CHARACTERIZATION OF SUBTRACTED cDNA CLONES AND ASSESSING THE FUNCTIONAL RELEVANCE OF A FEW STRESS RESPONSIVE CLONES

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PAK 2033

DEPARTMENT OF CROP PHYSIOLOGY
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To Christ

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UNIVERSITY OF AGRICULTURAL SCIENCES
BANGALORE

CERTIFICATE

This is to certify that the thesis entitled **'Characterization of subtracted cDNA clones and assessing the functional relevance of a few stress responsive clones'** submitted in partial fulfillment of the requirement for the degree of Doctor of Philosophy (Agriculture) in Crop Physiology to the University of Agricultural Sciences, Bangalore, is a record of research work carried out by **J. PATRICIA KALAIARASI** under my guidance and supervision and that no part of the thesis has been submitted for the award of any other degree**,** diploma, associateship, fellowship or any other similar titles.

Bangalore
June, 2008

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'Tis grace hath brought us safe thus far
And grace will lead us home.
John Newton, "Amazing Grace" (1779).

Place:Bangalore
Date: June 2008 *J. Patricia Kalaiarasi*

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ABBREVATIONS

DIP: Drought Induced Protein

ELIP: Early Light Induced Protein

Gs: Stomatal Conductance

ChlA: Chlorophyll A

ChlB: Chlorophyll B

GSI: Groundnut Stress Induced

Fig: Figure

U: Upper Primer

L: Lower Primer

F: Forward Primer

R: Reverse Primer

UTR: UnTranslated Region

ARP: Arabidopsis Randam Primer

μ: micro

ψ_w: Water Potential

INTRODUCTION

I. INTRODUCTION

The higher plants are sessile organisms, and therefore, demonstrate a high degree of homeostatic plasticity in response to nature's wrath, including abiotic and biotic stress factors. This optimises their growth and development in a manner that maximises opportunities for survival and reproduction. Plants are also exposed to stress factors that are anthropogenic in nature. All stress factors can be a menace for plants, preventing them from attaining their full genetic potential and limiting crop productivity, the world over.

Of these, drought is the most significant environmental stress factor, preventing crops from realising their yield potential. Abiotic stress is, in fact, the principal cause of crop failure, dipping the average yield for most major crops by as much as 50% (Bayer 1982, Bray et al., 2000). Drought alone may cause yield losses of as much as 15% (Dey et al., 1996). If crops can be redesigned to better cope with abiotic stress, agricultural production can be increased dramatically.

Recent advances in understanding the responses of plants to the external environment are of importance with respect to basic research. The advent of molecular genetic technology allows us to study these responses much more efficiently than in the past.

In response to stress, plants trigger a series of morphological, physiological, biochemical and molecular changes. When a plant is subjected to abiotic stress, a number of genes are turned on, resulting in an increased number of metabolites and proteins, some of which may confer a degree of protection to stress. A key achievement in breeding better crop under stress has been to understand changes in cellular, biochemical and molecular machinery in response to stress. Efforts in recent years have focused on isolation and characterisation of genes turned on during abiotic stress. Molecular and genomic analyses have facilitated gene discovery, and enabled genetic engineering using several functional and regulatory genes to activate specific or broad pathways related to drought tolerance in plants. Several lines of evidence indicate that molecular tailoring of genes has potential to overcome limitations in creating drought-tolerant transgenic plants.

Recent studies have increased our understanding of the regulatory networks controlling drought stress responses, and have led to practical approaches in engineering drought tolerance in plants. Use of modern molecular biology in elucidating the control mechanisms of abiotic stress tolerance, and engineering stress-tolerant crops is based on the expression of stress-related genes. Hence, genetic engineering, based on the introgression of genes known to be involved in stress response and putative tolerance, may produce quicker results in improving crop varieties.

The first step here is to identify candidate genes which alter the desired stress adaptive mechanisms. The pre requisite is to isolate and characterize the genes involved in tolerance mechanisms. Since the stress responsive genes are many emphases initially to characterize the stress responsive genes. Since the application of genomics approaches, the identification of candidate genes related to drought response has increased, thus fuelling this approach. Moreover generation of expressed sequence tags from cDNA libraries prepared from abiotic stress treated seedlings like *Arabidopsis*, rice, maize, barley and complete genome sequence information of rice and *Arabidopsis* provided a valuable resource for gene discovery coupled with employment of multi-parallel techniques such as expression profiling by microarrays, random and targeted mutagenesis, complementation and promoter-trapping strategies allow the identification of the key stress-responsive gene pools and in turn provide important clues for functional characterization of stress responsive stress.

With this background lot of cDNA libraries have been developed to understand the strategies employed by the crop plants. The diverse gene pool generated from these cDNA libraries has to be characterized to get a picture of the underlying stress mechanisms. It is noted that only 10% of the *Arabidopsis* genes isolated using the cDNA library has been characterized. So the functional characterization has been the most important issue for any progress. The debate now is whether it will be useful or relevant to characterize the stress genes from the adapted speices? Adapted species which have high level of basal tolerance may posses better adaptive mechanism and genes which are functionally and structurally different. With this focus, the major emphasis is to characterize the stress responsive genes from groundnut. Groundnut variety TMV-2 has

been proved to be drought tolerant crop in our lab. The transcriptome of groundnut TMV-2 has been well studied in our lab. Transcriptome profiling of groundnut has identified many genes with diverse functions. The urgent need of the hour is to validate these genes identified. Cloning of these stress genes is possible but for validation optimum expression is required. Thus expressing these genes under stress inducible promoter could be a better option.

It is against this backdrop, that this study has been undertaken. It is titled '**Characterisation of Subtracted cDNA Clones and Assessment of the Functional Relevance of Stress-Responsive Clones**'.

The study has four key objectives:

- Selecting clones based on size and expression analysis
- Obtaining full-length clones
    1. Genomic DNA
    2. cDNA
- Developing tranformants with constitutive and inducible promoters
- Characterising and analysing transformants for stress tolerance

II. REVIEW OF LITERATURE

Abiotic stress and its effects on plants in both natural and agricultural settings is a topic that has been receiving increasing attention. This is because of the potential impact of climate change on rainfall patterns and temperature extremes, salinisation of agricultural land by irrigation, and the need to maintain or increase agricultural productivity on marginal land. Globally, approximately 22% of the agricultural land is saline (FAO 2004). Areas under drought are expanding, and are expected to continue doing so (Burke *et al*., 2006).

Predictions of population growth outpacing agricultural production have been in circulation for the past 200 years (Malthus, 1817; Ehrlich, 1968). However, though world food supply has more than kept pace with demand, there are more than 850 million malnourished people in the world, the vast majority of these being in developing countries (FAO, 2006). Over the next 40 years, global demand for cereals will increase by 60% (Rosegrant and Cline, 2003) as the global population rises from 6.6 billion today to between 8.7 billion and 11.3 billion by 2050 (Bengtsson *et al*., 2006). Feeding the growing world population will pose significant challenges to agriculture.

Simultaneously, global climate change will pose additional challenges by considerably modifying the crop production environment. On the other hand, food productivity has been reducing due to the effect of abiotic stress. Therefore coping with the increasing food requirement is a major challenge for the nations. Increasing the yield of agricultural crops under drought conditions is also challenging because of the low heritability of the trait, the unpredictable nature of most periods of drought stress encountered in growing areas, and gaps in our understanding of drought biology. New approaches to improving the performance of crops grown under periodic drought stress are, therefore, being studied. Analysis of the biological, physiological and biochemical responses of plants to

the adverse effects of stress is being done in detail.

In the case of crop plants, the yield of genetically-altered plants under specific field conditions will determine whether or not a specific gene, metabolic, or signalling pathway is important. The challenge of abiotic stress research is to bridge the gap between such agronomic or eco-physiological experiments, and basic research in *Arabidopsis* and other model organisms, and thus to elucidate the molecular mechanisms by which plants sense and respond to abiotic stress.

2.1 Stress responses

Physiological and biochemical responses

Physiological and biochemical changes at the cellular level that are associated with drought stress include turgor loss, changes in membrane fluidity, and composition changes in solute concentration and protein-to-protein and protein-to-lipid interactions (Chaves *et al*., 2003).

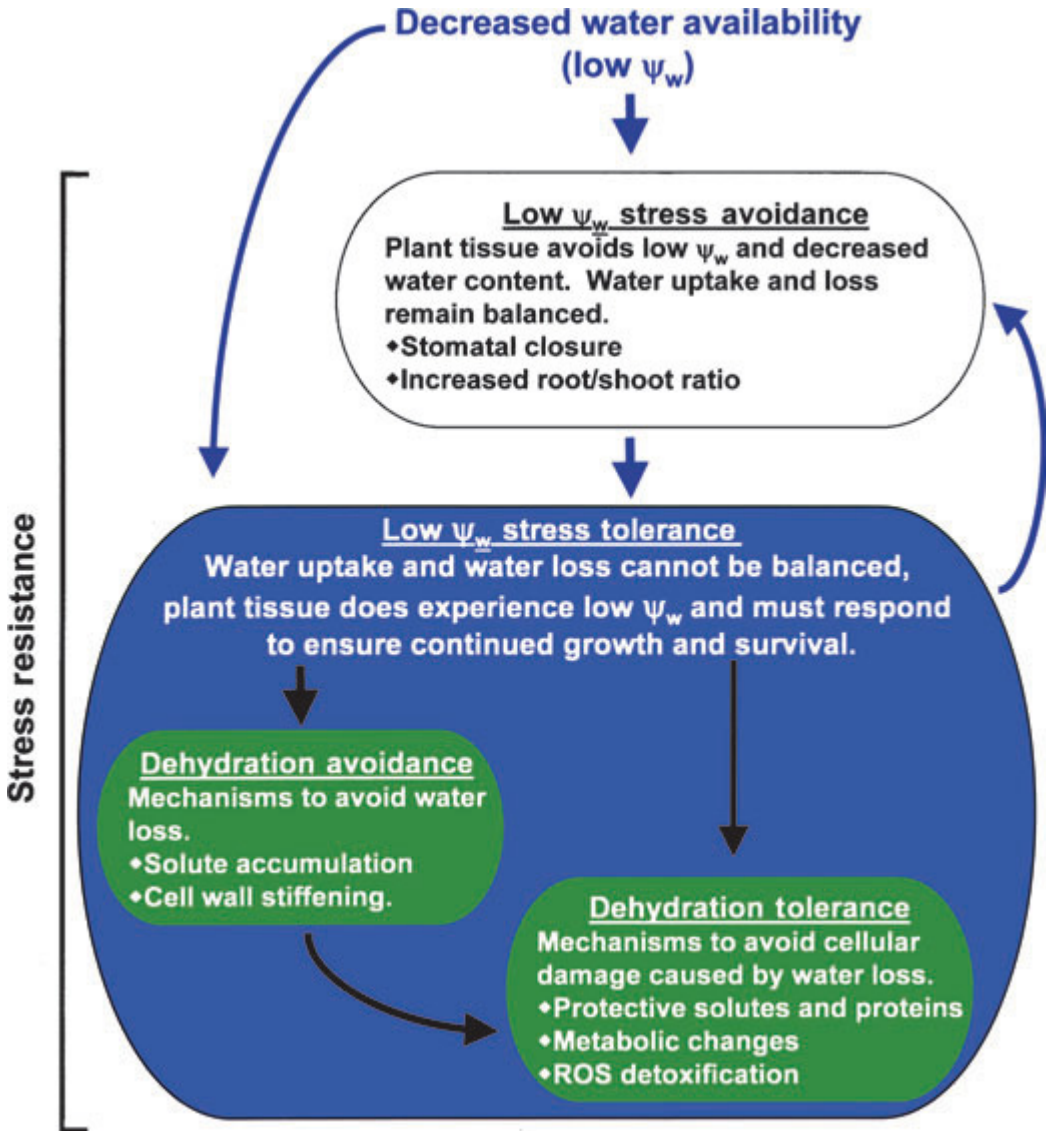
Physiological and biochemical changes

Constitutive phenotypic traits are present even in the absence of stress conditions. By contrast, adaptive traits such as osmotic adjustment and dehydration tolerance arise in response to water deficit (Seeraj and Sinclair, 2002). Reduction of photosynthetic activity, accumulation of organic acids and osmolytes, and changes in carbohydrate metabolism are typical physiological and biochemical responses to stress (Mahajan 2005).

2.2 Strategies for stress tolerance

To understand the responses to low water potential at the level of organism and cell, it is useful to consider the stress avoidance and stress tolerance terminology proposed by Levitt (1972), a modified version of which is presented in Fig (1). In most cases, the plant's first response is to avoid low water potential. Tissue ψ_w (Water potential) and water content are maintained close to the unstressed level by increasing water uptake or

limiting water loss such that the rates of water loss and water uptake remain balanced. Such a balance is achieved over the short term mainly by stomatal closure. Over the longer term, changes in root and shoot growth, leading to increased root/shoot ratio, tissue water storage capacity, and cuticle thickness, and water permeability are also important. Of these, changes in root growth to maximise water uptake are of the greatest importance for crop plants. In the case of mild stress or water stress of a limited duration, avoidance mechanisms by themselves can be sufficient to maintain plant performance (Kramer and Boyer, 1995). Under such conditions, modifications such as increased root growth or reduced stomatal conductance have potential to increase crop productivity. The trade off in such a case is the loss of photosynthesis caused by reduced stomatal CO_2 uptake or a shift of resources into root growth at the expense of photosynthesis reproductive tissues. Under severe drought or low-psi conditions, these mechanisms hold no good. Other mechanisms thus become important in maintaining plant function.



Versules et al.,2006

Figure 1: Conceptual diagram of stress tolerance/stress avoidance model of low- ψ_w responses.

Avoidance

When soil water content and water potential are low, the ψ_w of the plant tissue must also reduce, either through water loss or by adjustments to achieve a low ψ_w while avoiding water loss. Such adjustments are termed as 'dehydration avoidance' (Fig 1). The main mechanisms in dehydration avoidance are accumulation of solutes and cell wall

hardening. The accumulated solutes themselves do not interfere with cellular metabolic function (Yancey *et al*., 1982) and are, therefore, referred to as compatible solutes. Proline, glycine and betaine are few such compatible solutes.

Tolerance

Most dehydration tolerance mechanisms studied to date function primarily to protect cellular structure from the effects of dehydration. Several classes of protective proteins, most notably dehydrins and other late embryogenesis-abundant proteins, are known to accumulate in response to reduced tissue water content, either due to abiotic stress or during seed development (Close, 1997). Although the function of many dehydrins is not known, part of their function is to act as chaperones that protect protein and membrane structure (Bravo *et al*., 2003). Compatible solutes can also protect protein and membrane structure under dehydration. Another aspect of dehydration tolerance is the control of the level of ROS or limitation of the damage caused by reactive oxygen species (ROS). The sources, detoxification and role of ROS in stress are being extensively studied and have been well reviewed (Chen and Gallie, 2004; Laloi *et al*.,2004; Mori and Schroeder, 2004).

Integrated response

Several kinds of abiotic stress are united by the fact that at least part of their detrimental effect on plant performance is caused by distortion of plant water status. This occurs through drought, altered ion content and water uptake caused by salinity, or cellular dehydration caused by formation of extracellular ice during freezing stress.

It is now clear that many of the molecular events initiated by low ψ_w do not fit exclusively into one of the avoidance or tolerance categories shown in Fig 1. For example, accumulation of compatible solutes such as proline may play a role in dehydration avoidance by increasing the cellular solute content and thus maintaining high water content. At the same time, accumulation of proline has been proposed to play a role

in dehydration tolerance by protecting protein and membrane structure, regulating redox status, or acting as a scavenger of ROS (Hare *et al*., 1998; Hincha and Hageman, 2004, Vendruscolo *et al*., 2007). Likewise, dehydrin proteins may also act as 'hydrophilins' , and thus, may have a role in retaining water (dehydration avoidance) and protecting cellular structures (dehydration tolerance) (Close, 1997). Mechanisms, such as osmotic adjustments in the growing region of the root (dehydration avoidance) promote continued root growth at low ψ_w allowing roots to penetrate deeper into the soil and take up more water.

Given the overlapping functions of low ψ_w responses, it is perhaps not surprising that these responses are controlled by a complex regulatory network. This network responds to both external stimuli, such as loss of turgor, and internal stimuli such as production of ROS, sugar-sensing and hormonal stimuli, that reflect the metabolic and developmental status of the plant (Verslues and Zhu, 2005). Although many molecular components involved in the regulation remain uncharacterised, ABA is well known as a key regulatory factor in controlling responses to many types of abiotic stress, including low $\psi_{w.}$ ABA accumulates in response to abiotic stress, and regulates processes involved in all aspects of low-ψ_w response discussed above; ABA-regulated stomatal conductance and root growth (Schroder *et al*., 2001; Sharp and Le Noble, 2002) are important in avoidance of low ψ_w, ABA induced accumulation of compatible solutes can be crucial for dehydration avoidance. Thus, at the level of the organism, the main function of ABA appears to be to co-ordinate aspects of low-ψ_w response.

Although most of the biochemical factors necessary for stress tolerance acquisition are present in all species (Hare *et al*.,1997, Mizoguchi *et al*., 2000), the difference lies in how the stress is perceived and transduced into the series of responses (Mahajan *et al*., 2005).

2.3 Stress Signalling

When a plant is subjected to abiotic stress, a number of genes are turned on, resulting in increased levels of metabolites and proteins, some of which may confer a degree of protection during the stress. A key to progress towards breeding better crops under stress has been to understand the changes in cellular, biochemical and molecular machinery that occur in response to stress.

Signal perception

The perception of abiotic stress, and signal transduction to switch on adaptive responses are critical steps in determining the survival and reproduction of plants exposed to adverse environment. A cell is separated from its surrounding environment by a physical barrier, called plasma membrane. This membrane is permeable to lipid molecules such as steroids, and water-soluble materials such as ions and proteins. The physiological and biochemical changes are initiated once the extra-cellular material (ligand) interacts with the plasma membrane proteins (receptors). The stress signal serves as elicitor for the plant cell. The stress is perceived by receptors, and transduced to the cytoplasm for various downstream events. Following early cues from yeast, where it was shown that initial perception of water deficit was mediated through histidine kinase, a trans-membrane that functions as osmosensor (Posaa *et al*.,1996), Urao *et al*.,1999 were able to identify an osmosensor in *Arabidopsis* (AtHKT1). A recent study published in Nature now reveals that FCA, a nuclear RNA-binding protein, is a receptor for ABA that regulates flowering (Schroeder and Kuhn, 2006). FCA prevents the accumulation of FLC mRNA, a MADS box transcription factor that represses floral transition. The cloning of abiotic stress-inducible genes from the moss *Physcomitrella patens* led to the identification of the gene PpTSPO1 (Frank *et al*., 2007), encoding a protein homologous to the mammalian mitochondrial peripheral-type benzodiazepine receptor, and the bacterial tryptophane-rich sensory protein. Knockout mutants of this led to elevated H_2O_2, enhanced lipid peroxidation, and cell death, implying that PpTSPO1 is involved in stress adaptation.

Signal Transduction

Perceived signals are transduced downstream, resulting in generation of secondary messengers such as Ca^{2+}, ROS, lipases and inositol phosphatases, nitric oxides, fatty acids, phosphorylation and dephosphorylation mediated by protein kinases etc (Pandey *et al*., 2000, Iuan *et al*., 2002, Sanders *et al*., 1999, Takahashi *et al*., 2001 & Sapory *et al*., 1998, Shou *et al*.,2004, Wang *et al*., 2005). The interactions of secondary messengers with downstream compounds result in the relay of a series of transducing reactions, and activation of stress-specific transcription factors through a series of phosphorylation reactions. The end product of the complex series of reactions is the expression of stress responsive genes (SRGs).

The product of these SRGs finally leads to plant adaptation, and helps the plant to survive unfavourable conditions. Thus the plant responds to stress as individual cells, and synergistically, as a whole organism (Zhang *et al*., 2000, Ramanjulu and Bartles, 2002, Mahajan and Tuteja, 2005)

ABA, SA, and C_2H_4 induced by SRGs amplify the initial signals and initiate a second round of signals that may follow the same pathway or use an altogether different signaling cascade (Mahajan *et al*., 2005, Sanders *et al*., 1999, 2002, Leung *et al*., 1998, Finkelstein *et al*., 2002). Certain molecules, also known as accessory molecules, may not directly participate in signaling components, but may be added post-translationally to the signaling proteins like enzymes for myristoylation, glycosylation methylation and ubiquitination (Xiong and Zhu, 2001).

Stress responsive genes

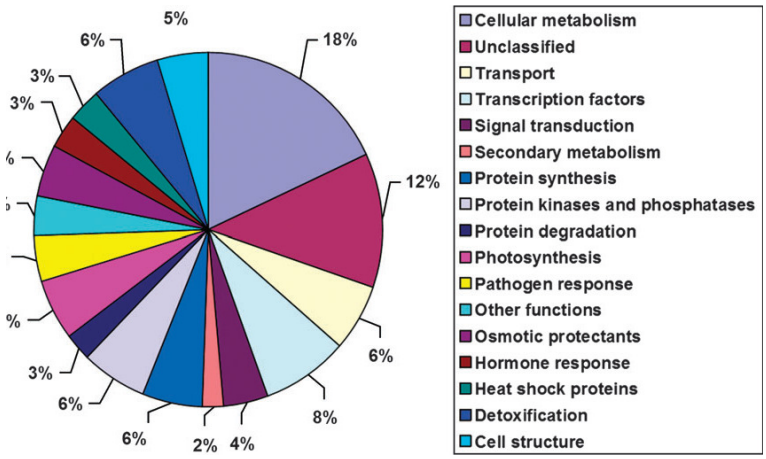
The abiotic stress-signaling cascade of events ends with the expression of SRGs, which may be classified as early and late induced genes. Early responsive genes, induced within minutes of stress signal perception, and often expressing various transcription factors, are

included in the list. Induction of these genes does not require synthesis of new proteins and signaling compounds are primed (Munniks *et al.,* 2001.).

In contrast, most of the late genes like RD, KIN, COR, and Lea are induced hours after the perception of signals and are mostly sustained. These are involved in detoxification, membrane stability and synthesis of osmolytes (Krishnamurthy *et al*., 1995).

Complex SRGs also include those that directly protect against stress. These are the proteins that function by protecting cells from dehydration (functional genes), and include enzymes responsible for osmoprotectants, such as Lea proteins, anti-freeze compounds, chaperones and detoxification enzymes. The second group of gene products is the regulatory genes, which include transcription factors, protein kinases and enzymes involved in phosphoinositol metabolism. These groups of gene products regulate expression of stress transduction pathways (Bhatnagar and Sharma, 2007).

With information gathered from the MIPS *Arabidopsis* database and interPro protein domain searches, Mahalingam *et al*, classified the known stress expressed sequence tags (ESTs) into 12 functional categories. Gorantla *et al*., 2007, classified these into 15 groups comprising cellular metabolism, unclassified, transport, transcription factors, signal transduction, secondary metabolism, protein synthesis, protein kinases and phosphatases, protein degradation, photosynthesis, pathogen response, other functions, osmotic protectants, hormone response, heat shock proteins, detoxification and cell structure (Fig 2).



Gorantla *et al*., 2007

Fig. 2: Functional classification of putative stress-responsive genes of rice

2.4 Role of functional genomic approaches in elucidating stress tolerance mechanisms

In response to abiotic stress, plants respond by altering their physiological, morphological, biochemical and molecular structures, leading to expression of many genes. Dissecting the mechanism that operates under stress is a major challenge. With advancements in gene discovery, transcriptome profiling and validation of genes by high throughput technologies, it has become possible to unravel the complex picture of stress tolerance. A precise review of these advancements follows.

2.4.1 Gene discovery

Among available approaches, an appropriately designed EST project offers a number of substantial advantages:

- It is often a much less expensive route to gene discovery than whole-genome sequencing
- It offers unambiguous identification of transcribed genomic sequences
- It results in a cDNA resource that can serve a broad scientific community

- It provides, at no additional cost, templates suitable for cDNA-based microarray applications
- It provides information about gene expression as a function of developmental stages, organ, and/or environmental parameters when plant material is harvested for RNA isolation
- It can reveal information about transcript properties, including untranslated region (UTR) structures, polyadenylation signals, and alternative splicing.

Because of these and other advantages, EST projects in commercially important plant species have been initiated in several crops (Michalek *et al*., 2001; Miller *et al*., 2001; Fedorova *et al*., 2002; Fernandes *et al*., 2002; Shoemaker *et al*., 2002; Van der Hoeven *et al*., 2002; Kikuchi *et al*., 2003; Ogihara *et al*., 2003; Ronning *et al*., 2003; Vettore *et al*., 2003; Ramı´rez *et al*., 2005). The extensive synteny among their genomes means that what is learned about any one of them increases our knowledge about all (Hulbert *et al*., 1990; Ahn *et al*., 1993; Paterson *et al*., 1995; Bennetzen and Freeling, 1997; Gale and Devos, 1998; Draye *et al*., 2001; Mullet *et al*., 2002; Bowers *et al*., 2003).

2.4.1.1 cDNA library

The identification of novel genes, determination of their expression patterns in response to stress, and a better understanding of their functions in stress adaptation will provide us with the basis for effective engineering strategies to improve stress tolerance (Cushman and Bohnert, 2000). Plant science has entered a new era following the completion of the entire genomic sequence of *Arabidopsis* and rice (*Oryza sativa*), representing model systems for dicot and monocot plants, respectively. Research during this post genomic era is targeted at identifying specific functions of plant genes and their expression profiles. Sequencing projects have produced not only increasing numbers of genomic sequences for many organisms, but also large numbers of ESTs and full-length cDNA sequences for plant species.

Rice cDNA libraries

Currently, over 75,000 rice EST sequences are available in the dbEST database (Reddy *et al*., 2002). Rabbani *et al*., 2003 generated about 1,700 independent cDNAs from cDNA libraries prepared from drought, cold, and high-salinity-treated rice plants. The expression of candidate genes selected by microarray analysis, and RNA gel-blot screened the genes to a total of 73 stress-inducible genes, including 58 novel unreported genes in rice. Among them, 36, 62, 57, and 43 genes were induced by cold, drought, high salinity, and ABA, respectively. A strong association in the expression of stress-responsive genes was found (15 genes responded to all four treatments). Of the 73 genes, 15 (20%) were of known function, while the 58 (80%) unregulated genes are hitherto unreported ones. 28 genes were constitutively expressed in all stresses and can be used as control genes for cDNA microarray and RNA gel blot analysis. Venn diagram analysis revealed greater crosstalk between signalling pathways for drought, ABA, and high-salinity stresses than between those for cold and ABA stresses or cold and high-salinity stresses in rice. Only 2 of 62 genes were specific to drought.

Gorantla *et al*., 2007 generated cDNA library, constructed from a drought-stressed leaf tissue of an indica cultivar, Nagina 22. 7794 cDNA sequences were obtained of which 5815 EST were identified. Analysis of 5815 EST led to the identification 1677 unique sequences. Of these, 334 exhibited no significant sequence homology with any known database. In order to further characterise the drought transcriptome, and to identify candidate genes associated with drought stress response, the rice data were compared with those for abiotic stress-induced sequences obtained from expression profiling studies in *Arabidopsis*, barley, maize and rice. The comparative analysis identified 589 putative stress responsive genes that are shared by these diverse plant species. 19% had no expression evidence for rice EST or cDNAs database. This 19% constitutes novel rice genes. Analysis of the N22 unigene set revealed that 57% of these have a candidate functional role assigned. These are classified into 15 functional groups, of which 65% are associated with metabolism, 18% with cellular communication and signal transduction,

4% with TFs, 8% with cellular defence and protein destination categories; the remaining 43% belong to genes that have expressional evidence but no assigned functional role.

Poaceace cDNA library: The Poaceae contains numerous species of importance to human nutrition. A thorough exploration of the transcriptome of this important plant family is an important step in understanding its fundamental biology, as well as in identifying genes that will continue to improve agricultural productivity. Defining the transcriptome of a complex, multicellular eukaryote is, however, a daunting challenge. The two most widely used and comprehensive approaches are whole-genome sequencing, and application of gene prediction algorithms (Mathe´ *et al*., 2002) and single-pass sequencing of cDNAs to obtain expressed sequence tags (Adams *et al*., 1991).

Pearl millet (*Pennisetum glaucum*), used as forage and grain crop is a stress tolerant species. Differentially regulated transcripts were identified in response to abiotic (salinity, drought and cold) stresses from subtracted cDNA libraries. A total of 2494 EST sequences were clustered and assembled into a collection of 1850 unique sequences with 224 contigs and 1,626 singleton sequences (Mishra *et al*.,).

Sorghum EST was developed under 13 different experiments, one of which was drought stress. 9965 ESTs with 1,517 singletons were obtained. The pools of genes in these libraries remain the same as the rice and *Arabidopsis* libraries (Pratt *et al*., 2005).

Maize EST: Of the 27,000 ESTs (Brendal *et al*., 2002) obtained from maize, 40% had homology with *Arabidopsis,* suggesting that maize whole genomes have to be sequenced.

Sugarcane EST: Sugarcane is a highly productive crop used for centuries as the main source of sugar, and recently, to produce ethanol, a renewable bio-fuel energy source. Drought-stress caused dramatic changes in the gene expression profile of sugarcane plants, with 93 genes being up or downregulated. Among the genes differentially expressed, transcription factor orthologs of the Myb, WRKY, NAC, and DREB proteins, which are known to play a role in drought responses of other systems (Narasuka *et al*.,

2004; Shinosaki *et al*., 2006; Abe *et al*.,1997; Tran *et al*.,2004), were upregulated. Sugarcane plants also selectively activated proteases in response to hydric stress, since a homologue to the cysteine proteinase RD19A precursor was induced. Sugarcane also undergoes cold stress: 20 novel genes induced under cold were identified.

Cotton EST: Approximately 185,000 *Gossypium* EST sequences comprising more than 94,800,000 nucleotides were amassed from 30 cDNA libraries constructed from a variety of tissues and organs under a range of conditions, including drought stress and pathogen challenges (Joshua *et al*., 2006). These libraries were derived from allopolyploid cotton (*Gossypium hirsutum*; AT and DT genomes) and two diploid progenitors, *Gossypium arboreum* (A genome) and *Gossypium raimondii* (D genome). ESTs were assembled using the Program for Assembling and Viewing ESTs (PAVE), resulting in 22,030 contigs and 29,077 singletons (51,107 unigenes). Further comparisons among the singletons and contigs led to recognition of 33,665 exemplar sequences that represent a non-redundant set of putative *Gossypium* genes containing partial or full-length coding regions and usually one or two untranslated regions (UTRs). The assembly, along with their UniProt BLASTX hits, GO annotation, and Pfam analysis results, are freely accessible as a public resource for cotton genomics.

Medicinal plants

CDNA libraries have been constructed from a number of medicinal plants. The details of these are presented in Table 1.

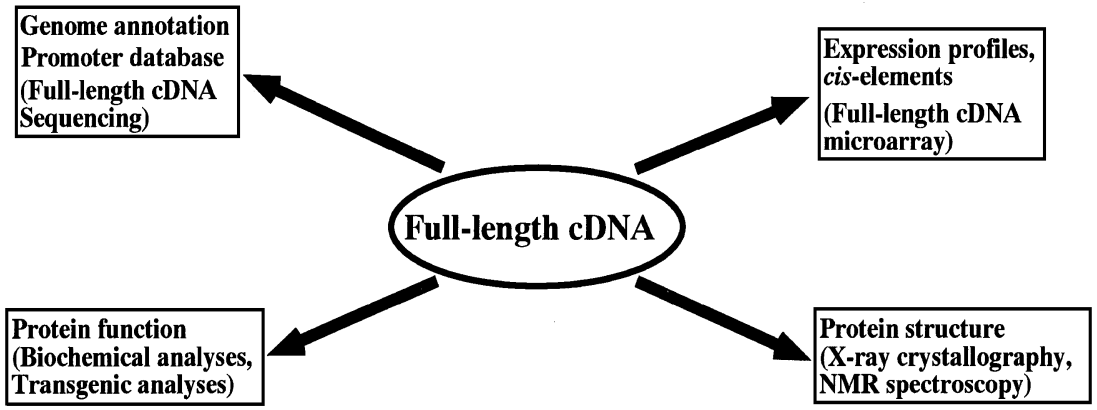
Table 1: Medicinal Plant cDNA library

Organism	Raw EST	Pre processed EST	Unigene	Consensus	Singleton
Nicotiana benthamiana	10774	10003	6389	1823	4566
Capsicum annuum	7529	7135	4115	1198	2917
Panax ginseng	29117	26843	13157	4883	8274
Sesamum indicum	3441	3331	1684	617	1067

Ipomoea batatas	3159	3073	926	464	462
Artemisia princeps var. orientalis	5087	4821	2995	715	2280
Physaliastrum japonicum	4992	4857	3200	749	2451
Allium victorialis var. *platyphyllum*	5083	4787	3108	825	2283

Full length cDNA libraries

EST projects are based on cDNA libraries in which most of the inserts are not full length. ESTs are useful for making a catalogue of expressed genes, but not for further study of gene function. Consequently, genome scale collections and full-length cDNAs of expressed genes are important in analysing the structure and function of genes and their products in this functional genomic era (Fig. 3).



Seki *et al.,* 2001

Fig 3: Application of full length cDNA to functional genomic

***Arabidopsis* cDNA libraries**

Assuming that the total number of *Arabidopsis* genes is about 26,000, the RAFL clones isolated should account for around 60% of all *Arabidopsis* genes. *Arabidopsis* full-length cDNA libraries have been constructed from plants grown under different conditions as reported previously (Seki *et al*., 1998, 2002a) by the biotinylated CAP trapper method using trehalose-thermoactivated reverse transcriptase (Carninci *et al*., 1996). 155,144

RIKEN *Arabidopsis* full-length (RAFL) clones were isolated and clustered into 14,668 non redundant cDNA groups (Seki *et al*., 2002a).

Using the biotinylated CAP trapper method, full-length cDNA libraries were constructed under cold and drought and various developmental tags (Seki *et al* in 2001). Microarray containing 1300 RAFL cDNAs was applied to identify new drought- or cold- inducible genes (Seki *et al*., 2001). 44 and 19 cDNAs for drought- and cold-inducible genes, respectively, were isolated, 30 and 10 of which were novel stress-induced genes that have not been reported as drought- or cold-induced genes previously. 12 stress induced genes were identified as target stress-inducible genes of DREB1A, and six of them were novel. All DREB1A target genes identified contained DRE (Yamaguchi-Shinozaki and Shinozaki, 1994) or DRE related CCGAC core motif sequences in their promoter regions.

Seki *et al* (2002) constructed the RAFL cDNA microarray containing 7000 *Arabidopsis* full-length cDNA to identify new drought-, cold-, high-salinity or ABA-inducible genes. 299 drought-inducible genes, 54 cold-inducible genes, 213 high-salinity-stress inducible genes, and 245 ABA-inducible genes were identified (Seki *et al*., 2002b, c). The products of the drought, high salinity or cold-stress inducible gene were classified into two groups (Seki *et al*., 2002b; Shinozaki and Yamaguchi-Shinozaki, 1999, 2000).

The first group includes functional proteins or proteins that probably function in stress tolerance. They are LEA proteins, heat shock proteins, KIN (cold inducible) proteins, osmo-protectant biosynthesis-related proteins, carbohydrate-metabolism-related proteins, water channel proteins, sugar transporters, potassium transporters, detoxification enzymes, proteases, senescence-related genes, protease inhibitors, ferritin, and lipid transfer proteins (Seki *et al*., 2002b). The second group contains regulatory proteins, that is, protein factors involved in the further regulation of signal transduction and gene expression that probably function in response to stress (Seki *et al*., 2002b, c; Shinozaki and Yamaguchi-Shinozaki, 1999, 2000). They include various transcription factors,

protein kinases, protein phosphatases, enzymes involved in phospholipid metabolism, and other signalling molecules, such as calmodulin-binding protein (Seki *et al*., 2002b, c).

Among the drought-, cold or high-salinity stress-inducible genes identified, 11% were transcription factor genes, suggesting that various transcriptional regulatory mechanisms function in drought, cold, or high salinity stress signal transduction pathways (Seki *et al*., 2002b, c).

Various genes involved in the metabolism of ABA, ethylene, JA, and auxin, and JA- or auxin-regulated genes have been identified as drought-inducible genes (Seki *et al*., 2002b), suggesting the link between ethylene, JA, and auxin, and drought-stress-signalling pathways.

Analysis of stress down-regulated and stress upregulated genes is important in understanding molecular responses to abiotic stresses. Many photosynthesis-related genes, such as ribulose 1,5-bisphosphate carboxylase small subunit (rbcS), chlorophyll a/b-binding protein (cab), and the components of photosystem I and II were found. These results are consistent with the report that water stress inhibits photosynthesis (Tezara *et al*., 1999).

Analysis of genes involved in the recovery process from drought stress and drought stress-inducible genes is also important, not only to understand molecular responses to abiotic stress, but also to improve the stress tolerance of crops by gene manipulation. The cDNA microarray analysis was applied to identify genes that are induced during the rehydration process after dehydration stress treatment and 152 rehydration-inducible genes were identified (Oono *et al*., 2003).

Kreps *et al*., 2002 performed global expression profiling of *Arabidopsis* plants subjected to cold, salt and osmotic stress. The combined results from all three stresses identified 2409 unigenes. About 30% of the transcriptome is sensitive to common stress conditions. The majority was stimulus-specific. 20 were identified as salt-specific with 50% of these

being oxidative stress enzymes. Steroid sulfotransferase At2g03760 had greatest (19fold) induction. Under cold stress, 42 genes were found to be specific, of which 20% were unknown or predicted to be involved directly in regulating the gene. ELIP had 232-fold increases at 27hr. Details of the ESTs are provided in Table 2.

Suppression subtractive cDNA libraries

Using a polymerase chain reaction (PCR)-based suppression subtractive hybridization, 1058 differently expressed genes were identified from 8cDNA libraries. Digital northern analysis revealed that 55% of the stress inducible genes are rarely transcripted in unstressed plants, and 17% are unique.

From our own lab, Gopalakrishna (2000) had isolated 126 differently expressed cDNA from SSH groundnut drought stress plants. The expression of these was studied using northern analysis. Few genes are strongly specific to groundnut drought stress. The expression of these genes in sunflower, a susceptible crop was analysed. They showed similar expression patterns, with minimal variance.

Using the above-mentioned technique, salt stress genes were recently isolated in tomato (Ouyang*et al*., 2006). 2447 cDNAs were obtained. One interesting finding is that both susceptible and tolerant genotypes had the same expression pattern. They were characterised based on the genes up- or down-regulated. This was similar to findings in rice (Walia *et al*., 2005). 201 differentially expressed genes were identified in 14 categories. Metabolism genes occupied the highest (25%) followed by transcription factors (23%).

One of the main advantages of SSH is that it normalises cDNA abundance so that cDNAs encoded by genes that are expressed infrequently, but nonetheless differentially, can be identified readily. The more uniform distribution of hybridization intensities obtained using the subtracted cDNA probe reflects the equalisation in concentration of the

individual species present at markedly-different concentrations in the initial unsubtracted library.

Table 2: Details of stress EST data for different crops

Crop	Stress	No. of genes identified	No of unique sequence	Known genes %	Unknown genes %	References
Arabidopsis	Cold salt osmotic (Mannitol)	2,409		65		Kreps et al., 2002
Arabidopsis	Combination of 19cDNA libraries constructed from *Arabidopsis* grown under different stress, hormone, and light conditions and from various developmental stages and plant tissues	14,688		60		Seki et al., 2004
Pennisetum	salinity, drought and cold	2,494				Rabi N. Mishra et al2007
Rice	drought-, cold-, and high-salinity	1700		20%	80%	Ashiq etal., 2003
Rice	Drought	5815	1677	80%	20%	Gorantla et al., 2007
Maize	Drought					

2.4.1.2 Gene trapping

Gene-trap systems contain an intron with multiple splicing acceptor and donor sites in each of three reading frames, in front of the promoter-less Beta Glucuronidase (GUS) coding region, allowing GUS expression when the insertion occurs in either an exon or intron. More recently, the green fluorescent protein (GFP) gene has been considered a reporter gene because of several desirable features (McElroy and Brettell, 1994; Heim *et al*., 1995; Gerdes and Kaether, 1996; Pang *et al*., 1996; Sallaud *et al*., 2003). First, the GFP assay does not require exogenous cofactors or substrates (Cody *et al*., 1993; Inouye and Tsuji, 1994; Prasher, 1995), as opposed to the GUS (Jefferson *et al*., 1987) and luciferase (LUC) (Williams *et al*., 1989) methods. Second, because the fluorescence of GFP is cell autonomous, it can be detected non-invasively (Chalfie *et al*., 1994), thereby allowing induction assays under a variety of environmental stress, such as temperature, metals, or drought (Allen *et al*., 1999; Li *et al*., 2001; Zimmer, 2002). Using single (Babiychuk *et al*., 1997) or bidirectional (Ryu *et al*., 2004) gene trap vectors, the function of the mutant can be studied.

2.4.1.3 Full-length Over eXpressor (FOX) gene hunting system

To elucidate the function of a large population of genes and to search the efficiency for the agriculturally useful genes, a group of scientists from Japan have used a novel technique called Full-length Over eXpressor (FOX) gene hunting system in rice. This system is useful in analysing a variety of gain function phenotypes from a large population of transgenic plants over expressing cDNA of interest and others with unknown or important functions. Using a specially designed binary vector called pRICE-FOX from pBIG2113-S (Fujita *et al*.,2007) under maize ubiquitin promoter and hpt

selection, 13,980 full length independent clones were transformed by high-speed electroporation. The FOX library was transformed into *Agrobacterium* by high-speed transformation. 12,000 FOX-rice lines were generated. Genomic PCR analysis indicated that an average number of genes integrated into a line is 1.04 (Nakamura *et al*., 2007).

The outcome of various gene discovery methods indicates that unknown genes still represent a very high percentage (20–30%) in all cDNA libraries of stress-treated plants. These need to be annotated in order to find likely functions and gain a comprehensive picture of tolerance mechanisms. Further insights into gene expression patterns need to be studied to ascertain their importance under abiotic stress. Several methods are discussed below.

2.4.2 Identification of a stress-responsive gene by transcript profiling

Microarray

Several methods are currently being employed to analyse the profiles of gene expression in plants. In recent years, the sequencing of entire genomes of numerous organisms has been completed, with *Arabidopsis* and rice leading the way in the plant kingdom (Goff *et al*., 2002;Yu *et al*., 2002). Genome expression analysis aims to provide a broad, unbiased survey of the transcriptome, and requires a true global coverage of complex genomes in a single microarray. Significant progress has been made towards this goal. GeneChip microarrays have been improved considerably since their invention. DNA microarray technology is one of the most powerful techniques recently developed to bridge the gap between sequence information and functional genomics. Microarrays are powerful tools for systematically analysing the expression profiles of a large numbers of genes, including stress-inducible and tissue-specific gene expressions, and changes in the expression profiles of mutants and transgenic plants (Eisen and Brown, 1999; Richmond and Somerville, 2000). GeneChip microarrays have been developed for *Arabiodopsis* (Town *et al*., 2002), maize (Hunter *et al*., 2002), rice (Zhu *et al*., 2003), barley (Wise *et al*., 2003), tomato, and wheat. Microarray technology allows for the determination of

transcript abundance for many or all transcripts in a genome, by comparing control and experimental states. This DNA chip-based technology arrays cDNA sequences on a glass slide at a density >1000 genes cm±2. These arrayed sequences are hybridized simultaneously to a two-colour, fluorescently-labelled cDNA probe pair prepared from RNA samples of different cell or tissue types, allowing direct and large-scale comparative analyses of gene expression. The strength of the fluorescent or radioactive signal from the captured targets reflects the abundance of the target molecules. A charged coupled device (CCD) or laser scanner is used to record the fluorescence. To avoid cross-hybridization problems, oligonucleotide microarrays such as the Agilent Oligo Microarray (Agilent Technologies, Inc., Palo Alto, CA, USA) and Affymetrix GeneChip Array (Affymetrix, Inc., Santa Clara, CA, USA) are considered powerful tools for the future (Tong Zhu, 2003).

Using 1501 cDNAs, 185 cDNA clones from the Rice Genome Project and 32 rice cDNAs of previously-reported, stress-inducible genes, a cDNA microarray was prepared. The resulting microarrays comprised a total of 1720 transcripts spotted on each glass slide. Data analysis revealed that the genes on the microarray showed differential expression profiles in response to various abiotic stresses. A total of 141 genes were identified as stress-inducible. Among them, 64, 75, 48, and 45 genes are candidates for cold-, drought-, high-salinity-, and ABA-inducible genes, respectively. A significant number of transcripts were exclusively up-regulated by the stresses, similar to the previous findings in *Arabidopsis* (Seki *et al*., 2002a). For example, 36/64 or 56% of the cold inducible genes were cold specific. Similarly, 28/75 or 37% of the drought-inducible genes were drought specific and so on. (Gorantla *et al*., 2007)

Digital northern:

Electronic northern or e-northerns allows one to study the expression profiles of a particular set of genes. As *Arabidopsis* libraries are derived from non-normalised cDNA libraries, they are useful for this purpose. The Botany Array resource provides biologist-

friendly tools to view and mine data. The results of the search are chronologically displayed in easy to understand graphics. The results can then be analysed for our purposes. Using the digital northern data, one can know whether the gene of our interest is expressed under our condition of interest or not. (Toufighi *et al*., 2005)

2.4.3 Functional characterisation of genes of interest via high-throughput techniques

2.4.3.1 Altering gene expression by gene inactivation techniques

One way of studying the function of a gene is by down regulation. Antisense constructs were developed by introducing the gene of interest in the reverse form, which conjugates with the native gene and brings in the down regulation. Antisense constructs of CCR, the key enzyme of lignifications, cinnomyl CoA Reductase (CCR) introduced in tobacco plants. Primary transformants exhibiting reduction in CCR activity had good correlation between the decrease in the steady state level of CCR mRNA and the level of CCR activity (van der Rest *et al*., 2006). Moreover, the less severely depressed lines exhibited a normal phenotype and a very slight reduction of the thioacidolysis yield, which is an indication of the abundance of the β4 linkages in lignin. The new lignin profiles observed in these lines support a role for CCR down regulation in improving wood properties of forest trees used in the pulp industry. This new lignin mutant offers an unique opportunity to explore the various roles of lignin's in plant development.

Mutations

One of the most important breakthroughs in the history of genetics was the discovery that mutations can be artificially induced in organisms (van Harten, 1998). Artificially-induced mutations, by physical and chemical mutagens, have greatly advanced the understanding of genetics of higher organisms. Starting in the late 1960s, the International Atomic Energy Agency (IAEA) and the Food and Agriculture Organization (FAO) of the United Nations sponsored extensive research on mutation induction and their application to breeding of food and industrial crops that resulted in the introduction

of new varieties of rice, wheat, barley, apples, citrus, sugar cane, banana, and others (more than 2500 officially released new varieties are to be found in the FAO/IAEA Mutant Varieties Database) (http://www-mvd.iaea.org/MVD/default.htm), resistant gene for MLO in Europe and Australia (http://www.crpmb.org/mlo/#Pifanelli). Mutation techniques have been used to improve almost all important traits, from tolerance to abiotic stress (i.e. salinity, cold, acidity) to disease resistances, from food and nutritional quality to market preference, and from plant structure to yield potential. Systematic development, characterisation and collection of chemically or physically-induced mutants came rather recently, when researchers began realising their potential in functional genomic studies (Li *et al*., 2001). For example, the IR64 mutant collection in the International Rice Research Institute comprises more than 38,000 M4 lines (http://www.iris.irri.org) (Wu *et al*., 2004). Compared with the T-DNA mutant lines, induced mutants are commonly more limited in number, because they are considered less straightforward in gene identification. Nevertheless, induced mutations are unique, and to some extent, irreplaceable in gene discovery. For example, most T-DNA insertions cause knock-out of genes, and no mutant can be recovered if such genes are essential for plant survival. Induced mutations are knock-down mutations and may be useful for gene discovery and gene function analyses of these types of genes. Besides, more T-DNA insertions are in the inter-generic region or in introns than in the exons, and thus are less useful for gene discovery. In rice, for instance, there are far more T-DNA insertion lines than rice genes; however, the lines with T-DNA insertions in exons are much fewer than the potential genes (http://signal.salk.edu/cgi-bin/RiceGE). In many other crops, it is not always possible to generate large numbers of such insertion mutants, because of resilience to transformation or inefficient regeneration protocol. But it is possible for almost all crops to generate induced mutants through physical or chemical mutagenesis. In addition, recent developments in biotechnology — especially in understanding the structure and function of plant genomes — confirm mutation induction as one of the most efficient and cost-effective tools for functional genomics projects dealing with both forward and reverse genetics strategies. Thus, induced mutants are bridging the so-called

phenotype gap (PhG) — the gulf between the available mutant resource and the full range of phenotypes.

The Rapid advances in molecular biology and DNA technologies has provided never-before- dreamed of possibilities to for increase ing both the efficiency and efficacy of mutation techniques in crop breeding and research. Firstly, for traits of which the controlling gene(s) is already known, the high throughput selection of mutations has become possible at the DNA level, e.g. using the TILLING (Targeting Induced Local Lesions IN Genomes (TILLING) technique (Henikoff *et al*., 2004), particularly, when simplified methods are applicable, i.e. detection of SNPs on agarose gels (Raghavan *et al*. 2007) and in ployploid crops (Slade *et al*., 2005). Secondly, for some important mutations, linked DNA tags are greatly facilitating its use in cross- breeding programs, e.g., using techniques such as marker-assisted selection for pyramiding various promising alleles into a breeding line. Thirdly, DNA technologies are also important for the set-up of an efficient mutation induction protocol, for example, identifying proper doses by assaying DNA damage for different crops and systems, which is indeed the very basis of any mutation induction project. The accumulating knowledge of DNA damage, repair and mutagenesis will also lead to knowledge-based design of mutation researches, which, together with the manipulation of these critical genes using transgenic tools, can greatly enhance the efficiency of mutation techniques.

TILLING

TILLING allows the identification of single base-pair (bp) allelic variations in a target gene in a high throughput manner. TILLING is a technique that adds significantly to the arsenal of reverse genetics tools that are available to researchers in capitalising on information provided by genome sequencing projects. It is efficient and cost effective, and mutants of any organism can be screened. TILLING complements other techniques well and, in the absence of site-directed mutagenesis in flowering plants, it is one of the few methods of detecting missense mutations in a high throughput manner available to

plant geneticists. Several TILLING reverse genetics projects are now underway in diverse plant species, including *Arabidopsis*, lotus, maize and *Brassica oleracea* and in some animal species. *Arabidopsis* TILLING projects (ATP) allow users to request mutations in genes of interest. ATP also develops software programmes (CODDLE www.proweb.org/input) to calculate the putative effect of induced or natural polymorphisms on gene function. TILLING competes easily with direct sequencing as a means of quickly identifying point mutations in genes of interest. However, improvements in pooled sequencing procedures, or the development of novel mutagenesis techniques may eventually make TILLING obsolete. At present, it remains the technique of choice for medium-to-high throughput reverse genetics in many organisms (reviewed in Gilchrist and Haughn, 2005).

Gene function may be revealed through analysis of mutants by either forward or reverse genetics. The classical or 'forward genetics' technique of acquiring mutants with desired phenotypes involves mutagenesis of a large number of wild-type seeds by treatment with chemical reagents or irradiation. Such treatments typically introduce single nucleotide changes or small deletions in the genome. As only two to three cells within each seed will ultimately give rise to the next generation of seeds, it is the mutations in these cells that will be carried forward into future generations (Lukowitz *et al*., 2001). Transposon insertion lines have been created and were used in forward genetics screens (Sundaresan *et al*., 1995). In addition, many groups have used plant transformation and insertion of the transferred-DNA (T-DNA) from *Agrobacterium* to create knockout alleles. One advantage of these approaches over chemical mutagenesis is that the chromosomal location of the inserted DNA can be easily identified as it provides a molecular tag with a known sequence. A disadvantage, however, is that often only one gene is disrupted per plant, requiring a much larger population of plants to be screened. These insertions generally also lead to imprecise chromosomal location of individual inserts by thermal asymmetric interlaced (TAIL) PCR technology (Liu and Whittier, 1995), subsequent sequencing of the resulting fragments, and deposition in databases. Users can now do in-

silico searches in these databases for lines in which a T-DNA has been inserted in a particular location of the genome. Sessions *et al*. (2002) were the first to report the generation and thorough characterisation of a relatively large insertion collection containing 52,964 T-DNA lines with flanking sequence and 15,000–18,000 of the *Arabidopsis* genes having insertions (the SAIL collection). At the Salk Institute, more than 225,000 independent insertion events have been created, and the precise location has been determined for T-DNA insertions in approximately 90,000 lines (Alonso *et al*., 2003) (The SIGnAL collection)

By conventional genetic screen, *Arabidopsis* Salt Overly Sensitive (SOS) mutants showing to growth inhibition by salt stress were identified (Zhu, 2001). The SOS mutant growth is impaired on media that are deficient in K+ and particularly hypersensitive to Na+ and Li+ ions (Zhu *et al*., 1998). Based on recent biochemical and physiological data, the role of SOS1 in K+ acquisition is indirect and SOS1 gene is identified as Na+/H+ antiporter, which maintains low concentration of Na+ in cytoplasm by pumping Na+ ions into acidic vacuoles (Shi *et al*., 2000). Subsequently, another locus, SOS2, and a third salt-tolerant locus, SOS3, were identified in *Arabidopsis* by Zhu *et al*. (1998).

Kinase domain and a regulatory domain are located in the SOS2 gene. The calcium-dependent kinase pathway of SOS3–SOS2 activates the Na+/H+ antiporter gene under excess of Na+. It, therefore, appears that SOS1, SOS2 and SOS3 encode regulatory components controlling K+ nutrition essential for ion homeostasis in plants under stress. Besides these, quite a number of T-DNA insertion-based mutants have been identified from ABA biosynthesis and signaling cascades (Umezawa *et al*., 2006, Mustilli *et al*., 2002; Y`oshida *et al*., 2002).

Gene silencing techniques

Anti-sense RNA (asRNA) technology was discovered in plant research in 1987. In this study Rothstein *et al*. (1987) have shown the inhibition of nopaline synthase gene in-tobacco cells, by expressing corresponding asRNA. Several gateway-ready vectors are

now available for anti-sense-mediated silencing. The information and construct resources are available at www.plantgenetics.rug.ac.be/gateway. Anti-sense methods, using either DNA or RNA, are straight forward techniques for probing gene functions. However the discrepancy lies in the fact that this process suffers from specificity and incomplete efficacy (Thakur, 2003).

Post transcriptional gene silencing (PTGS) is a nucleotide sequence-specific defence mechanism that can target both cellular and viral mRNAs. Anti-sense RNA complementary to the targeted mRNA was detected in virus. These RNA molecules were of a uniform length, estimated at 25 nucleotides, and their accumulation required either transgene sense transcription or RNA virus replication. Thus, the 25-nucleotide anti-sense RNA are likely synthesized from an RNA template and may represent the specificity determinant of PTGS. PTGS is an epigenetic phenomenon that results in the sequence-specific degradation of endogenous mRNAs. PTGS was first described in plants and referred to as co-suppression (van der Krol *et al*., 1990; Napoli *et al*., 1990).

The triggering molecule of PTGS is double-stranded RNA (dsRNA) (Waterhouse *et al*., 1998). This dsRNA is cleaved to produce small guide molecules namely siRNA (Martinez *et al*., 2002). The anti-sense strand of the siRNA associates with the RISC to target homologous RNA for degradation (Bartel, 2004). It has also been identified in several other organisms and is termed quelling in fungi and RNAi in animals (Burch-Smith *et al*., 2004).

Small/short interfering RNAs (siRNAs):

The Nobel Prize in physiology or medicine for 2006 was jointly awarded to Andrew Z Fire and Craig C Mello for their discovery of 'RNA interference-gene silencing by double-stranded RNA'

siRNAs are a class of double-stranded RNAs of 21-22 nucleotides in length, generated from dsRNAs. The siRNAs silence genes by promoting the cleavage of mRNAs with

exactly complementary sequences, or recruiting inhibitory proteins to, or directing the modification of DNA with exactly complementary sequences (reviewed in Novina and Sharp, 2004). They are implicated in a variety of processes, including defense against viruses, establishment of heterochromatin, silencing of transposons and transgenes, and post transcriptional regulation of mRNAs (reviewed in Jones-Rhoades *et al*., 2006)

miRNAs

MicroRNAs (miRNAs) are predominantly 21 nucleotides in length (Jones-Rhoades *et al*., 2006) and are derived from 70 to 500 nucleotide long single stranded primary transcripts (pri-miRNA) [Zhang *et al*., 2006]. Sometimes miRNA genes are located very close to each other, forming a cluster, and are transcribed together on the same pri-miRNA. Each miRNA forms an imperfect stem loop secondary structure on the pri-miRNA and these structures are recognized and released by the RNase III-like enzyme DICERLIKE 1 (DCL1) [Park *et al*., 2002; Reinhart *et al*., 2002]. The resulting shorter stem loop structures are the direct precursors of miRNA (pre-miRNA).

Further cleavage of the pre-miRNA by DCL1 liberates the miRNA/miRNA* duplex where the mature miRNA derives from one strand of the imperfect stem, while the miRNA* is from the other strand. The miRNA sequences are catalogued in the miRBase database (http://microrna.sanger.ac.uk), which contains over 800 plant miRNAs originating from 9 different species (release 9.0). The majority of these are identified in *Arabidopsis*, rice and poplar. This number appears certain to increase in the near future due to the application of high throughput sequencing technologies to sequence short RNA libraries from plants.

The main difference between miRNAs and siRNAs is the number of short RNA molecules generated from one locus. Only one miRNA duplex is produced from each miRNA locus but many siRNA duplexes are generated from each siRNA loc. Another common feature of siRNAs that distinguish them from miRNAs is that they are produced from long double- stranded RNA (dsRNA) molecules generated by RNA dependent RNA

polymerases (RDRs). Most siRNAs target the same locus they derive from, except the ta-siRNAs that target mRNAs from different loci, similarly to miRNAs. miR393 is involved auxin signalling and abiotic/biotic stress responses. miR393 targets four closely-related F-box auxin receptor genes, including transport inhibitor response1 (TIR1), which in turn targets AUX/IAA proteins for proteolysis by SCF E3 ubiquitin ligases in an auxin-dependent manner (Reyes, *et al*., 2007). ABA induces the accumulation of miR159 in association with the seed specific transcription factor ABI3. In turn, miR159 mediates cleavage of MYB101 and MYB33 transcripts, which encode positive regulators of ABA responses (Achard *et al*., 2004). This study also provides evidence to show how miR159 over-expression suppresses MYB33 and MYB101 transcript levels that lead to an ABA-hyposensitive response; transgenic plants over-expressing cleavage resistant forms of MYB33 and MYB101, on the other hand, are hypersensitive (Reyes *et al*., 2007).

Baohong Zhang identified 30 potential cotton miRNAs using a comparative genomic approach based on genomic survey sequence analysis and miRNA secondary structure. These cotton miRNAs belong to 22 miRNA families. EST analysis indicated that the predicted miRNAs were expressed in cotton plants. Based on the characteristic that miRNAs exhibit perfect or nearly perfect complementarity with their targeted mRNA sequences, a total of 139 potential miRNA targets were identified in cotton genome. A majority of these targets belong to transcriptional factors which regulate cotton growth and development, including leaf, root, stem, flowers, and even fibre development. Those miRNAs may also be involved in other cellular and metabolic processes, such as stress response, signal transduction, and secondary wall synthesis and deposition. At least 3 miRNA families (miR 396, 414, and 782) target callous synthase, fibre protein Fb23, and fibre quinone-oxido reductase, suggesting that miRNAs play an important role in cotton fibre differentiation and development.

2.4.3.2 Functional aspects of stress tolerance mechanisms identified through transgenic approaches

Down regulation studies help throw light on the function of the gene. It has been shown that only 10% of mutations cause phenotypic changes. This is due to the functional redundancy of genes due to gene duplication. Over expression offers an alternative way of functional validation. Initial attempts to develop transgenics (mainly tobacco) for abiotic stress tolerance involved 'single-action genes'.

2.4.3.2.1 Engineering transgenic abiotic stress-tolerant plants with functional genes

Osmoprotectants

One common mechanism is the accumulation of compatible solutes, which are low-molecular weight, highly-soluble compounds that are non-toxic at high concentrations. These molecules act as osmotic balancing agents accumulated in the plant cells in response to drought stress, and are subsequently degraded after stress relief. Osmoprotectants include amino acids, polyols, quaternary ammonium and tertiary sulfonium compounds, sugars such as mannitol, sorbitol and trehalose (Tabaeizadeh Z, 1998, Rontein *et al*., 2002)

Severe osmotic stress causes detrimental changes in cellular components. In stress-tolerant transgenic plants, many genes involved in the synthesis of osmoprotectants-organic compounds such as amino acids (e.g. proline), quaternary and other amines (e.g. glycinebetaine and polyamines) and a variety of sugars and sugar alcohols (e.g. mannitol, trehalose and galactinol) that accumulate during osmotic adjustment—have been used to date (Vincour and Altman 2005). Many crops lack ability to synthesise the special osmoprotectants that are naturally accumulated by stress tolerant organisms. It is believed that osmoregulation would be the best strategy for abiotic stress tolerance, especially if osmoregulatory genes could be triggered in response to drought, salinity and high temperature. Therefore, a widely adopted strategy has been to engineer certain osmolytes, or over-express such osmolytes in plants, as a potential route to breed stress-tolerant crops. Various strategies are being pursued to genetically engineer osmoprotection in plants. The first step involving obtaining stress tolerant transgenic plants has been to

engineer genes that encode enzymes for the synthesis of selected osmolytes (Bray 1993). This has resulted in a profusion of reports involving osmoprotectants such as glycine-betaine (Sakamoto *et al*. 1998, 2000; Holmstrom 2000; McNeil *et al*. 2000) and proline (Delauney and Verma 1993; Nanjo *et al*. 1999a; Zhu *et al*., 1998; Yamada *et al*., 2005).

Also, a number of sugar alcohols (mannitol, trehalose, myo-inositol and sorbitol) have been targeted for the engineering of compatible-solute overproduction, thereby protecting the membrane and protein complexes during stress (Tarczynski *et al*., 1993; Yang *et al*.,1996; Shen *et al*., 1997; Abebe *et al*., 2003; Holmstrom *et al*., 1996; Zhao *et al*., 2000; Garg *et al*., 2002; Cortina and Culia´n˜ez, 2005).Similarly, transgenics engineered for the overexpression of polyamines have also been developed (Roy and Wu 2001; 2002; Kumria and Rajam 2002; Waie and Rajam 2003; Anderson *et al*., 1998; Capell *et al*., 2004).

Early studies on changes in the carbohydrate metabolism of plants that are exposed to drought stress suggest that, under dry conditions, hydroxyl (OH) group of polyhydroxy compounds can form H bond with the polar heads of the membrane, and that these hydrophobic interactions are important for membrane stability (Chaves *et al*., 2003; Hincha *et al*., 2002; Villadsen *et al*., 2005).

Although the understanding of the mechanisms of sugar transduction and sugar regulation of gene expression are still incomplete (Smeekens, 1998), it appears that sugars favor gene expression of biosynthesis and storage of reserves and down regulates those associated with photosynthesis and reverse metabolism (Ho *et al*., 2001). Hexokinase plays a role in the mechanism by which plants sense the carbohydrate status and allocate reserves (Paul *et al*, 2001). In several plants, polyols, especially mannitol and sorbitol, accumulate up to 80% under drought (Rumpho *et al*., 1984). Mannitol works as an osmoprotectant and protects thiol-regulated enzymes against inactivation by hydroxyl radicals (Stoop *et al*., 1995).

The results of transgenic modifications of biosynthetic and metabolic pathways in most of the above-mentioned cases indicate that higher stress tolerance and the accumulation of compatible solutes may also protect plants against damage by scavenging of ROS, and by their chaperone-like activities in maintaining protein structures and functions (Hare *et al*., 1998; Bohnert and Shen, 1999; McNeil *et al*., 1999; Diamant *et al*., 2001).

Genetic manipulations of compatible solutes do not always lead to a significant accumulation of the compound (except in some cases of proline over-production; Chen and Murata 2002), thereby, suggesting that the function of compatible solutes is not restricted to osmotic adjustment, and that osmoprotection may not always confer drought tolerance. A recent review (Serraj and Sinclair 2002) shows that virtually none of the studies that tested the effect of osmotic adjustment on yield under water stress showed any benefit at all, since some benefit of osmotic adjustment may be in the ability of plants to maintain root growth under severe stress (Voetberg and Sharp, 1991). Another recent study with chickpea also reveals that osmotic adjustment provides no beneficial effect on yield under drought stress (Turner *et al*., 2007). The results of simulation modelling also suggest that changes in a given metabolic process, (Passioura 1977, 2007), may end up with little benefit for actual yield under stress (Sinclair *et al*. 2004). For agricultural practices, over-synthesis of compatible solutes should not account for the primary metabolic costs. Hence to minimise the pleiotropic effects, over-production of compatible solutes should be stress-inducible and/or tissue specific (Garg *et al*., 2002).

Late embryogenesis abundant (LEA) proteins

LEA proteins represent another category of high molecular weight proteins that are abundant during late embryogenesis and accumulate during seed desiccation and in response to water stress (Galau *et al*. 1987). Amongst the several groups of *LEA* proteins, those belonging to Group 3 are predicted to play a role in sequestering ions that are concentrated during cellular dehydration. These proteins have 11-mer amino acid motifs with the consensus sequence TAQAAKEKAGE repeated as many as 13 times (Dure

1993). Group 1 LEA proteins are predicted to have enhanced water-binding capacity, while Group 5 LEA proteins are thought to sequester ions during water loss. Constitutive overexpression of the *HVA1*, a Group 3 LEA protein from barley conferred tolerance to soil water deficit and salt stress in transgenic rice plants (Xu *et al*. 1996). Molecular analysis of the transgenic plants, over expressing hva1 in mulberry under a constitutive promoter via *Agrobacterium*-mediated transformation revealed the stable integration and expression of the transgene in the transformants. Transgenic plants were subjected to simulated salinity and drought stress conditions to study the role of hva1 in conferring tolerance. Transgenic plants showed better cellular membrane stability (CMS), photosynthetic yield, less photo-oxidative damage and better water use efficiency as compared to the non-transgenic plants under both salinity and drought stress. Under salinity stress, transgenic plants show manifold increase in proline concentration than non-transgenic plants. Under water-deficit conditions, proline is accumulated only in non-transgenic plants. Results also indicate that the production of HVA1 proteins helps in better performance of transgenic mulberry by protecting membrane stability of plasma membrane and chloroplastic membrane from injury under abiotic stress. Interestingly, it was observed that hva1 conferred different degrees of tolerance to transgenic plants towards stress conditions. Among lines analysed for stress tolerance transgenic line *ST8* was relatively more salt-tolerant, *ST30, ST31* more drought tolerant, and lines *ST11* and *ST6* responded well under both salinity and drought stress conditions as compared to non-transgenic plants. Thus hva1 appears to confer a broad spectrum of tolerance under abiotic stress in mulberry (Lal *et al*., 2007).

Transporter genes

An important strategy for achieving greater tolerance to abiotic stress is to help plants re-establish homeostasis under stressful environments, restoring both ionic and osmotic homeostasis. This continues to be a major approach to improve salt tolerance in plants through genetic engineering, where the target is to achieve Na+ excretion from the root, or its storage in the vacuole. Molecular and functional characterisation of aquaporins, has

revealed the significance of their regulation in response to these environmental stimuli. Early works have revealed that certain aquaporin isoforms show a strict tissue expression pattern, such as seed-specific a -TIP in bean (*Phaseolus vulgaris*) (Johnson, Höfte & Chrispeels 1990) or root-specific TobRB7 in tobacco (*Nicotiana tabacum*) (Yamamoto *et al*. 1991), whereas other isoforms are expressed throughout the plant. Transcriptional regulation of aquaporin genes by hormones such as ABA or GA 3 or environmental stimuli as diverse as low temperature, drought, salinity, light (daily rhythm), and nutrient deprivation or supply was also revealed. These properties have been assessed in many plant species including *Arabidopsis* (Liu *et al.,* 2003; Maathuis *et al.,* 2003; Jang *et al.,* 2004), rice (Lian *et al*., 2004), maize (Gaspar *et al* ., 2003; Lopez *et al* ., 2003, 2004), barley (Katsuhara *et al* ., 2002, 2003), radish (Suga *et al* ., 2003), and walnut tree (Sakr *et al.,*2003). An extensive review of this topic has been presented elsewhere (Maurel *et al*., 2002). A number of abiotic stress tolerant transgenic plants have been produced by increasing the cellular levels of proteins (such as vacuolar antiporter proteins) that control transport functions. For example, tomato (Gisbert *et al*., 2000) plants expressing the HAL1 gene showed a certain level of salt tolerance as a result of retaining more K+ than control plants under salinity stress.

Transgenic *Arabidopsis* and tomato plants over expressing AtNHX1 gene accumulated abundant transporters in the tonoplast and exhibited substantial salt tolerance.SOS1 locus in *Arabidopsis* cloned under CaMV35S promoter enhanced salt Na+ tolerance by providing greater proton motive force that is necessary for elevated Na+/H+antiporter activity.

Detoxifying genes

Under water stress, when there is excess light either in photosynthesis, or when light in photorespiration is not enough to cope up with excess energy, the production of reactive molecules (Reactive Oxygen Species) is exacerbated. The damage caused to the cell by these molecules (ROS) is called oxidative damage. These oxygen species are hydrogen

peroxide (H_2O_2), the super oxide (O_2.-) and hydroxyl (OH.) radicals and the singlet oxygen O_2. Depending on the nature of ROS, some are highly toxic and need to be rapidly detoxified. The mechanism by which ROS is scavenged is called antioxidant defence system or detoxifying system (Vranova *et al*., 2002). Many transgenics have been developed over expressing Apx, SOD, CAT, GST, GPx (Zhu *et al*., 1999; Sen Gupta *et al*., 1993; Oberschall *et al*., 2000). Overexpression of double gene CAT and Apx have also shown better tolerance than single gene transfer, moreover genotype and isozyme composition also have profound effect on relative tolerance (Rubio *et al*., 2002). Salt stress encounters oxidative stress due to reduction in photosynthesis. A gene, *ENH1* encoding for a chloroplast-localised protein with a PDZ domain at the N-terminal region and a rubredoxin domain in the C-terminal part has been identified (Zhu *et al*., 2007). Rubredoxins are known to be involved in the reduction of superoxide in some anaerobic bacteria. The *enh1-1* mutation causes enhanced accumulation of reactive oxygen species (ROS), particularly under salt stress. ROS also accumulate to higher levels in *sos2-1* but not in *sos3-1* mutants. The *enh1-1* mutation does not enhance *sos2-1* phenotypes. Also, *enh1-1* and *sos2-1* mutants, but not *sos3-1* mutants, show increased sensitivity to oxidative stress. These results indicate that ENH1 function in the detoxification of reactive oxygen species resulting from salt stress, by participating in a new salt tolerance pathway that may involve SOS2 but not SOS3.

Heat shock protein genes

The heat shock response, the increased transcription of a set of genes in response to heat or other toxic agent exposure is a highly conserved biological response, occurring in all organisms (Waters *et al*., 1996). The response is mediated by heat shock transcription factor (HSF) which is present in a monomeric, non-DNA binding form in unstressed cells and is activated by stress to a trimeric form which can bind promoters of heat shock genes. There have been a few reports on positive correlations between the levels of heat shock proteins and stress tolerance (Sun *et al*., 2001; Wang *et al*., 2005). Although the precise mechanism by which these heat shock proteins confer stress tolerance is not

known, a recent study demonstrated that in-vivo function of thermoprotection of small heat shock proteins is achieved via their assembly into functional stress granules (HSGs; Miroshnichenko *et al*., 2005)

2.4.3.2.2 Engineering transgenic abiotic stress-tolerant plants with upstream genes

However, the single gene approach overlooks the fact that abiotic stress tolerance is likely to involve many genes at a time, and that single-gene tolerance is unlikely to be sustainable. Moreover, in order to restore the cellular function and make plants more tolerant to stress, transferring a single gene encoding a single specific stress protein may not be sufficient to reach the required tolerance levels (Bohnert *et al*., 1995). To overcome such constraints, a second ‘‘wave’’ of transformation attempts to transform plants with the third category of stress-induced genes that would enhance tolerance towards multiple namely, regulatory proteins has emerged and gene encoding a stress inducible transcription factor that regulates a number of other genes is a promising approach (Yamaguchi-Shinozaki *et al*. 1994; Chinnusamy *et al*., 2005). Through these proteins, many genes involved in stress response can be simultaneously regulated by a single gene encoding stress inducible transcription factor (Kasuga *et al*., 1999), thus offering the possibility of enhancing tolerance towards multiple stresses including drought, salinity, and freezing. It is interesting to note that this ‘‘second wave’’ has also coincided with a better integration of genetic engineering and plant physiology.

Signal transduction genes

Signal transduction genes are genes involved in stress signal sensing and a cascade of stress-signalling. *A. thaliana* has been of recent research interest (Winicov and Bastola, 1997; Shinozaki and Yamaguchi-Shinozaki, 1999). Components of the same signal transduction pathway may also be shared by stress factors such as drought, salt and cold (Shinozaki and Yamaguchi-Shinozaki 1999). One of the merits for the manipulation of signalling factors is that they can control a broad range of downstream events that can result in superior tolerance for multiple aspects (Umezawa *et al*., 2006). Alteration of

these signal transduction components is an approach to reduce the sensitivity of cells to stress conditions, or such that a low level of constitutive expression of stress genes is induced (Grover *et al*., 1999). One merit for manipulation of signalling factors is that they can control a broad range of down stream events that can result in superior tolerance for multiple aspects (Umezawa *et al*., 2006)

Progress has recently been made in identification of cis - and trans -acting factors that mediate gene expression induced by variable external factors. However, in most cases, our understanding is limited in relation to the molecular signal transduction events that couple perception of the signal and changes in gene expression. It is now realised that protein phosphorylation / dephosphorylation has a very important role in signal transduction and also in regulating metabolic activities. Phosphorylation and dephosphorylation of proteins often serve as an "on-and-off" switch in the regulation of cellular activities (Luan, 2003). During the last decade, a large number of serine/threonine protein kinases have been isolated from plants. A significant portion of the sequenced *Arabidopsis* genome encodes protein kinases and protein phosphatases that catalyse reversible phosphorylation. For optimal regulation, kinases and phosphatases must strike a balance in any given cell. Only a very small fraction of the thousands of protein kinases and phosphatases in plants has been studied experimentally. Nevertheless, the available results have demonstrated critical functions for these enzymes in plant growth and development. Some of these belong to the same category as reported in animal systems, like MAP kinases, receptor kinases, cell division controlling kinases, whereas others are of very novel kind, like calcium-dependent protein kinases, Ca 2+ and calmodulin-stimulated protein kinases with visi-nin-like domain and some specific receptor kinases with defined functions in self-incompatibility, pathogen response and in regulating growth and differentiation (Sapory *et al*.,1998). In many cases, genes coding for these kinases have also been cloned and characterized. While serine/threonine phosphorylation is widely accepted as a predominant modification of plant proteins, the function of tyrosine phosphorylation, despite its overwhelming importance in animal

systems, had been largely neglected until recently when tyrosine phosphatases (PTPs) were characterised from plants. Similarly, protein phosphatases, also of different types, have been reported, purified, and their genes cloned. Kinases (*WEE1*) and phosphates (PASTICCINO2-anti phosphatase) involved in cell cycle has also been identified (Schutter *et al*., 2007; Costa *et al*., 2006).

Calcium-dependent protein kinases (CDPKs) are crucial sensors of calcium concentration changes in plant cells under diverse endogenous and environmental stimuli. 20 CDPKs were isolated from bread wheat, and .a comprehensive study was conducted on their structural, functional and evolutionary characteristics. Full-length cDNA sequences of 14 CDPKs were obtained using various approaches. Wheat CDPKs were found to be similar to their counterparts in rice in genomic structure, GC content, sub-cellular localisation, and subgroup classification (Li *et al*., 2008).

MAPK3 and MAPK6 are activated upon ozone exposure. Although the contribution of these MAPKs to control redox stress has been examined extensively, it remains unclear whether dual-specificity MKPs play an essential role in the regulation of these processes. Knockdown mutation in five MKPs revealed mpk2 plants showed a high level of ROS accumulation. A series of experimental data suggests that AtMKP2, a novel MKP protein in *Arabidopsis,* acts upon MPK3 and MPK6, and serves as a positive regulator of cellular responses to oxidant challenge (Lee and Ellis *et al*., 2007).

Transcription factors

An attractive target category for manipulation and gene regulation is the small group of transcription factors that have been identified as binding promoter regulatory elements in genes that are regulated by abiotic stress (Shinozaki and Yamaguchi-Shinozaki, 1997; Winicov and Bastola, 1997). Transcription factors activate cascades of genes that act together in enhancing tolerance towards multiple stresses. Over expression of ABF3 and ABF4 in *Arabidopsis,* demonstrated enhance tolerance by altered expression of rd29B, rab18, ABI1 and ABI2 (Kagaya *et al*., 2002) Dozens of transcription factors are involved

in plant responses to drought stress (Vincour and Altman, 2005; Bartels and Sunkar, 2005). Most of these fall into several large transcription factor families, such as AP2/ERF, bZIP, NAC, MYB, MYC, Cys2His2 zinc-finger and WRKY. Members of MYB, ERF, bZIP, and WRKY transcription factor families have been implicated in the regulation of stress responses (Schwechheimer *et al*., 1998; Singh *et al*., 2002; Liu *et al*., 2000). Over expression of DREB1A conferred tolerance to drought, salt and cold (Liu *et al*., 1998), whereas DREB1B conferred tolerance to freezing (Otosen *et al*., 1998). Jung *et al* (2008), showed that AtMYB44 belonging to the R2R3MYB sub-group was activated under abiotic stress, such as dehydration, low temperature and salinity by reduced expression of PP2Cs. zmDREB2A overexpression resulted in tolerance to drought, cold, salinity and heat stress in maize. It was observed that 28 genes were upregulated by the over expression of ZmDREB2A (Qin *et al*., 2007). In an extensive analysis, Fowler and Thomashow (2002) analysed CBF1 (DREB1b), CBF2 (DREB1c) and CBF3-expressing transgenic plants (DREB1a) and identified 41 downstream genes as CBF targets, among them AP2 transcription factor (RAP2.6) is subregulon of CBF regulon. In a recent attempt from Thomashow's group, 514 CBF2 target genes were identified as cold-responsive gene set using 24 K *Arabidopsis* microarray.

Very little is known about the functions of other major families of transcription factors that act under the influence of ethylene, jasmonic acid, salicylic acid and other phytohormones, conferring abiotic stress tolerance. Ethylene-responsive, element-binding protein (EREBP) transcription factor belong to the AP2-type family. These proteins are known to act under the influence of ethylene that mediates plant responses to biotic and abiotic stress. Park *et al*. (2001) identified stress-induced EREBP transcription factor (Tsi1), known to bind GCC and DRE/CRT boxes, in tobacco, which was induced by ethylene, methyl jasmonate, salicylic acid, salt treatment and wounding. Over-expressed transgenic lines of tobacco showed enhanced salt tolerance and resistance to pathogens.

Web based databases for stress genes

Plantstress.com, a web site is purely dedicated to abiotic stress. It provides updates on every area of abiotic stress. The website hosts updates on genes that are functionally validated either by mutation studies or transgenic approach. Around 400 genes have been validated (please refer full list in Appendix V), emphasising the importance attached to abiotic stress in our times.

However, only 15% of the genes identified through cDNA libraries have been validated thus far. Thus, a major area where advancement is called for is validation, either by down-regulation or over-expression. Functional validation by over-expression under constitutive promoters resulted in stunted growth and abnormal phenotypes (Shivakumar, 2006). This can be overcome by choosing the right kind of promoters for efficient and controlled expression of transgenes.

2.4.3 Promoters for efficient expression of stress-responsive genes

Choice of promoters

The term 'promoter' is used to designate a region in the genome sequence that is upstream to the gene transcription start site (TSS), although sequences that are downstream to the TSS may also affect transcription initiation. Promoter elements select the transcription initiation point, transcription specificity and rate. Depending on their distance from the TSS, terms such as 'proximal' (hundreds of nucleotides around the TSS) and 'distal' (thousands of nucleotides upstream to the TSS) are used to refer to these promoter elements. Proximal and distal promoters include sets of elements participating in the complex process of cell-, issue-, organ-, developmental stage- and environmental factor-specific regulation of transcription. Most promoter elements regulating TSS selection are localised in the proximal promoter.

An important aspect of transgenic technology is the regulated expression of transgenes. Tissue specificity of transgene expression is also an important consideration while deciding on the choice of the promoter so as to increase the level of expression of the

gene. Thus, the strength of the promoter, and the possibility of using stress-inducible, developmental-stage-, or tissue-specific promoters are also critical in tailoring plant response to these stresses (Bajaj *et al*. 1999). In plants, various types of abiotic stress induce a large number of well-characterised and useful promoters. An ideal inducible promoter should not only be devoid of basal level gene expressions in the absence of inducing agents, but the expression should also be reversible and dose-dependent. The transcriptional regulatory regions of drought- and cold-induced genes have been analysed to identify several cis-acting and trans-acting elements involved in the gene expression that is induced by abiotic stress (Shinwari 1999). Over expression of DREB1A under RD29A promoter showed better phenotype than constitutive expression under CaMV35S (Kasuga *et al*., 1999). A stress inducible expression of *Arabidopsis* CBF1 transgenic tomato expressed under ABRC1 promoter resulted in stress-induced expression of the gene.

Short direct-repeat (DR) DNA enhancer elements from plant viral intergenic regions were analysed for their ability, both individually and in combination, to influence transcription when inserted upstream from a minimal CaMV35S promoter. Synthetic promoters containing multiple copies and/or combinations of DR cassettes were tested for their effect upon reporter gene (luciferase) expression using an *Agrobacteria tumefaciens*-infiltration transient assay (*Nicotiana tabacum* SR1 leaves) and within stably transformed plants. Transgenic plants harbouring constructs containing different numbers or combinations of DR cassettes were further tested to look for tissue-specific expression patterns, and potential promoter responses to the infiltrations process. Multimerisation of DR elements produced enhancer activity that was in general additive, increasing reporter activity in direct proportion to the number of DR cassettes within the test promoter. In contrast, combinations of different DR cassettes often functioned synergistically, producing reporter enhancement markedly greater then the sum of the combined DR activities. Several DR constructs responded to *Agrobacteria* (lacking T-DNA) infiltration of transgenic leaves by an induction (2 elements) or reduction (1 element) in reporter

activity. Combinations of DR cassettes producing the strongest enhancement of reporter activity were used to create two synthetic promoters (SynPro3 and SynPro5) that drive leaf reporter activities at levels comparable to the CaMV35S promoter. Characterisation of these synthetic promoters in transformed tobacco showed strong reporter expression at all stages of development and in most tissues. The arrangement of DR elements within SynPro3 and SynPro5 appears to play a role in defining tissue-specificity of expression and/or *Agrobacterium* responsiveness (Cazzonelli *et al*., 2007).

Promoter database

To date, there are a number of databases with information on cis-acting elements that control transcription initiation by binding corresponding nuclear factors. These include transfac (Wingender *et al*., 2001), trrd (Kolchanov *et al*., 2001), ootfd (Ghosh, 2000), compel (Kel-Margoulis *et al*., 2002), plantcare (Lescot *et al*., 2002), place (Higo *et al*., 1999) and regsite (http://softberry.com). The last three databases are plant-oriented collections of transcription regulatory elements. The eukaryotic promoter database (epd) is the only established collection of sequences of eukaryotic pol ii promoters (Praz, *et al*., 2002). The latest release (#71) includes a total of 1402 entries, mainly of promoters from animals, with only about 200 from plant species.

Plantprom db, a plant promoter database, is an annotated, non-redundant collection of proximal promoter sequences for RNA polymerase II, with experimentally determined TSS from various plant species. The first release (2002.01) of PlantProm DB contains 305 entries including 71, 220 and 14 promoters from monocot, dicot and other plants, respectively. It provides DNA sequence of the promoter regions (-200: +51) with TSS on the fixed position +201, taxonomic/promoter type classification of promoters and Nucleotide Frequency Matrices (NFM) for promoter elements: TATA-box, CCAAT-box and TSS-motif (Inr). Analysis of TSS-motifs revealed that their composition is different in dicots and monocots, as well as for TATA and TATA-less promoters. The database serves as learning set in developing plant promoter prediction programs. One such program

(TSSP) based on discriminate analysis has been created by Softberry Inc. And the application of a support ftp: vector machine approach for promoter identification is under development. Plantprom DB is available at http://mendel.cs.rhul.ac.uk/ and http://www.softberry.com / (Shahmuradov *et al*.,2003)

2.4.4 Model systems for validating the stress genes

Chlamydomonas

Chlamydomonas has been a useful model system in the study of many eukaryotic processes at the molecular level (Harris 2001; Gutman and Niyogi 2004). It grows rapidly both photoautotropically and heterotropically, and is amenable for classical genetic analysis. It is haploid during vegetative growth and allows any mutation to be deducted immediately. *Chlamydomonas* has been shown to be suitable in over expression (Siripornadulsil *et al*., 2002; Kumar *et al*., 2005) or down regulation studies (Schroda *et al*., 2002; Shrager *et al*., 2003, Pazour and Witman 2000). The whole genome sequencing project of *Chlamydomonas* was completed in 2002 (Maul *et al*.,2002, Grossman 2005). It is suitable for abiotic stress studies as its genome responds at transcriptional and post transcriptional levels (Lilly *et al*., 2002). It is also suitable for rapid screening of transformed cells. As a photoautotropic system, it has added advantages over yeast in studying the functional relevance of stress genes. Our own group has exploited the advantages of chamy in studying the acclimatisation response to abiotic stresses as well the relevance of the CodA gene (Hema *et al*., 2007).

Resurrection plants

What promotes *Thellungiella* from a mere curiosity to a model system is its amenability to molecular techniques and, hence, its accessibility for genomic and post-genomic approaches. Indispensable molecular tools for Thellungiella are under construction in the

United States, Canada, China, and Japan. Altogether 1800 EST sequences from the Shandong ecotype have been deposited at the National Centre for Biotechnology Information under the names *T. halophila* and *T. salsuginea* (Wang *et al*., 2004), and their similarity to *Arabidopsis* sequences has been evaluated (Inan *et al*., 2004). More cDNA libraries from stressed plants have been created and were reported. For example, Barbara Moffatt's group constructed three cDNA libraries from cold, salt, and drought-treated Thellungiella, Yukon ecotype, as well as four libraries from PCR subtraction (two for cold, one for drought, and one for salt stress). A total of 6200 clones were sequenced, revealing approximately 50% redundancy. These libraries are available to interested researchers.

Resurrection plants are a group of desiccation tolerant plants that can withstand severe drought stress, and retain less than 5 per cent of the total water. They then revive completely to normal metabolism within 24 hours of watering (Graff, 1989). Some droughts genes isolated from the vegetative tissues of resurrection plants (Bartels and Nelson, 1994; Bloomstedt *et al.,* 1998a, 1998b; O'Mahony and Oliver, 1999) are also believed to be associated with desiccation-tolerance in the embryos of angiosperms (Dure, 1993; Bray, 1993). CDT1, a gene of known function from *Craterostigma plantagenium* (Furini *et al.,* 1997), cpm7 and HD-Zip (unknown function) has been isolated. *Sporobolus stapfianus, a* monocot resurrection plant has been used as model plant to isolate stress genes whose transcripts are present in very low expression profile alters during drought stress by the cold plaque screening technique. The characterisation of these clones identified a gene product encoding an elf, the protein translation factor, and a glycine-proline rich protein, which have not been previously associated with drought stress (Neale *et al.,* 2000).

Arabidopsis

Arabidopsis thaliana is a small member of the mustard family. Botanical information on it dates back to the 16th century. It has many advantages as a model system for plant molecular genetics: these include short life cycle, small size, small genome size, and well developed classical genetics. Many crop species have large genomes, often as a result of polyploidization events and accumulation of non-coding sequences during their evolution. Maize has a genome of approximately 2400 Megabase pairs (Mbp) – around 19 times the size of the *Arabidopsis* genome – with probably no more than double the number of genes, most of which occur in duplicate within the genome. Wheat genome is 128 times as large, and the human genome is 5 times the size of the *Arabidopsis* thus provides an alternative system to the commonly used crop plants for studies in the molecular genetics of plant physiology and development**.** It has a fast life cycle, produces numerous self progeny, has very limited space requirements, and is easily grown in a greenhouse or indoor growth chamber. It possesses a relatively small, genetically tractable genome that can be manipulated through genetic engineering more easily and rapidly than any other plant genome. Together with a newly developed means of creating gene knockout lines, it has made many basic biologists realise that *Arabidopsis* may be the best model system for basic research in the biology of all multi-cellular eukaryotes. A complete collection of knockout mutant seeds and full length genes of *Arabidopsis* is available to the public. With the completion of the *Arabidopsis* genome sequencing project, we now have in hand the sequence of approximately 25,500 genes in its genome. Sequence information and electronic expression of the genes under various developmental, stress and tissues is available to the public.

Medicago trunculata

Medicago trunculata is an important model organism in plant biology. In addition to its tractable genetic characteristics (small diploid genome and relatively short lifecycle), *M. truncatula* provides opportunities to investigate plant processes that are unique to legumes, such as interaction with the rhizosphere and formation of symbiotic relationships with nitrogen-fixing soil bacteria, and colonising arbuscular mycorrhizal

fungi. The GeneChip® Medicago Genome Array will be useful in genomic approaches in identifying underlying genetic mechanisms that are important for high crop yield, resistance to diseases and insects, and response to environmental factors (Farag *et al*., 2008).

Other crop plants

Among crop plants, a great deal of experimental work on various stress responses has been undertaken on *Oryza sativa* (Hossain *et al.,* 1996, Rivoal *et al.,* 1997) and *Zea mays* (kelly, 1989; kelly *et al.,* 1991). *Zeay mays* is a highly sensitive crop to flooding stress, while *Oryza sativa* is relatively tolerant (Perata and Alpi, 1993). The effects of water stress have been extensively analysed using *Oryza sativa* and T. *aestivum* (Mundy and Chua, 1998; Claes *et al.,* 1990; Nakagawa *et al.,* 1996; Pareek *et al.,* 1997). The experimentation in abiotic stress response utilising these and other species has evolved high-yield stress-sensitive cultivars, moderate-yield stress-tolerant cultivars and low-yield stress-tolerant wild relatives of the same specific plants. Sorghum is also an important crop that has been studied, on account of the fact that it is well-adapted to harsh environments, including high temperature, drought, and low nutrient availability, and has been investigated extensively with respect to important parameters such as C4 photosynthesis, drought resistance, variation in flowering time, and acid-soil resistance. Consequently, sorghum is an excellent model system in advancing our understanding of what is almost certainly the single most important group of plants with respect to human nutrition (Arumuganathan and Earle, 1991). With the advent of much better sequencing systems, the genome sequence of maize, sorghum and other crops are nearing completion, making it easy to use any crop plant as a model system.

Tolerant crops as model systems

All stress adaptive mechanisms may not operate in all plants to the same extent. Each type or species of plants may exhibit entirely different adaptive mechanisms to combat stress tolerance. Contrasting genotypes of species exhibit quantitative differences upon

stress in many known stress-responsive genes (Cellier *et al.,* 1998*;* Jayaprakash *et al.,* 1998). Stress-induced genes in contrasting species may also be qualitatively different. There is great emphasis in identifying novel stress response genes from stress tolerance species to improve the tolerance of stress-susceptible crops. Tolerant species may have specific adaptive mechanisms, and may express novel stress-responsive genes (Cushman and Bohnert, 2000). Identification of stress-responsive genes from a stress-adapted tolerant crop may be more advantageous .The stress response of sunflower is marginal, whereas tolerant crops such as groundnut maintained better membrane integrity, in both rapid and slow desiccation stress (Gopalakrishana, 2001). If tolerance crops have a similar but efficient and adaptive mechanism, the genes conferring resistance should be better and more efficient in tolerance crops compared to susceptible ones. A study by Waditee *et al.,* 2002 resulted in drastic tolerance of fresh water cyanobacteria to salinity, when a gene encoding Na^+/H^+ antiporter of halo-tolerant cyanobacteria was used. HSP101 from heat tolerant wheat cultivars have also been shown to be structurally different from the HSP of other monocots in their ATP binding domains (Campbell, 2001).

Partial cDNA coding putative phospholipase Da, cysteine protease, serine protease and a full-length cDNA encoding a LEA protein were cloned. Their expressions in response to progressive dehydration were compared between cultivars differing in their tolerance to drought. Differential gene expression patterns, according to either water intensity or the cultivar's tolerance to drought were observed. A good correspondence between the molecular responses and physiological responses as reported earlier in green houses and in the field were observed. Increases in PLDa and LEA transcript accumulation may contribute to reduced water loss. And protection of cellular prolonged expression of AHCP during drought stress may allow mobilisation of precursors in the synthesis of new proteins. Since target genes are differentially expressed they could be used as markers for screening groundnut genotypes (Drame *et al*., 2007).

The above discussion reveals that drought tolerance is indeed a complex trait. It calls for better, and clear understanding of the molecular and physiological processes involved in drought stress. With the advent of modern advanced molecular biology tools (*please refer Table 3*) such as sequencing and annotation, high screening and cloning and transformation techniques, it is possible to assign and validate the functions of genes. From the EST data and full-length cDNA microarray data, it is clear that 40% of the genes of the particular crop under a particular stress have no homology with the known databases. This indicates that, though common pathways exist for stress tolerance, specificity is still a factor to be reckoned with. The task of present-day scientists is to validate these genes, and develop need-based drought tolerant crops.

Table 3: Web resources related to drought

Crop	Details	Web resources	Reference
Sorghum	Stress microarray	http://fungen.org/Sorghum.htm and http:// cggc.agtec.uga.edu/cggc.	
Arabidopsis	14 668 RAFL cDNA clones	www.sciencemag.org/content/vol0/issue2002/images/data/1071006/DC1/table_s1.zip (Seki et al., 2002a).	Seki et al., 2002
Arabidopsis	The Arabidopsis promoter database	http://www.sciencemag.org/content/vol0/issue2002/images/data/1071006/DC1/table_s1.zip	Seki et al., 2002
Arabidopsis	RAFL cDNA clones	(http:// www.brc.riken.go.jp/lab/epd/Eng/index.html).	Seki et al., 2001
Arabidopsis	Stress inducible genes	http://pfgweb.gsc.riken.go.jp/pub_data/seki002/supplemental1.xls and http:// pfgweb.gsc.riken.go.jp/pub_data/seki003/supplemental1.	Seki et al., 2002
Plant stress	Discussions and articles on plant environmental stresses, their impact and mitigation in agriculture. Includes reference database, news, congress announcements and events.	http://www.plantstress.com	
	The Generation Challenge Programme has five sub programmes that span the spectrum of research in germplasm, genomics, bioinformatics and molecular breeding for agricultural development. The Generation Challenge Programme is focused on using genomics tools and discoveries to enhance drought tolerance in the staple crops of developing countries.	http://www.generationcp.org/	
/	provides services for resource data searching and presents Arabidopsis resource data (cDNAs,	http://rarge.gsc.riken.jp	

	transposon mutants and microarray experiments under various stress and hormone treatment conditions).		
/	The website of the Plant Root Genomics consortium is dedicated to root genetics and physiology. The aim of this consortium is to develop an understanding of the molecular mechanisms used by plant roots to acquire water and minerals from the soil and to elucidate the role roots play in adaptation to drought conditions.	http://rootgenomics.missouri.edu	
	Electronic annotation, Incorporates 6 sequences from 6 other databases	PIN-NREF	
	Alligns the high scoring pair, extensive information about each blast return	Http://fungen.org/genediscovery	
Sugarcane Rice	EST Ests	http://sucestfun. org,	
Chlamydomonas	Genome sequence	http://www.jgi.doe.gov/chlamy	Hema et al.,2007
Chlamydomonas	Microarray and other genomic resources	http:/genome.jgi-psf.org/chl-rez.infor.html.	Hema et al.,2007
Rice	Mutant lines	(http://signal.salk.edu/cgi-bin/RiceGE).	(Wu et al., 2004).
Gateway cloning vectors	Cloning vector	www.plantgenetics.rug.ac.be/gateway.)	
Plantprom DB	Plant promoter databases	http://mendel.cs.rhul.ac.uk/ and http://www.softberry.com/.	Shahmuradov et al.,2003

Rice mutants	55,000 TDNA tag with function of gene/promoter trap, with 20,000 flanking sequence tags(FSTs) resolved and align to Rice genome	(http://www.trim.sinica.edu.tw).	(Hsing et al. 2007), Yu et al.,2007

MATERIAL AND METHODS

III. MATERIAL AND METHODS

The aim of this investigation has been:

- to identify a few stress responsive genes in groundnut obtained via suppression subtractive hybridisation, using CLONETECH PCR-Select ^{TM} cDNA subtractive kit
- to construct full-length genes for stress responsive clones
- to functionally characterise and validate the full-length genes obtained for stress tolerance

Suppression subtractive hybridisation was done by Gopalakrishan R, 2001, when 126 different expressed clones were obtained. Of these 126, 93 clones were recombinant and 63 of these clones were sequenced and annotated. From the 63 sequenced and annotated clones, 10 stress-responsive ones were selected for the present study. To meet the objectives of the investigation, several experiments were conducted. The broad details of these experiments are described below:

3.0 Protocol for moisture stress imposition:

Plant establishment

Germinated groundnut seed (TMV20) were sown in battery pots containing 15-20 kg of 2:1 garden soil and sand mixture. All plant protection measures were taken to maintain healthy plants. Water status was maintained at 100% field capacity by regular watering until the plant reached 10-15 cm in height.

Stress treatment

Normal healthy groundnut plants maintained at 100% field capacity were subjected to gradual water stress. Soil moisture status (percentage of field capacity) as indication of stress was monitored gravimetrically. Well watered control groundnut plants were also maintained. Samples were collected when the plants showed slight wilting (50% FC) and

severe wilting (30% FC). Leaf material was collected from the control and stress samples (1g) and stored in -70^{o}C. For all RNA-based experiments, these RNA were used.

Measurement of soil field capacity

Plants were raised in known amount of soil: sand: manure mixture was verified for maximum water holding capacity. It was found that 1Kg of potting mixture can hold approximately 200 –250ml of water. To maintain at 100% field capacity, 200–250 ml of water was added to the pots. During stress imposition soil field capacity was monitored gravimetrically by checking the amount of water present in soil medium compared to maximum water it can hold. Water lost from containers having control plants was replenished regularly.

$$\%FC = \frac{\text{Water present in the soil medium}}{\text{Maximum water the soil can hold}} * 100$$

3.1 Selection of clones for construction of full-length gene

3.1.1 Selection of clones based on size and function

Based on the size and annotated data of the stress-responsive genes from suppression subtracted cDNA library, ten clones which were larger in size were selected.

3.1.2 Selection of clones based on expression pattern

3.1.2.1 Semi-quantitative RT-PCR analysis

Total RNA was extracted from the leaf tissues of groundnut stressed by withholding water for 1 week at different field capacities using phenol-chloroform method. Total RNA (5µg) was reverse transcribed to cDNA in a 10µl reaction mix containing 25 units of MMLV Reverse Transcriptase (MBI Fermentas), 1x reaction buffer, 10mM dNTP mix. The reverse transcription was performed at 42^{o}C for one hour. The enzyme inactivation was done at 90 ^{o}C for 5 minutes. 1µl of the cDNA mix was used for PCR amplification. The PCR was carried out in 20µl reaction mixture containing 1 unit of Taq DNA

Polymerase (Bangalore Genei) in 1x reaction buffer, 1.5mM $MgCl_2$, 2mMdNTP mix; 1µg of forward and reverse primers of the ten clones selected. The primer details are given in Appendix 1a. The conditions were initial denaturation of 95^0C for 4minutes, and indicated cycles of 95^0C for 4 min, 50-67^0C for 1min, and 72^0C for 1.5min, and final extension for 8min at 72^0C.

3.1.2.2 Electronic (e) northern to study the expression pattern of groundnut homolog in *Arabidopsis*

The *Arabidopsis* Genome Initiative (AGI) code of the groundnut homolog in *Arabidopsis* was obtained by blasting the eight sequences (39, 63, 75, 83,109,110,115 and Gdi15) in NCBI- Blast. The AGI code of the ten selected clones was then used to query the Expression Browser tool of the Botany Array Resource (BAR) at http://bbc.botany.utoranto.ca. This analysis provides insight into patterns of gene expression of a particular set of genes across all experiments in the Botany Array Resource (BAR) database. Expression analysis was conducted on the AtGen Express stress series of expression datasets for the ten sets of AGI code mentioned above under drought, salinity, cold, osmotic and heat stress at 0.5,1,3,6,12,24 hours of stress duration were analysed. The log2 values obtained were transformed to actual fluorescence values by the formula 2^log value. The fluorescence value obtained for each stress was plotted separately on the graph as fold increase over control against the different time periods. The graphs were studied for the expression pattern.

3.2 Different methods used for construction of full length clones

3.2.1 Construction of full length clones using genomic DNA

3.2.1.1 Construction of full length clones using ARP primers

Arabidopsis random primers (ARPs) designed by CDFD for the conserved stretch of *Arabidopsis* genome was used for amplification of genomic clone of the selected stress responsive genes. This primer sequence has M13 at 5' end, and the last 10 sequences are

random (*refer Appendix Ib*). Seven such primers were designed and designated as ARP1-ARP7. ARP 2 was not used. These primers were 26 nucleotides in length. The first 16-mer is common for all the primers (M13 sequence). The M13 sequence was added for easy and direct sequencing for PCR products. The DNA was extracted using the CTAB method. The detailed procedure of DNA extraction protocol is given in Appendix II f. 100ng of the genomic DNA of groundnut was taken. PCR reaction was carried out in 20µl reaction containing, 1x Taq Assay buffer, 2mM dNTPs, 1.5mM $MgCl_2$, 3 pmoles of forward and reverse primers and 3 units of Taq Polymerase. PCR conditions were: initial denaturation at 95^0C for 5 minutes, followed by 30 cycles of 95^0C for 1min, 55-65^0C for 1min, 72^0C for 2 min and final extension of 72^0C for 8min. The DNA was amplified with ARP primers and gene specific forward and reverse primers of the selected clones. The DNA was amplified also with only ARP primers, gene-specific forward, gene-specific reverse primer and gene-specific forward and reverse primers. The amplified product was run on the gel. The bands were compared among the sample and control reactions. The band present in sample reaction is considered polymorphic. This band is the band of interest as it is amplified out of the random and gene-specific primers.

3.2.1.2 Construction of full-length clone Walker Primers

It is a PCR-based directional genome walking method. The methodology was followed according to the protocol described in Biotechniques 2002, but with slight modifications. The authors of the book used biotinylated primers; however, owing to the prohibitive costs of designing biotinylated primers, we used non-biotinylated primers.

Genomic DNA was isolated from young leaves of groundnut using the CTAB method (Lodhi et al., 1994). 50ng of DNA were used as template to perform PCR reactions. The 5' and 3'genomic DNA fragment of Gdi15 was isolated by amplifying with gene specific primer along with the walker primers 1,2,3,4 (Appendix 1c) in four different PCR tubes, one each for each primer. PCR conditions were 94 ^{o}C for 1min, 50-55 ^{o}C for 1min, 72 ^{o}C for 4min for 30cycles. Once the reaction was over, the amplified products were run on the

gel and the maximum size of the polymorphic band was located. The band was eluted and cloned into T/A vector using the T/A cloning kit manual. Transformation was done by the KCM method (Chung and Miller, 1988). The recombinant DNA was transformed into DH5α *E.coli* competent cells. Competent cells were prepared using the $CaCl_2$ method according to the protocol explained. *E.coli* cells were plated on LB agar plates containing ampicilin (100µg/ml). After transformation the positive clones were selected.

The putative *E.coli* colonies containing recombinant vector on LB plates with 100µg/ ml of ampicilin selection were analyzed for presence of insert. The colonies were picked with tooth pick and dissolved in 20µl PCR reaction mix. The reaction mix contained single white colony dissolved in10µl of sterile water, 1x PCR buffer, 2mM dNTPs, 1.5mM $MgCl_2$, 1U Taq Polymerase. PCR reaction was carried out with 3 pmoles of M13 primers. The PCR conditions were initial denaturation of 95 ^oC for 10min, followed by 25cycles of 95 ^oC for 1min, 58 ^oC for 1min, 72 ^oC for 2min.The amplified fragment was analyzed on the gel and the positive colonies were confirmed based on the size of the product.

Each positive colony was grown in 3ml LB liquid containing 100µg /ml ampicilin overnight. Plasmids were isolated from grown *E.coli* cells by Alkaline Lysis method. (Sambrook *et al.,* 1989). Plasmids were digested with EcoRI (MBI Fermentas) restriction enzyme and analysed on 1% agarose gel electrophoresis in 1x TAE (Sambrook *et al.,* 1989). Approximate sizes of inserts were calculated based on the movement of known size markers on agarose gel, and the confirmed ones were sequenced for homology studies.

3.2.2 Construction of full-length clones using RNA

3.2.2.1 Gene Racer method

The GeneRacer^{TM} Kit ensures that only transcripts containing full-length cDNA ends are amplified. The advance protocol starts at the RNA level by specifically targeting only the

5'capped mRNA.

Drought stress was imposed as discussed earlier in this is chapter. RNA was isolated from severe stress (35%FC) by following the standard Phenol-Chloroform method (Appendix IIe). 5ug of total RNA was taken for the extraction of mRNA.

General protocol

1. RNA (mRNA or total) was treated with Calf intestinal phosphatase (CIP) for 1hr 30min at 50 ^{o}C. CIP removes the 5'phosphate from the partial transcripts. Without the 5'phosphate the GeneRacer^{TM} RNA Oligo is prevented from ligating. CIP does not affect the capped mRNA.
2. RNA is treated with tobacco acid pyrophosphatase (TAP) for 1hr at 37 ^{o}C, which removes the cap from the capped mRNA. This exposes the 5'phosphate and permits the ligation to the TAP treated mRNA with GeneRacer^{TM} RNA Oligo using T4 RNA ligase for 1hr at 37 ^{o}C.
3. cDNA template was synthesized using the GeneRacer^{TM} OligodT primer using Superscript^{TM} II.
4. 5' and 3' end of gene of interest was amplified using the cDNA template with GeneRacer^{TM} primers (Appendix1d) and gene specific primers.

3.2.2.2 Global Amplification of 'C' terminal ends

This is an improved method of Polymerase Chain Reaction (PCR) based methodology to generate large amounts of high quality complementary DNA (cDNA), from small amounts of initial total RNA. Global amplification of cDNA makes it possible to simultaneously clone many cDNAs directionally and also permit the rapid amplification of cDNA ends (RACE) from a limited amount of starting material. The priming of cDNA with an adaptor oligo-deoxythymidine primer and the ligation of modified nucleotide to the 3' end of the single stranded cDNAs, through the use of T4 RNA ligase, generates

known sequences on either end of cDNA population. This helps in the global amplification of cDNAs. The detail protocol, as described by Reddy *et al.,* 2002, was used with slight modification as discussed below.

cDNA synthesis

Total RNA was isolated from 15 days old ground nut leaves stressed for a week at 35%FW by withholding water. 5µg of the total RNA was used to synthesis the first strand cDNA with Superscript Reverse transcriptase (Gibco-BRL) and an adaptor OligodT primer with the following sequence: 5'-

TATAGATCTGCGGCCGCAAGCTTTTTTTTTTTTTTTTT-3' as per the manufacturer's protocol. The reaction mixture was heated at 95^{o}C for 3min then cooled on ice immediately. RNase (10mg/ml) was added and the mixture was incubated at 37^{o}C for 15min. the excess adaptor was PCR purified and eluted in 30µl sterile water.

Ligation:

A modified oligo nucleotide with the sequence 5'-GCTAGCATATGGGCCCGAATTCC-3' OH group blocked by a primary amine group was ligated to the 3' end of the first strand cDNA. 0.1µg of oligo nucleotide was used in the ligation mixture containing 20U of T4 RNA ligase in a 40µl final volume. The ligation was carried out at 25^{o}C for 15-20 hours, and the ligation mix was purified using PCR purification column and eluted with 50µl sterile water.

Amplification of the adaptor–ligated first strand cDNA using PCR

The adaptor ligated cDNA was amplified with a forward primer having the sequence 5'-GGAATTCGGGCCCCATATGCTAGC-3', which was complementary to the oligonucleotide ligated to 3' end and with reverse primer, 5'-TATAGATCTGCGGCCGCAAGCTT-3', which corresponds to the 5' heel region of the oligo-dT adaptor. PCR was done in a final volume of 100ul reaction containing 1xPCR

buffer, 200µM dNTPs, 5U Taq DNA Polymerase and 0.3 U of Pfu DNA Polymerase, 2µl of Adapator ligated cDNA template, and 200ng of each primer. PCR conditions were 1min at 94^{o}C, 2min at 68^{o}C, 1 min at 55^{o}C and 4min at 72^{o}C for 30 cycles. The PCR product was purified using QIAQuick PCR cDNA purification system.

RACE

Rapid amplification of cDNA ends (RACE) was done for clone Gsi110 coding for cinnomoyl CoA Reductase involved in lignin bio synthesis.

For Rapid amplification of 3' cDNA ends, the globally amplified double stranded cDNA was amplified with forward gene specific primer having sequence 5'-CACATCAAAACCCGTCTCCT-3' and reverse primer which corresponds to the 5'heel of oligodT adaptor and for Rapid amplification of 5' cDNA ends, the globally amplified cDNA ends were amplified with forward primer which was complementary to the 3' ligated oligo and gene specific reverse primer having sequence 5'-CGGTGTTTTCCACCTAGCAT-3'. The PCR reaction contained 2µl of globally amplified double stranded cDNA, 1x PCR buffer, 150ng Primer each, 200µM dNTPs ad 5U Taq Polymerase. The PCR conditions were, 1 min at 94^{o}C, 1 min at 55 ^{o}C and 4 min 72 ^{o}C for 30 cycles. The PCR products were resolved on 0.8% agarose gel containing ethidium bromide.

3.2.2.3 Subtracted cDNA screening for construction of cDNA clone

Total RNA extracted from leaves of stressed plant (30%) was used as the starter material. mRNA was isolated by using biotinylated oligodT primes. The poly A^{+} RNA bound to oligo dT was captures on streptavidin magnetic particles and then eluted. The poly A RNA was reverse-transcribed using oligodT to prime the synthesis, by a specific reverse transcriptase (power script from clontech) to obtain first strand cDNA. This reverse transcriptase has ability to add C residues, because of t terminal transferase activity. When the RT reaches the end of mRNA, 'C' residues are added. The C residues base pair

with the G cap' added during RT, which serves as an extended template. Template switching occurs enabling complete transcription of the mRNA. The resulting single stranded cDNA was made double stranded by Taq Polymerase and amplified for five cycles using oligo dT and Gcap primers, with final incubation at 72^0C for 10 to 15minutes. The double strand was digested with restriction enzyme Sfi and ligated to λ. This library was used to amplify the 3' and 5' end of Gsi39 and Gsi83. 3' RACE was performed using the oligodT & Gsi39/Gsi83 Forward and 5'RACE was performed using ssGcap primer and Gsi39/ Gsi83 Reverse primer. Primer details are given in appendix 1e.

PCR conditions followed are given below

94 for 4min

94 for 1min	
58 for 1 min	30 cycles
72 for 2min	

End

3.2.2.3.1 Analysis of the recombinant Gsi39 and Gsi83 with additional 3' and 5' ends

Cloning and confirmation of the Gsi 39 and Gsi 83 with additional 3' and 5' ends.

The amplififed product of Gsi 39 and Gsi 83 with additional 3' and 5' ends were eluted using the Gel Elution Kit(Sigma) cloned into T/A as described in Appendix 11a,b. The recombinant colonies and plasmids were screened as in Appendix IIc.

3.2.2.3.2 Construction of full-length gene of DIP and ELIP sequence and homology studies

The positive plasmids harboring the PCR products of 3' and 5' RACE Gsi39 and Gsi83 were isolated (Appendix IId) and sequenced using M13 F and M13 R sequence in

automated DNA sequencer, ABIPRISM. Homology search was done on deduced aminoacid sequence similarity/identity (WWW.ncbi.nlm.nih.gou/blastx) or longest open frame was deduced using ORF finder program (WWW.nlm.nih.gov/gorf.html). Sequence similarity search was performed using Blastp program. The Gsi 39 and Gsi83 with 3' and 5' ends were aligned using the Multiple Alignment Program of ClustalW. The alignment sequence was carefully examined. One stretch 5' sequence of DIP and ELIP was retained and the additional sequence at the 3' end was added to the 5' sequenced product of Gsi39 and Gsi83 to get the full sequence of Gsi 39 and Gsi83. The full sequence of DIP and ELIP was analysed in RNA Analyzer (http://rnaanalyzer.bioapps.biozentrum.uni-uerzburg.de/server.html) to retrieve general information of the sequence. This web interface helps to identify sequence characteristics like exon, UTRs, polyA signal, snRNP binding motifs etc.

3.2.2.3.3 Full-length amplification of DIP and ELIP clone from genomic DNA and cDNA

RNA was isolated as described earlier and Reverse transcribed to synthesis cDNA. The primers were designed in the UTR region of the DIP and ELIP so the full coding sequence can be amplified. The primer details are given in Appendix 1a. Using these primers the cDNA clone of DIP and ELIP were amplified.

Genomic DNA isolated as described earlier. 100ng of the DNA was used to amplify the genomic clone of DIP and ELIP using the same primers used for cDNA clone amplification. The amplified products of DIP and ELIP were cloned into T/A and confirmed for the presence of the gene in recombinant colonies.

3.2.2.3.4 Molecular and bio-informatics characterization of the DIP and ELIP gene

3.2.2.3.4.1 Expression Analysis of DIP and ELIP gene

Northern analysis

Total RNA was isolated from drought stressed and control groundnut plants. Stress was imposed as before and samples were collected at 80%, 70%, 60%, 50%, 40% and 30% FC. 15ug of the RNA from control and stress was blotted to nylon membrane and probed using DIP and ELIP gene. The probe of DIP and ELIP was prepared by amplifying the plasmid of DIP and ELIP with DIP and ELIP primer. The PCR component are same plasmid DNA 1µl, 1x Taq Buffer 5ul , 1mM dATP 1µl, 1mM dTTP 1µl, 1mM dGTP 1µl and radioactive dCTP 5µl, 3U of Taq polymerase and the volume made up to 50µl sterile water. The blots were washed and exposed to IP plates and the intensity was read in phospho Imager The detailed protocol is followed as in Appendix II.

Semi- quantitative RT-PCR

Total RNA was extracted from the leaf tissues of groundnut stressed by withholding water for 1 week at different Field Capacity by following phenol-chloroform method. Total RNA (5µg) was reverse transcribed to cDNA in a 10µl reaction mix containing 25 units of MMLV Reverse Transcriptase (MBI Fermentas), 1x reaction buffer, 10mM dNTP mix. The reverse transcription was performed at 42^{o}c for one hour. The enzyme inactivation was done at 90'c for 5 minutes. 1µl of the cDNA mix was used for PCR amplification. The PCR was carried out in 20µl reaction mixture containing 1 unit of Taq DNA Polymerase (Bangalore Genei) in 1x reaction buffer, 1.5mM $MgCl_2$; 2mMdNTP mix; 1µg of forward and reverse primers of the DIP and ELIP. The primer details are given in Appendix 1a.

3.2.2.3.4.2 Bioinformatics Analysis

Protein analysis

The protein sequence of the DIP and ELIP sequence was generated using the software DNA Star and specific motifs on the sequence identified using InterProScan Sequence Search tool, a protein functional analysis tool of EMBL EBI (http://www.ebi.ac.uk/Tools/InterProScan/). This tool is used to search for known motifs in a given protein sequence.

Phylogeny tree

NCBI database was scanned to identify the stress response DIP and ELIPs reported across plant species. These sequences were aligned by using the web http://bibiserv.techfak.uni- bielefeld.de/dialing and sequence alignment tool, Dialign 2.2.1 and the phylogeny tree was constructed

3.3 Cloning into binary vector

3.3.1 Cloning of DIP gene into binary vector

Binary Vectors

pBI121-35S-GUS

GUS gene was cloned under 35S promoter of 1.2 Kb and NOS terminator of 800bp. the Schematic map of pBI121-35S-GUS is given in Fig 4.

pBI121 –ABRE-GUS

The 4X-ABRE-35s promoter (A synthetic promoter which has four repeats of the core sequence (CC**ACGT**GG) of the Abscisic Acid Responsive Element (ABRE)) fused to the CaMV 35S (-70 to +1) minimal promoter) was prepared by annealing two synthetic oligo nucleotides followed by a fill in reaction with Taq DNA Polymerase. This fragment was sub cloned into the HindIII-BamH1 site of pBI121 from which the CaMV 35S promoter (upstream of GUS gene) was removed previously. The resultant plasmid was named as pBI121-ABRE-GUS (Fig. 5). This construct was obtained from Dr. Dibyendu. In the present study the enzymes used to digest the vector and gene were BamH1 and Sac1. The digestion was carried out with 1X Y-Tango buffer at 35C for 3hrs.

Ligation

Different DNA inserts were ligated into binary vectors in various independent DNA recombination experiments. The ligation reaction mixture was brought to 10ul (end volume) which comprised of 1ul of T4 DNA ligase buffer (10X), x ul of digested plasmid

vector, 5U of T4 DNA ligase (MBI-Fermentas) and y ul of DNA insert. The mixture was brought to 10ul with sterile distilled water and incubated at 16^0C for 16hrs. For a good ligation reaction, the amount of plasmid DNA vector must represent one third of the DNA insert in the ligation mixture. The subsequent transformation into E.coli and screening of the recombinant colonies are followed as before.

3.3.2 Gateway cloning of ELIP

BP Reaction for generation of Entry clone with ELIP

The ELIP gene was amplified using gene specific primers with attB1 and B2 sites Primer details are given below. The PCR product was purified and cloned into pDONR^{221} by BP clonase^{TM}-mediated recombination reaction and subsequently transformed to *E. coli* (DB3.1) cells to obtain the corresponding entry clones. Successful cloning of gene fragments was confirmed by PCR amplification using gene specific primers. The BP reaction resulted in an entry clone containing ELIP gene, flanked by attL sites.

LR Reaction for generation of Expression clone with ELIP

The ELIP gene from the entry clone was sub-cloned into suitable Gateway ready destination vector (pK7WG2.0) using LR clonase^{TM}-mediated recombination reaction. In LR reaction, attL sites in entry vector recombine with the attR sites in the destination vector. The final product of the reaction, the expression clone, will have regained the original attB sites and can be used for plant transformation studies. Successful LR reaction was confirmed by PCR amplification of these clones with *att*B primers. The confirmation of recombinant colonies and agro mobilization of ELIP gene in destination vector were followed as discussed earlier.

3.4 Development of transgenic tobacco plants.

3.4.1 *Agrobacterium tumefaciens* mediated transformation of tobacco

Leaf disc method of Agrobacterium mediated transformation was followed to deveope

transgenic tobacco (Variety KST19) plants (Horsch et al., 1985). Leaves from six week old palnts were excised and washed in running tap for an hour. The leaf was initially sterilized with 0.1% Bavistin and later with 0.1% $HgCl_2$ for 2-3 min and further washed thoroughly in sterile distilled water. Using a leaf punch discs were made. 10 leaf disc were culture in MS Morphogenesis media. This step referred as pre incubation and carried out for 48hours. The plates were kept under light in incubation chamber. Simultaneously 3-4 colonies of A.*tumifaciens* strain (GV2260) of bacteria habouiring either cDIPpBI121-35S orcDIPpBI121-ABRE were picked using sterile loop and transferred to 1ml LB medium with 50mg/L Kanamycin. This starter culture was grown at 28C for 16-18hrs. 100ul of overnight grown culture was inoculated into 25 ml AB minimal with 50mg/L Kanamycin and allowed to grow at 28^0C for 16hours. The grown of Agrobacterium was monitored by measuring the absorbance at 600nm. Cultures recording in absorbance of 0.6-0.8 were used for Co-cultivation of leaf discs. Pre incubated leaf disc were taken in a sterile in flask with agrobacterium culture and infected for 4-5min. the culture was drained off and leaf disc blotted on s sterile tissue paper to remove excess bacterium. The disc was transferred back to the respective plates and co cultivated for 48hoours.

After 48h, the disc was washed thoroughly in sterile water and blotted on tissue paper and transferred to fresh Petri plates with selection media (MS media + 0.2mg/l NAA + 2.0mg/L BA +500ml/L Cefotoxim +50mg/L Kanamycin). Kanamycin was used as selection marker, while cefotoxim was used as the bacteriostat. The regenerated shootlets were transferred to fresh deletion media and allowed for appreciable growth. The regenerated shootlets were then sub cultured on to half strength MS media for rooting.

Hardening and further growth of putative transforamants

The rooted plantlets were transplanted into small pots containing soilrit and covered with plastic bags to prevent dehydration. The plastic bags were gradually opened to decrease humidity, allowing hardening off, of the plants. After one week, the plastic bags were

removed and the plants transplanted to the pots containing soil and allowed to grow to maturity in the field

3.4.2 Molecular characterization of transformed tobacco plants

PCR analysis of primary transforamants of tobacco

Total genomic DNA we isolated from leaves of the npt resistant tobacco plants as well as untransformed control plants using c-TAB (cetyltimethyl ammoniuym bromide) method described by Doyle and Doyle 1990. The presences of transgene in putative transgenic plants are detected by PCR method with 4 different primer sets

a) DIP F and DIP R
b) DIPR and 35S F
c) DIP R and pRT100 F.

The expected amplified product size was about 600bp, 1.4kb and 948bp respectively. The PCR was performed in a total volume of 20 μ containing 2μl DNA, 3pmol each of primers, 2mM dNTPs, 1X PCR buffer, 1X MgCl2 and 1U Taq polymerase. The PCR reaction carried out by denaturing the template at 94^0C for 5 min followed by 30 cycles of amplification (1 min at 94^0C, 1 min at50-55 and 1 min at 72^0C) and by extension at 72^0C for 8 min.

Restriction endonulceases treatments

To confirm the transgenic nature of the DIP transformed tobacco plants, RE digestion was carried out. Genomic DNA digestion was carried out by appropriate restriction endonulceases after two around of amplification with 35s and DIP R and pRT100F and Gsi83L. The PCR fragment was purified using PCR Clean up Kit (Sigma). The purified sample was digested with BamH1 or Xba1 to get fragment size of 466,493bp and 434, 517 bp respectively. The schematic restriction digestion map is represented in fig 3. According to the criteria that the reaction buffer (10X) was $1/10^{th}$ of the end volume and

5U of restriction enzymes were used per 1µg of DNA to be digested. The DNA samples that were digested to completion were resolved on 0.8% agarose gel.

3.4.3 Physiological response of wildtype and DIP tobacco transgenics

3.4.3.1 Response of transgenics to water deficit

Photosynthetic rate: Photosynthetic gas exchange parameters were recorded using portable photosynthetic system (LICOR 6400, USA). The measurements were made at ambinent CO2 concentration, PPDF of 1200µmolesm^{-2}s^{-1} and temperature of 28+0.5. Various parameters such as photosynthetic rate, stomatal conductance, transpiration were recorded in replicates and the values were subjected to statistical analysis.

Measurement of Oxygen: Oxygen evolution and the rate of Oxygen evolved were determined by a Clark type Oxygraph Electrode Unit (Hansatech Instruments, Norfolk, England). The Oxygraph respirometer is designed to determine oxygen evolution and uptake measurements over a range of applications from studies of cellular and mitochondrial respiration to photosynthesis of chloroplast suspensions or leaf discs. The electrode comprises of a platinum electrode separated from a solid silver electrode by insulating material. The rate at which electrons move from the cathode is proportional to the concentration of oxygen that is available to receive them. The movement of electrons is an electrical current, which is then measured using a current-to-voltage converter. 10cm^2 leaf discs of wild type and DIP transgenic tobacco plants, grown under both control and drought stress conditions were used. Rate of oxygen evolved (µmols/m^2/s) from the leaf discs were determined under non-limiting CO_2 concentrations.

Moisture content and moisture retention capacity

Leaf discs were made from wild type and DIP tobacco transgenics. The fresh weight (FW) of the samples was measured. The leaf discs then were brought to full water holding capacity by saturating in water for 8-10 hours. The saturated leaf discs weight was again taken (SW). The leaf discs were allowed to dry under lab conditions. After 2,

4, 6, hours the fresh weight of the leaf discs were taken. The leaf discs were oven dried overnight. The dry weight was taken (DW). Various parameters such as moisture content and relative water content was calculated using the formula

MC= FW-DW/FW*100

MRC= FW at particular hour- DW/SW-DW*100

Total chlorophyll content (mg/g fresh wt) =[20.8 (A_{645}) + 8.02 (A_{663})]*[v/w]*1/ 1000

3.4.3.2 Response of transgenics to salinity and PEG stress

Membrane integrity in transgenic line

To study the membrane leakage, leaf segments at the end of stress (different levels of salinity stress (150 & 250 mM NaCl), PEG stress(-8 and -10 bars)) and were floated on deionised water for three hour and extent of electrolytes leaked into bathing medium was recorded at OD of 273nm. Subsequently, the leaf segments were boiled for 30 min and allowed to cool. The final reading was recorded and the loss of membrane integrity was determined using the formula

$$\text{Percent leakage} = \frac{\text{Initial OD}}{\text{Final OD}} * 100$$

Control samples were maintained for all control and transgenic plants. The leaf discs were maintained in water and EC was measured as discussed above.

The graphs were plotted as percent reduction over their respective controls (water)

3.4.3.3 Response of putative transforamants of Oxidative stress

The leaf discs from control and transgenic tobacco plants were flotted in 0.5µM methyl viologen and incubated in dark for two hours. Then the leaf discs are exposed to high

temperature 700-800µEin/m2/s for 4-5 hours. Quantification of stress is done by measuring the extent of chlorophyll reduction and the cell viability by TTC test

Leaf total chlorophyll content assay

30 days old control and trangenics leaf samples were used in this experiment, which has been subjected oxidative stress (methyl viologen induced) The 5 leaf discs were taken per transgenics in two replicates and incubated in test tubes containing mixture of acetone (80 %): DMSO (1:1) as described by Hiscox and Israelstam 1979. The tubes were placed in dark overnight in order to completely bleach the leaves. The absorbance of the extract was obtained at 645, 652, 663 nm respectively. Two replicates per line per treatment were sampled for this test. The pheophytin, degraded product of chlorophyll was recorded by taking ratios of OD at 553 and 663 nm. Initial chlorophyll values were taken for control and transgenic plants ie chlorophyll values of the control and transgenic plants before the stress. The graphs were plotted as percent reduction over the initial chlorophyll values.

Chlorophyll a=12.7(A_{663})-2.69(A_{645})}*{V/W}*1/1000

Chlorophyll b= 22.9 (A_{645})- 4.68(A_{663})}*{V/W}*1/1000

Estimation of cell viability by TTC assay

The TTC (2,3,5-triphenyl tetrazolium chloride) assay was done to measure the extent of cell viability. TTC solution was prepared by dissolving 0.4% TTC (Sigma Aldrich) in 50 mM sodium phosphate buffer (pH 7.4). Leaf segments (1 cm diameter) were prepared from the respective leaf of non-stressed and stressed plants and incubated in TTC solution at room temperature for 5 h while being shaken. The leaf segments were washed to remove unbound formazan and boiled with 5 ml of 2-methoxy ethanol until dry to extract the bound TTC. The absorbance was measured at 485 nm using a UV-2450 UV visible spectrophotometer (Shimadzu) (Towill and Mazur, 1975; Senthil-Kumar and Udayakumar, 2004).

3.4.3.4 Response of putative transforamants of Temperature stress

Leaf discs from control and transgenic tobacco lines expressing DIP gene were analyzed for their Chlorophyll stability. As high temperature disrupts chlorophyll stability, the control and transgenic plants were subjected to high temperature by incubated the leaf discs at boiling temperature for 30 minutes. The stressed leaf discs were incubated in DMSO: Acetone (80%) mix and the chlorophyll was estimated as before.

RESULTS

IV RESULTS

Abiotic stresses including drought are serious threats to the sustainability of crop yields accounting for more crop productivity losses than any other factor in rainfed agriculture. When a plant is subjected to abiotic stress, a number of genes controlling various biochemical, physiological and molecular mechanism are turned on, resulting in increased number of metabolites and proteins some of which confer tolerance to plants under stress. Use of modern molecular biology tools have helped in identification of genes that control these pathways. Contrasting genotypes of species exhibit quantitative differences upon stress in many of the known stress responsive genes (Cellier *et al.,* 1998*;* Jayaprakash *et al.,* 1998). On the other hand, stress induced genes in contrasting species may be qualitatively different. There is great emphasis in identifying novel stress response genes from stress tolerance species to improve the tolerance of stress susceptible crops. Tolerance species may have specific adaptive mechanism and express novel stress responsive genes (Cushman and Bohnert, 2000). Isolation of genes controlling these pathways from a tolerant genotype proves to be a better option.

Groundnut has proved to be a drought tolerant crop. Suppression subtractive hybridization was used to clone the genes, which are expressed under 25 per cent moisture combined with high light stress (Gopalakrishana, 2001). A subtracted library of clones representing differentially expressed stressed mRNA in stressed leaves compared to control mRNA was prepared as provided in the manual of CLONTECH PCR- Select^{TM} cDNA substractive Kit. Control (Driver) and stress (Tester) cDNA molecules were synthesized from the respective mRNAs and digested with Ras I enzyme to obtain shorter blunt end products. Two tester populations were created with different adaptors. Performing first hybridization led to equalization and enrichment of differentially expressed sequences. During the second hybridization, the two samples were mixed together without denaturing. Single strand subtracted tester cDNAs from two samples re-associate and form a new type of hybrid containing two different adaptors at the end.

Fresh denatured drivers were added to enrich subtracted molecules. The entire population was subjected to primary PCR using primers for adapters. The molecules containing single adaptor and those with no adaptor will not amplify. Due to the suppression PCR effect, the molecules containing similar adaptors at both the ends form a pan like structure that prevents their exponential amplification. Only molecules having two types of adaptors (Subtracted and annealed from two samples) would be amplified exponentially. Secondary PCR was performed with nested primers that were specific to adaptors with an aim to reduce the background and enrich further differentially expressed stressed cDNA molecules.

Enriched molecules after the second PCR were inserted into a T/A cloning vector using CLONTECH AdvanTAge PCR Cloning Kit. Since the insertion of any DNA fragment in the lacZ gene leads to inactivation of lac Z, *E. coli* colonies with recombinant vectors would be white in colour. 3ul of the secondary product resulted in 93 white colonies. The 93 colonies were confirmed by digestion and sequenced. The clones obtained were not full length as they were digested with Rsa1 enzyme in the first step itself. So the main objective in this study is to construct the full length clone of few of the SSH clones and to functionally validate them.

4.1 Selection of clones for construction of full-length gene

4.1.1 Selection based on size

The following stress responsive clones were selected from the groundnut suppression subtractive cDNA library by initial dot blot analysis (Data not presented). The clones were selected based on their function and size. The clones selected are Gsi4, Gsi39, Gsi46, Gsi63, Gsi75, Gsi83, Gsi109, Gsi110, Gsi115 and Gdi15.Table 4 show the genes selected with their name, function and size. Few clones of unknown function were also selected.

Table 4: Stress responsive clones selected based on size and function

Clone No	Size (bp)	Function of the Clone
Gsi4	300	Unknown
Gsi39	700	Early Light Induced Protein
Gsi46	400	Unknown
Gsi63	400	DEAD Box ATP dependent Helicase
Gsi75	650	Caffeic Acid o methyl transferase
Gsi83	450	Cold induced Protein
Gsi109	700	Myo-inositol 3 Phosphate synthase
Gsi110	700	Cinnomoyl coA Reductase
Gsi115	500	Alcohol dehydrogenase
Gdi15	600	Flavol 3-o glucosyltransferase

4.1.2 Selection of clones based on expression pattern

4.1.2.1 Semi-quantitative RT-PCR analysis

Total RNA was isolated using phenol-chloroform method. 5ug of this RNA was used to make cDNA with MMLV reverse transcriptase. The cDNA was used to amplify the genes using gene specific primers. The expression pattern was analysed. Clone numbers Gsi4, Gsi 83, Gsi110 and Gdi15 all are specific only to stress conditions. Gsi39, gsi63, Gsi109 and Gsi 115 showed increased expression over control and Gsi 46 and Gsi75 showed slight increase over control (Fig6).

4.1.2.2 e-northern analysis of groundnut homolog in *Arabidopsis*

The expression of the genes was further studied using the publicly available expression browser (www.bbc.utaronto.botany.ca). The Arabidopsis homolog of groundnut clone was got and its AGI code was identified. This code was used to get the expression pattern of the genes under drought, salinity cold, osmotic ad heat stresses in both shoot and root. The E-Value and AGI code are represented in table 5.

In shoots, At2g22240 (homolog of Gsi109) showed high level of expression in drought and osmotic with 157 and 45 fold increase in drought and osmotic stress respectively followed by Cold induced Protein (homolog of Gsi 83) with 55 and 27 fold increase. Cold induced Protein (homolog of Gsi 83) had nearly 24-27 fold increase over other genes in cold and salinity. Expression of other genes under all stresses was negligible when compared to homolog of Gsi83 and Gsi109 (Fig 7). All the genes showed increased expression under towards the later hours (6-12hours). Gsi39 showed least expression in all the stresses and homolog of Gsi115 (At1g77120) alone had very high expression under heat stress

In roots, Cold induced Protein (At1g20450-homolog of Gsi 83) showed high expression with 9, 1.9, 4 fold increase in cold, drought and osmotic stress respectively. Homolog of Gsi115 (At1g77120) showed up in heat stress as in shoots (13fold increase at 3 hour of

Table 5: Arabidopsis homolog of groundnut clones with their AGI code, E-value, % positive and % identity

Clone Name	AGI code	E-value	% Identity	% Positives
Gsi 39	At1g44575	0.56	30	35
Gsi 63	At1g20920	0.28	47	67
Gsi 75	AT1g77520	5.00E-05	25	32
Gsi 83	At1g20450	0.087	45	70
Gsi 109	At2g22240	4.00E-32	90	91
Gsi 110	At5g58490	5.00E-05	75	87
Gsi 115	At1g77120	6.00E-52	71	83
Gdi15	At2g36790	1.00E-18	41	62

The sequence of the selected cloens was basted against Arabidopsis genome to get their AGI code and E-Values. These AGI code were fed to the expression browser tool to get the expression under various stress.

stress). Gsi39 showed better expression in roots (Fig 8). Gdi 15 showed increased expression in roots at moderate stress level (6hours) in all stresses except drought stress. The fold increase in roots for all the genes were 1-5 with few exceptions, which is low compared to that of shoot.

4.2 Different methods for the construction of full length clone

4.2.1 Construction of full length clones using genomic DNA

4.2.1.1 ARP primers in construction of full length clone

ARP (*Arabidopsis* Random Primer) was designed by CDFD Hyderabad. These primers were used to amplify the genomic DNA of groundnut. Seven ARP primers were designed as ARP1-7. The primer details are given in previous chapter. Amplification products were obtained with ARP 1, 3, 4 & 7. Figure 9a shows the amplification of Gsi46, Gsi63 & Gsi75. Polymorphic bands of size 500-1.2kb were amplified. In Gsi 75, both forward and reverse showed high polymorphic bands and size upto 1-1.5kb were amplified. In case Gsi110, amplification of genomic DNA with ARP3 & Gsi110L resulted in a very prominent band of size 2kb, whereas ARP1 with GSi110U yielded a faint band around 1Kb (Fig 9b). Summary of the few of the Gsi clones amplified with various ARP primers are listed in Table 6. One major disadvantage of this method is the reproducibility of the bands. Moreover the specific bands after cloning failed to give amplification with gene specific primers.

4.2.1.2 Walker Primers in construction of full length clone

Walker primers are different from that of ARP primers; the last 4 sequence has N ie it can be any nucleotide. The primer details are given in materials and methods. In many cased these primers were used to pull out the promoter region of the genes (M.K Reddy et al., 2002) These primers were used for our study to pull out the full length genomic DNA clone of our interest.

Table 6: Summary of the various genes amplified with different ARP primers

Clone No	Size (Kb)	Function	Approximate sizes of the polymorphic band			
			ARP1	ARP 3	ARP 4	ARP 7
GSI 4-U	0.3	unknown			1.0	
Gsi 39 U	0.7	ELIP	0.5	-	-	-
Gsi 39 L			0.7	-	-	-
Gsi 46U	0.4	unknown	-	-	0.75	0.6
Gsi46L			-	-	0.7	1
Gsi 75U	0.65	Caffeic Acid o methyl transferase	0.9	-	-	-
Gsi 75L			1.0 & 0.8	-	-	-
Gsi 83U	0.45	Cold stress Protein	1,5 & 1.0	-	-	-
Gsi 83L			-	0.75	-	-
Gsi 109 U	0.7	Myo-inositol 3 phosphatase	0.75	-	-	-
Gsi 110 U	0.7	Cinnomoyl CoA Reductase	-	2	-	-
Gsi 110 L			-	1.2	1	-
Gsi 115 U	0.5	Alcohol dehydrogenase	1.2	-	-	-
Gsi 115 L			2	-	1.5	-

Four different Walker primers were designed and PCR annealing temperatures were standardized. Using these Walker primers, genomic amplification of Gdi15 was attempted. Specific band of 750bp was amplified with Walker primer 1 and Gdi15U and 1.5Kb band was amplified with Walker primer1 and Gdi15L (Fig 10a). The reaction was repeated (Fig 10b) to confirm the polymorphism and to elute the polymorphic band. The eluted band was cloned into T/A vector and transformed into *E.Coli* cells. The transformed colonies were checked for the presence of the Gdi15 genomic fragment by doing a colony PCR. Out of the 4 colonies obtained 2 were positive (Fig 11a). The plasmid was isolated from these two colonies and checked for amplification and digestion. Digestion with EcoR1 enzyme resulted in the release of the 750bp fragment (Fig 11b). But the PCR fragment cloned did not get amplification with gene specific primers. So we went ahead with the RNA based methods for isolation of 3' ad 5' ends of the stress genes.

4.2.2 Construction of full length clones using RNA

4.2.2.1 Gene Racer Kit method

RNA was isolated using the normal method. The RNA was used to construct the cDNA using the gene Racer protocol from Invitrogen. mRNA was isolated and treated with pyrophosphatase and Calf Alkaline phosphatase to remove all the truncated mRNAs to ensure that cDNA pool contains full length mRNAs. RNA oligo was added to the 5' end and the cDNA was amplified with the RNA oligo primer and OligodT primer. This pool was used to amplify the gene of our interest.

Gsi4, an unknown gene was amplified using the above cDNA pool. 5' RACE was performed with RNA oligo primer and Gsi4 lower primer. 250bp fragment was obtained. Similarly amplification of cDNA with OligodT and Gsi4 upper resulted in a fragment of 400bp (Fig 12). The fragments were cloned into T/A vector and sequenced. Sequencing results were aligned together to get the extended Gsi4 sequence (Plate1). The Blast analysis resulted in no homology with the existing database. RNA analyzer results

showed that the gene did not contain full coding sequence.

4.2.2.2 Global amplification of c Terminal ends

The methodology was followed according to Reddy et al., 2002. In this method, to the mRNA isolated from RNA of groundnut stressed leaves, adaptors were added at the 5'end. Using the adaptor primer and OligodT primer cDNA pool was made.

This cDNA pool was used to to pull out the 5' and 3' cDNA fragment of Gsi110. 5'RACE with Adaptor primer &Gsi110L and 3' RACE with OligodT & Gsi110U primer amplified approximately a 450bp and 300bp PCR product respectively (Fig13). Since the fragment size was less than the initial partial sequence of Gsi110 it was not considered for further work.

4.2.2.3 Subtracted cDNA screening for construction of cDNA clone

cDNA library using the SMART protocol was constructed earlier in our laboratory(Dr. Geetha Govind, 2006) . The mRNA was isolated from the control and stressed groundnut leaves. Subtraction was carried out as described in the SMART protocol. The SMART Protocol involves the addition of ssgcap at the 5' end and oligodT at the 3'end. The cDNA pool was amplified using these primers. This cDNA library was used for the puling of the cDNA clone of few genes.

Rapid Amplification of c terminal ends from SMART cDNA library

Initially three cDNA libraries were screened using Gsi39 gene specific primers. All the three pools of cDNA showed the presence of the Gsi39 gene (Fig 14). Once the presence of the gene was confirmed, 5' and 3' end was pulled out using the primer combination ssgcap +Gsi39L and OligodT +Gsi39U respectively. Both the RACE reaction yielded in 750bp fragment (Fig15).

The subtracted groundnut stressed cDNA library was screened for the presence of Gsi83. Once the presence of the gene was confirmed 5' and 3' end was pulled out using the

primer combination ssgcap +Gsi83 reverse and OligodT +Gsi83 Forward respectively. 450bp and 600bp fragment was amplified using the above set of primers to yield 3' and 5'end of the clone Gsi83 (Fig16).

4.2.2.3.1 Analysis of the recombinant Gsi39 and Gsi83 with additional 3' and 5' ends

Analysis of the recombinant Gsi39 with additional 3' and 5' ends

The 750bp fragment of 3'Gsi39 after elution and cloning into T/A vector was used to transform the *E.Coli cells*. The transformed white colonies were screened for the presence of the fragment by M13 primers and Gsi39 gene specific primer. Colonies 2,3,4,5 showed positive amplification of the desired fragment with both M13 and Gene specific primers (Fig17 a,b). Plasmids of these colonies were isolated and sent for sequencing.

Similarly, the 750bp fragment of 5'Gsi39 after elution and cloning into T/A vector was used to transform the *E.Coli cells*. The transformed white colonies were screened for the presence of the fragment by M13 primers and Gsi39 gene specific primer. All four colonies showed positive amplification with M13, Gene specific primers and ssgcap +Gsi39L primers (Fig18a). Plasmids of these colonies were isolated and reconfirmed for the presence of the fragment using all three primer set discussed above. All four plasmids showed positive amplification with M13, Gene specific primers and ssgcap +Gsi39L (Fig18b). The positive plasmids were sent for sequencing.

Analysis of the recombinant Gsi83 with additional 3' and 5' ends

The 450bp fragment of 3'Gsi83 after elution and cloning into T/A vector was used to transform the *E.Coli cells*. The transformed white colonies were screened for the presence of the fragment by OligodT +Gsi83 Forward primer and Gsi83 gene specific primer. Colonies 1, 2, 3, 4, 6 & 8 showed positive in both OligodT +Gsi83 Forward primer and Gene specific primer set (Fig19a). Plasmids from the positive colonies were

isolated and sent for sequencing.

Similarly, the 600bp fragment of 5'Gsi83 after elution and cloning into T/A vector was used to transform the *E.Coli cells*. The transformed white colonies were screened for the presence of the fragment by ssgcap +Gsi83 Reverse Primer and Gsi83 gene specific primer. Of the few colony screened one showed positive in Gene specific primers and ssgcap +Gsi83L (Fig19b). Plasmid of this colony was isolated and reconfirmed for the presence of the fragment using the two primer set discussed above. The positive plasmid was sent for sequencing.

4.2.2.3.2 Construction of full length gene of DIP and ELIP Sequence and homology studies

GSi 39 and Gsi83 with additional 3' and 5' ends were sequenced. The Gsi39 with addtional3' was aligned with Gsi39 with additional 5' end (plate 2a) was aligned using the multiple clustalW program to get the full length ELIP sequence. In the same way Gsi83 with additional 3' and 5' end was aligned to get the full length DIP sequence (Plate 2b).The final full length sequence is depicted in plate 3. The alignment of the full length Gsi 39 and Gsi83 sequence with their respective partial sequence is depicted in Appendix IIIc,d.The sequence was analysed in RNA analyzer a web based tool which predicts the exons, 3' and 5' UTRs and PolyA signal. Using this program we concluded that the sequence of DIP and ELIP were full length as they contained the Poly A signal and start codon. The results of the RNA analyzer of ELIP and DIP are shown in plate 4 and plate 5 respectively. The sequence was analysed in NCBI Blastx database. Gsi83 hit the cold or Drought Induced Protein (DIP) and Gsi39 to Early Light Induced Protein (ELIP).

4.2.2.3.3 Full length amplification of DIP and ELIP clone from genomic and cDNA

Primers were designed for the full length DIP and ELIP so that full coding sequencing can be amplified. The primers were used to amplify the full gene from cDNA and

Genomic DNA. cDNA and Genomic DNA clone of ELIP resulted in 750bp and 850bp respectively (Fig 20). cDNA and genomic clone of DIP yielded 586bp and 1.3Kb respectively (Fig21).

The full length DIP and ELIP amplified from cDNA and genomic DNA were PCR purified and cloned into T/A vector. The Genomic DNA (Fig 22a) and cDNA (Fig 22b) clone of ELIP colonies were screened for the presence of the insert by colony PCR using M13, Gene specific primers and M13 primers respectively. The positive colonies were sequenced and used for cloning and further studies. Like wise the cDNA and genomic clone of DIP were purified and cloned into T/A vector. The genomic and cDNA clone of DIP colonies were screened using gene specific primers (Fig 23a & b). Few colonies showed positives. The plasmids from the positive colonies were isolated screened for the presence of the gene using M13 and DIP gene specific primers. The positive plasmids were used for cloning and further studies. Sequence details of the genomic DNA clone of ELIP and DIP are given in plate (Plate 6)

4.2.2.3.4 Molecular and bio-informatics characterization of the DIP and ELIP gene

4.2.2.3.4.1 Expression analysis of DIP and ELIP

Expression analysis of DIP

Stress was imposed to healthy groundnut plants grown in battery pots. Gradual stress was given using Gravimetric method. The groundnut plants were maintained at well watered, 80%FC, 70%FC, 60%FC, 50%FC, and 30% FC. RNA samples were collected form control and groundnut plants maintained at various FC and extracted using the Phenol-Chloroform method and used for northern and semi quantitative RT-PCR. 15ug of the total RNA was transferred to the nylon membrane and probed using the DIP. The results indicate the DIP is expressed only under stress and it increases with increase in stress (Fig24a). RNA from control 50% and 30% FC plants were used for semi quantitative RT-PCR. RNA was Reverse transcribed and used for studying the expression pattern of DIP. DIP was expressed only under moderate (50%) and severe stress (30%) and not in control

(Fig24b). The expression of the DIP was studied under various PCR Cycles (Fig24c) using RNA control and 30%FC plant samples. Elf was used as loading control.

Expression analysis of ELIP

Stress was imposed to healthy groundnut plants grown in battery pots. Gradual stress was given using Gravimetric method. RNA samples were collected form control and groundnut plants maintained 30%FC. RNA was isolated from the 15days old groundnut control and stressed leaves (30%FC) using the Phenol-Chloroform method and used for northern and semi quantitative RT-PCR. 15µg of the total RNA was transferred to the nylon membrane and probed using the ELIP. ELIP is expressed even control but upregualted under stress treatment (fig 25a). As described earlier RNA was Reverse transcribed and used for studying the expression pattern of ELIP by Semi quantitative RT-PCR. ELIP expressed constitutively but upregulated under moderate and severe stress (Fig25b). Semi quantitative RT-PCR was performed with varying PCR Cycles (20, 25, 30, 35 cycles) using RNA control and 30%FC plant samples. Under stress the expression of ELIP gene was unregulated from 25 cycles were as in control expression increased after 30cycles (Fig25c). Elf was used as loading control.

4.2.2.3.4.2 Bio-informatics characterization of the DIP and ELIP

Protein analysis

The DIP and ELIP sequences were fed to SwissProt web browser to study the function of the protein. Plate 8 depicts the function of the protein and the protein sequence. DIP has two domains, one for electron transfer and the other involved in GTP hydrolysis. Predicted function of ELIP indicated that it has EGF domain involve din activation of TK, a Defensin domain and WFC domain for homeostasis (Plate7). DIP has a Fe-S domain involve din energy transfer (Plate8)

Phylogeny Analysis

Phylogenetic sequences tree was constructed for both DIP and ELIP. The homologous

sequences of DIP and ELIP across species were got from NCBI database. Then all the sequence was fed to ClustalW analyser to give the phylogenic tree. Plate 9, shows DIP is closely related to cold responsive gene of *Arabidopsis* and ELIP of groundnut falls into a different branch and has close similarity to Glycine max ELIP than to *Arbidopsis* ELIP.

4.3 Sub-cloning into Binary vector under constitutive and inducible promoters

4.3.1 Sub-Cloning of DIP into Binary vector

Both cDNA and genomic DNA clone of DIP was sub-cloned under constitutive(CaMV-35S) and inducible promoter (ABRE) of pBI121. The schematic map of the cloning strategy is depicted in Fig26.The vector and the DIP gene in T/A vector were restrict digested with BamH1 and sac1 enzyme to release the GUS and insert respectively. The released insert and the vector backbone of35S-pBI121 & ABRE-pBI121 were eluted and purified (Fig 27). The purified vector and insert were kept at 16'C for overnight ligation. The ligated product was transformed into E.coli cells and selected on Kanamycin selection media. The white colonies were screened for the presence of the gene using DIP gene specific primers by colony PCR (Fig28 a, b, c, d). Plasmids were isolated from the positive colonies and then mobilised into *Agrobacterium* strain Gv2260 by electroporation. The Agro colonies were also screened (Fig 29 a, b, c, d). Glycerol stocks of the positive colonies were used for plant transformations

4.3.2 Sub cloning of ELIP gene

Since ELIP gene did not have any compatibility with the available binary vector, Gateway cloning was done which uses the recombinase technology. For cloning into entry clone the gene should have attb sites. attb sites were added to the ELIP primers and primers were re-synthesised. The precise gateway cloning strategy is depicted in fig 30. The attb ELIP primers were used to amplify the plasmid of ELIP gene in T/A (Fig31). The amplified ELIP product was cloned into Entry clone and confirmed using gene specific primers. The colonies showed positive results (Fig32a). The plasmids where then cloned into destination vector and transformed into *E.Coli*. The colonies were once again

screened for the presence of ELIP gene using gene specific and attb-ELIP primer (Fig32b). The positive plasmid mobilised into *Agrobacterium* strain Gv2260. the agro mobilised colonies were confirmed using the Gene specific primers (Fig 33).

4.4 Development of transgenic tobacco plants

4.4.1 *Agrobacterium tumefaciens* mediated transformation of tobacco

The different recombinant plasmid constructs developed in the present study (pBI121-CaMV 35S-DIP and pBI121-ABRE-DIP) was tested for their performance using tobacco as the model system for plant transformation. Young leaves from the tobacco plants were harvested and washed in running tap water for 1 hour. The leaves were surface sterilised using Bavistin(0.1%) for 45 minutes and HgCl2(0.1%) for 2min. The surface sterilized leaf material was cut into small leaf disc and pre incubated for 2 days, then co cultured with the overnight grown *Agroabcterium* culture containing the recombinant DIP in pBI121 vectors for 2min . After 2 days they were selected on media containing Kan+ and Rif+. The resistant shootlets were sub-cultured and rooted in half strength MS. The well rooted plants were then hardened and transferred big pots and maintained in green house (Fig 34). The starting material was 60 lines per plant but eventually due to contamination we were able to maintain 4 wildtype tobacco plants (non-transformed), 5 tobacco plants under 35S-pBI121 and 2 plants of DIP under ABRE- pBI121 in green house. These plants were subjected to molecular and physiological analysis.

NOTE: The control (non-transformed) were labelled as 'WT', 5 plants of cDNA clone of DIP in 35S-pBI121 were labelled as D1,D2,D3,D4,D5 and 2 plants of cDNA DIP in ABRE- pBI121 were labelled as D5, D6

4.4.2 Molecular characterization of transformed tobacco plants

PCR analysis of primary transforamants of tobacco

The presence of transgene DIP in the primary transforamants was confirmed by PCR amplification of genomic DNA samples. The primers specific to DIP gene was used for

PCR analysis of the tobacco transformed with pBI121-CaMV 35S-DIP and pBI121-ABRE-DIP. In addition to the positive control (Plasmid of CaMV 35S-DIP); the 586bp amplified product was obtained in tobacco plants transformed with pBI121 35S-DIP and pBI121-ABRE-DIP (Fig 35). The primers specific to CaMV 35S promoter & DIP (Fig 36a) and internal primer of 35S promoter & DIP (Fig 36b) were used for confirmation of T-DNA region of 35S driven DIP gene expressing plants. 1.4Kb and 948bp PCR product amplified only in 35Sdriven DIP gene expressing plants. To further confirm the nature of the 35Sdriven DIP gene expressing plants the PCR product (948bp) amplified using internal primer of 35S promoter & DIP gene was digested with BamH1 and Xba1. BamH1 and Xba1 cut this region (948bp) of 35S driven DIP gene into 466bp, 493bp and 434bp, 517bp respectively (Fig 37).

4.4.3 Physiological screens to assess the relevance under drought stress

4.4.3.1 Measurement of Photosynthetic gas exchange parameters

Photosynthetic gas exchange parameters were recorded using portable photosynthetic system (LICOR 6400, USA) was recorded on the day of stress imposition (control) and 48 hours after gradual drought stress (water status of the stress pots were around 72-76% FC in different pots). The measurements were made at ambient CO2 concentration, PPDF of 1200µmolesm^{-2}s^{-1} and temperature of 28+0.5. Lines D1, D6, D7 maintained better assimilation and gs under stress condition (Fig38). Except for D4 which showed ambiguous results all the primary transforamants showed high gs and high assimilation than wildtype under control and stress conditions.

4.4.3.2 Rate of oxygen evolution

Rate of oxygen evolution is the indication of the primary reaction of photosynthesis taking place in the leaf. Oxygen evolution was determined by a Clark type Oxygraph Electrode Unit (Hansatech Instruments, Norfolk, England). Rate of Oxygen evolution was recorded on the day of stress imposition (control-100%) and 48 hours after gradual drought stress (water status of the stress pots were around 72-76% FC in different pots).

Leaf punches of 10cm were taken from control and drought stressed tobacco transformants. The leaf punches were placed in closed chamber of the instrument. The chamber is connected to a computer. So rate of oxygen ($nmol_{02}$ evolved /m^2/s) evolved can be directly computed. The rate of oxygen evolved in transgenics is higher than the wildtype under control and stressed conditions (Fig 39).

4.4.2.3 Moisture retention capacity

The moisture retention capacity was calculated to assess ability of the excised leaf discs of wildtype and trangenics to hold water under stress conditions. 5 leaf punches in two replicates was taken per wildtype and transgenic plant. The fresh weight of the leaf discs were taken and then put in water for saturation overnight. The saturated weight was taken. The leaf discs were allowed to dry gradually in lab conditions. After 2, 4, 6 hour of gradual drying the weight of the leaf punches was taken. The dry weights of the leaf discs of the wildtype and transgenic were taken and moisture retention capacity was calculated. Transgenic lines D1, D6 and D7 retained more water under stress conditions than the control (Fig 40).

4.4.2.4 Membrane Leakage

The extent of stress induced damage to cellular machinery was assessed by electrical conductivity of solution containing 5 leaf discs (Sullivan's test). The leaf disc were floated on different Nacl concentration of 150mM and 250mM and PEG stress (-8bars and -12 bars) for 16hours. After the initial period of stress, the EC was measured at 273nm absorbance. Then the leaf discs were boiled for 15min and again absorbance was measured. The percent increase in electrolyte leakage over the control was calculated. All the putative transforamants had less electrolyte leakage than the control except D2. Whereas under PEG induced osmotic stress there was no significant difference in electrolyte leakage between the wildtype and trangenics (Fig 41a, b).

4.4.2.5 Response of putative transforamants to Oxidative stress

High light combined with methyl viologen stress creates oxidative stress. The leaf discs from wildtype and transgenics were floated in methyl viologen (2µM). After 2hours of dark incubation the leaf discs were exposed to high light for 4 hours. The extent of oxidative stress damage on the wildtype and transgenics was then assessed by measuring the chlorophyll content and cell viability test using 0.1%TTC. Transgenic lines D4, D5, D6, and D7 had less percent reduction in chlorophyll and also high tissue viability than the wildtype (Fig42a, b).

4.4.2.6 Response of putative transformants of Temperature stress

High temperature disrupts the chlorophyll stability. The extent of effect of temperature on chlorophyll pigment stability was assessed by DMSO: Acetone method. The leaf discs were floated in beaker containing water. High temperature stress (80^0C) was given by placing the beaker containing the leaf discs in water bath maintained at -80^0C for 30-45 minutes. Then the leaf discs were incubated in DMSO:Acetone over night. The chlorophyll absorbance was taken at 663,645, and 652 nm. The reduction in chlA, chlB, total chlorophyll was less in case of putative transformants. Percent reduction in chlorophyll was less in case of primary tobacco transformants than the wildtype (Fig 43).

DISCUSSION

V. DISCUSSION

Plant growth and productivity is adversely affected by nature's wrath in the form of various abiotic and biotic stress factors. All these stress factors are a menace for plants and prevent them from reaching their full genetic potential and limit the crop productivity worldwide. Abiotic stress in fact is the principal cause of crop failure world wide, dipping average yields for most major crops by more than 50% (Bray *et al*., 2000)

Research into the plant response to water stress is becoming increasingly important, as most climate change scenarios suggest an increase in aridity in many areas of the globe (Burke *et al*., 2006, IPCC 2007). On a global basis, drought in conjunction with coincident high temperature and radiation, poses the most important environmental constraints for plant survival and productivity (Boyer, 1982). Plants respond and adapt to drought stress by altering a large number of genes, as a result, cellular, physiological and biochemical processes are modified. Understanding the functions of these stress-inducible genes helps to unravel the possible mechanisms of stress tolerance. Of late, functional genomic approaches triggered a major paradigm from single gene discovery to thousands of genes by using multi-parallel high throughput techniques. With near completion of genome sequence information of *Oryza* and *Arabidopsis* and rapidly growing databases, complex traits like drought are now amenable to a detailed molecular analysis using genomic tools.

ESTs, a catalogue of stress genes

Expressed sequence tag (EST) provides direct approach for discovery genes associated with drought stress. this has been demonstrated in barley (Michalek *et al*., 2002) maize (Fernandes *et al*., 2002; Brendal *et al*., 2002) rice (Reddy *et al*.,2002, Markandeya *et al*., 2005, Gorantala *et al*., 2007, Rabbani *et al*.,2003), *Pennisetum* (Mishra *et al*., 2006) sorghum (Pratt *et al*., 2005), *Arabidopsis* (Seki *et al*.,2001, 2002). The annotation and comparative analysis of these stress ESTs have identified many genes associated with or

having potential role in drought stress tolerance. These genes provide starting point for understanding the nature and molecular mechanisms of plant response and tolerance to drought (Kreps *et al*., 2002; Gorantala *et al*., 2007). Thus suggesting the emphasis in this area of research and how complex transcriptome is. EST analysis has uncovered numerous novel genes and transcriptional activators, the master switches that influence the expression of cascades of genes associated with stress response. These specific transcriptome is diverse and range from number of functional genes to transcriptional regulators which coordinate the expression of functional genes. The lacuna is that these SRGs are not functionally validated. A major challenge for the coming decade is the functional analysis of this large set of genes.

Adapted species a better option for dissecting genes for stress tolerance

Species differ in their biological physiological and biochemical responses they put forth to rescue themselves from the adverse effects of stress. Genes responding to a particular stress vary between species and even genotypes due to the fact that certain genotypes have efficient signal perception and transcriptional changes that lead to successful adaptations and eventually tolerance. Species do differ in their responses to various stresses though there is cross talk among the stresses. Pie diagram analysis of genes expressed under various stresses showed cross talk as well as specificity to a particular stress (Rizksky *et al*., 2002). Thus discovering genes from a tolerant genotype would be a better option. Resurrection plants are a group of desiccation tolerant plants that can withstand severe drought stress and revives completely to normal metabolism within 24 hours of watering (Graff, 1989). But the growth rates of resurrection plants are very less. In crop plants growth is important in addition to adaptive strategies. Genes from drought tolerant crop will have genes that are functionally and structurally different from the susceptible species (Waditee *et al*., 2002). With this hypothesis, few crop plants were analysed for their stress tolerance. From our own lab, groundnut, finger millet, horse gram had been proved to be better adapted to drought (Gopalakrishna, 2001, Geetha Govind, 2006). Thus isolating stress specific genes from groundnut and finger millet was

undertaken in our own lab by either Suppression subtracted cDNA library or through SMART cDNA library construction (Gopalakrishna, 2001, Geetha Govind, 2006, Prakash, 2007).

Functional characterization of stress genes by high throughput techniques

The current trend in stress-biology is to use large-scale genomics data to scrutinize and revalidate the protective mechanisms of these genes either by testing the putative function of the genes by forward genetics or by the transfer of several of the stress-responsive genes into crops to enhance the tolerance levels (transgenic). In order to reveal their putative functions various highthroughput methods like gene inactivation or over expression were used. There are two main complementary approaches developed for identifying mutations in target genes, namely TILLING (Targeted Induced Local Lesions IN Genomes) and T-DNA insertion mutant lines. Both had relevance and importance. Many reports say only 10% of the knockout mutants revealed any phenotype [Fraser *et al*., 2003]. It is possible that the lack of phenotypes in knockouts could be explained by our inability to detect small phenotypic changes. Functional redundancy due to the extensive duplication of the Arabidopsis genome is also likely (Brown *et al*., 2000; vandepoele *et al*., 2002). Overexpression offers an alternative and complementary strategy and less affected by functional redundancy. Thus we preferred to validate by over expression, since the relevance of the genes can be studied in the background of other stress genes expressed.

With this background the present study was under taken to clone and characterize a few clones from suppression subtracted cDNA library constructed by Gopalakrishana (2001) and to functionally validate them. This requires initially, confirming their stress responsive nature. Having established their stress responsive nature our efforts has been to develop full length clones. Since the gene has to be expressed on an inducible promoter the emphasis has been to clone the full length clones under constitutive and

stress specific promoters. And finally to develop tranformants as a test tool to validate the stress relevance of target genes based on physiological screens.

Validation of Stress responsiveness of the subtracted cDNA clones

Insights into gene expression patterns and functions coupled with stress tolerance can be explored by EST-based cDNA arrays. Transcriptome analysis using cDNA microarrays (Chen *et al*., 2002, Sreenivasulu *et al*., 2006) are novel approaches to identify higher number of transcripts and pathways related to stress tolerance mechanisms at the global level. One of the major technical merits of microarray analysis is its high sensitivity in the detection of mRNAs. Expression profile of rice ESTs (Gorantala *et al*., 2007) Sorghum (Pratt *et al*., 2005) and various other crop plants have been studied using microarray.

We studied the expression profile of the groundnut suppression subtracted cDNA clones identified in our lab using the web based expression browser (www.bbc.utaronto.botany.ca) which utilises the microarray data of *Arabidopsis* cDNAs. Expression profile of *Arabidopsis* homolog of groundnut clones expressed under drought cold, salinity, osmotic, and heat stress was studied. Gsi 83 homolog showed very high expression pattern followed by homolog of Gsi 109 in shoots under drought, cold, salinity, and osmotic stress. In roots the expression of homolog of Gsi115 and Gdi15 was high in addition to homolog of Gsi 83. Microarray data coupled with qualitative real time (qRT-PCR) or semi quantitative RT-PCR or RNA gel blot analysis would reconfirm the stress responsiveness of the genes (Rabbani *et al*., 2003). Semi quantitative RT-PCR results showed that Gsi 4, Gsi83, Gsi 110 showed high level expression under moderate and severe stress. Stress inducible genes identified by semi- quantitative RT-PCR analysis are grouped into highly expressed only under stress and genes upregulated under stress. Gsi83, Gsi110, Gsi4 comes under first category whereas Gsi63, Gsi46, Gsi75, Gsi109, GSi39 and Gdi15 fall into the second category.

Comparative analysis of e-northern and semi-quantitative RT-PCR

Comparison of e-northern and semi quantitative RT-PCR results shows the difference in expression pattern of the stress responsive genes under study. Except for Gsi83 and Gsi109, the level of other genes remained low in e-northern analysis. But RT-PCR analysis in groundnut showed that Gsi110 is expressed only under severs stress, Gsi115 and Gsi63 are unregulated under moderate stress and Gsi39 upregulated under severe stress. These results of RT-PCR do not coincide with the e-northern analysis. Suggesting that, the genes upregulated under stress may differ in susceptible and resistant species. Thus the importance of these genes under stress may be more as they are expressed in a drought adapted species. Another possibility is, the groundnut clones Gsi39, Gsi63 and Gsi83 had very low level of homology to the *Arabidopsis* indicating that they represent different gene with similar biochemical functions different from that of *Arabidopsis* genes. Since groundnut is an adapted species we hypothesis that it is relevant to study the importance of the stress responsive genes of this species.

Development of Full length gene as a prelude to functional validation

Full length cDNAs of the expressed genes are important for the analysis of the structure and function of genes. To validate the gene functionally various methods are followed like mutation studies, down regulation by antisense RNA technology and by over expression studies. Overexpression studies is a better option as it studies the function of the gene against the background of other stress genes expressed. Consequently, for functional validation by over expression studies a prerequisite is that the selected stress genes should have the full coding sequence. So our major emphasis was to construct full length gene of few stress responsive clones identified from the subtracted cDNA library.

Different methods (DNA and RNA based) were followed to obtain the full length clones. The advantages of isolating full length gene from genomic DNA is that along with the genomic sequence of the gene of interest, the intron regions can be amplified successfully. These regions could help in better transcription of the gene or the splicing of

the gene which helps in better expression of the gene of our interest. Identification of unknown regions can be achieved through screening of genomic DNA library using identified DNA probes. However, construction and screening of libraries are time consuming and laborious procedure (Rishi *et al*., 2004). Moreover accuracy depends on how much DNA sequence information is available for DNA probes. Advancements in the PCR technique have helped researchers to reduce this time and avoid the use of radioactive probes.

Genome walking to isolate genomic clone of stress genes

Genome walking is a relatively fast, reliable and general approach to sequence or clone DNA adjacent to a known region. Numerous modification has been made to the existing protocols for the amplification of flanking regions such as inverse PCR (Ochama *et al*., 1998), vectorette PCR (Arnold and Hodgson, 1991), adaptor specific PCR (Universal Genome Walking Kit, Clontech), Anchored PCR (Roux *et al*., 1990) etc. we used in the present study primers (*Arabidopsis* Random Primers (ARP)) designed by Dr.Nagaraju of CDFD, Hyderabad to amplify the gene of our interest. We also used walker primers to amplify the genomic clone of the stress responsive genes. Reddy *et al*., 2002 has been successful in isolating the 5' flanking regions of the Glyoxylase gene of *Brassica* using directional genome walking with walker primers. We were successful in amplifying fragment size ranging from 500- 2kb using ARP as well Walker primers, but there was some noise in the results we obtained. This could be overcome by using biotinylated gene specific primers as used by Reddy *et al*., 2002 to pull out fragments specific to our gene of interest. Thereby non specific amplification can be avoided.

Rapid Amplification of c terminal ends for isolation of cDNA clone of stress genes

Attempts were made to isolate the full length gene of the stress responsive clone's selected using cDNA. Three methods were tried, Global amplification, Gene Racer and SMART cDNA library construction kit. All the three protocols follow the basic principle of RACE PCR. 3' 5' RACE is a commonly practiced to isolate the 3' and 5' end of the

cDNA clone. SRF2 gene involve in freezing tolerance has been isolated using the 3'5' RACE kit using Rouche Kit (Lievens *et al*., 2004). Though the methods have produced successful results they have their own disadvantages. In Global Amplification of c terminal ends there are chances of getting smaller fragments as truncated mRNAs are not eliminated as in case of Gene Racer method. Gene Racer Kit employs the use of many enzymes and purification steps where our mRNA can yields are very less. But SMART utilizes the Powerscript Reverse Transcriptase property of terminal transferase activity ie it adds 'c' residues when the RT reaches the end of mRNA. Thus SMART is a one step reaction with less complication. SMART library developed by Geetha Govind (2006) was screened by us using the Kit primers. We were successful in amplifying the 3' and 5' ends of Gsi 83 and Gsi 39. Finally we were able to clone 586bp and 878bp of full length cDNA clone of DIP and ELIP.

Gene name	Initial sequence length	Full cDNA sequence length	Additional cDNA sequence amplified	Genomic sequence length	Additional genomic sequence	Introns
DIP	271	586	315	1189	603	2
ELIP	636	878	242	910	38	1

Importance of genomic clone was well elucidated. Expression of maize PEPC genomic sequence on its natural promoter resulted in substantially higher transcript levels, signifying the importance of genomic clone (Tagu, 1990). In view of its importance, we isolated 1189bp and 910bp of DIP and ELIP genomic DNA clone respectively. Table below gives the details of the full length clones obtained. The genomic clone of DIP had 2 introns whereas ELIP had small introns of 38bp.

Both RNA and DNA based methods to clone full length genes was successful in our studies. DNA based methods of full length amplification involve repeated confirmation as it involves random primers. Moreover all the specific bands amplified using the gene of interest and random primers had to be sequenced for better results. Though RNA based

method has its disadvantages in terms of elaborate procedure and it is more specific with less noise (non specificity). For better results use of gene specific biotinylated primers in case of DNA as well as RNA based methods would yield more specific 5' and 3' DNA or RNA fragments of target genes.

Characterization of DIP and ELIP gene

To characterize the full length gene clones in this study, we subjected the sequence to various web based tools like Blast analysis, RNA analyser, InterPRO and ClustalW analysis. RNA anlayser scans the sequence for the polyA signal, 3'and 5' UTR, ORF and Start codon. DIP and ELIP sequence had both poly A signal and start codon with 3' and 5' UTRs confirming that they are full length gene.

Characterization of Drought Induced protein (DIP)

The function of a gene can be predicted based on the protein sequence. The protein sequence of DIP and ELIP were analysed using InterPRO protein database. DIP is related to cytoskeltal mechanism as it has tubulin domain and a electron transfer domain which could involve in some transport or energy metabolism. Homology analysis related DIP to a Cold responsive protein SRC1 of soyabean. SRC1 encodes Steroid Receptor Coactivator which is expressed under cold stress. DIP has 80-100bp homology to the dehydrins. It has been proposed that, during cellular dehydration, dehydrin proteins play an important role in maintenance of the structure of other proteins, vesicles, or endomembrane structures, in the sequestration of ions such as calcium, in binding or replacement of water, and functioning as molecular chaperones The multiple targets of dehydrins (euchromatin, cytosol, cytoskeleton) suggest that the direct consequences of dehydrin activity are biochemically diverse. The high level of expression of DIP protein under stress conditions could be attributed to the TTTCTTCTCT stretch in the 5' UTR which is a cis active element conferring high level of transcription in *Lycoperscium*. In addition it has GARE domain, GA responsive element. GA has been proved to be involved in cold stress. Thus, suggesting the role of DIP in cold stress. E-northern

analysis of homolog of DIP in *Arbaidopsis* showed 60fold increase over control in cold and 30 fold increase under drought at later hours of stress. 5' UTR, homology and e-norhtern analysis data infer that DIP might have a role in drought and cold in maintaining the cytoskeleton structure.

Characterization of Early Light Induced protein (ELIP)

ELIP is very well characterized in *Arabidopsis*. ELIPs (Early Light-Induced Proteins) are thylakoid proteins, encoded by nuclear genes, expressed in plants exposed to stress (Meyer and Kloppstech, 1984; Adamska, 2001). ELIPs are synthesized in the cytoplasm, imported into the chloroplast, and inserted in thylakoids via a pathway involving cpSRP43 (Hutin *et al*., 2002). ELIPs have three transmembrane domains. In a study on winter acclimation of bearberry (Arctostaphylos uva-ursi), Zarter *et al*. (2006) found a correlation between the persistent retention of zeaxanthin and the up-regulation of ELIP type proteins. They suggested that ELIPs could be involved in long-term acclimation of plants to extreme stress conditions by interacting with this persistent pool of zeaxanthin. Expression of ELIP has been shown under high light and cold. 5' UTR region of the ELIP sequence was analysed using the PlantCare program. The promoter predicting tool showed a light responsive element (I-box) suggesting the fact that it is induced under high light. Predicted protein domains shows an EGF1 domain a membrane bound protein domain, confer with the fact that ELIPs are membrane bound and involved in energy dissipation.

Both the genes, DIP and ELIP could be involved not only in drought stress but also in cold and high light stress respectively.

Relevance of promoters

Promoters play key roles in conferring temporal, spatial, chemical, developmental, or environmental regulation of gene expression. They usually contain essential cis-acting elements that interact with transcription factors, for regulation of transcription upon

perception of upstream signals. Promoters could be constitutively active or become active only upon induction, and may have differential strength under different conditions. Constitutive expression of DREB1a resulted in severe growth retardation under normal growing conditions (Kasuga *et al*. (1999)). In contrary, expression of DREB1a gene under the control of a stress-inducible promoter rd29A gave rise to minimal effects on plant growth under normal growing conditions and provided even greater tolerance to abiotic stress treatments. It is interesting to note that although both ABF4 and ABF3 play an essential role in drought tolerance, yet their constitutive overexpression resulted in stunted phenotype in ABF4 line and no visible growth inhibitions in ABF3 overexpression lines in comparison to wild type plants (Kang *et al*., 2002).These results together with the results from our own lab on P5CS under constitutive promoter resulting in abnormalities (Shivakumar *et al*., 2006)) suggests that promoters that are subject to specific regulations are useful for manipulating foreign gene expression in plant cells, tissues, or organs with desirable patterns. Two options are available 1) promoter of gene of highly expressed gene (rd29A, Hsp promoter) or 2) Synthetic promoters. There are many stress responsive cis elements known so far. DREB (CCGAC), MYB (CCAAT) ABRE (GTGGC) are well known and validated *cis* elements which can be used as promoters for the gene of our interest.

ABRE promoter - a better choice

Plants have the ability to monitor and adjust to their adverse environments, although the degree of adjustability or tolerance to specific stresses varies from species to species. The acclimation/adaptation process is, in large part, mediated by the plant hormone Abscisic acid (ABA). The hormone level increases under common stress conditions to trigger metabolic and physiological changes (Ingram and Bartels, 1996; Shinozaki and Yamaguchi-Shinozaki, 2000; Zhu, 2002). The adaptations entail changes in gene expression patterns. Numerous genes involved in the acclimation/adaptation processes are up- or down-regulated under stress conditions. Although not all of them are subjected

to ABA-regulation, expression of a large number of them is controlled by ABA. Promoter analyses of ABA/stress-responsive genes revealed that a DNA sequence element consisting of ACGTGGC is important for ABA-regulation. Transcription factors regulate the expression of ABA /stress responsive genes via the consensus element, which is generally known as "Abscisic acid Response Element" (ABRE). Many bZIP class of TF fall into this category. Four ABFs are identified which differ in the flanking region. The role of the ABFs is well studied. ABF1 expression is induced by cold, ABF 2 and ABF3 by high salt, and ABF4 by cold, high salt and drought. Many Lea class of proteins such Em1a, rd29, HVA1 are activated by the ABFs. Thus ABRE was our choice of promoter for the present study. In this angle we cloned the DIP and ELIP under constitutive as well 4xABRE promoter. 4xABRE promoter was designed by Dibyendu Sengupta of Bose Institute and validated in our lab (Shivakumar, 2006). It is a synthetic promoter which has 4 repeats of the core sequence ACGT of the Abscisic acid response element (ABRE) fused to the CaMV 35S (-70 to +1) minimal promoter.

The approach has been to over express DIP both constitutively and on drought stress synthetic promoter ABRE and subsequently evaluate the stress response of the trangenics. The choice species for such approaches has been *Arabidopsis* or tobacco and recently *chlamydomonas* (Hema *et al*., 2007). Tobacco is best situated plant species to study stress responses than the others. In this view transgenics were developed expressing DIP under inducible and constitutive promoter

Validation of stress responsive nature of DIP transgenics

Molecular characterization of the transgenics

The T_0 plants were assessed for the presence of the putative gene (DIP). All the 35S-DIP as well as ABRE-DIP transformed putative plants showed the presence of the gene confirming they are putative transformants. Amplifying the T-DNA region could confirm the gene integration in the transgenics. Amplification of the T-DNA region with both 35s and DIP and internal primer yielded positive results. An alternate method to southern, to

confirm the gene transformed by restriction digestion was done. The amplified T-DNA region of the putative tranformants was restrict digested with Xba1 and BamH1 to release two fragments. The restriction profile of the 35s transformed plants resulted in the desired fragments size.

Physiological characterization of the transgenics

Two approaches to quantify the stress responsiveness of the DIP transgenics are often employed. One is empirical approach based on phenotype ie abnormalities and other one is to study the various physiological and biochemical parameters. The transgenic will show visual phenotypic differences under stress only when relevant upstream regulatory genes are expressed or when an important water relations gene is over expressed. Often visible phenotype is not seen when functional genes are expressed. Therefore to assess the subtle difference it is necessary to assess the response using different physiological stress screens.

Under drought stress conditions, photosynthetic machinery gets affected. The first response of virtually all the plants to water deficit is the closure of their stomata to prevent the transpirational water loss. Stomatal closure in response to a water deficit stress primarily results in decline in the rate of photosynthesis. Very severe drought conditions results in reduction in photosynthesis due to decline in Rubisco activity. So we measured the gs and rate of photosynthesis on exposure to drought stress in wildtype and trangenics. Rate of photosynthesis and gs was significantly higher in transgenics than the wildtype (Fig 38). The transgenics maintained the chlorophyll stability (Fig 43) under stress conditions compared to wildtype indicating that function of DIP gene as dehydrin is protecting the protein structures.

Membrane leakage is another parameter to assess the damage of stress on plants. The extent of damage caused by Nacl on tissue membrane was studied by treating the wildtype and trangenics to different concentrations of NaCl. The results shows transgenics have less membrane leakage than the wildtype (Fig 41)

Another aspect of dehydration tolerance, and of tolerance to other abiotic and biotic stresses, is the control of the level of reactive oxygen species (ROS) or limitation of the damage caused by ROS. The sources of ROS under stress, mechanisms of ROS detoxification and the role of ROS in stress signalling are all active areas of current research and have been extensively studied and reviewed (Apel and Hirt, 2004; Camp *et al*., 2003; Chen and Gallie, 2004; Corpas *et al*., 2001; Foyer and Noctor, 2003; Hung *et al*., 2005). Methyl viologen coupled with high light induced oxidative stress was imposed on the wildtype and transgenics and cell viability as well as percent chlorophyll reduction over control was estimated. The transgenic lines which showed less chlorophyll reduction (D4, D5, D6, and D7) had more tissue viability (Fig 42). The tolerance to oxidative stress could be DIP gene is able to maintain the photosynthetic machinery as evident from the gas exchange measurements.

Comparison of 35S-DIP and ABRE-DIP transgenics

Comparison of the stress response of the 35S-DIP and ABRE-DIP gave mixed results. ABRE Reduction in Photosynthetic and gs under stress over control in ABRE-DIP transgenics was less compared to the 35S-DIP transgenic (Fig 38). Reduction in chlorophyll stability and cell viability on exposure to oxidative stress was less in ABRE-DIP transgenics (Fig 42). Line 1 (D6) of ABRE- DIP transformed plant showed better stress tolerance in all the physiological screens than the 35S-DIP transformed plants. The average performance of the ABRE-DIP showed better stress tolerance compared to the average performance of the 35S-DIP transformed putative transgenics.

All the physiological assays proved that the transgenics though not all but few performed significantly better than the wildtype conferring the importance of DIP gene in stress tolerance. The physiological screens are best studied in T_1 generation plants, where replicated data of the confirmed transgenic lines will provide accurate picture of the relevance. These experiments are in progress.

Outcomes of this study, from the stress specific subtracted cDNA library we identified

stress genes which are over expressed and those expressed only under stress.

Comparative analysis with *Arabidopsis* homolog suggests that among these stress responsive genes some are specific to groundnut signifying stress importance.

Gene constructs under a stress inducible promoter are a potential tool to improve stress tolerance in susceptible species. The full length cDNA and genomic clone of DIP and ELIP were isolated and cloned under constitutive and stress inducible promoter.

Over expression studies indicate that DIP is potential candidate gene.

SUMMARY

VI. SUMMARY

Global climate change predicts increased abiotic stress and evaporation of moisture from soils could reduce yields in lower latitudes. To maintain the world food production with the increasing population, will need to rise food production by approximately 70%.This dramatic and sustained increase in demand for food production will need to be achieved at a time when world agriculture faces the new challenge of global climate change.

Part of the solution to meet the challenges of nourishing a growing human population in an era of global climate change may emanate from the natural abilities of plants to cope with unfavourable environmental conditions. As sessile organisms, plants had to develop various biochemical and physiological mechanisms to respond and adapt to these stresses and thus acquire stress tolerance. Adaptation to stress has been suggested to be mediated by both preexisting and induced defences (Bray *et al*., 2000; Hasegawa *et al*., 2000; Pastori and Foyer, 2002).

Indeed, the ability to respond and ultimately adapt to abiotic stress may be a driving force in speciation (reviewed by Lexer and Fay, 2005). Species differ in their ability to tolerate stress depends at the level of perception and the effective means to transduce the signal perceived to express the genes that could impart tolerance. It has been shown that species have better tolerance mechanisms and the genes that are expressed in the tolerant species will be structural and functionally different from the susceptible species.

Thus a closer look into genes expressed in the drought tolerant crop would answer a few complexities in drought tolerance. Resurrection plants could be a option one can think off. Though they are really a good drought tolerant model yet the growth rates are low. Yield component is one thing a scientist as well as farmers eye on. In this angle research has gone into drought tolerant crop plants. Various researchers have identified genes which are differentially expressed under various abiotic stresses.

Thus understanding the major players involved in drought tolerance will shed light on the mechanisms of drought tolerance. To achieve this goal of understanding the drought tolerance various molecular biology tools has come handy. The advancement in the areas of gene discovery, transcriptome profiling using microarray, promoter trapping and efficient transformation techniques had hastened our understanding of mechanisms involve in drought tolerance. cDNA libraries provide a major source of these differentially expressed genes. EST database is developed for almost all the crop plants in recent years, barley (Michalek *et al*., 2002) maize (Fernandes *et al*., 2002; Brendal *et al*., 2002) rice (Reddy *et al*.,2002, Markandeya *et al*., 2005, Gorantala *et al*., 2007, Rabbani *et al*.,). *Pennisetum* (Chandru *et al*., 2006) sorghum (Pratt *et al*., 2005) ,*Arabidopsis* (Seki *et al*.,2001, 2002). Gene discovery gas left us with large sets of genes in different crops. Though, it has been validated in terms of expression, annotation and expression under different stresses and crops, yet the main lacuna is that they are not functionally validated. It is observed that only around 10% of *Arabidopsis* genes are validated, suggesting that much more insight into the area of drought can be achieved by validating many more genes. Functional validation of the huge set of genes from different crop species is the challenge of today's era.

Several methods of validation exist to understand the function of these large set of genes. Down regulation by mutation and over expression studies have helped to understand the function of these genes. In plants, there are several recent examples in which knockouts, in these cases knockouts of TF genes, failed to generate informative phenotypes. Gene functions can be studied clearly only when they are over expressed. Even when the knockout mutant produce clear informative phenotype, over expression can still be valuable because it can completely produce unexpected phenotype and shed light on aspects of the function of the gene that would otherwise be missed.

With this background, the major goal of the present study was to characterize the selected stress specific subtracted cDNA clones developed earlier by Gopalakrishna (2001) and assess the functional relevance of a few genes selected for stress tolerance. Based on the

initial expression studies, ten stress responsive genes were selected and these clones were used for the present study. The first objective was to screen the suppression subtracted cDNA library for stress responsive genes and reconfirm their expression using RT-PCR and e-northern analysis. For any functional characterization full-length genes are a must. So the second main objective is to isolate the full length of few of the stress responsive genes selected using the genomic or cDNA tool. Finally, to develop transgenics over expressing the stress responsive genes under the constitutive and inducible promoter and to study their relevance under stress.

Salient findings of this present study

Characterization of the selected genes by expression analysis

Suppression subtracted cDNA clones were stress responsive as demonstrated in RT-PCR. e- Northern analysis of the Arabidopsis homolog of groundnut clones selected showed these genes to be stress responsive. Based on the RT-PCR data, selected genes can be grouped into genes expressed only under stress (Gsi4, Gsi83, Gsi110) and those that are up regulated under stress (Gsi109, Gsi115, Gsi63, Gsi46, Gsi39, Gsi75 and Gdi15). The genes which expressed only under stress can be used as marker genes to indicate whether the plants are stressed or not and it could be value genes to be manipulated. Comparison of the *Arabidopsis* homolog of groundnut stress genes revealed that these genes are expressed under stress. Homolog of Gsi83 and Gsi109 showed several fold increase over the control. Whereas other genes where only 3-4 fold increase over control in *Arabidopsis.* It was noticed that homolog of Gsi110 which is highly expressed only under stress was not highly expressed in *Arbidopsis*. Same result was seen with homologues of Gsi39, Gsi115, Gsi63 which were upregulated under stress, was not highly expressed in *Arabidopsis*. Gsi83 expression was deceased after 12 hours of stress whereas in groundnut its expression was very high under moderate and severe stress. These findings throw light on the fact that genes expressed in response to stress in groundnut is different compared to the *Arabidopsis* Thus comparison between susceptible species and tolerant species provide crucial leads.

Construction of full length genes using DNA and cDNA based methods

For any functional validation, the genes have to be full length. The stress responsive genes selected were of partial length. So to isolate the 3' and 5' ends of the selected stress genes various DNA and cDNA based protocols were used.

DNA based methods attempted are 1) ARP primers designed by Dr. Nagaraju 2) Walker Primers designed by Dr.M.K.Reddy. Amplification product size of 500-2000bp was obtained using the ARP/Walker and gene specific primers. These methods provided some lead in isolation of full length clone. But there was noise in the results obtained. The noise can be reduced with little modifications in the protocols.

cDNA based methods used were Gene Racer Kit from Invitrogen, Global amplification protocol and SMART cDNA library screening. We were able to obtain 3' and 5' end using the first two methods. But the sequence was not full length. With the third method, SMART cDNA screening isolated the 3' and 5' ends of two clones Gsi83 and Gsi39 was successful and the sequence was full length.

Both the DNA and cDNA based methods are a potential options for isolation of full length clones but the still better choice is cDNA based method because of less noise. The noise in DNA and cDNA based methods can be reduced by the use of biotinlyated gene specific primers.

Analysis of the cDNA and genomic full length clone of DIP and ELIP

The additional sequence obtained at the 3' and 5' end of ELIP was 193bp and 140bp and for DIP was 56bp and 301bp respectively. The total additional sequence for ELIP was 242bp and for DIP was 315bp. For efficient transcription of the gene it is proved that the genomic clone of the gene is better option. So the genomic DNA clone of ELIP and DIP were isolated by designing the primer for the full length cDNA sequence. Using these

primers we amplified around 910bp for ELIP and 1189bp for DIP. ELIP had one intron of 28bp and DIP had two introns.

Predicted function of DIP and ELIP for better understanding of these genes

The phylogenitic analysis of ELIP showed it to be closely related to *Glycine max* ELIP than to *Arabidopsis* ELIP1 or 2. DIP was closely related to SRC1 of *Glycine max* and cold responsive protein of *Arabidopsis*. The predicted protein structure shows ELIP to be membrane bound. It is similar to *Arabidopsis* ELIP which is also membrane bound and it could be involved in energy dissipation. DIP protein had tubulin domain suggesting it to be involved in cytosketal machinery.

Sub cloning into stress specific promoter and its importance

Phytohormone ABA regulates several stress responses. Control of ABA-regulated gene expression is mediated via the ABRE cis-acting element PyACGTGGC, which is bound by bZIP transcription factors known as ABRE-binding (AREB) proteins or AREB factors (ABFs; Choi *et al*., 2000; Uno *et al*., 2000). Synthetic promoter with ABRE elements could be potential promoter. With this background the synthetic ABRE promoter developed by Dibyendu Sengupta, Bose Institute and validated by Shivakumar (2006) was used to develop inducible constructs. So the DIP and ELIP were sub-cloned into ABRE, as ABA is a well known important signal in stress. The DIP gene was sub cloned into binary vector pBI121 under 35S and ABRE promoter.ELIP was sub cloned into binary vector by Gateway cloning strategy as it did not have compatible enzyme sites with any of the known binary vector.

Developing transgenics expressing DIP under constitutive and inducible promoter for stress tolerance

The binary constructs with DIP and ELIP mobilised into *Agrobacterium* strain GV2260 strain and transformed into model plant tobacco for functional validation.

The putative transgenics expressing DIP showed PCR positives for DIP gene. T-DNA region of the 35S transformed plants were amplified with CaMV 35S promoter and

Dip primers. All the five 35S-DIP transformed plants showed the presence of the T-DNA region. Restriction digestion of the T-DNA region with Xba1 and BamH1 resulted in restriction fragments of desired size.

The putative tranformants were analysed for the stress tolerance using various physiological assays. Rate of photosynthesis and gs was significantly higher in transgenics than the wildtype; D6 and D7 had less reduction in photosynthesis and gs on exposure to stress conditions. The transgenics maintained the chlorophyll stability under stress conditions compared to wildtype indicating that function of DIP gene as dehydrin is protecting the protein structures. Percent reduction in cell viability and chlorophyll was less in the transgenic lines D4, D5, D6 and D7 compared to the wildtype. The transgenics had less leakage under salinity stress. The ABRE-DIP transformed putative transformants performed better than the 35S-DIP transformed putative tranformants in case of less reduction in photosynthetic rate, gs, cell viability, and chlorophyll stability. These physiological screens performed on the putative DIP transgenics showed that DIP has a role in drought tolerance.

Future perceptive

Characterization of DIP and ELIP gene in terms of expression studies under various stresses need to be studied. Isolation of the protein of DIP and ELIP would give the protein structure and clear function of these genes. The putative transgenics overexpressing DIP gene has to be advanced to T_1 generation and studied for their stress tolerance. Comparison of transgenics expressing the genomic clone of DIP and ELIP in comparison with the cDNA clone has to be done, which helps us to understand the importance of expressing genomic clone. RIKEN has full length genes of most of the *Arabidopis* clones. By procuring the full length homolog of DIP and ELIP from

Arabidopsis and comparative functional validation can be done to assess whether genes from adapted species like groundnut are more efficient.

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VII. REFERENCES

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APPENDICES

Appendix

Appendix 1-Primer details of the genes used for the present study

Appendix 1a- Gene specific primers

Primer Name	Length	Sequence	Product Size (bp)	Tm
GSI4 U	21	5'CATGCATGCTTAGTTTTCTTC 3'	178	56
GSI4(L)	21	5'AGCACCATATTTTACTCCTTA 3'		
GSI39(U)	20	5'CCCTATGACCCGCACTCCTA 3'	526	55
GSI39(L)	19	5'ACCACCCTTAACATACTCG 3'		
GSI46(U)	20	5'ACCACCCCTCAGGATACGAA 3'	210	52
GSI46(L)	17	5'TGACCAGGAACATTTTT 3'		
GSI63(U)	20	5'ACTCGCTGGCCTTCCTACCC 3'	284	67
GSI63(L)	22	5'GAACCCTAAACCCCTAATCTCC 3'		
GSI75(U)	18	5'CCAGAGAACGGGAAGGTG 3'	216	55
GSI75(L)	19	5'CTCAATGATGGATGGAAGG 3'		
GSI83(U)	19	5'TGAGCAGCAGCACAAGGAG 3'	184	50
GSI83(L)	24	5'GCAAGCAGTAATGGCGGAGAAGAT 3'		
Gsi 109(U)	20	5'AATTACGGCAAGGCTTGGTA 3'		55
Gsi109(L)	20	5'CCCTCTTAGCCGCACCTATT 3'		
Gsi110(U)	20	5'CACATCAAAACCCCTCTCCT 3'		58
Gsi110(L)	20	5'CGGTGTTTTCCACCTAGCAT 3'		
Gsi115(U)	20	5'TACCGAAGACTCGGATCCAC 3'		
Gsi115 (L)	20	5'AACCAAAGCCCGGTTCTACT 3'		55
GDI15(U)	19	5'GCTGAGCAGAGAATGAATG 3'	375	57
GDI15(L)	23	5'TGATAATAAACAAAGAACAAACA 3'		
Gn ELIP F [Gateway]	50	5'GGGGACAAGTTTGTACAAAAAAGCAGGC TGGAGTGCAATTTGATACACAC3'	850	55
Gn ELIP R [Gateway]	58	5'GGGGACCACTTTGTACAAGAAAGCTGGGTCT ATACATACACTAAGATAAGATAAAAGG 3'		
Dip-F	27	5'CAGGATCCGAGATCAAAACAGAGTGTG 3'	580	50
Dip-R	25	5'CGGAGCTCACACAAGAATGAAAGTG 3'		
ELIP-F	28	5'GGAGCTCGGAGTGCAATTTGATACACAC 3'	750	55
ELIP-R	36	5'CGGAGCTCTATACATACACTAAGATAAGATAA AAGG 3'		

Appendix Ib ARP primers

Primer name	Length (bp)	Sequence	Standardized annealing temperature(^{0}c)
ARP1	26	ACAATTTCACACAGGACGACTCCAAG	55-63
ARP 3	26	ACAATTTCACACAGGAGACCATTGCA	55-65
ARP 4	26	ACAATTTCACACAGGAGCTAGCAGAC	55-65
ARP 5	26	ACAATTTCACACAGGAATGGTAGTCT	55-65
ARP 6	26	ACAATTTCACACAGGATACAACGAGG	55-65
ARP 7	26	ACAATTTCACACAGGATGGATTGGTC	55-65
ARP 8	26	ACAATTTCACACAGGATGGTAAAGGG	55-65

Appendix Ic- Walker primers

Primer name	Length (bp)	Sequence	Standardized Annealing temperature(^{0}c)
Walker 1	29	CTAATACGACTCACTATAGGG$(AGCT)_4$ATGC	50-55
Walker 2	29	CTAATACGACTCACTATAGGG$(AGTC)_4$GATC	50-55
Walker 3	29	CTAATACGACTCACTATAGGG$(AGTC)_4$TAGC	50-55
Walker 4	29	CTAATACGACTCACTATAGGG$(AGTC)_4$CTAG	50-55

Appendix 1d Gene Racer primers

Primer name	Length (bp)	Sequence	Tm(^{0}c)
GeneRacer™ OligodT	25	5’ GCTGTCAACGATACGCTACGTAACG 3’	76
Nested OligodT	25	5’ CGCTACGTAACGGCATGACAGTG 3’	72
GeneRacer™ RNA Oligo	23	5’CGACTGGAGCACGAGGACACTGA 3’	74
GeneRacer™ RNA Oligo complementary	26	5’ GGACACTGACATGGACTGAAGGAGTA 3’	78

Appendix Ie **SMART Primers**

OligodT	5’attcgagctctagaggccgaggcggccgacatg$(t)_{30}$3’
ssGcap	5’aagcagtggtctagaaccaattggccattacggccggg3’

Appendix II

Protocols used in the present study

IIa. **Preparation of competent E.coli cells**

The E.coli strain DH5α-10C was used to prepare competent cells for routine transformation experiments to multiply different plasmids used in the current study. From a freshly streaked LB plate (Luria Bertani: Bacto Yesat extract (5g/ L): Bacto-tryptone (10g/L); NaCl (10g/L); pH-7.0; Agar-1.5%), a single colony was inoculated into 3ml LB broth and grown overnight at 37C on a rotary shaker at 200rpm. The overnight growth culture (1ml) was inoculated to a sterile 1L flask containing 200ml of 2X-YT medium (Bacto yeast extract (10g/L); Bacto-Tryptone (16g/L) and NaCl (10g/L),pH-7.0) and was grown on a rotary shaker at 200rpm. The groeth of the culture was monitored every 30min by measuring the OD at 600nM (Spectronic Genesys II). When the OD reached at around 0.6, the culture was cooled on a ice bath for 30min. the cells were pelleted by centrifuging at 3000rpm for 10min at 4C. the pellet was resuspended in 10ml of pre-chilled TSB buffer (Transformation and storage Buffer:10%PEG4000, 5% DMSO , 10mM MgCl2, 10mM MgSO4, 10% (v//v) Glycerol, 50%(v/v) LB medium, pH 6.1) and centrifuged at 3000rpm for 10min at 4^{o}C. The pellet was resuspended in fresh 10ml TSB and incubated on ice for 10min. The cells were further aliquot into pre-chilled sterile microfuges and shock frozen in liquid nitrogen and stored at -80^{o}C until further use.

IIb. Transformation of plasmids constructs into E.Coli

One micro liter plasmid DNA(25-50ng/µl) or ligated plasmid DNA mixture and 20µl of filter sterilized 5xKCM (0.5M KCl,0.15M CaCl2 and 0.25M MgCl2) was made upto 100µl using sterilized distilled water and added to one aliquot of competent cells (100µl) and carefully mixed. The mixture was placed on ice for 20min and then at room temperature for 10min. later LB medium (800µl) was added to transformed cells and further incubated at 37 ^{o}C (250rpm) for 1hr. Aliquots (100-200µl) of the diluted cells were spread on appropriated selection plates and incubated at 37^{o}C overnight.

IIc. Screening of recombinant clones

The recombinant clones (in pBI121-35S and pBI121-ABRE) were initially identified based on blue/white selection (since the vector had the lacZ interrupted by the multiple cloning site were the appropriate gene fragments were sub clones) on the selection plate supplemented with Xgal (40ul of X-gal (20mg/ml in dimethyl formamide) to each plate) and IPTG (4ul of IPTG (200mg.ml in water) to each plate). The white colonies were further tested for their recombinant nature by Colony PCR (Instead of DNA as template the entire white colony was used as the template (indirect template) in the PCR mixture) by using appropriate gene specific primers. A typical colony PCR was performed to that of the standard PCR except that the initial denaturization was carried out for 6-7 min. the PCR positive colonies were further confirmed by their recombination by restriction digestion with appropriate restriction endonucleases and observed for the expected fragment by agarose gel electrophoresis.

IId. Plasmid DNA isolation (mini-prep)

The plasmid DNA was isolated by alkaline-lysis method (Sambrook et al., 1989) with some modifications. The bacterial colony (E.Coli) containing the appropriated plasmid was inoculated in 2ml LB medium (Luria Bertani: Bacto Yesat extract (5g/ L): Bacto-tryptone (10g/L); NaCl (10g/L); pH-7.0) with appropriate antibiotics and allowed to grow for 16hrs at 37^{o}C and 200rpm. The bacteria were centrifuged (5000rpm, 5min, 4^{o} C) and the pellet suspended in 200µl of solution I(25mM Tris,pH-8.0;10mM EDTA and 50mM Glucose) and further incubated on ice for 10min without shaking. 200µl of Solution II (0.2M NaOH and 1%(w/v) SDS(always prepared freshly)) was added to the suspension, carefully mixed and further incubated for 10min on ice. In order to obtain high quality plasmid DNA mini-prep, 150µl of solution III (5M Potassium Acetate (60ml), glacial Acetic Acid (11.5ml) and water (28.5ml)) was added to the suspension and carefully mixed to avoid breaking of DNA. The mixture was again incubated on ice for 10min and centrifuged at 12,000rpm for 15min at 4^{o}C. the supernatant, which contains the plasmid DNA was carefully take and mixed with 1µl of RNase A (10mg/ml) and incubated at

37^{o}C for 45min. further, equal volume of Tris saturated Phenol: Chloroform (1:1) was added, vortexed and centrifuged at 12,000rpm for 10min at 4^{o}C. to the supernatant equal volume of chloroform: isoamyl alcohol (24:1) was added and centrifuged at 12,00rpm for 10min at 4^{o}C. the plasmid DNA in the supernatant was then precipitated with 0.1 volume of 3M Sodium Acetate (pH-5.2) and 2V of ethanol and keep at -70 ^{o}C for 1hr.

The plasmid DNA was recovered in the pellet after centrifugation (12,00rpm for 10min at 4^{o} C). The plasmid DNA was washed twice in 70% (v/v) ethanol and air dried at RT. The dry pellet was dissolved in 30µl of sterile distilled water and stored at -20C until further use. A test gel (0.8% agarose gel) of 10 µl of plasmid prep was made to monitor the purity of extraction. The OD was measured at 260nm to determine the DNA concentration.

IIe. Restriction endonucleases treatments

Plasmid DNA digestion was carried out by appropriate restriction endonulceases according to the criteria that the reaction buffer (10X) was 1/10th of the end volume and 5U of restriction enzymes were used per 1µg of DNA to be digested. A double digestion was possible per reaction only when both restrictions enzymes use the same buffer (or any compatible buffer) otherwise the digestion were performed chronologically. The DNA samples that were digested to completion were resolved on 0.8% agarose gel.

IIf. **Preparation of electro competent *Agrobacterium tumifaciens***

Electroporation competent *A.tumifaciens* (GV2260) cells were prepared according to shen and Ford, 1989. a single colony of *A.tumifaciens* (GV2260) was inoculated in 3ml of LB medium, incubated at 28C for 16h and 250rpm and resuspended into fresh LB medium (50ml) and further grown to an OD600 of 0.5. The cell culture was cooled on ice for 30min and centrifuged (5000rpm, 4C) for 5min. the pellete was washed successfully in 1.0, 0.5, 0.2 and 0.1 culture volumes of cold 10% (v/v) glycerol and suspended in

0.01v of 10% (v/v) Glycerol. Aliquots (40ul) of the last suspension were made, shock frozen in liquid nitrogen and stored at -70C as electrocompetent *A.tumifaciens.*

Transformation of plasmids into Agrobacterium via electroporation

The frozen *A.tumifaciens cells* (GV2260) were thawed on ice and 40ul aliquot was transferred to a pre cooled 2mm electroporation cuvette (Bio-Rad Laboratories Ltd). About 1-2 ul (100ng/ml) of specific recombinant plasmid DNA (generally in pBI121 binary vector) to the component cells and carefully mixed in a pre cooled electrocompetent cuvettes. An electric pulse was applied using a Gene Pulser ^{TM} with pulse controller unit. Good transformation efficiencies were obtained at field strength of 12.5 kV/cm, a capacitance of 12mF and resistors of 400 in parallel with the sample. One ml of LB medium was then added to the transformed cells and further incubated in a glass tube and shaken at 250rpm (28C) for 3 hours. Aliquots of 100ul of the cell were finally spread on LB agar medium containing appropriate antibiotics and incubated at 28^0C for 48hours.

IIg. Isolation of total RNA

Total RNA was extracted according to the protocol described by Datta et al., 1989. The RNA was precipitated selectively using Lithium Chloride ($Licl_2$). All care was taken to use RNAse free material for RNA work according to instructions (Sambrook et al., 1989). Solutions were treated with 0.1% DEPC (Diethyl pyrocarbonate) except for Tris solutions. Tris solutions were prepared from DEPC treated Rnase free water and autoclaved before use. All glassware's, that were used for RNA extraction, were baked at 200•c for 4-8 hours.

The detailed protocol followed for RNA extraction is given below,

Leaf material (1g) was taken from the plant, frozen in the liquid nitrogen and either used immediately or stored in –70°c.

↓

Liquid nitrogen freeze material was taken into pestle and ground to fine powder in liquid nitrogen.

↓

10ml of extraction buffer was added and homogenized properly (0.1M Tris Hcl pH 9.0,0.25M Sucrose, 0.2M Nacl and 10mM Mgcl2)

↓

10ml of phenol (waters saturated): chloroform mixture (1:1) v/v was added and ground.

↓

1ml of potassium EDTA and 1ml of 10% SDS were added sequentially. The homogenized solution was transferred to centrifuge tubes.

↓

144µl of mercaptaethanol were added and shook at 4^{o}c for 20 min.

↓

Centrifuge at 10,000 rpm for 30 min and supernatant was separated into fresh centrifuge tube. Equal volume of phenol (water saturated): chloroform mixture (1:1) v/v was added to aqueous phase, vortexed and spun at 15,000 rpm for 15min at 4^{o}c.

↓

Aqueous phase was transferred to fresh centrifuge tube and equal volume of chloroform: isoamyalcohol (49:1) was added and centrifuge at 15,000 rpm for 15 min at 4^{o}c.

↓

Aqueous phase was transferred to fresh centrifuge tube and 8M $LiCl_2$ was added to a final concentration of 3M and kept in 4^{o}c for 20 hours.

↓

Centrifuge at 15,000 rpm for 30 min at 4^{o}c

↓

Supernatant was discarded and precipitate was washed with 5ml 2M $LiCl_2$ and with 5ml of 75% ethanol by centrifuging at 15,000rpm for 20min at 4.c

↓

The pellet was air dried and dissolved in 100µl of DEPC water.

Estimation of RNA was done spectrophotometrically by diluting 1µl of RNA in 1ml of DEPC water at 260 nm. The purity was analyzed 260/280 ratio as well as visualizing on a formaldehyde denaturing gel.

IIh. Northern analysis

Separation of RNA and transfer to membrane

Total RNA (20 µg) was extracted following formaldehyde denaturing gel electrophoresis (Sambrook et al., 1989). Gel was washed with DEPC treated water for three times, 20 minutes each time. RNA was blotted to hybond nitrocellulose membrane (Amersham) through capillary transfer using 20X SSC (Sambrook et al., 1989) and cross-linked to membrane with UV cross- linker (Amersham) at 0.4 joules for 10 minutes.

Probe preparation

The plasmid DNA was amplified using gene specific primers of the six selected clones (clone no.90, 106,109,110,115,126). This PCR amplified product purified using the High Pure PCR Purification Kit (ClONTECH) and was used as probes.

Fifty nanograms of each PCR amplified plasmid was radiolabelled with α^{32}PdCTP following the instruction provided in the random labelling Kit (GIBCO-BRL). Radiolabelled probe was purified by passing through G-50 sephadex column and unincorporated nucleotides were removed. The counts were taken in the Liquid scintillating counter (WALLAC).

Prehybridization, hybridization and washings

Membrane containing the cross-linked RNA was transferred to the hybridization bottle with 15ml of prehybridization buffer (0.5M Sodium Phosphate pH 7.2,7% SDS and 1mM EDTA, Church and Gilbert, 1984). Prehybridization was done for 6-8 hours at 60^0C.

Individual probes were boiled, immediately frozen on ice and added to the prehybridization buffer in such a way that the count in the solution was 0.5 to 1.0 million counts per ml. Hybridization was done at 60^0C for 16-20 hours.

Blots were washed with 2X SSC and 0.1%SDS at 37^0C for 10 minutes, 2X SSC and 0.1%SDS at 65^0C for 10 minutes, 1X SSC and 0.1%SDS at 65^0C for 10 minutes and a high stringency wash with 0.2X SSC and 0.1%SDS at 65^0C for 10 minutes. The blot was exposed on a Kodak X-ray film for 10 days and developed.

II i. DNA extraction

Genomic DNA was extracted from the leaf tissue harvested from primary transforamants as described by Doyle and Doyle (1989), with some modifications. The plant material (500mg) was ground in liquid nitrogen and dissolved in 7.5ml of pre-warmed (65^oC). DNA extraction buffer (3.5%cTAB; 100mM Tris-Hcl,pH-8.0; 20mM EDTA,pH-8.0;1.4M Nacl; 0.2% mercaptoethanol (should be added in situ before DNA extraction)) and further incubated at 65 in water bath for 1hour. The mixture was spinned at 5000rpm for 20minutes. RNase treatment was given to the supernatant at 37C for 45minutes.To the RNase treated mixture equal volume of chloroform-isoamyl alcohol (24:1) was added and centrifuged at 5000rpm for 15minutes. To The supernatant $1/7^{th}$ volume of chilled isopropyl alcohol was added and incubated at 4C for 1-2hours. The DNA was pelleted by centrifuging at 8000rpm for 20minutes. The supernatant was discarded and the pellet was washed with 75% alcohol at 8000rpm for 15minutes. The washed pellet was air dried and dissolved in sterile distilled water. The quality and quantity of the DNA was quantified in nanodrop.

Appendix III

Appendix IIIa

Alignments of ELIP and Gsi39

```
ELIP            GGAGTGCAATTTGATACACACTAAGAACCTTTCTGCGGTTTCACTCACAAGGTTATTAGT 60
Gsi39           --------ATTTGATACACACTAAGAACCTTTCTGCGGTTTCACTCACAAGGTTATTAGT 52
                        **************************************************

ELIP            AACATACCAATCTAGCAATGGCTGTTTCATCTTATGCTATGCAATCTATCCTTGCAAGCC 120
Gsi39           AACATACCAATCTAGCAATGGCTGTTTCATCTTATGCTATGCAATCTATCCTTGCAAGCC 112
                ************************************************************

ELIP            CTATGACCCGCACTCCTAGCAGGTCAAGGGTGAACCAATTTGGTGTTCCTGCTCTTTACA 180
Gsi39           CTATGACCCGCACTCCTAGCAGGTCAAGGGTGAACCAATTTGGTGTTCCTGCTCTTTACA 172
                ************************************************************

ELIP           TGCCTAACATGAGAAGGAATGCTAGCCTTAGTGTTCGATCCATGGCTGAGGAAGAGCAGA 240
Gsi39           TGCCTAACATGAGAAGGAATGCTAGCCTTAGTGTTCGATCCATGGCTGAGGAAGAGCAGA 232
                ************************************************************

ELIP            AAGAACAATCAACTGAACCTACAAGCCCAGTTACACCACCACCACCAAAGCCTCAGCCTC 300
Gsi39           AAGAACAATCAACTGAACCTACAAGCCCAGTTACACCACCACCACCAAAGCCTCAGCCTC 292
                ************************************************************

ELIP            GTTCACCAAAGATGAGCACAAAGTTCAGTGATGTGTTGGCTTTCAGTGGGCCAGCACCTG 360
Gsi39           GTTCACCAAAGATGAGCACAAAGTTCAGTGATGTGTTGGCATTCAGTGGGCCAGCACCTG 352
                ************************************ ******************** 

ELIP            AGAGGATCAATGGAAGGCTTGCAATGATTGGATTTGTAGCAGCATTAGCAGTGGAACTAT 420
Gsi39           AGAGGATCAATGGAAGGCTTGCAATGATTGGATTTGTAGCAGCATTAGCAGTGGAACTAT 412
                ************************************************************

ELIP            CCAACGGTCAAGATTTGTTGTCACAGATATCCAATGGTGGGATCCCATGGTTCTTGGGGA 480
Gsi39           CCAACGGTCAAGATTTGTTGTCACAGATATCCAATGGTGGGATCCCATGGTTCTTGGGGA 472
                ************************************************************

ELIP            CTAGTGTGGTGCTGTCCCTTGCTTCATTGATCCCACTGTTTCAAGGTGTTAGTGTTGAGT 540
Gsi39           CTAGTGTGGTGCTGTCCCTTGCTTCATTGATCCCACTGTTTCAAGGTGTTAGTGTTGAGT 532
                ************************************************************

ELIP            CAAAATCTGGTGGATTCATGTCCTCAGATGCTGAGCTTTGGAATGGTAGATTTGCCATGT 600
Gsi39           CAAAATCTGGTGGATTCATGTCCTCAGATGCTGAGCTTTGGAATGGTAGATTTGCCATGT 592
                ************************************************************

ELIP            TGGGCTTGATTGCTCTTGCTTTCACCGAGTATGTTAAGGGTGGTACCCTTGTGTAATCAT 660
Gsi39           TGGGCTTGATTGCTCTTGCTTTCACCGAGTATGTTAAGGGTGGT---------------- 636
                ******************************************

ELIP            TTTTAAGCCTCCAGTATTCTTATATTACCTTTTATCTTATCTTAGTGTATGTATAGAATT 720
Gsi39           ------------------------------------------------------------


ELIP            TTATAAAGGGATTTTTCTGAGATAAATTCATGATCATAATATCATA 766
Gsi39           ---------------------------------------------
```

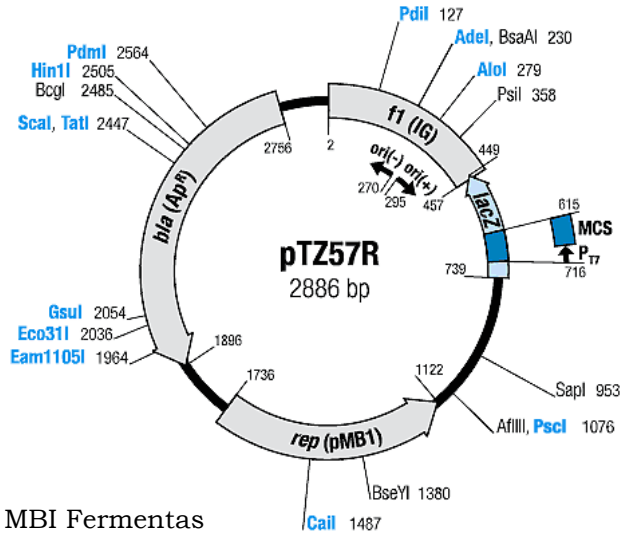
Appendix IIIb

Alignment of DIP and Gsi39

```
DIP             AAGCAGTGGTCTAGAACCAGAGTGGCCATTACGGCCGGGGAGATCAAAACAGAGTGTGTG
Gsi_83          ------------------------------------------------------------

DIP             TCACAAAGAAACAACCAAAAACTCGATCAGAGAAGAGAAAAGAGAAAAACAAACAAGATG
Gsi_83          ------------------------------------------------------------

DIP             TCAGGAATCATGCACAAGATCGAGGAGACCCTCCACATGGGAGGGAACAAGAAAGATCAG
Gsi_83          ------------------------------------------------------------

DIP             GAGCACAAGGGAGAGAGCCATGGCAGCGATCAGTACAAGCAGGGAGAGCACCACCAACAG
Gsi_83          ------------------------------------------------------------

DIP             CAGCAGCACTACGGTGGTGGTGGTGTTGGTGTTGGTGGAGAGCACCATGGTAGTGGAGGA
Gsi_83          ------------------------------------------------------------

DIP             GTGTACGGAGGAGAACATGGCCACGTGCAAGAGCACCACCCAGGTGAGCAGCAGCACAAG
Gsi_83          -------AGAGAGAACATGGCCACGTGCAAGAGCACCACCCAGGTGAGCAGCAGCACAAG
                       ************************************************
DIP             GAGGGTCTTGTAGACAAGATCAAGGATAAGATCCACGGCGGCGACGGTGCCGAGGGTGAG
Gsi_83          GAGGGTCTTGTAGACAAGATCAAGGATAAGATCCACGGCGGCGACGGTGCCGAGGGTGAG
                ************************************************************
DIP             AAGAAGAAGGAGAAGGATAAGAAGAAGGGCGAACATGGCCACGACCATGGCCATGACAGC
Gsi_83          AAGAAGAAGAAGAAGGATAAGAAGAAGGGCGAACATGGCCACGACCATGGCCATGACAGC
                ********* ************************************************
DIP             AGCAGCAGCAGCGACAGTGATTAAATCTTCTCCGCCATTACTGCTTGCTGCACCTTTCTT
Gsi_83          AGCAGCAGCAGCGACAGTGATTAGATCTTCTCCGCCATTACTGCTTGCTGCACCTTTCTT
                ********************** ***********************************
DIP             CTATGTCAAGGTGCATGGGGTCACAGTTGTGAGGGGCTTGGTTTTACACTTTCATTCTTG
Gsi_83          CTATGTCAAGGTGCATGGGGTCACAGTTGTGAGGGGC-----------------------
                ***********************************
DIP             TGTGAACCAAAAAAAAAAAAAAAAAGATC
Gsi_83          -----------------------------
```

APPENDIX IV: Vectors

A) T/A Cloning Vector

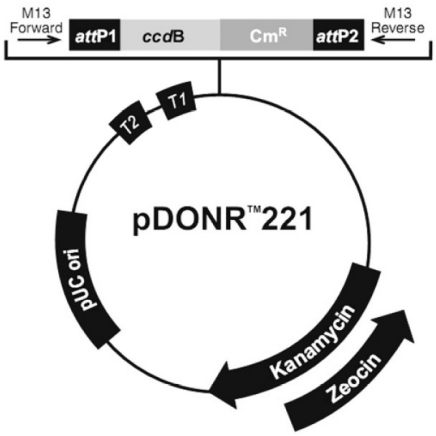


MBI Fermentas

Map of T/A cloning vector pTZ 57R/T

T/A cloning vectors are convenient systems for the cloning of PCR products. The single 3´-T overhangs at the insertion site greatly improve the efficiency of ligation of a PCR product into the plasmids by preventing recircularization of the vector. The pTZ57R/T was obtained from MBI Fermentas. Initially the PCR products were prepared with A overhang and cloned into the T/A vectors adopting manufacturers protocols.

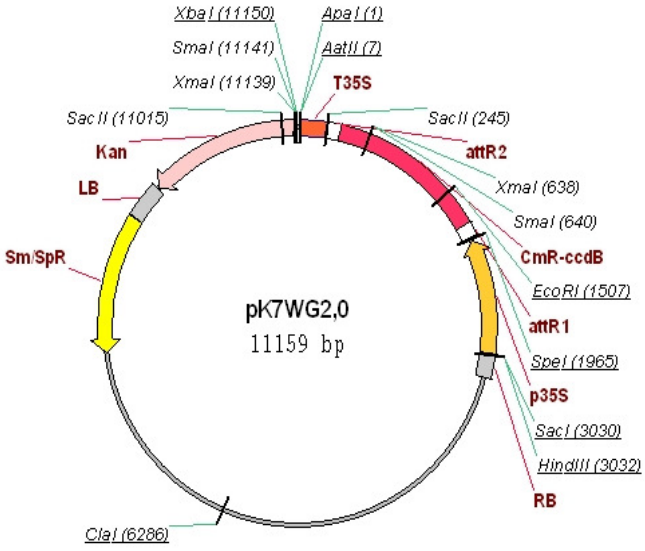
B) Gateway Donor Vector



Map of Gateway Donor vector pDONR^{TM}221

The attP sites of the Donor vector can recombine with the attB sites of the insert of choice and this recombination reaction mediated by the BP clonase enzyme create an entry vector with the insert of choice. This is the primary step of gateway cloning technique.

C) Gateway Destination Vector



Map of Gateway Destination vector, pK7WG2.0

Gateway compatible destination vector, pK7WG2.0, having plant selection marker genes *npt* (kanamycin). The attL sites of the entry clone can recombine with the attR sites of the destination vector, to generate an expression clone with the insert of choice. Acknowledgement-http://www.psb.ugent.be/gateway/ Karimi *et al*., (2002).

Appendix V

Genes for stress resistance in transgenic/mutant plants

Genes encoding enzymes that synthesize osmotic and other protectants

Gene	***Gene Action***	***Species***	***Phenotype***	***Reference***	***ID****
	Dehydrin protein accumulation	Tobacco	Cold tolerance and possible antioxidative effect	Masakazu et al. 2003	6602
	Accumulation of different compatible solutes	Tobacco	Photosystem cold tolerance	Parvanova et al., 2004	7334
adc	Arginine decarboxylase	Rice	Reduced chlorophyll loss under drought stress	Capell et al. 1998	5607
adc	Polyamine synthesis	Rice	Drought resistance	Capell et al., 2004	7290
Apo-Inv	Apoplastic invertase	Tobacco	Salt tolerance, high "osmotic pressure"	Fukushima et al., 2001	5202
AtGolS2	Galactinol and raffinose accumulation	Arabidopsis	Reduced transpiration	Taji et al. 2002	5884
AtHAL3	Phosphoprotein phosphatase	Tobacco	Improved salt, osmotic and Lithium tolerance of cell cultures	Yonamine et al., 2004	6947
AtHAL3a	Phosphoprotein phosphatase	Arabidopsis	Regulate salinity and osmotic tolerance and plant growth	Espinosa-Ruiz et al. 1999	4601
ATP-PRT	ATP-phosphoribosyltransferase	Alyssum	His accumulation and Nickel tolerance	Ingle et al., 2005	7942
AtTPS1	trehalose-6-phosphate synthase	Tobacco	Drought resistance; sustained photosyntehsis	Almeida et al., 2007	8668
BADH-1	Betaine aldehyde dehydrogenase	Carrot	Salinity tolerance	Kumar et al., 2004	7353
BADH-1	Betaine aldehyde dehydrogenase	Maize	Salinity tolerance	Wu et al., 2007	9117
BADH-1	Betaine aldehyde dehydrogenase	Tobacco	Heat tolerance in photosynthesis	Xinghong Yang, et al., 2005	7858
BADH-1	Betaine aldehyde dehydrogenase	Tobacco	Salinity tolerance	Yang et al., 2008	9160
BADH-1	Betaine aldehyde dehydrogenase	Tomato	Maintenance of osmotic potential	Moghaieb et al. 2000	5094
betA	Choline dehydrogenase (glycinebetaine synthesis)	Maize	Drought resistance at seedling stage and high yield after drought	Ruidang et al., 2004	7409
betA	Choline dehydrogenase (glycinebetaine synthesis)	Tobacco	Increased tolerance to salinity stress	Lilius et al. 1996	3287
CHIT33, CHIT42	Endochitinase synthesis	Tobacco	Salt and metal toxicity resistance (& disease)	Dana et al., 2006	8504
CMO	Choline monooxygenase (glycine betaine synthesis)	Tobacco	Better in vito growth under salinity and osmotic (PEG6000) stress	Yi-Guo Shen et al. 2002	6285
codA	Choline oxidase (glycine betaine synthesis)	Arabidopsis	Increased stress tolerance	Huang et al. 2000	4731
codA	Choline oxidase (glycine betaine synthesis)	Arabidopsis	Salt tolerance in terms of reproduction	Ronan et al., 2003	6822
codA	Choline oxidase (glycine betaine synthesis)	Arabidopsis	Seedlings tolerant to salinity stress and increased germination under cold	Hayashi et al. 1997, Alia et al. 1998	4571
codA	Choline oxidase (glycine betaine synthesis)	Brassica juncea	Tolerance to stress induced photoinhibition	Prasad and Saradhi 2004	7094
codA	Choline oxidase (glycine betaine synthesis)	Rice	Increased tolerance to salinity and cold	Sakamoto et al. 1998	3859

codA	Choline oxidase (glycine betaine synthesis)	Rice	Recovery from a week long salt stress	Mohanty et al., 2003	6347
codA	Choline oxidase (glycine betaine synthesis)	Tobacco	Freezing toleance	Parvanova et al., 2004	7334
codA	Choline oxidase (glycine betaine synthesis)	Tomato	Chilling tolerance in yield and oxidative stress tolerance	Eung-Jun et al., 2004	7416
codA	Choline oxidase (glycine betaine synthesis)	Tomato	Chilling tolerance	PARK et al., 2007	8969
COR15a	Cold induced gene	*Arabidopsis*	Increased freezing tolerance	Steponkus et al. 1998	5618
COX	Choline oxidase (glycine betaine synthesis)	Rice	Salt and 'stress' tolerance	Su et al. 2006	8227
Ect A…ect C	Edtoin accumulation in chloroplasts	Tobacco	Salt and cold tolerance	Rai et al. 2006	8090
GS2	Chloroplastic glutamine synthetase	Rice	Increased salinity resistance and chilling tolerance	Hoshida et al. 2000	4792
IMT1	Myo-inositol o-methyltransferase (D-ononitol synthesis)	Tobacco	Better CO2 fixation under salinity stress. Better recovery after drought stress.	Sheveleva et al. 1997	3660
LWR1, LWR2	Solute accumulation (proline)	Arabidopsis	Growth, osmotic adjustment, water status	Verslues and Bray, 2004	7352
M6PR	Mannose-6-phosphate reductase	Arabidopsis	Mannitol accumulation under salt stress leading to salt tolerance	Zhifang and Loescher, 2003	6343
M6PR	Mannose-6-phosphate reductase	Arabidopsis	Mannitol accumulation and salt tolerance due to chloroplast protection	Sickler et al., 2007	8850
mt1D	Mannitol-1-phosphate dehydrogenase (mannitol synthesis)	Arabidopsis	Increased germination under salinity stress	Thomas et al. 1995	5620
mt1D	Mannitol-1-phosphate dehydrogenase (mannitol synthesis)	Petunia	Chilling tolerance	Chiang et al., 2005	7715
mt1D	Mannitol-1-phosphate dehydrogenase (mannitol synthesis)	Tobacco	Increased plant height and fresh weight under salinity stress	Tarczynski et al. 1993	2383
mt1D	Mannitol-1-phosphate dehydrogenase (mannitol synthesis)	Tobacco	No contribution to sustained growth under salinity and drought stress.	Karakas et al., 1997	3492
mt1D	Mannitol-1-phosphate dehydrogenase (mannitol synthesis)	Wheat	Drought and salinity tolerance of calli and plants	Abebe et al., 2003	6533
mt1D & GutD	Mannitol-1-phosphate dehydrogenase & glucitol-6-phosphate dehydrogenase	loblolly pine	High salt tolerance due to mannitol and glucitol accumulation	Tang et al., 2005	7614
mtlD	Mannitol-1-phosphate dehydrogenase (mannitol synthesis)	Populus tomentosa	Salinity tolerance	Hu et al., 2005	7946
Osm1 …Osm4	Osmotin protein accumulation	Tobacco	Drought and salt tolerance in plant water status and proline accumulation	Barthakur et al., 2001	5560
Osmyb4	Cold induced transcription factor	Arabidopsis	Accumulation of compatible solutes	Mattana et al., 2005	7915
Osmyb4	Specifically cold inducible	Tobacco	Freezing and Chilling tolerance	Candida et al., 2004	6922
Osmyb4	Cold induced transcription factor	Tomato	Drought but not cold resistance	Vannini et al., 2007	8978
OsP5CS2	Highly homologous to P5CS	Rice	Cold and salinity tolerance	Hur et al., 2004	7264
otsA	Trehalose-6-phosphate synthase (trehalose synthesis)	Tobacco	Increased leaf dry weight and photosynthetic activity under drought. Increased carbohydrate accumulation.	Pilon-smits et al. 1998	3101

otsB	Trehalose-6-phosphate synthase (trehalose synthesis)	Tobacco	Increased leaf dry weight and photosynthetic activity under drought. Increased carbohydrate accumulation.	Pilon-smits et al. (1998)	3101
P5CR	Pyrroline carboxylate reductase (proline accumulation)	Soybean	Antioxidants activity under stress	Kocsy et al., 2005	7797
P5CR	Pyrroline carboxylate reductase (proline accumulation)	Soybean	Amino acid accumulation	Simon-Sarkadi et al., 2006	8612
P5CS	Pyrroline carboxylate synthase(proline synthesis) (tomato)	Citrus	Osmotic adjustment and drought resistance	Molinari et al., 2004	7361
P5CS	Pyrroline carboxylate synthase(proline synthesis)	Petunia	Drought resistance and high proline	Yamada et al., 2005	7750
P5CS	Pyrroline carboxylate synthase(proline synthesis)	Potato	Salinity tolerance	Hmida-Sayari et al., 2005	7864
P5CS	Pyrroline carboxylate synthase(proline synthesis)	Rice	Increased biomass production under drought and salinity stress	Zhu et al. 1998	3871
P5CS	Pyrroline carboxylate synthase(proline synthesis)	Rice	Reduced oxidative stress under osmotic stress	Hong Zong Lie et al., 2000	5562
P5CS	Pyrroline carboxylate synthase(proline synthesis)	Rice	Resistance to water and sainity stress	Su and Wu, 2004	7034
P5CS	Pyrroline carboxylate synthase(proline synthesis)	Soybean	Resistance to osmotic stress and heat	De Ronde et al. 2001	5767
P5CS	Pyrroline carboxylate synthase(proline synthesis) (tomato)	Soybean	Drought resistance, high RWC, high proline	De Ronde et al., 2004	7383
P5CS	Pyrroline carboxylate synthase(proline synthesis) (tomato)	Sugarcane	Drought resistance via antioxidant role of proline	Molinari et al., 2007	8859
P5CS	Pyrroline carboxylate synthase(proline synthesis)	Tobacco	Increased biomass production and enhance flower development under salinity stress	Kishor et al. 1995	3078
P5CS	Pyrroline carboxylate synthase(proline synthesis)	Tobacco	Freezing tolerance	Parvanova et al., 2004	7334
P5CS	Pyrroline carboxylate synthase(proline synthesis)	Wheat	Drought resistance due to antioxidative action	Vendruscolo et al., 2007	9069
P5CS	Pyrroline carboxylate synthase(proline synthesis) (tomato)	Yeast	Reduced growth under none-stress and some promoted growth under mild stress	Maggio et al., 2002	6181
pdc1	Pyruvate decarboxylase overexpression	Rice	Increased submergence tolerance	Quimio et al. 2000	4747
pdc1; pdc2	Pyruvate decarboxylase overexpression	Arabidopsis	Hypoxic stress survival	Ismond et al. 2003	6669
PpDHNA	Dehydrin protein accumulation	Moss	Salt and osmotic stress tolerance	Saavedra et al. 2006	8082
PPO	Polyphenol oxidases suppression	Tomato	Drought resistance	Thipyapong et al., 2004	7267
SAMDC	S-adenosylmethioninedecarboxylase (polyamine synthesis)	Rice	Better seedling growth under a 2 day NaCl stress	Malabika and Wu, 2002	6252
SAMDC	S-adenosylmethioninedecarboxylase (polyamine synthesis)	Tobacco	drought, salinity, Verticillium and Fusarium wilts resistance	Waie and Rajam, 2003	6538

SMT	selenocysteine methyltransferase	Arabidopsis, Indian Mustard	Selenium hyperaccumulation tolerance	Danika et al., 2004	7175
SPE	Spermidine synthase	Arabidopsis	Chilling, freezing, salinity, drought hyperosmosis	Kasukabe et al., 2004	7277
spe1-1; spe2-1	Spermidine non-accumulating	Arabidopsis	Decreased salt tolerance	Vasuki and Astrid, 2004	7089
SST/FFT	Fructan accumulation	Potato	Reduced proline accumulation at low water status	Knipp and Honermeier, 2006	8144
TPS; TPP	Trehalose synthesis	Arabidopsis	Drought, freezing, salt and heat tolerance	Miranda et al., 2007	9094
TPS1	Trehalose synthesis	Tomato	Drought, salt and oxidative stress tolerance	Cortina and Culiáñez-Macià, 2005	7788
TPS1	Trehalose synthesis	Potato	Delayed wilting under drought	Stiller et al., 2008	9146
TPS1 & TPS2	Trehalose synthesis	Tobacco	Maintenance of water status under drought stress	Karim et al., 2007	8913
TPSP	Trehalose synthesis	Rice	Drought, salt and cold tolerance expressed by chlorophyll fluorescence	In-Cheol Jang et al., 2003	6389
WCOR15	Cold induced gene	Tobacco	Increased freezing tolerance	Shimamura et al. 2006	8067
WCOR410	Dehydrin	strawberry	Freezing tolerance	Houde et al., 2004	7362

Late embryogenesis abundant (LEA) related genes

Gene	***Gene Action***	***Species***	***Phenotype***	***Reference***	***ID****
DQ663481	Lea gene	Tobacco	Drought resistance via cell membrane stability	Wang et al., 21006	8510
HVA1	Group 3 LEA protein gene	Oat	Delayed wilting under drought stress	Maqbool et al., 2002	6146
HVA1	Group 3 LEA protein gene	Oat	Salinity tolerance in yield/plant	Oraby et al., 2005	7971
HVA1	Group 3 LEA protein gene	Rice	Dehydration avoidance and cell membrane stability	Babu et al., 2004	7030
HVA1	Group 3 LEA protein gene	Rice	Drought and salinity tolerance	Rohila et al. 2002	6185
HVA1	Group 3 LEA protein gene	Wheat	Increased biomass and WUE under stress	Sivamani et al. 2000	4781
HVA1	Group 3 LEA protein gene	Wheat	Improved plant water status and yield under field drought conditions	Bahieldin et al., 2005	7618
OsLEA3-1	Lea protein	Rice	Drought resistance for yield in the field	Xiao et al., 2007	8926
Rab17	LEA protein	Arabidopsis	Resistance to osmotic and salinity stress	Figueras et al., 2004	7204
ME-leaN4	LEA protein	Lettuce	Enhanced growth and delayed wilting under drought. Salt resistance	Park et al., 2005	7671
ME-leaN4	LEA protein	Chinese cabbage	Drought and salt resistance	Park et al., 2005	7794

Various regulatory genes

Gene	***Gene Action***	***Species***	***Phenotype***	***Reference***	***ID****
--	Overexpression of glycerol-3-phosphate acyltransferase - inducing cis-unsaturation of chloroplast phosphatidylglycerol	Tobacco	Chilling sensitivity	Sakamoto et al., 2004	6816
--	Overexpression of nicotianamine synthase	Tobacco & Arabodopsis	Heavy metal tolerance by chelation	Kim et al., 2005	8006
AB13	Transcription factor	Arabidopsis	Enhanced freezing tolerance	Tamminen et al. 2001	5217
ABF3	Transcription factor	Rice	Drought resistance	Oh et al., 2005	7780
ADC	Arginine decarboxylase overexpression	rice	Polyamine accumulation and salt resistance in biomass accumulation	Roy and Wu, 2001	5561
ADH1	alcohol dehydrogenase	Rice	Submergence tolerance	Rahman et al. (001	5688
ADH1	alcohol dehydrogenase	Arabidopsis	No effect on hypoxic stress survival	Ismond et al. 2003	6669
ADH1; ADH2	alcohol dehydrogenase	Maize	Chilling tolerance	Peters and Frenkel, 2004	7468
AhPDF	Defensin	Arabidopsis	Zinc toxicity tolerance	Mirouze et al., 2006	8404
AHK1	Regulatory gene	Arabidopsis	Down-regulation of stress responsive genes	Phan Tran et al., 2007	9164
ALDH3	aldehyde dehydrogenase	Arabidopsis	Tolerance to dehydration, NaCl, heavy metals (Cu2+ and Cd2+), MV, and H2O2	Sunkar et al. 2003	6715
ALDH3I1 & ALDH7B4	aldehyde dehydrogenase	Arabidopsis	Salt, dehydration and oxidative stress tolerance	Kotchoni et al. 2006	8303
Alx8	High APX2 and ABA	Arabidopsis	Drought resistance	Rossel et al, 2006	8164
AREB1	ABA hypersensitivity	Arabidopsis	Dehydration survival	Fujita et al. 2005	8099
AREB1	ABRE-dependent ABA signaling	Arabidopsis	Drought resistance	Fujita et al. 2006	8099
ArsC	Arsenate reductase	Tobacco	Cadmium tolerance	Dhankher et al. 2003	6638
ars4;ars5	Thiol synthesis	Arabidopsis	Arsenic tolerance	Sung et al., 2007	8729
ASR1	Undetermined	Tobacco	Decreased water loss; salt tolerance	Perlson et al., 2004	7462
AtAOX1	alternative oxidase (AOX) pathway of plant mitochondria	Arabidopsis	Cold acclimation	Fiorani et al. 2005	8085
AtATM3	Regulating nonprotein thiols (NPSH) levels	Arabidopsis	Cadmium tolerance	Kim et al. 2006	8253
AtBCB	Blue copper-binding protein	Arabidopsis	Suppress aluminum root absorption	Ezaki et al. 2001	5664
AtCBF1-3	Transcription factor	potato	Promoter driven freezing tolerance in yield	Pino et al., 2007	9022
AtCPK23	serine/threonine kinase	Arabidopsis	Stomatal regulation	Ma and Wu, 2007	9082
AtGluR2	Transcription factor	Arabidopsis	Calcium utilization under ionic stress	Kim et al. 2001	5172
AtGSK1	Homologue of GSK3/shaggy-like protein kinase	Arabidopsis	Salt tolerance in whole plant and root growth	Piao et al., 2001	5526
ATHB6	Transcription factor	Arabidopsis	Cell division and /or differentiation in developing organs	Soderman et al. 1999	5617
AtMT2a AtMT3	Metallothioneins synthesis in guard cells	Faba bean	Cadmium tolerance through antioxidative action	Lee et al., 2004	7417
Atnoa1	Impaired Nitric Oxide synthesis	Arabidopsis	Salt tolerance	Zhao et al., 2007	8866
AtPCS1	Phytochelatin synthesis	Arabidopsis	Paradoxically showed hypersensitivity to Cd stress	Lee et al., 2003	6387

AtPCS1	Phytochelatin synthesis	Arabidopsis	Arsenic tolerance and cadmium hypersensitivity	Li et al., 2004	7513
AtPCS1	Phytochelatin synthesis	Tobacco	Cadmium tolerance	Pomponi et al. 2006	8092
AtPCS1	Phytochelatin synthesis	Indian mustard	As and Cd tolerance	Gasic et al., 2007	8912
---	Phytochelatin synthesis (3 genes)	Tobacco	Cadmium tolerance	Wawrzyski et al., 2006	8372
ATP-PRT	Free His accumulation	Alyssum	Nickel tolerance	Ingle et al., 2005	7812
AtPCS1	Phytochelatin synthesis	Indian Mustard	Cadmium and zinc tolerance	Gasic and Korban, 2007	8815
AtRabG3e	Intracellular vesicle trafficking	Arabidopsis	Salt and osmotic stress tolerance	Mazel et al., 2004	6975
atRZ-1a	Zinc finger glycine-rich RNA-binding proteins	Arabidopsis	Negative effect on germination and seedling growth under salt stress	Kim et al., 2007	9026
ATTS244 and ATTS405	FtsH protease protecting photosystem	Arabidopsis	Heat tolerance	Chen et al. 2006	8472
AZF1, AZF2, AZF3, and STZ	Cys2/His2-Type Zinc-Finger Proteins	Arabidopsis	Reduced growth and drought resistance	Sakamoto et al., 2004	7351
atRZ-1a	zinc finger-containing glycine-rich RNA-binding proteins (GR-RBPs)	Arabidopsis	Freezing tolerance	Yeon-Ok et al., 2005	7773
AtSZF1 & AtSZ	CCCH-type zinc finger proteins, involved in salt stress responses	Arabidopsis	Salt tolerance	Sun et al., 2007	9025
BNCBF5- and 17 17	CBF/DREB1-like transcription factors	Brassica napus	Freezing tolerance and photosynthetic capacity	Savitch et al., 2005	7926
CAbZIP1	Plant development (dwarf phenotype)	Arabidopsis	Disease, drought and salt tolerance	Lee et al. 2006	8477
cad 1-3	Phytochelatin deficiency	Arabidopsis	Cadmium sensitivity, low photosynthesis, low nutrient status	Larsson et al., 2001	5382
cad 1-3	Phytochelatin deficiency	Arabidopsis	Cadmium sensitivity	Larsson et al. 2002	5873
CAP2	Transcription factor	Tobacco	Drought and salt tolerance	Shukla et al. 2006	8470
CaPF1	Transcription factor	Virginia pine	Antioxidant activity and metal tolerance	Tang et al., 2005	8022
CaPIF1	Cys-2/His-2 zinc finger protein	Tomato	Chilling and disease resistance	Seong et al., 2007	8716
CBF1	Transcription factor	Arabidopsis	Cold tolerance	Jaglo-Ottosen et al. 1998	5611
CBF1	Transcription factor	Tomato	Chilling tolerance, antioxidative action and activation of catalase (CAT1 gene)	Tsai-Hung Hsieh et al. 2002	6124
CBF1	Transcription factor	Populus spp.	Cold tolerance	Benedict et al. 2006	8344
CBF1; CBF3	Transcription factor	Arabidopsis	Cold tolerance	Novillo et al., 2004	7114
CBF3	Transcription factor	Rice	Drought and salt resistance	Oh et al., 2005	7780
CBF4	Transcription factor	Arabidopsis	Drought and freezing tolerance (via activation of C-repeat/dehydration responsive element)	Haake et al., 2002	6232
Wcbf2	Transcription factor	Tobacco	Freezing tolerance	Takumi et al., 2008	9208
CBL1	Ca sensing protein	Arabidopsis	Salt and drought tolerance & cold sensitivity	Cheong et al. 2003	6707

CBL1	Ca sensing protein	Arabidopsis	Salt and drought resistance – reduced transpiration	Albrecht et al., 2003	6823
CBL1	Ca sensing protein	Arabidopsis	Salt tolerance	Wang et al., 2007	9136
CBP20	cap binding complex	Arabidopsis	Loss of function (recessive) induces drought resistance	Papp et al., 2004	7420
CGS	Cystathionine--synthase	Indian Musta	Selenium tolerance due to enhanced Se Volatilization	Van Huysen et al., 2003	6888
CIT1	Mitochondrial citrate synthase	Canola	Increased root citrate exudation and Al tolerance	Anoop et al. 2003	6720
CpMYB10	Glucose sensitive and ABA hypersensitive	Arabidopsis	Desiccation and salinity tolerance	Villalobos et al. 2004	7173
cpSL	Selenocysteine lyase (mouse)	Brassica juncea	Selenium tolerance	Garifullina et al. 2003	6702
CRYOPHYTE/ LOS4	RNA helicase	Arabidopsis	Cold tolerance and heat sensitivity	Gong et al., 2004	7509
CUP1	metallothionein accumulation	Tobacco	Arbuscular mycorrhiza decreases cadmium phytoextraction in transgenic	Janoukova et al., 2005	7764
Cys	Enhanced cysteine synthase activity	Tobacco	Tolerance to sulfur containing pollutants	Masaki Noji et al., 2001	5429
desC	Acyl-lipid 9-desaturase	Tobacco	Increased fatty acid unsaturation and chilling tolerance	Orlova et al., 2003	6519
DREB	Transcription factor	Arabidopsis	Increased tolerance to cold, drought and salinity	Kasuga et al. 1999	4534
DREB1A	Transcription factor	Paspalum grass	Salinity and dehydration tolerance	Phan Tran et al., 2008	9167
DREB1 or OsDREB1	Transcription factor	Rice	Drought, salt and cold tolerance with reduced growth under non-stress	Ito et al. 2006	8176
DREB1A	Transcription factor	Tobacco	Drought and cold tolerance	Kasuga et al., 2004	7091
DREB1A	Transcription factor	Tobacco	Salinity tolerance and dwarfing	Cong et l., 2008	9162
DREB1A	Transcription factor	wheat	Delayed wilting under drought stress	Pellegrineschi et al., 2004	7443
DREB2A	Transcription factor	Arabidopsis	Drought resistance	Sakuma et al. 2006	8302
EhCaBP	Calcium binding protein	Tobacco	Enhanced germination and seedling growth under salinity	Pandey et al. 2002	5743
ERA1	Farnesyltransferase	Canola	Antisense promote drought resistance in the field	Wang et al., 2005	7775
FAD3 & FAD8	Increased fatty acid desaturation	Tobacco	Drought resistance	Meng et al., 2005	8020
FAD7	Increased fatty acid desaturation under COR15a	Tobacco	Cold tolerance	Khodakovskaya et al. 2006	8260
FER	Root development and physiology	tomato	Possibly, enhance iron nutrition	Hong-Qing Ling et al., 2002	6235
Ferritin	Iron storage protein	rice	Increased iron and zinc accumulation in grain	Vasconcelos et al., 2003	6393
FLD	Flavodoxin expression in chloroplasts	Tobacco	Resistance to heat, high irradiation, water deficit, UV	Tognettia et al. 2006	8446
FLD	Flavodoxin from bacteria expressed in chloroplasts	Tobacco	Iron deficiency tolerance	Tognetti et al., 2007	8993

FRO2	Ferric chelate reductase responsible for reduction of iron at the root surface.	Arabidopsis	Iron deficiency tolerance	Connolly et al., 2003	6884
Gal	Raffinose hydrolysis	Petunia	Antisense expressed increased freezing tolerance	Pennycooke et al., 2003	6825
GASA4	Chaperone?	Arabidopsis	Heat tolerance	Ko et al., 2007	9084
Gli1	Mutant lack glycerol catabolism	Arabidopsis	Accumulated glycerol confers dehydration resistance	Eastmond, 2004	6974
GhDREB1	Transcription factor	Tobacco	Chilling tolerance, negatively regulated by gibberellic acid	Shan et al., 2007	9012
GPAT	glycerol-3-phosphate acyltransferase of chloroplasts	Rice	Chilling tolerance.	Ariizumi et al., 2002	6190
GR-RBP4	Transcription factor	Arabidopsis	No effect on cold tolerance	Kwak et al., 2005	7989
HAL1	Promote K+/Na+ selectivity	Tomato	Salt tolerance in growth and fruit production	Rus et al., 2001	5483
HAL1	Promote K+/Na+ selectivity	Watermelon	Salt tolerance in growth	Ellul et al. 2003	6775
HAL2 (Yeast)	Promote K+/Na+ selectivity	Tomato	Salt tolerance in calli and rooting	Arrillaga et al. 1998	4537
Hb	hemoglobin	Alfalfa	Modulation of NO under hypoxia?	Dordas et al. 2003	6758
HMA5	Cu-transporting P-type ATPase	Arabidopsis	Copper tolerance	Andrés-Colás et al. 2006	8081
HOS9	Transcription factor	Arabidopsis	Cold tolerance, growth	Zhu et al., 2004	7289
HOS10	Transcription factor	Arabidopsis	Cold tolerance, growth	Zhu et al., 2005	7806
HOT2	Encode a chitinase-like protein	Arabidopsis	Salt tolerance	Kwon et al., 2007	8640
HsfA2	Transcription factor	Arabidopsis	Resistance to environmental stresses	Ayako et al., 2006	8534
HsfA2	Heat-inducible transactivator	Arabidopsis	Heat tolerance	Charng et al., 2007	8645
HvCBF4	Transcription factor	Rice	Drought, salt chilling tolerance	Oh et al., 2007	9023
IMPDH	Root Inosine-5'-monophosphate dehydrogenases	Soybean	Reduced Al uptake in root	Ermolayev et al., 2003	6853
ISPS	Isoprene synthesis	Poplar trees	Silencing the gene cause susceptibility to heat	Behnke et ak., 2007	8983
ISPS	Isoprene synthesis	Poplar trees	Overexpression induce heat tolerance	Sasaki et al., 2007	9027
JERF3	Jasmonate and ethylene-responsive factor 3	Tobacco	Salinity tolerance	Wang et al., 2004	7419
JERF1	Jasmonate and ethylene-responsive factor 1	Tobacco	Salinity tolerance	Zhang et al., 2004	7472
JERF1	Jasmonate and ethylene-responsive factor 1	Tobacco	Salt and cold tolerance	Wu et al., 2007	9036
ERF1	Jasmonate and ethylene-responsive factor 1	Wheat	Multiple stress tolerance	Xu et al., 2007	9135
KAPP	Kinase-associated protein phosphatase	Arabidopsis	Salt (Na+) tolerance	Manabe et al., 2008	9204
LeGPAT	Glycerol-3-phosphate acyltransferase	Tomato	Photosystem chilling tolerance	Sui et al., 2007	9038
lew2	Wilting allele; cellulose synthesis complex	Arabidopsis	Drought resistance	Chen et al. 2005	7774
MBF1c	Transcriptional coactivator multiprotein bridging factor	Arabidopsis	Tolerance to bacterial infection, heat, osmotic stress	Suzuki et al., 2005	8028
ME	NADP-malic enzyme which converts malate and NADP to pyruvate, NADPH, and CO2	Tobacco	Reduced stomatal conductance	Laporte et al. 2002	5895
NADP-ME 2	NADP-malic enzyme	Arabidopsis	Salt tolerance	Liu et al., 2007	8864
MerA	Mercuric ion reductase	Plants	Mercury tolerance	Bizily et al., 2003	6383
MerA; MerB	mercuric ion reductase and organomercurial lyase respectively	Plants	Mercury tolerance	Ruiz et al. 2003	6670

MerA9; MerA1	mercuric ion reductase	Populus deltoides	Mercury tolerance	Che et al. 2003	6706
merApe9	mercuric ion reductase	Tobacco	Mercury tolerance by mercury volatilization	He et al. 2001	5692
MerB	Organomercurial lyase	Plants	Mercury tolerance	Bizily et al., 2003	6383
MIZ1	Hydrotropism of root	Arabidopsis	Dehydration avoidance?	Kobayashi et al., 2007	8816
MKK9	MAP Kinase	Arabidopsis	Salt resistance in germination	Alzwiya et al., 2007	8979
MsPRP2	Transcription factor	Alfalfa	Increased salinity tolerance	Winicov and Bastola 1999	5622
na	nicotianamine synthase- metal chelating	tobacco	Nickel tolerance	Douchkov et al., 2005	7578
naat	Nicotianamine aminotransferase activity	Rice	Fe efficiency, high phytosiderophores secretion, grain yield	Michiko Takahashi et al., 2001	5384
NahG	salicylate hydroxylase expression	Arabidopsis	Reduced leaf necrosis under salt stress	Borsani et al., 2001	5432
NPK1	mitogen-activated protein kinase	Maize	Frost tolerance without hardening	Shou et al., 2004	7045
NPK1	mitogen-activated protein kinase	Maize	Drought resistance of photosynthesis	Shou et al., 2004	7156
NtC7	Trans-membrane protein, osmotic adjustment	Tobacco	Resistance to osmotic stress	Tamura et al., 2003	6385
NtGDI1	GDP dissociation inhibitor	Arabidopsis	Promote Al release from roots	Ezaki et al. 2001	5664
OsCDPK7	Transcription factor	Rice	Increased cold salinity and drought tolerance	Saijo et al. 2000	4866
OsCIPK01–OsCIPK30	Calcineurin B-like protein-interacting protein kinases	Rice	Cold salt and drought tolerance	Xiang et al., 2007	8984
OsCOIN	RING finger protein	Rice	Cold, salt and drought tolerance and overexpression of P5CS	Liu et al., 2007	9037
OCPI1	Transcription factor	Rice	Drought resistance in yield	Huang et al., 2007	8873
OPBP1	Transcription factor	Tobacco	Salinity and disease tolerance	Guo et al., 2004	7418
OsSbp	Calvin cycle enzyme sedoheptulose-1,7-bisphosphatase	Rice	Tolerance of photosynthesis to salt	Feng et al., 2007	9005
OsDREB1A	Transcription factor	Arabidopsis	Drought, salt, freezing tolerance	Dubouzet et al., 2003	6429
OsMYB3R-2	MYB homeodomain, and zinc finger proteins	Arabidopsis	Drought, salt, freezing tolerance	Dai et al., 2007	8803
OsiSAP8	Stress/zinc finger protein	Rice	Salt drought and cold tolerance	Kanneganti and Gupta, 200	9181
OsPTF1	Transcription factor	Rice	Tolerance to P deficiency	Yi et al., 2005	7861
OsUGE-1	Raffinose synthesis	Arabidopsis	Resistance to abiotic stresses	Liu et al., 2007	9071
P6	Ferritin	Tabacco	Iron Deficiency tolerance	Robin et al., 2006	8397
PARP1; PARP2	Poly(ADP-ribose) polymerase	Arabidopsis; Brassica	Silencing induces drought and heat tolerance	Block et al., 2004	7514
PDH45	DNA helicase 45	Pea	Salinity tolerance in yield	Sanan-Mishra et al., 2005	7526
PLD?1	Modulation of COR genes	Arabidopsis	Cold tolerance	Rajashekar et al., 2006	8456
RGS1	Regulation of G-protein signalling	Arabidopsis	ABA mediated root elongation and drought resistance	Chen et al. 2006	8331
S851	Encodes 8 sphingolipid desaturase in cell membranes	Arabidopsis	Aluminum tolerance	Ryan et al., 2007	9029
SacB	Fructan synthesis	Tonacco	Freezing tolerance	Parvanova et al., 2004	7334
SCABP8	Interacts with SOS2	Arabidopsis	Salt tolerance	Quan et al., 2007	8908

SCOF-1	Transcription factor	Arabidopsis, Tobacco	Increased cold tolerance	Kim et al. 2001a	5218
ShMTP1	CDF Mn+2 sequestering protein	Arabidopsis	Mn+2 tolerance	Delhaize et al. 2003	6551
See2	Senescence-associated legumain gene	Maize	Role in N-use under N-deficiency	Donnison et al., 2007	8630
shn	Increased epicuticular wax	Arabidopsis	High cuticular resistance	Aharoni et al., 2004	7363
SL	Se-Cys lyase	Arabidopsis	Selenium tolerance	Pilon et al., 2003	6472
SIZ1	SUMO E3 ligase	Arabidopsis	Basal heat tolerance	Chan Yul Yoo et al., 2006	8577
SNAC1	Stomatal activity	Rice	Drought and salt tolerance	Hu et al., 2006	8447
SPS	sucrose phosphate synthase	Arabidopsis	Increased sucrose synthesis and freezing tolerance	Strand et al., 2003	6525
SRK2C	Protein kinase	Arabidopsis	Osmotic stress/drought resistance	Umezawa et al., 2004	7475
STO	Protein binds to a Myb transcription factor	Arabidopsis	Salt tolerance	Shuuichi and Takano 2003	6736
STOP1	Zinc finger protein	Arabidopsis	Aluminum tolerance	Luchi et al., 2007	8925
Sto1	Reduced ABA accumulation	Arabidopsis	Better growth under salt stress	Ruggiero et al., 2004	7355
TaPP2Ac-1	catalytic subunit (c) of protein phosphatase 2A	Tobacco	Drought resistance; maintain RWC and membrane stability	Xu et al., 2007	8658
TaSTK	serine/threonine protein kinase	Wheat	Salt tolerance	Ge et al., 2007	8923
TaSrg6	Transcription factor	Arabidopsis	Drought resistance	Tonga et al., 2007	8870
TERF1	ERF transcription activator	Tobacco	ABA sensitivity and drought resistance	Zhang et al., 2005	8033
Tsi1	Transcription factor	Tobacco	Increase osmotic stress tolerance	Park et al. 2001	5350
uvi1	Transcription factor	Arabidopsis	UV-B tolerance due photoreactivation of CPDs and dark repair of (6-4) photoproducts,	Tanaka et al., 2002	6024
VuNCED1	Involved in ABA biosynthesis	Creeping bent grass	Salinity and drought resistance	Aswath et al., 2005	8019
WXP1	Epicuticular wax accumulation	Alfalfa	Drought resistance in maintained leaf water status and delayed wilting	Zhang et al., 2005	7772
WXP1;WXP2	Epicuticular wax accumulation	Arabidopsis	Drought and freezing tolerance	Zhang et al., 2007	8865
wft1, wft2	Fructan synthesis	Ryegrass	Freezing tolerance	Hisanoa et al., 2004	7271
ZIF1	Zn sequestration	Arabidopsis	Zinc tolerance	Haydon and Cobbett, 2007	8802
ZmDREB2A	Encodes HSP & LEA proteins	Arabidopsis	Drought and heat tolerance	Qin et al., 2007	8829
ZPT2-3	Encodes a Cys2/His2-type zinc finger protein	Petunia	Dehydration tolerance	Shoji et al., 2004	6920
MtZpt2	zinc finger protein	Medicao	Recover Root growth under salt stress	Merchan et al., 2007	8911

Hormone regulating genes

Gene	***Gene Action***	***Species***	***Phenotype***	***Reference***	***ID****
ABI1, ABI2	ABA regulation	Arabidopsis	Heat tolerance	Larkindale et al., 2005	7783
ABA2	ABA regulation	Arabidopsis	Tolerance to various prolonged stresses?	Lin et al., 2007	8734
ABA27	ABA hypersensitivity	Wheat	Freezing tolerance	Kobayashi et al., 2008	9155
hab1 group	ABA hypersensitivity	Arabidopsis	Dehydration avoidance	Saez et al., 2006	8435

ACC	ACC de-aminase	Tobacco	Increased flooding (waterlogging) tolerance	Grichko and Glick 2001	5214
AtMYB44	ABA sensitivity	Arabidopsis	Stomatal closure	Jung et al., 2008	9205
AtNCED3	Increased ABA synthesis	Arabidopsis	Reduced transpiration and drought resistance	Iuchi et al., 2001	5527
AtPP2CA	Reduce ABA sensitivity	Arabidopsis	Induce cold sensitivity	Tahtiharju and Palva, (2001)	5437
EIN2	Ethylene and ABA signaling pathways	Arabidopsis	Salt and osmotic stress responsees	Wang et al., 2007	8975
Eto 1-1	Ethylene over-production	Arabidopsis	Reduced ABA sensitivity and greater transpiration	Tanaka et al., 2005	7859
CYP707A3	Regulate ABA levels	Arabidopsis	Dehydration and rehydration responses	Umezawa et al. 2006	8245
LE-ACS6	1-aminocyclopropane-1-carboxylate (ACC) synthase (tomato)	Tobacco	Responsible for visible Ozone damage symptoms	Nakajima et al., 2002	6007
LLA23	Reduced ABA sensitivity	Arabidopsis	Drought and salt resistance	Yang et al., 2005	8026
NTHK1	Ethylene receptor	Arabidopsis	Salt sensitivity	Cao et al., 2007	8733
PSAG12-IPT	Over production of cytokinins	*Petunia*	Delayed leaf senescence (not tested under stress)	Clark et al., 2004	6904
PLD alpha	Phospholipase D (alpha) expression	*Arabidopsis*	Increase sensitivity to ABA and reduce transpiration	Sang et al. 2001	5635
rolD	ACC deaminase	*Canola*	Lower ethylene and Nickel tolerance	Stearns et al., 2005	7929
SAG12:ipt	Increased cytokinin biosynthesis	*Arabidopsis*	Flooding tolerance and recovery	Nguyen Huynh et al., 2005	7659
sp12 and sp5	ABA overproduction	Tomato	High water-use efficiency, low transpiration and greater root hydraulic conductance	Thompson et al., 2007	8805
tos1	Increased ABA sensitivity	tomato	Hypersensitive to osmotic stress and exogenous ABA	Borsani et la., 2002	6330
ZmACS6	Ethylene synthesis	*Maize*	Non-functional mutant expressed drought induced senescence	Todd et al., 2004	7471
35S::ZOG1	Trans-zeatin O-glucosyltransferase gene	*Tobacco*	High cytokinin in root under drought stress	Havlova et al., 2008	9200

Oxidative stress related genes

Gene	***Gene Action***	***Species***	***Phenotype***	***Reference***	***ID****
'AO'	Ascorbate oxidase	Tobacco & Arabidpsis	Salt sensitivity in germination, photosynthesis, & seed yield	Yamamoto et al., 2005	7744
ALR	Aldose/aldehyde reductase	Tobacco	Drought and UV-B tolerance	Hideg et al., 2003	6524
ALR	Aldose/aldehyde reductase	Tobacco	Cold and cadmium stress tolerance	Hegedüs et al., 2004	7098
'AO'	Ascorbate oxidase	Tobacco	Reduced redox state of ascorbate and increased ozone susceptibility	Sanmartin et al., 2003	6545
ApGPX2 and AcGPX2	Glutathione peroxidase (GPX)-like proteins	Arabidopsis	Oxidative stress, drought and salt resistance	Gaber et al. 2006	8466
Apx	Ascorbate peroxidase	Cotton	Photoprotection under chilling stress	Kornyeyev et al. 2003	6769
Apx	Ascorbate peroxidase	Tomato	Chilling and salt tolerance	Wang et al., 2005	7630

Apx1	Ascorbate peroxidase	Arabidopsis	Heat tolerance	Shi et al. 2001	5715
APX2	Ascorbate peroxidase	Arabidopsis	High light and drought tolerant mutant	Rossel et al. 2006	8164
Apx3	Ascorbate peroxidase	Tobacco	Increased protection against oxidative stress	Wang et al. 1999	4531
Apx3	Ascorbate peroxidase	Tobacco	Drought resistance in photosynthesis	Juqiang Yan et al. 2003	6614
AtMDAR1	Monodehydroascorbate reductase; Ascorbate regeneration	Tobacco	Ozone, salt and polyethylene glycol tolerance	Eltayeb et al., 2007	8814
CAT	Wheat catalase	Rice	Reduction in rice H2O2 under chilling stress	Matsumura et al., 2002	6218
DHAR	regeneration of ascorbate	Arabidopsis	Salt tolerance	Ushimaru et al., 2006	8492
DHAR	regeneration of ascorbate	Tobacco	tolerance to ozone, drought, salt, and PEG	Elsadig et al. 2006	8297
Gly1; gly2	Glutathione-based detoxification of methylglyoxal.	Tobacco	Salt tolerance	Singla-Pareek et al. 2006	8261
GmTP55	Antiquitin-like protein	Soyban Tobacco	Resistance to drought, salt and oxidative stress	Rodrigues et al. 2006	8330
GPX	Glutathione peroxidase	Tobacco	Chilling and salt resistance	Kazuya et al., 2004	6921
GST	glutathione S-transferase overexpression	Arabidopsis	No whole-plant salt resistance despite antioxidant activity	Katsuhara et al., 2005	7793
GST	glutathione S-transferase overexpression	Cotton	No whole-plant salt resistance and no antioxidant activity	Light et al., 2005	8032
GST	glutathione S-transferase overexpression	Rice	Salt and chilling resistance	Zhao and Zhang, 2006	8555
GST/GPX	Glutathione S-transferase with Glutathione peroxidase	Tobacco	Increased stress tolerance	Roxas et al. 2000	
katE	Escherichia coli catalase	Tobacco	Salt tolerance by hydrogen peroxide scavenging	Al-Taweel et al., 2007	9030
MsFer	Ferritin (iron storage)	Tobacco	Increased tolerance to oxidative damage caused by excess iron	Deak et al. 1999	5608
ndhCKJ	NAD(P)H dehydrogenase	Tobacco	Photosystem function under heat stress	Wang et al. 2006	8353
Nt107	Glutathione S-transferase	Tobacco	Sustained growth under cold and salinity stress	Roxas et al. 1997	5616
NtPox	Gluthathione peroxidase	*Arabidopsis*	Protect against Al toxicity and oxidative stress	Ezaki et al. 2001	5664
parB	Glutathione S-transferase	*Arabidopsis*	Protect against Al toxicity and oxidative stress	Ezaki et al. 2000	4728
parB	Glutathione S-transferase	*Arabidopsis*	Protect against Al toxicity and oxidative stress	Ezaki et al. 2001	5664
SOD	Mn superoxide dismutase	Alfalfa	tolerance to freezing stress	McKersie et al. 1993	5615
SOD	Mn superoxide dismutase	Alfalfa	tolerance to water deficit	McKersie et al. 1996	3345
SOD	Mn superoxide dismutase	Alfalfa	winter survival	McKersie et al. 1999	3894
SOD	Mn/Fe superoxide dismutase	Alfalfa	Background dependent increased photosynthesis under drought stress	Maria et al., 2002	6103
SOD	Cu, MN, Fe. Zn-SOD	Alfalfa, rye grass	Increased winter hardiness	McKersie 2001	5614
SOD	Mn superoxide dismutase	Arabidopsis	Salt tolerance	Wang et al., 2004	7266
SOD	Mn superoxide dismutase	Canola	aluminum tolerance	Basu et al., 2001	5684
SOD+CAT	Mn superoxide dismutase +	Chinese cabbage	Salt tolerance	Tsenga et al., 2007	9086

SOD	Mn superoxide dismutase	Rice	Reduced injury and sustained photosynthesis under PEG stress	Wang et al., 2005	7724
SOD+APX	Mn superoxide dismutase +	Tal fescue	Heavy metals tolerance	Lee et al., 2007	9122
SOD	Cu/Zn superoxide dismutase	Tobacco	Retained photosynthesis under chilling and heat stress	Gupta et al. 1993	5609
SOD	Cu/Zn superoxide dismutase	Tobacco	enhanced tolerance to salt, water, and PEG stresses,	Badawi et al., 2004	7033
SOD	Fe superoxide dismutase	Tobacco	Protected plants from ozone damage	Van Camp et al. 1994	5621
SOD	Mn superoxide dismutase	Tobacco	Reduced cellular damage under oxidative stress	Bowler et al. 1991	5606
SOD	Mn superoxide dismutase	Tobacco	tolerance to Mn deficiency	Yu et al. 1999	4512
SOD	Cu/Zn superoxide dismutase	Tobacco, Tomato	No protection seen against superoxide toxicity	Tepperman and Dunsmuir 1990	5619
TFT7	A 14-3-3 protein with antioxidative function?	Tomato	Salinity tolerance	Wu and Shi, 2008	9189
vtc-1	Ascorbate deficient mutant	Arabidopsis	Sensitivity to salinity stress	Huang et al., 2005	7990
vtc1, vtc2, npq1, cad2	reactive oxygen metabolism mutants	Arabidopsis	Heat tolerance/sensitivity	Larkindale et al., 2005	7783

Genes encoding for molecular chaperones

Gene	***Gene Action***	***Species***	***Phenotype***	***Reference***	***ID****
APG6	Chloroplast structure	Arabidopsis	Heat tolerance	Myouga et al. 2006	8474
AtDjA2 & atDjA3	J-domain molecular chaperone family	Arabidopsis	Heat tolerance	Li et al., 2007	9034
AtMTP3	Metal tolerance protein	Arabidopsis	Zinc tolerance	Arrivault et al. 2006	8307
Atsbp1	Selenium binding protein	Arabidopsis	Selenium tolerance	Agalou et al., 2005	7899
atRZ-1a	RNA chaperone protein	Arabidopsis	Cold tolerance	Kim and Kang 2006	8338
BiP	Endoplasmic reticulum binding protein (BiP)	Tobacco	Maintenance of plant water status under drought stress and antioxidative defence	Alvim et al., 2001	5433
CaHSP26	Chloroplast (CP)-localized small heat shock protein	Tobacco	Protection of PSII and PSI during chilling	Guo et al. 2007	8673
hs	Heat shock transcription factor	Arabidopsis	Increased thermotolerance in transgenic plants	Lee et al. 1995	5612
Hsp101	Heat shock protein	*Arabidopsis*	Decreased Thermotolerance in Hsp101 deficient (hot1) mutant	Hong and Vierling 2000	5363
Hsp101	Heat shock protein	*Arabidopsis*	Manipulated themotolerance in transgenic plants	Queitsch et al. 2000	4733
Hsp101	Heat shock protein	Rice	Heat tolerance in plant growth	Katiyar-Agarwal et al. 2003	6430
Hsp17.7	Heat shock protein	Carrot	Increased or decreased thermotolerance	Malik et al. 1999	4526
Hsp70	Heat-inducible antisense HSP70	*Arabidopsis*	Increased thermotolerance in transgenic plants	Lee and Schoof 1999	5613
LeHSP100/ClpB	Chloroplast HSP	*Tomato*	Heat tolerance	Yang et al. 2006	8468
mHSP22	Mitochondrial small HSP	*Arabidopsis*	Heat tolerance (high leaf mass after heat stress)	Rhoads et al., 2005	7619

P5CR	Inducible heat shock promoter (IHSP)	Soybean	Increased proline accumulation	de Ronde et al. 2000	4936
pBE2113/ hiC6	Overexpressed HIC6 cryoprotective protein	Tobacco	Freezing tolerance; reduced membrane injury	Honjoh et al., 2001	5531
S1pt::ECS	glutamylcysteine synthetase	Arabidopsis	Metal tolerance	Li et al. 2006	8310
TLHS1	Overexpressed class I cytosolic small HSP	Tobacco	Seedling thermotolerance	Park and Hong 2002	5811
wx	Control amylose synthesis	Rice	Increased amylose content at low temperature	Hirano and Sano 1998	5610

Genes encoding proton pumps, antiporters and ion transporters

Gene	***Gene Action***	***Species***	***Phenotype***	***Reference***	***ID****
AhHMA4	Low Cd & Zn in cytoplasm	Arabidopsis	Cadmium & Zinc tolerance	Courbot et al., 2007	8917
ALMT1	aluminum-activated malate transporter	Arabidopsis	Aluminum tolerance	Hoekenga et al. 2006	8362
ALMT1	aluminum-activated malate transporter	Arabidopsis	Aluminum tolerance	Kobayashi et al., 2007	9138
ALMT1	aluminum-activated malate transporter	Barley	Aluminum tolerance	Delhaize et al., 2004	7428
ALMT1	aluminum-activated malate transporter	Wheat/tobacco	Aluminum tolerance	Takayuki et al., 2004	7024
ALS1	Encodes an ABC transporter-like protein	Arabidopsis	Aluminum tolerance; Al transport & sequestration	Larsen et al., 2007	8872
ALS3	Encodes an ABC transporter-like protein	Arabidopsis	Aluminum tolerance	Larsen et al., 2004	7515
ALS3	Encodes an ABC transporter-like protein	Arabidopsis	Aluminum tolerance	Gabrielson et al. 2006	8219
AqpL1	Aquaporin-like	Tobacco	Enhance osmotic water uptake by leaf cells	Ding et al., 2004	6970
AtCAX2;AtCAX4	Cation/proton antiporters	Tobacco	Cadmium, zinc, manganese resistance	Korenkov et al., 2007	9093
Atchx21	Putative Na+/H+ antiporter	Arabidopsis	Sodium concentrations in plant, root growth, plant size	Hall et al. 2006	8231
AtHKT1	Reduction in Sodium in root	Arabidopsis	Salt tolerance	Horie et al. 2006	8335
AtHKT1	Sodium and Potassium transporter	cells	Reduced sodium accumulation	Tomoaki et al. 2005	8080
AtMGT1	Mg++ transporter protein	Nicotiana benthamiana	Magnesium deficiency tolerance	Deng et al., 2006	8568
AtMRP4	Stomatal guard cell plasma membrane ABCC-type ABC transporter,	Arabidopsis	Drought susceptibility due to loss of stomatal control	Markus et al., 2004	7252
AtMTP11	Encode proteins of the cation diffusion facilitator (CDF) family	Arabidopsis	Manganese tolerance	Delhaize et al., 2007	8981
AtNHX1	Vacuolar Na+/H+ antiporter	Arabidopsis	Salt tolerance	Yokoi et al., 2002	6120
AtNHX1	Vacuolar Na+/H+ antiporter	Brassica napus	Salt tolerance, growth, seed yield and seed oil quality	Zhang, Hong-Xia et al., 2001	5596
AtNHX1	Vacuolar Na+/H+ antiporter	Buckwheat	Salt tolerance	Chen et al., 2008	9168
AtNHX1	Vacuolar Na+/H+ antiporter	Cotton	Salt tolerance in photosynthesis and yield	He et al., 2005	8007
AtNHX1	Vacuolar Na+/H+ antiporter	Tall fwscue	Salt tolerance	Zhao et al., 2007	9070
AtNHX1	Vacuolar Na+/H+ antiporter	Tomato	Salt tolerance, growth, fruit yield	Apse et al., 1999	5523
AtNHX1	Vacuolar Na+/H+ antiporter	Wheat	Salt tolerance for grain yield in the field	Xue et al., 2004	7270
AtNHX2 AtNHX5	Vacuolar Na+/H+ antiporter	Arabidopsis	Salt tolerance	Yokoi et al., 2002	6120
AtPcr1	Membrane protein	Arabidopsis	Cadmium tolerance (exclusion?)	Song et al., 2004	7256

AtPDR12	ABC transporter	Arabidopsis	Lead tolerance	Lee et al., 2005	7781
AtPDR8	Plasma membrane efflux pump	Arabidopsis	Cadmium and lead tolerance (exclusion)	Kim et al., 2007	8830
AtZIP1	Zinc transporter	Barley	Increased grain zinc & iron content	Ramesh et al., 2004	7254
AVP1	AVP1 proton pump overexpression	Arabidopsis	Salt tolerance in growth and sustained plant water status	Gaxiola et al., 2001	5597
BOR1	Boron transporter	Arabidopsis	Boron deficiency tolerance	Kyoko et al. 2006	8352
CAX	Tonoplast localized transporter	Tobacco	Cation transport selectivity	Koren'kov et al., 2007	8591
CAX1	Vacuolar Ca2+/H+ antiporter	Arabidopsis	Freezing tolerance after cold acclimation	Catalá et al., 2003	6871
cNHX1	Vacuolar Na+/H+ antiporter	Citrus	Possibly, heat-induced chilling tolerance response	Porat et al., 2002	6127
GhNHX1	Vacuolar Na+/H+ antiporter	Arabidopsis (cotton)	Salt tolerance	Wu et al., 2004	7179
GmCAX1	Cation/proton antiporter	Arabidopsis	Salt tolerance	Luo et al., 2005	8023
HKT1	Potassium transporter	Wheat	Salt tolerance in growth and improved K+/Na+ ratio	Laurie et al., 2002	6229
HvAACT1	Citrate transporter	Tobacco	Aluminum tolerance	Furukawa et al., 2007	9024
HvPIP2;1	PIP2 plasma membrane aquaporin Over-expression	rice	Increased susceptibility to salt stress	Katsuhara et al., 2004	6818
IRT1	Divalent cation transporter	Arabidopsis	Iron uptake by root and elimination of iron deficiency	Vert et al., 2002	6117
NRT2.1	Nitrate transporter	Arabidopsis	Root architecture and nitrate uptake under N stress	Remans et al. 2006	8252
NtAQP1	PIP1 plasma membrane aquaporin	Tobacco	High root hydraulic conductance and reduced plant water deficit under drought stress	Siefritz et al., 2002	6116
NtPT1	Phosphate transporter	Rice	Phosphate acquisition	Park et al., 2007	8810
OsNHX1	Vacuolar Na+/H+ antiporter	rice	Salt tolerance	Fukuda et al., 2004	7017
OsSOS1	Plasma membrane Na+/H+ exchanger	rice	Salt tolerance	Martínez-Atienza et al., 2007	8735
PcSrp	Serine rich protein (enhancing ion homeostasis?)	Finger millet	Salt tolerance	Mahalakshmi et al. 2006	8359
PgTIP1	Tonoplast intrinsic protein	Arabidopsis	Salt tolerance; root dependant drought tolerance	Peng et al., 2007	8992
Pht1, Pht1;4	Phosphate acquisition by roots	*Arabidopsis*	Phosphate efficiency	Shin et al., 2004	7253
PIP	Plasma membrane aquaporin overexression	Soybean, lettuce	Downregulated by arbuscular mycorrhisa causing water conservation	Porcel et al. 2006	8246
PIP1;4 & PIP2;5	Plasma membrane aquaporin overexression	Tobacco	Excessive water loss and retarded seedling growth under drought stress	Jang et al., 2007	8974
PIP1b	Plasma membrane aquaporin overexression	Tobacco	No effect under salt and negative effect under drought stress	Aharon et al. 2003	6659
PIP1bn	Plasma membrane aquaporin overexression	Tobacco	Tolerance to osmotic stress	Yua et al., 2005	7863
PIP2;2	Plasma membrane aquaporin knockout	Arabidopsis	Reduced hydraulic conductivity of root cortex cells	Javot et al., 2003	6382

RWC3	Aquaporin overexpression	Rice	Maintenance of leaf water potential and transpiration under 10 h PEG stress	Lian et al., 2004	7177
SOD2	Vacuolar Na+/H+ antiporter	Arabidopsis	Salt tolerance; higher plant K/Na ratio	Gao et al., 2004	6924
SOD2	Vacuolar Na+/H+ antiporter	Rice	Salt tolerance	Zhao et al. 2006	8088
SOS1	Na+-H+ antiporter	Arabidopsis	Protect K+ permeability during salt stress	Qi and Spalding, 2004	7350
SOS3	Sodium accumulation in roots	Arabidopsis	Salt tolerance	Horie et al. 2006	8335
SOS4	Involved in the synthesis of pyridoxal-5-phosphate which modulates ion transporters	Arabidopsis	Salt tolerance through Na+/K+ homeostasis	Shi et al., 2002	5931
SsNHX1	Vacuolar Na+/H+ antiporter	Rice	Salt tolerance	Zhao et al. 2006	8216
SsVP-2	Vacuolar Na+/H+ antiporter	Arabidopsis	Salt tolerance	Guo et al. 2006	8166
SULTR1;2	High affinity root sulfate transporter	Arabidopsis	Selenate sensitivity	El Kassis et al., 2007	8800
TNHX1 and H+-PPase TVP1	Vacuolar Na+/H+ antiporter	Arabidopsis	Salt tolerance	Brini et al. 2007	8697
TsVP	Vacuolar Na+/H+ antiporter	Tobacco	Salt tolerance	Gao et al., 2006	8462
YCF1	Sequester glutathione-chelates of heavy metals into vacuoles	Arabidopsis	Heavy metal and salt tolerance	Koh et al. 2006	8172
ZntA	Regulation of Cd, PB and Zn pump	Arabidopsis	Cd and Pb resistance; reduced metal accumulation	Lee et al., 2003	6824

Others

Gene	***Gene Action***	***Species***	***Phenotype***	***Reference***	***ID****
eibi1	Wilty mutant, defective cuticle	Wild barley	Drought susceptible	Chen et al., 2004	7282
Als1	Aluminum sensitivity	rice	Reduced growth under Al stress	Ma et al., 2005	7865
Calcineurin (mouse)	Ca2+- and calmodulin-dependent serine/threonine phosphatase	Rice	salt stress tolerance via controlled Na+ accumulation	Ma et al., 2005	7776
dgd1-2	Inability to accumulate digalactosyldiacylglycerol	Arabidopsis	Heat sensitive	Chen et al. 2006	8345
GDH	Bacterial Glutamate dehydrogenase expression	Tobacco	Maintaining high leaf water potential under drought stress	Mungur et al., 2006	8575
GDH	Bacterial Glutamate dehydrogenase expression	Maize	Drought resistance	Lightfoot et al., 2007	8899
LeGPAT	Increased unsaturated fatty acid contents	Tomato	Chilling tolerance	Sui et al., 2007	8961
Rf1	Fertility restorer	Rice	Improved fertility at low temperature	Toshiyuki and Hidemasa, 2005	7605
sfr4	Low sugar content in cells?	Arabidopsis	Freezing susceptible mutant	Uemura et al., 2003	6534
SGR	Stay-green	Rice	Chlorophyll retention	Jiang et al., 2007	9089
	Thaumatin gene	Tobacco	Salt and disease tolerance	Rajam et al., 2007	8766
	Fructan accumulation	Potato	Increased proline accumulation under drought stress	Knipp et al. 2006	8144
	Overexpression of Glyoxalase Pathway Enzymes	Tobacco	Zinc toxicity and other metal tolerance	Singla-Pareek et al. 2006	8169
	Co-suppression of fatty acid desaturase - increased dienoic fatty acids	Rice	Heat tolerance	Sohn and Back, 2007	8770
	isopentenyltransferase	Tobacco	Delayed senescence and yield under drought	Rivero et al., 2007	9149