2 µl dNTP mix (10 mM)

   ➢ 1 µl RevertAid H minus M-MuLV Reverse Transcriptase (200 units/µl)

6. The content was mixed gently and centrifuged.

7. The reaction mix was incubated for 60 min. at 42°C.

8. Terminated the reaction by heating at 70°C for 15 min.

### 3.4.3.2. Confirmation of first strand cDNA

Three samples of single stranded cDNA were amplified by normal PCR with endogenous housekeeping gene Actin specific primers.

**Table 4. Actin gene specific primers used for cDNA confirmation and normalization**

| Orientation | Name | Sequential information (5' to 3') | No. of bases |
|---|---|---|---|
| Forward | Act F | TCCATCTTGGCATCTCTCAG | 20 |
| Reverse | Act R | GTACCCGCATCAGGCATCTG | 20 |

### Procedure

Master mix was prepared as indicated in the Table 5. given below and spun briefly for proper mixing. Then aliquot of the master mix was added to each tube and cDNA template was added separately. PCR reaction was done as per the PCR profile given in Table 6.

**Table 5. Composition of reaction mixture for PCR used for first strand cDNA amplification and normalization**

| Components | Volume per reaction (µl) |
|---|---|
| 10X reaction buffer A | 2 µl |
| MgCl$_2$ (25 mM) | 2 µl |
| dNTP mix (10 mM) | 1.5 µl |
| Forward primer | 1.5 µl |
| Reverse primer | 1.5 µl |
| Sterile distilled water | 10.1 µl |
| Taq. DNA polymerase | 0.4 µl |
| cDNA | 1 µl |
| Total | 20 µl |

**Table 6. Temperature profile for first strand cDNA amplification and normalization with actin primers**

| Sl. No. | Step | Temperature | Time | No. of cycles |
|---|---|---|---|---|
| 1. | Initial denaturation | 94°C | 4 min | 1 |
| 2. | Denaturation | 94 °C | 45 sec | |
| 3. | Annealing | 55 °C | 1 min | 25 |
| 4. | Extension | 72 °C | 2 min | |
| 5. | Final extension | 72 °C | 8 min | 1 |
| 6. | Final hold | 4 °C | ∞ | |

PCR product was stored at -20 °C, and then electrophoresed using 1.2 % agarose gel electrophoresis and the gel image was documented.

### 3.4.3.3. Quality analysis of amplified cDNA by agarose gel electrophoresis

1.2 per cent agarose gel was prepared and electrophoresis was performed to check the quality of cDNA.

**Materials for agarose gel electrophoresis**

1. Agarose (GeNei, Low EEO)
2. 50X TAE buffer (pH 8.0)
3. 6X Loading/Traking dye
4. Electrophoresis unit, power pack (Biorad), casting tray, comb
5. Ethidium bromide solution ($0.3\mu g/ml$)
6. Gel documentation and analysis system (Biorad)

**Procedure**

1. 800 ml of 1X TAE buffer was prepared by diluting 50X stock solution with double distilled water. This buffer was used to fill the electrophoresis tank and to prepare the gel.
2. The open ends of gel casting tray were scaled with cello tape placed on a horizontal platform. The comb was placed at one end properly.
3. 1.2 per cent agarose was dissolved properly in 1X TAE buffer by boiling in a microwave oven.
4. The solution was poured into the gel casting tray and allowed to solidify.
5. After the gel was completely set (20 to 30 min. at room temperature), the comb and cello tape were removed.
6. The gel placed in the electrophoresis tank containing 1X TAE buffer (with the wells near the cathode) such that it is submerged to a depth of 1 cm.

7. 2 μl of 6X gel loading dye was mixed with 20 μl of amplified product and then loaded in the wells. λ DNA/*Eco* RI + *Hind* III double digest (Bangalore GeNei) marker was loaded in the first well.

8. The cathode and anode were connected to the power pack and the gel was run at a constant voltage of 85 volts.

9. The power was switched off when the tracking dye moved more than half of gel from the well.

10. The gel placed in a gel documentation system, bands were visualized under UV light and the gel image was documented.

### 3.4.3.4. Normalization of cDNA

While estimating cDNA with nanodrop, there is the interference of dNTPs, left over RNA, degraded RNA nucleotides, and cDNA, all of which contribute to the nanodrop reading and may result in fluctuating values. cDNA normalization decreases the prevalence of high abundance transcripts and equalizes transcript concentrations in the sample. For normalization of cDNA, endogenous control actin was used. The dilutions up to 1:5 (cDNA: distilled water) were made to equalize intensity of bands in all the three cDNA samples in 1.2 per cent agarose gel electrophoresis and gel picture was documented.

### 3.5.1. Primer designing and validation for real-time PCR

Primers were designed and validated for six genes such as *CO I (Cytochrome oxidase subunit I), PKDP (Protein kinase domain protein), bZIP1 (Basic leucine Zipper), AP2-EREBP (APETALA2 and ethylene-responsive element binding protein), Hsp20 (Heat shock protein 20) and COC1 (Circadian oscillator component).* The gene sequences used for the study was obtained from Baburao, (2012) are given in Appendix 5. IDT PrimerQuest tool (http://eu.idtdna.com/primerquest/home/index) was used for designing gene specific primers according to the criteria required for

real-time PCR and IDT OligoAnalyzer 3.1 tool (https://eu.idtdna.com/calc/analyzer) was used for validating designed primers based on parameters like homodimer, heterodimer, Tm mismatch, GC content and hairpin loop structures.

## 3.5.1.2. Parameters to be considered during primer designing for real time PCR

### A. Template Considerations

i. Amplicons should ideally be between 50-150 bases in length

ii. For RNA targets, select primers spanning exon-exon junctions

iii. Consideration of template secondary structure is important

### B. Primers Considerations

i. The optimal length of primers is generally accepted as 18–24 bases in length

ii. Melting temperatures (Tm) between 59 and 68°C, with an optimal Tm of 62–64°C

iii. Optimal real-time PCR annealing temperatures are 55°C to 60°C

iv. An ideal amplicon should be between 80 and 150 bps

v. GC content should be between 50 to 60 percent

vi. The last five nucleotides at 3' end contain no more than two G+C residues

vii. Repeats should be avoided because they promote mispriming. If unavoidable, the maximum number should be 4 di-nucleotides

viii. Higher 3' stability will improve the efficiency of the primer

ix. Having 1 to 2 GC clamps are ideal, as it allows the primer to bind strongly to the template strand, making it more specific

**Table 7. List of genes and their respective primers designed for expression analysis for real-time PCR**

| Sl.no. | Gene name | Oligo name | Sequence 5'-3' | No. of Bases | Amplicon length |
|---|---|---|---|---|---|
| 1 | *CO I* | SP1F | CTCCTAGTCGGCCTGATTTC | 20 | 108 |
| | | SP1R | CATGAGCAGTAGCATCCTTGA | 21 | |
| 2 | *PKDP* | SP2F | CGTTGATAGTCGCCGCTAAA | 20 | 109 |
| | | SP2R | TTTAAGAGGCGGGAATGGTG | 20 | |
| 3 | *bZIP1* | SP3F | GAGCGTACTCTGTCCCATTTAG | 22 | 115 |
| | | SP3R | GTTCCAGCGATGAGGTTGT | 19 | |
| 4 | *AP2-EREBP* | SP4F | AGGTAAAGCCCGAGCAATTC | 20 | 101 |
| | | SP4R | GCATCGGTGAATGGTGGTATAA | 22 | |
| 5 | *Hsp20* | SP5F | TGTGTGTCACCACGCTTTA | 19 | 119 |
| | | SP5R | CCTCGCATAGACCCATTCATC | 21 | |
| 6 | *COC1* | SP6F | CACCTCATGACGATGCAAGA | 20 | 101 |
| | | SP6R | GAGCTTGCTCACTCCTTCAA | 20 | |

### 3.6.1. Standardization of annealing temperatures

Annealing temperature for each primer was standardized by using gradient PCR from SureCycler 8800 from Agilent technologies and the same annealing temperature was used in real-time PCR. The PCR product was stored at -20 °C, and then electrophoresed using 1.2 per cent agarose gel electrophoresis and the gel image was documented.

### 3.7.1. Quantitative real-time Polymerase Chain Reaction (qRT-PCR) assay

A real-time polymerase chain reaction is a molecular technique based on the polymerase chain reaction (PCR). It differs from normal PCR by the measurement of the amplified PCR product at each cycle throughout the PCR reaction.

### 3.7.1.1. General precautions taken during real-time PCR assay

1. Prior to use, ensure that the reagent is evenly mixed by gently inverting several times without creating bubbles. Uneven reagent mixture will result in inadequate reactivity.
   - Do not mix by vortexing.
   - When stored at -80°C SYBR Premix Ex Taq II could develop a white to pale yellow precipitate. Gently hand-warm and permit to stand protected from light at room temperature concisely, and then invert several times to dissolve the precipitate completely.
   - The presence of precipitate is indicative of uneven reagent composition; make sure the reagent is evenly mixed before use.
2. Place reagents on ice when preparing the reaction mixture.
3. SYBR green is sensitive to light; avoid exposure to strong light when preparing the reaction mixture.

4. Use fresh disposable tips to avoid contamination between samples when preparing or dispensing reaction mixtures.

### 3.7.1.2. Internal controls

For data normalization, the transcripts of stably expressed (reference genes) genes are commonly used. In rice the house keeping gens like *18S rRNA, 25S rRNA, ACT11, UBC, UBQ10, UBQ5, GAPDH, elF-4a, eEF-1a* and *b-TUB* have served as good reference genes (Jain *et al.*, 2006). In the present study housekeeping gene *Actin* as internal control earlier reported by Yamanouchi *et al.* (2002).

### 3.7.1.3. Setting baseline and threshold level

The points of measurement in terms of baseline and threshold level .should be accurately determined to reflect the quantity of a particular target within a reaction. During PCR, changing the reaction conditions and environment can influence fluorescence. In general, the level of fluorescence in any well corresponds to the amount of target present in it. Fluorescence levels may fluctuate due to changes in the reaction medium creating a background signal. The background signal is most evident during the initial cycles of PCR prior to significant accumulation of the target amplicon. During these early PCR cycles, the background signal in all wells is used to determine the "baseline fluorescence" across the entire reaction plate. For data analysis, more accurate measurements of fluorescence are needed which are attained when the target amplification is sufficiently above the background signal. The Applied Biosystems Sequence detection software sets the default baseline from 3-15 cycles if the baseline needs to be adjusted, it is important to determine assay emerges earliest above the baseline. Therefore, baseline was not changed an set default between 3-15 cycles. The threshold value is a numerical value assigned for each run which reflects a statistically significant point above the calculated baseline. It was ensured that threshold line would be placed in the exponential phase to increase the

precision and quality of the exponential data and same baseline and threshold default setting was used for all PCR reactions.

### 3.7.1.4. Real-time PCR reaction mix and thermal profile

Master mix was prepared as indicated in the Table 8. given below and spun briefly for proper mixing. Then aliquot of the master mix was added to each well in PCR plates. PCR profile was followed as per the provided by DSS Takara Bio India given in Table 9. A dissociation stage was added to check primer dimers if any. For each treatment and for each primer, three biological replicates cDNA were used.

**Table 8. Real-time PCR reaction mix (20 μl)**

| Reagent | Volume |
|---|---|
| SYBR Premix Ex Taq II (Tli RNaseH Plus) (2X) | 10 μl |
| PCR Forward Primer (10 μM) | 1 μl |
| PCR Reverse Primer (10 μM) | 1 μl |
| ROX Reference Dye (50X) | 0.4 μl |
| Template | 2 μl |
| sterile distilled water | 5.6 μl |
| Total | 20 μl |

**Table 9. The thermal profile used for real time PCR**

| Sl. No. | Step | Temperature | Time | No. of cycles |
|---------|------|-------------|------|---------------|
| 1. | Initial denaturation | 95°C | 30 sec | 1 |
| 2. | Denaturation | 94°C | 5 sec | |
| 3. | Annealing | 55-58°C | 35 sec | 40 |
| 4. | Dissociation stage | 95°C | 15 sec | |
| | | 55-58°C | 30 sec | 1 |
| | | 95°C | 15 sec | |

### 3.7.2.1. Semiquantitative real-time PCR

After real-time PCR semiquantitative PCR was performed for further confirmation of gene expression levels in agarose gel electrophoresis. The PCR product obtained after real-time PCR reaction was loaded in 1.2 per cent agarose gel electrophoresis and gel pictures was documented.

### 3.7.2.1. Relative quantification analysis

The two most commonly used methods to analyze data from reel-time PCR experiments are absolute quantification and relative quantification. Absolute quantification determines the input copy number, usually by relating the PCR signal to a standard curve and the relative quantification relates the PCR signal of the target transcript in a treatment group to that of other samples such as an untreated control. In this experiment, the relative quantification approach was used to measure the

expression level of target genes in absolute control ($T_1$) and other treatments such as water stressed ($T_2$) and water stressed + *Pf* treated ($T_3$).

The mathematical model Comparative $\Delta$ Ct method (Livak and Schmittgen, 2001) was used to determine relative expression ratio (fold change). In real-time PCR, fluorescence was recorded at each cycle to monitor the generation of amplified product. For proper calculation of initial target levels, differences in efficiency of amplification must be taken into consideration. Even small differences in amplification efficiencies will get added up making large apparent differences in mRNA levels. The absolute quantification requires a setup of standard curves from which PCR efficiency will be deduce; the disadvantages of standard curves are (i) the extra efforts and cost needed to set up additional samples (ii) Non matching efficiency due to presence of inhibitors and serial dilutions. The relative quantification with PCR efficiency correction was adopted to calculate the fold change expression. In the present study relative quantification was done to analyze fold change expression in target genes by keeping *Actin* gene as internal control.

### 3.8.1. *In silico* identification of putative transcription factor binding sites

### 3.8.1.1. Orthologue search

Orthologue search was done against *Arabidopsis thaliana* genome by using RGAP database to obtain AGI codes for further *in silico* analysis. The option called "Orthologue groups" in pseudomolecules tool of RGAP database (http://rice.plantbiology.msu.edu/annotation_pseudo_ortho.shtml) was used to retrieve AGI codes against *Arabidopsis* genome. First hit from the results was used as AGI code for identification of TFBSs in AthaMap and STIFDB2 databases.

### 3.8.1.2. Identification of TFBSs from AthMap database

TFBSs were identified using AthaMap database, a genome-wide map of TFBSs in *Arabidopsis thaliana*. AGI codes obtained from orthologue search were used to identify TFBSs. AthaMap provides two parameters to determine functionally significant transcription factors. The Matrix score ≥ 10 and Threshold ≥5 were selected as potential TFBSs using "Search" tool in AthaMap. The restriction to 20% by entering a "20" in the text field restriction was done to restrict the number of binding sites on the gene. The default upstream and downstream region of all genes to search is -500 and 50 bps respectively. The region of -500 bps already covers the area in which most of the regulatory sequences are found within the upstream region of *Arabidopsis thaliana* genes.

### 3.8.1.3. Identification of TFBSs from STIFDB2 database

STIFDB V2.0 (Stress Responsive Transcription Factor Database Version 2.0) is a comprehensive collection of biotic and abiotic stress responsive genes in *Arabidopsis thaliana* and *Oryza sativa* L. with options to identify plausible transcription factor binding sites in their promoters. The database suggest that Z-score for 100bp and its 5'UTR regions can be seen above 2.0 and for 1000bp and its 5'UTR regions can be seen above 1.5. The experimentally determined stress inducible TFBSs were identified from 1000bp data of query gene.
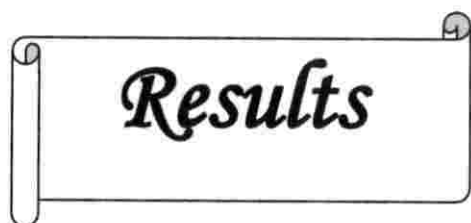
### 3.8.1.4. Cross validation

Cross validation is an approach by which the sets of scientific data generated using two or more methods are critically assessed. AthaMap database provides the data about all categories of TFs factors involved in growth and development and also the TFs responses for stress signals. STIFDB2 contains the TFs responses predominantly for stress signals. The identified transcription factor binding sites from

both AthaMap and STIFDB2 databases were cross validated manually to make prediction robust and also to screen stress inducible TFBSs from other binding sites.

### 3.8.1.4. Identification of combinatorial *cis*-regulatory elements

A "Co-localization" function allows the search for user-defined potential combinatorial elements. A set of pre-calculated known combinatorial elements is already incorporated in AthaMap (Steffens *et al.*, 2006). List of *cis*-regulatory elements by co localizing two transcription factors of either same family or different by keeping minimum and maximum spacer 0 and 20 bps respectively.

# Results

# 4. RESULTS

The results of the study entitled 'Validation of identified genes for water stress in rice (*Oryza sativa* L.) mediated by *Pseudomonas fluorescens*' was undertaken during the period from 2014 to 2016 at the Centre for Plant Biotechnology and Molecular Biology, College of Horticulture, Kerala Agricultural University, Vellanikkara are presented in this chapter.

## 4.1. Measurement of biometric parameters

Drought was induced in plants during its reproductive stage two weeks after the panicle initiation (Plate 3). Observations were taken on shoot length, root length, fresh weight, dry weight and 1000 seed weight after the harvest. Statistical analysis was carried out using ANOVA and since there was significant differences between treatments. Duncan's multiple range statistical analysis had been carried out for noted observations and validated by using WASP - Web Agri Stat Package 2.0 online software. Maximum shoot length, root length, fresh weight, dry weight and 1000 seed weight were obtained in control plants ($T_1$) followed by water stressed + *Pf* treated plants ($T_3$) (Table 10).

### 4.1.1. Shoot length

$T_1$ recorded highest shoot length (111.500 cm) and it was significantly superior over the other treatments such as $T_3$ (91.380 cm) and $T_2$ (98.860 cm). $T_3$ and $T_2$ were on par to each other (Table 10).

### 4.1.2. Root length

Root length of 15.400 cm was observed in $T_2$ which the lowest was observed value whereas the root length of $T_1$ (26.660 cm) recorded as highest and was on par with $T_3$ (24.000 cm) (Table 10).

### 4.1.3. Fresh weight

$T_1$ was noticed with maximum fresh weight (45.620 g) followed by $T_3$ (34.940 g) and $T_2$ (27.420 g). It was also found superior over other treatments (Table 10).

### 4.1.4. Dry weight

The dry weigh of $T_1$ was found highest (29.500 g) followed by $T_3$ (14.400) and in $T_2$ it was lowest among the three (11.800 g) (Table 10).

### 4.1.5. Yield

The average yield of grain per each plant was 3.196, 1.482 and 1.994 g. in control, water stressed and water stressed + *Pf* treated plants respectively. Control showed highest yield and was superior over other two treatments but $T_3$ showed better yield compared to $T_2$ (Table 10).

### 4.1.6. 1000 seed weight

Thousand seed weight of T1, T2 and T3 were 24.520, 19.100 and 21.220g respectively. $T_1$ was found significantly superior over the other treatments $T_2$ and $T_3$ (Table 10).

Plate 1. Transplanted seedlings 15 days after sowing



Plate 2. Pots were maintained under CRD (Plants 30 days after sowing)

**Plate 3. Plants 65 days after sowing**

**($T_1$: Control, $T_2$: Water stressed, $T_3$: Water stressed + *Pf* treated)**
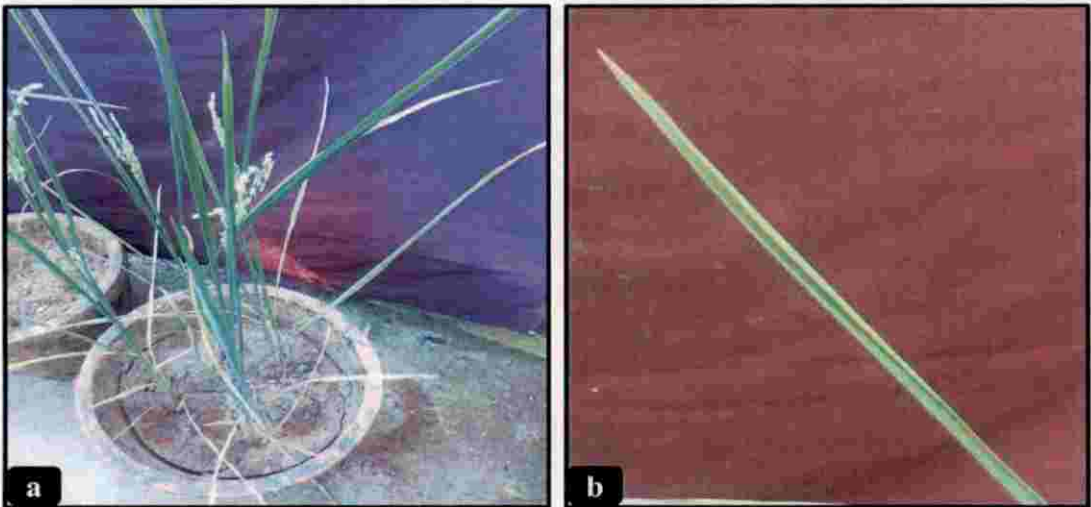


**Plate 4 (a and b). Ninety per cent leaf rolling at the score of 7.0 used for total RNA isolation**

**Table 10. Measurement of biometric parameters**

| Treatment | Shoot length(cm) | Root length(cm) | Fresh wt.(g) | Dry wt.(g) | Yield/ panicle (g) | 1000 seed wt. (g) |
|---|---|---|---|---|---|---|
| Control (T₁) | 111.500 | 26.660 | 45.620 | 29.500 | 3.196 | 24.520 |
| Water stressed (T₂) | 91.380 | 15.540 | 27.420 | 11.800 | 1.482 | 18.700 |
| Water stressed + *Pf* treated (T₃) | 98.860 | 24.000 | 34.940 | 14.400 | 1.994 | 21.220 |
| CD (1%) | 11.592 | 3.041 | 10.248 | 12.584 | 0.297 | 1.747 |

## 4.2. Similarity search of identified TDFs

The sequences showed good homology with known proteins. Searching the TIGR rice database showed that a 159 bp fragment corresponding to sequence 1 has homology with cytochrome c oxidase subunit 1 (CO I), sequence 5 of 161 bp found similarity with protein kinase domain protein (PKDP). Sequence 3 (680 bp) and sequence 6 (228 bp) were found as bZIP1 (basic leucine zipper 1) and COC1 (Circadian oscillator component) transcription factors coding genes from Plant transcription factor database. For sequence 4 (276 bp) the BLASTX analysis was done using DRTF database and was found as AP2-EREBP (APETALA2-ethylene responsive element binding proteins). Sequence 5 (484 bp) found as Hsp20 (Heat shock protein 20) from RGAP database. Further details of BLASTX analysis showed in Table 11. This showed that these six genes are already reported in rice genome.

**Table. 11. Sequence homologies of the TDFs with known genes based on BLASTX from different databases**

| Sequence | Gene description | Sequence length (bp) | Hit score | E value | Accession Id | Database |
|---|---|---|---|---|---|---|
| Sequence 1 | Cytochrome c oxidase subunit 1 | 159 | 150 | 0.00 | Os12g0561000 | TIGR, rice |
| Sequence 2 | Protein kinase domain containing protein | 161 | 129 | 0.00 | OS08G39170 | TIGR, rice |
| Sequence 3 | bZIP family protein (bZIP1) | 680 | 83 | 2e-17 | CT833525 (Genbank) | PlantTFDB |
| Sequence 4 | APETALA2-ethylene responsive element binding protein | 276 | 79 | 0.00 | OsIBCD031146 | DRTF |
| Sequence 5 | Heat shock protein 20 | 484 | 230 | 4e-25 | Os01g04380.1 | RGAP |
| Sequence 6 | Circadian clock associated 1 | 228 | 97 | 3e-06 | AY885936 (Genbank) | PlantTFDB |

## 4.3.1. Isolation and spectrophotometric analysis of total RNA

Total RNA was isolated from three treatments using TRIzol method. The samples were run on 0.7 percent agarose gel in 1X MOPS buffer. Three intact bands corresponding to 28S, 18S and 5S rRNA + tRNA were obtained (Plate 5), indicating the good quality RNA.

Total RNA from the three samples quantified spectrophotometrically is given in Table 12. The ratio of OD260/OD280 for the samples was greater than 1.8 indicating pure RNA free from DNA and protein contamination. The ratio for OD260/OD230 was greater than 1.0 indicating that the samples were free from polysaccharides and polyphenols.

**Table 12. Qualitative and quantitative analysis of total RNA**

| Name of sample | Absorbance | | Quantity of RNA(ng/µl) |
|---|---|---|---|
| | 260/280 | 230/280 | |
| Absolute Control (T$_1$) | 1.93 | 1.01 | 1847.9 |
| Water stressed (T$_2$) | 1.97 | 1.00 | 2218.0 |
| Water stressed + *Pseudomonas* treated(T$_3$) | 1.94 | 1.03 | 2002.8 |
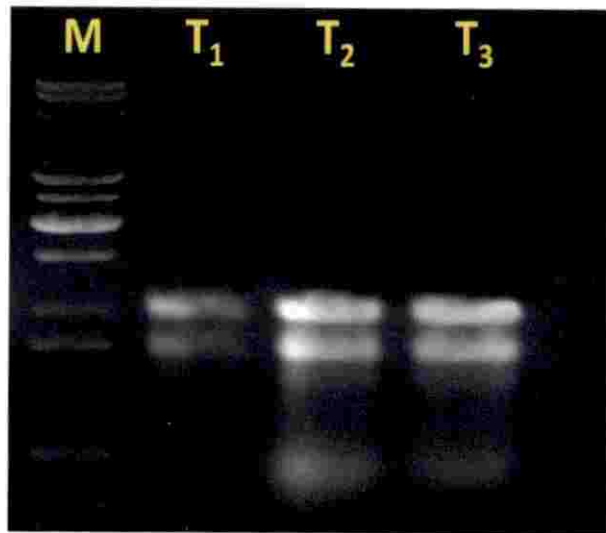
(Ladder: Invitrogen 1 kb plus)

($T_1$: Control, $T_2$: water stressed, $T_3$: Water stressed + *Pf* treated)

**Plate 5. Gel electrophoresis showing total RNA isolated from leaf samples of different treatments**

## 4.3.2. Reverse Transcriptase Polymerase Chain Reaction and confirmation of cDNA

About 1000 ng/µl of total RNA from all the treatments was used for reverse transcriptase PCR analysis. The first strand cDNA was synthesized from the above RNA samples using Thermo Scientific RevertAid H Minus First Strand cDNA kit (Thermo Scientific) as per the manufacturer's guidelines. OligodT primers were used to synthesize first strand cDNA. First strand cDNA was confirmed by amplification with actin gene specific primers. The PCR product was resolved on 1.2 per cent agarose gel electrophoresis and visualized in gel documentation system (Plate 6).

### 4.3.3. Normalization of cDNA

First strand cDNA was normalized by amplifying with endogenous control actin gene specific primers. The band intensity of all three cDNA samples was uniform when visualized in 1.2 per cent agarose gel electrophoresis (Plate 7).

### 4.4. Primer designing for real-time PCR

The details of primers designed for the six differentially expressed genes are given in Table 13. The primers were designed by using IDT PrimerQuest tool (http://eu.idtdna.com/primerquest/home/index) and the designed primers were validated using IDT OligoAnalyzer 3.1 tool (https://eu.idtdna.com/calc/analyzer). The primers were designed as per the criteria required for real-time PCR.

### 4.5. Standardization of annealing temperatures by using gradient PCR

Annealing temperature for each primer was standardized by using gradient PCR. Expected band size was obtained on 1.2% agarose gel at specific annealing temperatures. The details of annealing temperatures are given in Table 14.
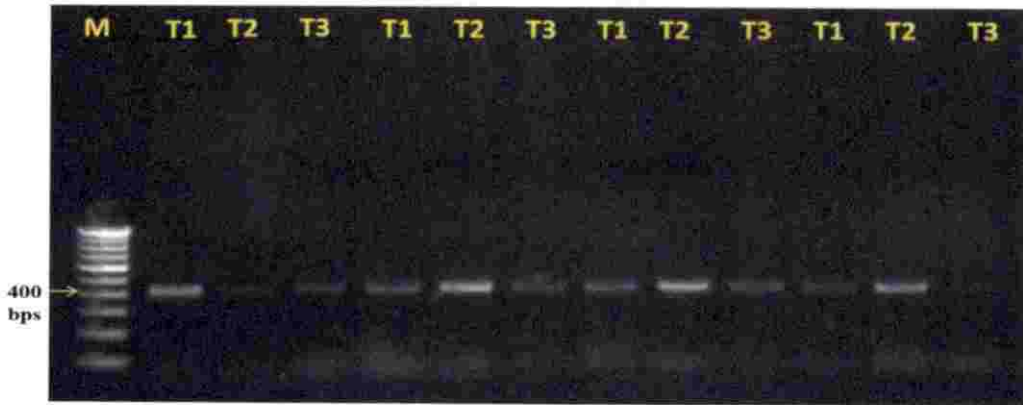
(Ladder: Genei (100 bp); Amplicon size: ~420 bp)

(T$_1$: Control, T$_2$: water stressed, T$_3$: Water stressed + *Pf* treated)

**Plate 6. Confirmation of cDNA using *Actin* gene specific primers in four replications of each treatment**



(Ladder: Genei (100 bp); Amplicon size: ~420 bp) (T$_1$: Control, T$_2$: water stressed,

T$_3$: Water stressed + *Pf* treated, B: Blank)

**Plate 7.  Normalized cDNA with *Actin* gene specific primers**

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**Table 13. List of genes and their respective primers designed for expression analysis in real-time PCR**

| Sl.no. | Gene name | Oligo name | Sequence 5'-3' | No. of Bases | Amplicon length |
|--------|-----------|------------|----------------|--------------|-----------------|
| 1 | *CO I* | SP1F | CTCCTAGTCGGCCTGATTTC | 20 | 108 |
|   |        | SP1R | CATGAGCAGTAGCATCCTTGA | 21 |     |
| 2 | *PKDP* | SP2F | CGTTGATAGTCGCCGCTAAA | 20 | 109 |
|   |        | SP2R | TTTAAGAGGCGGGAATGGTG | 20 |     |
| 3 | *bZIP1* | SP3F | GAGCGTACTCTGTCCCATTTAG | 22 | 115 |
|   |        | SP3R | GTTCCAGCGATGAGGTTGT | 19 |     |
| 4 | *AP2-EREBP* | SP4F | AGGTAAAGCCCGAGCAATTC | 20 | 101 |
|   |        | SP4R | GCATCGGTGAATGGTGGTATAA | 22 |     |
| 5 | *Hsp20* | SP5F | TGTGTGTCACCACGCTTTA | 19 | 119 |
|   |        | SP5R | CCTCGCATAGACCCATTCATC | 21 |     |
| 6 | *COC1* | SP6F | CACCTCATGACGATGCAAGA | 20 | 101 |
|   |        | SP6R | GAGCTTGCTCACTCCTTCAA | 20 |     |

**Table 14. Standardized annealing temperatures for gene specific primers**

| Sl.no | Gene name | Oligo name | Annealing temperature ($^0$C) |
|-------|-----------|------------|-------------------------------|
| 1 | *CO I* | SP1 | 56 |
| 2 | *PKDP* | SP2 | 57 |
| 3 | *bZIP1* | SP3 | 58 |
| 4 | *AP2-EREBP* | SP4 | 58 |
| 5 | *Hsp20* | SP5 | 55 |
| 6 | *COC1* | SP6 | 57 |

## 4.6.1. Quantitative real-time Polymerase Chain Reaction (qRT-PCR) assay

Relative quantification was carried out in this study. The primers were designed for six differential genes such as *CO I, PKDP, bZIP1, AP2-EREBP, Hsp20* and *MYB. Actin* was used as endogenous control.

The gene expression at different treatments (Absolute control, water stressed and water stressed + *Pf* treated) was studied for all the six genes after normalizing cDNA samples with reference gene. The Ct values obtained were used for comparative Δ Ct method. The values obtained using this method was used to plot graph.

## 4.6.2. Threshold cycle values

The real-time PCR data were expressed as the cycle number necessary to achieve a threshold cycle value (Ct) and analyzed with the comparative Ct method (ΔΔ Ct). The reported Ct values were the averages of three biological replicates, and each biological replicate entailed of three replications. Actin was used as reference gene to analyze the expression data. The average Ct values of reference gene and target genes are listed in table 15.

**Table 15. The average Ct values of reference gene and target genes**

| Name of gene | Treatment | Average Ct values |
|---|---|---|
| *Actin* | Control | 23.57 |
| | Water stressed | 23.41 |
| | Water stressed + *Pf* treated | 23.94 |
| *CO I* | Control | 20.22 |
| | Water stressed | 19.57 |
| | Water stressed + *Pf* treated | 19.50 |
| *PKDP* | Control | 24.27 |
| | Water stressed | 24.04 |
| | Water stressed + *Pf* treated | 24.13 |
| *bZIP1* | Control | 24.68 |
| | Water stressed | 23.93 |
| | Water stressed + *Pf* treated | 22.61 |
| *AP2-EREBP* | Control | 25.00 |
| | Water stressed | 24.02 |
| | Water stressed + *Pf* treated | 24.08 |
| *HSP20* | Control | 21.55 |
| | Water stressed | 20.95 |
| | Water stressed + *Pf* treated | 19.88 |
| *COC1* | Control | 25.61 |
| | Water stressed | 24.41 |
| | Water stressed + *Pf* treated | 24.09 |

### 4.6.3. Amplification plots

Amplification plots (Delta Rn vs Cycle) were obatained for all the six genes and for all different treatments for each gene. The points on the baseline where the plots for all the three treatments coincide have been considered as the Ct value (Fig. 1 to Fig. 6).

### 4.6.4. Dissociation curve analysis

Dissociation curves were obtained for all the six genes in different treatments. The curves were obtained as a single dominant peak denoting that there was specific gene amplification. The temperatures at which the double stranded DNA gets dissociated (Tm) are given in Table 16. and plots for dissociation curves are shown from Fig 8 to Fig 15.

### 4.6.5. Semiquantitative PCR

Agarose gel electrophoresis (1.2 per cent) was done after the real-time PCR reaction. The bands of actin gene showed uniform intensity in all three treatments (Absolute control, water stressed and water stressed + *Pf* treated) and the target genes showed difference in intensity of bands which shows the differential expression of genes in different treatments. The band intensity was high in water stressed + *Pf* treated sample (Plate 8).

**Table 16. Melting temperatures (Tm) were obtained for different genes under different treatments dissociation curves**

| Name of gene | Treatment | Tm ($^0$C) |
|---|---|---|
| *Actin* | Control | 74.3 |
| | Water stressed | 74.0 |
| | Water stressed + *Pf* treated | 74.3 |
| *CO I* | Control | 74.0 |
| | Water stressed | 74.3 |
| | Water stressed + *Pf* treated | 74.3 |
| *PKDP* | Control | 74.8 |
| | Water stressed | 75.0 |
| | Water stressed + *Pf* treated | 74.7 |
| *bZIP1* | Control | 82.8 |
| | Water stressed | 82.5 |
| | Water stressed + *Pf* treated | 82.8 |
| *AP2-EREBP* | Control | 76.4 |
| | Water stressed | 76.1 |
| | Water stressed + *Pf* treated | 75.9 |
| *Hsp20* | Control | 80.1 |
| | Water stressed | 80.2 |
| | Water stressed + *Pf* treated | 80.1 |
| *COC1* | Control | 74.5 |
| | Water stressed | 75.5 |
| | Water stressed + *Pf* treated | 75.1 |

**Fig 1. Amplification curve for *Actin* gene** ( ' ━ ' $T_1$, ' ━ ' $T_2$ and ' ━ ' $T_3$ )



**Fig 2. Amplification curve for *CO I* gene** ( ' ━ ' $T_1$, ' ━ ' $T_2$ and ' ━ ' $T_3$ )

**Fig 3. Amplification curve for *PKDP* gene**

( ' — ' T$_1$, '—' T$_2$ and '—' T$_3$)



**Fig 4. Amplification curve for bZIP gene** ( ' — ' T$_1$, '—' T$_2$ and '—' T$_3$ )
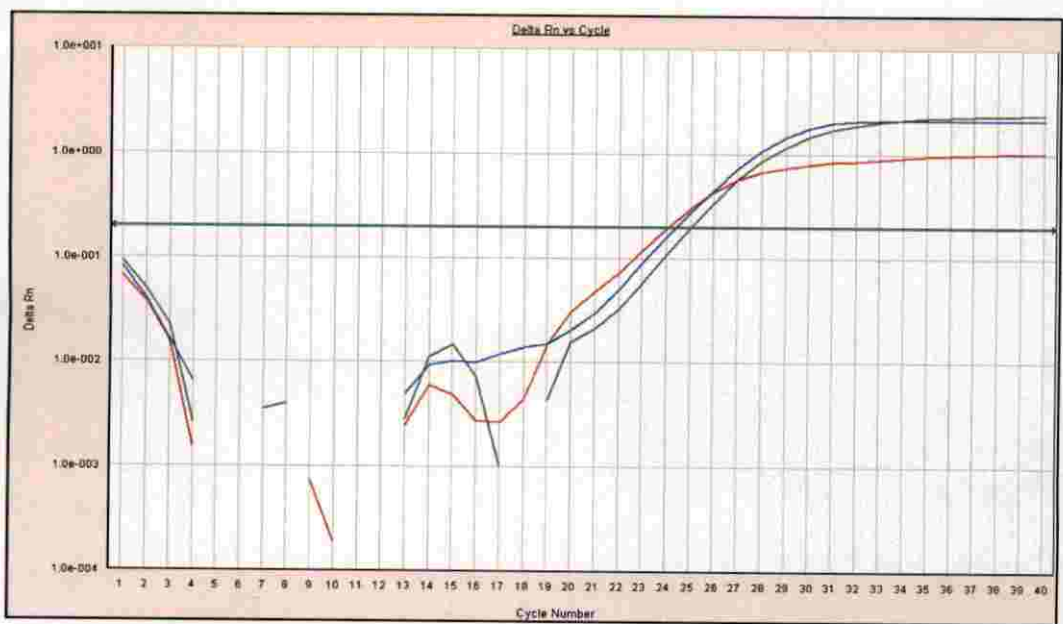
**Fig 5. Amplification curve for *AP2-EREBP* gene**

( ' — ' T$_1$, ' — ' T$_2$ and ' — ' T$_3$ )



**Fig 6. Amplification curve for *Hsp20* gene**　　( ' — ' T$_1$, ' — ' T$_2$ and ' — ' T$_3$ )
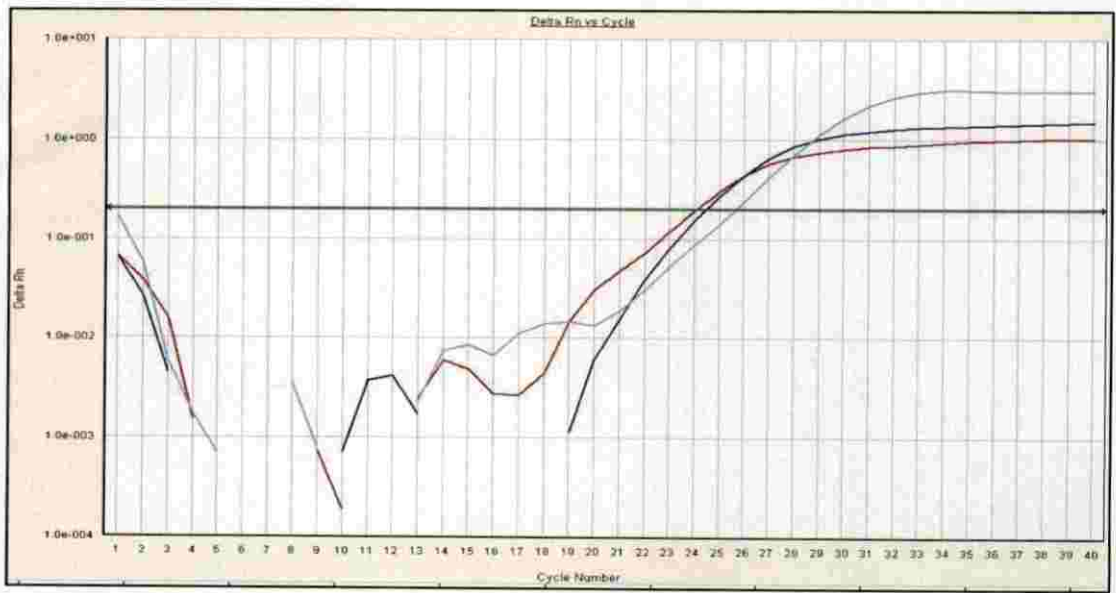
Fig 7. Amplification curve for *COC1* gene   ( ' — ' T$_1$, ' — ' T$_2$ and  ' — ' T$_3$ )
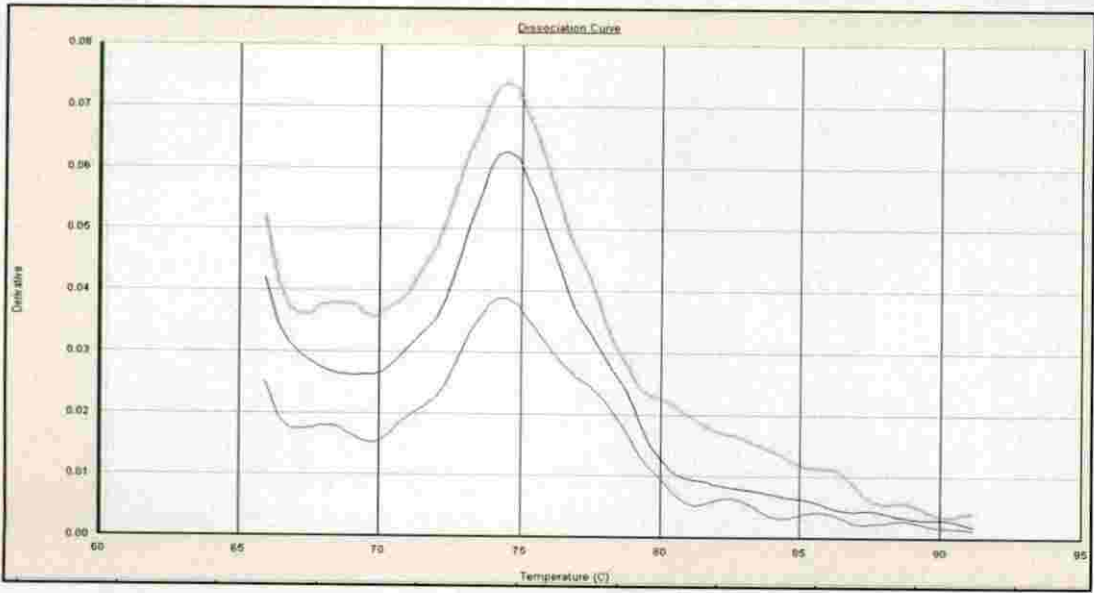


Fig 8. Dissociation curves for *Actin* gene   ( ' — ' T$_1$, ' — ' T$_2$ and  ' — ' T$_3$ )
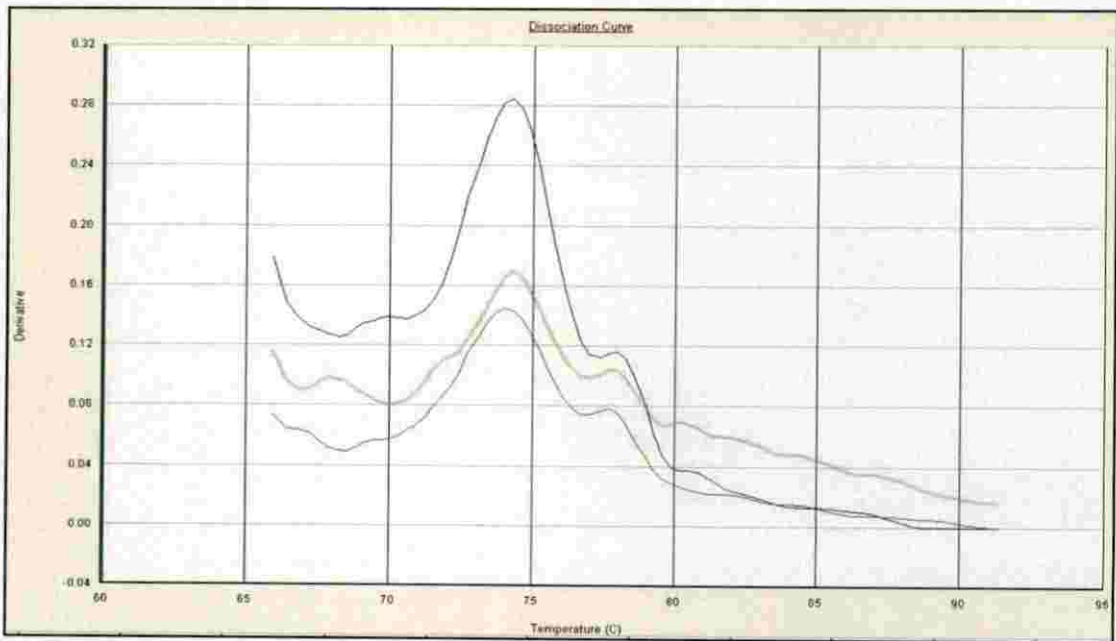
**Fig 9.** Dissociation curves for *CO I* gene ( ' ━ ' T₁, '━' T₂ and '━' T₃ )
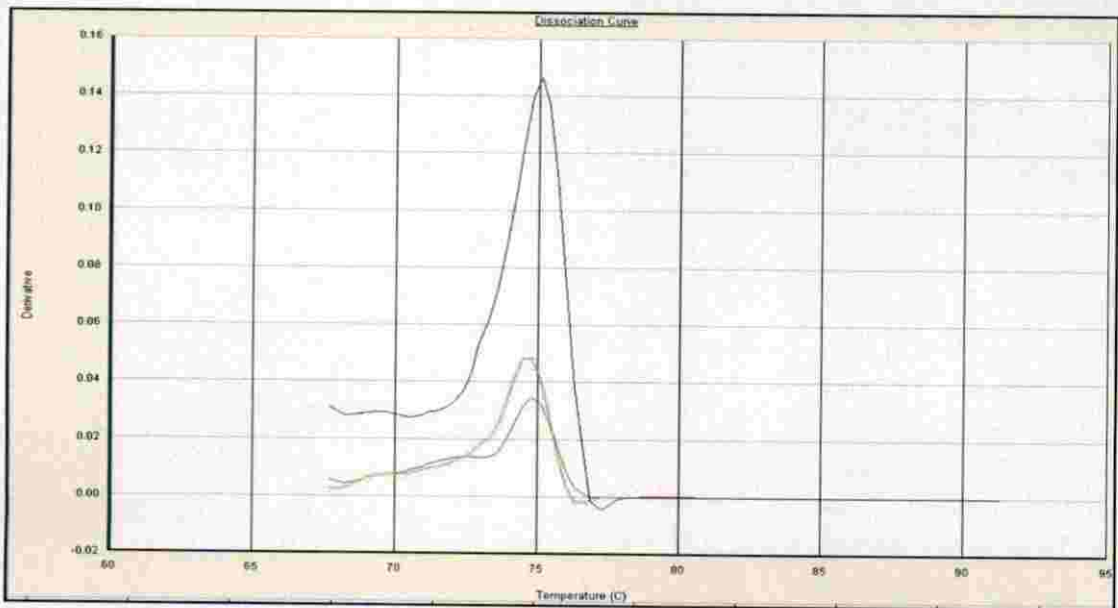


**Fig 10.** Dissociation curves for *PKDP* gene ( ' ━ ' T₁, '━' T₂ and '━' T₃ )
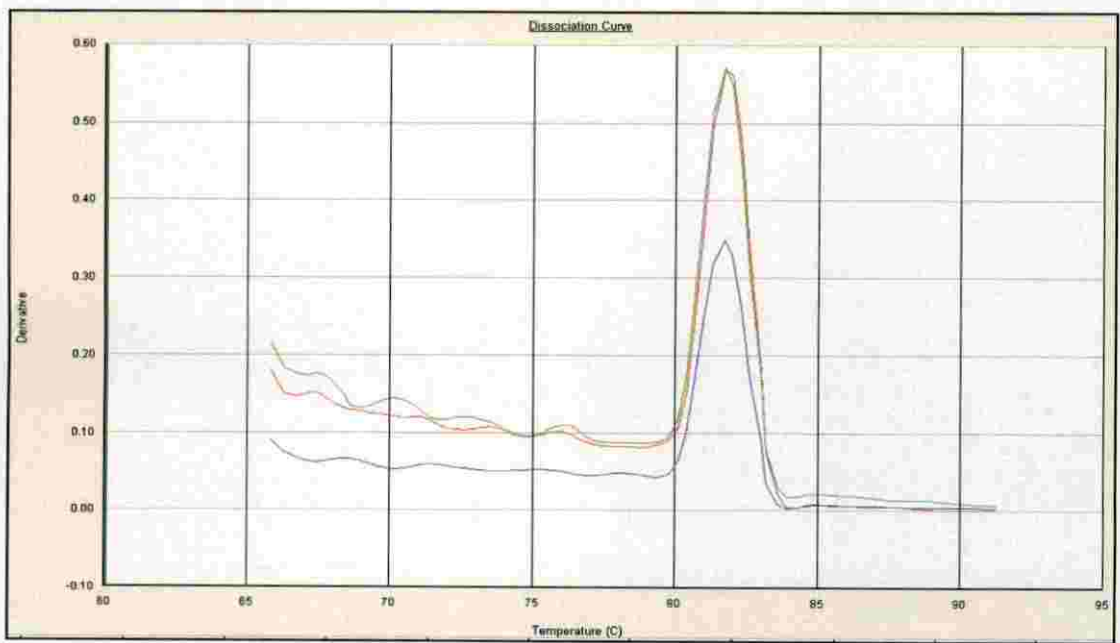
**Fig 11.** Dissociation curves for *bZIP1* gene ( ' — ' T$_1$, ' — ' T$_2$ and ' — ' T$_3$ )
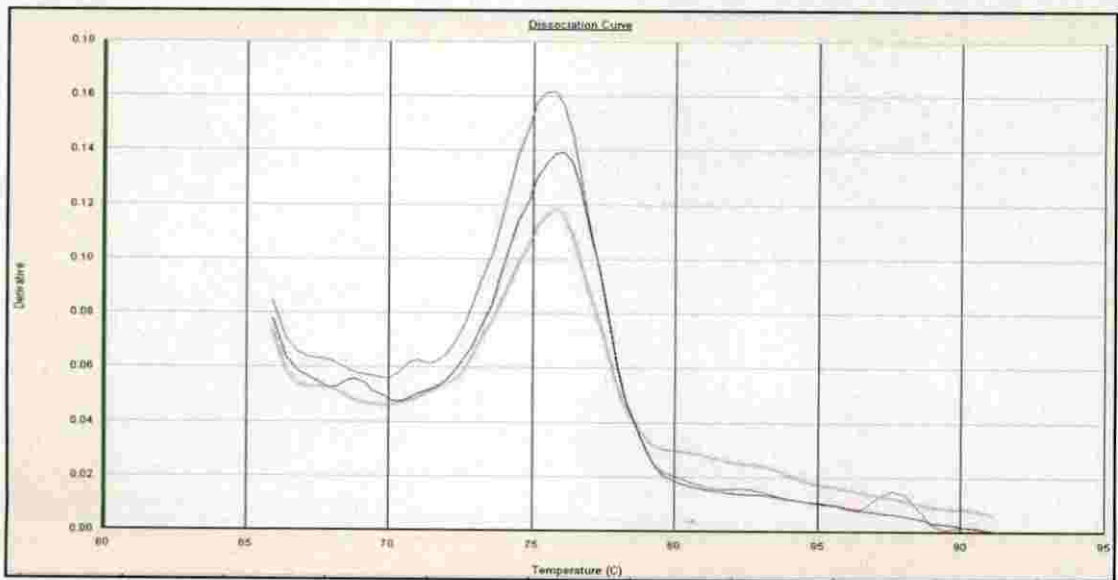


**Fig 12.** Dissociation curves for *AP2-EREBP* gene ( ' — ' T$_1$, ' — ' T$_2$ and ' — ' T$_3$ )
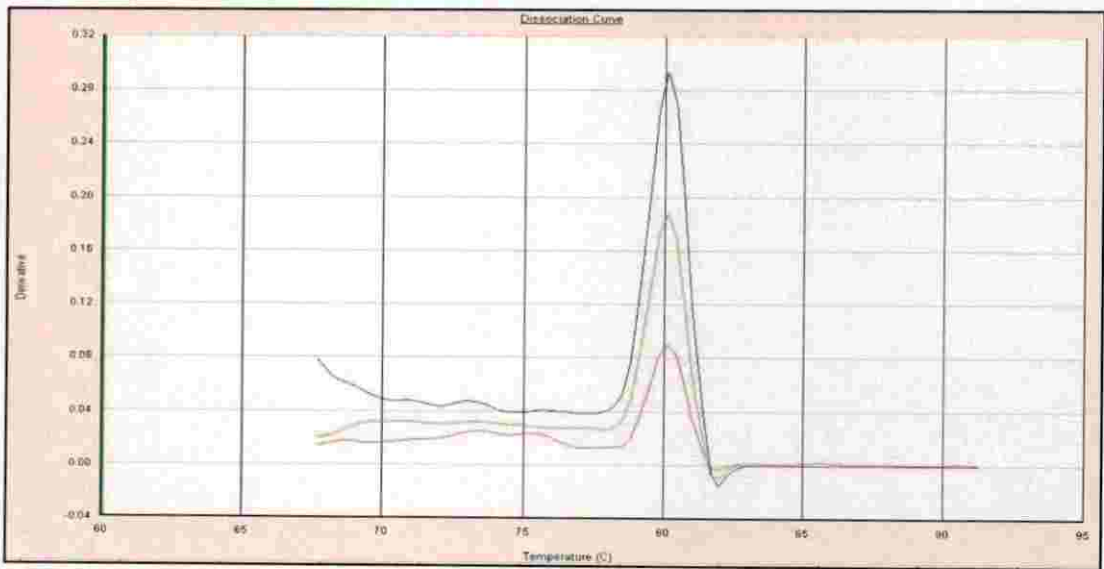
**Fig 13. Dissociation curves for *Hsp20* gene** ( ' — ' $T_1$, '—' $T_2$ and '—' $T_3$ )
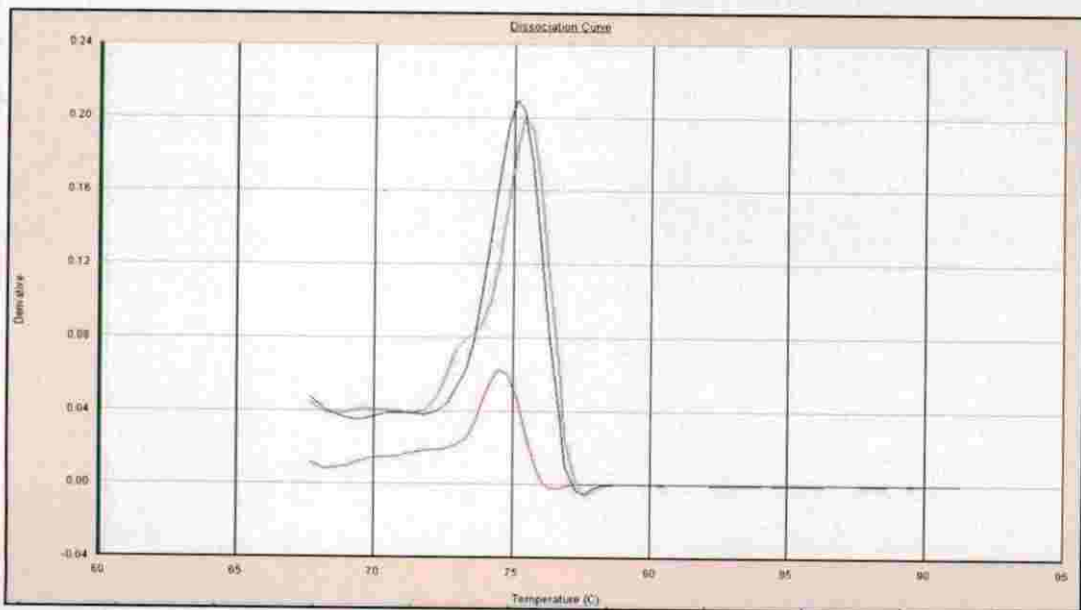


**Fig 14. Dissociation curves for *COC1* gene** ( ' — ' $T_1$, '—' $T_2$ and '—' $T_3$ )
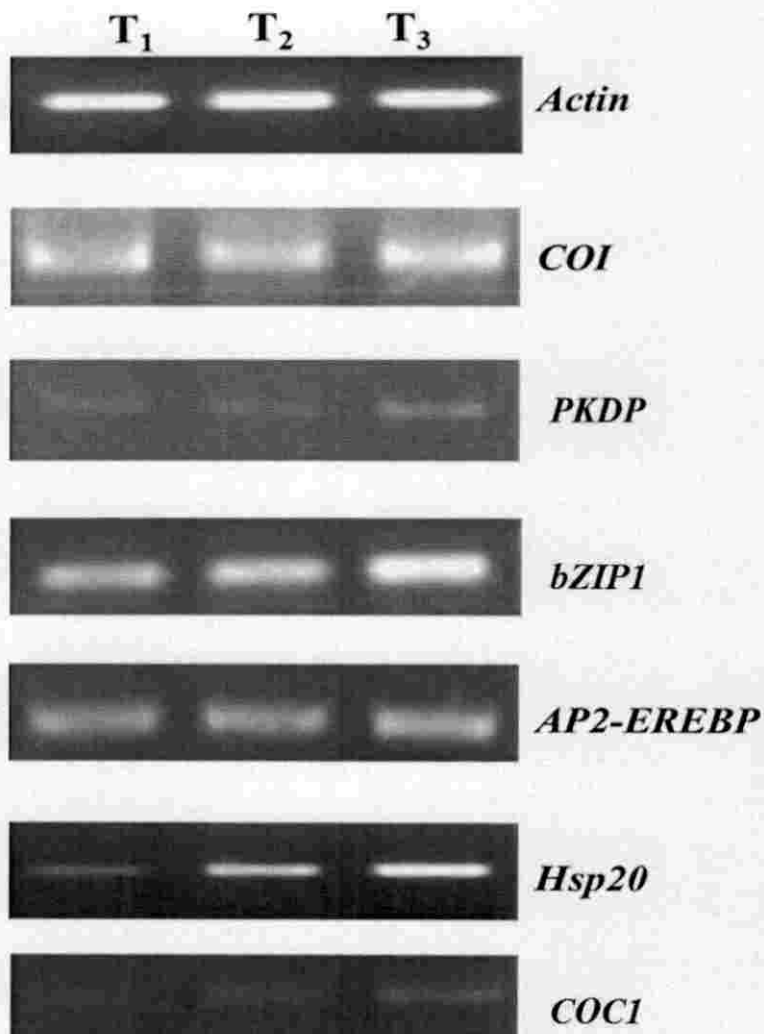
Plate 8. Semiquantitative real-time PCR analysis showing differential gene expression of genes (*CO I, PKDP, bZIP1, AP2-EREBP, Hsp20* and *COC1*) in different treatments such as Absolute control ($T_1$), Water stressed ($T_2$) and water stressed + *Pf* treated ($T_3$); the expression of each gene was compared relative to its expression in control ($T_1$).

### 4.6.6. Relative gene expression analysis

Relative gene expression studies were conducted by using real-time PCR for all the six genes using comparative Δ Ct method. The relative gene expression were plotted against three treatments (control, water stressed, water stressed + *Pf* treated) keeping actin as endogenous control.

### 4.6.6.1. Relative expression analysis of *CO I*

When normalized with the endogenous control, the expression of CO I gene had shown 2.36 folds increase in water stressed + *Pf* treated plants than that of control and a slight increase in *CO I* expression (1.4 fold) was found in water stressed plants. Detail of relative expression analysis of *CO I* gene is given in the Fig 15.

### 4.6.6.2. Relative expression analysis of *PKDP*

In case of *PKDP* gene 2 fold increase in relative expression was observed in water stressed + *Pf* treated plants when normalized with the endogenous control. In water stressed plants a minor increase in *PKDP* expression was obtained. Details of relative expression analysis of *PKDP* gene is given in the Fig 16.

### 4.6.6.3. Relative expression analysis of *bZIP*1

The expression of *bZIP1* gene showed 6 folds increase in water stressed + *Pf* treated plants when compared with that of control plants and 1.51 fold increase in gene expression was found in water stressed plants. A detail of relative expression analysis of bZIP1 gene is given in the Fig 17.

### 4.6.6.4. Relative expression analysis of *AP2-EREBP*

In water stressed + *Pf* treated plants there was 2.81 fold increase in *AP2-EREBP* gene expression than that of control and an increase in gene expression of

2.54 fold was found in water stressed plants also. Details of relative expression analysis of *AP2-EREBP* gene is given in the Fig 18.

### 4.6.6.5. Relative expression analysis of *Hsp20*

The relative expression of *Hsp20* gene was shown highest in case of water stressed + *Pf* treated plants followed by the water stressed plants. There were 4.56 and 1.36 folds increase in gene expression level found in water stressed + *Pf* treated plants and water stressed plants respectively. Detail of relative expression analysis of *Hsp20* gene is given in the Fig 19.

### 4.6.6.6. Relative expression analysis of *COC1*

In water stressed + *Pf* treated plants, the *MYB* gene showed 4 fold increase in gene expression which was observed as highest and water stressed plants showed 1.47 fold increase than that of control. Details of relative expression analysis of *MYB* gene is given in the Fig 20.
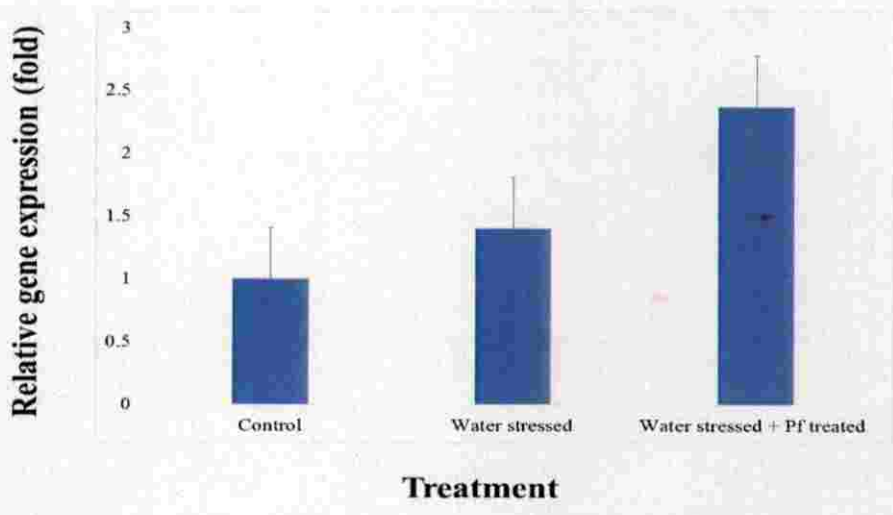
**Fig 15. Relative expression analysis of *CO I* gene normalized with *Actin* as endogenous control**
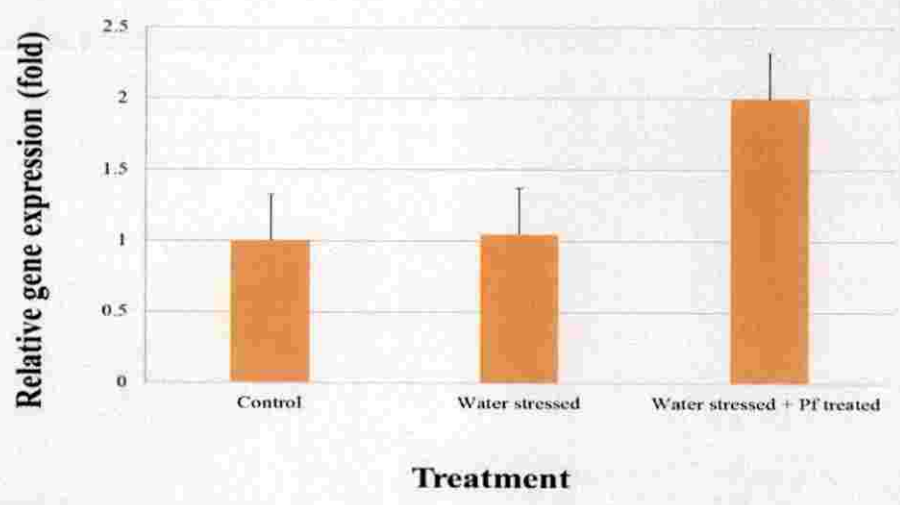


**Fig 16. Relative expression analysis of *PKDP* gene normalized with *Actin* as endogenous control**
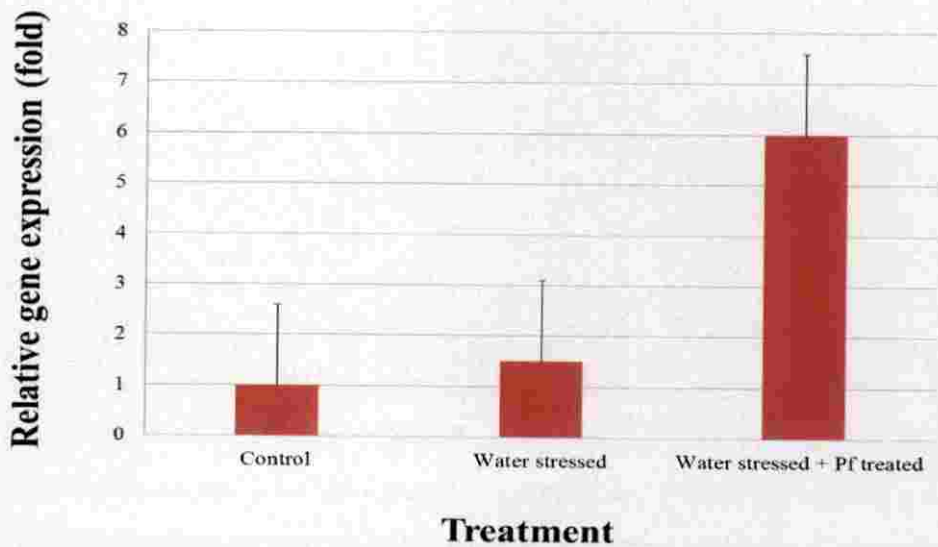
**Fig 17. Relative expression analysis of *bZIP1* gene normalized with *Actin* as endogenous control**
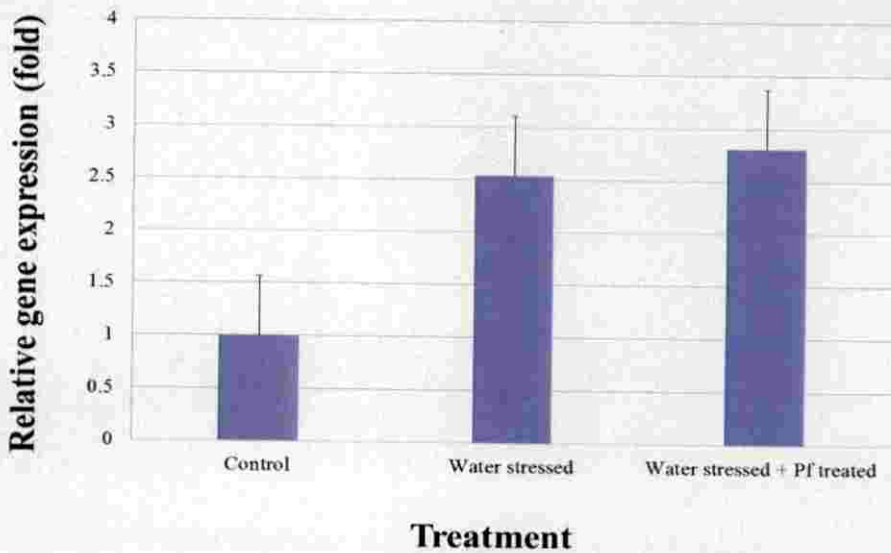


**Fig 18. Relative expression analysis of *AP2-EREBP* gene normalized with *Actin* as endogenous control**
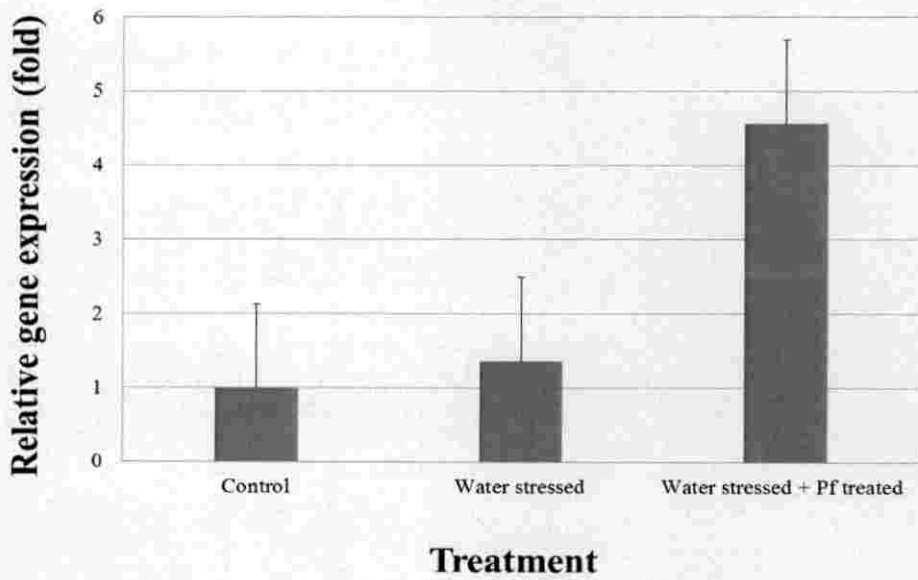
**Fig 19. Relative expression analysis of *Hsp20* gene normalized with *Actin* as endogenous control**
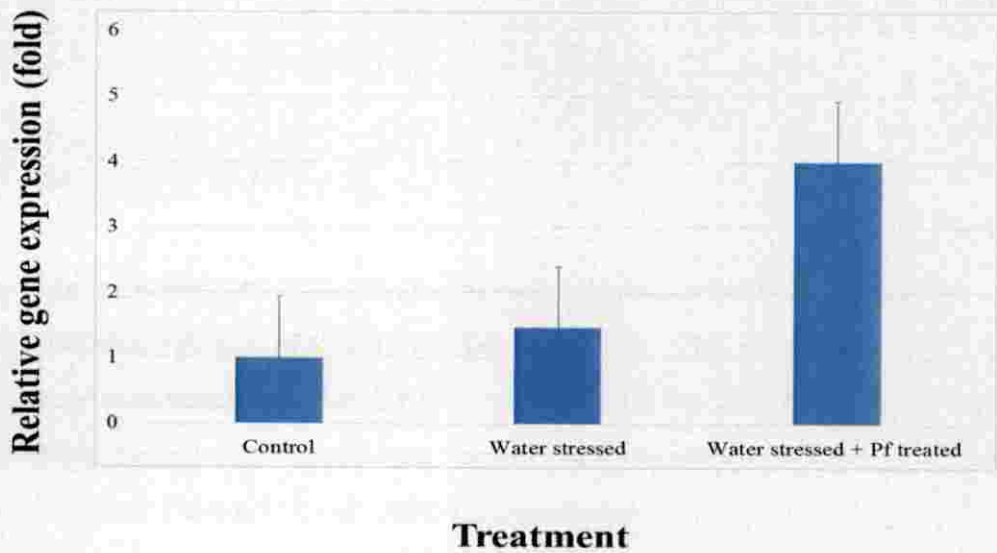


**Fig 20. Relative expression analysis of *COC1* gene normalized with *Actin* as endogenous control**

## 4.7.1. Orthologue search

Orthologue search was done against *Arabidopsis thaliana* genome using RGAP database to obtain AGI codes. The AGI codes are required for accessing in AthaMap and STIFDB2 databases to identify TFBSs and combinatorial *cis*-regulatory elements. Details of AGI codes are listed in Table 17.

**Table 17. List of rice genes and their corresponding orthologues from *Arabidopsis* genome**

| Name of gene | Acc. ID (Rice) | AGI code (*Arabidopsis*) |
|---|---|---|
| *CO I* | Os12g0561000 | AT2G07687 |
| *PKDP* | Os08g39170.1 | AT5G61310 |
| *bZIP1* | OsIBCD024362 | AT3G19290 |
| *AP2-EREBP* | OsIBCD031146 | AT5G05410 |
| *Hsp20* | Os01g04380.1 | AT1G53540 |
| *COC1* | Os08g06110.3 | AT1G01060 |

### 4.7.2. Identification of putative transcription factor binding sites

The compiled data using AthaMap and STIFDB2 which are web based tools for database-assisted identification of transcription factor binding sites and cross validated for existence of common TFBSs in both AthamMap and STIFDB2 databases. Combinatorial *cis*-regulatory elements for stress responsive genes were identified using Colocalization tool of AthaMap database.

### 4.7.3. Identification of TFBSs in AthaMap database

The curated data comprises TFBSs for six stress responsive genes of rice which was found orthologue with *Arabidopsis thaliana* genome. The major transcription factor families like AP2/EREBP, bHLH, bZIP, ABI3/VP1, HSF and MYB (Table 18) were identified using AthaMap.

### 4.7.4. Identification of TFBSs in STIFDB2 database

Stress inducible TFBSs were identified for four genes such as *bZIP1, AP2-EREB, Hsp20* and *COC1*. The TF families WRKY, MYB, HSF, bZIP and ARF were mostly found. The promoter region of *bZIP1* gene bound by MYB, bZIP, WRKY and HSF transcription factors at different binding positions (Table 19 and Fig. 21). MYB, bHLH, bZIP, HSF were found on promoter region of *AP2-EREBP* gene, MYB transcription factor was found to bind multiple sites of gene promoter (Table 20 and Fig. 22). Upstream region of *Hsp20* gene contains HSF, WRKY, bHLH transcription factors and MYB found on 5' UTR region (Table 21 and Fig. 23). The transcription factors MYB and ARF were predicted to bind 5' UTR region and bHLH, bZIP, WRKY, HB and HSF found on upstream region on promoter of *COC1* gene (Table 22 and Fig. 24).

**Table 18. Predicted TFBs in promoter regions of *CO 1*, *PKDP*, *bZIP1*, *AP2-EREBP*, *Hsp20* and *COC1* genes using AthaMap database**

| Sl. no. | Gene name | Gene orientation | Gene position | TF Family | TF | TF orientation | Max. score | Threshold | cis elements | TFBS position |
|---|---|---|---|---|---|---|---|---|---|---|
| 1 | *CO 1* | Forward | 3311651-3312648 | Trihelix | At5g28300 | Reverse | 10.64 | 5.11 | gttacggca | 3312205-3312214 |
| | | | | bHLH | PIF5 | Reverse | 11.33 | 6.41 | gcaggtgcca | 3312313-3312322 |
| | | | | bHLH | PIF5 | Reverse | 11.33 | 6.41 | gcaggtgcca | 3312313-3312322 |
| | | | | AP2/EREBP | RAV1(2) | Forward | 11.21 | 4.98 | actacctgatg | 3312311-3312322 |
| | | | | AP2/EREBP | TOE2(2) | Reverse | 11.05 | 5.56 | taacctccg | 3312366-3312375 |
| | | | | AP2/EREBP | TOE2(2) | Forward | 11.05 | 5.56 | taacctccg | 3312366-3312375 |
| | | | | SBP | AtSPL3 | Reverse | 11.69 | 5.09 | taattgtacgacttt | 3312408-3312423 |
| | | | | bHLH | MYC3 | Reverse | 10.11 | 5.27 | caacgtg | 3312439-3312446 |
| | | | | bHLH | MYC3 | Forward | 10.11 | 5.27 | caacgtg | 3312439-3312446 |
| | | | | MYB | AtMYB61 | Forward | 11.85 | 5.65 | gccaccatac | 3312466-3312475 |
| | | | | ABI3/VP1 | AtLEC2 | Reverse | 10.73 | 5.41 | gtgcatgta | 3312537-3312546 |
| | | | | MYB | AtMYB61 | Reverse | 11.85 | 5.65 | cggtggttg | 3312560-3312569 |
| | | | | MYB | MYB55(2) | Forward | 12.9 | 6.36 | cggtggttg | 3312561-3312570 |
| | | | | MYB | MYB52(2) | Forward | 11.47 | 5.2 | cggtggttg | 3312561-3312570 |
| | | | | AP2/EREBP | TOE2(2) | Reverse | 11.05 | 5.56 | taacctccg | 3312829-3312838 |
| | | | | WRKY(Zn) | WRKY1 | Forward | 12.26 | 8.48 | tttgaccg | 3312837-3312846 |
| | | | | MYB | MYB52(2) | Forward | 11.47 | 5.2 | agaacgaac | 3312905-3312914 |
| | | | | MYB | MYB52(2) | Forward | 11.47 | 5.2 | gaacgaacg | 3312909-3312918 |

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| | | | Family | TF | Direction | | | Sequence | Coordinate |
|---|---|---|---|---|---|---|---|---|---|
| **PKDP** | 5618551-5620860 | Reverse | AP2/EREBP | RAP2.3(1) | Reverse | 13.19 | 6.71 | atgcggctc | 5620404-5620413 |
| | | | AP2/EREBP | ATERF1(1) | Reverse | 12.97 | 5.42 | atgcggctc | 5620404-5620413 |
| | | | MYB | MYB46(2) | Forward | 10.58 | 6.15 | agttcggtg | 5620531-5620540 |
| | | | MYB | P | Reverse | 10.19 | 4.94 | gggttgct | 5620546-5620554 |
| | | | GARP | ARR10 | Forward | 12.04 | 5.23 | agagattcgtg | 5620575-5620586 |
| | | | MYB | AtMYB61 | Reverse | 11.85 | 5.65 | tggtggtgg | 5620585-5620594 |
| | | | AP2/EREBP | DEAR3(1) | Reverse | 11.84 | 4.93 | gtgacggcg | 5620622-5620631 |
| | | | AP2/EREBP | RAP2.6(2) | Reverse | 11.29 | 5.77 | aaggcggtg | 5620707-5620716 |
| | | | AP2/EREBP | DREB2C(2) | Reverse | 12.45 | 7.3 | aaggcggtg | 5620707-5620716 |
| | | | MYB | MYB46(2) | Reverse | 10.58 | 6.15 | tacctaact | 5620766-5620775 |
| | | | MADS | AGL1 | Forward | 14.15 | 5.44 | gttgcaagaattg gaaa | 5620866-5620883 |
| | | | MADS | AG | Forward | 14.56 | 6.32 | gttgcaagaattg gaaa | 5620866-5620883 |
| | | | NAC | TaNAC69(2) | Forward | 19.93 | 6.05 | atggttaactgtca cacgtctct | 5620897-5620920 |
| | | | NAC | ANAC55(1) | Forward | 10.55 | 5.25 | cacacgtct | 5620910-5620919 |
| | | | bZIP | TGA1a | Reverse | 10.45 | 5.21 | cacacgtct | 5620910-5620919 |
| | | | ARF | ETT(2) | Reverse | 11.71 | 5.73 | tctcgacaa | 5621020-5621029 |
| | | | Trihelix | At5g28300 | Forward | 10.64 | 5.11 | ttaccgggg | 5867187-5867196 |
| | | | Trihelix | At5g28300 | Forward | 10.64 | 5.11 | tttacggtg | 5867216-5867225 |
| | | | bZIP | TGA1 | Forward | 10.84 | 5.81 | ataatgacgtc | 5867326-5867337 |
| | | | bZIP | TGA2(2) | Reverse | 11.69 | 7.23 | aatgacgtc | 5867327-5867336 |
| | | | bZIP | STF1 | Forward | 12.43 | 7.74 | aatgacgtcat | 5867328-5867339 |
| | | | bZIP | STF1 | Reverse | 12.43 | 7.74 | aatgacgtcat | 5867328-5867339 |
| | | | bZIP | bZIP60(1) | Reverse | 12.03 | 5.33 | aatgacgtc | 5867329-5867338 |

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| | | | | Family | TF | val1 | val2 | Sequence | Position |
|---|---|---|---|---|---|---|---|---|---|
| | | | | bZIP | bZIP60(1) | Forward | 5.33 | 12.03 | aatgacgtc | 5867329-5867338 |
| | | | | bZIP | TGA1 | Forward | 5.81 | 10.84 | atgacgtcata | 5867330-5867341 |
| | | | | bZIP | TGA2(2) | Reverse | 7.23 | 11.69 | tgacgtcat | 5867331-5867340 |
| | | | | MYB | MYB46(2) | Forward | 6.15 | 10.58 | ggttaggta | 5867345-5867354 |
| | | | | MYB | MYB111(1) | Forward | 5.95 | 11.35 | ggttaggta | 5867345-5867354 |
| | | | | | | | | | tcaaccattttgga | |
| 3 | *bZIP1* | Forward | 6687146 - 6690129 | MADS | AG | Reverse | 6.32 | 14.56 | aag | 6686661-6686678 |
| | | | | C2H2(Zn) | ID1 | Reverse | 5.71 | 11.02 | aagagacaaa | 6686675-6686685 |
| | | | | MYB | MYB55(2) | Reverse | 6.36 | 12.9 | caaccactc | 6686928-6686937 |
| | | | | MYB | MYB46(3) | Forward | 5.14 | 10.94 | caaccactc | 6686928-6686937 |
| | | | | GARP | ARR10 | Reverse | 5.23 | 12.04 | cgaatcttcac | 6776237-6776248 |
| | | | | MYB | MYB52(2) | Reverse | 5.2 | 11.47 | aaacaaacg | 6776953-6776962 |
| | | | | bZIP | TGA1a | Reverse | 5.21 | 10.45 | ccacgtcgc | 6776974-6776983 |
| | | | | bZIP | bZIP60(2) | Forward | 5.82 | 12.68 | ccacgtcgc | 6776974-6776983 |
| | | | | bZIP | TGA1a | Reverse | 5.21 | 10.45 | ccacgtcgc | 6776974-6776983 |
| | | | | MYB | RVE1(1) | Reverse | 5.95 | 10.28 | ataatatct | 6777098-6770107 |
| | | | | AP2/EREBP | ATERF1(1) | Forward | 5.42 | 12.97 | ggcggcagc | 6777157-6770166 |
| | | | | AP2/EREBP | ATERF1(2) | Forward | 5.42 | 12.97 | aggcggca | 6777155-6770164 |
| | | | | AP2/EREBP | RAV1 | Forward | 5.01 | 10.65 | cagcaacacac | 6777162-6770173 |
| | | | | C2H2(Zn) | ID1 | Forward | 5.71 | 11.02 | ttgtcgtct | 6777172-6770182 |
| | | | | | | | | | cttctctggagtttt | |
| | | | | AP2/EREBP | WRI1 | Forward | 6.03 | 19.35 | cgcagt | 6777192-6770213 |
| | | | | HSF | HSFB2a(2) | Forward | 5.2 | 10.7 | ttctggatc | 6777291-6770300 |
| | | | | HSF | HSFB2a(2) | Reverse | 5.2 | 10.7 | ttctggatc | 6777291-6770300 |

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|---|---|---|---|---|---|---|---|
| 4 | AP2-EREBP | EREBP | Forward | 6774304-6775847 | | | Forward |
| | AB13/VP1 | REM1(2) | Forward | 12.31 | 6.11 | tctccacgc | 6775729-6775738 |
| | NAC | ANAC55(1) | Reverse | 10.55 | 5.25 | atacgtata | 6775820-6775829 |
| | MYB | MYB59 | Forward | 10.18 | 4.74 | atttggtc | 6775997-6775006 |
| | MYB | MYB59 | Forward | 10.18 | 4.74 | atttggtc | 6775997-6775006 |
| | AP2/EREBP | ORA47(1) | Reverse | 12.93 | 8.19 | tggtcggtc | 6776000-6776009 |
| | AP2/EREBP | DEAR3(2) | Reverse | 11.86 | 5.92 | ggtcggtca | 6776002-6776011 |
| | WRKY(Zn) | ZAP1 | Reverse | 12.26 | 8.48 | gacgctaa | 6776003-6776011 |
| | WRKY(Zn) | WRKY18(1) | Forward | 11.77 | 6.28 | acgctaagg | 6776004-6776013 |
| | WRKY(Zn) | WRKY12 | Reverse | 12.11 | 6 | cgctaaggc | 6776005-6776014 |
| | WRKY(Zn) | WRKY38(1) | Reverse | 11.82 | 5.94 | cgctaaggc | 6776005-6776014 |
| | GARP/ARR-B | ARR14(1) | Reverse | 10.99 | 6.35 | ccgaatctc | 6776017-6776026 |
| | GARP/ARR-B | ARR11(1) | Reverse | 11.16 | 6.11 | ccgaatctc | 6776018-6776027 |
| | HSF | HSFB2a(1) | Forward | 10.67 | 5.24 | aaaagtgca gaagaagaaact | 6776019-6776028 |
| | NAC | ANAC81 | Forward | 21.7 | 9.67 | gataaaaacaaa | 6776041-6776065 |
| | C2H2(Zn) | ID1 | Reverse | 11.02 | 5.71 | aaagaacaaa | 6776083-6776093 |
| | GARP | ARR10 | Forward | 12.04 | 5.23 | gcttagatatt | 6776237-6776248 |
| | MYB | RVE1(1) | Reverse | 10.28 | 5.29 | tagatattt | 6776240-6776249 |
| | MYB | CCA1(1 | Reverse | 10.46 | 6.44 | tagatattt | 6776240-6776249 |
| | ARF | ETT(2) | Forward | 11.71 | 5.73 | tgtcgatcg | 6776378-6776387 |
| | AP2/EREBP | RAV1(2) | Reverse | 11.21 | 4.98 | ctcacctgatt | 6776682-6776693 |

| | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| **Hsp20** | 19981814-19983752 | Forward | AP2/EREBP | DEAR3(1) | Reverse | 11.84 | 4.93 | gtttggtg | 19983870-19983879 |
| | | | AP2/EREBP | ORA47(1) | Forward | 12.93 | 8.19 | cgccgacct | 19983871-19983880 |
| | | | HSFC1(2) | HSFC1(2) | Forward | 11.41 | 5.57 | agaaacttc | 19983913-19983922 |
| | | | HSFC1(2) | HSFC1(2) | Reverse | 11.41 | 5.57 | agaaacttc | 19983913-19983922 |
| | | | HSF | HSFB2a(1) | Forward | 10.67 | 5.24 | agaaacttc | 19983913-19983922 |
| | | | HSF | HSFB2a(1) | Reverse | 10.67 | 5.24 | agaaacttc | 19983913-19983922 |
| | | | AP2/EREBP | RAV1(2) | Forward | 11.21 | 4.98 | gcacctgaga | 19983927-19983938 |
| | | | HSFC1(2) | HSFC1(2) | Reverse | 11.41 | 5.57 | agaaacttc | 19984025-19984034 |
| | | | other | TBP | Forward | 10.98 | 5.16 | cctataaat tagctgtacgaac | 19984057-19984068 |
| | | | SBP | AtSPL3 | Reverse | 11.69 | 5.09 | ca | 19984078-19984093 |
| | | | AP2/EREBP | RAP2.3(3) | Forward | 12.95 | 7.09 | gacgccgtc | 19984270-19984279 |
| | | | bHLH | OsbHLH66 | Forward | 14.81 | 8.24 | cgcacgtgttc | 19984354-19984366 |
| | | | AP2/EREBP | DEAR3(2) | Reverse | 11.86 | 5.92 | gtcggttac | 19984584-19984593 |
| | | | Trihelix | At5g28300 | Reverse | 10.64 | 5.11 | gttaccgtg | 19984588-19984597 |
| 6 **COC1** | 33666 - 37840 | Reverse | Trihelix | At5g28300 | Forward | 10.64 | 5.11 | ccgtgaaa | 37299-37308 |
| | | | AP2/EREBP | RAV1(1) | Forward | 10.65 | 5.01 | ctccagcagaa | 37321-37332 |
| | | | AP2/EREBP | DEAR3(1) | Forward | 11.84 | 4.93 | caccgcag | 37383-37392 |
| | | | AP2/EREBP | RAP2.3(3) | Forward | 12.95 | 7.09 | cgcagccaa | 37386-37395 |
| | | | MYB | RVE1(1) | Reverse | 10.28 | 5.29 | aaaatctct | 37681-37690 |
| | | | bZIP | TGA1a | Forward | 10.45 | 5.21 | acacgtggt | 37944-37953 |
| | | | bZIP | TGA1a | Reverse | 10.45 | 5.21 | acacgtggt | 37944-37953 |
| | | | bHLH | MYC3 | Reverse | 10.11 | 5.27 | acgtggt | 37945-37952 |
| | | | bHLH | MYC3 | Forward | 10.11 | 5.27 | acgtggt | 37945-37952 |
| | | | bHLH | MYC4 | Forward | 11.04 | 7.04 | acgtggt | 37945-37952 |

| bHLH | MYC4 | Reverse | 11.04 | 7.04 | acgtggt | 37945-37952 |
|---|---|---|---|---|---|---|
| bHLH | MYC2 | Reverse | 10.97 | 5.76 | acgtggt | 37945-37952 |
| bHLH | MYC2 | Forward | 10.97 | 5.76 | acgtggt | 37945-37952 |
| MYB | RVE1(1) | Reverse | 10.28 | 5.29 | tgatattgt | 37951-37960 |
| HSFC1(2) | HSFC1(2) | Forward | 11.41 | 5.57 | agaagcttc | 38015-38024 |
| HSFC1(2) | HSFC1(2) | Reverse | 11.41 | 5.57 | agaagcttc | 38015-38024 |
| bZIP | TGA1a | Reverse | 10.45 | 5.21 | tcacgtagg | 38033-38042 |
| MYB | AtMYB61 | Reverse | 11.85 | 5.65 | gtgtggtgg | 38051-38060 |
| MYB | AtMYB61 | Reverse | 11.85 | 5.65 | tggtggtcc | 38054-38063 |
| NAC | ANAC55(1) | Forward | 10.55 | 5.25 | ttacgtgta | 38071-38080 |
| AP2/EREBP | ABI4(2) | Forward | 12.9 | 8.03 | cggtgccag | 38123-38132 |
| AP2/EREBP | RAV1(2) | Reverse | 11.21 | 4.98 | tgccagctgtg | 38125-38136 |
| MYB | AtMYB61 | Reverse | 11.85 | 5.65 | tgatggtgt | 38134-38143 |
| SBP | AtSPL3 | Reverse | 11.69 | 5.09 | atggtgactgctc | 38136-38151 |
| GATA | AGP1 | Reverse | 10.11 | 5.45 | ggatctggg | 38164-38173 |

**Table 19. Predicted TFBSs identified for *bZIP1* gene using STIFDB2 database**

| No: | Transcription Factor | Z - Score | Start Residue | End Residue | Chromosome Location | Orientation | Cis elements | Stress signals |
|---|---|---|---|---|---|---|---|---|
| 1 | Myb_box5_MYB | 2.3759 | 17 | 22 | 5'UTR | Reverse | [C/T]AAC[A/T/G/C][A/G] | Dehydration, Wounding |
| 2 | Myb_box5_MYB | 2.3759 | -485 | -480 | Upstream | Reverse | [C/T]AAC[A/T/G/C][A/G] | Dehydration, Wounding |
| 3 | Myb_box5_MYB | 2.3759 | -217 | -212 | Upstream | Forward | [C/T]AAC[A/T/G/C][A/G] | Dehydration, Wounding |
| 6 | Myb_box2_MYB | 3.2611 | -218 | -213 | Upstream | Reverse | CC[T/A]ACC | Dehydration, Wounding |
| 7 | Myb_box1_MYB | 2.4906 | -485 | -480 | Upstream | Reverse | (T/C)AAC[G/T]G | Dehydration, Wounding |
| 8 | G_box2_bZIP | 3.2030 | 61 | 66 | 5'UTR | Reverse | TGACG[T/C] | ABA, DROUGHT |
| 9 | C_ABRE_bZIP | 4.2939 | -864 | -859 | Upstream | Forward | CGCGTG | ABA, DROUGHT |
| 10 | W_box_WRKY | 2.5427 | -289 | -284 | Upstream | Forward | (T)TGAC[C/T] | Biotic stress (pathogen attack), Abiotic Stress (wind, rain, hail) |
| 12 | HSE1_HSF | 1.1837 | -208 | -201 | Upstream | Forward | TTC(A/T/G/C)(A,T,G,C)G AA,GAA(A/T/G/C)(A,T,G ,C)TTC | Drought, Cold, heavy-metal stress, oxidative stress |
| 13 | Myb_box3_MYB | 2.4754 | -376 | -371 | Upstream | Reverse | TAACTG | Dehydration, Wounding |

**Table 20. Predicted TFBSs identified for *AP2-EREBP* gene using STIFDB2 database**

| No: | Transcription Factor | Z-Score | Start Residue | End Residue | Chromosome Location | Orientation | *Cis*-elements | Stress signals |
|---|---|---|---|---|---|---|---|---|
| 1 | Myb_box5_MYB | 2.0282 | -406 | -401 | Upstream | Reverse | [C/T]AAC[A/T/G/C][A/G] | Dehydration, Wounding |
| 2 | Myb_box5_MYB | 2.0282 | -501 | -496 | Upstream | Forward | [C/T]AAC[A/T/G/C][A/G] | Dehydration, Wounding |
| 3 | Myb_box5_MYB | 2.0282 | -897 | -892 | Upstream | Reverse | [C/T]AAC[A/T/G/C][A/G] | Dehydration, Wounding |
| 4 | Myb_box5_MYB | 2.0282 | -839 | -834 | Upstream | Reverse | [C/T]AAC[A/T/G/C][A/G] | Dehydration, Wounding |
| 5 | Myb_box5_MYB | 2.0282 | -175 | -170 | Upstream | Forward | [C/T]AAC[A/T/G/C][A/G] | Dehydration, Wounding |
| 6 | Myb_box5_MYB | 2.0282 | -962 | -957 | Upstream | Forward | [C/T]AAC[A/T/G/C][A/G] | Dehydration, Wounding |
| 7 | Myb_box5_MYB | 2.0282 | -349 | -344 | Upstream | Reverse | [C/T]AAC[A/T/G/C][A/G] | Dehydration, Wounding |
| 8 | G_box_bHLH | 3.3422 | -434 | -429 | Upstream | Reverse | CACGTG | Nacl, ABA, Drought |
| 9 | Myb_box2_MYB | 2.9249 | -985 | -980 | Upstream | Reverse | CC[T/A]ACC | Dehydration, Wounding |
| 10 | Myb_box1_MYB | 2.1151 | -406 | -401 | Upstream | Reverse | (T/C)AAC[G/T]G | Dehydration, Wounding |
| 11 | Myb_box1_MYB | 2.1151 | -962 | -957 | Upstream | Forward | (T/C)AAC[G/T]G | Dehydration, Wounding |
| 12 | Myb_box1_MYB | 2.1151 | -349 | -344 | Upstream | Reverse | (T/C)AAC[G/T]G | Dehydration, Wounding |
| 13 | Myb_box1_MYB | 2.1151 | -897 | -892 | Upstream | Reverse | (T/C)AAC[G/T]G | Dehydration, Wounding |

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| | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| 14 | Myb_box1_MYB | 2.1151 | -839 | -834 | Upstream | Reverse | (T/C)AAC[G/T]G | Dehydration, Wounding |
| 15 | G_box2_bZIP | 2.1139 | -363 | -358 | Upstream | Reverse | TGACG[T/C] | ABA, DROUGHT |
| 16 | HSE1_HSF | 0.7710 | -159 | -152 | Upstream | Forward | TTC(A/T/G/C)(A,T,G,C)GAA, GAA(A/T/G/C)(A,T,G,C)TTC | Drought, Cold, heavy-metal stress, oxidative stress |
| 17 | HSE1_HSF | 0.7710 | -164 | -157 | Upstream | Forward | TTC(A/T/G/C)(A,T,G,C)GAA, GAA(A/T/G/C)(A,T,G,C)TTC | Drought, Cold, heavy-metal stress, oxidative stress |
| 18 | Myb_box3_MYB | 2.1170 | -927 | -922 | Upstream | Forward | TAACTG | Dehydration, Wounding |

**Table 21. Predicted TFBSs identified for *Hsp20* gene using STIFDB2 database**

| No. | Transcription Factor | Z - Score | Start Residue | End Residue | Chromosome Location | Orientation | *Cis* elements | Stress signals |
|---|---|---|---|---|---|---|---|---|
| 1 | HSE1_HSF | 1.7737 | -125 | -118 | Upstream | Forward | TTC(A/T/G/C)(A,T,G,C)GA A,GAA(A/T/G/C)(A,T,G,C) TTC | Drought, Cold, heavy-metal stress, oxidative stress |
| 2 | Myb_box5_MYB | 2.9589 | 28 | 33 | 5'UTR | Reverse | [C/T]AAC[A/T/G/C][A/G] | Dehydration, Wounding |
| 3 | W_box_WRKY | 2.3797 | -579 | -574 | Upstream | Reverse | (T)TGAC[C/T] | Biotic stress (pathogen attack), Abiotic Stress (wind, rain, hail) |
| 4 | G_box_bHLH | 3.5978 | -242 | -237 | Upstream | Forward | CACGTG | Nacl, ABA, Drought |
| 5 | GCC_box_AP2_ERE BP | 4.5484 | -548 | -543 | Upstream | Reverse | GCCGCC | Cold, Drought |

**Table 22. Predicted TFBSs identified for *COC1* gene using STIFDB2 database**

| No | Transcription Factor | Z - Score | Start Residue | End Residue | Chromosome Location | Orientation | *Cis* elements | Stress signals |
|----|----------------------|-----------|---------------|-------------|---------------------|-------------|----------------|----------------|
| 1 | Myb_box5_MYB | 2.7037 | 325 | 330 | 5'UTR | Forward | [C/T]AAC[A/T/G/C][A/G] | Dehydration, Wounding |
| 2 | G_box_bHLH | 3.1949 | -111 | -106 | Upstream | Forward | CACGTG | Nacl, Aba, Drought |
| 3 | DREB_AP2_EREBP | 2.5805 | -129 | -124 | Upstream | Reverse | GCCGCC | Cold, Drought |
| 4 | G_box2_bZIP | 2.0155 | -138 | -133 | Upstream | Reverse | TGACG[T/C] | ABA, Drought |
| 5 | Myb_box1_MYB | 2.7584 | 325 | 330 | 5'UTR | Forward | (T/C)AAC[G/T]G | Dehydration, Wounding |
| 6 | AuxRE_ARF | 2.6096 | 246 | 251 | 5'UTR | Forward | TGTCTC | Auxin |
| 7 | AuxRE_ARF | 2.6096 | 126 | 131 | 5'UTR | Reverse | TGTCTC | Auxin |
| 8 | HBE_HB | 1.9873 | -859 | -851 | Upstream | Reverse | HB | ABA, Drought |
| 9 | HSE1_HSF | 1.4109 | -183 | -176 | Upstream | Forward | TTC(A/T/G/C)(A,T,G,C)GAA, GAA(A/T/G/C)(A,T,G,C)TTC | Drought, Cold, heavy-metal stress, oxidative stress |
| 10 | W_box_WRKY | 2.0661 | -532 | -527 | Upstream | Forward | (T)TGAC[C/T] | Biotic stress, Abiotic Stress (wind, rain, hail) |
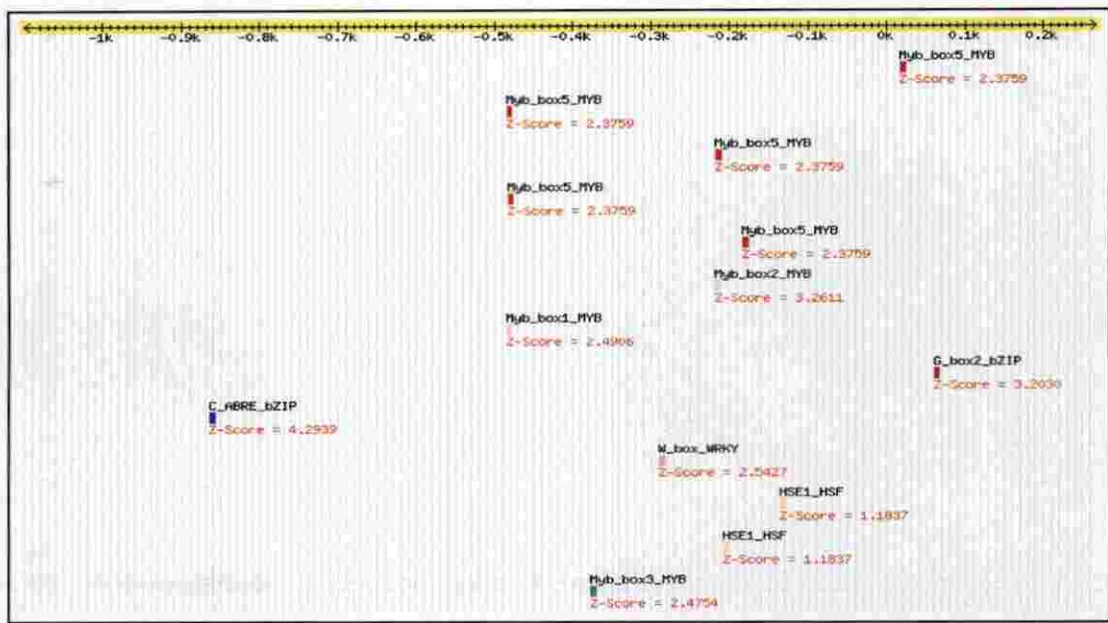
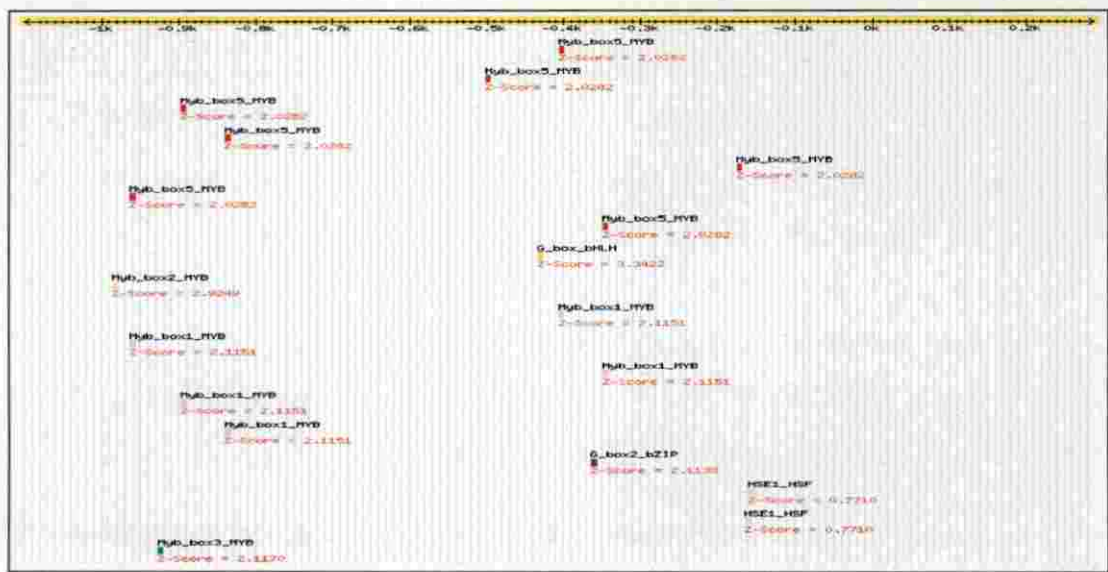**Fig 21. 1000bp TF map of *bZIP1* (AT3G19290) gene**



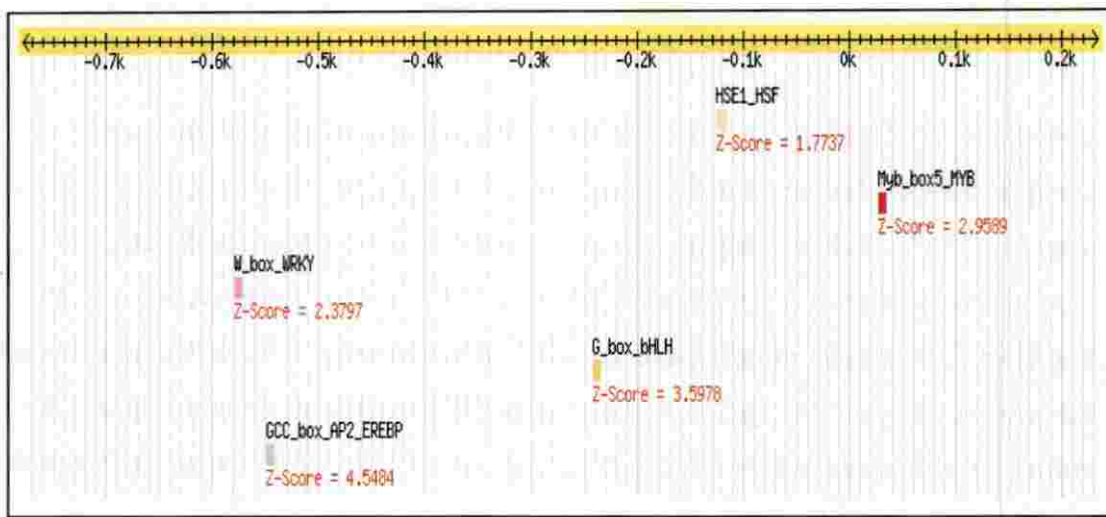**Fig 22. 1000bp TF map of *AP2-EREBP* (AT5G05410) gene**
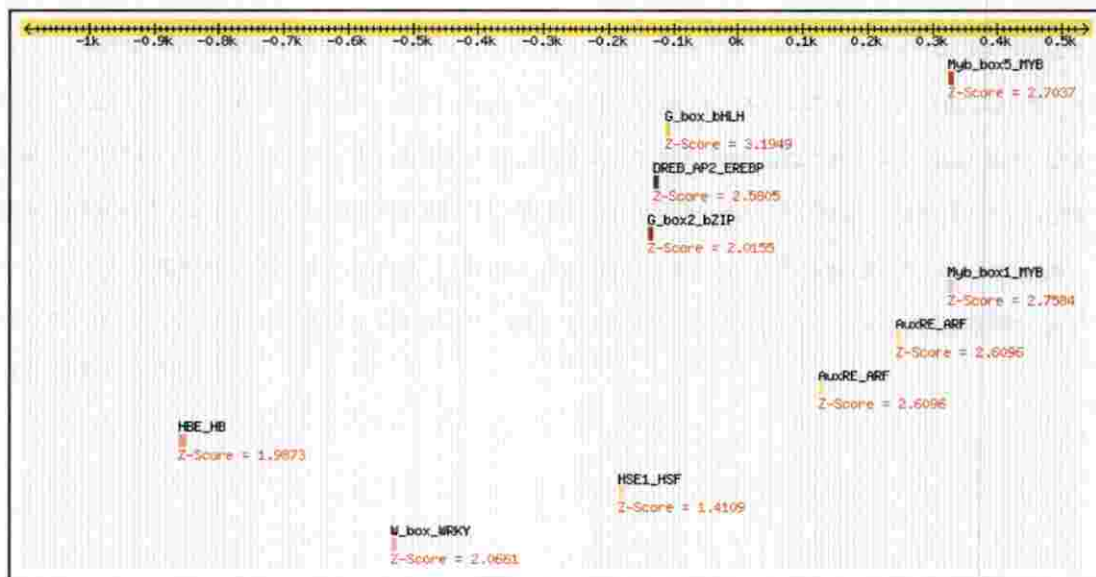
Fig 23. 1000bp TF map of *Hsp20* (AT1G53540) gene



Fig 24. 1000bp TF map of *COC1* (AT1G01060) gene

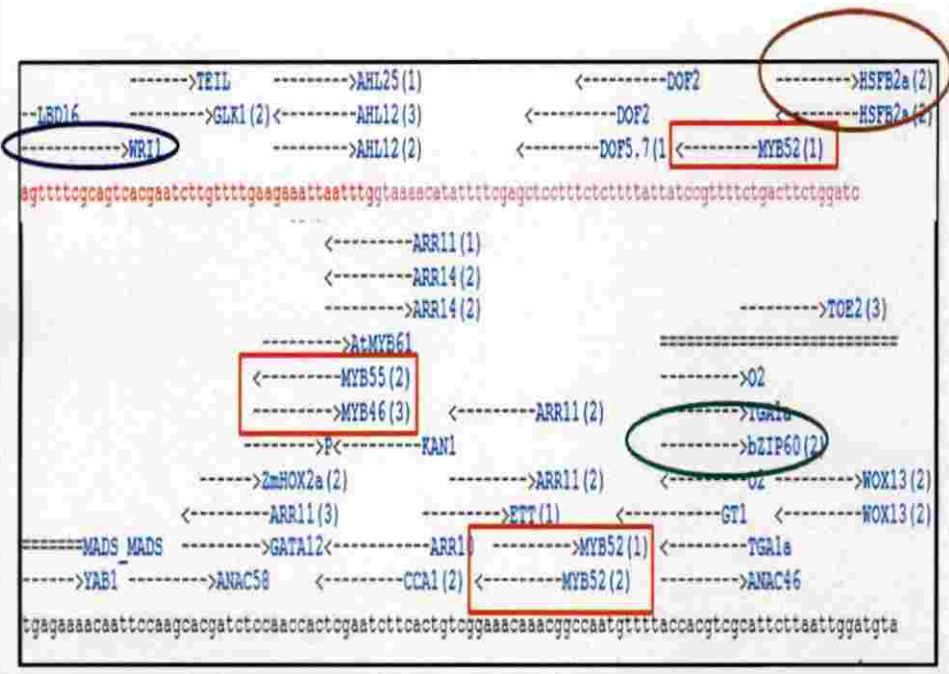### 4.7.5. Cross validation with identified transcription factor binding sites

Few transcription factors in *bZIP1* (AT3G19290) *AP2-EREBP* (AT5G20060), *Hsp20* (AT1G5354) and *COC1* (AT1G01060) genes were found in both AthaMap and STIFDB2 databases, when cross validated AthaMap database with STIFDB2. In gene bZIP1, the transcription factors MYB, HSF, bZIP and WRKY were localized in both AthaMap and STIFDB2 databases (Fig. 25). The transcription factors HSF, bZIP and MYB found common in *AP2-EREBP* gene promoter (Fig. 26). In gene *Hsp20*, the transcription factors HSF, WRKY and bHLH/PIF5 (PIF5 belongs to bHLH TF family mentioned in AthaMap database) (Fig. 27). The transcription factors AP2-EREBP/DEAR3, bHLH/MYC3, HSF and bZIP/TGA1a were localized in both AthaMap and STIFDB2 (Fig. 28).

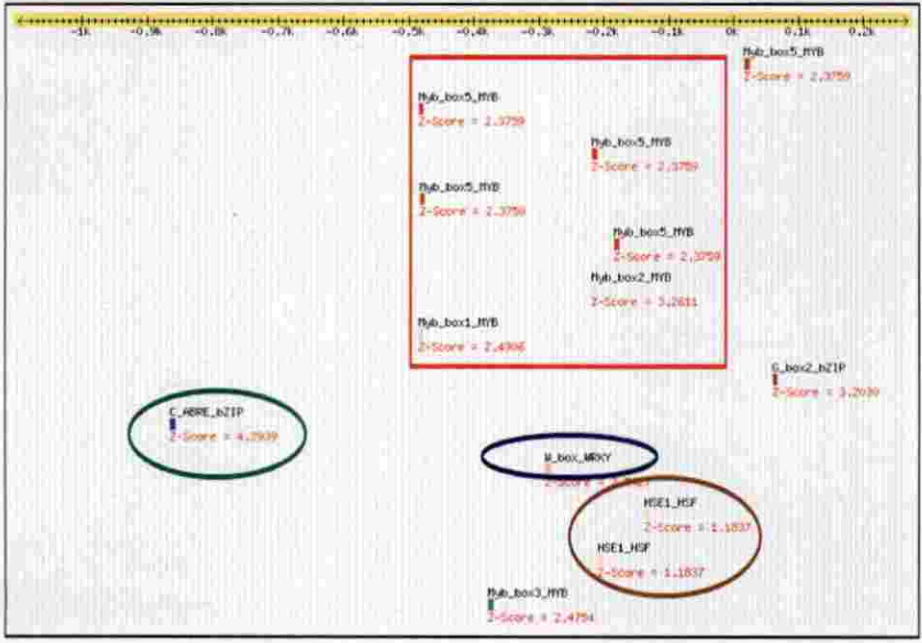### 4.8. Identification of combinatorial *cis*-regulatory elements

From the data curated, the combinatorial *cis*-regulatory elements for three genes viz, *CO I*, *AP2-EREBP* and *Hsp20* were identified. Out of 27 transcription factor families mentioned in the AthaMap, that contains families and sub families, the transcription factors belonging to MYB, WRKY, bHLH and Trihelix families which are mainly responsible for drought stress signals, were predicted as co-associated. Curated data of transcription families, sub families, identified from AthaMap are provided in Table 23.

**Table 23. Identified combinatorial *cis*-regulatory elements for *CO I*, *AP2-EREBP* and *Hsp20* genes using AthaMap database**

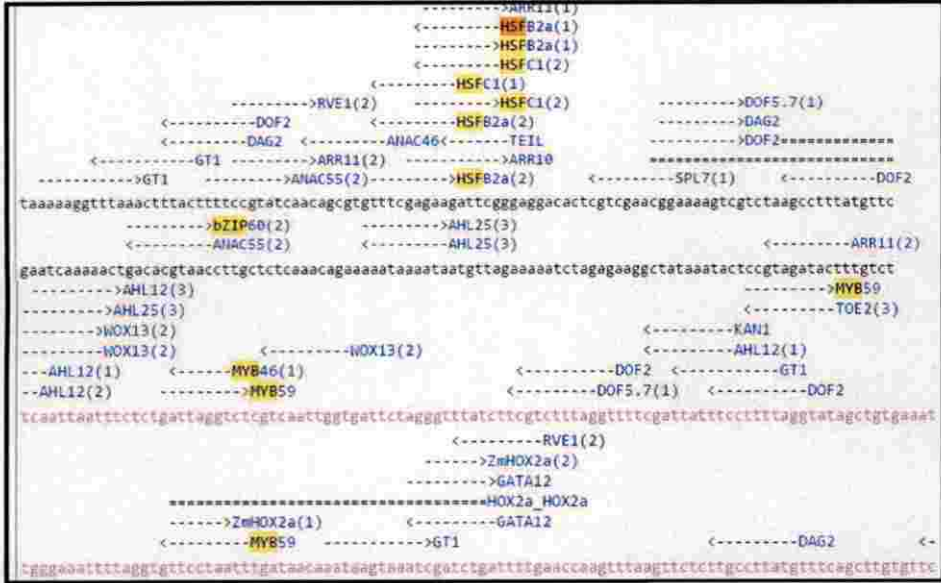| Sl.No | Query gene | Transcription Factor Family (TFF) | | Transcription Factor (TF) | | Spacer | Sequence | |
|---|---|---|---|---|---|---|---|---|
| | | TFF 1 | TFF2 | TF1 | TF2 | | TF1 | TF2 |
| 1 | *CO I* | MYB | MYB | MYB83 | MYB59 | 1 | gttggt | aagttggtc |
| | | | | | | 1 | cattag | atcattagg |
| | | | | | | 2 | acctaa | aaacctaat |
| | | | | | | 2 | gtgactc | aacctttgg |
| 2 | *AP2-EREBP* | MYB | MYB | MYB83 | MYB59 | 1 | tttggt | aatttggtc |
| | | | | | | 5 | ggtcgg | atttggtc |
| | | | | | | 1 | agttgg | aagttggta |
| | | | | | | 1 | tttggt | tatttggta |
| 3 | *Hsp20* | Trihelix | bHLH | At5g28300 | OsbHLH66 | 20 | gcgcacgtgttc | tttaccgg |
| | | bHLH | bHLH | MYC2 | OsbHLH66 | 19 | acgtgttc | gcgcacgtgttc |
| | | bZIP | bZIP | TGA1 | bZIP60(1) | 1 | tcacgtcatt | ttcacgtca |
| | | ABI3/VP1 | NAC | REM1(1) | TaNAC69(2) | 2 | gcttcaact | atgcttcaactgattcacgtcat |

**Fig 25. Common TFs found in** *bZIP1* **gene promoter; the transcription factors MYB, HSF, bZIP and WRKY were localized in both AthaMap and STIFDB2 databases**
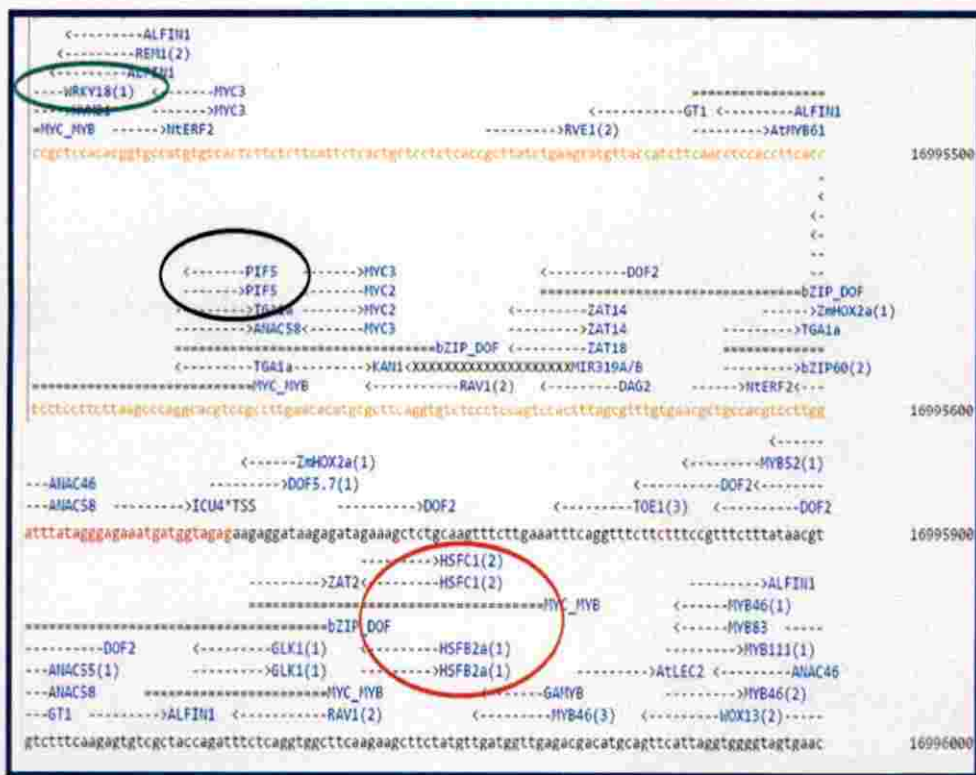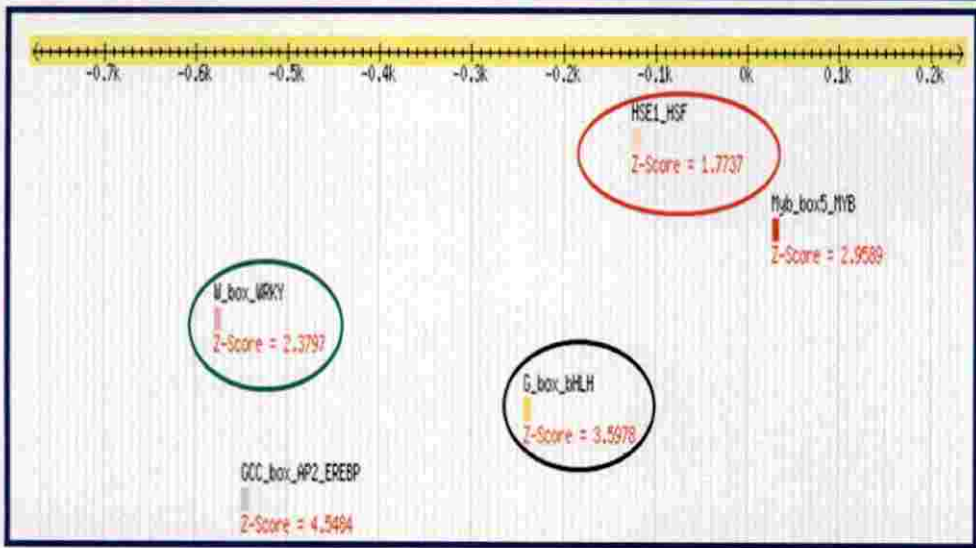
**AthaMap**
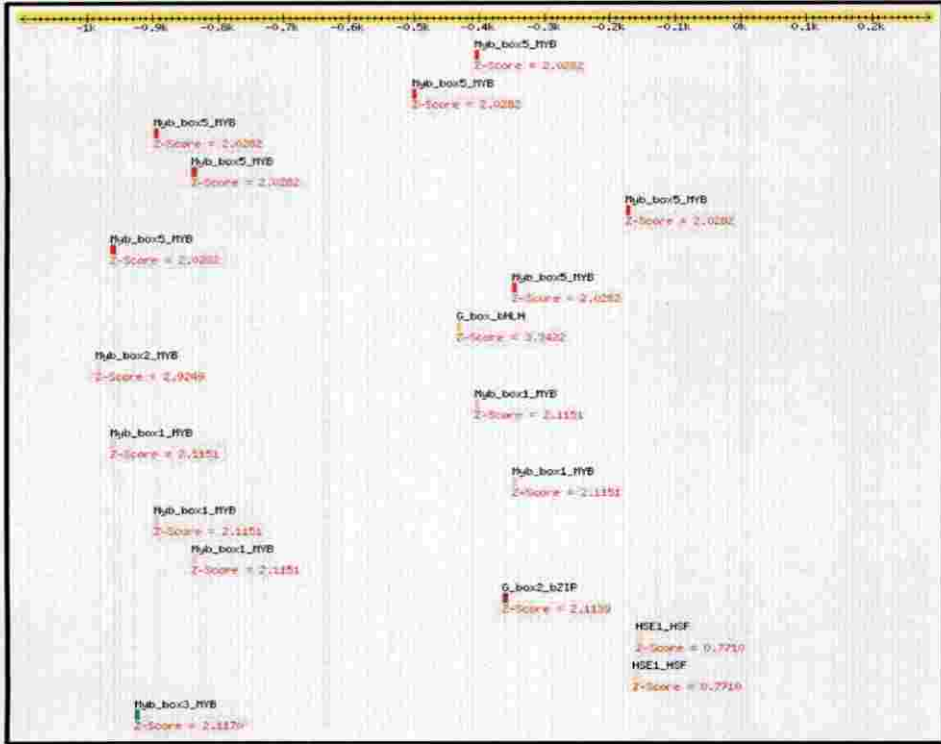
**STIFDB2**

Fig 26 Common TFs found in *AP2-EREBP* gene promoter; the transcription factors HSF, bZIP and MYB were localized in both AthaMap and STIFDB2 databases
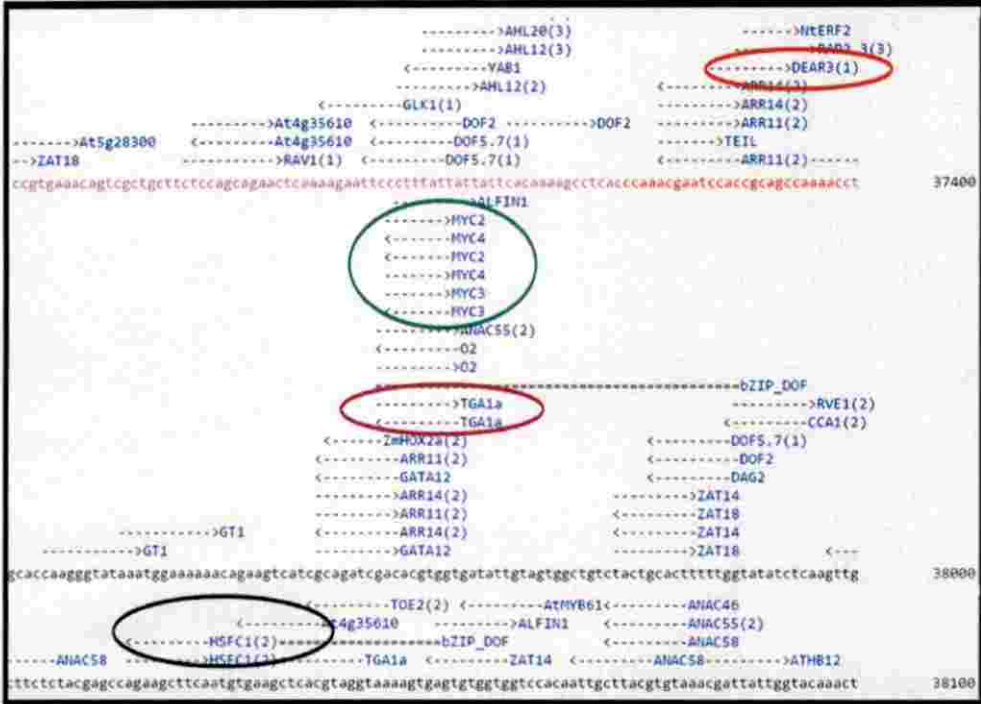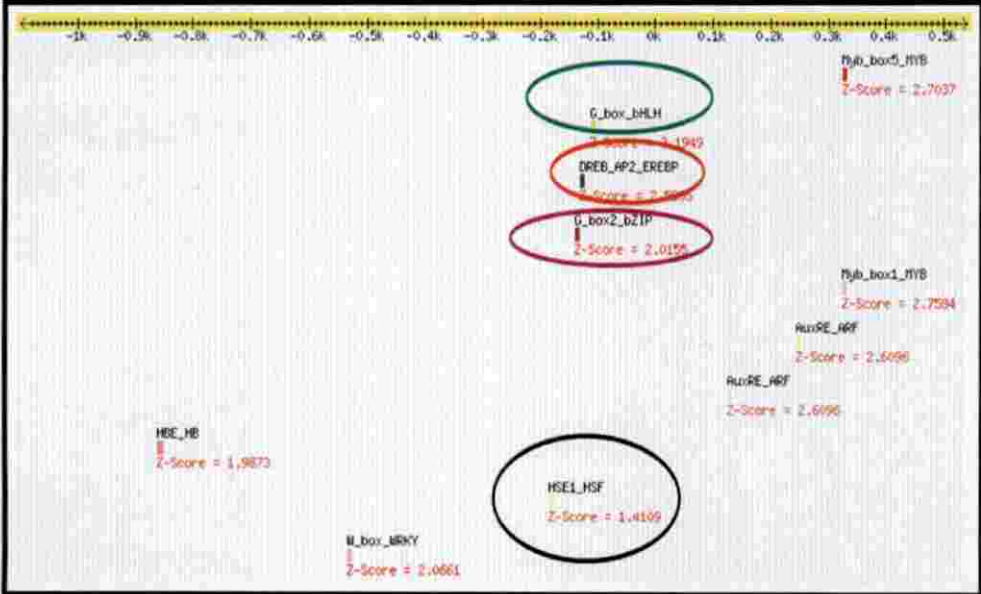
Fig 27. Common TFs found in *Hsp20* gene promoter; the transcription
factors HSF, WRKY and bHLH/PIF5 were localized in both
AthaMap and STIFDB2 databases

**Fig 28. Common TFs found in *COC1* gene promoter; the transcription
factors AP2-EREBP/DEAR 3, bHLH/MYC3, HSF and
bZIP/TGA1a were localized in both AthaMap and STIFDB2
databases**

# Discussion

# 5. DISCUSSION

This study was under taken to validate the differentially expressed genes which are expressed at reproductive stage of rice for water stress by the influence of *Pf* (Baburao, 2012) and to identify putative transcription factor binding sites and combinatorial *cis*-regulatory elements. The results presented in the study are discussed in this chapter.

## 5.1. Analysis of biometric parameters

Biometric parameters were measured in three treatments. There was significant difference in shoot length, root length, fresh weight, dry weight, yield and 1000 g seed weight between the treatments. The significant increase in shoot length, root length, fresh weight, dry weight, yield and 1000 g seed weight was found in absolute control plants than that of water stressed and water stressed + *Pf* treated plants. But in *Pf* + water stress plants showed noteworthy increase in shoot length, root length, fresh weight, dry weight, yield and 1000 g seed weight than water stressed plants.

Baburao, (2012) analyzed biometric parameters in vegetative stage for the Matta Triveni (PTB 45) rice variety. It has been observed that maximum shoot length, root length, no. of tillers, fresh weight, dry weight and recovery in *Pf* treated + water stressed plants and showed significantly superior over other two such as absolute control and water stressed. In present study the biometric parameters were analyzed after harvest and water was withheld in reproductive stage, in which the absolute control plants were superior over water stressed and water stressed + *Pf* treated plants. It may be due to high susceptibility of rice to water stress during the reproductive stage. Kamoshita *et al.* (2008) reported that plant growth will be resumed after the vegetative stage drought. This resumed growth will affect the development of sink size and source supply. Manjunatha *et al.* (2015) observed

reduced accumulation of photosynthates in the reproductive parts (seed formation and grain filling) during reproductive stage due to water stress conditions.

The effects of *Pseudomonas fluorescens* have been analyzed in relation with drought resistance and its systemic tolerance for water stress. *P. fluorescens* is a Plant Growth Promoting Rhizobacteria (PGPR). It promotes plant growth and development by facilitating the uptake of nutrients from the environment (Kandasamy *et al.*, 2009). Effect of PGPR on plant growth and development is manifested as increase in germination rates, root growth and leaf area. Chlorophyll content, magnesium, nitrogen and protein content are also improved as an effect of PGPR application. Other effects of PGPR include increase in hydraulic activity, tolerance to drought and salt stress, shoot and root weight. They also delay the leaf senescence (Lucy and Glick, 2004). PGPR mediated plant growth enhancement was reported by many workers (Kloepper *et al.*, 1988; Peer *et al.*, 1989; Hergarten *et al.*, 1998; Glick *et al.*, 1999; Polyanskaya *et al.*, 2000; Saravanakumar *et al.*, 2009). The growth promotional activity of *P. fluorescens* in rice has been revealed previously under different conditions such as laboratory, glass house and field conditions. However, the molecular basis of host plant - PGPR interaction in promoting plant growth is less understood yet. Baburao, (2012) identified six differentially expressed genes in rice variety Matta Triveni a popular variety of Kerala susceptible to drought with the application of *P. fluorescens*. Real time PCR was used in this study to analyze the gene expression levels of differentially expressed genes that are expressed in water stressed plants under the influence of *P. fluorescens*.

## 5.2. Extraction and quantification of total RNA

The total RNA was isolated from three different treatments with TRIzol (or TRI Reagent) method. TRIzol is a single phase solution made of phenol and guanidiniumisothiocyanate. This is a single step method wherein the TRI reagent solubilizes the biological sample and at the same time it denatures the protein.

Further, on adding chloroform, there is phase separation (much like extraction with phenol:chloroform:isoamyl alcohol). Here the protein is extracted to the organic phase. DNA is obtained at the interface whereas RNA can be extracted from the aqueous phase. The reagent is named as TRIzol as all the three components namely, RNA, DNA, and protein can be purified from a single sample. This method is more of use in those cells or tissues which contain high level of endogenous RNases or when it is practically difficult to separate the cytoplasmic RNA from the nuclear RNA. The method is also an effective procedure to isolate small RNAs including microRNAs and piwi-associated RNAs. Isolation of endogeneous, small interfering RNAs can also be done with the same procedure.

While disrupting cells and dissolving the cell components during sample homogenization, it is TRIzol that maintains the integrity of RNA. Addition of chloroform followed by centrifugation will separate DNA, RNA and proteins. Aqueous phase will have the RNA and the organic phase will have the DNA and protein. RNA will be present only in the aqueous phase. From the aqueous phase, RNA is recovered by precipitating with isopropanol. It is then washed with 70% ethanol and stored in DEPC water at $-80^0$C freezer.

Formaldehyde-agarose gel electrophoresis and spectrophotometric analysis of samples helps to know the quality and quantity of RNA. Good quality of RNA samples are indicated by the presence of clear, intact and distinct bands. RNA quality can also be analyzed by checking the presence of bands for 28S, 5S rRNA and tRNA over the gel. A value between 1.8 and 2 for the absorbance ratio of 260/280 is considered to be best for RNA. The value less than 1.8 indicates that the sample consists of proteins, while a value above 2.0 indicates the possible contamination by polysaccharides and polyphenols (Sambrook and Russel, 2001). The quantity determined was about 2000 ng/µl in each sample which was a good quantity required for first cDNA synthesis. Earlier Shankar (2009), Baburao (2012), Katiyar *et al.*

(2012), Kumar *et al.* (2015) isolated RNA from leaf samples in rice using TRIzol method.

## 5.3. Reverse Transcription Polymerase Chain Reaction and confirmation of cDNA

Various molecular biology detection procedures including the RT-PCR assays require good quality and quantity RNA for effective and accurate results. RT-PCR can be used for various studies such as gene expression analysis, molecular characterization of genes, cloning *etc.* Baburao (2012) used RT-PCR to analyze expression levels of genes in rice mediated by PGPR. Recently Babitha *et al.* (2015) used RT-PCR to analyze *EcbZIP60* gene which is a bZIP family transcription factor, Sajeevan and Nataraja (2016) used RT-PCR Molecular cloning and characterization of a novel basic helix–loop–helix-144 (bHLH144) like transcription factor from *Morus alba*. In this study, RT-PCR was used to study the expression levels of induced genes by *Pseudomonas fluorescens.* Initially the first strand cDNA was synthesized using Thermo Scientific RevertAid First Strand cDNA Synthesis Kit. RevertAid Reverse Transcriptase (RT) is a recombinant M-MuLV RT and has a consistent activity at temperature ranging from 42 to 50°C which is suitable for synthesis of cDNA. RiboLock RNase Inhibitor is available with the kit. This prevents the degradation of RNA templates. The poly A tail of mRNA was selectively annealed using oligo (dT)18 primer. Using RNA as the template strand, cDNA was synthesized whereas actin, the housekeeping gene was used to synthesize second strand cDNA.

### 5.4. Primer designing and validation for real-time PCR

For relative gene expression analysis in the present study, the gene specific primers were designed for *CO I, PKDP, bZIP1, AP2-EREBP, Hsp20 and COC1* genes. The IDT PrimerQuest tool (http://scitools.idtdna.com/Primerquest/) was used

for designing primers for real-time PCR. Primers were designed based on conserved sequences present for these genes. The IDT PrimerQuest tool is one of the most frequently used IDT SciTools® programs that enables to design primers and probes for a wide variety of applications. The advantage of using the PrimerQuest program lies in its customizable designs, which allows users to adjust reaction conditions (e.g., primer, salt, and $Mg^{2+}$ concentrations). The program allows the use of a specific primer or probe sequence and also it specifically gives the design regions. Earlier Samadder *et al.* (2008) used IDT PrimerQuest tool for designing primers for *GUS* and *NOS* genes in rice for real-time PCR analysis. The primers used for the reference gene (rice Act1) were first designed by Yamanouchi *et al.* (2002).

### 5.5. Normalization of cDNA

While estimating cDNA with nanodrop, there is the interference of dNTPs, left over RNA, degraded RNA nucleotides, and cDNA, all of which contribute to the nanodrop reading and may result in fluctuating values. cDNA normalization is important to reduce the presence of high amount of transcripts and maintains equal amount of transcript concentrations in the sample (Vandesompele *et al.*, 2002; Huggett *et al.*, 2005). For normalization of cDNA, endogenous control actin was used and amplified in PCR with standardized thermal profile. The bands from the three samples showed een uniform intensity in agarose gel. When the cDNA samples were analyzed in real time PCR with actin gene specific primers, the Ct values gave nearly similar values in absolute control (23.57), water stressed (23.41) and water stressed + *Pf* treated (23.94) which denotes cDNA samples from all the three samples were normalized . Xu *et al.* (2015) used rice actin gene as the reference gene to normalize the target gene expression which was calculated using the relative quantization method ($2^{-\Delta\Delta CT}$).

## 5.6. Real-time PCR analysis

Real-time PCR record data during each cycle of the amplification process as it proceeds. It allows making a highly specific, sensitive and reproducible quantification of the initial amount of DNA template based on the fluorescence during each cycle of PCR. During the proceeding cycles, the increase in fluorescence is proportional to the amount of produced PCR product. Initially, there is only slight change in the fluorescent signals, and this describes the baseline. Detection of the accumulated target is possible only above the baseline. The cycle at which the fluorescence emitted by the target is above the threshold, known as the threshold cycle (Ct), characterize the reactions. The threshold level is always above the baseline and within the exponential region of the amplification curve. High amount of target DNA implies a higher increase in the fluorescent signal and a lower Ct and vice versa (Dorak, 2006).

SYBR® Green chemistry is a real-time PCR analysis procedure. Whenever high specificity is required, SYBR® Green is recommended for real-time PCR as this reagent efficiently suppresses primer-dimer and non-targeted product amplification. The minor groove of double-stranded DNA acts as the region for the SYBR Green dye to bind to the DNA. The intensity of fluorescence increases as the dye binds to double-stranded DNA and it further increases with production of more double-stranded amplicons. SYBR Green dye can bind any double-stranded DNA molecule.

In the present study, real-time PCR assay was performed to determine relative expression levels of differential genes from three different treatments in rice variety Matta Triveni. Relative quantification was carried out to analyze expression patterns of genes and absolute quantification was done to analyze dissociation curves.

Relative quantification was carried out with cDNA samples synthesized from total RNA isolated from leaves for all the three treatments (absolute control, water

stressed and water stressed + *Pf* treated) after imposing water stress. A housekeeping gene was selected as an endogenous control for normalizing the data. The gene expression data was derived for different treatments after normalizing the data with the expression of endogenous control.

In the present study, the SYBR Premix Ex Taq II (DSS Takara Bio India Pvt. Ltd) was used for detection of amplification in real-time PCR. This dye is non-specific in binding to DNA and hence can detect any double stranded DNA present in the reaction mixture. To ensure the specificity of the reaction, dissociation curve of amplified product was obtained during absolute quantification. The curves were obtained as a single dominant peak denoting that there was no non-specific amplification in any of the genes. Presence of multiple peaks indicates the presence of non-specific products and primer dimers.

### 5.6.1. Relative gene expression analysis

The relative quantification was done to analyze the gene expression at transcript level relative to another reference sample as control. During real-time PCR, changes in sample gene expressions are measured based on a reference sample.

In the present study, the relative quantification was carried out using comparative Ct method (Livak and Schmittgen, 2001). This method does not require a standard curve and hence is useful for assaying large number of samples. The changes in gene expression can be calculated as a relative difference between an experimental and calibrator sample in the comparative Ct method (Shankar, 2009). The control sample, which is maintained without any treatments or those samples which are stable throughout the experiment, is known as the calibrator sample. After relative quantification for the gene expression, the threshold cycle data which was obtained was normalized with the threshold cycle data, in the study, for expression of

an endogenous control gene. The endogenous control is the reference gene, mostly housekeeping gene and has a stable expression throughout the treatment.

### 5.6.1.1. Relative expression analysis of *CO I* gene

*CO I* is a mitochondrial DNA encoded subunit of respiratory complex IV, which is a biological catalyst in the electron transport chain of mitochondrial oxidative phosphorylation. CO I (Cytochrome c oxidase subunit I) protein is involved in regulation of carbohydrate, nitrogen and energy metabolism. Yan *et al.* (2005) reported that *CO I* protein act as a scavenging agent of reactive oxygen species and is involved in processing of mRNA and proteins. It is also involved in maintaining cytoskeleton stability. There are very few reports which are providing information about CO I gene involved in drought stress. Sanchari and Parekh (2015) observed that 18 drought responsive genes in rice were up-regulated at a rate of four times across all tissues and stages and *CO I* was one among those genes. Here in this study, the *CO I* gene was up-regulated both in water stressed plants as well as water stressed + *Pf* treated plants. Water stressed + *Pf* treated plants showed 2.36 fold increase in gene expression levels and there was also a slight increase in level of gene expression (1.4 fold) for water stressed plants.

### 5.6.1.2. Relative expression analysis of *PKDP* gene

Real-time PCR was employed to examine *PKDP* gene expression, in response to various treatments namely, control, water stressed and water stressed + *Pf* treated plants. Results demonstrated that *PKDP* is a positive regulator of water stress tolerance under the influence of *Pseudomonas fluorescens* treatments. It has been showed that there was 2 fold increase in relative gene expression in water stressed + *Pf* treated plants (Fig. 17).

The *PKDP* is structurally conserved domain with a catalytic function of Protein kinases (Scheeff and Bourne, 2005). Protein kinases are involved in the

phosphorylation reaction, wherein a phosphate group is transferred onto proteins. This functions as switch for many cellular processes, including metabolism, transcription, cell cycle progression and various others. Embryonic development and many physiological responses are also switched on or off with the help of various protein kinases.

Plant-specific serine/threonine kinases included under the SnRK2 family are involved in plant response to abiotic stresses and abscisic acid (ABA)-dependent plant development. Members of SnRK2s have been classified into three groups. Group 1 comprises of kinases that are not activated by ABA whereas group 2 comprises of kinases which are not activated or activated very weakly by ABA. Those kinases that are strongly activated by ABA are grouped under group 3. ABA-dependent kinases belong to group 3 and are the major regulators of plant response to ABA. Direct phosphorylation of different downstream targets such as SLAC1, KAT1, AtRbohF, and transcription factors required for the expression of stress responsive genes are responsible for the regulation of the plant response to ABA via SnRK2s pathways. Group 2 and 3 kinases have certain similar cellular functions and act as positive regulators of plant responses to water deficit. Kulik *et al.* (2011) reported their role in complementing the ABA-dependent kinases in plant defense against environmental stress.

Another group of protein kinases in plants are the calcium-dependent protein kinases (CDPKs). They are involved in tolerance to abiotic stresses and in seed development. In rice, the functions of few CDPKs have been identified by Asano *et al.* in 2012. The expression of OsCPK9 in different organs in response to different treatments such as PEG6000, NaCl and ABA treatments were examined by quantitative real-time PCR. Abiotic stress tolerance, spikelet fertility and sensitivity were positively regulated by OsCPK9 (Wei *et al.*, 2014).

Growth under drought kinase (GUDK) is a drought-inducible receptor-like cytoplasmic protein kinase which is required for the grain yield under drought as well as well-watered conditions in rice plants (Ramegowda *et al.*, 2014). Induction of GUDK during drought was revealed by quantitative PCR (qPCR) analysis at different growth stages in wild rice in the seedling root, vegetative leaf, and flag leaf. It had shown 3-fold, 1.5-fold, and 2-fold increases in transcript levels respectively as compared with respective controls maintained at flooded conditions (Ramegowda *et al.*, 2014).

### 5.6.1.3. Relative expression analysis of *bZIP1* gene

The bZIP transcription factor family is an important component in the abscisic acid (ABA) signaling pathway which is activated during abiotic stress in plants. Liu *et al.* (2014) analyzed the expression pattern of OsbZIP71, a bZIP family transcription factor in real-time PCR and suggested its potential role in ABA-mediated drought and salt tolerance in rice.

Present study revealed the expression of *bZIP1* was induced by *Pf* treatments under water stress which showed *bZIP1* is a positive regulator and the importance of *Pseudomonas fluorescens* as a signaling molecule. The TFs encoded by *bZIP1* genes are involved in the plant development processes. To activate downstream gene expression, the bZIP transcription factors interact with ABA-responsive elements (ABREs). These are *cis*-acting small DNA regions present in the promoter region of ABA-inducible genes. Hence, these bZIP transcription factors are assigned as ABRE-binding factors (ABFs) or ABRE-binding proteins (AREBs) (Yamaguchi, 2005).

### 5.6.1.4. Relative expression analysis of *AP2-EREBP* gene

In this study, the gene had been expressed 2.71 fold increase in water stressed + *Pf* treated plants than that of control. In water stressed plants, the gene expression was not much different than in the control plants (Fig. 19).

Various stress-related responses and developmental processes in plants are highly influenced by AP2-EREBPs (APETALA2-ethylene responsive element binding proteins) transcription factors. AP2- EREBP genes is a multigene family playing key roles throughout the plant life cycle by regulating several developmental processes including determination of floral organ identity and leaf epidermal cell identity control. They also form a part of the defense mechanisms employed by the plants to respond to various biotic and abiotic stresses. Verma *et al.*, (2016) reported its role in various hormone-related signal transduction pathway including ABA, ethylene, cytokinin and jasmonates.

### 5.6.1.5. Relative expression analysis of *Hsp20* gene

Plant Hsps are expressed during a fascinating range of developmental processes, which indicates they may have important roles in the absence of stresses The transcription levels of *Hsp20* gene was significantly enhanced by *Pf* under water stress, which showed the gene may play roles in water stressed conditions in plants especially during reproductive stage and *Pseudomonas fluorescens* may indirectly influence the expression of *Hsp20*. The results obtained showed that the *Hsp20* gene was expressed under water stressed as well as in water stressed and *Pseudomonas fluorescens* augmented conditions. The relative gene expression of *Hsp20* gene was found upregulated in both water stressed and water stressed + *Pf* treated conditions. Water stressed + *Pf* treated plants showed 4.56 fold increase in gene expression and 1.36 fold increase in water stressed plants. The present study showed *Hsp* gene regulated by *Pseudomonas fluorescens* as an inducer which can increase the expression levels.

Previously Sarkar *et al.* (2009) conducted expression analysis based on real-time PCR showed that 19 sHsp genes were upregulated by high temperature stress. Besides heat stress, expression of sHsp genes was up or downregulated by other abiotic and biotic stresses. In addition to stress regulation, various sHsp

genes were differentially upregulated at different developmental stages of the rice plant. In 2009 Ouyang *et al.* 2009 reported the identification and characterization of 39 *OsHsp20* genes in rice and they have used real-time PCR to perform a characterization of the normal and heat shock-induced expression of selective *OsHsp20* genes.

### 5.6.1.6. Relative expression analysis of *COC1* gene

The *COC1* gene was expressed and showed 4 fold increase in gene expression. It was expressed highest in water stressed + *Pf* treated plants while in water stressed plants showed 1.47 fold increase than that of the control (Fig. 21).

*COC1* gene belongs to the MYB (Myeloblastosis) gene family is featured by highly conserved MYB DNA-binding domain. It is one of the richest groups of transcription factors in plants. MYB transcription factors play an important role in various functions in plant development and metabolism. They are also involved in hormone signal transduction, disease resistance and abiotic stress tolerance (Katiyar *et al.,* 2012). Genome-wide analysis revealed 155 *MYB* genes in rice and 197 in *Arabidopsis.* MYB family genes possess relatively more introns in the middle than at the C- and N-terminals of the genes. In rice and *Arabidopsis,* MYB-genes without introns are highly conserved. Several MYBs are up-regulated by various abiotic stresses both in rice and *Arabidopsis.*

### 5.7. Role of *Pseudomonas fluorescens* influencing gene expression during water stress

Induced systemic tolerance is a phenomenon whereby resistance against subsequent abiotic stress is induced at the whole plant level in response to colonization of the roots by certain plant growth-promoting rhizobacteria like *Pseudomonas fluorescens.* Compared with the relative means of information in model plant species such as *Arabidopsis,* our understanding of the molecular mechanisms

underlying systemic tolerance in economically important cereal crops is still in its infancy. This study had focused on the abiotic determinants and host defense responses underlying *Pseudomonas fluorescens* activated induced systemic tolerance in rice, the most important food crop worldwide and a key model for molecular genetic studies of water stress in monocotyledonous plants.

The study showed that colonization by the well characterized biocontrol agent *Pseudomonas fluorescens* strain *Pf1* renders tolerance to water stress. The data also reveal that this *Pf1* is not based on direct activation of basal resistance mechanisms but rather acts for a pronounced multifaceted cellular defense platform. Moreover, the present study demonstrated that systemic tolerance by *Pseudomonas fluorescens* enhances gene expression levels of stress induced transcription factors like bZIP, MYB (*COC1*) and stress induced proteins like Hsp20 which are involved in ABA dependent signaling pathway. Vleesschauwer *et al.* (2008) demonstrated the ability of WCS374r a strain of *Pseudomonas fluorescens* to trigger induced systemic tolerance in rice against the leaf blast pathogen *Magnaporthe oryzae* which was a study with respect to biotic stress. However, the studies regarding the systemic tolerance induced by *Pseudomonas fluorescens* for abiotic stress are very few. The current study demonstrated the influence of *Pseudomonas fluorescens* under water stress for the high level expression of genes involved in ABA mediated signaling pathway that provides tolerance to the plants especially during reproductive stage.

## 5.9.1. Orthologue search

The Os (*Oryza sativa*) accession IDs obtained from the BLAST analysis was used to search orthologue gene against *Arabidopsis thaliana* genome to obtain AGI (Arabidopsis Genome Initiative) codes. RGAP database was used to identify orthologes for rice genes. The AGI codes obtained were used for identification of TFBSs and combinatorial *cis*-regulatory elements in AthaMap and STIFDB2 database which are specifically required for accession.

An orthologue gene is a gene which is present in two different species and performs similar function. Orthologue search can be useful for cross-genome comparative analysis of abiotic stress-responsive genes across different plant species. Earlier, Sanchari and Parekh (2015) used RGAP database for searching homology for orthologs for 13 uncharacterized genes to understand functional role of those uncharacterized genes.

### 5.9.2. TFBSs and combinatorial *cis*- regulatory elements

It is important to understand the basic process and mechanism of the transcription factors and improve upon their stress response for better crop productivity. It will also be important to know the Transcription factors binding sites and combinatorial control of gene expression and help with combating stress using stress responsive adaptive mechanisms at molecular level.

The regulation of various biological processes in the living systems, especially during different adverse conditions are brought about by the change in the expression of different genes which in turn is the result of the binding of specific transcription factors in their respective TFBSs.

Earlier Sanchita *et al.* (2013) identified TFBSs using the data from seven plants of solanaceae family having differentially expressed genes during different time periods of salt stress. The data was retrieved from the public domain. Up and down expression of genes were revealed which might be due to binding of transcription factors in the promoter region. TFBSs were predicted by utilizing the promoter regions of differentially expressed genes.

Stress Responsive Transcription Factor Database V.2. 0 (STIFDB2) provides information on stress-responsive genes from *Arabidopsis thaliana, Oryza sativa* subsp. *japonica* and *O. sativa* subsp. *indica*. A total of 31 TFs were identified for 15 different stress signals that affect plants was also compiled (Naika *et al.*, 2013).

The data compiled in this study can be categorized into two classes: (i) Identification of TFBSs and their cross validation (ii) Identification of Combinatorial cis-regulatory elements. Here, the first class of data refers to list of TFBSs for six stress responsive genes from AthaMap and three from STIFDB2, analysis of significant TFBSs and cross validation between two databases. The six genes having maximum number of transcription factor families, they are WRKY, MYB, HSF, bZIP, ARF, AP2/EREBP, bHLH, and ABI3/VP1 which are having high significant score and threshold showed in AthaMap. Second class of curated data refers to a list of cis-regulatory elements by co-localizing of two transcription factors of two same or different families for three genes (*CO I, AP2-EREBP* and *Hsp20*) using co-localization function in AthaMap. The transcription factors belonging to MYB, WRKY, bHLH and Trihelix families which are mainly responsible for drought stress signals, were predicted as co-associated.

The binding of transcription factors in TFBSs is important for the regulation of various biological processes which shows changes in the gene expression during adverse conditions.

**Future line of study**

The validation of important genes involved in systemic tolerance will establish the role of PGPR under water stress in sustainable management of rice ecosystem. Results obtained could be very well utilized for improving drought tolerance in other susceptible varieties of rice and also the variety Matta Triveni can be further investigated by field trails to understand further for its tolerance to water stress with multiple applications of *Pseudomonas fluorescens*.

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# Summary

# 6. SUMMARY

Rice (*Oryza sativa* L.) is one of the major food crops for about 65 percent of the world's population. It is the staple food for an expansive part of the world, particularly in Asia. Plants are sessile and therefore they cannot escape the adverse environmental factors such as biotic and abiotic stresses. Abiotic stress can be the negative effect of non-living factors on the living organisms during specific environmental condition. Drought is a worldwide issue, obliging global crop creation and quality seriously. The global climate change has made this situation more serious.

The study on "Validation of identified genes for water stress in rice (*Oryza sativa* L.) mediated by *Pseudomonas fluorescens*" was carried out at the Centre for Plant Biotechnology and Molecular Biology (CPBMB), IT – BT Complex, College of Horticulture, Vellanikkara during period from August, 2014 to July, 2016. The study was intended to analyze quantitative expression of identified genes for water stress in rice, *in-silico* identification of stresses inducible Transcription Factor Binding Sites (TFBSs) and combinatorial cis-regulatory elements.

The plants of rice variety Matta Triveni (PTB-45) were given three treatments. Plants without any treatment served as absolute control ($T_1$). Water was withheld in second treatment ($T_2$) and in third treatment water was withheld in plants for which *Pseudomonas fluorescens* was applied ($T_3$). Plants were given two applications of *Pseudomonas fluorescens* (*Pf*), i.e., seed treatment and foliar spray. Water was withheld continuously for 15 days during reproductive stage (panicle initiation stage) one week after foliar spray. Plants exhibited leaf rolling score as per IRRI, SES was taken for total RNA isolation and biometric parameters were recorded after harvest.

Biometric parameters were measured in three treatments. There was significant difference in shoot length, root length, fresh weight, dry weight, yield and

1000 g seed weight between treatments. There was significant increase in shoot length, root length, fresh weight, dry weight, yield and 1000 g seed weight in control plants than that of water stressed and water stressed + *Pf* treated plants. But in *Pf* + water stress plants showed noteworthy increase in shoot length, root length, fresh weight, dry weight, yield and 1000 g seed weight than water stressed plants.

Total RNA was isolated from 65 days old rice plants after drought induction using TRIzol reagent. Total RNA was used to synthesize first strand cDNA using Thermo Scientific RevertAid H Minus First Strand cDNA kit (Thermo Scientific) as per the manufacturer's guildlines. The cDNA was normalized using actin, which is a housekeeping gene. The real-time PCR primers were designed for six differentially expressed genes such as *COI, PKDP, bZIP1, AP2-EREBP, Hsp20* and *COC1* using IDT PrimerQuest software and the designed primers were validated using IDT OligoAnalyzer 3.1 tool. The target genes were analyzed in real-time PCR by keeping actin as endogenous control. Comparative $\Delta$ Ct method was used to analyze the expression levels of genes. Results revealed that all these genes were upregulated in water stressed + *Pf* treated plants. *COI, PKDP, bZIP1, AP2-EREBP, Hsp20* and *COC1* genes were found to have 2.3, 2, 6, 2.8, 4.5 and 4 fold increases in relative expression levels respectively in the same treatment as compared to control. After real-time PCR, semiquantitative real-time PCR was performed for further confirmation of gene expression levels in agarose gel electrophoresis.

In this study, an *in-silico* analysis of the data of differentially expressed genes of rice under water stress mediated by *Pseudomonas fluorescens* was performed to find out the TFBSs. The AGI codes of differentially expressed genes were utilized for the prediction of TFBSs by performing orthologue search against *Arabidopsis* genome in RGAP (Rice Genome Annotation Project) database. Further, TFBSs were identified by AthaMap database, a genome-wide map of TFBSs in *Arabidopsis thaliana,* and STIFDB2 (Stress Responsive Transcription Factor Database V2.0)

database which is a comprehensive collection of biotic and abiotic stress responsive genes in *Arabidopsis* and *Oryza sativa* L. The Matrix score $\geq 10$ and Threshold $\geq 5$ were selected as potential TFBSs using "Search" tool in AthaMap and Z-score with above 1.5 were indicated as potential TFBSs in STIFDB2. The significant TFBSs were analyzed based on the parameters provided by databases and were cross validated. The results revealed that the WRKY, MYB, HSF, bZIP, ARF, AP2/EREBP, bHLH, Trihelix and ABI3/VP1 TF families and their respective TFBSs were predicted as functionally significant. The combinatorial *cis*-regulatory elements for three genes viz, *CO I, AP2-EREBP* and *Hsp20* were also identified. The transcription factors belonging to MYB, WRKY, bHLH and Trihelix families which are mainly responsible for drought stress signals and were predicted as co-associated. These predicted TFBSs would be responsible for the change in expression of genes under water stress.

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# Appendices

# Appendix I

## Buffers and dyes used in gel electrophoresis

1. 6x Loading / tracking dye

| | |
|---|---|
| Bromophenol blue | 0.25% |
| Xylene cyanol | 0.25% |
| Glycerol | 30% |

The dye was prepared and kept in fridge at 4°C

2. Ethidium bromide (intercalating dye)

The dye was prepared as a stock solution of 10mg/ml in water and was stored at room temperature in a dark bottle.

3. 50x TAE buffer (pH 8.0)

| | |
|---|---|
| Tris base | 242.0 g |
| Glacial acetic acid | 57.1 ml |
| 0.5M EDTA (pH 8.0) | 100 ml |
| Distilled water | 1000 ml |

The solution was prepared and stored at room temperature

4. 10X MOPS buffer (pH 7)

| | |
|---|---|
| 200mM MOPS (pH 7) | 41.85 gm |
| 80mM Sodium acetate | 6.56gm |
| 10 mM EDTA | 3.725 |
| DEPC water | 1000 ml |

The solution was prepared in DEPC autoclaved water and stored at room temperature.

## Appendix II

**Primers used for real-time PCR**

| Sl.no. | Gene name | Oligo name | Sequence 5'-3' | No. of Bases | Amplicon length |
|--------|-----------|------------|----------------|--------------|-----------------|
| 1 | *CO I* | SP1F | CTCCTAGTCGGCCTGATTTC | 20 | 108 |
| | | SP1R | CATGAGCAGTAGCATCCTTGA | 21 | |
| 2 | *PKDP* | SP2F | CGTTGATAGTCGCCGCTAAA | 20 | 109 |
| | | SP2R | TTTAAGAGGCGGGAATGGTG | 20 | |
| 3 | *bZIP1* | SP3F | GAGCGTACTCTGTCCCATTTAG | 22 | 115 |
| | | SP3R | GTTCCAGCGATGAGGTTGT | 19 | |
| 4 | *AP2-EREBP* | SP4F | AGGTAAAGCCCGAGCAATTC | 20 | 101 |
| | | SP4R | GCATCGGTGAATGGTGGTATAA | 22 | |
| 5 | *Hsp20* | SP5F | TGTGTGTCACCACGCTTTA | 19 | 119 |
| | | SP5R | CCTCGCATAGACCCATTCATC | 21 | |
| 6 | *COC1* | SP6F | CACCTCATGACGATGCAAGA | 20 | 101 |
| | | SP6R | GAGCTTGCTCACTCCTTCAA | 20 | |

## Appendix III

**Composition of reaction mixture for cDNA confirmation and normalization with actin gene specific primers**

| Components | Volume per reaction (µl) |
|---|---|
| 10X reaction buffer A | 2 µl |
| MgCl$_2$ (25 mM) | 2 µl |
| dNTP mix (10 mM) | 1.5 µl |
| Forward primer | 1.5 µl |
| Reverse primer | 1.5 µl |
| Sterile distilled water | 10.1 µl |
| Taq. DNA polymerase | 0.4 µl |
| cDNA | 1 µl |
| Total | 20 µl |

**Thermal profile for cDNA confirmation and normalization**

| Sl. No. | Step | Temperature | Time | No. of cycles |
|---|---|---|---|---|
| 1. | Initial denaturation | 94°C | 4 min | 1 |
| 2. | Denaturation | 94 °C | 45 sec | |
| 3. | Annealing | 55 °C | 1 min | 25 |
| 4. | Extension | 72 °C | 2 min | |
| 5. | Final extension | 72 °C | 8 min | 1 |
| 6. | Final hold | 4 °C | ∞ | |

## Appendix IV

### Composition of reaction mixture for real-time PCR

| Reagent | Volume |
|---|---|
| SYBR Premix Ex Taq II (Tli RNaseH Plus) (2X) | 10 µl |
| PCR Forward Primer (10 µM) | 1 µl |
| PCR Reverse Primer (10 µM) | 1 µl |
| ROX Reference Dye (50X) | 0.4 µl |
| Template | 2 µl |
| sterile distilled water | 5.6 µl |
| Total | 20 µl |

### The thermal profile for real time PCR

| Sl. No. | Step | Temperature | Time | No. of cycles |
|---|---|---|---|---|
| 1. | Initial denaturation | 95°C | 30 sec | 1 |
| 2. | Denaturation | 94°C | 5 sec | 40 |
| 3. | Annealing | 55-58°C | 35 sec | |
| 4. | Dissociation stage | 95°C | 15 sec | 1 |
| | | 55-58°C | 30 sec | |
| | | 95°C | 15 sec | |

## Appendix V

>**AB027123**

AAGTCGACCCACGCGTCCGCCTCCTCTCCCTCGCGGCGGCGGCGCGGCGT
CTATCCTCCTCCACCGGCGGCAAGGCATCTGAGCTCAACAGCAAGCAAAT
GGCAGGTGGCAGAATTGCACATGCGACCCTCAAGGGGCCGAGCGTGGTG
AAGGAGATCTGCATTGGGCTCACCCTTGGGCTGGTCGCTGGTGGTCTGTG
GAAGATGCATCACTGGAACGAGCAGAGGAAGACTAGATCCTTCTACGAC
ATGCTCGAGAAGGGCCAGATCAGTGTTGTCGTCGAGGAGTAGTCTTTTGT
GTTGAAAGTCTTTTGCCTTTGTTTTTTGCAAGTTATTTCTGAAACTCTCTGG
AGAGCATGACAAAGCTAGTGCCGTGGAGATTGTCATGAAAAATAAGCAT
AGCAATTTGAGAACATTTGATCTCGTTGAATTTGCTTCTTTTCTTGTATTAT
CTTAAAATCTGTCTTTAATATATGCCAGTAACAGCTTAAGGGATTGGATG
GCTTCCCCCTGACAAAAAAAAAAAAAAAAAAAA

> **OS08G39170**

ATGGCTGGGC ACAAGATAGC ACACGCAACT TTAAAAGGCC CAAGTGTT
GTGAAGGAGTTA GTTATTGGTC TCACACTGGG ACTAGCGGCT GGTGGG
CTATGGAAGATGCA TCACTGGAAC GAGCAGAGGA AAACAAGAGT CTTC
TATGATTTGCTTGAGA GAGGCGAGAT TGGTGTTGTT GTTACCGAGG AAT
AG

**>OsIBCD024362**

CCCGTAATATGTGTAGGAGACTTCTAGAAGATAAGCTTTTTTTTTTTCATG
CAGGCACTGTCGCGAGTTCAAGAGTTCCTTGGAGTTCCAGCGATGAGGTT
GTCCAGCAGGCATGTGAAGATCCACACAAGCCCTCTTCCTGACCTTGTTG
ATAATTGGGAGGAAGTGAGCGAGAATCTAAATGGGACAGAGTAGCTCGT
TTGTTGATGGTGCAAGCTGGTTACTTCACCAGTTCACAGAAAAAGCATTTT
ACCGATGCATGTAGTACAAAATAATGCCATGCTGCAATAACCATGAGTTT
AACTTGCGCACCACTTCGGACAACGGATTCGTAGAGAACCGGAAAGAGA
CCATCAACAAAACGAGCGTACTCTGTCCCATTTAGATTCTCGCTCACTTCC
TCCCAATTATCAACAAGGTCAGGAAGAGGGCTTGTGTGATCTTCACATGC
CTGCTGGACAACCTCATCGCTGGAACTCCAAGGAACTCTTGAACTCGCGA
CAGTGCCTGCATGAAAAAAAAAAAGCTTATCTTTCTAGAAGATCTCCTAC
AATATTCTCAGCTGCCATGGTTGAAAGCTCCCTCGGGCTCCTCCTGTTCCG
AACTGACGCTTACCGGAACGTGACGCTACCTCGTAACGTGGCGCTTCTCA
ATAGCTCACGCCGTGTAAGGCT

**>OsIBCD031146**

CCAGGCAAAGGAAGGAATTTAAGAAGGGCTGGCTGATTTATCCGTATTCA
TAGTATTCGCCCTAATTCTTCAGATTTTGCAGGATATGAGTTTTAATCACC
TGCACCTATACAGACTCGTTTAAAACTTTTACGCACGAAAGGATACATTG
AATGGACTGCAGGTAAAGCCCGAGCAATTCAAGTTTTACAACCTATTAAG
CCAAGTGTGCCGGTTCTGGGAGCGATCGCCGCAGGTGGTTTATACCACCA
TTCACCGATGCTGTCGATCATATCG

>OS01G04380

TTTCGAGCGGCGCCCGGGGCGGTAAGTGGCCCGGGTGGAGAGGAGCAGC
GGCAGGTTCTTGAGGAGGTTTAGGTTGCCGGAGAATGCTAAGGTGGAGG
AGGTGAAGGCGGGGGTGGAGAACGGGGTGCTCACTGTGACTGTGCCCAA
GGTGGAGGTGAAGAAGTCTGAGGTTAAGGCCATTGAAATCTCTGGAAACT
AAGAGTTTCGAAAAAGATAATATTGTGTCAAGGCCATGTGTGTCACCACG
CTTTTCGTGAATGTGTTTGGTCTCGGTTGGAGCTTGTTTTCTTTATCTTCGC
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>Os08g06110

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ACGAGGCAAGCTTCAAGGCAAGTTGAAGGAGTGAGCAAGCTCAAGCTGG
ATTTGGAGCTTGGCTGCAGGATGCAACCATCTCCCTCTCATGCGGTTCCTG
CCCGA

# VALIDATION OF IDENTIFIED GENES FOR WATER STRESS
# IN RICE (*Oryza sativa* L.) MEDIATED BY *Pseudomonas fluorescens*

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## ABSTRACT OF THE THESIS

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# MASTER OF SCIENCE IN AGRICULTURE

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# ABSTRACT

Rice is the staple food for an expansive part of the world, particularly Asia. It is grown under diverse water regimes ranging from poorly drained waterlogged situations to well drain or water stressed upland conditions. Drought is one of the major abiotic stresses that adversely affect crop plants limiting their growth and yield potential. It disrupts the ionic and osmotic equilibrium of the cell. Susceptibility to water stress in rice is more pronounced at the reproductive stage and causes the greatest reduction in yield when stress coincides with the irreversible reproductive processes.

Transcriptome analysis was carried out in Matta Triveni (PTB 45) to understand the influence of PGPR under water stress tolerance. Matta Triveni is a popular short duration (100-105 days) rice variety of Kerala, recommended for rainfed ecosystem especially in lowlands during the first crop and irrigated conditions during summers (third crop). With growing emphasis on expanding area under upland rice in the state, the need for an early, profusely tillering, drought tolerant variety is on the rise. Matta Triveni though high yielding is found to be adversely affected by drought. Hence, the present study was performed to assess the impact of application of *Pseudomonas fluorescens* in inducing tolerance to drought in variety Matta Triveni.

In the present study the already identified differentially expressed genes such as *Cytochrome oxidase subunit I (CO I), Protein kinase domain protein (PKDP), bZIP1 (basic Leucine Zipper), AP2-EREBP (APETALA2 and ethylene-responsive element binding protein), Hsp20 (Heat shock protein 20) and COC1 (Circadian oscillator component)* which are involved in ABA dependent and independent signal transduction pathways under water stress were validated using real-time PCR.

Seedlings of rice variety Matta Triveni were given three treatments. Unstressed plants were maintained as absolute control. Water was withheld in second treatment and in the third treatment water was withheld in plants for which *Pseudomonas fluorescens* Pfl (KAU strain) was applied. Plants were given two applications of *Pseudomonas fluorescens* (*Pf*), i.e., seed treatment and foliar spray. Water was withheld continuously for 15 days during reproductive stage (panicle initiation stage) after foliar spray. Plants exhibited leaf rolling which was scored as per IRRI, SES and was used for total RNA isolation. Biometric parameters were recorded after harvest.

Total RNA was isolated from 65 days old rice plants after drought induction using TRIzol reagent. The first strand cDNA was synthesized by reverse transcriptase PCR from the above RNA samples. The cDNA was normalized using *actin*, which is a housekeeping gene. The real-time PCR primers were designed for six differentially expressed genes such as *COI, PKDP, bZIP1, AP2-EREBP, Hsp20 and COC1* using IDT PrimerQuest software and the designed primers were validated using IDT OligoAnalyzer 3.1 tool. The target genes were analyzed by keeping actin as endogenous control. Comparative $\Delta$ Ct method was used to analyze the expression levels of genes. Results revealed that all these genes were upregulated in water stressed + *Pf* treated plants. *COI, PKDP, bZIP1, AP2-EREBP, Hsp20* and *COC1* genes were found to have 2.3, 2, 6, 2.8, 4.5 and 4 fold increases in relative expression levels respectively in the same treatment as compared to control.

*In-silico* analysis was performed to identify the Transcription Factor Binding Sites (TFBSs) and combinatorial *cis*-regulatory elements for *COI, PKDP, bZIP, AP2-EREBP, Hsp20 and MYB* genes which are expressed in *Pf* treatments. TFBSs were identified by AthaMap database, a genome-wide map of TFBSs in *Arabidopsis thaliana*, and STIFDB2 database which is a comprehensive collection of biotic and abiotic stress responsive genes in *Arabidopsis* and *Oryza sativa*. The significant TFBSs were analyzed based on the parameters provided by databases and were cross

validated. The results revealed that the WRKY, MYB, HSF, bZIP, ARF, AP2/EREBP, bHLH, Trihelix and ABI3/VP1 TF families and their respective regulatory elements were predicted as functionally significant. The combinatorial *cis*-regulatory elements for three genes viz, *CO 1, AP2-EREBP* and *Hsp20* were also identified. The transcription factors belonging to MYB, WRKY, bHLH and Trihelix families which are mainly responsible for drought stress signals and were predicted as co-associated.

The study had shown that gene expression in plants lacking adequate water can be remarkably influenced by microbial colonization. The activation of the ABA dependent signaling pathway induced by colonization of *P. fluorescens* might be a key element for induced systemic tolerance. The predicted TFBSs and combinatorial *cis*-regulatory elements would be responsible for the upregulation of genes under water stress.