A COMPARATIVE STUDY OF THE GENE EXPRESSION AT THE mRNA LEVEL DURING ABIOTIC STRESS IN THE SEEDLINGS OF CONTRASTING INDICA RICE VARIETIES IR64 AND RASI USING HETEROLOGOUS cDNA PROBES

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DEPARTMENT OF BIOTECHNOLOGY
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BANGALORE

CERTIFICATE

This is to certify that the thesis entitled **"A COMPARATIVE STUDY OF THE GENE EXPRESSION AT THE mRNA LEVEL DURING ABIOTIC STRESS IN THE SEEDLINGS OF CONTRASTING INDICA RICE VARIETIES IR-64 AND RASI USING HETEROLOGOUS cDNA PROBES"** submitted in partial fulfillment of the requirements for the degree of **MASTER OF SCIENCE in BIOTECHNOLOGY** to the University of Agricultural Sciences, GKVK, Bangalore, is a record of bona-fide research work done by him during the period of his study in the University, and under my guidance and supervision and the thesis has not previously formed the basis for the award of any degree, diploma, associateship, fellowship or any other similar titles.

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INTRODUCTION

I. INTRODUCTION

Rice is the most important cereal crop of the world. This crop is grown in diverse ecosystems and extensively in the tropics. Rice is the staple food of majority of the people in South & East Asian nations, parts of South America and Africa. The present production of rice in the world falls well short of the demand. To meet the ever increasing demand, continuous improvement in the quality and productivity of this cereal is vital.

Several biotic and abiotic factors are important constraints in increasing the quality and yield of rice. Biotic stresses in the form of pests and diseases considerably affect rice productivity. Abiotic stresses however have been shown to cause more harm to the rice crop than biotic stresses. The major abiotic stresses which significantly hamper rice yields are drought, salinity, floods, extremes of temperature and metal toxicity.

Minimizing crop losses by abiotic stresses especially drought and salinity is an important area for the overall improvement of rice. A thorough understanding of the responses of the rice plant to abiotic stresses is fundamental for developing a strategy to make the rice plant more hardy.

Efforts have been made to improve the performance of rice crop under limiting environmental conditions through traditional breeding programs and agronomic practices. Strategies for the evaluation of rice for drought and salinity tolerance using field screening and multi-location testing have been developed. These approaches have also been able to distinguish rice varieties into susceptible and tolerant ones. The development of molecular linkage maps and the use of molecular markers, of late, is helping selection and breeding for drought resistance. Molecular markers linked to root traits, osmotic adjustment and other stress tolerant characters are now being identified and used for selection and breeding.

Initial work in understanding the effects of abiotic stress on rice was done at the whole plant level. The role, interactions and alterations of root-shoot characteristics in response to stress in rice has been studied. Later work focused on the physiology of stress. The effects of drought and salinity on the physiological processes like metabolism, growth and development has come forth.

The molecular responses of plants to abiotic stresses is a complex phenomenon. However, advances in molecular biology offer new tools to investigate changes in plants, at the cellular and molecular level, in response to abiotic stresses.

Many genes have been identified and characterized to have a definite role in the response of plants to salinity and drought, and are induced by a complex mechanism of stress perception and signal transduction events. Stress related gene products have a role in moisture stress tolerance such as signaling molecules, regulatory proteins, protection of cellular structures, synthesis of osmoprotectants, ion sequestration, chaperon activity and protein stabilization, protein degradation, scavenging of accumulated toxins (especially reactive oxygen species), promotion of damage repair mechanisms, anti-pathogen activity and others.

Changes, in the tissue specific gene expression, are fundamental to the responses that occur during salinity and drought and influence many of the short and long term cellular changes that determine stress resistance or susceptibility. Northern Blot analysis, using stress related cDNA probes, offers a simple but powerful tool to monitor alterations in gene expression in roots and shoots, in response to salinity and water deficit, while comparing a susceptible and tolerant variety.

A comparative study of the gene expression, at the transcript level, in the roots and shoots of two contrasting *Indica* rice varieties IR64 (a susceptible variety) and Rasi (a tolerant variety), using heterologous stress related cDNA probes for important downstream events, during salinity and desiccation stress was done. The study involved four components:

1. Varieties : IR64 (a susceptible variety) and Rasi (a tolerant line).
2. Probes : MnSOD, FeSOD, Cat2, cAPx, MCP, HSP 70, CyP, TL-protein (heterologous cDNAs)
3. Stresses : Salinity and Desiccation.
4. Tissues : Roots and Shoots.

An attempt has been made in the present study to understand the role and complex interactions of these four components during the molecular changes that occur in rice as a result of abiotic stress.

The specific objectives of the study were ;

a. To correlate the expression pattern (at the mRNA levels) of genes under study with their role in abiotic stress tolerance or susceptibility in IR64 (susceptible variety) and Rasi (tolerant variety).

b. To compare the differences in the expression of genes encoding stress proteins during salinity and desiccation.

c. To assess the gene expression pattern in root and shoot during different stages of salt and dehydration.

REVIEW OF LITERATURE

II. REVIEW OF LITERATURE

Life cannot exist without water. It forms an important constituent of the plant and animal cell and is present to the extent of 80 to 90%. Water is essential for plants due to the following reasons:

1. It is the major component of protoplasm. If the protoplasm is dehydrated, it ceases to be active and the protoplasm looses its physical and chemical properties. Water maintains turgidity of cells.
2. Water is an universal solvent. The intake of minerals and nutrients from the external medium into the cell is only in the dissolved form.
3. Water serves as the medium for translocation of minerals from the soil to leaves through the xylem and food manufactured by the leaves to other plant parts via phloem.
4. Water also plays an important role in the transport of plant harmones.
5. Plant movements (especially of certain organs) are caused by changes in water content of cells.
6. Water is directly involved in the biochemical reactions that take place in plant cells. Hydrolysis of macromolecules takes place by the addition of water. Water is the source of hydrogen for the reduction of carbon-dioxide during photosynthesis. Water is one of the products of cellular respiration. All these reactions are influenced by the availability of adequate and good quality water.

Since water plays such an important role in plants, its deficit severely affects cellular functions, plant growth & development and reduces yields. However the plant devises a number of changes that occur at the whole plant, physiological, cellular, biochemical and molecular levels in an attempt to cope with moisture stress. The literature reviewed here will focus on the molecular responses of plants to water deficit, induced by specific abiotic stresses (salinity and drought), with emphasis on rice and the role of genes whose expression has been investigated in this study.

Salinity Stress in Rice

Salinity refers to the presence of various salts in soil and irrigation water in concentrations that affect the growth and yield of rice plants. Sodium chloride (common salt), is often the dominant salt present in saline soils. Saline-alkaline and sodic soils may have excess of chlorides, sulphates and bicarbonates of sodium, calcium and potassium in addition to other inorganic ions.

Saline soils have a soil water conductivity of 4 deci-seimen/meter and exchangeable sodium percentage of not less than 15. This translates into nearly 2.56 g/L of total dissolved salts in an extract or if all the salt is NaCl, an ionic concentration of 44.14 mM.

The irrigation water in majority of the rice growing areas is generally of marginal or poor quality (EC of 2-5ds/m or more). Though water is present it is unavailable to plants because the osmotic potential of soil is altered. To exclude salts and minimize ion toxicity, water must be imported against a free energy gradient. However, if water is taken up freely the endogenous salt concentration rises.

Macromolecular assembly and enzyme activity associated with shaping and maintaining each cell can proceed only within a properly constituted ionic environment. The inorganic ions selectively neutralize charges on macromolecular surfaces and simultaneously permit formation of intramolecular bridges that determine the final conformation of many proteins. The same ions also determine the availability of free water around enzymes and their substrates and thus the rate of catalysis. Finally, ionic gradients, setup at considerable cost to the plant cell, constitute free energy gradients that can be tapped to direct the flow of organic molecules within and between cells [Claes *et al.*,1990].

An extracellular ion excess invariably disrupts the ionic balance intracellularly. With the influx of salt, proteins may denature or aggregate leading to a loss of function, gradient-driven pumps may reverse and thus block the normal redistribution of symported molecules, membrane fluidity and consequently, the activity of some membrane components may change, and even the entry of water may be restricted. Some ions may have additional secondary effects. For example, increasing amounts of intercellular Na+ can lead to decreases in the concentration of K+ [Ben-Hayyim *et al.*,1987; Binzel *et al.*,1988]. This, in turn, reduces the rate of photosynthesis [Pier and Berkowitz, 1987], and, based on studies with bacteria can accelerate polysome decay and degradation of the free ribosomal proteins [St. John and Goldberg, 1980]. Salt imposed stress has been shown to have an impact even before ions enter the cell. Extracellular Na+ (or mannitol), for example, can leach Ca+2 from root cell plasmalemma, and as a result of membrane destabilization, increases K+ efflux [Cramer *et al.*, 1985].

These are only the immediate problems facing the cell. If the stress is prolonged, normal maintenance processes are impaired because general protein synthesis [Hurkman and Tanaka, 1987] and metabolism [Criddle *et al.*,1989] both decline. Denatured proteins may form inactive complexes with otherwise functional proteins. Enzymes may be poisoned when inorganic cofactors are displaced by incoming salts.

Rice is a salt sensitive plant. Relatively speaking, this species is more sensitive to salt stress at the seedling stage and the reproductive stage [Lutts *et al.*, 1995]. Excess salt leads to reduced seed germination and poor seedling vigor. During the vegetative phase, premature senescence of leaves and reduced number of tillers can occur. During the reproductive stage, the number of spikelets per panicle gets significantly reduced [Lutts *et al.*, 1996]

Drought and Rice

Rice cultivation in tropical areas is mostly dependent on seasonal rainfall. Vagaries of tropical monsoon renders the growth and yield of rice crop uncertain. The modern high yielding varieties of rice in particular are unable to attain their full genetic potential in the absence of adequate and good quality water.

Drought occurs when there is insufficient soil water to be taken up by the plants over a period of time to meet its transpirational requirements. Sustained drought results in complete loss of free water and will result in desiccation and dehydration. Concentration of solutes in the cell leads to drop in cellular water potential. Loss of turgor leads to changes in the cell volume and membrane area. The crucial cell wall plasma membrane continuum is lost. An osmotic shock can cause extensive cell damage through disruption of membrane integrity and leakage of cellular contents. Cellular water deficit causes extensive damage to functional proteins and increases formation of misformed proteins. Impairment in the normal metabolic pathways leads to formation of toxic and highly reactive byproducts such as the reactive oxygen species. Many other cellular changes similar to those occurring during salt stress are also observed during drought.

In Rice, at the plant level, drought affects several developmental processes. Seed germination is non-uniform. At the vegetative stage, canopy photosynthetic rates decrease drastically. Root growth is affected. Leaf rolling and leaf scorching is observed. At the reproductive stage, drought causes pollen sterility, small, thin and deformed anthers. Drought during anthesis causes inhibition of anther dehiscence and pollen germination, reduced pollen viability, failure of the panicle to exert the flag leaf, resulting in loss of grain set. Water constraint during ripening causes incomplete grain filling [O'Toole and Moya, 1981].

Molecular Responses of Rice to Salinity and Moisture Stress

Osmotic stress (such as salinity and Drought) leading to water deficit elicit complex molecular responses in plants. The events described here are common to all plants and also apply to Rice.

The molecular responses of plants to water deficit is dependent upon the type of stress (salinity/ drought), severity of stress (mild/ moderate or severe) and duration of stress (sporadic or chronic). A gradual onset of stress allows cellular mechanisms to adopt better while a sudden severe stress results in cellular damage and activates repair mechanisms. Plant factors such as genotype/variety, developmental stage (seed/seedling/vegetative or reproductive stage) and organ (root/ shoot etc.) exposed to stress also influences the nature of response [Bray, 1997].

Molecular events during water deficit has been investigated using four major approaches [reviewed in Ingram and Bartels, 1996] :

1. Examining tolerant systems such as seeds and resurrection plants.
2. Analyzing mutants from genetic model species.
3. Analyzing the effects on agriculturally relevant plants.
4. By the targeted expression of drought related genes *in vivo* using transgenic plants.

The responses of plants to water deficit at the molecular level normally occur in the following sequence [Bray, 1993] :

1. Cellular perception of the stress.
2. Signal transduction events.
3. Alterations in the gene expression.
4. The role of gene products induced by salinity and drought in stress avoidance or tolerance.

Cellular Perception of Water Deficit

Plant responses to biotic stimulus from the environment is predominantly mediated by signaling molecules and receptors. Water deficit on the other hand is a physical stimulus. A number of cellular changes as a consequence of moisture stress have now been directly linked to the perception of moisture stress by plant cells. The important ones among them are:

1. Alterations in the concentration of solutes and solvent in the cell.

Water deficit causes an immediate drop in the cellular water potential due to the concentration of solutes. The levels of sodium, calcium, potassium, chlorine, ammonium, magnesium, sulphate and phosphate rise in relation to water. This altered solute potential affects the cytoplasmic buffering capacity and activates a number of proteins. Some of these proteins are initiators of complex signaling pathways, leading to altered gene expression.

2. Changes in the cell wall plasma membrane connections.

In a turgid cell, the cell wall and plasma membrane are closely associated. Certain transmembrane glycoproteins and cell wall lectins are part of the cell wall-plasma membrane continuum. During moisture stress the cell tends towards flaccidity and this can activate mechanosensing elements due to the break in the cell wall-plasma membrane continuum. Integrins, Fibronections and stretch activated channels are the well characterized mechanosensing elements and their role in animal cells and fungi are well understood. Some such potential signal perceiving molecules in plants are the Wall Associated Kinases (WAK-3) and Lectin like Receptors (Ath. Lec. RK-1) of *Arabidopsis*, Tangled Gene of Maize etc. [reviewed in Miller *et al.*, 1997].

3. Denaturation of Proteins

Water deficit can lead to the accumulation of misformed and damaged proteins. This could be a signal for the activation of pathways involved in degradation of defective proteins and synthesis of molecules essential for stabilization of existing proteins [Belknap *et al.*,1992].

4. Disruption of Membrane Integrity

Disruption or membrane integrity leads to leakage of cellular content. This can directly activate pathways for repair mechanisms [reviewed in Bray.,1997].

5. Accumulation of Toxins

Impairment in the normal biochemical pathways in a plant cell as a result of stress, leads to the accumulation of toxic metabolic by-products and highly reactive intermediates (especially the reactive oxygen species) which can cause extensive cell damage. Cellar increases in the levels of these toxins during stress may activate toxin scavenging pathways [reviewed in Inze and Van Montagu, 1995].

6. Osmosensors

Receptors involved in the mechanism by which bacterial and yeast cells sense osmotic cells have been identified. These molecules have been termed as osmosensors.

The prokaryotic 'two-component' signal transduction systems are composed of a transmembrane sensor (eg. *E.coli* osmosensor EnvZ) which can recognize environmental stimuli and a cytosolic 'response-regulator' which can be activated by the sensor by phosphorylation, to act as a transcriptional activator [Bourret *et al.,* 1991].

An osmosensing mechanism in the budding yeast (*Saccharomyces cerevisiae*) involves both a two-component signal transducer (Slnlp, Ypdlp and Ssklp) and a MAP kinase cascade (Ssk2p/Ssk22p, Pbs2p and HOGlp). This mechanism is active under high osmolarity conditions leading to the High Osmolarity Glycerol (HOG) synthesis response [Posas *et al.*, 1996].

A similar osmosensing mechanism is expected to operate in higher plants in response to water deficit. An Arabidopsis SLN1 homolog, ATHK1, was recently shown to compliment yeast Sln1 mutants and function as osmosensors in yeast [Shinozaki and Yamaguchi-Shinozaki,1997]. An Arabidopsis ethylene-responsive gene ETR1 was found to function similar to the prokaryotic two-component regulators [Chang *et al.*, 1996]. Cloning of other osmosensors involved in the signal perception of water stress in plants is in progress based on the knowledge of osmosensors in yeast and bacteria [Shinozaki and Yamaguchi-Shinozaki,1997].

Signal Transduction Events

Following the cellular perception of water loss, a signaling mechanism must be activated to induce specific genes. Not all stress related gene are induced under the same conditions or in the same cell types, and thus there appears to be several different signaling mechanisms [Bray,1997]. It appears that dehydration triggers the production of abscisic acid (ABA) [reviewed in Giraudat *et al.*,1994], which in turn induces various genes. Several other genes that are induced by water stress are not responsive to exogenous ABA treatment. These findings suggest the existence of ABA Independent and ABA Dependent signal transduction cascades between the cellular perception of water deficit and the expression of specific genes. Genetic analysis of osmotic and cold stress signal transduction in Arabidopsis has revealed the interactions and convergence of the ABA dependent and ABA independent pathways [Zhu *et al.*,1997]. Dissecting the complex network of osmotic stress signaling is a highly stimulating and active area of research in stress physiology.

Abscisic Acid (ABA) and Moisture Stress Signaling.

ABA is a plant harmone. It is involved in leaf senescence, seed development and bud dormancy and response of vegetative tissues to environmental stress. ABA is a sesqiuterpenoid, with Mevalonic Acid as its precursor. ABA biosynthesis occurs via a carotenoid pathway. Many genes and intermediates in ABA biosynthesis have been identified [reviewed by Giraudat *et al.,* 1994].

Water deficit in the soil leading to drop in root water potential quickly triggers ABA biosynthesis. It appears that the genes involved in ABA biosynthesis are the first to be activated by drought. The biochemical signals of the onset of water deficit is then conveyed to shoots in the form of ABA via the xylem. The role of ABA in root elongation during drought has been linked to the root osmotic adjustment especially by the accumilation of proline by the activation of Δ`-Pyrroline-5-Carboxylate Synthase, and influencing the wall yielding properties of root cell by the action of Xyloglucan ExoTransferase. ABA can also help the plant to cope with drought by reducing leaf expansion and there by reducing leaf surface area for water loss. ABA controls stomatal aperture by rapidly regulating ion transporters, causing an outwardly directed potassium flux, coupled by increase in the cytosolic calcium levels, causing a drop in guard cell turgor leading to stomatal closure [reveiwed in Giraudat *et al.*, 1994].

There is evidence that ABA can be recognised both inside and outside the cell, though the exact nature of receptors remains elusive [Bray,1997].

A number of water stress inducible genes are up-regulated by the exogenous application of ABA [Ingram and Bartels,1996]. Endogenous levels of ABA increase significantly in many plants under drought and high salinity conditions [Giraudat *et al.*, 1994]. There exists two ABA dependent signalling pathways for gene induction during water stress. In one of the ABA-dependent pathways (Fig. No.1), water stress inducible genes do not require protein biosynthesis for their expression [reviewed by Shinozaki *et al.*,1996]. These dehydration inducible genes contain potential ABREs (ACGTGGC) in their promoter regions. An ABRE functions acts a cis-acting DNA element involved in ABA regulated gene expression. ABREs were first identified in wheat *Em* and rice *rab* genes, and the ABRE-DNA-binding protein EmBP-1 was shown to encode a bZIP protein. Also, a coupling element is required to specify the function of the ABRE, constituting an ABA responsive complex in the regulation of the HVA22 gene [Shen and Ho, 1995]. However it has not been resolved how ABA activates bZIP proteins to bind to ABRE and initiate transcription of ABA inducible genes.

There are several cis acting elements other than ABRE that function in ABA responsive gene expression not only under water stress conditions but also in seed desiccation. The Sph box and GTGTC motifs regulate ABA and VP1-dependent expression of the maize C1 gene, whose product is an MYB-Related transcription factor [reviewed in McCarty, 1995].

In one of the two ABA dependent pathways, biosynthesis of protein factors is necessary for the expression of water stress inducible genes (Fig. No. 1). The induction of an Arabidopsis drought inducible gene rd22 is mediated be ABA and requires protein biosynthesis for its ABA dependent expression [Shinozaki *et al.*,1996]. The rd22 bp1 gene is induced by salt and drought stress.

Several bZIP transcription factors from rice, maize and Arabidopsis plants respond to cold, dehydration and exogenous ABA treatment. These bZIP proteins bind to G-box like sequences. These results suggest that ABA inducible bZIP proteins are also involved in one of the ABA-dependent pathways. Many stress and ABA inducible genes encoding various transcription factors have now been reported.

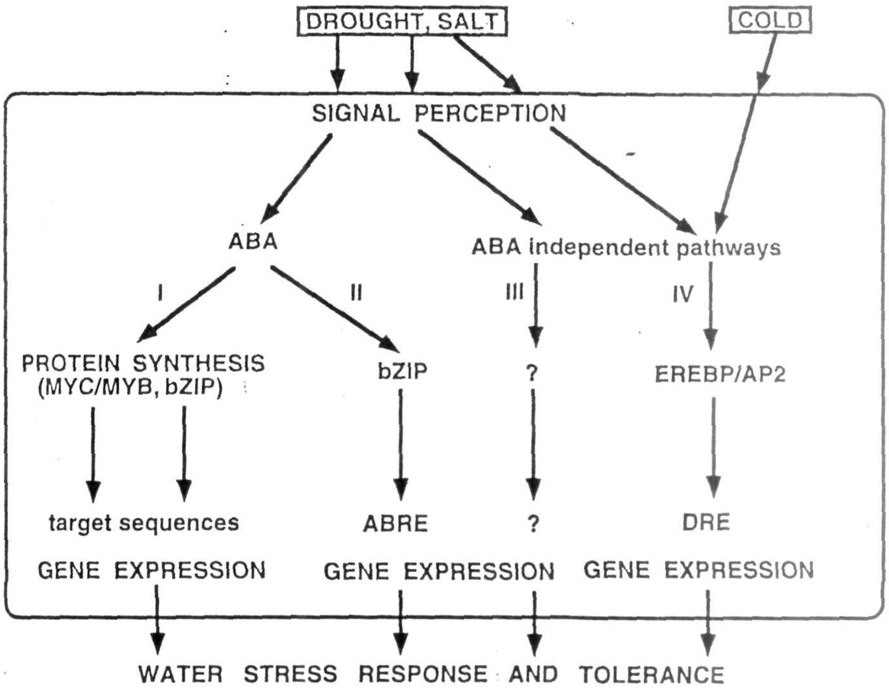


Fig.No.1: Signal Transduction Pathways between the perception of water-stress signals and gene expression. (Shinozaki etal., 1997).

These transcription factors are thought to function in the regulation of ABA-inducible genes, which respond to water stress rather slowly after the production of ABA inducible transcription factors (Fig. No 1) [reviewed by Shinozaki *et al.*,1996].

ABA Independent Gene Expression During Water Stress

Several genes are induced by drought, salt and cold in aba (ABA-Deficient) or abi (ABA-Insensitive) Arabidopsis mutants. This suggests that these genes do not require ABA for their expression under cold or drought conditions but do respond to exogenous ABA. These genes include rd29-A (or lti78 and cor78), kin1, cor6.6 and cor47. Among them the expression of a drought inducible gene for rd29A was extensively analysed. At least two separate regulatory systems function in gene expression during drought. One is ABA-dependent and the other is ABA independent. A nine base pair conserved sequence, TACCGACAT, termed Dehydration Responsive Element (DRE) is essential for the regulation of the induction of rd29A. The rd29A promoter also contains ABRE, which probably functions in ABA responsive expression [Zhu *et al.*,1997]. Protein factors that specifically interacts with DRE sequence have been identified and several cDNAs have been cloned for DRE binding proteins. All these proteins contain a conserved DNA binding motif [reviewed in Shinozaki *et al.*,1997].

Intermediate signaling molecules in the signal transduction cascades from the sensing of water deficit to the induction of various genes not yet been extensively studied. The role of Calcium and IP3 as second messengers in the stomatal response to water deficit have been demonstrated [reviewed in Cote`, 1995].

Several Protein kinases, Calmodulins, G-proteins and transcription factors have been identified in various signal transduction pathways in plants. AtPLC1, ATCDPK1, ATCDPK2 , components of the MAPK cascades and other proteins have been found to be induced by a variety of environmental stresses such as light, freezing, drought, high salinity, heavy metals, mechanical stimuli etc. [reviewed in Shinozaki *et al.*,1996]. Molecular and genetic approaches are providing valuable insights into the complex nature of signaling events during salinity and drought.

The Role of Gene Products Induced by Salinity and Drought

Salinity and Water deficit have shown to induce the expression of a number genes. These gene products have either a regulatory role in gene expression or a functional role in the adaptive responses of plant cells to the stress. The role of water deficit induced gene products in recognition of stress and in regulating gene expression has been dealt with in the previous section. The functional role of gene products will be describe as follows with emphasis on the genes whose expression have been monitored during salinity and drought, in the present study.

Osmotic Adjustment

The cellular water potential Ψw is dependent on the pressure/turgor potential Ψp and osmotic potential Ψs according to the relationship $\Psi w=\Psi p-\Psi s$. Due to water deficit, both Ψw and Ψs decrease (i.e. become more negative) and Ψw decreases at a greater rate than Ψs. However to facilitate drop in Ψw, without the actual loss of water or in other words to conserve moisture, solutes are actively accumulated in the system during the course of cellular water loss (thereby decreasing Ψs) and this process is termed as Osmotic Adjustment or Osmoregulation. Several compatible solutes are accumulated during water stress. These compatible solutes are highly water soluble, carry no net charge at neutral pH and are extremely hydrophilic. They are retained by the plasmamembrane even against a large concentration gradient. They however do not alter the structure or inhibit the function of cellular enzymes.

The osmo-protectants accumulated not only contribute to a drop in cellular Ψw, but also maintain cell volume, protect membranes, macromolecules, enzyme activity and stabilize protein tertiary structures. Compatible solutes like mannitol for example act as substrates for oxidation by hydroxyl radicals, thereby reducing cellular damage during salinity and water deficit [Garcia et al., 1997].

The major solutes involved in cellular osmoregulation are ;

1. Proline [reviewed in Taylor, 1996] : an amino acid.
2. Glycine Betaine [Grumet and Hanson, 1986] : a quaternary ammonium compound.
3. Sugar alcohols like Sorbitol, Mannitol [Tarczynski *et al.*, 1993] and cyclic polyols like pinitol etc.
4. Carbohydrates like Trehalose, Polyfructose, Glucose, Sucrose etc. [Garcia *et al.*, 1997].
5. Inorganic ions like potassium, calcium etc.

The biosynthetic pathway of the major osmolytes have been dissected and studied extensively. The genes encoding key enzymes regulating their biosynthesis during moisture stress have been cloned and in many cases over-expressed in transgenic plants to asses stress tolerance.

Ion Homeostasis

Normally there is a constant ionic flux, into and out of the plant cells, in a controlled fashion, with a net flux adjusted to accommodate the cytoplasmic and organellar buffering requirements. In plant cells exposed to high salinity, the kinetic steady states of ion transport for Na+ and Cl- and other ions such as K+ and Ca2+, are disturbed [Binzel *et al.*,1988].

High apoplastic levels of Na+ and Cl- alter aqueous and ionic thermodynamic equilibria, resulting in hyper osmotic stress, ionic imbalance and toxicity (reviewed in previous sections). Thus it is vital for plants to reestablish cellular ion homeostasis for metabolic functioning and growth, to adapt to osmotic stress in saline environment. Intracellular compartmentation of ions and active exclusion against prevailing ionic gradients are the important means to achieve ion homeostasis [Hasegawa *et al.*,1995].

The transport protein that mediate ion flux can be generally classified as Pumps, Carriers and Channels [Sussman and Harper, 1989]. Pumps directly utilize metabolic energy for the vectorial transport (e.g. Na+-ATPase, Ca2+-ATPase etc.). Carriers couple uphill transport of one solute to the downhill movement of another, either in the same (Symporter) or opposite (Antiporter) direction (e.g. Na+/H+ antiporter and K+/H+ symporter). Channels on the other hand mediate passive transport, i.e. movement down a free energy gradient (e.g. K+ inward), although the movement may be electrophoretic flux, resulting from an energy dependent process [reviewed in Niu *et al.*,1995]

The energy dependent flux of most ions in plant cells is mediated by the $\Delta\mu H+$ (H+ electrochemical gradient), that generates the pH gradient (ΔpH) and is principally responsible for $\Delta\Psi$ (membrane potential) [Sze, 1985]. Under typical physiological conditions, homeostatic concentrations of ions in the cytosol are 100-200mM K+, 1-10mMNa+, 1-10 mM Cl- and 100-200 nM Ca2+ [Binzel *et al.*,1988]. During salinity, the uptake of Na+ occurs passively across the membrane (mediated by membrane channel protein). The efflux of Na+ is presumably due to the activities of a Na+/H+ antiporter [Niu *et al.*,1995]. Cl- uptake is assumed to be coupled to a H+ symporter.

The exact mechanism of Na+ influx across the plasmamembrane is unknown. Na+ acts a competitor of K+ uptake. System-2 which mediates uptake at higher external K+ concentrations (in mM) has a less pronounced K+/Na÷ selectivity. There have been suggestions that Na+ uptake may also occur through outwardly rectyfying cation channels [reviewed in Niu *et al.*, 1995]. Any regulatory process that decreases the open probability of these outward rectifying cat ion channels would reduce both the entry of Na+ into the cell and the leakage of K+ out of the cell thus hypothetically representing a mechanism of adaptation to NaCl stress. Also at the whole plant level, it is generally accepted that increased K+/Na+ selectivity during uptake and reduced translocation from the root to shoot contribute to overall salt tolerance.

Genotypes that are most adapted to salt tightly regulate ion uptake across the plasmamembrane at a rate that is compatible with the capacity for vacoular compartmentation [Binzel *et al.*, 1988]. Thus transport process at the plasma membrane and tonoplast that regulate ion influx and efflux, particularly those involved in the control of Na+ uptake and compartmentation are of crucial importance to salinity adaptation [Niu *et al.*, 1995]. Active efflux of Na+ from the cytosol, across the plasmamembrane and out of the cell or across the tonoplast and in to the vacoule, has been linked to the ΔpH generated by antiporters located in these membranes. The gene expression and physiological responsiveness of plasma membrane H+-ATPase to NaCl is positively correlated with salt tolerance, since halotolarent cells and plants exhibited higher transcript levels and or pump activities than intolerant counter parts [Braun *et al.*, 1986]. The plasma membrane H+ ATPase mRNA accumulation is induced only during NaCl adaptation and low mRNA levels were seen after adaptation [Niu *et al.*, 1993]. In tobacco cells, a 70-kD (subunit B) mRNA of the vacoular H+ATPase accumulated in response to NaCl treatment and the salt induced tonoplast H+ATPase activity has been linked to stress adaptation [Narasimhan *et al.*,1991].

Little is known about the intracellular uptake and vacoular compartmentation of Cl- [Binzel *et al.*, 1988]. Na+ influx depolarizes the ΔΨ across the plasma membrane and, Cl- can be taken up passively through an anion channel. Vacoular compartmentation of Cl- is mediated by channels, with transport by the electrophoretic flux generated by proton pumps across the protoplast under saline conditions. Efficient vacuolar compartmentation of Cl- is an essential adaptation for NaCl tolerance [Niu *et al.*, 1995].

By the differential screening of cDNAs derived from Mercuric Chloride treated and untreated plants, a transcriptionally activated cDNA clone, designated as CHEM 8 (345 bp) and sharing high structural homology to an Arabidopsis protein was identified in maize [Didierjean *et al.*, 1996]. This gene has also been shown to be present in tobacco, mammals, drosophilla, bacteria and yeast, and is homologous to an evolutionarily conserved gene family of Membrane Channel Protein [Yamamoto *et al.*, 1990]. This protein is believed to be involved in the uptake and transport of ions by plant cells. CHEM 8 was constitutively expressed in maize, but the transcript levels increased upon heat stress, mercuric chloride treatment, salinity, polluted rain water and UV radiation. This protein is believed to maintain ion homeostasis in plants during abiotic stresses..However its exact role is yet to be deciphered [Didierjean *et al.*, 1996]. Hence the movement of Na+ and Cl- into the cell and during sub cellular compartmentation is a function of the presence and activity of membrane channel proteins.

The 7a cDNA from Pea encodes a polypeptide with characteristic features of ion channels while the RD28 cDNA (Arabidopsis) and H2-5 cDNA (*C.plantagineum*) encode putative water channel proteins [Ingram and Bartels,1996]. Their levels are upregulated during drought.

Degradation of Defective Proteins

Abiotic stress especially drought and salinity severely affect the structural and functional properties of proteins (discussed in the previous sections). Imbalance of ion during stress create conditions which does not permit the formation the formation of intra-molecular bridges that determine the final conformation of many proteins. These mis-formed and non-functional proteins tend to accumulate. Heavy salt influx, leads to loss of function and denaturation of existing proteins. Salinity in bacteria can accelerate polysomal decay and degradation of freed ribosomal proteins. The prolonged stress leads to impairment of general protein synthesis. Accumulated defective proteins may form inactive complexes with otherwise functional proteins. This accumulation of defective proteins is a burden to the cell and have to be degraded to restore normal cellular activities [Claes *et al.*,1990].

Genes encoding proteins with sequence similarity with proteases, and which are induced by drought have been isolated from both Pea [Guerrero *et al.*,1990] and Arabidopsis [Shinozaki *et al.*,1993]. One of the functions of these enzymes could be to degrade the proteins irreparably damaged by the effects of drought [Ingram and Bartels, 1996]. During early drought in Arabidopsis, there is an increase in the levels of mRNA encoding Ubiquitin extension protein [Shinozaki *et al.*,1993], a fusion protein from which active Ubiquitin is derived by proteolytic processing. Ubiquitin is a small highly conserved protein, found in all eukaryotes. Within the cell, Ubiquitin is covalently linked to substrate proteins often targeting them for degradation via the Ubiquitin Pathway.

Heat induced marker genes transcribed from a maize poly-ubiquitin promoter has been demonstrated in rice [Christensen A.H., *et al.*,1992]. Increased levels of poly-ubiquitin mRNA was found in response to $HgCl_2$, wounding, cold and heat stress in maize [Didierjean *et al.*,1996].

Chaperone Activity

A system subjected to a short non-lethal heat treatment will subsequently be able to withstand a higher temperature and survive, whereas it would not be able to do so without the pretreatment. This survival at high temperatures is the result of acquired thermo-tolerance and is predominantly mediated by a set of proteins called Heat Shock Proteins (HSPs). The heat shock response is common to bacteria, animals and plants and is considered to be an adaptive mechanism to help the cell, organ or organism to cope with an unfavorable temperature condition [reviewed in Morimoto *et al.*,1994].

A diverse group of at least 11 different classes of proteins that show transient increased expression in response to a rapid rise in temperature [Nover and Scharf, 1997]. Many of the different classes of heat shock proteins function in protein metabolism as molecular chaperones. Some function in biogenesis by assisting in folding process and translocation into organelles; others are involved in the assembly and disassembly of oligomers and proteolysis of damaged or misfolded proteins [Morimoto *et al.*,1994], translocation of preproteins across membranes and even in gene regulation [reviewed in Rassow *et al.*,1995].

The HSP 70 (proteins of ~70 kD) class of heat shock proteins is a major protein induced by heat shock and are often linked to the characteristics of acquired thermo-tolerance in Arabidopsis [Lee *et al.*,1996]. One of the ways in which HSP 70 might function in acquired thermo-tolerance would be to stabilize heat-liable proteins and super-molecular structures [Minton *et al.*,1982] and prevent them from becoming irreversibly denatured or damaged. HSP 70 can prevent irreversible aggregation of non-native proteins by binding to determinants not normally solvent accessible in native conformations. The binding of HSP 70 non polar regions in non native peptides can prevent coalescence into an insoluble aggregate, thereby maintaining the peptide in a form that may subsequently refolded into a native conformation. Their role as chaperones of non native nascent polypeptides has many facets, including assisting at several stages along the protein biogenesis pathway, stabilizing folded proteins, and in participating in degradative process of abnormally folded polyeptides. Since several classes of HSPs are evolutionarily highly conserved, they have become important tools in our understanding of molecular evolution of organisms [Guy *et al.*,1998].

Several abiotic stresses apart from high temperatures including salinity and drought hamper the post translational processing of proteins and formation of functional proteins. Hence there is a need for HSPs to fulfill the role of chaperones during stress. By employing the differential screening of cDNAs derived from Mercuric Chloride treated and untreated plants of maize, a transcriptionally activated cDNA clone, designated as CHEM 3 was obtained. On sequencing it showed a very high degree of homology to a maize heat shock protein (HSP 70) [Rochester *et al.*,1986]. Subsequent studies indicted a substantial transcriptional activation of this gene by heat an UV light. Moderate to low transcriptional activation of this gene was observed in response to other environmental stresses [Didierjean *et al.*,1996].

In another search for stress related genes in maize, during heavy metal toxicity, a differential screening of mercuric chloride treated maize cDNA library was performed. Among the positive clones, one, designated as CHEM 7 was found to be expressed at a significantly higher level in stressed plants. CHEM 7 showed complete identity to maize Cyclophilin (CyP) [Didierjean *et al.*,1996]. A protein from mammalian thymocytes which specifically binds to cyclosporin A (CsA) was identified [Handschumacher *et al.*, 1984] and termed as cyclophilin. Later CyP was found to be identical to a porcine peptidyl - prolyl *cis-trans* isomerase (PPIase or rotomase), that catalyses the *invitro cis-trans* isomerization of proline peptide bonds in oligopeptides and accelerates the slow folding of several proteins. *In vivo*, this class of enzymes is believed to play an important role in accelerating the rate at which proteins fold in to their native conformation. CyP in mammals have a role in immuno-suppression (especially T-cell mediated immune response) and is widely used in human organ transplantation to prevent allograft rejection.

In higher plants, such as tomato, maize, bean, *Brassica napus*, cDNAs encoding proteins that are highly homologous to the mammalian CyP have been isolated [Marivet *et al.,* 1992, Didierjean *et al.*,1996].

It is now suggested that CyP might play a role during stress conditions as CyP mRNA synthesis is significantly stimulated by chemical stresses in maize and bean plants. Apart from a good basal level of expression, this gene is transcriptionally activated upon treatment of seedlings of maize with 170mM NaCl, 4°C and 42°C, UV Light and polluted water [Marivet *et al.*, 1992; Didierjean *et al.*,1996].

Under stress, it is suggested that higher amounts of CyP might be needed to accelerate the folding steps of the nascent polypeptide chains in order to decrease the risk of proteolytic degradation of partially folded chains [Gasser *et al.*, 1990; Marivet *et al.,* 1992; Didierjean *et al.*,1996].

Enzymes involved in the scavenging of Active Oxygen Species (AOS)

One of the important mechanisms by which plants are damaged during adverse environmental condition is by the excess production of AOS, such as superoxide radical, singlet oxygen, hydrogen peroxide, hydroxyl radical and hydroperoxyl radical. Environmental adversities such as ozone, sulfur dioxide, heavy metals, high temperature, chilling, UV light, drought, salinity etc. can lead to oxidative stress and the sensitivity of plants to stress is shown at the cellular level by the appearance of oxidative degradation products [Inze and Van Montagu, 1995].

Tolerance to desiccation and salinity was found apparently to be related to the ability to maintain a balance between free radical production and scavenging reactions [Bewley, 1979]. To maintain this balance, the levels of enzymes involved in free oxygen radical scavenging is vital during stress.

Superoxide is formed by the univalent reduction of oxygen to superoxide. Several enzymes like NADPH oxidase, xanthine oxidase, aldehyde oxidase, peroxisomal oxidases etc. catalyze the partial reduction of oxygen. The non enzymatic source of superoxide is the membrane located electron transport chains (ETC) of chloroplast, mitochondria, endoplasmic reticulum and plasmalemma [Van Montagu *et al.*, 1995]. Most environmental stress destabilize membranes, affecting the normal ETC. The electrons, instead of reaching the designated acceptor from the respective donor, are misdirected, and can reach oxygen, resulting in the formation of superoxide. In the chloroplast, superoxide radicals are produced by the reaction of molecular oxygen at Photosystem I via the Mehler reaction. Oxygen generated in the chloroplasts during photosynthesis can accept electrons passing through the photosystems. Univalent transfer of electrons to oxygen is more likely to occur from the electron acceptor of photosystem I, and more particularly from ferredoxin [Asada and Takahashi, 1987]. During salinity and drought, due to reduced stomatal conductance, sufficient CO2 is not available for the NADPH generated during the light reaction to be used up for carbohydrate biosynthesis.

However there is a demand for NADP to be recycled and hence NADPH oxidase activity increases, resulting in superoxide as a byproduct.

Superoxide is highly reactive and can directly inactivate important macromolecules of the cell. Efficient scavenging of super oxide is vital for a plant cell to survive and function normally, especially during stress. Plants have evolved non-enzymatic and enzymatic protection mechanisms to scavenge superoxide radicals [Inze and Van Montagu, 1995].

The non-enzymatic mechanisms to scavenge superoxide is by the antioxidants. Ascorbic Acid (Vitamin C), α-tocopherols, carotenoids, sulphydryl antioxidants (glutathione, dithiothreitol, cysteine) etc. [Alscher, 1989], in the reduced forms can directly act as antioxidants. Their levels increase under exposure to stress. Apart from a direct role, they activate the transcription of a panoply of stress genes, such as Cu-Zn SOD genes [Herouart *et al,* 1993;1994] and also genes encoding enzymes of the phenyl-propanoid pathway [Wingate *et al.*, 1988].

Superoxide dismutases (SOD) are metallo-enzymes that catalyze the dismutation of superoxide, according to the following reaction:

$$2O_2^{\cdot -} + 2H+ \rightarrow H_2O_2 + O_2$$

The isozymes of SOD differ according to their metal cofactor ;

a. Cu-Zn SOD : it is the major isoform. It is present in both the cytoplasm and chloroplast. Cu-Zn SOD are classified in rice as: [Kaminaka *et al*, 1997].
   1. Cu-Zn SOD I : it is the major isozyme in leaves and is plastidic. Chloroplast SOD is localized in the stroma and in the interthylakoid space. It is encoded by the nuclear genome and has a signal peptide for subcellular targetting. Its expression is enhanced by paraquat in light.
   2. Cu-Zn SOD II, III and IV : found mostly in seed embryos, etiolated seedlings and very low levels in leaves. The mRNA

levels are enhanced mostly by heat shock, chilling, paraquat treatment and drought [Perl-Treves and Gulan, 1991].

b. MnSOD

The manganese SOD is widely distributed among prokaryotic and eukaryotic organisms. In eukaryotes it is most often found in the mytochondrial matrix [Bowler *et al.*, 1989]. In plants, MnSOD encoding nuclear gene was first isolated and characterized from tobacco by Bowler in 1989. The protein was highly homologous to eukaryotic MnSODs from other organisms and also contained a N-terminal leader sequence resembling a transit peptide for mitochondrial targeting .

Production of superoxide radicals from the mitochondrial respiratory chain is known to occur from NADPH dehydrogenase and from ubiquinol-cytochrome c reductase. In times of high respiratory activity, a concomitant need for SOD activity within the mitochondria is expected [Bowler *et al.*,1989]. This has been proved by the dramatic induction of MnSOD during tissue culture (especially with higher sucrose and glucose levels), for protection against severe oxidative stress. MnSOD mRNA accumulation was also observed in response to *Pseduomonas syringae* infection. Hence MnSOD is induced whenever there is increased production of superoxide in the mitochondria [Bowler *et al.*,1989]. It has been suggested that superoxide radicals trigger a specific molecule in each subcellular compartment, which is capable of acting as a signal to induce nuclear gene(s) encoding for the particular superoxide dismutase(s) associated with that compartment.

During abiotic stress, in the mitochondria, electrons from the respiratory ETC are in part deviated to oxygen [Asada and Takahasi, 1987].

Regulation of MnSOD expression occurs mainly at the level of transcription [Scandalios, 1987; Bowler *et al.*,1989]. During stress condition, induction at the mRNA level may not always be accompanied by an increase in protein, possibly due to enchanced protein turnover [Williamson and Scandalios.,1992]. Transcriptional activity is a valid parameter to asses MnSOD gene induction, during stress, in different parts of the plant and this approach has been successfully used [Herouart *et al.*,1994]. MnSOD expression is induced by ethylene, salicylic acid and sucrose [Bowler *et al.*, 1989].

Succinate dehydrogense activity (involved in Krebs cycle of respiration, conversion of Succinate to Fumaric acid, with reduction of FAD to $FADH_2$) coincide with MnSOD expression in most parts of the plant, confirming that superoxide production in mitochondria is primarily caused by the leakage of electrons from the ETC. Respiratory rates are also generally correlated to cytochrome oxidase activity. This is the terminal enzyme, catalyzing the reduction of oxygen to water. MnSOD and cytochrome oxidase activity are proportional [Bowler *et al.*, 1989].

Paraquat (a herbicide), chilling, heat shock stress, increases electron leakage from mitochondria and chloroplast ETCs, and cause a dramatic increase in MnSOD mRNA levels [Tsang *et al.*,1991].

Since the formation of AOS accelerate senescence, MnSOD levels are low in mature senescent leaves of barley [Casano *et al.*,1994].

Higher MnSOD mRNA levels in younger leaves has been attributed to the reduced sensitivity response of older leaves to photooxidation [Casano *et al.*,1994].

The effects of ABA on the accumulation of Sod3 (MnSOD) in maize indicated the induction in steady state levels of Sod3.2, Sod3.3 and Sod3.4. The response of Sod 3.3 gene to ABA was also similar to Lea genes and the accumulation of this transcript is a time dependent increase in the presence of ABA [Zhu and Scandalios, 1994].

Transgenic tobacco lines overexpressing MnSOD in the chloroplast, were shown to be more tolerant to photooxidative stress and methylviologen [Slooten *et al.*,1995]. The transgenic plants were shown to enhance the stromal antioxidant system.

Trasnsgenic alfalfa overexpressing MnSOD in the chloroplast, tended to have reduced injury from water deficit stress. Field trials indicated the yield and survival of transgenic plants were better [McKersie *et al.*,1996].

c. FeSOD

The iron SOD is present in prokaryotes and within the plastids of some plants [Bannister *et al.*,1978]. The FeSOD is similar to the MnSOD in its primary, secondary and tertiary structures. Since FeSOD is very similar to MnSOD and is believed to have arisen from the same ancestral enzyme, whereas Cu-Zn SOD appears to have evolved separately [Bannister *et al.*,1987]. This metallo-protein catalyzes the dismutaion of superoxide radicals to hydrogen peroxide and oxygen in the chloroplast . [Van Camp *et al.*1990] have characterized FeSOD cDNAs from plants.

Formation of AOS in the chloroplast is enhanced when carbon assimilation is inhibited. Oxidative stress in the chloroplast, for example arises when high irradiance is combined with chilling temperatures, drought or heat [Bowler *et al.*,1992]. Salt stress is another condition that that may give rise to plastidic oxidative stress, as described earlier.

Antioxidant enzymes activity (including FeSOD) increases in response to stress, and there is a correlation of salt tolerance with antioxidant enzyme levels. [Gosset *et al.*,1994; Olmos *et al.*,1994; Hernandez *et al.*, 1995].

Overproduction of SOD in the chloroplast enhances tolerance to various abiotic stress [McKersie *et al.*,1996; Foyer *et al.*,1994; Sen Gupta *et al.*, 1993]. Transgenic tobacco plants overproducing either FeSOD or MnSOD in the chloroplasts indicate that FeSOD provides better protection against methyl viologen dependent oxidative stress than MnSOD [Van Camp *et al.*,1996]. FeSOD overexpressing transgenic tobacco were more tolerant to salt stress than control [Van Camp *et al*, 1996].

Chloroplasts have plastidic Cu-Zn SOD in addition to FeSOD [reviewed in Bowler *et al.*, 1994]. IAA and ABA had no effect on sodB (FeSOD) expression levels in tobacco. However there exists a discrepancy between the harmone induced changes in the FeSOD transcript levels and the constant activity of FeSOD enzyme and this has led to suggestions that SOD activity is regulated post-transcriptionally, for example, by regulating efficiency of translation and /or protein stability [Kurepa *et al.*,1997].

Catalases

Catalases and peroxidases are the two major systems for the enzymatic removal of hydrogen peroxide. Catalases dismutate H_2O_2 to H_2O and O_2. Catalases have been mainly associated with the removal of H_2O_2 in the peroxisomes.

Plant cells have many potential sources of H_2O_2 during environmental stress:

a. By the dismutation of superoxide.
b. The major source of H_2O_2 in the peroxisomes are the various oxidases, which include acyl Co-A oxidase, glycolate oxidase and urate oxidase. Acyl Co-A oxidase is involved in the β oxidation pathway of fatty acids. Glycolate oxidase converts glycolate produced in the chloroplast during photorespiration to glyoxylate and urate oxidase is involved urea metabolism. Glycolate oxidase is found in peroxisomes of photosynthetic cells, which are in close contact with chloroplast and mitochondria.

c. Oxidases not present in the peroxisomes such as Oxalate oxidase, diamine oxidase and polyamine oxidase.

Environmental stress enhance H_2O_2 production in the cell. The toxicity of H_2O_2 is by three mechanisms:

1. H_2O_2 is a weak oxidant and can inactivate some enzymes directly oxidizing their sulphydril groups. E.g. glyceraldehyde-3-phosphate dehydrogenase (of the glycolytic pathway), fructose-bi-phosphatase and sedoheptulose bisphosphate (of the Calvin's cycle).
2. H_2O_2 reacts with ketoacids, releasing CO_2. Therefore H_2O_2 reduces CO_2 assimilation efficiency at high temperatures.
3. By the formation of OH. Hydroxyl radical is one of the most highly reactive radicals known to chemistry and can react instantaneously with proteins, lipids and DNA, causing rapid cell damage. Hydroxyl radicals are formed by the following reactions [reviewed extensively in Van Montagu *et al.*,1995 and Inze and Van Montagu, 1995] ;
   a. Haber Weiss reaction

   $$O_2^{-} + H_2O_2 \rightarrow OH^{-} + OH^{\bullet} + O_2$$

   b. Fenton- type reaction

$$H_2O_2 + \text{Reduced metal ion (} Fe^{2+}/Cu^{+}) \rightarrow OH^{-} + OH^{\bullet} + \text{oxidisedmetal ion}(Fe^{3+}/Cu^{2+})$$

The participation of catalase in photorespiration suggests that catalase is a determining factor for the protection of photosynthesizing cells against oxidative stress. As mentioned earlier, stress like drought and salinity limits CO_2 availability to photosynthesizing tissues due to reduced stomatal conductance, thus increasing photo respiration and increased H_2O_2 production [Willekens *et al.*,1995].

The generation of superoxide radicals in leaf peroxisomes have been proved [del Rio LA *et al.*,1992]. H_2O_2 can generate the highly reactive hydroxyl radical (OH) in the presence of Fe(II) and O_2.

Except CAT-3 of maize [Scandalios *et al.*,1980], all other plant catalases have been localized in the peroxisomes. The catalase seems to be a nuclear encoded protein with a conserved tri-peptide at the extreme carboxy terminus for peroxisomal or glyoxysomal targeting.

Molecular analysis of the catalase gene family and expression have been done in cotton, tobacco, castor bean and maize [reviewed in Willekens *et al.*,1995]. Among the catalases, Cat2 has been characterized to be rapidly induced by environmental adversities and is predicted to have a protective role [Willekens *et al.*,1994] during stress.

Cat 2 levels in maize leaves have been attributed to the reduced photo respiratory activity of C4 plants [Scandalios *et al.*,1994].

Catalases have been grouped into three classes based on their expression profile. Class I catalase is high in photosynthetic tissues and is positively regulated by light. Class II expression is highest in vascular tissues and are upregulated during environmental stress. Class III is found mainly in seeds and very young seedlings. Class I catalases are involved in the removal of H_2O_2 produced during photorespiration in photosynthesizing tissues. Class III are involved in H2O2 removal that is produced during fatty acid degradation in glyoxysomes.

Photo oxidative inactivation of catalases has been demonstrated in several species during light stress [reviewed in Willekens *et al.*,1995] and during high photo-respiratory activity there is a continuos demand for new synthesis of this enzyme, to compensate for losses [Fierabend and Engel,1986]

Chilling stress and high temperature can also cause photo-inhibition of photosynthesis and photo-inactivation of catalases [Fierabend J *et al.*,1992]. Tobacco mutants containing 40% more catalase activity showed only a 10% reduction in net photosynthesis, when shifted from 30 to 38°C compared to 20% of wild type plants [Willekens *et al.*,1994].

Studies on the transcriptional regulation of different antioxidant enzymes in response to ozone, Cat2 transcripts accumulated much faster and hence Cat2 is thought to be an important defense against environmental stress [Willekens *et al.*,1994].

During abiotic stress, the H_2O_2 produced can suppress CO_2 fixation and chloroplast enzymes containg sulphydryl groups were more markedly inhibited [Tanaka *et al.*,1982].

H2O2 is produced rapidly at the cell membrane during pathogen attack and has been implied in the activation of hypersensitive reaction by acting as a messenger molecule [Schraunder *et al.*,1992].

Sodium chloride at low concentrations of 0.5M or less stimulated the activities of catalase in the leaves of *Halimione portulaciodes* (L), a halophyte [Kalir and Paljakoff-Mayber, 1981] indicating that it has a protective role against oxygen toxicity caused by free radicals during salinity and other extreme climatic conditions.

Peroxidases

Peroxidases, along with catalases, forms the major system for the enzymatic removal of hydrogen peroxide. Peroxidases are classified according to their substrate specificity. Glutathione peroxidases (GPx) are vital for the removal of H_2O_2 in the cytosol and mitochondria of mammals [Thomas *et al.*,1990]. In plants Ascorbate peroxidases (APx) are crucial for the removal of H_2O_2. Three different isozymes have been characterized that are associated with the removal of H_2O_2 in the cytosol, stroma, and thylakoid of chloroplasts [Cressen *et al.*,1994].

The chemical reaction that is catayzed by peroxidases can be viewed in two different ways. On the one hand, peroxidases can be seen as enzymes that consume reduced substrates for the removal of H_2O_2. On the other hand, the oxidation of specific substrates could be the main function, in which case, the consumption of H_2O_2 ,as an oxidant, should be regarded as a rather fortuitous side effect [Willekens *et al.*,1995]. The peroxidase family in plants is far from being fully characterized and the physiological role of many plant peroxidases remain unclear [Welinder *et al.*,1993]. Yet, it is currently thought that the primary function of a large group of peroxidases lies in biosynthetic oxidation reactions, such as lignification and suberisation. These peroxidases generally do not have a high substrate specificity *in vitro* and are mostly referred to as guaiacol peroxidases, because they can use guiacol as an artificial substrate. The subcellular location has not been determined for all of them, but several isoforms are found in the extracellular matrix and in the vacoule.

Extracellular scavenging of H_2O_2 could be particularly relevant during biotic stress. One of the first events in plant pathogen interactions is the oxidative burst, i.e. a sudden and dramatic increase in the production of various reactive oxygen intermediates, including H_2O_2 by the host plant [Mehdy ,1994]. By analogy with the phagocyte oxidative burst in mammals, this oxidative burst in plants is believed to have direct anti-microbial activity. Although this strategy may be deliberately suicidal, with the aim of isolating the invading pathogen from living tissue, extracellular peroxidases could be crucial for the protection of neighboring host cells [Willekens *et al.*,1995].

Ascorbate peroxidases (Apxs) are the most important enymes for protection against H_2O_2 in the cytosol and chloroplasts [Asada, 1992]. They have a high specificity for ascorbate as a substrate and are almost exclusively found in the photosynthesizing organisms. Their primary function in chloroplasts is to remove H_2O_2 that is produced during the Mehler reaction, i.e. electron leakage from the photosystems to oxygen. The Mehler reaction will generate superoxide, which will be dismutated to O_2 and H_2O_2 by SODs. Effective scavenging of partially reduced oxygen species produced during photosynthesis will therefore require the concerted action of SOD and Apx. The fact that chloroplastic Apx occurs both as a thylakoid-associated and a stromal isoform emphasizes the importance of removing H_2O_2 directly at its site of formation [Miyake *et al.*,1993].

The monodehydroascorbate that is produced by APx can be directly reduced at the expense of NAD(P)H by monodehydroascorbate reductase. Monodehydroascorbate can also disproportionate spontaneously to ascorbate and dehydro-ascorbate. Ascorbate is regenerated from dehydroascorbate by a glutathione dependent dehydroascorbate reductase and the glutathione in turn is recycled by glutathione reductase. This cycle of ascorbate consumpion and regeneration is named the ascorbate-glutatione cycle or Halliwell-Asada pathway. Clearly each of the downstream activities for ascorbate regeneration is potentially limiting for APx activity in the chloroplast [reviewed in May *et al.*,1998].

With the exception of monodehydroascorbate reductase, all enzymes of the ascorbate-glutathione cycle were present in the cytosol of plant cells. The importance of APx for cytosolic H_2O_2removal is underscored by the fact that other H_2O_2 scavenging enzymes such as catalase or glutathione peroxidase (GPx) are absent in the cytosol of plants [Willekens *et al.*,1995].

The chloroplastic and cytosolic isozymes of APx show some distinct biochemical properties. The chloroplastic isozyme is more specific to ascorbate as electron donor, has a much shorter stability in ascorbate depleted medium and is more sensitive to various inhibitors than the cytosolic isozymes. Interestingly, 3-amino-1,2,4-triazole (3-AT), which is often used as an inhibitor of catalase, also inhibits APx. Again, the chloroplastic isozyme is more sensitive to 3-AT than the cytosolic one [Asada *et al.*, 1992]. The structural homology between cytosolic (cAPx), stromal (sAPx), and thylakoid APx (tAPx) has not been elucidated. Sequence data are only avaliable for the cytosolic isozyme which is also the best characterised isozyme in terms of expression. Drought, heat shock, paraquat (a superoxide generating xenobiotic), and ethephon (a precursor of ethylene) caused a strong increase in the steady state transcript levels of the cAPx from pea [Mittler and Zilinskas, 1992]. Apx activity was not induced to the same level as APx messenger. Surprisingly, immunodetected cAPx levels did not change in response to these stresses. This may suggest that only a portion of the cytosolic APx pool in pea exists as an active enzyme and that activation occurs upon stress treatment. Heat shock, paraquat and ethephon are also inducers of cAPx mRNA accumulation in Arabidopsis. The promoters of cAPx from pea and Arabidopsis show extensive similarity in the region around the TATA box. Both genes process heat shock elements (HSEs) a short distance upstream from the TATA box, and there is evidence that their induction by heat is regulated through traditional heat shock factors (HSFs) [Asada *et al.*,1992]

cAPx mRNA levels in Arabidopsis leaves was elevated on exposure to the air pollutants ozone and sulphur dioxide [Kubo *et al.*,1995].

Proteins with other roles

Changes in protein pattern of plant cells subjected to stress can be complex [Singh *et al.*, 1985]. In general, between 8 and 25 proteins show notable increases in abundance in salt stress suspensions or roots. Between 4 and 75 proteins decrease in amount disproportionately [Singh *et al.*, 1985; Hurkman and Tanaka 1987; Ramgopal 1987; Wincov *et al.*, 1989]. A numerically similar but molecularly distinct set of proteins can be induced by drought or PEG treatments [Singh *et al.*, 1985; Vartanian *et al.*, 1987]. The genes upregulated by drought stress and encoding polypeptides of known or unknown functions has been reviewed extensively in Ingram and Bartels [1996].

The genes encoding Late Embryogenesis Abundant (LEA) proteins are consistently represented in differential screens for transcripts with high levels during water deficit due to moisture and ionic stress in plants. A general structural features of the LEA protein is their biased amino acid opposition, which results in highly hydrophilic polypeptides and lacking in cysteine and tryptophan residues and located predominantly in the cytoplasm.

The LEA proteins are divided in to many groups based on the conserved domains as described from the dot matrix analysis with proteins from cotton [Dure *et al.*,1989]. Group 1 of LEA proteins (D-19 family) have enhanced water binding capacity (e.g. Em Protein of Wheat). Group 2/ Rab/dehydrin LEA proteins (D-11) family resembles chaperones and have been identified in rice, barley, maize and several dicots. Group 3 LEA proteins (D-7) family are predicted to have a role in ion sequestration. Group 4 LEA (D-113) proteins may be involved in the preservation of cellular structures. Group 5 of the LEA proteins have a role in ion sequestration. However there is no direct experimental evidence that LEA proteins can protect the cell and ameliorate the effects of drought stress [reviewed in Ingram and Bartels, 1996].

Several salt responsive proteins have been characterized from plant species such as rice [Claes *et al.*,1990] and tobacco [Singh *et al.*,1985]: Osmotin in dicots [Singh *et al.*, 1987], salT in rice roots [Claes *et al*,1990], rab21 in rice [Mundy and Chua, 1998] now termed as dehydrin. Upregulation of several other proteins, whose roles are not yet clear have been reported [Claes *et al.*,1990] in rice.

Osmotin, a thaumatin-like protein was characterized and found to be associated with osmotic adaptation in plant cells [Singh *et al.*,1987]. A cDNA clone (CHEM 4) coding for a Thaumatin like protein (TL-p) was isolated from a mercuric chloride treated maize cDNA library by differential screening [Burkard *et al.*,1992]. CHEM 4 was one of the heavy metal stress induced proteins, with a high structural homology to Thaumatin [hence referred to as Thaumatin-like, TL], a sweet tasting protein isolated from the fruits of *Thaumatococcus danielli* [Loeve *et al.*,1972]. TL proteins have also been found in tobacco, tomato, potato, barley and wheat [Burkard *et al.*,1992]. Thaumatin and TL proteins have been grouped under PR-5 family. This family also includes Osmotin and Permatin. Though TL-proteins accumulate when the plant is attacked by a pathogen, it has no antifungal activity [Ogata *et al.,*1992]. This PR-5 protein also has a sweet taste. In grapes, it accumulates at very high levels in conjugation with the onset of sugar accumulation and berry softening [Tattersall *et al.*,1997].

Though no α-amylase or protease inhibitor activity is associated with TL-p, it was found to inhibit the growth of *Trichoderma viride* and *Candida albicans.*

The chemical structure of CHEM4 indicates a terminal hydrophobic signal peptide of 25 amino acid residues and is suspected to be localised in the intercellular spaces like most other acidic PR proteins [Burkard *et al.*,1992]. The CHEM4 TL-p is rather small and hence this stress induced TL-p family might be specific for monocotyledonous plants. This TL-p protein was found to be upregulated by NaCl treatment (170mM) at the third hour of salinity in maize and reaching peak accumulation by 24 hours of stress [Burkard *et al.*,1992].

MATERIAL AND METHODS

III MATERIAL AND METHODS

The experiments were conducted at The Rockefeller Foundation funded Rice Transformation Laboratory, National Centre for Biological Sciences, Tata Institute of Fundamental Research, Indian Institute of Science Campus, Bangalore. The material and methods employed for the study are described in this chapter.

I Collection of Plant Material

a. Raising of Rice Seedlings

Seeds of IR64 and Rasi, the two varieties of *Indica* rice chosen for the study were dehusked and good seeds were selected. These were surface sterilized using 70% ethyl alcohol for 1 minute and 2% sodium hypochlorite for 20 minutes. Surface sterilized seeds were washed repeatedly with sterile distilled water to remove traces of sterilizing agents.

Circular sterile filter papers were placed in autoclaved plastic petriplates and moistened with 20ml sterile distilled water in the laminar flow hood. About 25 surface sterilized seeds were placed in each plate, and the lid was covered and the plates were incubated at room temperature.

The seeds on an average took 2 days for germination. After germination the seedlings were allowed to grow for one week. The plates were constantly monitored for contamination. Since the plant material was to be used for RNA extraction, plates with any sign of contamination was discarded. Petriplates were irrigated whenever necessary.

Nine day old seedlings were used for inducing salt and dehydration stresses.

b. Induction of Salt Stress

For the induction of salt stress, the water in the petriplates, containing 9 day old seedlings was replaced with 150mM NaCl solution. One, two, four, eight and sixteen hours were the different duration of salt stress chosen for the study. Samples were collected by excising the endosperm and separating the seedling into root and shoot. The plant material was immediately frozen in liquid nitrogen and stored at -80°C for RNA isolation later on.

c. Induction of moisture stress

Moisture stress was induced by allowing nine day old seedlings to desiccate gradually in inflated plastic bags at room temperature. Loss of weight of the seedlings was constantly monitored. Plastic bags were changed frequently to decrease humidity inside the bag. When the seedlings recorded 30% and 40% weight loss, samples were collected by excising the endosperm and separating the seedlings into root and shoot and freezing them.

d. Controls

Unstressed nine day old seedlings of Rasi and IR64, collected in the same manner as described above, were used as controls.

The plant samples at all stages were handled with gloves and RNase free instruments to avoid RNase contamination.

II. Isolation of Total RNA

b. Preparation for RNA extraction

The following precautions were taken to inhibit ribonucleases:

1. All glassware and heat resistant materials (Pestle and mortar, forceps etc.) were baked overnight at 200℃ in an oven.

2. 0.1% DEPC (diethylpyrocarbonate) was added to all solutions (except those containing Tris), incubated overnight after thorough shaking and then autoclaved.
3. All plastic-ware were treated with 10% H_2O_2 overnight, autoclaved and dried properly before use.
4. Clean disposable gloves were used at all stages of RNA extraction

b RNA Isolation

Single Step Method of RNA Isolation by Acid Guanidium Thiocyanate Phenol-Chloroform Extraction [Sacchi et.al.,1987] was employed to isolate total RNA. The procedure consisted of the following steps:

1. 0.5 to 1 gm of tissue was ground in liquid nitrogen using a pestle and mortar to make a fine powder.
2. To this 6 ml of freshly prepared Extraction Buffer (Appendix I.1) was added and homogenized.
3. To the homogenate taken in a centrifuge tube, the following reagents were sequentially added and mixed thoroughly after addition of each reagent:
    a. 1ml of 2M sodium acetate (pH 4)
    b. 10ml of phenol (saturated with DEPC treated water)
    c. 2ml of chloroform: isoamyl alcohol (49:1) mixture.

1. This was incubated on ice for 15min and centrifuged at 8000rpm for 12min at 4°C.
2. The aqueous phase was carefully transferred to a fresh centrifuge tube and 10ml of isopropanol was added, mixed well and incubated at -20°C for 1hour.
3. The tube was centrifuged at 14,500rpm for 20min at 4°C.

4. The pellet was re-suspended in 3ml of extraction buffer and 3ml of isopropanol was added, mixed well and incubated at -20°C for 1hour.
5. The tube was centrifuged at 14,500rpm for 20min at 4°C.
6. The pellet was washed with 1ml of 75% ethanol, centrifuged at 14,500rpm for 15min at 4°C.
7. The pellet was dissolved in DEPC treated water and stored at -80°C.

a. Determination of RNA concentration

3µl of the RNA extract was taken in 1ml of DEPC treated water for spectrophotometric quantification and purity analysis. Absorbance at 260nm and 280nm was taken using a 'Spectronic Genesis-5' Spectrophotometer. RNA concentrations were determined based on the relationship that an OD of 1 at 260nm corresponds to 40µg of RNA. RNA purity was assessed by calculating the A260/A280 ratios (Table No 1). The ratio should be close to 2 for a good RNA extraction.

d. Checking of RNA integrity by Submarine Agarose Gel Electrophoresis.

A 100ml 1.2% formaldehyde agarose gel was cast by melting 1.2g of agarose (RNase free) in 73.3ml of DEPC treated water. Just before pouring the gel, 10ml of 10X MOPS/EDTA (Appendix I.2) and 16.7ml of Formaldehyde (2.2M) was added.

30µg of RNA was taken in 25µl of the gel loading dye (Appendix I.3), mixed well and heated at 65°C for 15min on a dry bath and snap cooled on ice before loading on to the gel.

Table No 1 : A_{260}/A_{280} values of RNA extracts

Sample	A_{260}	A_{280}	A_{260}/A_{280}	Con.(µg/µl)
I. Salt Stress				
A.IR64 Roots				
0 Hours	0.311	0.185	1.68	4.15
1 Hour	0.412	0.232	1.78	5.49
2 Hours	0.339	0.179	1.89	4.52
4 Hours	0.792	0.397	1.99	10.56
8 Hours	0.306	0.163	1.88	4.08
16 Hours	0.303	0.184	1.65	4.04
B.IR64 Shoots				
0 Hours	0.742	0.391	1.89	9.89
1 Hour	1.088	0.549	1.98	14.51
2 Hours	0.538	0.283	1.90	7.17
4 Hours	0.686	0.359	1.91	9.15
8 Hours	0.263	0.137	1.92	3.51
16 Hours	0.502	0.296	1.69	6.69
C. Rasi Roots				
0 Hours	0.304	0.164	1.85	4.05
1 Hour	0.280	0.162	1.73	3.73
2 Hours	0.340	0.182	1.87	4.53
4 Hours	0.376	0.226	1.66	5.01
8 Hours	0.699	0.324	2.16	9.32
16 Hours	0.668	0.280	2.38	8.91
D.Rasi Shoots				
0 Hours	0.485	0.266	1.82	6.46
1 Hour	0.678	0.349	1.94	9.04
2 Hours	0.490	0.270	1.81	6.53
4 Hours	0.340	0.190	1.79	4.53
8 Hours	0.448	0.278	1.61	5.97
16 Hours	0.265	0.140	1.89	3.53

II. Desiccation				
A. IR64 Roots				
0%	0.220	0.109	2.02	2.93
30%	0.126	0.061	2.06	1.68
40%	0.368	0.177	2.08	4.91
B. IR64 Shoots				
0%	0.322	0.163	1.97	4.29
30%	0.313	0.165	1.89	4.17
40%	0.178	0.092	1.93	2.37
C. Rasi Roots				
0%	0.424	0.217	1.95	5.65
30%	0.607	0.297	2.04	8.09
40%	0.187	0.111	1.68	2.49
D.Rasi Shoots				
0%	0.706	0.352	2.00	9.41
30%	0.451	0.227	1.99	6.01
40%	0.616	0.312	1.97	8.21

3μl of 0.24kb to 9.5kb RNA ladder from GIBCO BRL, containing a mixture of 6 synthetic poly(A) tailed RNAs (0.5μg each) of sizes 9.49kb, 7.46kb, 4.40kb, 2.37kb, 1.35kb and 0.24kb, was used as a marker for these gels (Fig. No. 2).

Horizontal or Submarine Agarose Gel Electrophoresis system was used. 1X MOPS/EDTA was used as the electrode buffer. A potential difference of 5 to 10 volts per cm (distance between the electrodes) was used for the anionic run.

The two prominent RNA bands of sizes 4.7kb and 1.9kb correspond to 28s and 18s ribosomal RNA respectively (fig No 2). Faint bands of 2.9kb (23s chloroplast rRNA) and 1.5kb (16s chloroplast rRNA) can also be visualized. 5s rRNA is about 120bp and runs faintly below the dye front. The 240bp RNA size marker comigrates with the Bromo Phenol Blue dye front. The smear below the dye front also represents degraded RNA apart from tRNA and a small mRNA population. The rest of the RNA is the mRNA population. DNA (contamination) stays in the well and hardly moves. A good RNA extract, when run on a gel, shows minimum or no DNA in the well, distinct rRNA bands, prominent smear up to the dye front and a faint fuzzy band below the dye front (Fig. No.2).

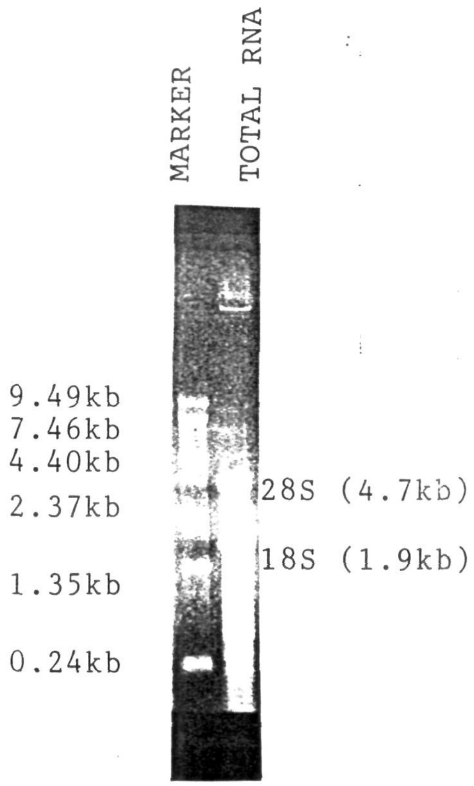


Fig.No.2: Sample RNA agarose (denaturing gel) with size markers.

III Northern Blotting

30μg of total RNA of each sample was separated on a 1.2% formaldehyde agarose gel for the purpose of blotting.

Salt Stress (150mM) Root Samples/ Salt Stress Shoot Samples were loaded in the same gel in the following order

Lane Number	Sample
1	IR-64 0 hour
2	RASI 0 hour
3	IR-64 1 hour
4	RASI 1 hour
5	IR-64 2 hours
6	RASI 2 hours
7	IR-64 4 hours
8	RASI 4 hours
9	IR-64 8 hours
10	RASI 8 hours
11	IR-64 16 hours
12	RASI 16 hours

Dehydration Stress Roots or Dehydration Stress Shoot Samples were loaded in the same gel in the following order:

Lane Number	Sample
1	IR-64 0 %
2	RASI 0 %
3	IR-64 30 %
4	RASI 30 %
5	IR-64 40 %
6	RASI 40 %

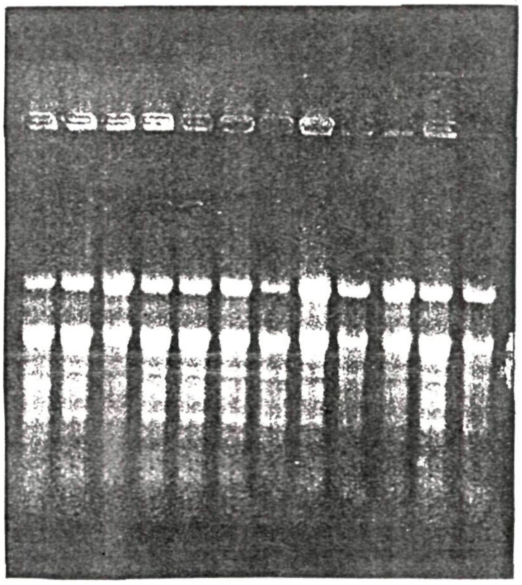


Fig.No.3.03 : ROOTS-1

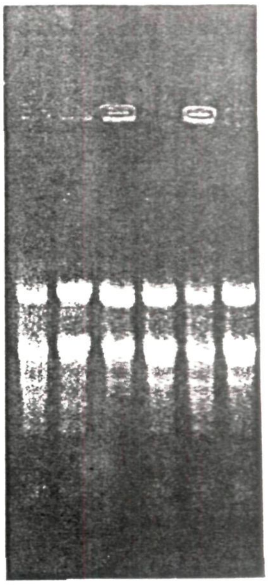


Fig.No.3.01
ROOTS-2

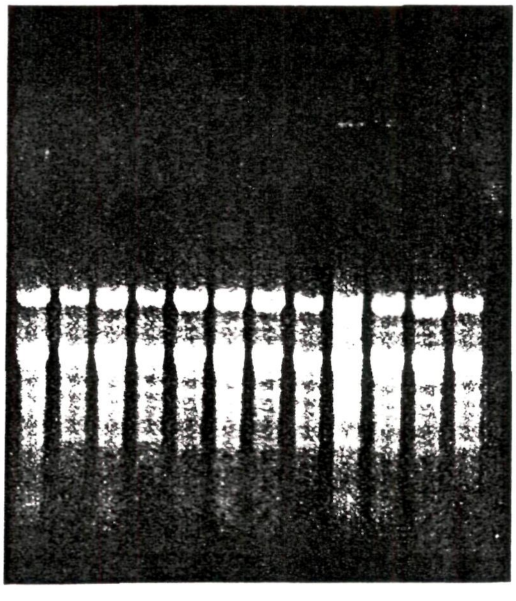


Fig.No.3.04 : SHOOTS-1

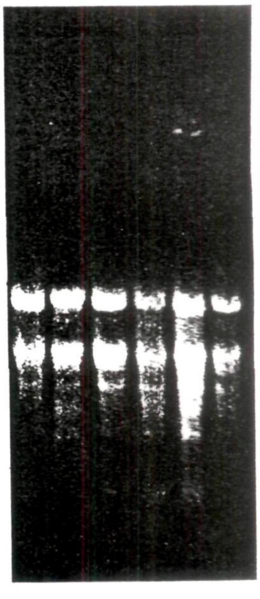


Fig.No.3.02

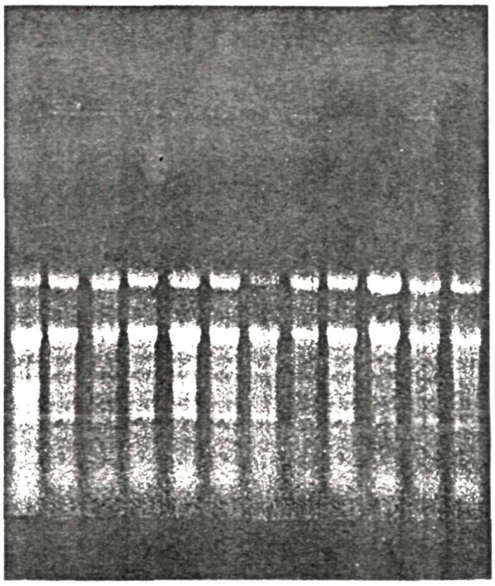


Fig.No.3.07 : ROOTS-3

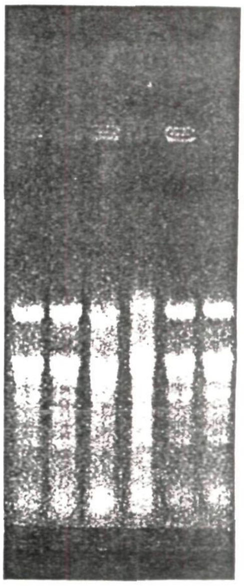


Fig.No.3.05

ROOTS-4

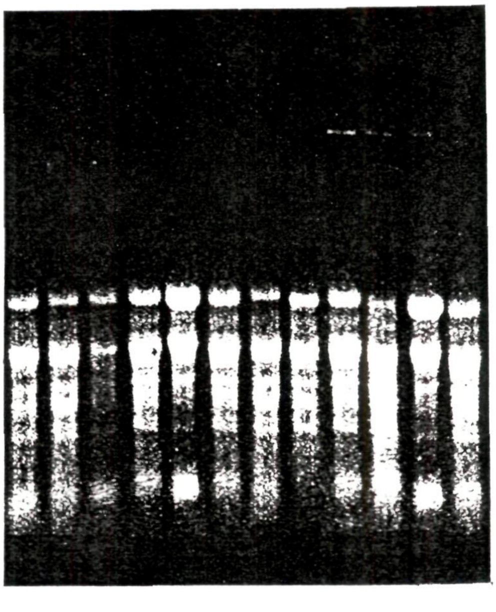


Fig.No.3.08 : SHOOTS-3

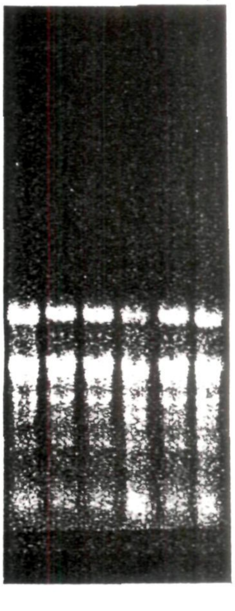


Fig.No.3.06

SHOOTS-4

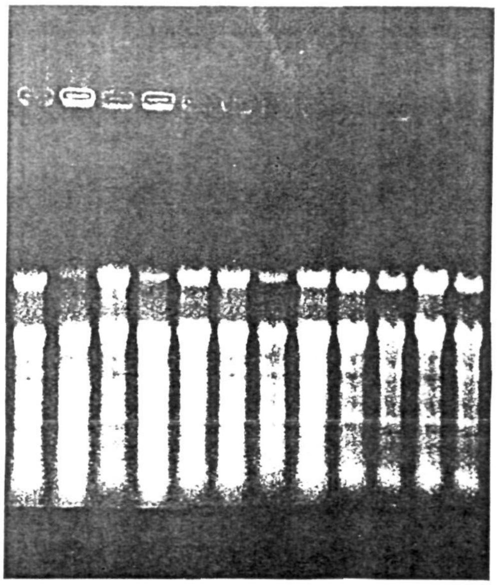


Fig.No.3.11 : ROOTS-5

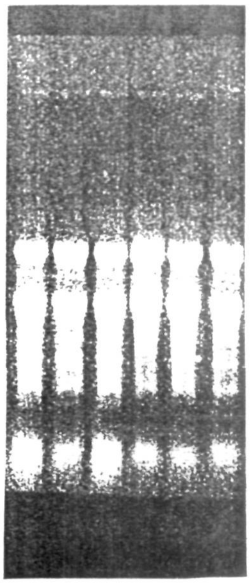


Fig.No.3.9
ROOTS-6

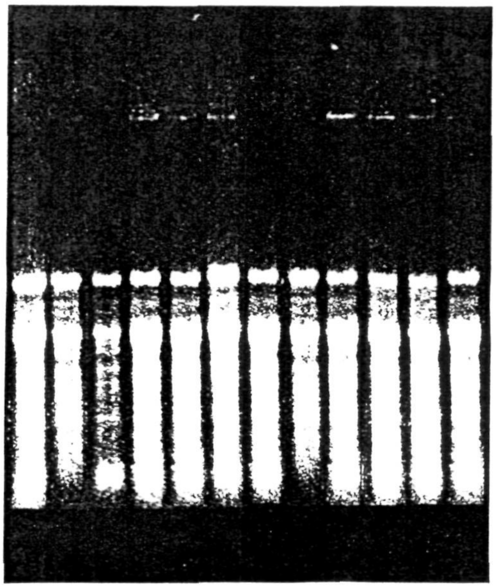


Fig.No.3.12 : SHOOTS-5

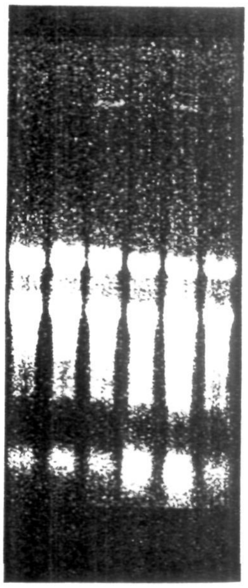


Fig.No.3.10
SHOOTS-6

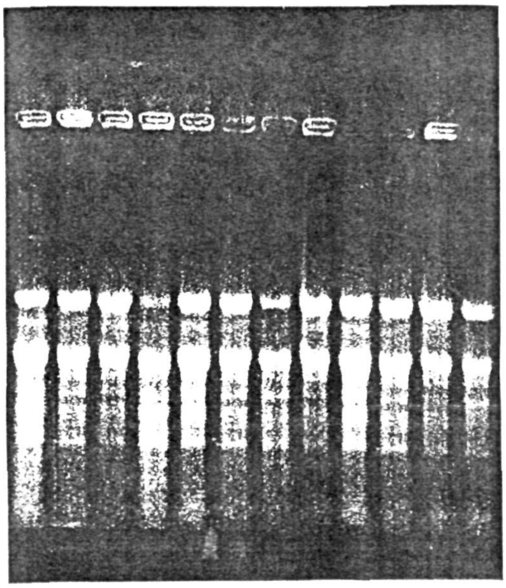


Fig.No.3.15 : ROOTS-7

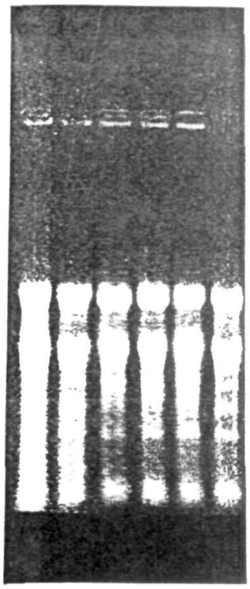


Fig.No.3.13
ROOTS-8

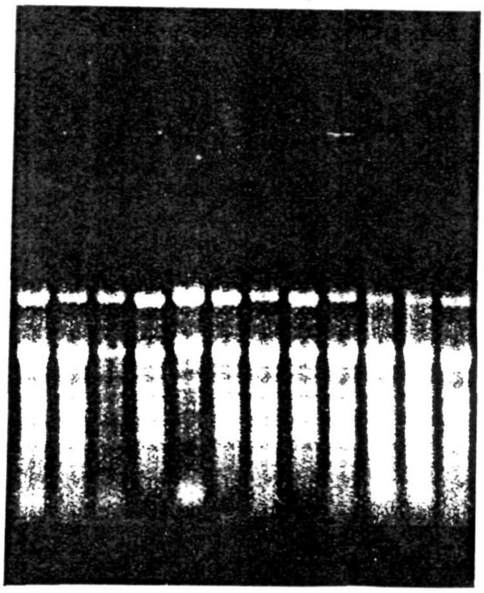


Fig.No.3.16 : SHOOTS-7

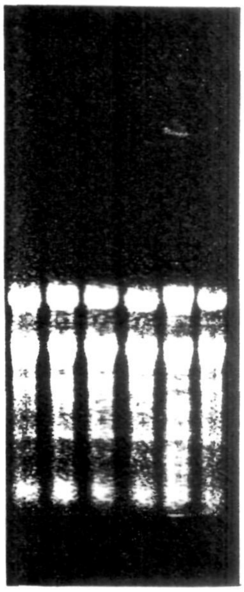


Fig.No.3.14
SHOOTS-8

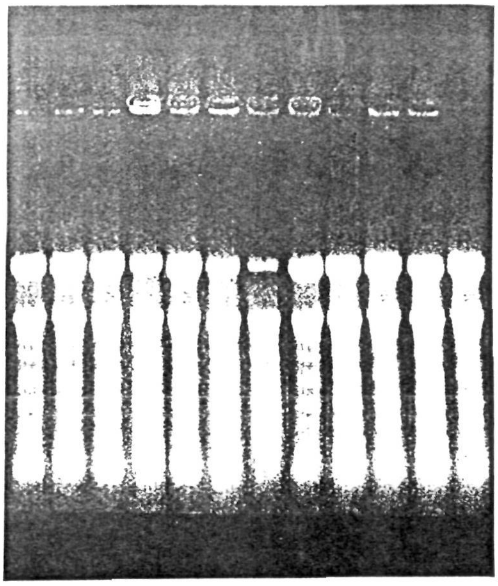


Fig.No.3.19 : ROOTS-9

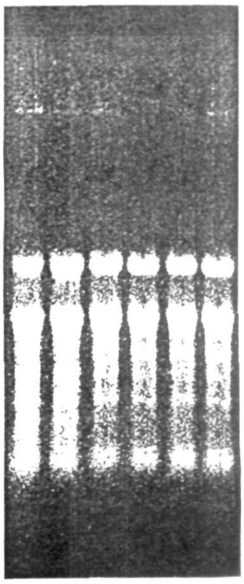


Fig.No.3.17

ROOTS-10

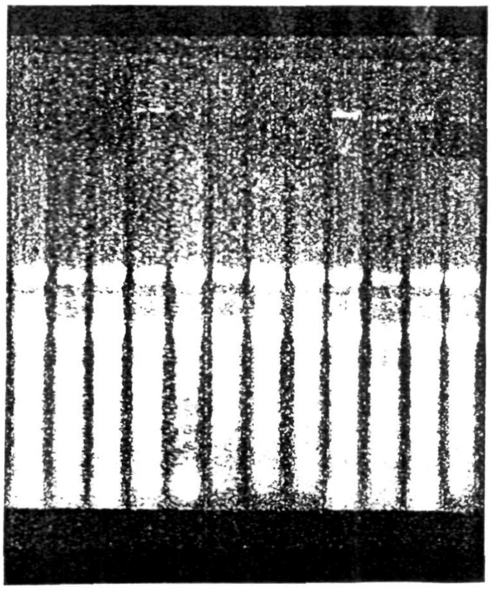


Fig.No.3.20 : SHOOTS-9

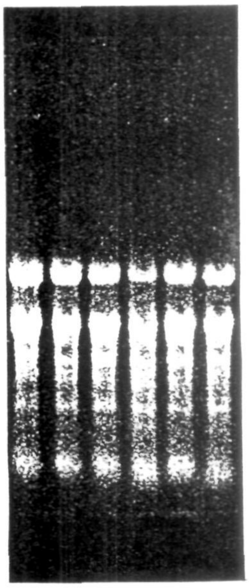


Fig.No.3.18

SHOOTS-10

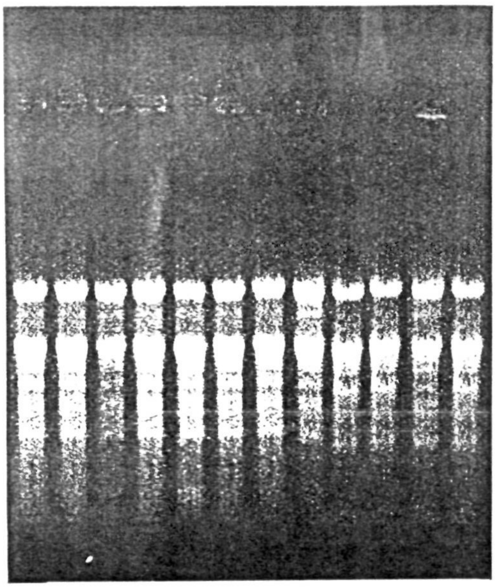


Fig.No. 3.23 : ROOTS-11

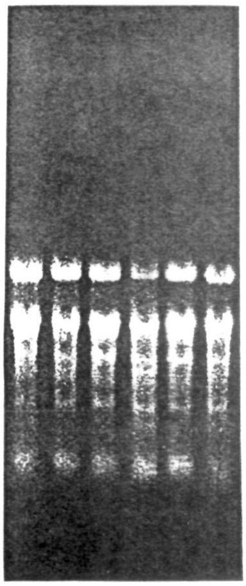


Fig.No: 3.21
ROOTS-12

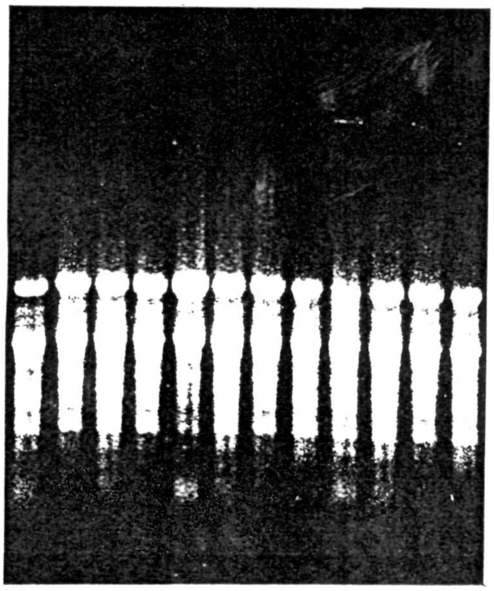


Fig.No.3.24 : SHOOTS-11

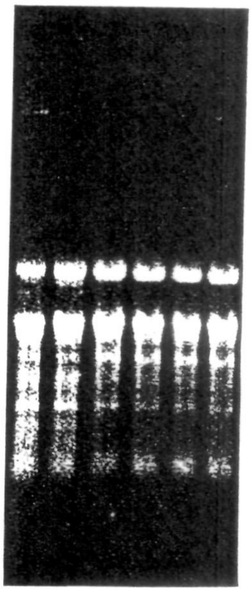


Fig.No.3.22
SHOOTS-12

Fig. No. 3.01 to 3.24 are the photographs of the gels from the northern blots of which results have been obtained.

After electrophoresis, the gel was placed in a baked glass baking dish and kept for gentle rocking in DEPC treated water for 30min, to remove formaldehyde.

The gel was then placed on an elevated horizontal surface in a baked glass baking dish. Two strips of Whatman 3 MM paper were used as wicks. The dish was filled with 10X SSC (Appendix I.4) and the Whatman paper was also moistened with the same buffer.

The gel was then placed inverted on the paper and strips of parafilm were placed below the four edges of the gel. Air bubbles form between the gel and the filter paper were teased out using a baked glass rod.

Hybond N (Amersham) nylon membrane was cut exactly to the same size as the gel, soaked in autoclaved milliQ water for 5min and laid out on the gel. Air bubbles between the gel and the membrane were removed.

Four sheets of 3MM Whatman paper cut to the exact size as the gel were placed on top of the membrane and over it, a stack of filter papers about 6" thick was placed. Weight of about 500g was placed over this. The transfer was allowed to take place overnight (Fig. No.4)

Next morning the stack of filter papers were removed and the locations of the wells were marked out on the membrane using a soft pencil. The membrane was cut at the right hand side top corner to recognize the orientation. The membrane was then gently peeled of the gel and rinsed briefly in 2X SSC and allowed to dry at room temperature.

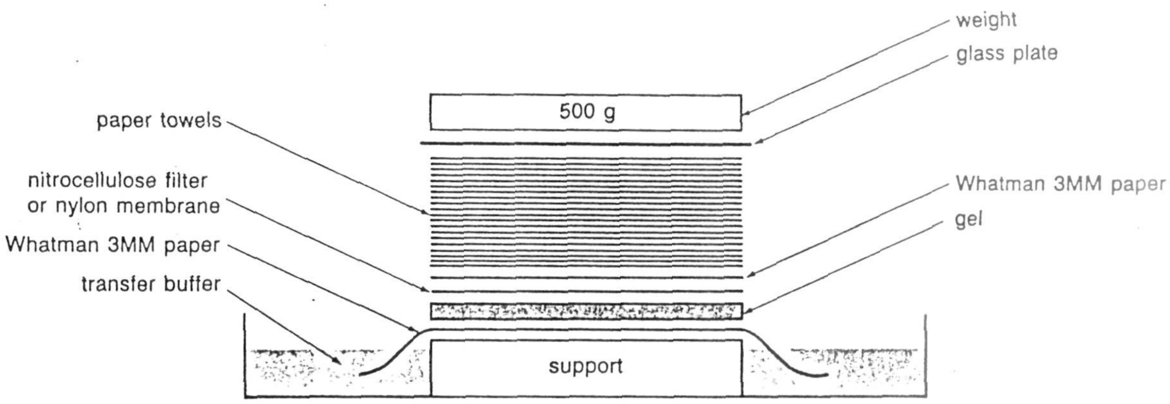


Fig.No.4 : Setup for Northern Blotting

The dried membrane was exposed to Ultra Violet radiation (254nm) of 1200mJ per square cm. in an Amersham UV Cross Linker to fix the RNA to the Nylon Membrane.

The membrane was then sealed on a plastic bag and stored for use later on .

IV Preparation of Probes

a. MnSOD , FeSOD, Cat-2 and cAPx Probes

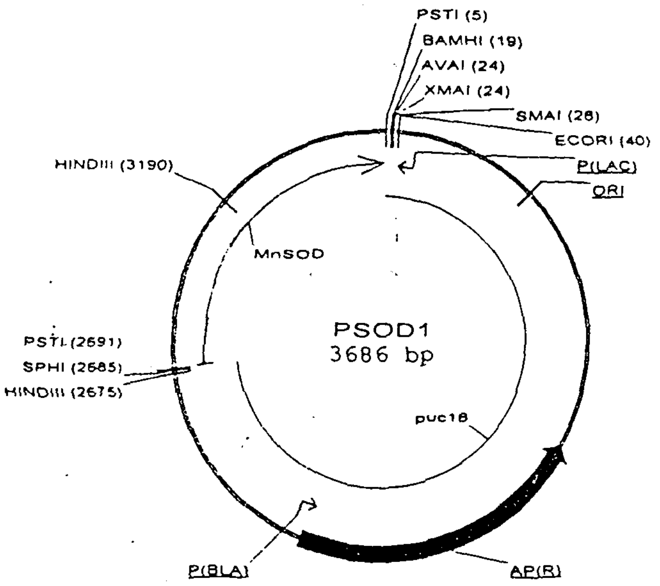
The probes were the kind gift of Prof. Marc van Montagu, VIB, University of Gent, Belgium. The 1kb MnSOD (Manganese Super-Oxide Dismuatse) cDNA fragment [Bowler *et al.*,1989] and 980bp FeSOD (Iron Super-Oxide Dismutase) cDNA fragment [Van Camp *et al.*,1990] were inserts in pSOD1 (Fig. No.5.1) and pSOD2 (Fig. No. 5.2) puc18 plasmids and released by digestion with *PstI* (Fig. No.5). The Catalase-2 cDNA (1150bp) probe [Willekens *et.al.*,1994] and cAPx (906bp) cDNA probe were present in a pbluescriptII KS+ backbone (pCat2, Fig. No.6.1 and ApxBLU, Fig. No.6.2 respectively). The plasmids were maintained in E. coli cultures and supplied as stab cultures.

b. Culturing of Bacteria

Bacteria were inoculated from stab cultures to Luria Bertani Medium (LB Liquid Media, Appendix II.A) containing 60µg/ml Ampicillin (Appendix II.C) in a test tube and incubated overnight in a shaker at 37° C. When the growth of bacteria reached late log phase (approximately), the culture was used for plasmid isolation.

c. Minipreparation of plasmid DNA by Alkaline Lysis Method

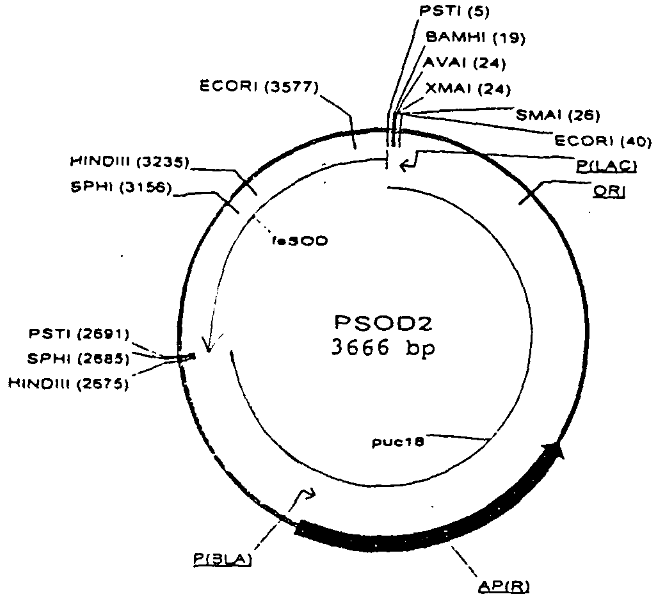
The protocol followed was a modification of the methods given in Maniatis *et al.*[1989] and is as follows:



cut out fragment with PstI

Bowler et al., EMBO vol. 8, 31-38, 1989

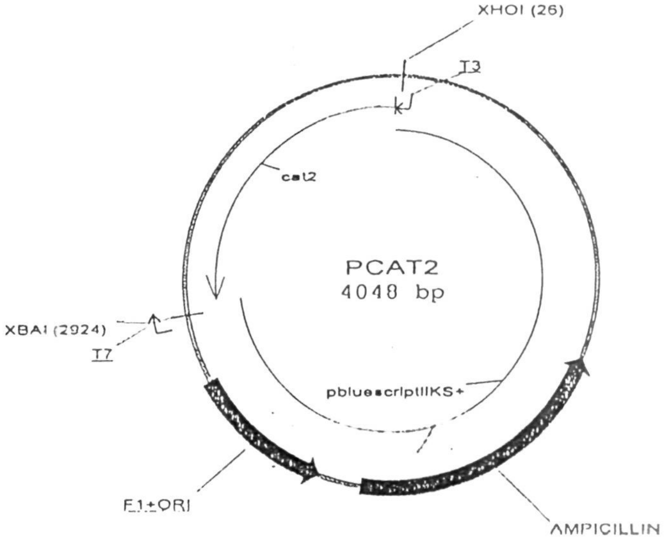
Fig.No.5.1 : PSOD1



cut out fragment with PstI

Van Camp et al., PNAS vol. 87, 9903-9907, 1990

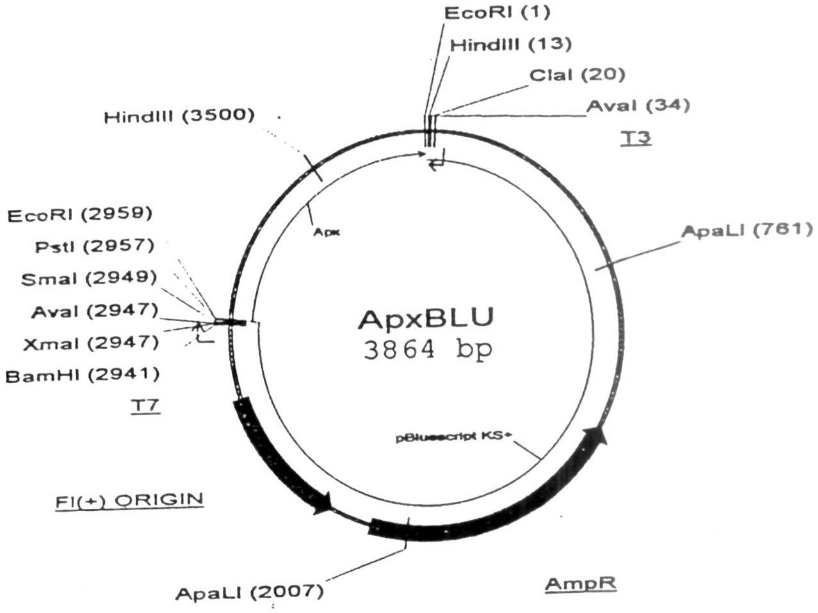
Fig.No.5.2 : PSOD2



cut out fragment with XhoI/XbaI

Willekens et al., FEBS letters vol. 352, 79-83, 1994
Fig. 1, b (3' end)

Fig.No.6.1 : PCAT2



RNA probe: PstI + T3 DNA polymerase

1. 1.5ml of the bacterial culture was taken in a eppendorf and centrifuged at 10000rpm for 3 min at 4°C.
2. The media was removed and the bacterial pellet was resuspended completely in 100μl of ice cold Solution I (Appendix II.B.1)
3. 200μl of freshly prepared solution II (Appendix II.B.2) was added and mixed gently by inverting the tube several times.
4. 150μl of ice cold solution III (Appendix II.B.3) was added and the eppendorf vortexed gently and incubated on ice for 5min.
5. The eppendorf and its contents were centrifuged at 12,000rpm for 7min at 4°C and the supernatant was transferred to a fresh tube.
6. To this 500μl of Tris equilibrated Phenol (pH 8. Appendix II.B.7) was added, mixed by shaking and centrifuged at 12,000rpm for 7min at 4°C.
7. The aqueous phase was taken in a fresh tube and 500μl of chloroform was added and mixed and centrifuged at 12,000rpm for 7min at 4°C.
8. To the aqueous phase 1ml of 100% ethanol was added and incubated at -80°C for 30min to precipitate the DNA.
9. This was centrifuged at 14,000rpm for 20min at 4°C and the supernatant was discarded.
10. The pellet was washed with 70% ethanol.
11. The pellet was air dried for 10min at room temperature and then dissolved in autoclaved milliQ water and stored at -20°C.

1μl of the plasmid extract was loaded in 0.8% agarose gel with ethidium bromide to asses the quality and for approximate visual quantification.

d. Restriction Digestion of plasmid DNA

The plasmids isolated were digested with the restriction enzymes prescribed in the respective plasmid maps (Fig. No. 5 and 6) to release the insert.

MnSOD and FeSOD fragments of sizes 1kb and 980 base pairs respectively were released upon digestion of the puc18 vector (pSOD1 and pSOD2) harboring the insert, with *PstI* (Fig. No.7.1and 7.2). The Pst*I* restriction enzyme (obtained from *Provedentia suatii*) recognises the sequence $\begin{array}{l}\text{CTGCA}\overset{\downarrow}{\text{G}}\\ \underset{\uparrow}{\text{G}}\text{ACGTC}\end{array}$ and cuts at the places indicated. The respective assay buffer (Appendix II.B.8) was used for the reaction mixture. The digestion reaction was carried out at 37°c for 1.5 hours. The reaction mixture was run on a gel (Fig. No.7.1 and 7.2).

The Cat2 fragment of size 1150bp was released upon restriction digestion of the pBluescriptII KS+ vector harboring the insert (pCAT-2), with *XhoI* and *XbaI*. The *XbaI* restriction enzyme (source *Xanthomonas badrii*) recognises the sequence $\begin{array}{l}\overset{\downarrow}{\text{T}}\text{CTAGA}\\ \text{AGATC}\underset{\uparrow}{\text{T}}\end{array}$ for cutting at the places indicated. The *XhoI* restriction enzyme (source *Xanthomonas holcicola*) recognises the sequence $\begin{array}{l}\overset{\downarrow}{\text{C}}\text{TCGAG}\\ \text{GAGCT}\underset{\uparrow}{\text{C}}\end{array}$ and cuts at the places indicated. The "One-Phor-All Buffer"(Amersham) was used for the reaction mixture. The digestion was carried out at 37°c for 1.5 hours. (Fig. No.7.3)

The Apx fragment size 906bp was released upon restriction of the pBluescript KS+ vector containing the insert with *EcoRI*. The *EcoRI* has $\begin{array}{l}\overset{\downarrow}{\text{G}}\text{AATTC}\\ \text{CTTAA}\underset{\uparrow}{\text{G}}\end{array}$ as the recognition sequence and is obtained from *Escherichia coli*. The digestion was carried out at 37°c for 1.5 hours using the respective buffer for the reaction mixture. (Fig. No.7.4)

e. DNA Agarose Gel Electrophoresis

A 0.8% agarose gel was used to run the restriction digestion reaction mixtures. The gel was cast in TAE(1X) Buffer (Appendix II.B.4) and contained 0.5 µg/ml Ethidium Bromide (Appendix II.B.9).

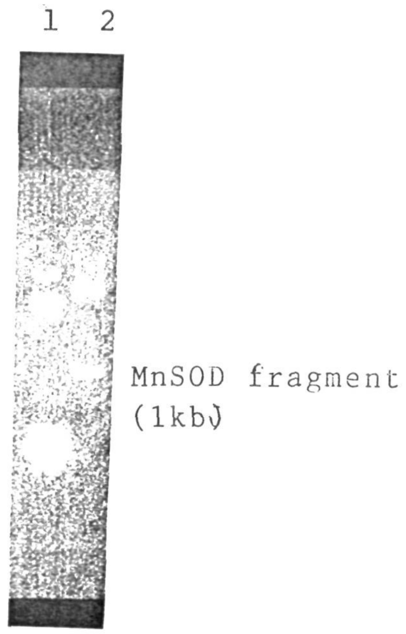


Fig.No.7.1 : Lane1:PSOD1 Plasmid
Lane2:PSOD1 cut with PstI
and treated with RNase

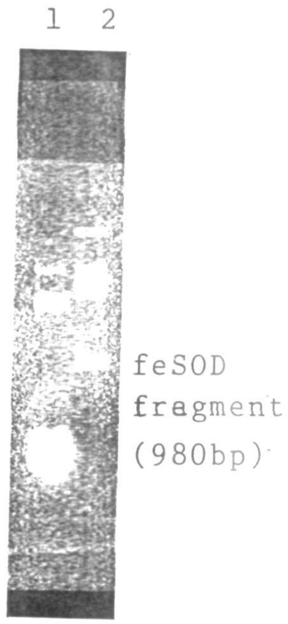


Fig.No.7.2:
Lane1:PSOD-2
Lane2:PSOD2 cut
with PstI and
treated with RNase

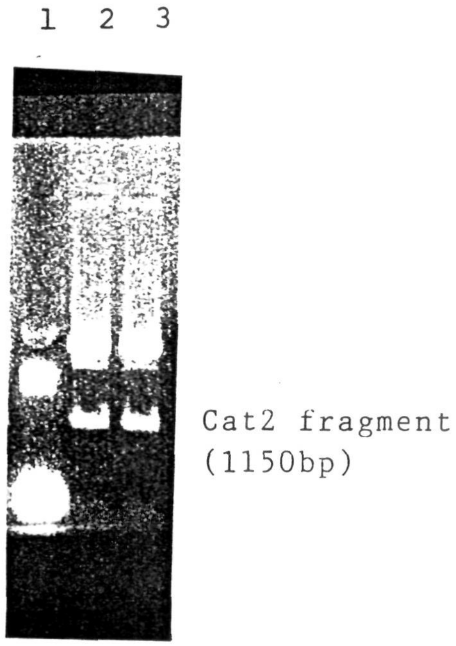


Fig.No.7.3 : Lane1:PCAT2
Lane2&3:PCAT2 cut with
XhoI/XbaI and
treated with RNase

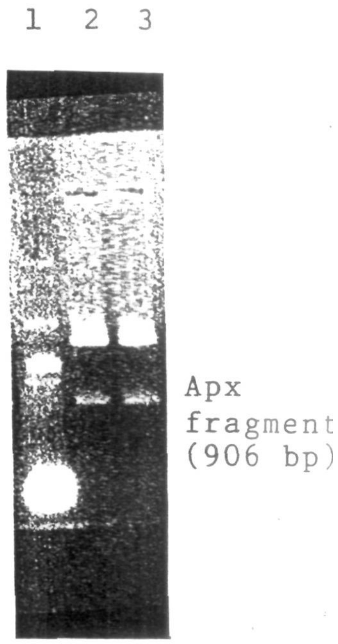


Fig.No.7.4
Lane2:Apx BLU
Lane2&3:Apx BLU cut
with EcoRI and

The gel loading dye consisted of Bromo Phenol Blue and Glycerol (Appendix II.B.6). 1X TAE was used as the electrode buffer. A typical horizontal/submarine DNA agarose gel electrophoresis apparatus was used. A potential difference of 5 to 10 volts/cm distance between electrodes was used. The photographs of the gels in which the reaction mixture was run are presented in Fig. No. 7.1 to 7.4.

f. Purification of MnSOD, FeSOD, Cat-2 and Apx probes.

QIAEX II Gel Extraction Kit (from Qiagen) was used to extract and purify the DNA from agarose gels. The kit consists of a phenol free buffer (QX I) in which the gel bit containing the DNA is first solubilized. QIAEX II (silica particles) are then used to adsorb DNA, in the presence of high salt. All non nucleic acid impurities such as agarose, proteins, salts and Ethidium Bromide are removed in the subsequent washing steps. Pure DNA is eluted in water or TE.

The protocol followed was as follows;

1. The DNA band in the gel corresponding to the released insert was excised using a clean sharp scalpel.
2. The gel slice was weighed in an eppendorf tube and 300µL of QX I buffer was added to every 100mg of the gel.
3. QIAEX II (silica particles) was re-suspended by vortexing for 30 seconds and 10µL was added and mixed.
4. The tube was incubated at 50°c for 10 minutes to solubilize the agarose and bind the DNA. The tube was vortexed every 2 min to keep QIAEX II in suspension.
5. The tube was centrifuged at 13,000rpm for 30 sec and the supernatant was discarded.
6. The pellet was washed with 500µL of QX I buffer to remove residual agarose contaminants.

7. The pellet was washed twice with 500µL of buffer PE to remove residual salt contamination.
8. The pellet was air dried for 10 to 15min or until it became white.
9. The DNA was eluted in water by resuspending the pellet.
10. The tube was centrifuged for 30 sec at 13,000rpm and the supernatant transferred carefully to a clean tube.

The eluted fragment (a small volume) was run on a 0.8% agarose gel with Ethidium Bromide to confirm the elution (Fig. No.10).

g. Preparation of Membrane Channel Protein, Heat Shock Protein, Cyclophilin and Thaumitin DNA probes.

These probes were the kind gift of Didierjean *et al.*, Institute of Plant Molecular Biology, Strasbourg, France. These were cDNA probes obtained from maize and cloned into pBSKS+ in *EcoRI* site. MCP probe used was 345 bp while the HSP probe was 680 bp. The Cyclophilin and Thaumatin probes were 595bp and 700bp respectively.

E.coli (DH5α strain) were transformed with pBSKS+ containing the respective inserts for the purpose of cloning.

Competent cells of DH5α were prepared according to the Chung *et.al.*, [1989] protocol as follows.

1. DH5α cells were inoculated in LB (Appendix II.A) and grown overnight at 37°c in an incubator shaker.
2. 250µL of the overnight DH5α culture in LB was inoculated in 50 ml of 2xTY medium (Appendix II 2.)and incubated in a shaker at 37°c.
3. The OD of the media was constantly monitored till it reached 0.45.
4. The cells were then spun down at 5000rpm at 4°c for 10min in a centrifuge tube.

5. The pelleted cells were re-suspended in 1/20th the volume of Transformation Storage Buffer (Appendix II.D).
6. Aliquots of 100μL each were made.
7. These aliquots were incubated on ice for 10 min, frozen in liquid Nitrogen and stored at -80°C.

Transformation.

1. The competent cells were thawed on ice.
2. 1μL of the DNA was taken in 99μL of KCM (Appendix II. D.2).
3. The DNA in KCM was added to 100μL of competent cells.
4. The eppendorfs were incubated on ice for 20min and then at room temperature for 10min.
5. 1ml of SOC (Appendix II.A.3) was added and the tube was incubated at 37°c for 60min.
6. The cells were spun down at 5000rpm for 10min and resuspended in 100μL of the supernatant.
7. The entire 100μL was plated on LBA (Appendix II.A.4) plates with Ampicillin (60μg/ml).
8. Untransformed cells were used as control and did not show growth on LBA plates with Ampicillin.
9. Cells transformed with pBSKS+ containing the inserts showed growth on LBA Amp. plates.

h. Culturing of Transformants

A single colony of each of the transformants was picked up and inoculated in LB broth (Appendix II 1) with Ampicillin (60μg/ml) and incubated O/N at 37°C in a incubator shaker.

i. Plasmid Isolation

Plasmid isolation from the overnight cultures were done by the mini preparation of Plasmid DNA by Alkaline Lysis Method (as described in IV c).

j. Restriction Digestion of Plasmid DNA

The plasmids isolated were digested with *EcoRI* restriction enzyme. The pBSKS+ vector released fragments of the expected sizes upon digestion (Fig. No. 8.1 to 8.4).

k. Agarose Gel Electrophoresis

The reaction mixture was run on a 0.8% Agarose gel. The photograph of the gel is given in Fig. No. 8.1to 8.4.

l. Purification of MCP, HSP, Cyclophilin and Thaumatin Probes.

The DNA band from the agarose gel corresponding to the released fragment was excised from the gel and the DNA eluted out and purified as described in section IV.f. The purified fragments were run on a gel (Fig. No.10)

V. Radioactive Labeling of DNA Probes.

The probes used for the study (MnSOD, FeSOD, Cat2, Apx, MCP, HSP, Cyclophilin and Thaumatin-like protein) were labeled using Megaprime DNA Labeling System (Amersham) using $\overset{32}{\alpha}$P dCTP from Amersham.

The Megaprime DNA Labeling System uses the random primer mediated DNA labeling method. Here random sequence nanomers are used to prime DNA synthesis on a denatured template DNA at numerous sites along the length. The primer template complex is a substrate for the Klenow fragment of DNA Polymerase I., by substituting a radiolabelled

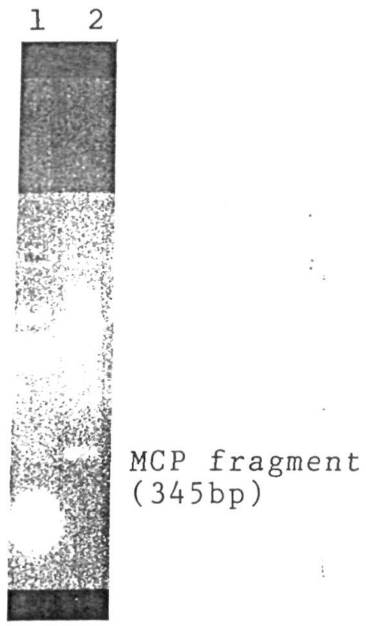


Fig.No.8.1 Lane1:PBSKS(+) Plasmid
Lane2:PSKS(+) cut with
EcoRI

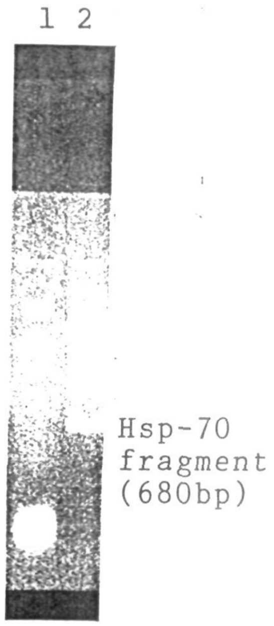


Fig.No.8.2
Lane1:PBSKS(+) Plasmid
Lane2:PBSKS(+) cut with
EcorI

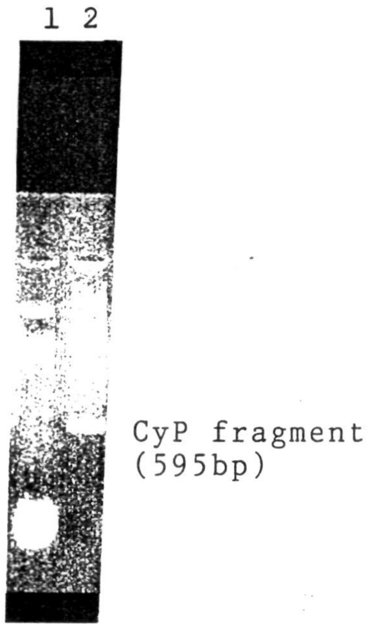


Fig.No.8.3 Lane1:PBSKS(+) Plasmid
Lane2:PBSKS(+) cut with
EcoRI

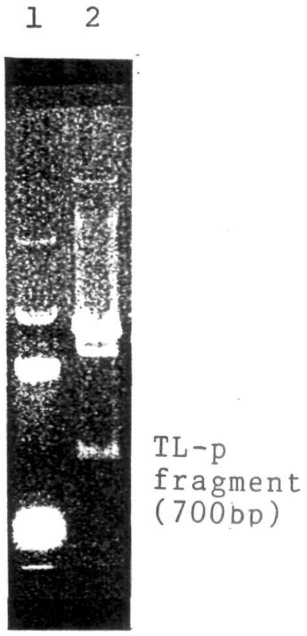


Fig.No.8.4 Lane1
Lane1:PBSKS(+) Plasmid
Lane2:PBSKS(+) cut with
EcoRI

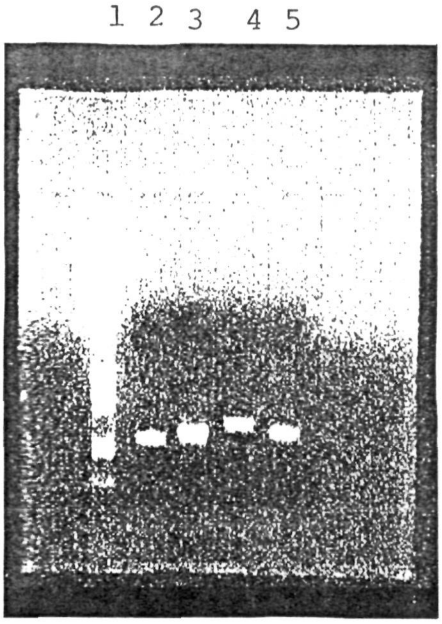


Fig.No.9 : Purified MnSOD,FeSOD,Cat2 and Apx ; cDNA fragments (Lane 2,3,4 & 5)

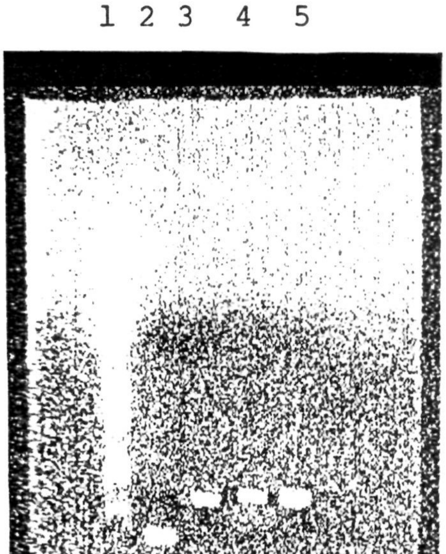


Fig.No.10 : Purified MCP,Hsp,CyP and TL-p cDNA fragments (Lane 2,3,4 & 5) nucleotide for a non radioactive equivalent in the reaction mixture, the newly synthesized DNA is made radioactive.

The DNA fragments were first quantified spectrophotometrically and by taking the Absorbance of 2µL in 1ml water at 260nm using a Spectronic Genesis-5 spectrophotometer. The concentrations were estimated using the relationship that an OD of 1 corresponds to 50µg of DNA. The absorbance values, respective DNA concentrations and volumes used for the labeling reaction are given in table no 2.

The protocol used was as follows:

1. 50ng of the DNA fragment was taken.
2. MilliQ water for a final reaction volume of 50µL was added.
3. 5 µL of primer was added.
4. The DNA was denatured by heating at 95°C for 5 min, spinning the tube briefly and allowing it to cool gradually (to facilitate primer annealing) to room temperature.
5. The following nucleotide tri-phosphates were added:
    dATP : 4µL
    dTTP : 4µL
    dGTP : 4µL
5. $\alpha^{32}P$ labeled dCTP (5µL) was added.

2µL of Klenow enzyme was added and the tube was incubated at 37°C for 1 hour.

a. Purification of Radiolabelled probes using Sephadex G-50 Spin Column Chromatography.

G-50 spin column chromatography was employed to separate to separate unincorporated nucleotides from the labeled DNA. This step helps to clean the probe and reduce background while probing.

Table No. 2: Spectrophotometric quantification of purified cDNA probes for labeling.

NO.	cDNA Probe	A_{260} (2µl in 1ml)	Con. (ng/µl)	Volume used
1	MnSOD	0.002	50	1
2	FeSOD	0.003	75	1
3	Cat2	0.004	100	0.5
4	Apx	0.005	125	0.5
5	MCP	0.002	50	1
6	HSP	0.002	50	1
7	Cyclophilin	0.004	100	0.5
8	Thaumatin	0.003	75	1

The procedure followed was as described in Maniatis *et.al* [1989].

1. The bottom of a 1ml disposable syringe was plugged with small amounts of sterile glass wool.
2. Sephadex G-50 (equilibrated with 1x TE) was added to the syringe.
3. The syringe was inserted in a disposable plastic tube and centrifuged in a swinging bucket rotor at 1500 rpm for 1min at room temperature.
4. More resin was added and centrifugation repeated till the volume reached 0.9 ml and did not change even after centrifugation (1500 rpm, room temperature, 1 min).
5. 100 µl of TE was added and centrifuged (1500 rpm, room temperature, 1 min). This was repeated 4 times to ensure equilibration of the column.
6. The volume of the labeled DNA mixture was made up to 100 µl using 50µl of TE.
7. An eppendorf was placed below the mouth of the spun column inside the plastic tube.
8. The labeling reaction mixture (now 100µl) was added to the column and centrifuged at 1500 rpm for 1 min at room temperature.
9. The purified labeled DNA eluted out was collected in the eppendorf.

VI. Probing of Northern Blots

1. Pre-hybridization:

Pre-hybridization was done to block non specific sites on the blot for the probe to bind, and thus reduce background. Freshly prepared modified version of Church-Gilbert Hybridization solution (Appendix I.5) was preheated to 60°C and added to the northern blot in a plastic cover. About 4ml of the solution for every 10 *sq.cm.* of the blot. Air bubbles were carefully removed as far as possible. The bag was sealed using a plastic bag sealer. The plastic bag was placed in an Amersham Hybridization Oven with shaking and incubated for 6 hrs at 60°C.

2. Hybridization:

The labeled purified DNA was denatured by heating at 95°C for 5 min. and snap cooling on ice before being used for hybridization. The plastic bag containing the blot and pre-hybridization was cut at a corner and the denatured radio-labeled purified probe (100μl) was added directly to the pre-hybridization solution. The bag was then sealed at the cut corner and hybridization was allowed at 55 to 60°C (depending on the probe) for about 18 hrs. with rocking in the hybridization oven.

3. Washing:

The removal of probe hybridized to sequences that are not a perfect match was achieved through controlled-stringency washes by varying the temperature and ionic strength of the washing solution. The progress of washing was monitored using a Geiger Muller Counter and washing was stopped when to background was the least. The normal sequence of washing steps was as follows;

a. Low stringency rinse: The blot was washed with 2X SSC and 0.1% SDS at room temperature for 15 min with rocking.
b. Moderate stringency wash: 1X SSC and 0.1% SDS for 10 min at room temperature was used.
c. High stringency washes: 1X SSC and 0.1% SDS for 30 min at 60°C. The blot was checked using a Geiger Muller hand held counter to decide washing time and temperature of this wash.

After washes, the blot was packed in a very thin plastic pouch and sealed (to avoid drying) before exposure.

VII. Detection of Signals

A FUJIFILM Fluorescent Image Analyzer FLA-2000 (Phosphorimager) was used to detect signals from the probed northern blots with the help of Fuji Image Plate (IP sheet).

a. Principle: The Imaging Plate (IP) is a two dimensional sensor of radioactive energy. The IP consists of an image-sensing layer composed of fine Photostimulable Phosphor Crystals ($BaFBr:Eu2+$) bonded to a special backing material. When exposed to a probed Blot emitting radioactivity the IP accumulates and stores the irradiated radioactive energy. The IP sheet is then scanned with a He-Ne Laser beam. It then emits luminescence in proportion to the recorded radiation intensity. This luminescence is detected by a photo-multiplier tube (PTM) and converted to electrical signals. Image data are recorded as digital values on the Hard Disc inside the Analyzing Unit (Computer) for further. The image recorded on the IP is read as high resolution digital data up to 50μm per pixel (20 pixel/sq.mm).

All the image data recorded on an IP can be erased and the general isotope-use IP can be re-used.

b. Exposure

The IP sheet was erased by exposure to bright light from 15 Watt fluorescent lamps using a 'IP Eraser' for 40 min. The erased IP sheet was placed in a X-Ray Film Cassette with its sensitive surface facing the blot under normal room light conditions and exposed to 0.5 to 12 hours depending on the counts recorded from the Blot. The recommended exposure time, however, is 1/20th of the X-Ray Film exposure time.

c. IP Reading

The Phosphorimager and analyzing unit were switched on. The IP sheet was stuck on to the IP stage and this was placed in the Main Unit. The Macintosh Analyzing Unit (Computer) connected to the main unit and having the software required for IP reading was booted.

"Image Reader" version V-1.4E software was employed for importing data of the image read with IP Reader (FLA 2000) to the computer. "L-Process" software of the analyzing unit was used to process the image obtained by the Image Reader. This software can execute a variety of filtering to considerably reduce background and clarify the signals. Since the computer's hard disc memory (RAM) permitted processing of a small sized image, the region of the blot where signals were obtained could only be subjected to processing. The refined processed images are shown in Fig. Nos. 12.01 to 12.30.

"Image Gauge" software was used to quantify the intensity of bands. The "Quant Mode" tool was used to select the bands and read the band intensity as Phospho-luminescent units (PSLs). The luminescence emitted by the IP Sheet is proportional to the recorded radiation intensity from each band. The data obtained is presented in table nos. 3.01 to 3.30.

Presentation and Analysis of the Relative Transcript levels

The transcript levels have been quantified as phospho-luminiscent units (PSLs). The actual PSL values of each band (minus the background values) recorded have been given under **IR64** and **RASI** columns respectively (Table Nos. 3.01 to 3.30).

To enable comparison of the transcript levels of control samples with the stress ones of a particular variety, the percentage differences have been presented in the **Str-Ctl%** column (Table No. 3.01 to 3.30). These values have been obtained by using the formula ;

$$\text{Str-Ctl\%} = \frac{\text{PSL value of stress sample - PSL value of control sample of the same variety}}{\text{PSL value of control sample of the same variety}} \times 100$$

IR64 and Rasi transcript levels, at a that particular stage of stress have been compared and the percentage differences, of one another, have been presented as **I-R%** or **R-I%** values (Table No. 3.01 to 3.30). The values have been obtained using the formulae ;

$$\text{I-R \%} = \frac{\text{IR64 PSL value - Rasi PSL value at a particular stage of stress}}{\text{Rasi PSL value at that stage of stress}} \times 100$$

$$\text{R-I \%} = \frac{\text{Rasi PSL value - I R64 PSL value at a particular stage of stress}}{\text{IR64 PSL value at that stage of stress}} \times 100$$

Normalization of the quantified transcript levels have been done by taking the susceptible variety (IR-64) control value as 100. All other values have been expressed in relation to this value [similar to Umeda and Uchimiya, 1994]. The normalized values have been used for drawing graphs and has been tabulated under the **Graph** column heading of IR-64 and Rasi respectively (Table No. 3.01 to 3.30 and Fig. No. 13.01 to 13.30). the following formula was used to calculate Graph values ;

$$\text{Graph Value} = \frac{\text{Sample Value - IR64 control value}}{\text{IR64 control value}} \times 100$$

As a convention, to indicate that equal amounts of RNA have been loaded in each lane, the 28S rRNA band of each gel which has been blotted and probed with the respective cDNA probe has been shown along with the signals from northern blots.

VIII. Stripping and Re-probing of Northern Blots.

Northern Blots were stripped of the radio-labeled probe by washing the membrane using boiling TE with 0.1%SDS for 15 min and the absence of signals was confirmed using the Phosphor-Imager. These blots were pre-hybridized and re-hybridized as outlined in the previous sections. The gel/blots figure numbers and the probes used are given in table no.4.

Table No. 4: Gel / Blot Figure Numbers and Probes Used

cDNA Probe used	Blot Numbers			
	Dehydration Stress		Salt Stress	
	Roots	Shoots	Roots	Shoots
MnSOD	3.01	3.02	3.03	3.04
FeSOD	3.05	3.06	3.07	3.08
Cat2	3.09	3.10	3.11	3.12
Apx	3.13	3.14	3.15	3.16
MCP	3.17	3.18	3.19	3.20
HSP	3.21	3.22	3.23	3.24
CyP	3.05	3.06	3.07	3.08
TL-p		3.14		3.16

EXPERIMENTAL RESULTS

IV. EXPERIMENTAL RESULTS

Experiments were conducted to compare the differences in the pattern of gene expression between IR64 (susceptible) and Rasi (tolerant) *Indica* rice varieties, during salinity and moisture stress in roots and shoots. A salt stress of 150mM for 1,2,4,8 and 16 hours was applied. Seedlings were allowed to loose 30% and 40% moisture for desiccation stress. 30µg of total RNA, from the roots and shoots of stressed and control samples, in Northern Blots were probed with heterologous stress related cDNA probes. Signals were detected and quantified using a phosphorimager The results obtained from different blots using each of the probes are presented in this chapter.

As a convention, the 28S rRNA (fig nos. 11.01 to 11.30) bands have been shown along with the signals (fig nos.12.01 to 12.30, to indicate that equal amounts of total RNA were loaded in each lane. The order of loading is as follows;

Salt Stress		Desiccation Stress	
Lane Number	Sample	Lane Number	Sample
1	IR-64 0 hour	1	IR-64 0 %
2	RASI 0 hour	2	RASI 0 %
3	IR-64 1 hour	3	IR-64 30 %
4	RASI 1 hour	4	RASI 30 %
5	IR-64 2 hours	5	IR-64 40 %
6	RASI 2 hours	6	RASI 40 %
7	IR-64 4 hours		
8	RASI 4 hours		
9	IR-64 8 hours		
10	RASI 8 hours		
11	IR-64 16 hours		
12	RASI 16 hours		

Dehydration stress:

MnSOD transcript levels registered an increase in response to dehydration in roots and shoots of both of IR64 (susceptible variety) and Rasi (tolerant variety). IR64 had 17% more transcripts, than Rasi at moderate (30%) dehydration in the roots. There was no upregulation of MnSOD mRNA levels in both IR64 and Rasi at 30% desiccation in the shoots. Rasi accumulated higher transcripts at 40% dehydration in both roots and shoots by as much as 5 and 14.5% respectively (Fig. No. 12.01 and 12.02, Fig. No. 13.01 and 13.02, Table No. 3.01 and 3.02).

Salinity stress

Sudden increases in the MnSOD mRNA levels (by 18%) of IR64 roots was observed in response to 1hr of 150mM salinity. Similar increases in Rasi was observed after 2 hrs. Peak transcript levels were observed in IR64 roots at the 2nd hour. In the roots of both the varieties, the transcript levels of MnSOD reached levels similar to that of control by the 16th hour. Similar pattern was observed in the shoots of both the varieties during salt stress at the last time point of salt stress (Fig. No. 12.03 and 12.04, Fig. No. 13.03 and 13.04, Table No. 3.03 and 3.04).

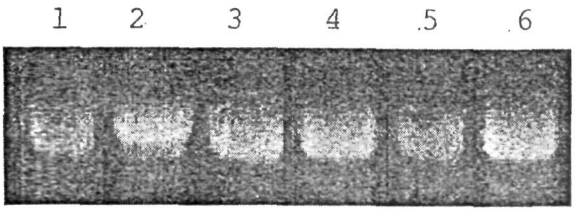


Fig. No. 11.01: 28S rRNA Roots-2

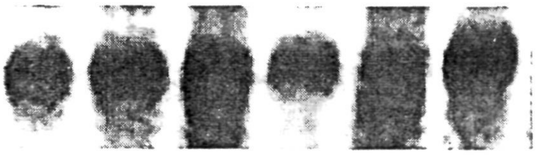


Fig. No. 12.01: MnSOD transcripts: Dehydration Stress: Roots

Stress	IR-64	Str-Ctl %	I-R %	Graph		RASI	Str-Ctl %	R-I %	Graph
0%	4169	0.00	3.89	100.00		4013	0.00	-3.74	96.26
30%	4940	18.49	17.14	118.49		4217	5.08	-14.64	101.15
40%	4897	17.46	-4.78	117.46		5143	28.16	5.02	123.36

Table. No. 3.01

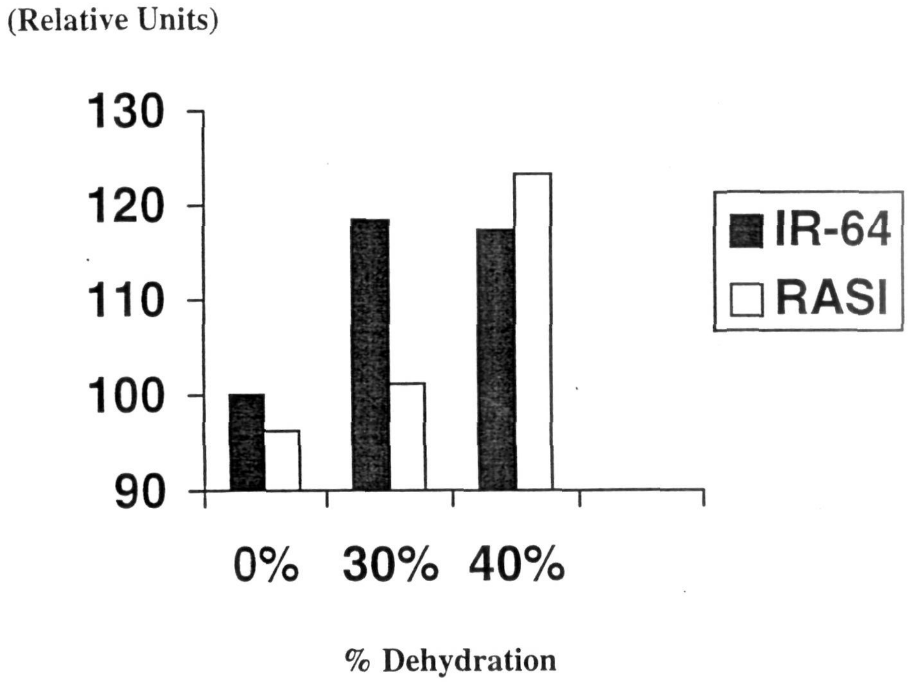


Fig. No. 13.01

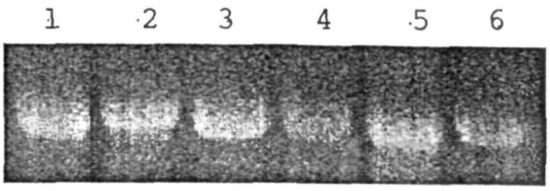


Fig. No. 11.02: 28S r RNA: Shoots-2

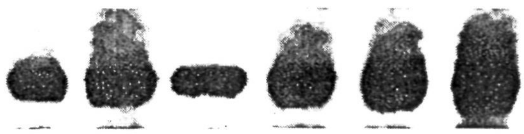


Fig. No. 12.02: Mn SOD Transcripts: Dehydration Stress : Shoots

Stress		IR-64	Str-Ctl %	I-R %	Graph		RASI	Str-Ctl %	R-I %	Graph
0%		4312	0.00	-11.15	100.00		4853	0.00	12.55	112.55
30%		4183	-2.99	-13.05	97.01		4811	-0.87	15.01	111.57
40%		5126	18.88	-12.60	118.88		5865	20.85	14.42	136.02

Table No. 3.02

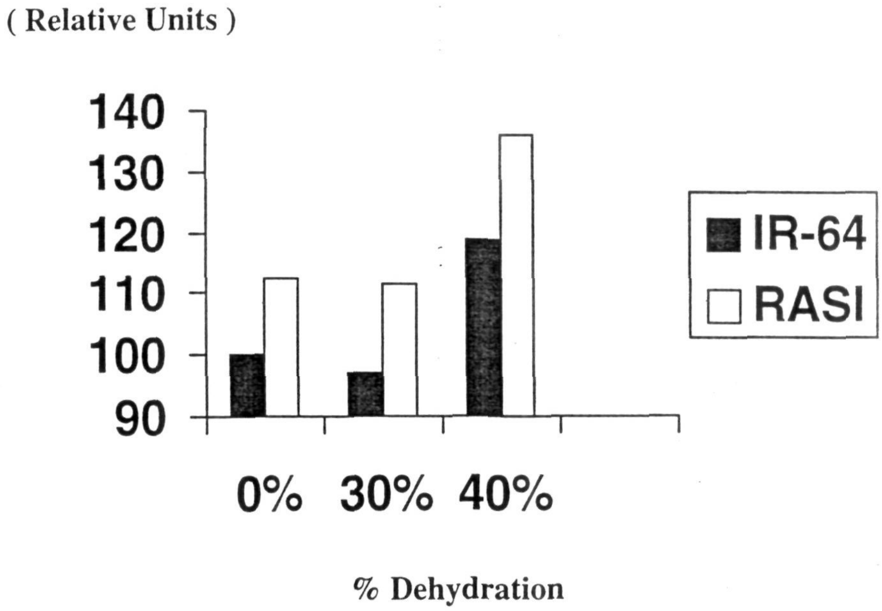


Fig. No. 13.02

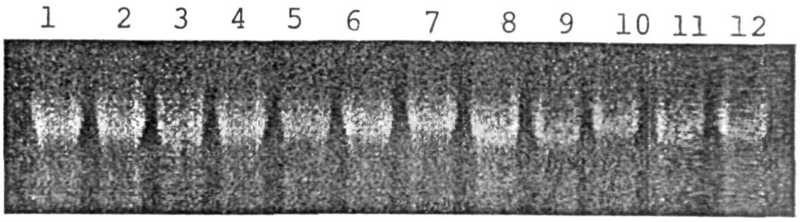


Fig. No. 11.03: 28S rRNA: Roots-1

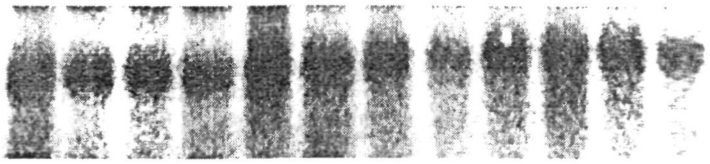


Fig. No. 12.03: MnSOD transcripts: Salt Stress: Roots

Time (hrs)		IR-64	Str-Ctl %	I-R %	Graph		RASI	Str-Ctl %	R-I %	Graph
0		4108	0.00	0.61	100.00		4083	0.00	-0.61	99.39
1		4850	18.06	15.78	118.06		4189	2.60	-13.63	101.97
2		4979	21.20	2.62	121.20		4852	18.83	-2.55	118.11
4		4302	4.72	0.82	104.72		4267	4.51	-0.81	103.87
8		4692	14.22	8.46	114.22		4326	5.95	-7.80	105.31
16		4153	1.10	1.32	101.10		4099	0.39	-1.30	99.78

Table. No. 3.03

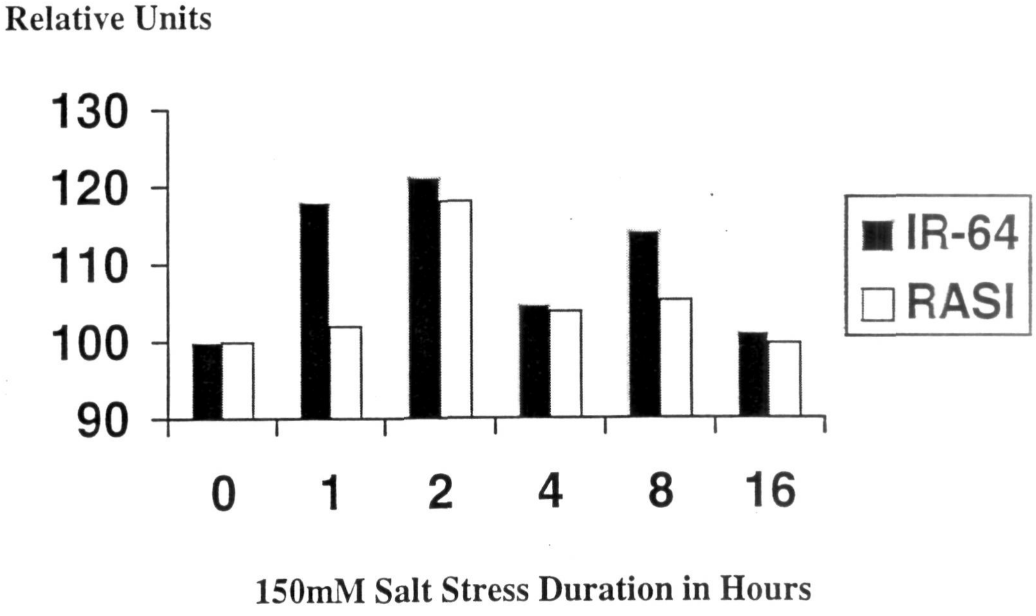


Fig. No. 13.03

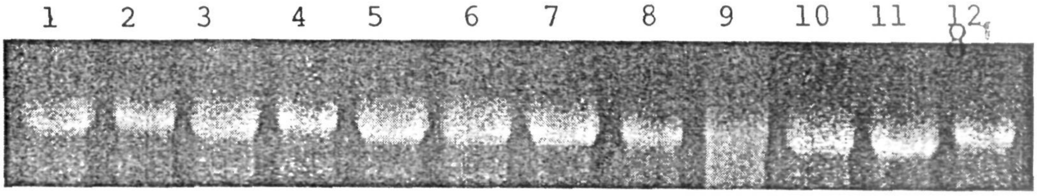


Fig. No. 11.04: 28S rRNA: Shoots-1

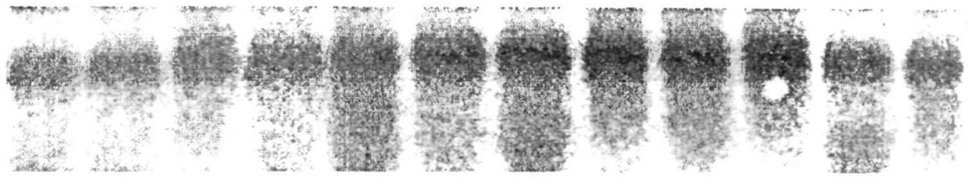


Fig. No.12.04: MnSOD transcripts: Salt Stress: Shoots

Time (hrs)		IR-64	Str-Ctl %	I-R %	Graph		RASI	Str-Ctl %	R-I %	Graph
0		4696	0.00	-5.05	100.00		4946	0.00	5.32	105.32
1		5222	11.20	-1.79	111.20		5317	7.50	1.82	113.22
2		5403	15.06	-9.01	115.06		5938	20.06	9.90	126.45
4		5838	24.32	3.20	124.32		5657	14.38	-3.10	120.46
8		5819	23.91	4.04	123.91		5593	13.08	-3.88	119.10
16		4950	5.41	3.45	105.41		4785	-3.26	-3.33	101.90

Table. No.3.04

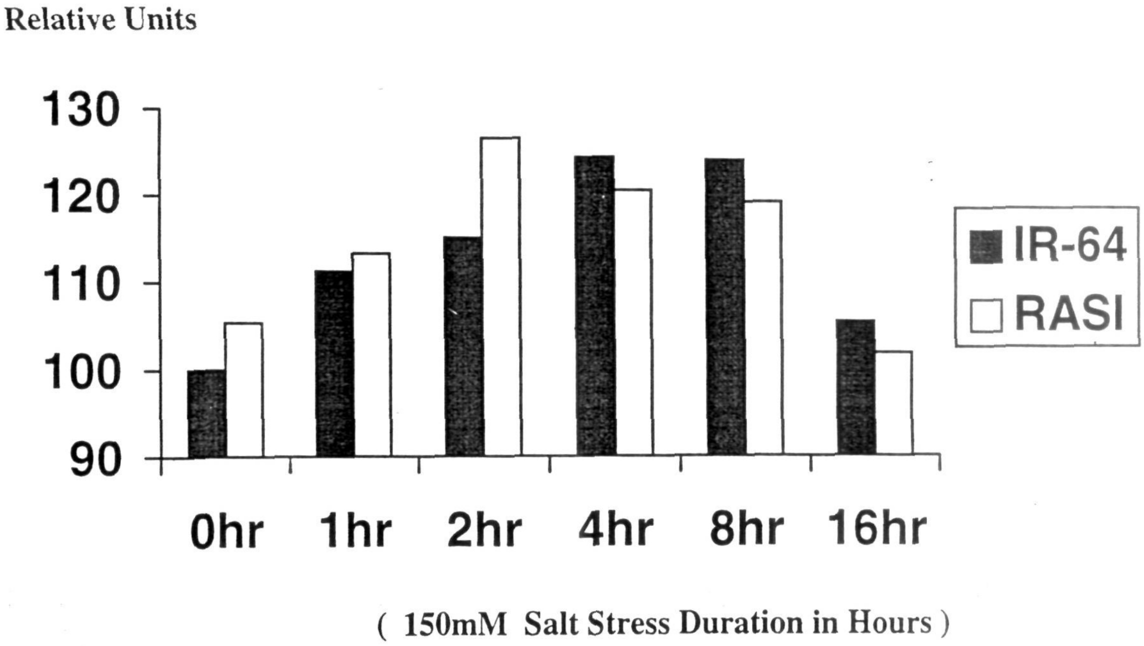


Fig. No. 13.04

mRNA levels of FeSOD during stress

Dehydration Stress

The transcript levels of FeSOD in the roots of Rasi (tolerant variety) was downregulated at 30% dehydration, while it increased by 28% in IR64 (susceptible variety). The levels of IR64 FeSOD mRNA was similar to that of control at 40% desiccation. In Rasi it was upregulatd by about 35%. In the shoots of Rasi, the FeSOD transcript levels were always higher than IR64. It was higher by as much as 60% at 30% desiccation (Fig. No. 12.05 and 12.06, Fig. No. 13.05 and 13.06, Table No. 3.05 and 3.06).

Salinity

The FeSOD gene expression was induced at the 1st hour of salt stress itself in the roots of both the varieties. There after the transcript levels some what stabilized. In the shoots the induction of FeSOD genes was delayed. However Rasi was able to upregulate gene expression more quickly than IR64, with peak transcript level of 38% at the 4th hour of salinity. IR64 on the other hand recorded peak transcript levels at the 8th hour (Fig. No. 12.07 and 12.08, Fig. No. 13.07 and 13.08, Table No. 3.07 and 3.08).

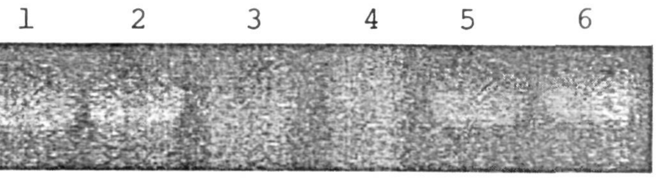


Fig. No.11.05: 28S rRNA: Roots-4

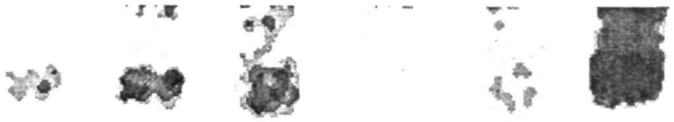


Fig. No.12.05: FeSOD transcripts: Dehydration Stress: Roots

Stress		IR-64	Str-Ctl %	I-R %	Graph		RASI	Str-Ctl %	R-I %	Graph
0%		2468	0.00	-14.72	100.00		2894	0.00	17.26	117.26
30%		3172	28.53	48.57	128.53		2135	-26.23	-32.69	86.51
40%		2596	5.19	-26.23	105.19		3519	21.60	35.55	142.59

Table. No. 3.05

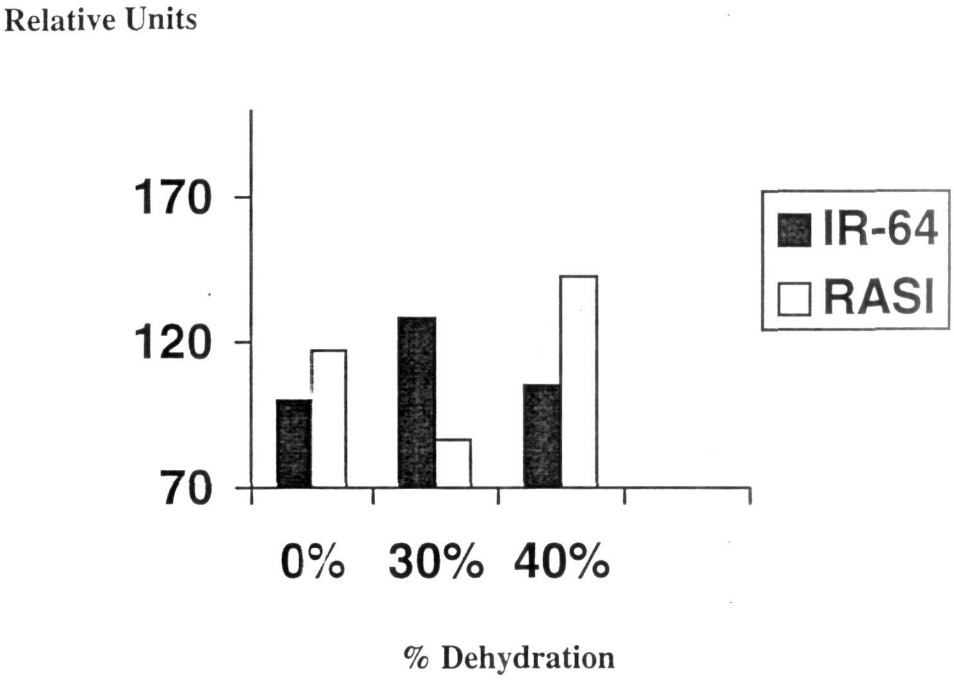


Fig. No.13.05

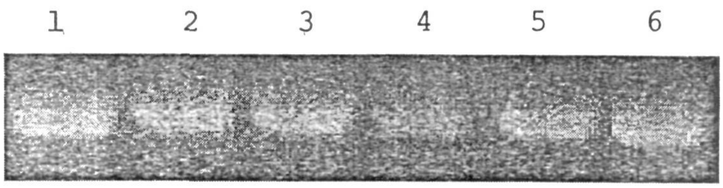


Fig. No. 11.06: 28S rRNA: Shoots-4

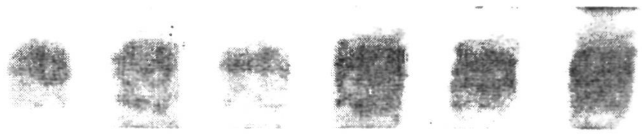


Fig. No. 12.06: FeSOD transcripts: Dehydration Stress: Shoots

Stress		IR-64	Str-Ctl %	I-R %	Graph		RASI	Str-Ctl %	R-I %	Graph
0%		2435	0.00	-14.41	100.00		2845	0.00	16.84	116.84
30%		2117	-13.06	-37.68	86.94		3397	19.40	60.46	139.51
40%		3442	41.36	-8.68	141.36		3769	32.48	9.50	154.78

Table. No. 3.06

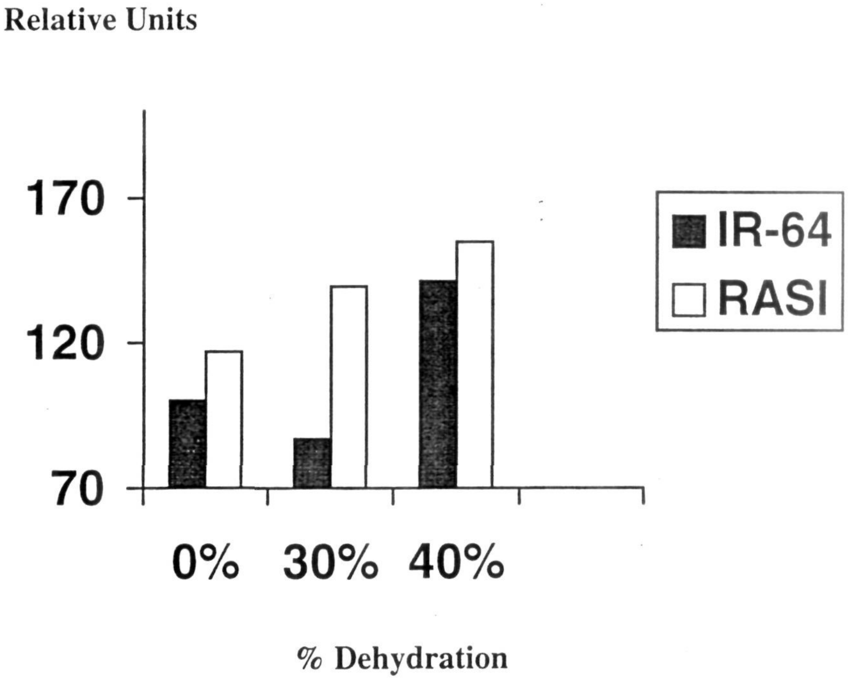


Fig. No. 13.06

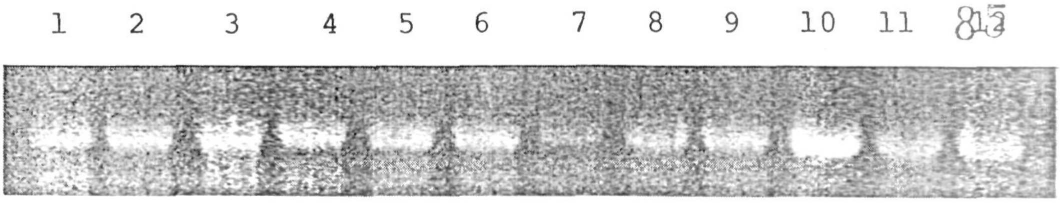


Fig. No. 11.07: 28S rRNA: Roots-3

Fig. No. 12.07: FeSOD transcripts: Salt Stress: Roots

Time (hrs)		IR-64	Str-Ctl %	I-R %	Graph		RASI	Str-Ctl %	R-I %	Graph
0		2540	0.00	-25.97	100.00		3431	0.00	35.08	135.08
1		4153	63.50	4.03	163.50		3992	16.35	-3.88	157.17
2		4329	70.43	-5.15	170.43		4564	33.02	5.43	179.69
4		4343	70.98	-2.62	170.98		4460	29.99	2.69	175.59
8		3734	47.01	-6.58	147.01		3997	16.50	7.04	157.36
16		3774	48.58	-4.89	148.58		3968	15.65	5.14	156.22

Table. No. 3.07

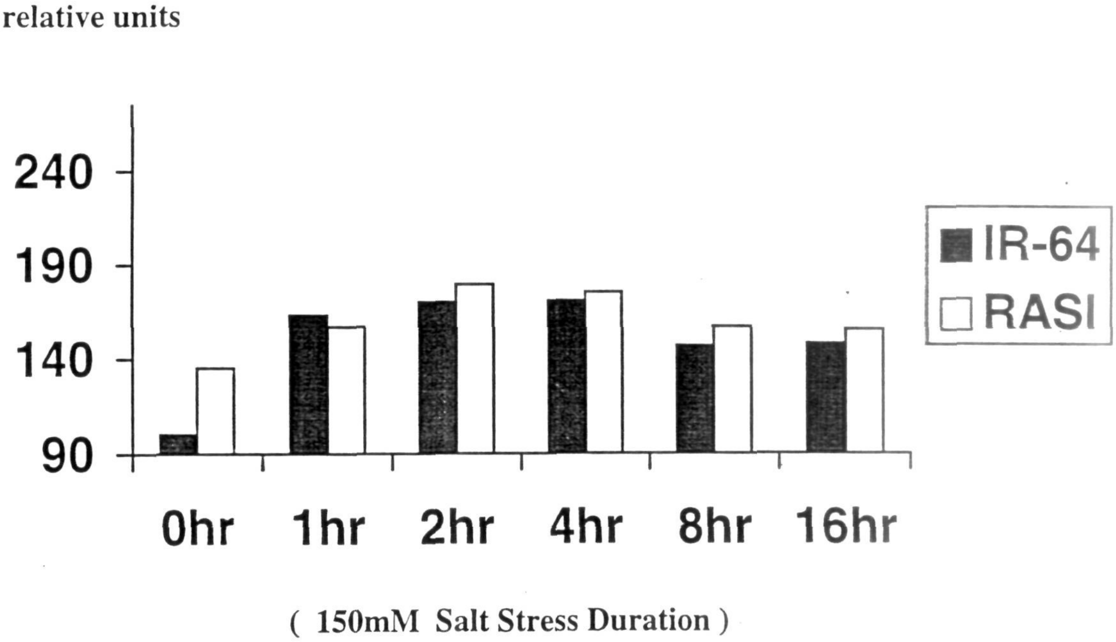


Fig. No.13.07

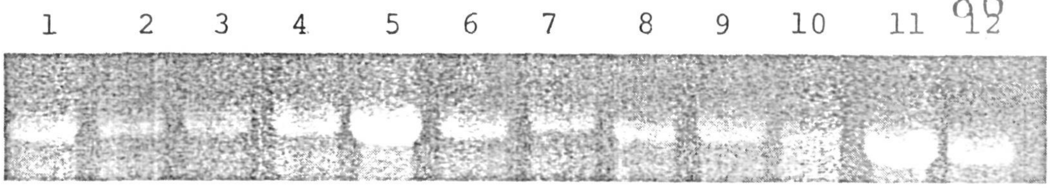


Fig. No. 11.08: 28S rRNA: Shoots-3

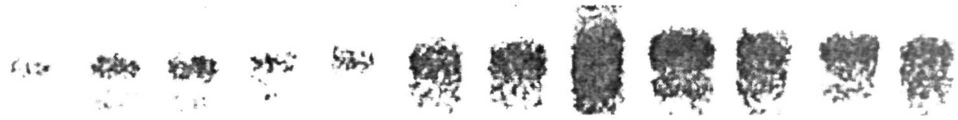


Fig. No. 12.08: FeSOD transcripts: Salt Stress: Shoots

Time (hrs)		IR-64	Str-Ctl %	I-R %	Graph		RASI	Str-Ctl %	R-I %	Graph
0		1797	0.00	-12.26	100.00		2048	0.00	13.97	113.97
1		2308	28.44	6.07	128.44		2176	6.25	-5.72	121.09
2		2158	20.09	-27.46	120.09		2975	45.26	37.86	165.55
4		3408	89.65	-21.83	189.65		4360	112.89	27.93	242.63
8		4595	155.70	27.43	255.70		3606	76.07	-21.52	200.67
16		3220	79.19	-2.54	179.19		3304	61.33	2.61	183.86

Table. No.3.08

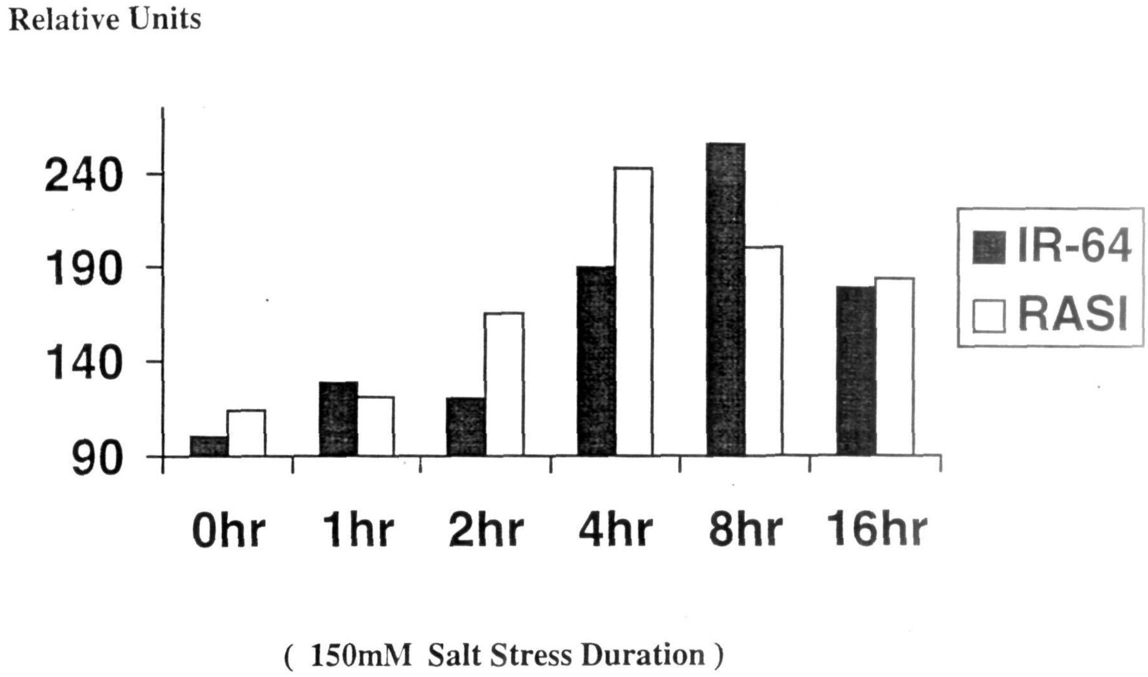


Fig. No. 13.08

Effects of salinity and drought on the accumulation of Cat2 transcripts

Desiccation

In both the varieties an increase in the transcript levels of Cat2 was observed in the root in response to desiccation. Though there was not much of a difference between the two varieties at 30% moisture loss, IR64 had 23% more Cat2 mRNA at 40% dehydration (Fig. No. 12.09, Fig. No. 13.09, Table No. 3.09).

The shoots of tolerant variety (Rasi) was able to quickly accumulate Cat2 transcripts at moderate level of (30%) dehydration, while IR64 failed to do so. At 30% dehydration Rasi had 24% more Cat2 mRNA than IR64. At an advanced stage of dehydration (40%), the transcripts were similar in both the varieties (Fig. No. 12.10, Fig. No. 13.10 and, Table No. 3.10).

Salt Stress

In the roots of IR64 (susceptible variety) and Rasi (tolerant variety), there were no changes in the transcript levels of Cat2 during stress. In the shoots how ever upregulation of Cat2 mRNA accumulation was observed. Both IR64 and Rasi recorded peak transcript levels at the 8th hour of salt stress, Rasi had 15% more Cat2 mRNA than IR64 (Fig. No. 12.11 and 12.12, Fig. No. 13.11 and 13.12, Table No. 3.11 and 3.12).

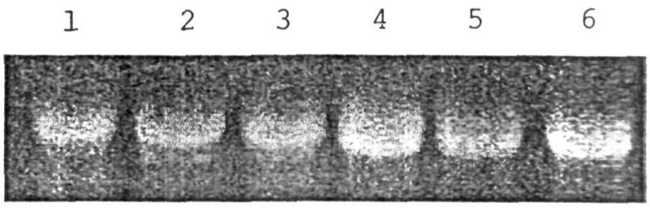


Fig. No. 11.09: 28S rRNA: Roots-6

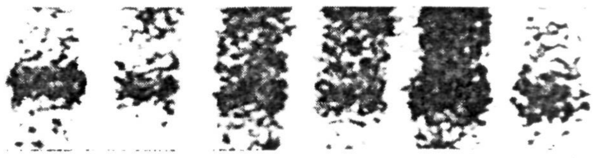


Fig. No. 12.09: Cat2 transcripts: Dehydration Stress: Roots

Stress		IR-64	Str-Ctl %	I-R %	Graph		RASI	Str-Ctl %	R-I %	Graph
0%		1393	0.00	19.78	100.00		1163	0.00	-16.51	83.49
30%		2052	47.31	-0.68	147.31		2066	77.64	0.68	148.31
40%		2537	82.12	23.28	182.12		2058	76.96	-18.88	147.74

Table. No. 3.09

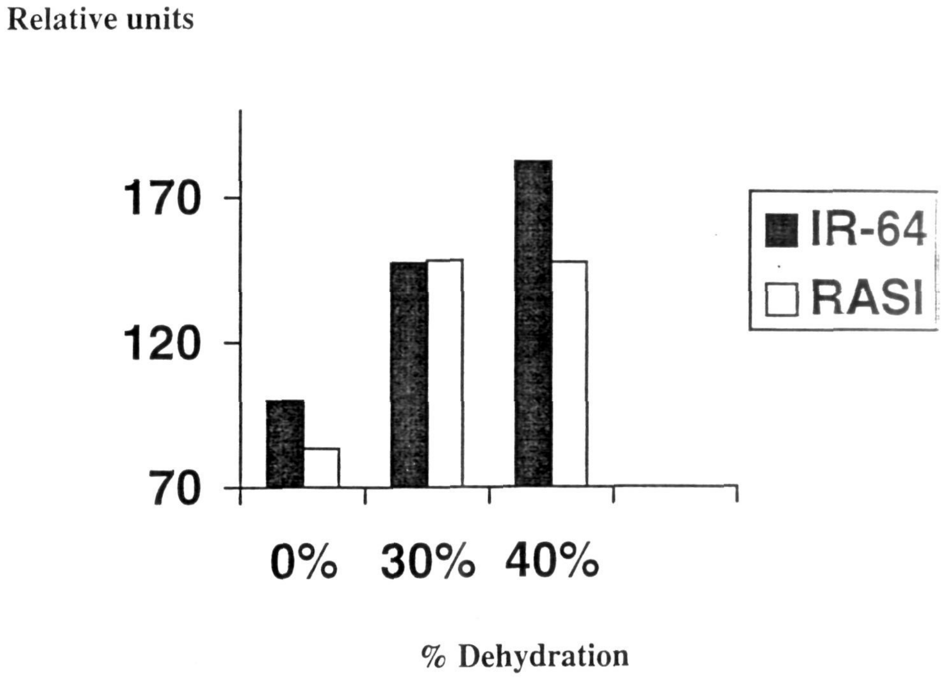


Fig. No. 3.09

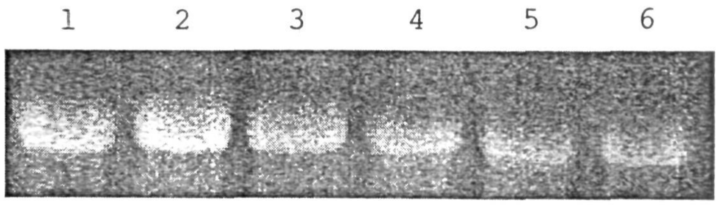


Fig. No. 11.10: 28S rRNA: Shoots-6

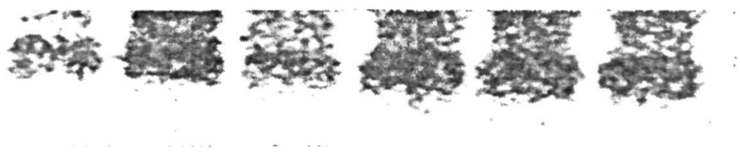


Fig. No. 12.10: Cat2 transcripts: Dehydration Stress: Shoots

Stress		IR-64	Str-Ctl %	I-R %	Graph		RASI	Str-Ctl %	R-I %	Graph
0%		1492	0.00	-1.78	100.00		1519	0.00	1.81	101.81
30%		1586	6.30	-19.45	106.30		1969	29.62	24.15	131.97
40%		2003	34.25	2.72	134.25		1950	28.37	-2.65	130.70

Table No. 3.10

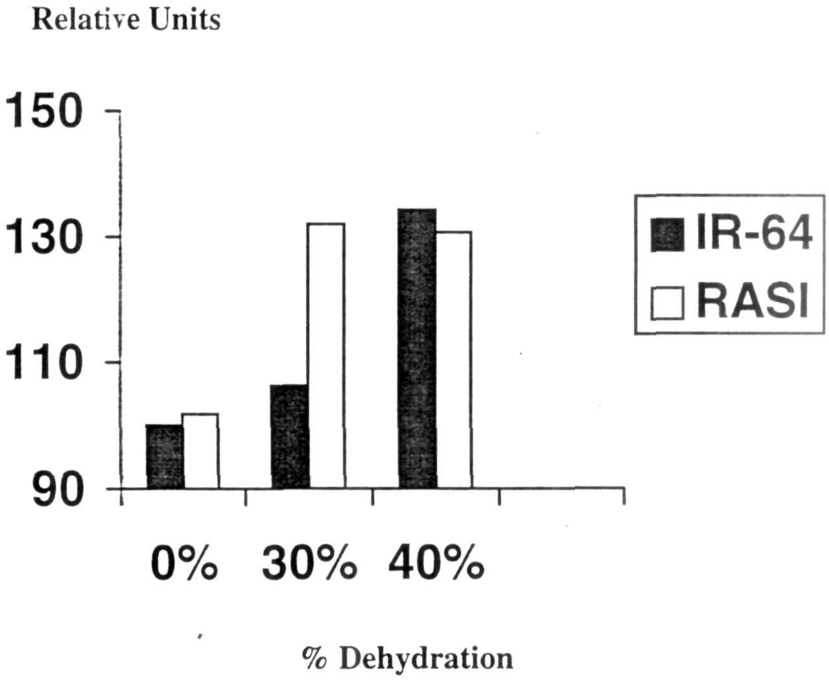


Fig. No. 13.10

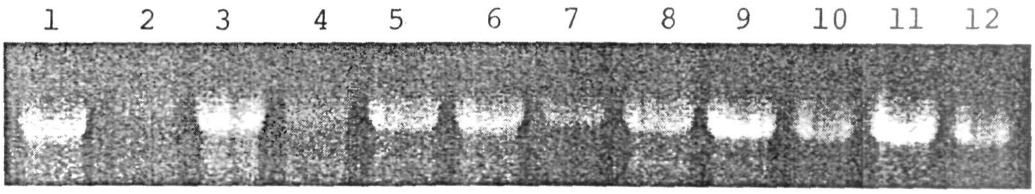


Fig. No. 11.11: 28S rRNA: Roots-5

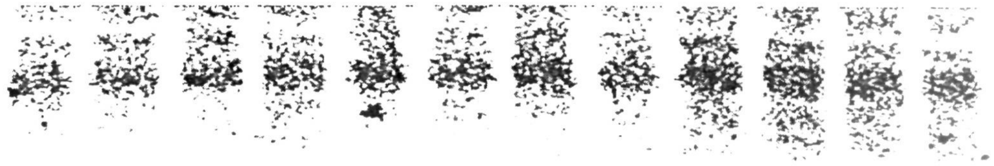


Fig. No. 12.11: Cat2 transcripts: Salt Stress: Roots

Time (hrs)		IR-64	Str-Ctl %	I-R %	Graph		RASI	Str-Ctl %	R-I %	Graph
0		967	0.00	3.87	100.00		931	0.00	-3.72	96.28
1		914	-5.48	-5.68	94.52		969	4.08	6.02	100.21
2		948	-1.96	-5.11	98.04		999	7.30	5.38	103.31
4		1010	4.45	3.48	104.45		976	4.83	-3.37	100.93
8		999	3.31	1.63	103.31		983	5.59	-1.60	101.65
16		937	-3.10	-0.32	96.90		940	0.97	0.32	97.21

Table. No. 3.11

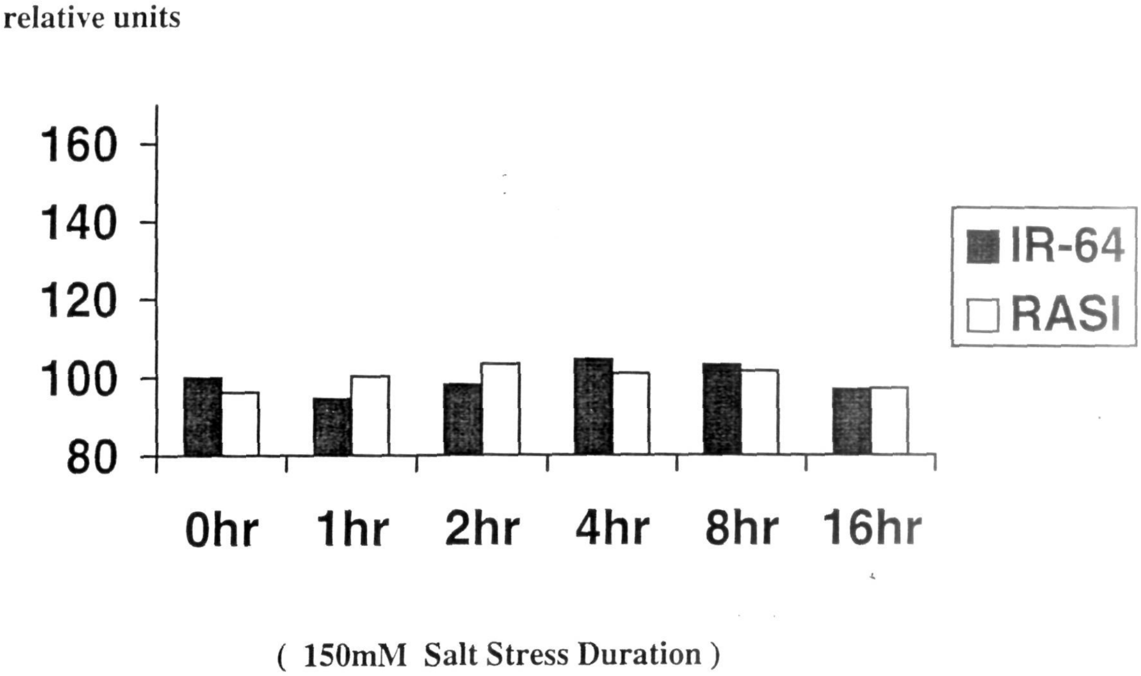


Fig. No. 13.11

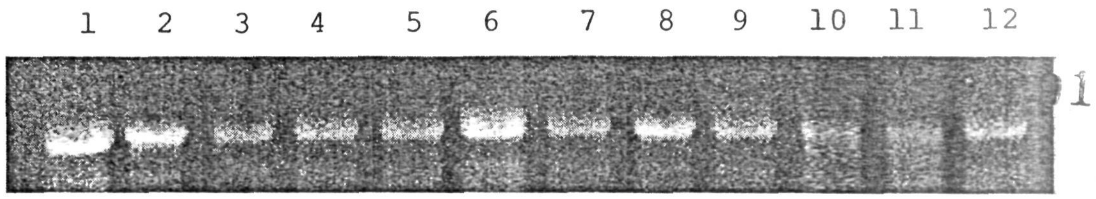


Fig. No. 11.12: 28S rRNA: Shoots-5

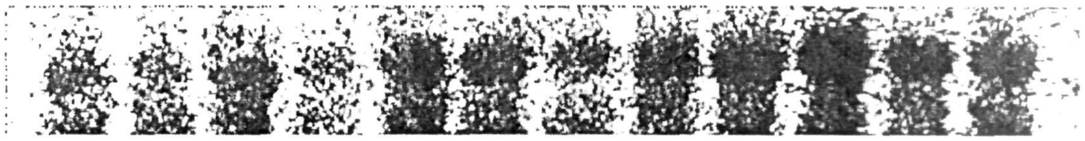


Fig. No. 12.12: Cat2 transcripts: Salt Stress: Shoots

Time (hrs)		IR-64	Str-Ctl %	I-R %	Graph		RASI	Str-Ctl %	R-I %	Graph
0		807	0.00	5.22	100.00		767	0.00	-4.96	95.04
1		924	14.50	12.68	114.50		820	6.91	-11.26	101.61
2		1037	28.50	3.49	128.50		1002	30.64	-3.38	124.16
4		897	11.15	-10.12	111.15		998	30.12	11.26	123.67
8		1103	36.68	-12.74	136.68		1264	64.80	14.60	156.63
16		1073	32.96	1.13	132.96		1061	38.33	-1.12	131.47

Table No. 12.12

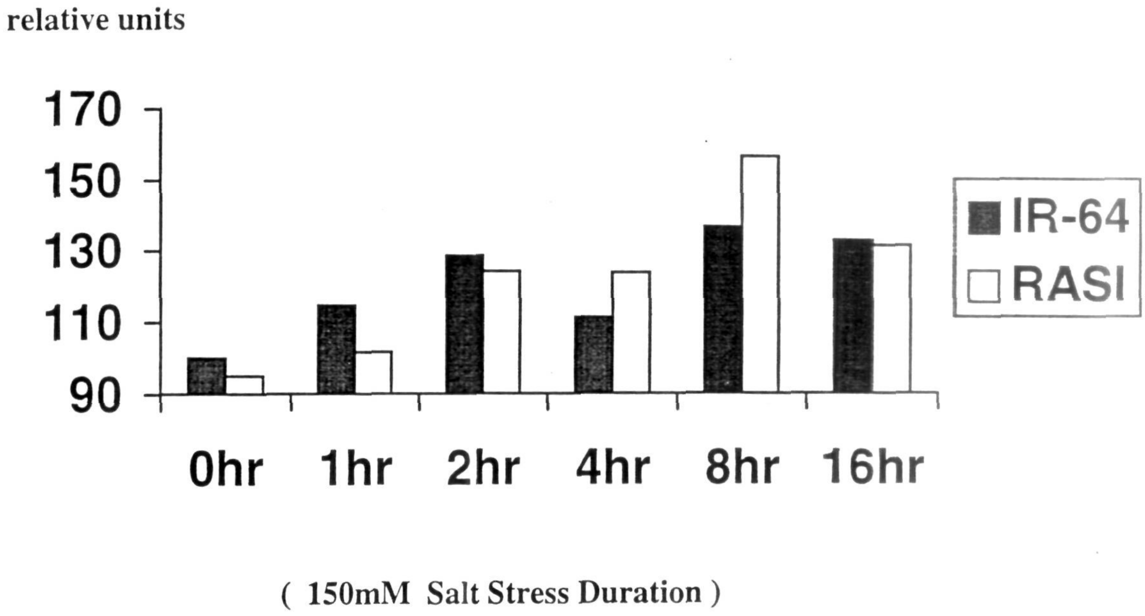


Fig. No.13.12

Influence of drought and salinity on the steady state level of cApx transcripts

Moisture Stress

Gradual increases of cAPx mRNA levels are observed in both roots and shoots of IR64 and Rasi during water deficit. At 30% water loss in the roots, both IR64 and Rasi had about 20-30% higher transcript compared unstressed roots. At 40% water loss Rasi had 15% more transcripts than IR64. In the shoots there was no change in response to moderate dehydration. At an advanced stage of dehydration only marginal increases of 10-12% in the cAPx mRNA levels was seen (Fig. No. 12.13 and 12.14, Fig. No. 13.13 and 13.14, Table No. 3.13 and 3.14).

Salt Stress

In the roots Rasi had higher cAPx mRNA levels and at all the time points during salinity. IR64 cAPx transcript levels reached a plateau from the 2nd hour on wards. Rasi on the other hand had maximum cAPx mRNA levels at the 16th hour of salt stress (42% more than control and 30% more than IR64).

The cAPx gene induction was marginal in the shoots during salinity. Differences of 20% or less was observed (Fig. No. 12.15 and 12.16, Fig. No. 13.15 and 13.16, Table No. 3.15 and 3.16).

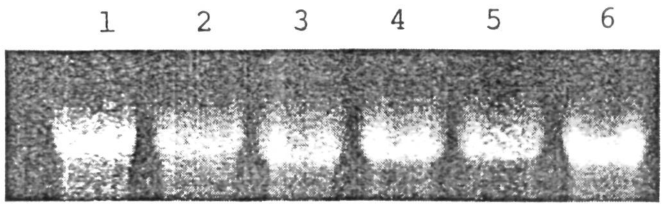


Fig. No. 11.13: 28S rRNA: Roots-8

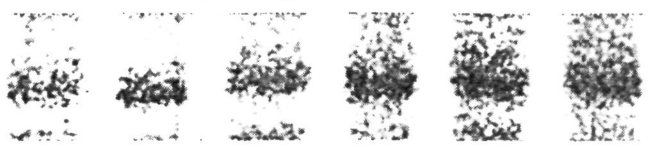


Fig. No. 12.13: Apx transcripts: Dehydration Stress: Roots

Stress		IR-64	Str-Ctl %	I-R %	Graph		RASI	Str-Ctl %	R-I %	Graph
0%		915	0.00	-5.48	100.00		968	0.00	5.79	105.79
30%		1179	28.85	0.60	128.85		1172	21.07	-0.59	128.09
40%		1380	50.82	-13.15	150.82		1589	64.15	15.14	173.66

Table No. 3.13

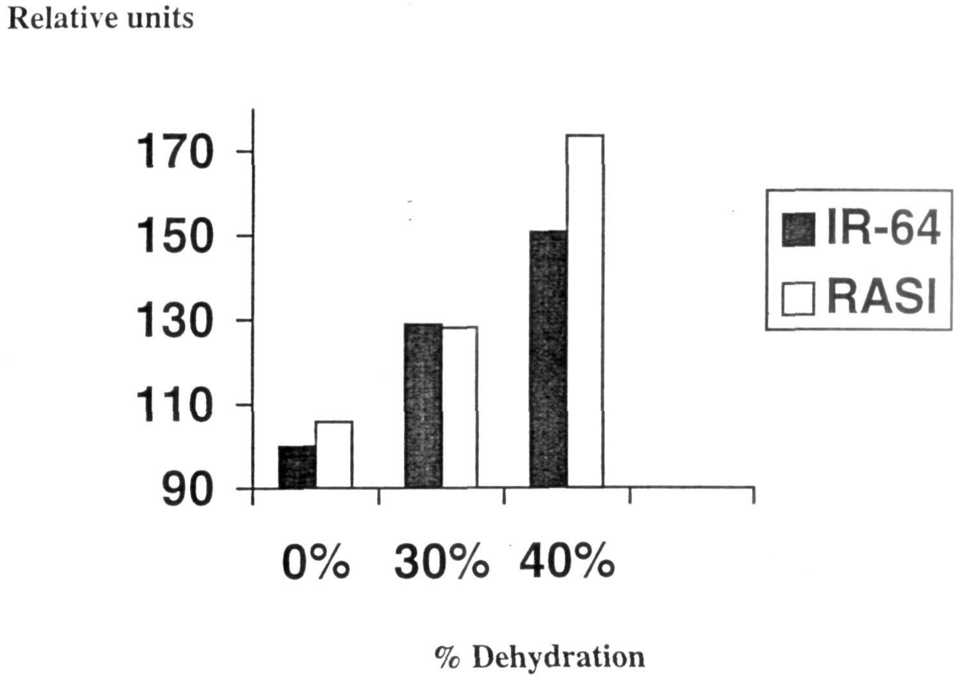


Fig. No. 13.13

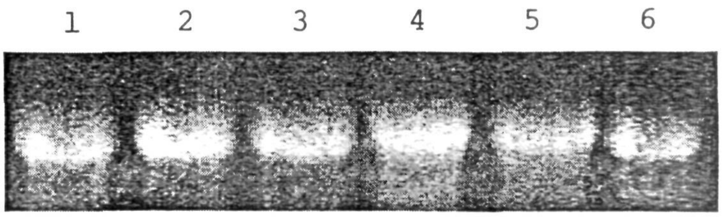


Fig. No. 11.14: 28S rRNA: Shoots-8

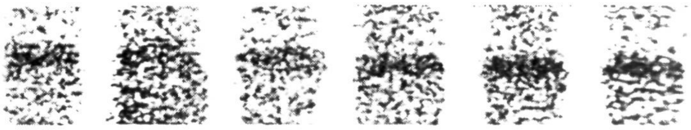


Fig. No. 12.14: Apx transcripts: Dehydration Stress: Shoots

Stress		IR-64	Str-Ctl %	I-R %	Graph		RASI	Str-Ctl %	R-I %	Graph
0%		910	0.00	-1.19	100.00		921	0.00	1.21	101.21
30%		904	-0.66	-3.62	99.34		938	1.85	3.76	103.08
40%		1009	10.88	-2.42	110.88		1034	12.27	2.48	113.63

Table No. 3.14

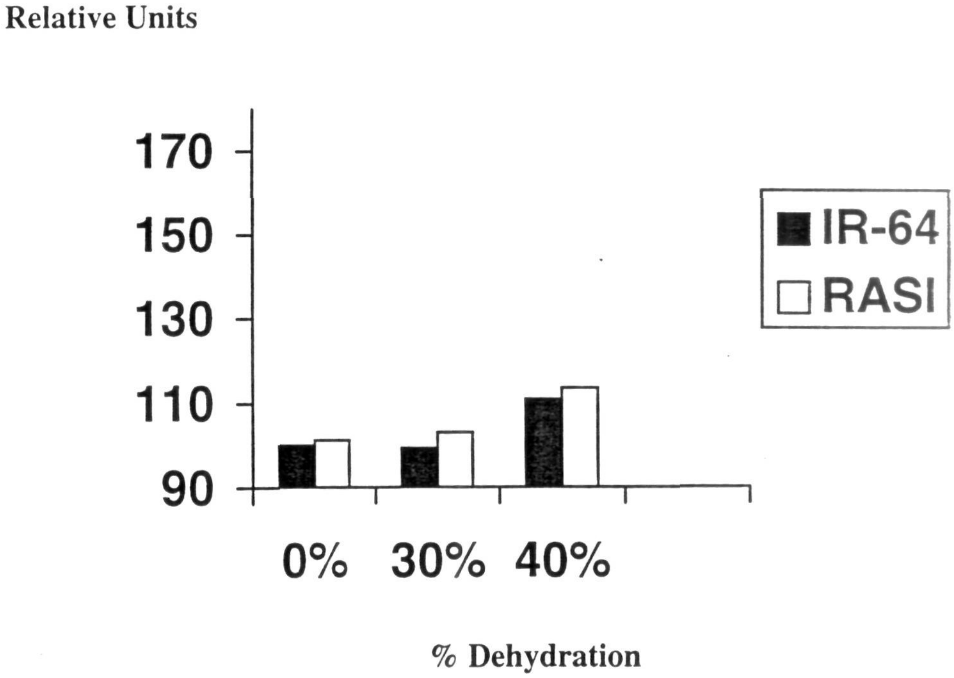


Fig. No. 13.14

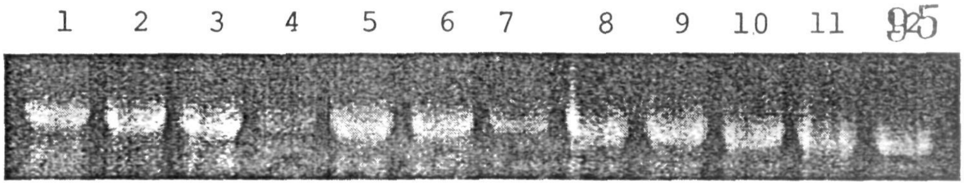


Fig. No. 11.15: 28S rRNA: Roots-7

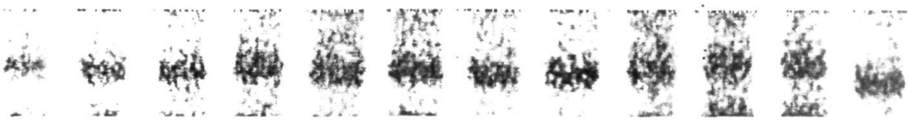


Fig. No. 12.15: Apx transcripts: Salt Stress: Roots

Time (hrs)		IR-64	Str-Ctl %	I-R %	Graph		RASI	Str-Ctl %	R-I %	Graph
0		609	0.00	-15.42	100.00		720	0.00	18.23	118.23
1		733	20.36	-13.97	120.36		852	18.33	16.23	139.90
2		873	43.35	-7.52	143.35		944	31.11	8.13	155.01
4		769	26.27	-8.12	126.27		837	16.25	8.84	137.44
8		835	37.11	-7.02	137.11		898	24.72	7.54	147.45
16		792	30.05	-22.81	130.05		1026	42.50	29.55	168.47

Table. No. 3.15

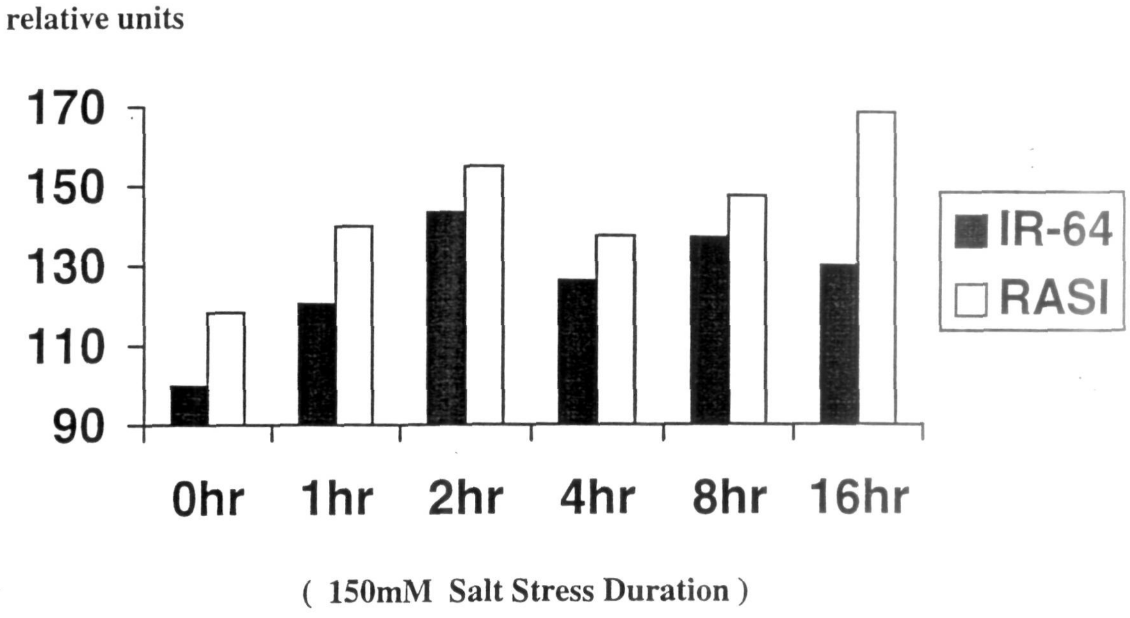


Fig. No. 13.15

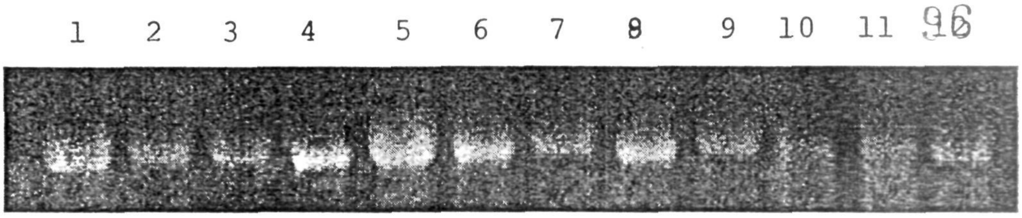


Fig. No. 11.16: 28S rRNA: Shoots-7

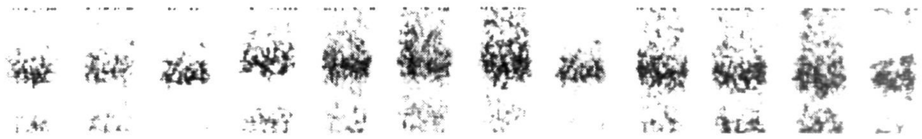


Fig. No. 12.16: Apx transcripts: Salt Stress: Shoots

Time (hrs)		IR-64	Str-Ctl %	I-R %	Graph		RASI	Str-Ctl %	R-I %	Graph
0		1035	0.00	-0.10	100.00		1036	0.00	0.10	100.10
1		1011	-2.32	-15.54	97.68		1197	15.54	18.40	115.65
2		1160	12.08	8.41	112.08		1070	3.28	-7.76	103.38
4		1262	21.93	2.85	121.93		1227	18.44	-2.77	118.55
8		1191	15.07	0.25	115.07		1188	14.67	-0.25	114.78
16		1134	9.57	-5.74	109.57		1203	16.12	6.08	116.23

Table. No. 3.16

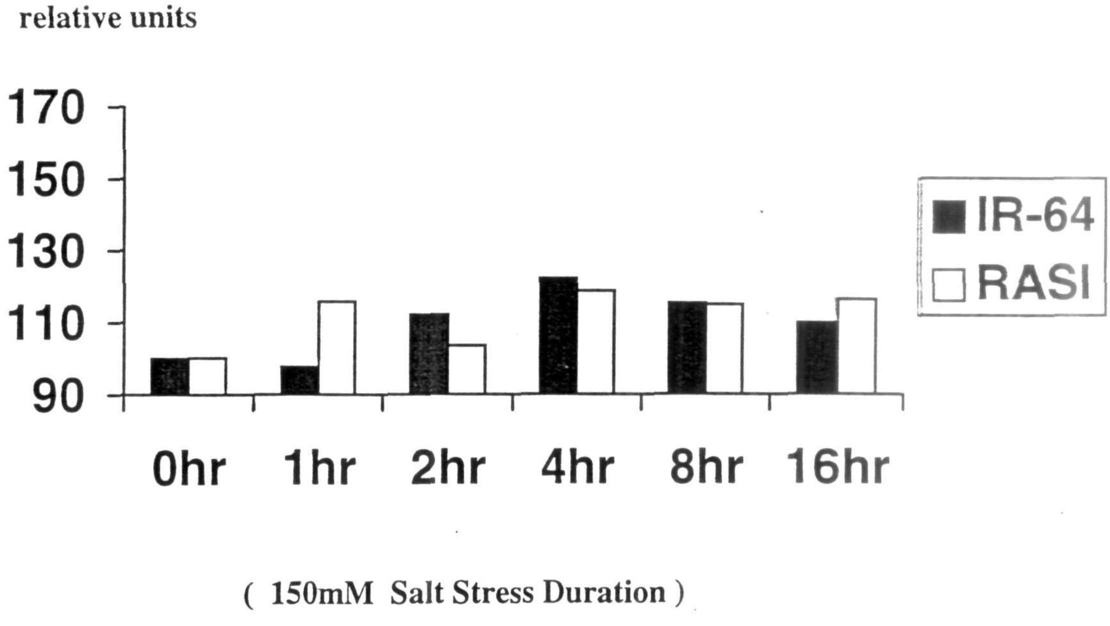


Fig. No. 13.16

Induction of Membrane Channel Protein gene expression during stress

Water Deficit

There were no differences in MCP mRNA levels in the roots and shoots during water deficit between IR64 and Rasi and in comparison to controls (Fig. No. 12.17 and 12.18, Fig. No. 13.17 and 13.18, Table No. 3.17 and 3.18).

Osmotic Stress

There were no changes in the accumulation of MCP mRNA in the shoots during salinity. In the roots however IR64 had considerably higher transcript levels in response to 150mM salt at the 2nd hour itself (60% compared to control). The increase in MCP mRNA in the tolerant variety (Rasi) was delayed and much less compared to IR64 (Fig. No. 12.19 and 12.20, Fig. No. 13.19 and 13.20, Table No. 3.19 and 3.20).

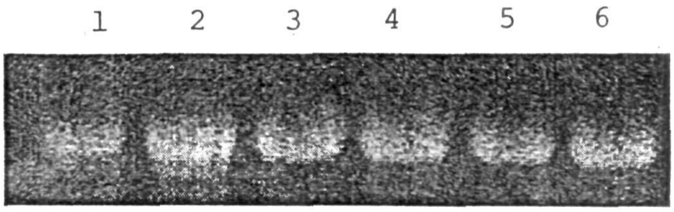


Fig. No. 11.17: 28S rRNA: Roots-10

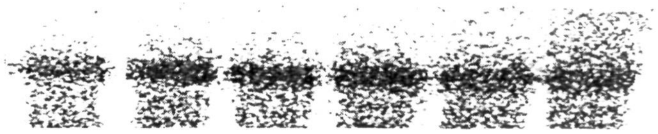


Fig. No. 12.17: MCP transcripts: Dehydration Stress: Roots

Stress		IR-64	Str-Ctl %	I-R %	Graph		RASI	Str-Ctl %	R-I %	Graph
0%		1245	0.00	-0.48	100.00		1251	0.00	0.48	100.48
30%		1244	-0.08	-0.96	99.92		1256	0.40	0.96	100.88
40%		1260	1.20	-0.32	101.20		1264	1.04	0.32	101.53

Table No. 3.17

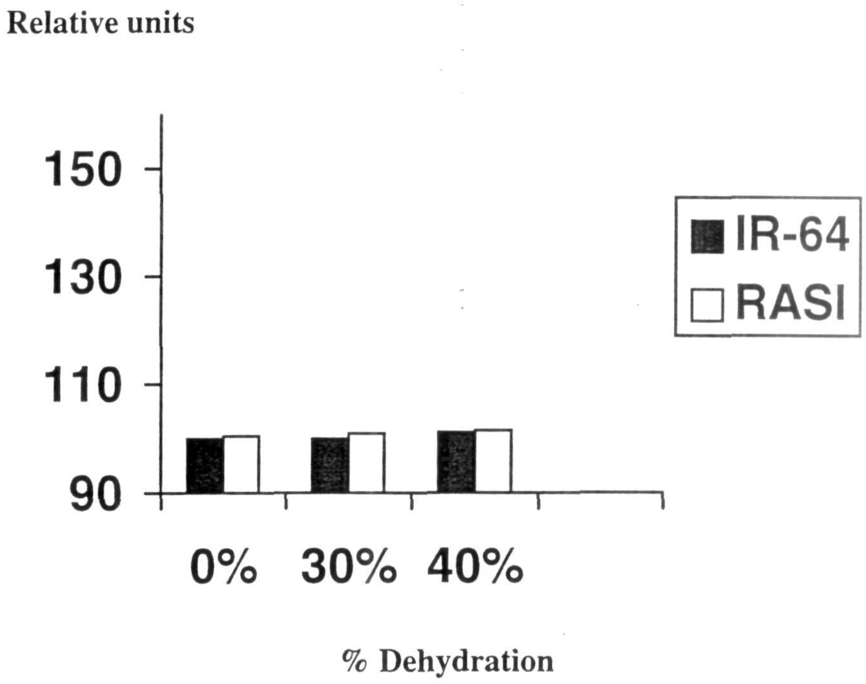


Fig. No. 13.17

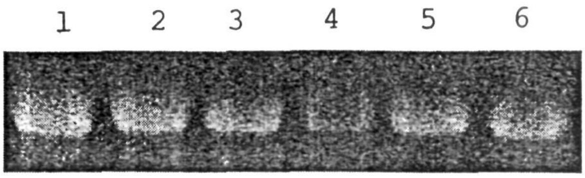


Fig. No. 11.18: 28S rRNA: Shoots-10

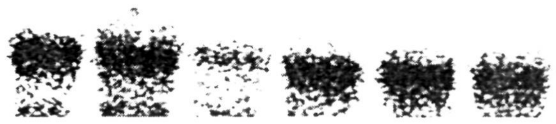


Fig. No. 12.18: MCP transcripts: Dehydration Stress: Shoots

Stress		IR-64	Str-Ctl %	I-R %	Graph		RASI	Str-Ctl %	R-I %	Graph
0%		1247	0.00	-1.27	100.00		1263	0.00	1.28	101.28
30%		1171	-6.09	-8.59	93.91		1281	1.43	9.39	102.73
40%		1301	4.33	-0.23	104.33		1304	3.25	0.23	104.57

Table No. 3.18

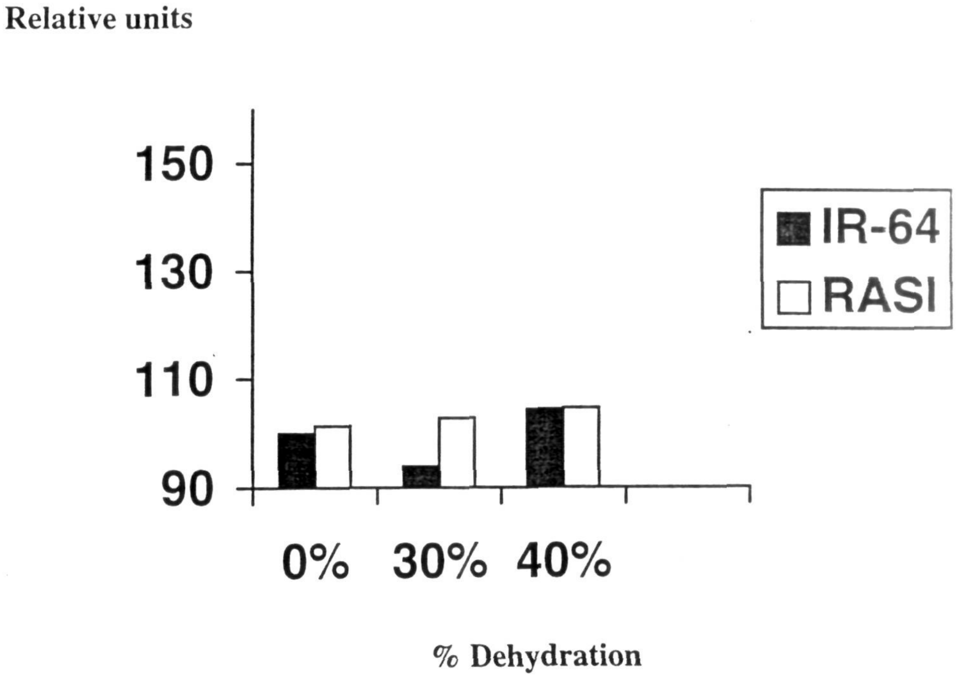


Fig. No. 13.18

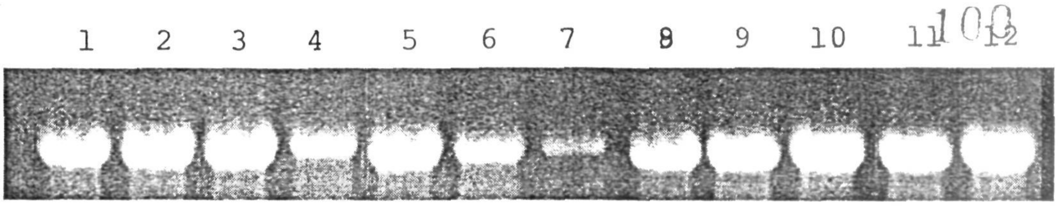


Fig. No. 11.19: 28S rRNA: Roots-9

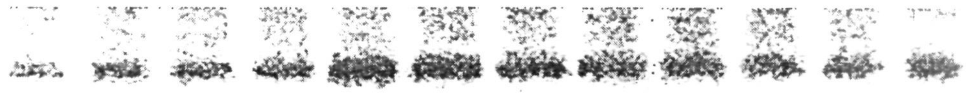
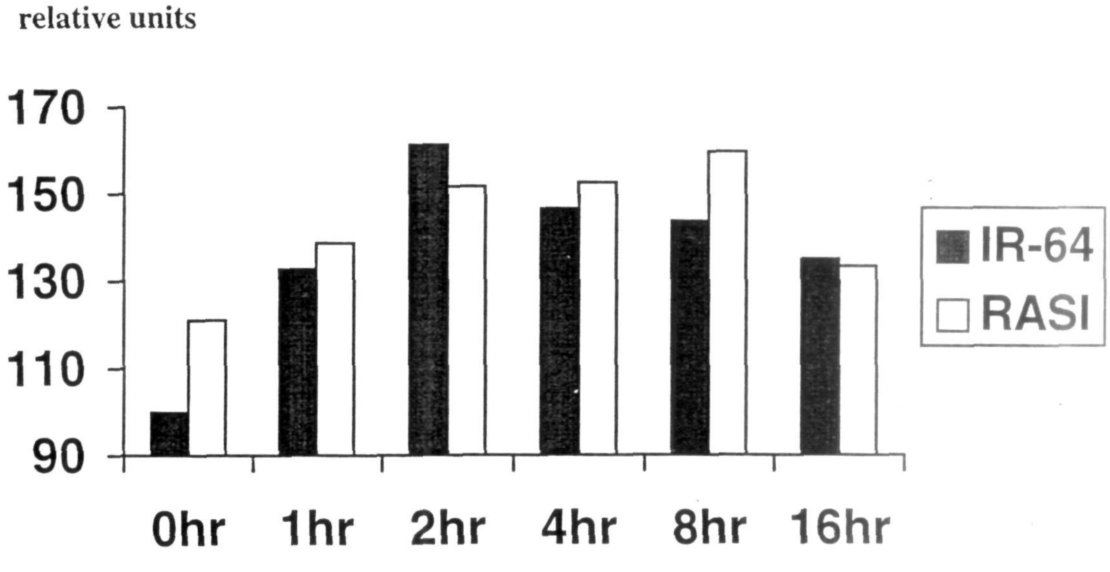


Fig. No. 12.19: MCP transcripts: Salt Stress: Roots

Time (hrs)		IR-64	Str-Ctl %	I-R %	Graph		RASI	Str-Ctl %	R-I %	Graph
0		580	0.00	-17.38	100.00		702	0.00	21.03	121.03
1		770	32.76	-4.35	132.76		805	14.67	4.55	138.79
2		935	61.21	6.25	161.21		880	25.36	-5.88	151.72
4		850	46.55	-3.95	146.55		885	26.07	4.12	152.59
8		833	43.62	-10.04	143.62		926	31.91	11.16	159.66
16		783	35.00	1.29	135.00		773	10.11	-1.28	133.28

Table. No. 3.19



(150mM Salt Stress Duration)

Fig. No. 3.19

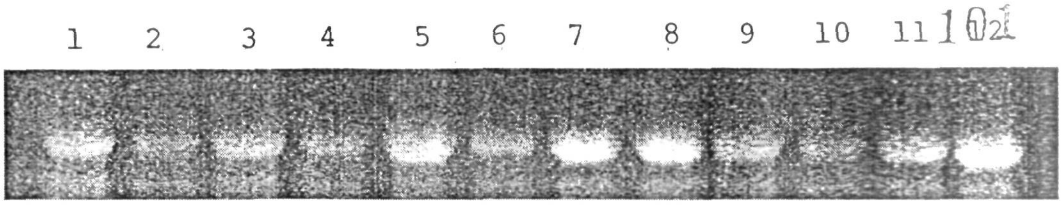


Fig. No. 11.20: 28S rRNA: Shoots-9

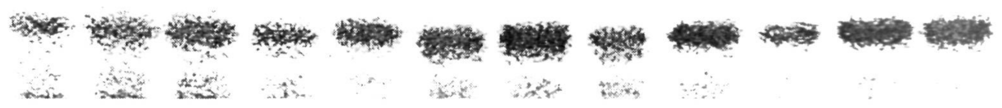


Fig. No. 12.20 : MCP transcripts: Salt Stress: Shoots

Time (hrs)		IR-64	Str-Ctl %	I-R %	Graph		RASI	Str-Ctl %	R-I %	Graph
0		1669	0.00	-0.30	100.00		1674	0.00	0.30	100.30
1		1692	1.38	1.32	101.38		1670	-0.24	-1.30	100.06
2		1689	1.20	0.60	101.20		1679	0.30	-0.59	100.60
4		1709	2.40	2.46	102.40		1668	-0.36	-2.40	99.94
8		1693	1.44	1.38	101.44		1670	-0.24	-1.36	100.06
16		1739	4.19	0.87	104.19		1724	2.99	-0.86	103.30

Table. No. 3.20

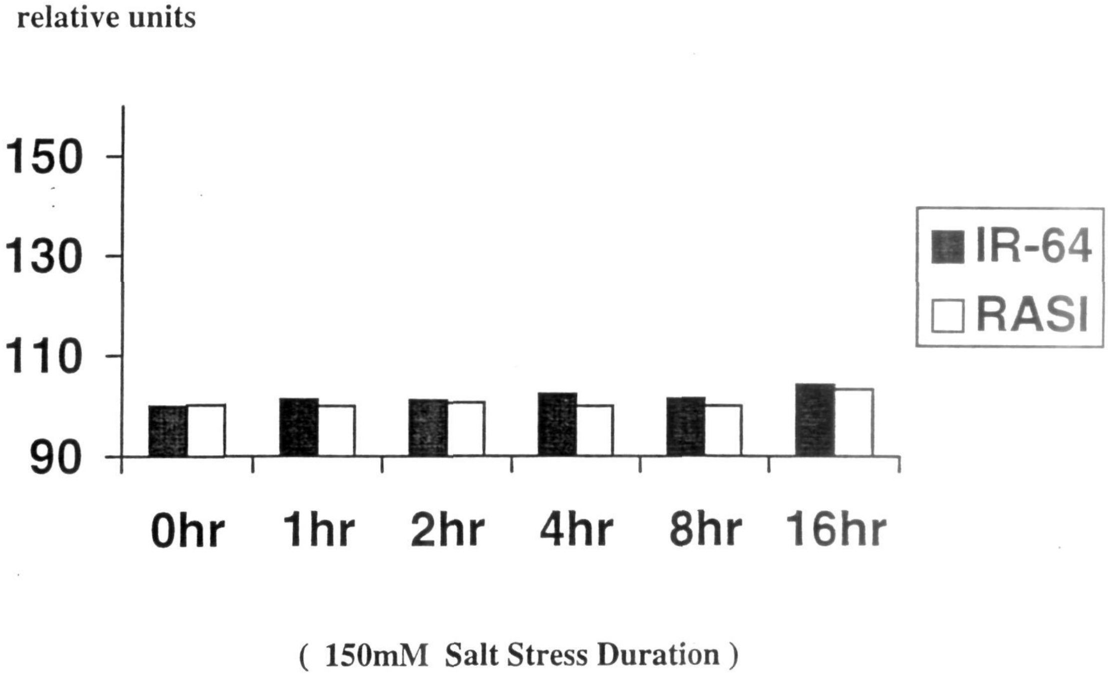


Fig. No. 13.20

HSP-70 transcript levels during stress

Desiccation

In the roots, response to 30% water loss, the HSP-70 mRNA levels incresed by 37% in the susceptible variety (IR64) while it was just 12% in the tolerant variety. In both the varieties the transcript varieties increased by about 45% at advanced stage of desiccation (Fig. No. 12.21, Fig. No. 13.21, Table No. 3.21).

An increase of 73% was observed in HSP-70 mRNA of IR64 at 30% moisture loss in the shoots. The increase was much less in Rasi (40%). There were no differences in the transcript levels of HSP-70 at 40% moisture loss in both the varieties (Fig. No. 12.22, Fig. No. 13.22, Table No. 3.22).

Salt Stress

Dramatic induction of the HSP-70 gene was noticed as early as 1 hour of salt stress in the roots. The induction was delayed in Rasi and noticed at the 8th hour of salinity (Fig. No. 12.23, Fig. No. 13.23, Table No. 3.23).

In the shoots also induction of the HSP-70 gene occurred at the 2nd hour of salinity in the susceptible variety. At the later stage of salt stress, Rasi had higher HSP-70 transcript levels compared to IR64, by 30% (Fig. No. 12.24, Fig. No. 13.24, Table No. 3.24).

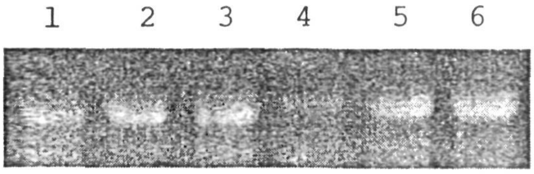


Fig. No. 11.21: 28S rRNA: Roots-12

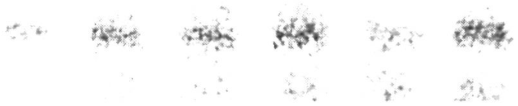


Fig. No. 12.21: HSP transcripts: Dehydration Stress: Roots

Stress		IR-64	Str-Ctl %	I-R %	Graph		RASI	Str-Ctl %	R-I %	Graph
0%		977	0.00	-16.28	100.00		1167	0.00	19.45	119.45
30%		1336	36.75	2.14	136.75		1308	12.08	-2.10	133.88
40%		1409	44.22	-17.79	144.22		1714	46.87	21.65	175.44

Table. No. 3. 21

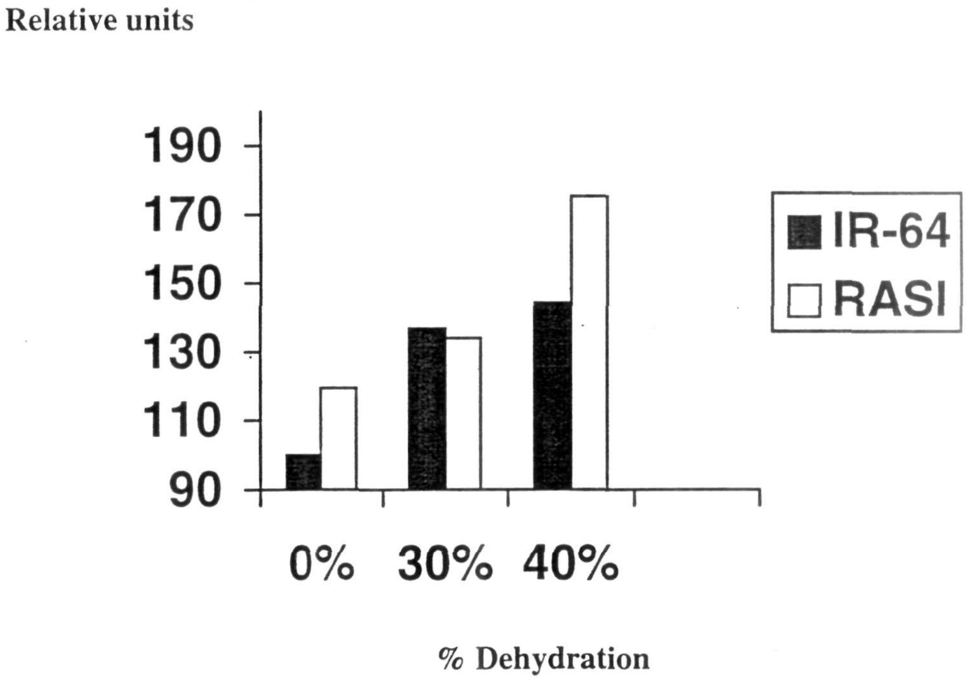


Fig. No. 13.21

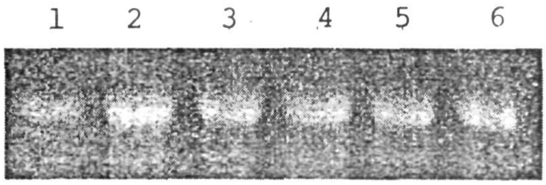


Fig. No. 11.22: 28S rRNA: Shoots-11

Fig. No. 12.22: HSP transcripts: Dehydration Stress: Shoots

Stress		IR-64	Str-Ctl %	I-R %	Graph		RASI	Str-Ctl %	R-I %	Graph
0%		948	0.00	-8.76	100.00		1039	0.00	9.60	109.60
30%		1647	73.73	12.65	173.73		1462	40.71	-11.23	154.22
40%		1629	71.84	0.12	171.84		1627	56.59	-0.12	171.62

Table. No. 3.22

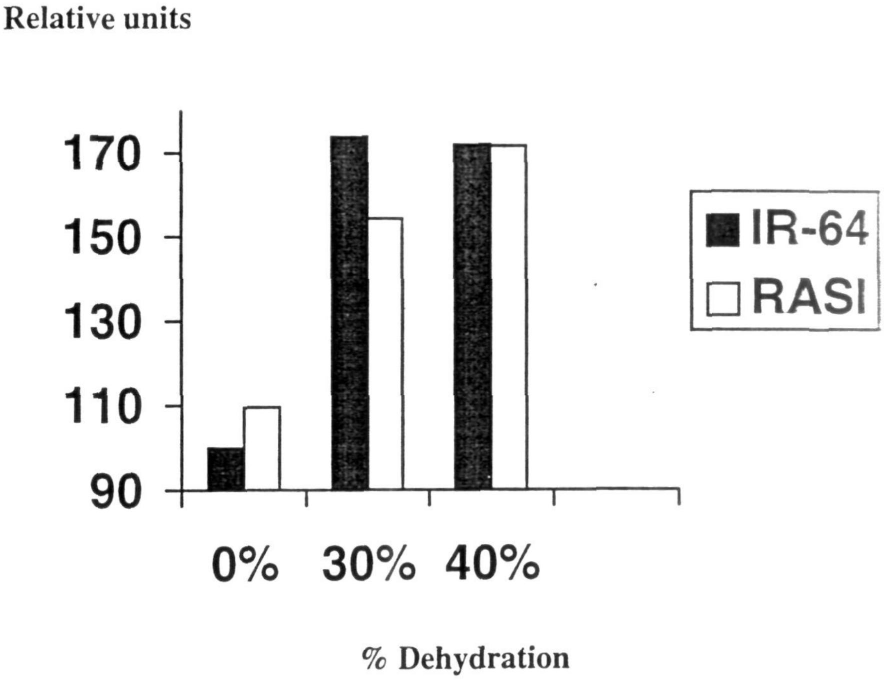


Fig. No. 13.22

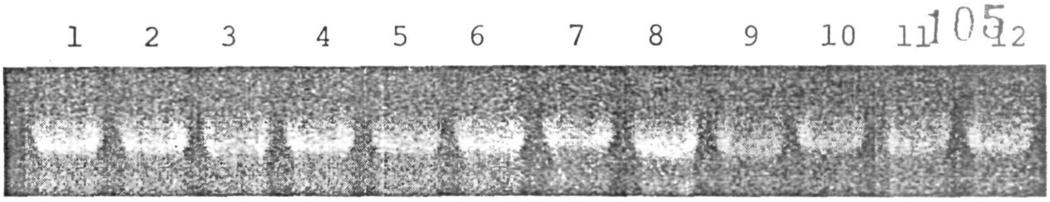
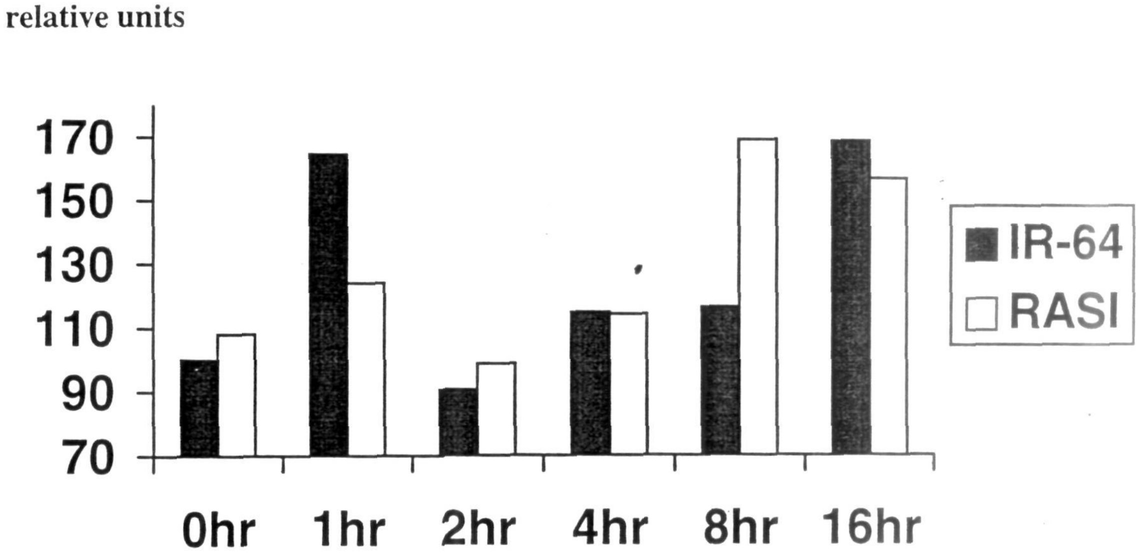


Fig. No. 11.23: 28S rRNA: Roots-11

Fig. No. 12.23: HSP transcripts: Salt Stress: Roots

Time (hrs)		IR-64	Str-Ctl %	I-R %	Graph		RASI	Str-Ctl %	R-I %	Graph
0		1724	0.00	-7.41	100.00		1862	0.00	8.00	108.00
1		2834	64.39	32.62	164.39		2137	14.77	-24.59	123.96
2		1564	-9.28	-8.16	90.72		1703	-8.54	8.89	98.78
4		1977	14.68	0.56	114.68		1966	5.59	-0.56	114.04
8		2004	16.24	-31.25	116.24		2915	56.55	45.46	169.08
16		2908	68.68	7.70	168.68		2700	45.01	-7.15	156.61

Table. No. 3.23



(150mM Salt Stress Duration)

Fig. No. 13.23

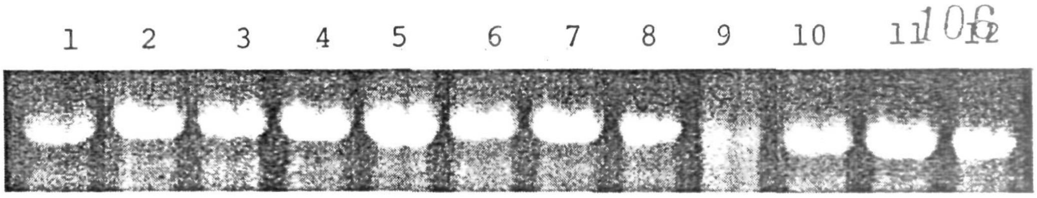


Fig. No. 11.24: 28S rRNA: Shoots-11

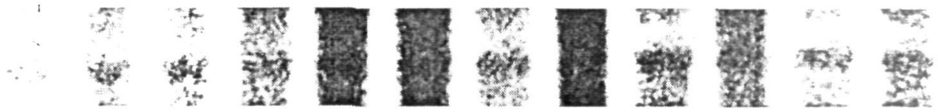


Fig. No. 12.24: HSP transcripts: Salt Stress: Shoots

Time (hrs)		IR-64	Str-Ctl %	I-R %	Graph		RASI	Str-Ctl %	R-I %	Graph
0		919	0.00	-30.43	100.00		1321	0.00	43.74	143.74
1		1278	39.06	-20.37	139.06		1605	21.50	25.59	174.65
2		2253	145.16	8.84	245.16		2070	56.70	-8.12	225.24
4		1713	86.40	-20.10	186.40		2144	62.30	25.16	233.30
8		1943	111.43	11.92	211.43		1736	31.42	-10.65	188.90
16		1612	75.41	-22.80	175.41		2088	58.06	29.53	227.20

Table No. 3.24

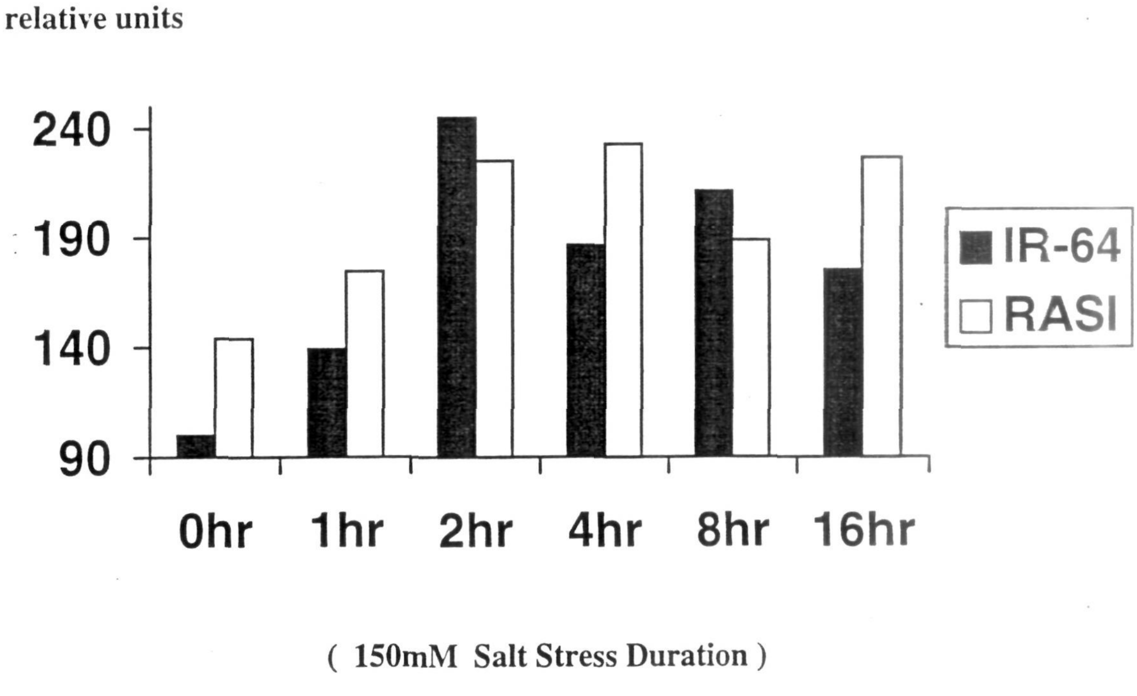


Fig. No. 13.24

Changes in the transcription of Cyclophilin gene during abitoc stress

Salinity and dehydration failed to transcriptionally activate the cyclophilin gene. Almost no differences were observed both in IR64 and Rasi, in roots and shoots, in the CyP mRNA levels during desiccation and salt stress (Fig. No. 12.25, 12.26, 12.27 and 12.28, Fig. No. 13.25, 13.26, 13.27 and 13.28, Table No. 13.25, 13.26, 13.27 and 13.28).

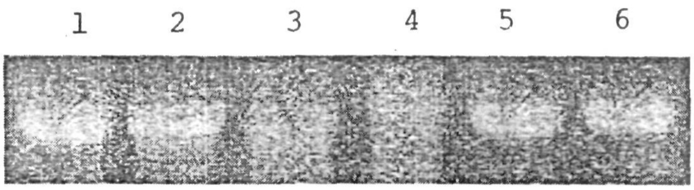


Fig. No. 11.25: 28S rRNA: Roots-4

Fig. No. 12.25: Cyclophilin transcripts: Dehydration Stress: Roots

Stress		IR-64	Str-Ctl %	I-R %	Graph		RASI	Str-Ctl %	R-I %	Graph
0%		2338	0.00	1.74	100.00		2298	0.00	-1.71	98.29
30%		2264	-3.17	-3.25	96.83		2340	1.83	3.36	100.09
40%		2297	-1.75	-12.86	98.25		2636	14.71	14.76	112.75

Table. No. 3.25

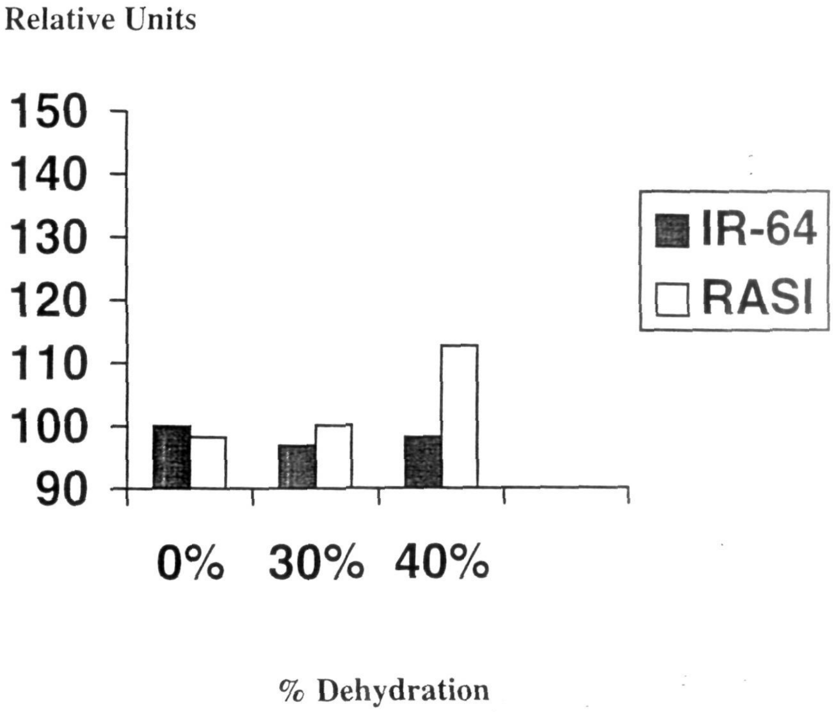


Fig. No. 13.25

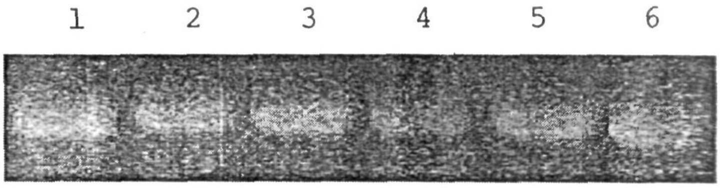


Fig. No. 11.26: 28S rRNA: Shoots-4

Fig. No. 12.26: Cyclophilin transcripts: Dehydration Stress: Shoots

Stress		IR-64	Str-Ctl %	I-R %	Graph		RASI	Str-Ctl %	R-I %	Graph
0%		2515	0.00	0.52	100.00		2502	0.00	-0.52	99.48
30%		2664	5.92	-0.63	105.92		2681	7.15	0.64	106.60
40%		2612	3.86	-3.22	103.86		2699	7.87	3.33	107.32

Table. No. 3.26

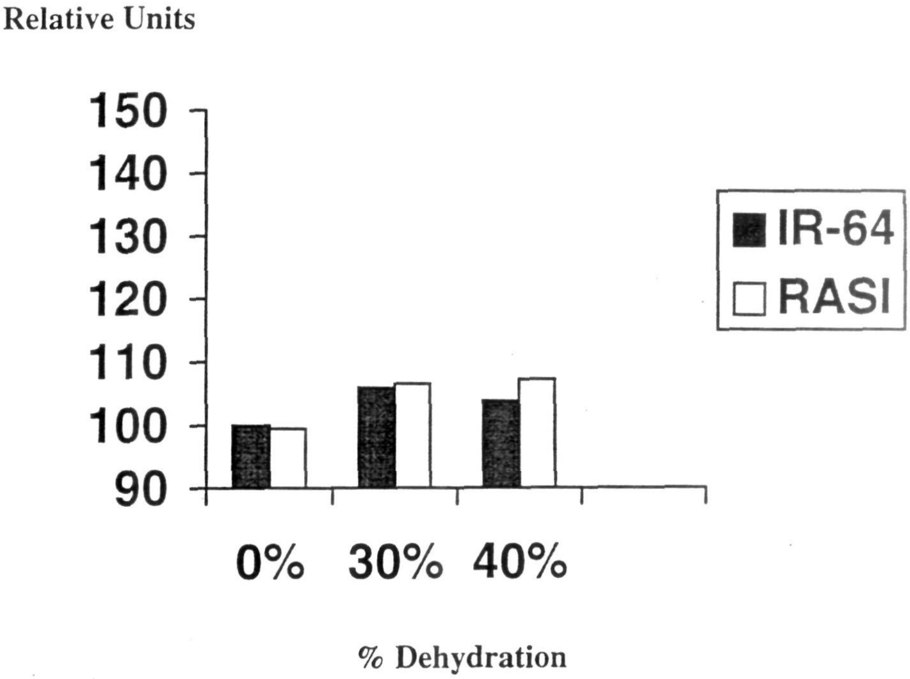


Fig. No. 13.26

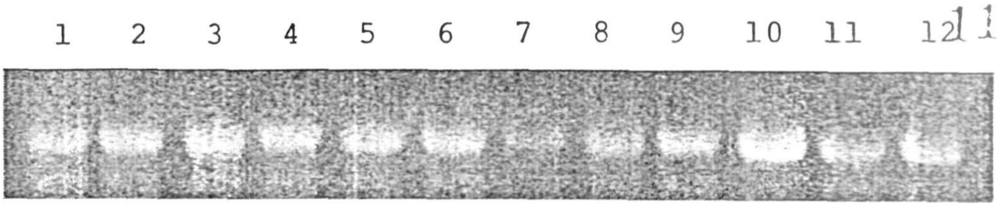


Fig. No. 11.27: 28S rRNA: Roots-3

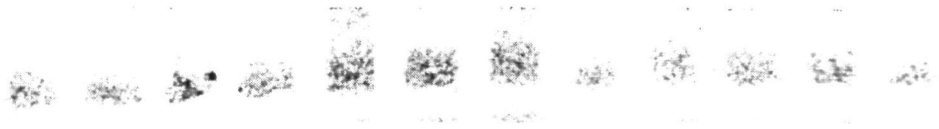
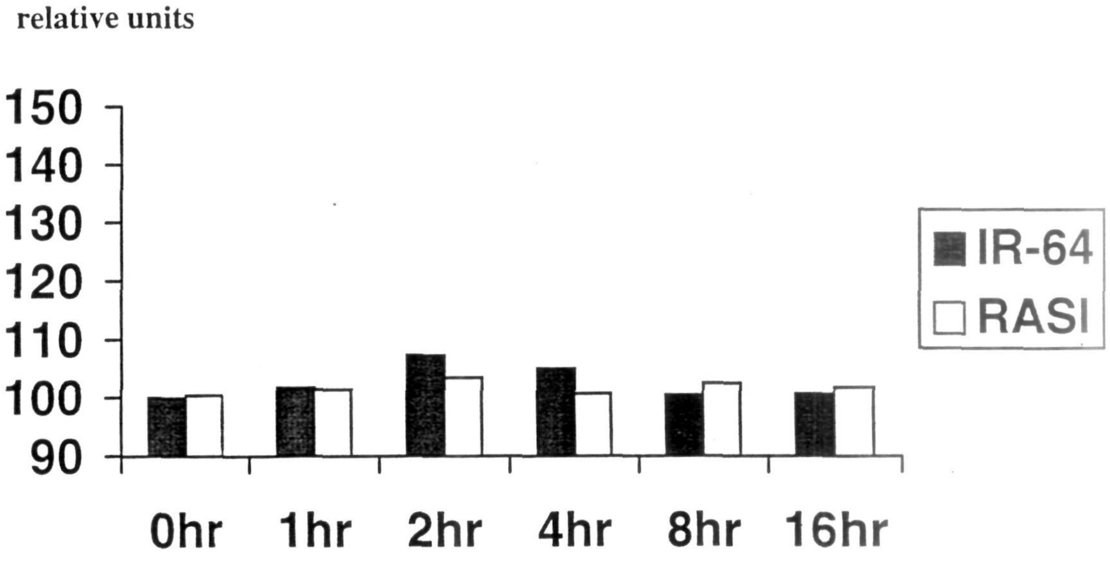


Fig. No. 12.27: Cyclophilin transcripts: Salt Stress: Roots

Time (hrs)		IR-64	Str-Ctl %	I-R %	Graph		RASI	Str-Ctl %	R-I %	Graph
0		2053	0.00	-0.44	100.00		2062	0.00	0.44	100.44
1		2091	1.85	0.29	101.85		2085	1.12	-0.29	101.56
2		2204	7.36	3.72	107.36		2125	3.06	-3.58	103.51
4		2158	5.11	4.25	105.11		2070	0.39	-4.08	100.83
8		2065	0.58	-2.04	100.58		2108	2.23	2.08	102.68
16		2071	0.88	-1.05	100.88		2093	1.50	1.06	101.95

Table. No. 3.27



(150mM Salt Stress Duration)

Fig. No. 13.27

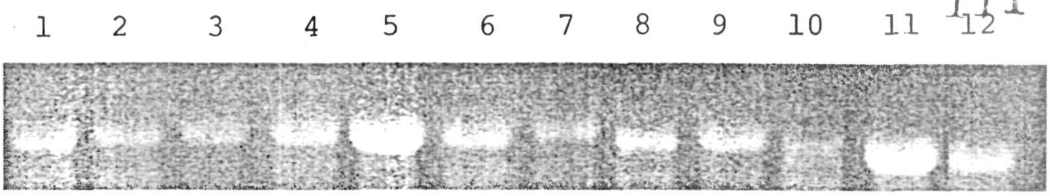


Fig. No. 11.28: 28S rRNA: Shoots-3

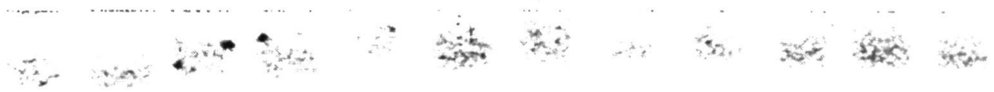


Fig. No. 12.28: Cyclophilin transcripts: Salt Stress: Shoots

Time (hrs)		IR-64	Str-Ctl %	I-R %	Graph		RASI	Str-Ctl %	R-I %	Graph
0		2344	0.00	1.21	100.00		2316	0.00	-1.19	98.81
1		2273	-3.03	-4.70	96.97		2385	2.98	4.93	101.75
2		2210	-5.72	-5.88	94.28		2348	1.38	6.24	100.17
4		2421	3.28	-10.20	103.28		2696	16.41	11.36	115.02
8		2176	-7.17	-10.08	92.83		2420	4.49	11.21	103.24
16		2430	3.67	0.87	103.67		2409	4.02	-0.86	102.77

Table. No. 3.28

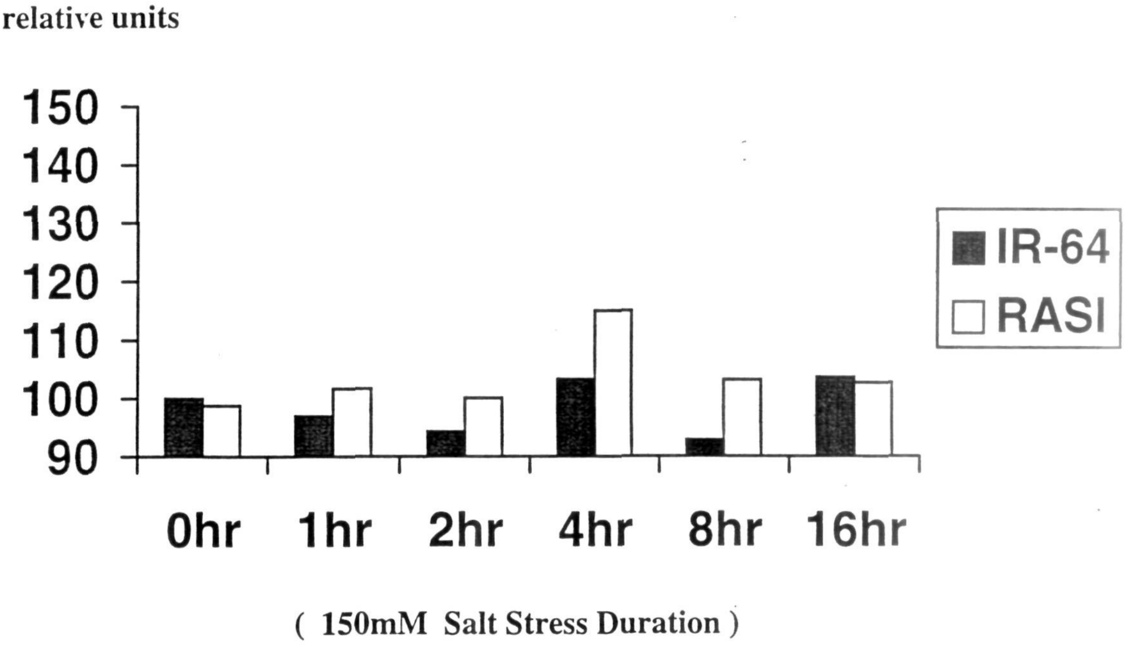


Fig. No. 13.28

Thaumatin-like Protein (TL-Protein) mRNA levels

Expression of TL-protein was observed only in the shoots. There was almost no expression of this gene in the roots.

Water Loss

Upregulation of the TL-protein gene was observed only in IR64 during dehydration especially at 30% desiccation, where the TL-protein mRNA level was 22% more than control. In the tolerant variety the gene expression was marginally suppressed (by 7%) in response to advanced drought (Fig. No. 12.29, Fig. No. 13.29, Table No. 3.29).

Salt Stress

Salinity elicited greater response in the susceptible variety with respect to increases in TL-protein mRNA levels. In both the varieties, gene expression was stimulated by 25-32% at the 2nd hour of salt stress. There after there was a suppression of gene expression in both the varieties. The down regulation of gene expression of TL-protein was to a greater extent in the tolerant variety. The TL-protein mRNA levels were 25% less than control in Rasi in response to 16hours of 150mM salt stress (Fig. No. 12.30, Fig. No. 13.30, Table No. 3.30).

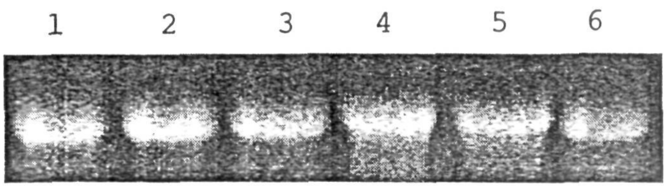


Fig. No. 11.29: 28S rRNA: Shoots-8

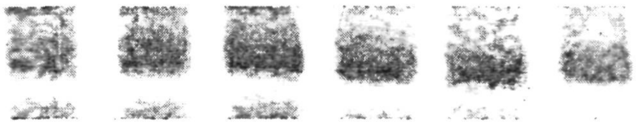


Fig. No. 12.29 Thaumatin transcripts: Dehydration Stress: Shoots

Stress		IR-64	Str-Ctl %	I-R %	Graph		RASI	Str-Ctl %	R-I %	Graph
0%		1350	0.00	-8.54	100.00		1476	0.00	9.33	109.33
30%		1649	22.15	7.71	122.15		1531	3.73	-7.16	113.41
40%		1554	15.11	13.68	115.11		1367	-7.38	-12.03	101.26

Table No. 3.29

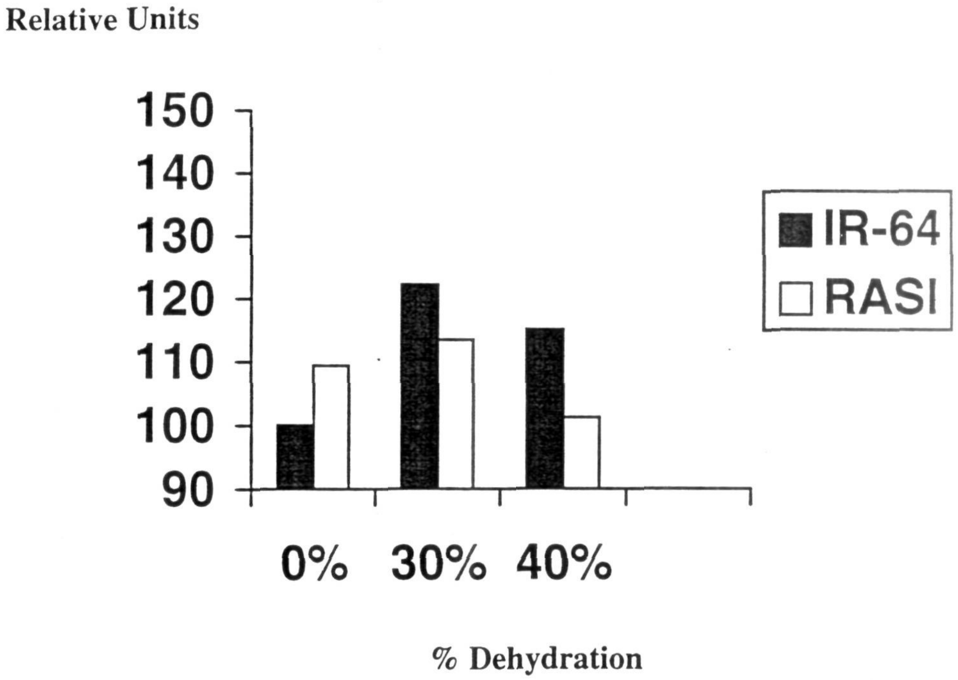


Fig. No. 13.29

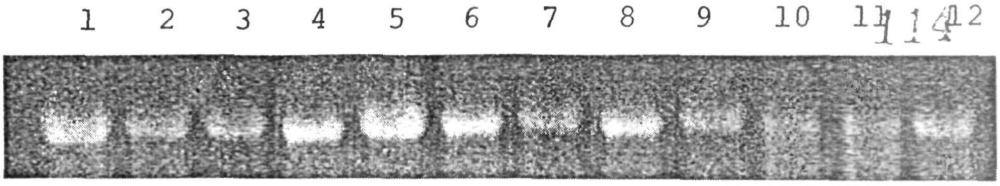


Fig. No. 11.30 28S rRNA : Shoots-7

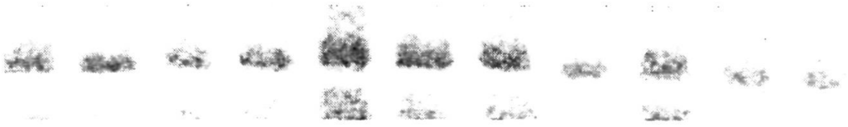
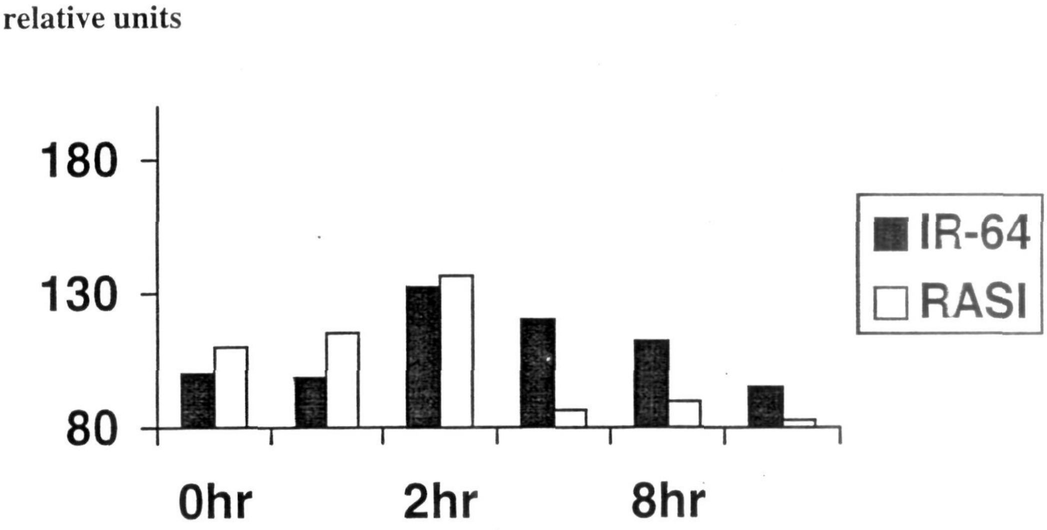


Fig. No. 12.30: Thaumatin transcripts: Salt Stress: Shoots

Time (hrs)		IR-64	Str-Ctl %	I-R %	Graph		RASI	Str-Ctl %	R-I %	Graph
0		1217	0.00	-8.91	100.00		1336	0.00	9.78	109.78
1		1200	-1.40	-14.47	98.60		1403	5.01	16.92	115.28
2		1612	32.46	-3.13	132.46		1664	24.55	3.23	136.73
4		1463	20.21	39.20	120.21		1051	-21.33	-28.16	86.36
8		1365	12.16	25.00	112.16		1092	-18.26	-20.00	89.73
16		1158	-4.85	15.11	95.15		1006	-24.70	-13.13	82.66

Table. No. 3.30



(150mM Salt Stress Duration)

Fig. No. 13.30

DISCUSSION

V. DISCUSSION

Salinity and drought are the major constraints for the improvement of rice quality and productivity. There have been several reports on the effects of salinity and drought in rice [O'Toole and Moya, 1981; Yoshida, 1981; O'Toole and Namuco, 1983; Lutts *et al.*,1995 and others]. The biochemical, physiological, cellular, morphological and whole plant responses of plants to moisture stress have also been well documented. The complex molecular responses of plants to salinity and drought induced water deficit have only been studied in the recent past [reviewed extensively in Bray, 1993,1997; Ingram and Bartels.1996 and others]. Several salinity, drought and other abiotic stress induced genes have been identified, isolated, characterized and cloned. With the knowledge of their roles during stress in experimental plants and by using them as cDNA probes, the molecular responses of important field crops to salinity and drought can be investigated.

In the present study, the tissue specific expression pattern of important stress related genes at the transcript level, during different stages of salinity and drought, using heterologous cDNA probes, have been monitored in a susceptible (IR64) and tolerant (Rasi) genotypes of *Indica* rice.

The heterologous cDNA probes used correspond to genes involved in key downstream responses of plant cells to abiotic stresses. MnSOD and FeSOD are involved in the dismutation of superoxide in the mitochondria and chloroplast respectively. Cat2 and cAPx are involved in the scavenging of hydrogen peroxide in the peroxisomes and cytoplasm respectively. Membrane channel proteins are involved in regulating ion movement across membranes. Hsp-70 has been implied several protective roles in plant cells during stress. CyP is involved in assisting protein folding during stress, while Thaumatin like protein are associated with fruit ripening and plant pathogen interactions. The expression of these genes in response to several abiotic stress factors in tobacco, Arabidopsis and maize have been studied.

Among the abiotic stresses, salinity and drought are important in the context of *Indica* rice and they cause more harm to the rice crop than other factors. However salt stress and dehydration show a high degree of similarity with respect to physiological, biochemical, molecular and genetic effects [Cushman *et al.*, 1990]. This remarkable commonality is possibly due to the fact that sub-lethal salt stress conditions, only to an extent that ionic interactions do not contribute much in causing effects, is ultimately an osmotic effect which is apparently similar to that brought about by water stress and to an extent by cold as well as heat stress [Hurkman and Tanaka,1988; Hurkman,1990; Almegeura *et al.*,1995]. Several workers have used sub-lethal salt concentrations in the range of 150 to 200mM, in different plants, to demonstrate the transcriptional activation of several stress related genes [Van Camp *et al.*,1996; Kalir and Poljakoff-mayber,1981; Gosset *et al.*,1996; Didierjean *et al.*, 1996; Zhu *et al.*, 1997, Van Montagu *et al.*, 1990 and others].

During water deficit studies, the stress conditions applied in the laboratory may not accurately represent those that occur in the field. Frequently, laboratory stresses are rapid and severe, whereas stress in the field often develops over an extended period of time [Radin, 1993]. A novel technique for induction of water stress has been used in the present study. This method ensures gradual desiccation of seedlings as against the rapid water loss by the use of other techniques. It is very likely that the molecular responses observed here mimic those that occur in the field during stress. Moderate (30%) water loss is a stage where the seedlings are actively responding to avoid the effects of desiccation. Forty percent is an advanced stage of moisture loss which only hardy varieties are able to cope with. Few plant species survive desiccation beyond 40%.

In addition to the induction by stress, the expression of water deficit associated genes is controlled with respect to tissue, organ and developmental stage, and may be expressed independently of the stress condition [Bray, 1993]. There have been several reports on the differences in tissue specific gene expression during moisture stress [reviewed in Ingram and Bartels,1996].

During salinity and drought stress, root tissues are perhaps the first to be affected. Hence, during the onset of stress, the molecular responses of roots and shoots are expected to be different. The molecular changes associated with roots during moisture stress are more to do with osmotic adjustment, increased water intake, synthesis of the signaling molecule (ABA), regulation of ion uptake and sub-cellular compartmentation etc. Shoots on the other hand respond by attempting to reduce the transpirational water loss, changes in the photosynthetic physiology etc. in addition to the other changes. A study of the changes at the gene expression level, in the roots and shoots separately, could therefore reveal vital differences in the tissue specific responses.

Expression of MnSOD gene during stress

Superoxide radicals are ubiquitously generated in many biological oxidations within all compartments of the cell [reviewed in Fridovich, 1978]. The mitochondrial electron transport chain is one of the major sources of the superoxide radical. Young plant tissues consist of actively dividing cells with high mitochondrial respiratory activity. Hence, to keep superoxide levels under check, the mitochondrial isozyme of SOD, MnSOD gene appears to be constitutively expressed. Green leaves, roots and calli of rice in particular were found to have good basal level of MnSOD transcripts [Kaminaka *et al.*, 1997]. The present study showed that unstressed samples of roots and shoots of both IR64 (susceptible variety) and Rasi (resistant variety) seedlings had high levels of MnSOD transcripts with no remarkable differences between the varieties. However, during stress, discrete differences were observed in the extent of MnSOD transcript accumulation in these two varieties

There have been reports where MnSOD transcript levels have been linked to the extent of oxidative stress experienced by mitochondria. This has been demonstrated in plants subjected to environmental stress where MnSOD gene is transcriptionally activated due to the generation of superoxide radicals.

In response to 30% dehydration, MnSOD levels of IR64 (susceptible variety) roots showed a sudden increase, unlike Rasi, in comparison to control. The susceptible variety IR64, therefore appears to be experiencing oxidative stress earlier in the form of increased superoxide generation, while the tolerant variety RASI appears unaffected at this stage of desiccation. However, only at a higher level of desiccation (40%), does RASI (tolerant variety) need to upregulate the MnSOD gene (Fig. No. 12.01, Fig. No 13.01and table no. 3.01).

The MnSOD transcript levels of shoots in both varieties remain unchanged even at 30% dehydration. Hence, desiccation at the initial stage, seems to elicit a greater response in the roots than the shoots in terms of transcriptional activation of the MnSOD gene. At 40% water loss though there is an appreciable increase in the MnSOD mRNA levels of both varieties. But there appears to be no difference in the extent to which oxidative stress is experienced by mitochondria of shoots of both varieties at this advanced stage of desiccation, as implied by the transcript levels of MnSOD (Fig. No. 12.02, Fig. No 13.02and table no. 3.02).

In the root tissues, during salt stress, IR64 appears to be experiencing a higher degree of superoxide generation. This is evident from the fact that IR64 root MnSOD transcript levels shoot up by 18% on exposure to just 1hr of 150mM NaCl. A similar level of salt stress seems to have no effect on Rasi. Only after a 12hr treatment RASI mRNA levels of MnSOD increases. 150mM NaCl is a mild stress. This level of salt is not lethal, but only elicits stress responses. Prolonged exposure to this sub-lethal levels of stress, enables seedlings to get adapted. This is due to several other tolerance and adaptive mechanisms could begin to operate (osmotic adjustments, ion sequestration and compartmentaion, for example). A decrease in the MnSOD mRNA levels after 2 hrs during salt stress indicates that both IR64 and Rasi may not need to express the gene at higher levels any more due to a suppression in the excessive generation of superoxide in the root mitochondria due to these adaptive mechanisms (Fig. No. 12.03, Fig. No 13.03 and table no. 3.03).

Upregulation of the MnSOD gene expression in the shoots appears to be slower when compared to roots. This is because salinity affects roots more quickly than shoots. MnSOD transcript levels were higher only between the second and eighth hour of NaCl stress. By the sixteenth hour of salt stress, both IR64 and Rasi shoots seem to have been be able to overcome oxidative stress (Fig. No. 12.04, Fig. No 13.04 and table no. 3.04).

During stress conditions, increased mRNA levels of MnSOD have not always been accompanied by an increase in the protein. This has been attributed to enhanced protein turnover [Williamson and Scandalios *et al*,1992]. IR64 seedling appears to be experiencing a greater demand for MnSOD, possibly due to enhanced mitochondrial oxidative stress, as compared to Rasi. However, the increase in transcript levels are only marginal. With MnSOD predicted to have a very short half life during stress, this increase may not be sufficient to compensate for the turnover and effectively meet the demand.

Studies during stress on the levels of superoxide being generated in mitochondria of these two *Indica* rice varieties, MnSOD enzyme levels, turnover rates kinetics of superoxide scavenging and the levels of antioxidants could further clarify this point.

Fe SOD transcript levels during drought and salinity

FeSOD catalyses the dismutation of superoxide radicals to hydrogen-peroxide and oxygen, in the chloroplast. The electron transport chain of the photosynthetic apparatus is a well documented source of superoxide radical. especially during stress [Bowler *et al.*,1992].

Maintenance of photosynthetic rates, even during stress is vital for a crop to minimize the reduction in yields. However there is a greater risk of superoxide being generated in the chloroplast when the photosynthetic machinery is active. In a tolerant variety like Rasi, the growth rates are maintained during stress. Hence it is likely to have more active but at the same time better protected photosynthetic machinery.

Though increases in FeSOD transcripts is a good indication of the extent of oxidative stress in the chloroplast. Efficient and timely transcriptional activation of this gene is also a good indicator of the system's ability to scavenge the excessive superoxide being generated. In Rasi shoots where the chloroplasts may be more active, show higher FeSOD mRNA, during all stages of desiccation (Fig. No. 12.06, Fig. No 13.06 and table no. 3.06). This could be an important factor which contributes to the drought tolerance of Rasi. This is also because tobacco plants (transgenics) with higher Fe SOD levels were able to more efficiently cope with salinity and other stresses [Van Camp, 1996].

Fe SOD mRNA in Rasi were higher than that of IR-64 up to the fourth hour of salt stress in shoots. This ability of Rasi shoots to also react quickly to salinity perhaps enables it to be immune to the toxic effects of superoxide radicals in the chloroplasts. Decrease in the transcript levels of Fe SOD in both varieties from the 8th hour onwards indicates adaptations to the sub-lethal levels of salt (Fig. No. 12.08, Fig. No 13.08 and table no. 3.08).

It seems that plastidic SODs, in rice, can be present in non-photosynthetic tissues and in other plastids in addition to chloroplast, and have been detected in rice in roots, calli and etiolated seedlings, [Kaminaka et al., 1997]. The transcripts of FeSOD detected in roots in the present study may also have a similar role. FeSOD is a nuclear encoded protein. Discrepancy between the transcript levels and constant activity of FeSOD has led to suggestions that the final enzyme status is regulated post-transcriptionally [Jasmina Kurepa et al., 1997]. Whether the transcripts are ultimately used for FeSOD synthesis in the roots can only be proved by enzyme assays and or Western Analysis. However transcriptional activation of the gene even in roots of rice during salinity and drought is evident from the results of this study (Fig. No. 12.05 and 12.07, Fig. No 13.05 and 13.07, table no. 3.05 and 3.07).

Effects of Salinity and Drought on the accumulation of Cat2 transcripts

Catalase has been associated with the removal of H_2O_2 in the peroxisomes. Cat2 of tobacco, is rapidly induced by environmental stress and is predicted to have a protective role. Catalase is a determining factor for the protection of photosynthesising cells against oxidative stress resulting from photorespiration. Since 150mM is a mild stress and roots are non photosynthesizing tissues, Cat2 mRNA levels remained unaltered throughout stress (Fig. No. 12.09 and 12.11, Fig. No 13.09 and 13.11 and table no. 3.09 and 3.11). In shoots however Cat2 gene was transcribed at higher levels during salinity. The very gradual increase in mRNA accumulation in both varieties can be attributed to the reduced photorespiratory activity of rice under light limiting conditions in the present study.

Reduction in the moisture content of tissues by up to 30-40% is a much severe from of stress compared to 150mM salinity. Hence mechanisms by which superoxide and subsequently H_2O_2 are generated tend to be more active. Higher levels of catalase in peroxisomes is needed to aviod damages. Increased transcription of Cat2 gene of both IR64 and Rasi, in the shoots during desiccation fulfills this role. The increased levels of Cat2 mRNA at 30% water loss itself indicates that Rasi shoots are better equipped to deal with the stress (Fig. No. 12.10. Fig. No 13.10 and table no. 3.10).

Peroxisomes are closely associated with chloroplasts. H_2O_2 arising out of the superoxide scavenged in the plastids and by photorespiration, are removed by Cat2 here. However if removal of both superoxide and H_2O_2 is inefficient, the highly reactive hydroxyl radicals can be formed by the Haber-Weiss reaction. It is in the shoots that such an event is more likely to occur since the chloroplasts are active. Much higher levels of both FeSOD and Cat2 mRNA at 30% desiccation in Rasi suggests that hydroxyl radical generation may be adequately suppressed. IR64, with lower levels of both species of transcripts is likely to be highly prone to the occurrence of Haber-Weiss reaction..

Influence of drought and salinity on the steady state level of cAPX transcripts

Ascorbate Peroxidases are the most important enzymes for protection against H_2O_2 in the Cytosol and Chloroplasts. Although catalases are capable of efficiently scavenging H_2O_2, it is unable to protect the cytoplasm from peroxide related damage due to its peroxisomal location and relatively low Km [Asada, 1992].

The cytosolic APx (cAPx) is the best characterized isozyme in terms of expression. Drought, heat shock, paraquat and ethephon caused a strong increase in cAPx transcript levels in pea [Mittler and Zilinskas, 1992]. Similar results have been obtained during salinity and water deficit in *Indica* rice seedlings.

Since cAPx transcript levels increase dramatically both in IR64 and Rasi roots, in response to desiccation (Fig. No. 12.13, Fig. No 13.13 and table no. 3.13), it may be assumed that generation of AOS within the cytosol and or their leakage from organelle(s) is enhanced by factors that cause oxidative stress (such as salinity and drought).

The induction of cAPx mRNA accumulation in the shoots of IR64 and Rasi seedlings were only moderate (Fig. No. 12.14 and 12.16, Fig. No 13.14 and 13.16, table no. 3.14 and 3.16). This could indicate that H_2O_2 is generated to a greater extent in the peroxisomes, rather than cytoplasm in the shoots. This is because of the photosynthetic activity of the shoots, which is absent in roots tissues. Enhanced Cat2 levels only in shoots during stress supports this viewpoint. Hence catalases and not cAPx have a greater role in shoots.

A similar trend was observed in the roots and shoots of IR64 and Rasi during salinity. There appears to be a greater need for the cytoplasmic H_2O_2 scavenging enzyme in the roots rather than shoots, during salinity. cAPx mRNA levels increased by up to 45% in both the varieties in roots (Fig. No. 12.15, Fig. No 13.15 and table no. 3.15) as against moderate increases of about 20% in the shoots (Fig. No. 12.16, Fig. No 13.16 and table no. 3.16). Rasi roots had consistently higher cAPx transcripts throughout during salinity as compared to IR64 indicating effective scavenging of H_2O_2 in the tolerant variety of *Indica* rice. A comparative study of the appearance of oxidative degradation products in the cytoplasm of IR64 and Rasi will further clarify this point.

Induction of membrane channel protein (MCP)transcripts during stress

The expression of the gene encoding membrane channel protein was examined using the CHEM8 cDNA probe of maize. This heavy metal responsive gene was shown to be upregulated by various abiotic stresses in maize [Didierjean *et al.*,1996]. Membrane channel proteins are involved in the regulation of solvent and ion movement across across membranes [Yamamoto, 1990].

MCP was constitutively expressed in maize [Didierjean *et al.*,1996]. A similar basal level of expression was detected in the present study. This suggests that MCP have a role in the movement of ions even in normal plant cells.

Regulation of the ion uptake and intracellular compartmentation is vital , especially under saline conditions [Binzel *et al.*,1988]. There were no changes in the transcript levels of the MCP gene during drought (both in the roots and shoots) and during salinity in shoots (Fig. No. 12.17, 12.18 and 12.20, Fig. No 13.17, 13.18 and 13.20 and table no. 3.17, 3.18 and 3.20). Roots in particular need to efficiently regulate the expression of MCP gene to prevent salt entering the system. Changes in the expression of MCP gene was observed only in the roots of IR64 and Rasi (Fig. No. 12.19. Fig. No 13.19 and table no. 3.19).

Channel proteins mediate the passive transport of ions [Niu *et al.*,1995]. A greater presence of this protein could facilitate free entry of sodium and chlorine ions into the tissues. Quick increases of MCP transcripts during salinity in IR64 roots (61% by the second hour itself) suggests that the susceptible variety is unable to control ion entry efficiently into the system. Rasi on the other hand tightly regulates MCP gene expression and therefore minimizes ion entry into roots. Peak mRNA levels of just 31% compared to control supports this hypothesis (Fig. No. 12.19, Fig. No 13.19 and table no. 3.19).

Studies on the intracellular levels of salt in the tissues of these two varieties during salinity should provide better clues regarding regulation of ion entry into the roots of these two varieties.

Hsp-70 transcript levels and their role during salinity and water deficit

Several abiotic stresses (apart from high temperature), including salinity and drought hamper the post translational processing of proteins and the formation of functional proteins. Hence there is a need for HSPs to fulfill the role of chaperones during stress. Hsp-70 performs several other cellular functions in cooperation with specific soluble and membrane bound proteins. Partner proteins are predicted to determine the multiple functions of HSPs [Joachim et al.,1995]. Hsp-70s however are best known for their participation in protein folding.

Hsp-70 appears to have small basal level of expression in *Indica* Rice seedlings. This is evident from transcript levels detected in control samples. The induction of this gene during stress is dramatic and exceeds that of any other gene monitored in the present study.

Shoots of IR64 in particular, both during salinity and moisture stress, recorded very high transcript levels of Hsp-70. A 70 to 75% increases in Hsp-70 mRNA was observed in response to dehydration in IR64 while it was just 40 to 45% in Rasi, as compared to controls. It appears that the protein folding process needs assistance to a greater extent in the susceptible variety. The tolerant variety on the other hand may be able to perform these routine processes without any special assistance, during mild stresses. But, during extreme stress, Rasi Hsp-70 mRNA levels are on par or higher than that of IR64. This could ensure good support to the protein biosynthetic apparatus at a higher level of desiccation (Fig. No. 12.22, Fig. No. 13.22 and table no. 3.22).

150mM salinity appears to be a mild stress for Rasi and hence there is much less accumulation of Hsp-70. Increases of up to 150% in the susceptible variety in response to just 150mM salt concentration in the shoots by itself indicates the severety of stress felt by IR64 (Fig. No. 12.24, Fig. No. 13.24 and table no. 3.24).

The expression pattern of Hsp-70 in rice seedlings is differentially influenced by variety, tissue type and nature of environmental stress. Similar observations have been made [Guy *et al.* 1998] in spinach.

Changes in the transcription of cyclophilin (Cyp) gene during abiotic stress

CyP is believed to play an important role in accelerating the rate at which proteins fold into their native conformation. The transcriptional activation of this gene in response to salinity (170mM), heat, U.V, HgCl2 and other stress in maize indicates that CyP may play an important role in plants during stress conditions [Marivet *et al.*, 1992]. The role of Cyp in rice during salnity and water deficit was scrutinized in the present study. Basal level of CyP transcripts have been detected in maize and bean.[Marivet *et al.*, 1992]. Rice also appears to express this gene constitutely. However salinity and drought stress did not alter the mRNA levels of CyP gene in both IR64 and Rasi tissues. Very meager and inconsistent increases in the CyP mRNA was observed. CyP may not play a significant role in the response of rice seedlings to salinity and drought in the present study conditions (Fig. No. 12.25, 12.26, 12.26 and 12.27, Fig. No. 13.25, 12.26, 12.27 and 12.28, table no. 3.25, 3.26, 3.27 and 3.27).

Thaumatin-like proteins(TL-proteins) mRNA levels

There have been reports of the synthesis of PR proteins in plants in response to abiotic stress also. The changes in mRNA levels of TL-proteins of the PR-5 family was examined in rice seedlings. Almost no expression of the TL-protein was observed in roots. It appears that the expression of TL-proteins in rice is shoot-specific. This TL-proteins which is similar to osmotin and permatin and having antifungal properties, may be synthesized specifically to ward off opportunistic shoots specific fungal pathogen in rice.

It is predicted that certain signal transduction events are common in the response of plants to biotic and abiotic stresses. This could be the major reason for changes in the gene expression of PR proteins like TL-protein, during salinity and drought.

Upregulation of gene expression was observed only in the susceptible variety during drought and salinity. There were moderate increases in the mRNA levels during early salinity in the tolerant variety but gene expression was subsequently suppressed to a similar extent (Fig. No. 12.29 and 12.30, Fig. No. 13.29 and 13.30, table no. 3.29 and 3.30).

H_2O_2 also synthesized during pathogen attack is required for salicylic acid biosynthesis and is also implied to have a role as a signaling molecule for activation of genes involved in plant pathogen interactions. Efficient scavenging of H_2O_2 in the tolerant variety may therefore suppers the expression of PR genes (including TL-proteins). Lower H_2O_2 levels in the tolerant variety (Rasi) also alters the red-ox balance in the cytoplasm of plant cells which is vital for the stability of specific defense-related mRNA transcripts could be the reason for the down regulation of TL-protein gene expression during advanced drought and salinity (Fig. No. 12.29 and 12.30, Fig. No. 13.29 and 13.30, table no. 3.29 and 3.30).

The exact role of PR proteins synthesized during abiotic stress remains to be clarified. They may only contribute indirectly to stress tolerance or susceptibility. Studies on the levels of other important classes of PR proteins is also essential to predict their role in rice seedlings during abiotic stresses.

The expression of a large number of genes is induced or altered in plants in response to salinity and drought [reviewed in Ingram and Bartels,1996]. The exact role of a good number of these genes during stress is still speculative. Among the genes that have been characterized, the specific mechanisms by which their expression is regulated by drought and salinity is yet to be completely elucidated. It is evident from the present study that the expression of genes is differentially regulated in the roots and shoots of these two varieties (IR64 and Rasi), in response to salinity and drought. The upstream events like stress perception and signal trandsuction events which are prerequisites for the alterations in gene expression as a result of stress needs to be dissected and compared in these two varieties. Examination of the expression pattern of genes involved in these upstream events and other important down stream events is essential to strengthen our understanding of the molecular factors that contribute to stress tolerance or susceptibility in Rasi and IR64 respectively.

SUMMARY

VI. SUMMARY

Environmental stresses like drought and salinity elicit complex molecular responses in plants. Changes in gene expression are fundamental to the responses that occur during salinity and water deficit and they control many of the short and long term responses that determine stress tolerance and susceptibility. The present work attempted to compare the alterations in the tissue specific gene expressions, at the transcript level, during mild salinity and moderate and advanced drought, in two agronomically important but contrasting *Indica* rice lines IR64 (a susceptible variety) and Rasi (a tolerant variety). An attempt has been made to interpret the data obtained and attribute the differences observed to stress susceptibility or tolerance.

One week old rice seedlings, grown under controlled conditions, were subjected to 150mM NaCl stress and gradual desiccation (using a novel technique). Root and shoot tissues were separately used for total RNA extraction and Northern blotting. These blots were probed with heterologous stress related cDNA probes of genes involved in active oxygen scavenging (MnSOD, FeSOD, Catalase and Ascorbate peroxidase), regulation of ion movement (Membrane Channel Protein), protein folding and stabilization (HSP and CyP) and Pathogenesis-Related proteins (Thaumatin-like protein). The expression of these genes was monitored at different stages of salinity and drought stress. The transcript levels of the respective genes were detected and quantified using the highly sensitive phosphor-imager.

The present study has revealed certain interesting and vital differences in the accumulation of the transcripts of the genes under study in the roots and shoots of the susceptible and tolerant varieties.

Gene products involved in similar functions but differing in sub-cellular locations seem to be differentially regulated depending on the variety and tissue type, during stress.

MnSOD, activity is localised in the mitochondria. The pattern of MnSOD gene expression observed implies that IR64 (susceptible variety) roots experience a greater oxidative stress in the form of superoxide generation in the mitochondria, during early stages of drought and salinity unlike Rasi (tolerant variety).

FeSOD, which is involved in the scavenging of superoxide in the chloroplasts, transcript levels was quickly and efficiently up-regulated by Rasi (tolerant variety) shoots in response to salinity and drought. This could indicate a more active but at the same time a better protected photosynthetic machinery in Rasi (tolerant line) in spite of the stress.

Delayed induction of Cat2 gene in shoots can be attributed to the reduced photo-respiratory activity of rice seedlings in the present experimental conditions like low illumination and the mild salinity treatment. Efficient transcription of Cat2 in Rasi (tolerant variety) shoots at 30% water loss itself implies effective scavenging of hydrogen peroxide.

Higher levels of both FeSOD and Cat2 mRNA at 30% desiccation in Rasi (tolerant variety) suggests that the generation of highly reactive hydroxyl radical by the Haber-Weiss reaction is greatly suppressed. Lower transcript levels of both these genes in IR64 makes it highly prone to the formation of and subsequent damage by hydroxyl radicals.

Dramatic induction of the cytoplasmic ascorbate peroxidase gene in the roots of both the varieties indicates that the generation of Active Oxygen species within the cytosol and/or their leakage from organelle(s) is enhanced by factors that cause oxidative stress (such as salinity and drought). Consistently higher cAPx transcript levels were observed during all stages of salinity and advanced drought in the tolerant variety (Rasi).

Moderate increases in the mRNA levels of the genes involved in AOS scavenging, during salinity and drought in the present study, as compared to several fold increases in other studies, is due to the mild salt stress (150mM), low light intensity during stress treatments and modest metabolic activity at the seedling stage.

At the level of ion sequestration, increased accumulation of MCP transcripts in the roots of susceptible variety (IR64) could indicate the inability of IR64 to tightly regulate passive entry of sodium and chlorine ions during salinity.

Large HSP-70 transcript accumulation in the shoots was observed in the susceptible line indicating the severity of stress felt by IR64. It appears that the protein folding process, especially in shoots of the susceptible variety (IR64), needs greater assistance as a result of even mild salinity and moderate dehydration.

Very meager and inconsistent changes in CyP mRNA levels observed during the present study could suggest that CyP may not play a significant role in the response of the seedlings of both varieties to stress. Prolonged exposure to stress or studies at later stages of the rice plant, may reveal differences, if any.

Early induction but suppression of gene expression later on during different stages of salinity and drought was observed for the TL-protein of PR-5 family. PR proteins only seem to have an indirect role during stress responses of plants. Greater suppression of TL-protein gene in the resistant (Rasi) rice variety could be due to efficient scavenging of the signaling molecule H_2O_2 and alterations in red-ox balance which regulate the stability of specific defense related mRNA in plants.

The tissue specific expression of genes involved in key downstream responses in specific organelles and cytoplasm of plants cells during salinity and drought have been investigated and compared in IR64 (susceptible variety) and Rasi (tolerant variety) of *Indica* rice. Other cellular events which influence and or complement gene expression in conferring tolerance to salinity and drought need to be examined in these two varieties. Upstream events like the perception of stress and signal transduction events which regulate gene expression needs to be dissected and compared in IR64 and Rasi (tolerant line). The present work has yielded valuable information for a microcosm of stress related events under mild salinity and moderate to severe drought. Further studies on the expression of other stress related genes during salinity and drought at different developmental stages in these two varieties could identify precise differences in the adaptive attempts of these two varieties and provide us a better picture of what makes IR64 a susceptible or Rasi a tolerant variety.

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VII. REFERENCES

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APPENDICES

APPENDICES

I. RNA Isolation and Analysis

1. Extraction Buffer used in RNA Isolation

   10ml of Extraction Buffer consisted of ;

   a. 4.72g of Guanidium Thiocyanate
   b. 250µl of 1M Sodium Citrate (pH 7)
   c. 0.5% Lauryl Sarcosine
   d. 72µl of β Mercaptoethanol
   e. Volume made up to 10ml using DEPC treated water

2. 10 X MOPS / EDTA buffer

   a. 0.2M MOPS (3-N-morpholinopropane sulfonic acid)
   b. 50mM Sodium Acetate
   c. 10mM EDTA

   pH was adjusted to 7, DEPC treated, autoclaved and stored in the dark.

3. RNA Gel Loading buffer

   750 µl deionised formamide
   240 µl formaldehyde
   150 µl 10 x MOPS / EDTA
   200 µl 50% Glycerol
   10 µl 10mg / ml ethidium bromide
   0.5 mg bromophenol blue

4. 10 x SSC

1.5M NaCl
0.15M Sodium Citrate
pH was adjusted to 7, DEPC treated and autoclaved

5. Church-Gilbert Hybridization Solution

1mM EDTA
0.5M Na_2HPO_4 , pH 7.2
7% SDS

6. Stripping Solution for Northern Blots

   a. TE
   b. 0.1% SDS

II Media and reagents used for probe preparation

A. Media

1. Luria Bertani Liquid Media (LB)

1 liter of LB was prepared as follows;
To 950ml of deionised H_2O, the following was added;

Bacto-Typtone	10g
Bacto Yeast Extract	5g
NaCl	10g

The contents were dissolved, pH was adjusted to 7 using 5N NaOH (about 0.2ml) and volume made up to 1 litre with deionised water. The media was sterilized for 20 minutes at 15 lb/sqin at 121°C.

2. 2 X Y T Medium

For 1 litre of medium, to 900ml of deionised H2O, the following were added

Bacto-Tryptone	16g
Bacto-Yeast Extract	10g
NaCl	5g

pH was adjusted to 7 and volume made up to 1 liter with 5N NaOH and sterilized at 15 lb/ sq-inch for 20 min (121°C).

3. SOC Medium

1 liter of SOC was prepared by dissolving the following in de-ionised water

Bactotryptone	20g
Bacto Yeast Extract	5g
NaCl	0.5g

To this 10ml of 250mM solution of KCl was added, pH adjusted to 7 with 5N NaOH, volume made up to 1 liter and autoclaved. Just before use 5ml of a sterile solution of 2M $MgCl_2$ and 20ml of 1M sterile glucose solution was added.

4. LB Agar (LBA)

LB was prepared as described in section IIA1 and 15g / liter of Bactoagar was added before autoclaving.

B. Reagents used for Plasmid Isolation by Alkaline Lysis

1. Solution I

50mM Glucose
25mM Tris Cl (pH 8)
10mM EDTA (pH 8)
Autoclaved for 15min at 10lb / sq.inch and stored as 4°C

2. Solution II

0.2N NaOH
1% SDS
Freshly prepared before use

3. Solution III (100ml)

5M Potassium Acetate	60ml
Glacial Acetic Acid	11.5ml
Water	28.5ml

(The resulting solution would be 3M with respect to Potassium and 5M with respect to acetate)

4. TE (Tris - EDTA) Buffer : pH 8

10mM Tris.Cl (pH 8)
1mM EDTA (pH 8)

5. TAE (Tris-acetate EDTA) Buffer

1 liter of 50X TAE stock;
424g Tris base
57.1 ml Glacial Acetic Acid
100ml 0.5M EDTA (pH 8)
Working solution = 1X

6. 6X Gel Loading Dye

0.25% Bromophenol Blue
30% Glycerol
Stored at 4°C.

7. Preparation of tris-equilibrated phenol (pH 8)

Phenol was melted at 68°C, 0.1% 8-hydroxy quinoline was added and the phenol was first equilibrated with deionised water. The volume of phenol was measured and 1g of Tris salt was added for every 14ml of phenol, fresh water was added and equilibrated again till the pH reached 8. The aqueous phase was replaced with 100mM TrisCl (pH 8) and the phenol was stored at 4°C in the dark.

8. Assay buffers used for restriction digestions ;

EcoRI and PstI : Buffer B (Bangalore Genie)
XhoI and XbaI : One-phor-all buffer (Amersham)

9. Ethidium Bromide Stock Solution : 10mg/ml.

C. Ampicillin

Stock Solution 50mg/ml in Water.
Filter sterilized and stored at -20°C.
Working concentration (relaxed plasmids): 60µg/ml.

D. Reagents for transformation of Bacteria.

1. Transformation and Storage Solution (TSS)

TSS consisted of LB Broth with;

10% (w/v) Polyethylene Glycol (molecular weight 8000)
5% (v/v) Dimethyl Sulfoxide
10mM $MgCl_2$
10mM $MgSO_4$
10% glycerol.

TSS was filter sterilized using a 0.45filter & stored at 4°C.

2. 5X KCM

   0.5M KCl

   0.15M $CaCl_2$

   0.25M $MgCl_2$