Studies on Water Stress-responsive Proteins and Enzymes in Rice *(Oryza sativa* L.): Identification and Partial Characterization of Aldose Reductase

A thesis
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By

B. Karuna Sree

Department of Plant Sciences
School of Life Sciences
University of Hyderabad
Hyderabad-500046, India

Enrol. No. 92LSPH13

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To
My Parents and Husband

DECLARATION

I hereby declare that the work presented in the thesis entitled ***"Studies on Water Stress-responsive Proteins and*** *Enzymes* ***in*** *Rice (Oryza* ***sativa L.): Identification and Partial Characterization of Aldose Reductase"*** has been carried out by me under the supervision of **Prof.Arjula Ramachandra Reddy** in the Department of Plant Sciences, School of Life Sciences, University of Hyderabad and that this work has not been submitted for degree or diploma of any other University or Institute.

B. Karuna 7/5/98

B. Karuna Sree

A Reddy 7/5/98

Prof. A. Ramachandra Reddy
Supervisor
Dept. of Plant Sciences

Department of Plant Sciences
School of Life Sciences
University of Hyderabad
H> derabad-500 046
INDIA

CERTIFICATE

This is to certify that **B. Karuna Sree** has carried out the research work embodied in the present thesis entitled ***"Studies on Water Stress-responsive Proteins and Enzymes in Rice (Oryza sativa L.): Identification and Partial Characterization of Aldose Reductase"*** under my supervision in the Department of Plant Sciences, School of Life Sciences, University of Hyderabad for the submission of degree of Doctor of Philosophy.

This work has not been submitted for the award of degree or diploma of any other University or Institute.

Prof. A. Ramachandra Reddy
Supervisor
Dept. of Plant Sciences

Head
Dept. of Plant Sciences

Head, Dept. of Plant Sciences
School of Life Sciences

Dean
School of Life Sciences

DEAN
School of Life Sciences
University of Hyderabad
Hyderabad-500 046

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Karuna Sree

Abbrevations

ABA	Abscisic Acid
Abi	ABA insensitive mutants
APS	Ammonium Persulphate
APX	Ascorbate peroxidase
AR	Aldose reductase
BCIP	5-bromo 4-Chloro-3-Indolyl phosphate
Cas	Cold acclimation-specific
Cor	Cold responsive
DAG	Days after germination
DAP	Days after pollination
EDTA	Ethylenediamine tetra acetic acid
GA	Gibberlic acid
GR	Glutathione reductase
GSRP	Generalised stress responsive protein
GSSG	Oxidised glutathione
HPLC	High performance liquid chromatography
HSP	Heat shock protein
kDa	Kilodaltons
LEA	Late Embryogenesis Abundant
MOPS	4-Morpholinepropanesulfonic acid
NBT	Nitroblue tetrazolium
nm	nano meters
PAGE	Poly acrylamide gel electrophoresis
PEG	polyethylene glycol
PVP	Polyvinyl prrrolidine
RAB	Responsive to ABA
RD	Desiccation responsive
SDS	Sodium dodecyl Sulphate
SOD	Superoxide dismutase
TEMED	N,N,N,N (Tetramethyl) Aminomethyl ethylene Diamine

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1. INTRODUCTION

Introduction

Rice, the most important food crop of the world, is grown in a wide range of ecosystems and varying water regimes. The crop is naturally exposed to a variety of environmental stress conditions, the most important being drought, salinity, submergence, extreme thermal environments and metal toxicity. Drought in rice is an important constraint that incurs large yield losses. About 45% of the world's rice grown in rainfed low land and upland conditions is often exposed to chronic and sporadic periods of drought (IRRI, 1993). In Eastern India drought became a major constraint to improve rice yields in lowland areas (Widawasky and O'Toole, 1990). The growing human demand for food along with economical problems faced by developing nations makes breeding for high yielding crops with built in resistance against such environmental constraints an important necessity. Furthermore, most abiotic stress resistance traits are polygenic and quantitative, thus making them less suitable for genetic engineering than simple inherited traits. The interactions of a number of component responses that may differ in different crops mostly contribute to drought tolerance. Besides, performance of drought resistant plants under controlled environments rarely correlate with performance at field level largely due to enormous variations in environmental conditions.

Most of the progress made in improving crop performance in water limiting conditions was through breeding programs. Such breeding-based approaches make use of empirical data and correlation between yield and stress tolerance. Other approaches to improve drought tolerance are aimed at deciphering the physiological, biochemical and molecular basis of drought stress response in crop plants. Efforts to understand the genetic and molecular basis of stress response mechanisms in plants in general and rice in particular began much later. The new technologies have not only provided the much-needed impetus but have also answered the long-standing questions on drought response mechanisms and associated traits. Engineering of the drought tolerant genes for

improvement of drought resistance in crop plants requires generation of an extensive information base for each component trait.

The ability of plants to tolerate water deficit is determined by biochemical pathways that help in the acquisition and retention of water, protect organellar functions and maintain ion homeostasis of the plant cells. Significant progress was made in documenting stress-induced changes such as protein profiles, synthesis of osmotically active compounds like sugars, proline etc.. root/shoot ratios, patterns of gene regulation and enzyme activities in a selected few plants, rice being one of them. Precise understanding of these mechanisms requires a rigorous analysis of stress-related genes and their protein products. Numerous reports have described the induction of proteins under stress in a large number of plant species (Ingram and Bartels, 1996; Bohnert and Jenson, 1996: Moons *et al,* 1995; Bohnert *et al.,* 1995; Bartels and Nelson, 1994; Bray, 1993; Bartels *et al..* 1990; Close *et al,* 1989 and Mundy and Chua, 1988). Of these, many have been biochemically characterized and their aminoacid sequences have been either determined or deduced from the cDNA sequences. However, the possible function of stress induced proteins and their role in stress resistance process remains to be established in crop plants.

ABA is a phytohormone with an important role in diverse physiological processes such as seed development, stomatal movement and plant adaptation to environmental stress (Skriver and Mundy. 1990). It acts as a signal molecule and controls gene expression both al the transcriptional and translational levels in stress phenomena (Chandler and Robertson. 1994). ABA receptors have not yet been identified but the characterization of the downstream elements of the ABA regulatory pathways are known. ABA inhibitors and mutants were helpful in the identification and unraveling of the some of the stress tolerance mechanisms (Giraudat *et al,* 1994).

Oxidative damage to cellular components is a major biochemical manifestation of stress response in plants. Drought stress response in plants lead to the generation of oxygen free radicals that cause lipid peroxidation and also damages the DNA and proteins (Bowler *et al,* 1992). Much of the stress injury to plants is associated, at least initially, with oxidative damage at cellular level. The H_2O_2 levels increase during water

depletion and affect photosynthetic rates. Plants have evolved specific defense mechanisms to keep these deleterious reactions to a minimum level by the upregulation of antioxidant enzymes (Bowler *et al,* 1992; Allen. 1995). Antioxidant pathways are activated by various stresses such as drought. abscisic acid, cold and salt stresses. The major antioxidant enzymes include superoxide dismutase (SOD), ascorbate peroxidase (APX) and glutathione reductase (GR).

This study is aimed mainly at the identification of certain proteins and enzymes belonging to the osmolyte pathways and antioxidant pathways in plants. Major emphasis of our study is on determination of possible function of stress induced proteins in rice. Specific objectives include:

a) Analysis of the stress induced root/shoot growth ratios

b) Identification of stress induced polypeptides

c) Purification, characterization and immunological analysis of the stress-induced 15-kDa polypeptide in different tissues under different stresses and

d) Characterization of stress induced 23 kDa polypeptides

e) Determination of possible functions of stress induced proteins and identification of enzyme activity and

f) Analysis of changes in antioxidant enzymes and determine the stress induced changes in the isozyme profiles of SOD and APX enzymes.

2. LITERATURE REVIEW

Literature Review

Most crops are exposed to sporadic and chronic periods of drought. Most of the traits contributing to drought tolerance are complex and our understanding of molecular basis and the control mechanism is rather limited. Plant responses to water deficit can be analyzed systematically by identifying the traits related to drought tolerance followed by their physiological, cellular, biochemical and molecular analysis. Precise understanding of these factors requires a vigorous analysis of stress-related biochemical changes, the associated genes, protein products and their role in stress tolerance. Such stress-induced effects include changes in growth rates, accumulation of compatible solutes, for instance polyols and proline, and changes in gene expression. The relevant literature on such aspects of drought response in plants is reviewed below.

2.1 Natural mechanisms of drought tolerance:

The natural mechanisms by which plants can withstand drought conditions are classified into three main groups (Jones *et al.*, 1981):

a) Drought escape: Plants complete their life cycle before a severe water deficit develops and are often termed as ephemerals. A significant feature of these ephemerals is the rapid development characterized by the formation of underground organs or buds followed by the death of arial parts under drought conditions.

b) Drought avoidance: These plants can withstand well in prolonged rainfall deficient periods by maintaining high water potential in tissues with a well established deep root system, leaf movements, increased leaf wax deposition and a controlled water loss during water deficit periods.

c) Drought tolerance: These are true drought tolerant plants which can withstand drought conditions at low tissue water potential by maintaining solute accumulation and tissue elasticity. Xerophytes and halophytes accumulate solutes during normal and

drought conditions while some mesophytes of this class accumulate solutes during water deficit.

Certain resurrection plants, for example the African resurrection plant, *Craterostigmaplantagineum* exhibit protoplasmic resistance. This is accomplished by an orderly loss of water accompanied by dehydration of the cytoplasm in such a way that the metabolic machinery can be maintained and eventually restored following rehydration.

Stress response mechanisms include a series of changes in physiological, biochemical and molecular pathways in addition to phenotype alteration. Molecular biology of stress response mechanisms, induction of genes and their products in a number of crop species have been extensively reviewed (Ingram and Bartels, 1996; Bohnert *et al.*, 1995; Bohnert and Jensen. 1996; Bartels and Nelson. 1994; Bray, 1993; Skriver and Mundy, 1990). Relevant aspects of stress responses in plants and their consequences are reviewed below.

2.2 Morphological changes during water stress:

Cell division and growth in plants are known to be sensitive to water stress. Under reduced water availability the leaf area is decreased which in turn reduces water loss and thus providing an adaptive mechanism. In addition to reducing the rate of water use, there is evidence for an increased access to soil water utilization. which in turn lead to an increase in root/shoot growth ratios, for e.g. maize (Sharp and Davies, 1979) and soybean (Creelman *et al.*, 1990). Root elongation is important for efficient water uptake particularly for seedlings growing in dry soil. It was reported that solutes that are accumulated in shoots, were translocated into roots thereby promoting root elongation (Sharp and Davies, 1979). This was suggested to be an adaptive feature and roots might have a higher capacity for osmotic adjustment than shoots.

The solutes involved in osmotic adjustment were mostly free amino acids, glucose, fructose and sucrose, which account for most of the increased dry weight under stress (Meyer and Boyer, 1981). The physiology and molecular biology of stress response mechanisms in plants are extensively reviewed. The molecular basis of the root

elongation has been studied in soybean seedlings by Creelman *et al*., (1990). The process of root elongation during water deficit is reported to be mediated by altered levels of ABA. Increased root/shoot growth ratios have been reported under water deficit and also by exogenous ABA. There are numerous reports on the accumulation of endogenous ABA during water deficit (Cohen and Bray. 1990). In seedlings grown at low water potential the ABA increased by 5 to 10 folds than well-watered soybean seedlings. ABA is formed affect the root growth more than water deficit, suggesting possible involvement of ABA in the differentiation of roots and shoots in response to water deficit.

The role of increased endogenous ABA in maintaining primary root growth and inhibition of shoot growth of maize seedlings at low water potentials has been reported (Saab *et al*.. 1990). The ABA deficient *vp5* mutant of maize, and wild type seedling exposed to ABA inhibitor (fluridone) grown at low water potentials show inhibition of root elongation and promotion of shoot elongation compared to their respective controls at the same water potential. These results confirm that the ABA accumulation plays a role in both the maintenance of primary root and shoot elongation at low water potentials.

2.3 Physiological adaptations:

Mechanisms of water stress: Water stress develops in plant when the demand exceeds the supply of water resulting into a stress gradient potential which is developed between the soil or soil-root interface and transpiring leaves. Since leaf is directly involved in the production of assimilate for growth and yield, leaf water potential is the common immediate measure of plant stress response.

The relationship between water potential (Ψw), pressure potential (turgor potential (Ψp) and osmotic potential (Ψs) is expressed as

$$\Psi w = \Psi p + \Psi s$$

As the water deficit develops, both water potential and osmotic potential decrease (becoming more negative) at different rates. Water potential is reduced at a greater degree compared to the osmotic potential (Kermode and Bewley, 1987). The difference between these two is the pressure potential. When the pressure potential equals to zero, then water

potential equals to osmotic potential. Thus the maintenance of turgor depends on the difference between osmotic and water potential. The osmotic potential and cell wall elasticity is in turn affected by other factors such as solute accumulation, cell wall size, and osmotic volume and cell wall thickness. As the water is being removed from the cell during water deficit, osmotic potential is reduced due to the simple effect of solute concentration. However, solutes are actively accumulated in the system during the course of cellular water loss, and such an accumulation of solutes during the development of water stress is termed as "osmotic adjustment" or "osmoregulation". Osmoregulation allows maintenance of higher osmotic potential at a given leaf water potential. The solutes involved in such osmotic adjustment vary from plant to plant and osmoregulatory processes in turn govern the stress tolerance mechanisms.

2.4 Accumulation of proline, a compatible osmolyte, during water deficit:

Proline is the most frequent and extensively accumulated amino acid in a wide variety of plants under different stress conditions. Accumulation of proline upon dehydration or due to water deficit and increase in osmotic pressure has been recorded in many organisms ranging from bacteria to eukaryotes. Proline accumulation is thought to be a primitive response of all living organisms under stress conditions (Measures. 1975). In barley, the accumulation of free proline begins rapidly upon exposure of leaves to dehydration and the free proline increase depends on the length of exposure to water deficit (Singh *et al,* 1973; Sivaramakrishnan *et al*., 1988).

The rate of proline accumulation during water deficit changes depending on the leaf water status. Rajagopal *et al.,* (1977) showed that, the proline content of un irrigated field grown wheat reaches maximum at mid day, which correlates closely with the time of minimum relative water content of those leaves. Proline accumulation not only occurs during water deficit but also during cold (Charest and Phan, 1990) and salt stress (Chu *et al.,* 1976). Some halophytes such as *Triglochin maritima* (Stewart and Lee, 1974) accumulate free proline upto 123 fig/mg fresh weight.

The phytohormone ABA and proline accumulation are two of the widely recognized metabolic responses to stress in plants (Hanson and Hitz, 1982). Proline also accumulates by the application of exogenous ABA but only in certain species (Stewart. 1980). In wilted leaves of barley, ABA accumulation precedes proline accumulation (Stewart and Voetberg. 1985; Stewart *et al.*, 1986). In salt stressed barley leaves, proline does accumulate in the absence of ABA accumulation (Stewart and Voetberg, 1985) suggesting that these two could be independent events.

The role of proline accumulation in adaptation to water stress is not yet clearly understood (Stewart and Hanson, 1980; Aspinall and Paleg, 1981). The role of proline accumulation in stress metabolism has been suggested to be osmoregulation. Proline accumulation may also lead to protection of enzymes from denaturation (Paleg *et al.*. 1984; Rajendrakumar *et al.*. 1994).

Substantial differences in accumulation of proline have been observed between plants during water stress. Studies on barley (Singh *et al*, 1973), in rice (Mali and Mehata, 1977) and in *Sorghum* (Sivaramakrishnan *et a J.*. 1988) revealed that plants, which accumulate more proline. were found to survive well under stress condition than those with low proline levels did. On the contrary, Ilahi and Dorffling (1982) reported a negative correlation between proline accumulation and drought resistance in barley. Ibarra-Cabalero *et al.*, (1987) reported that proline accumulation caused by drought stress was not an indication of drought stress resistance but rather a symptom of it. The reduced nitrate reductase activity coincided with the increased proline concentration during water stress in *Sorghum* (Sivaramakrishnan *et al.*, 1988).

2.5 Stress-responsive proteins:

Stress responsive changes in gene expression in general and protein profiles in particular have been targeted for intensive investigation, although in a few selected plants. Changes in gene expression, both quantitative and qualitative, occur in plants when exposed to abiotic stress and consequently new proteins are synthesized with the simultaneous disappearance /decline of some others (Bewely and Larsen, 1982). There

are numerous reports regarding the induction of proteins by various abiotic stress conditions in plants: water stress induced proteins in maize, rice and sunflower (Gomez *et al.,* 1988, Mundy and Chua, 1988; Cellier *et al.*. 1998), ABA responsive proteins in *Arabidopsis* and maize (Hong *et al.*, 1992; Conlay *et al.*, 1997). salt stress responsive proteins in rice and tobacco (Moons *et al.*, 1995; Singh *et al.*, 1987;) cold stress induced proteins in Prunus, rice (Arora and Wisniewski,1994; Hahn and Walbot. 1989); anaerobic stress induced proteins in rice, (Kadowaki *et al.*. 1988) heat shock proteins in maize and rice (Heikkila *et al.* 1984; Singla and Grover. 1993). and auxin induced proteins in mung been seedlings (Dhindsa *et al.*. 1987).

Although, there is a clear decline in overall protein synthesis, the synthesis of certain proteins increases substantially during water stress. Several water stress induced proteins have been characterized from many crop plants including maize (Gomez *et al.*, 1988), pea (Guerrero and Mullet. 1988). rice (Mundy and Chua. 1988). sunflower (Cellier *et al.*, 1998), *Populus* (Pelah *et al.*, 1997). tomato (Bray. 1988: Ho and Mishkind, 1991), *Brassica* (Vartanian *et al.*. 1987), *Arabidopsis* (Yamaguchi-Shinozaki *et al.*, 1992), and barley (Close *et al.*, 1989). However, not many such induced proteins were analyzed for their role in stress tolerance. Important classes of stress responsive proteins that are relevant to the present study are reviewed below.

2.5.1 RAB (responsive to abscisic acid) proteins:

The well-known phytohormonc ABA mediates a number of physiological processes in plants including seed maturation and response to environmental stresses (Skriver and Mundy, 1990; Hetherington and Quatrano, 1991). Mundy and Chua (1988) characterized a novel rice gene called *RAB 21* encoding a basic glycine rich polypeptide of mol. wt 16.5 kDa that was induced in response to water stress and ABA called as RAB21. This *RAB21* mRNA and protein were induced in rice roots, shoots by water stress and ABA. It was also induced by salt treatment. It is localized in cytosol and independent of *de nova* protein synthesis indicating that the stress response mechanisms are mediated by preformed cellular factors. The primary sequence of RAB 21 protein (now referred as RAB 16) contains duplicated conserved domains, each consisting of A and B repeats. The RAB 16 proteins are encoded by atleast four tightly linked genes and

localized in about a 30 kb region on the rice chromosome (Shinozaki *et al.*. 1989). The four proteins encoded by these genes are highly homologous (65-92%) and share conserved domains. All *RAB* genes except *RabD* are expressed in mature embryos.

The sequence comparison of promoter regions of the *rab* genes revealed two conserved sequence motifs. One of the motifs was 80% homologous to the transcription-modulating SP1 binding site (Briggs *et al.,* 1986), the other motif was similar to the cAMP-responsive element (Deutsch *et al.,* 1988). The transgenic tobacco plant, expressing a chimeric gene containing the 5`sequence of the rice *rab-16B* fused to the GUS reporter gene was analyzed (Shinozaki *et al,* 1990). The GUS expression was seen in zygotic embryos starting form 10 days after flowing and increased until seed maturation at 25 days after flowering. Its expression was parallel to the accumulation of endogenous ABA levels. The 482-bp sequence of 5* end of rice *rab-16B* gene promoter is responsible for the developmentally regulated expression in embryos. However, this is not due to the ABA responsive expression observed in the vegetative tissues of a transgenic plant. This analysis further reveals the basis of tissue specific response of a plant gene to stress.

ABA and water stress induced 15 kDa glycine rich protein was characterized from maize (Gomez *et al.*, 1988). It is reported to contain an RNA binding domain (Mortenson and Dreyfuss. 1989). This protein was localized in the scutellar epidermal cells of the embryo and is induced during water stress and wounding. Another ABA responsive *Rab 17* gene encoding a highly phosphorylated protein was reported in maize embryos (Vilardell *et al.,* 1990). ABA treatment was also shown to induce the synthesis of RAB-17 mRNA and protein in calli. Interestingly, the RAB-17 protein was found to be highly phosphorylated only in embryos and its putative casein type kinase phosphorylatable sequence showing homology to rice Rab 16 was identified (Mundy and Chua, 1988).

An ABA responsive gene *rab 28* encoding a protein of mol.wt 28-kDa, was isolated from maize, (Pla *et al.,* 1991). Sequence alignment revealed homology to LEA D-34 protein from cotton (Baker *et al,* 1988). The rab 28 mRNA was accumulated by the application of exogenous ABA both in embryos and leaves. The *cis* regulatory element, CCAAGTGG involved in the expression of *rab 28* gene in maize during water and ABA

stress (Pla *et ah,* **1991) was identified.** It accumulates during embryogenesis **and** is induced in vegetative tissues during dehydration stress in maize. Immunoelectron microscopy revealed that the Rab 28 protein accumulates in nucleolus of different cell types and its expression was restricted to provascular tissues in embryos. Another ABA responsive gene, *rab* 75, expressed in wheat roots during water stress was isolated (King *et al.,* 1992). The *rah 15* showed a significant sequence homology with that of other members of RAB family, dehydrins and LEA proteins, from both monocots and dicots.

Hong *et al.,* (1992) characterized an ABA responsive protein, HVA1 in barley aleuerone layers. The induction of this protein by ABA. drought, salt, cold and heat treatments is organ specific and is developmentally regulated. It was not detected in endosperm. However. it accumulates in aleuronc layers and embryos starting 25 days after anthesis. It's induction by ABA application to leaf tissues is restricted to leaf base. The HVA1 homologous gene is present in different graminae species such as rice, rye, wheat, oat and maize. Tissue specific accumulation of RAB proteins under stress needs to be analyzed in detail.

Rao *et al.*. (1993) reported the induction of ABA responsive proteins of mol. wts 45, 23 and 15-kDa in rice seedlings. The 23-kDa was immunologically similar to the Rab 16 protein (Mundy and Chua, 1988) The 23-kDa protein was also induced in rice calli exposed PEG stress and ABA (Reddy *et al,* 1993). The heat and ABA induced 110 kDa protein from young rice seedlings showed an immunological cross reactivity with yeast anti-HSP 104 antibodies (Singla and Grover, 1993). Interestingly this protein was constitutive!}' present in the flag leaf of 90 day old plant and was not affected by heat shock and ABA.

2.5.2 Dehydins:

Dehydrins represent a group of closely related proteins whose abundance in cereal-seedlings significantly increases during a gradual non-lethal dehydration condition (Close *et al.,* 1989). There are several reports on dehydrin proteins in several plants such as wheat, *Populus*, barley and maize **(Labhilili** *et al.,* 1995; **Pelah** *et al.,* 1997; Close and Chandler, 1990; Close *et al,* **1989).** These dehydration-induced proteins, called **dehydrins,** were found **to** be **very similar to** each other and also **to a previously identified**

rice proteins induced by ABA and salt (Mundy and Chua 1988). Further, they were found to have some similarity to a cotton embryo protein (Baker *et al.*. 1988). Dehydrins are hydrophilic, glycine-rich. cysteine and tryptophan-free and contain a repeating unit occurring twice in each protein. In dehydrating seedlings. the mRNAs corresponding to each of the dehydrin cDNAs are abundantly produced. The aminoacid sequence of dehydration induced proteins from barley revealed that they correspond to a protein that is produced in abundance in response to dehydration in corn seedlings (Close *et al.*, 1989). Antibodies raised against a maize dehydrin cross-react with polypeptides accumulated in dehydrating barley and wheat. Barley dehydrin shows homology with dehydrins present in dehydrating wheat and other grass seedlings. These results indicate the similarity among dehydrin proteins and mRNA sequences from different plant genera (Close and Chandler, 1990).

Bradford and Chandler (1992) showed that a North American wild rice, *Zizania palustries* withstands dehydration up to less than 10% moisture content. However, seeds of paddy rice *{Oryza sativa L.)* readily tolerate desiccation to less than 5% water content. Interestingly, both dehydrins and ABA accumulation are not directly involved in dehydration response of *Zizania* seeds. Further, Still *et al.,* (1994) analyzed the role *of* dehydrin expression. ABA and sucrose accumulation in desiccation tolerance during embryogenesis of *Oryza sativa* and *Zizania palustris.* The results revealed that accumulation of dehydrins. ABA and sucrose did not have role in desiccation sensitivity of *Zizania* seeds. On the contrary there are several reports on dehydrin accumulation conferring tolerance in wheat, *Populus* and sunflower (Labhilili *et al.,* 1995; Pelah *et al.,* 1997; Cellier *et al.* 1998). Accumulation of dehydrin proteins during salt (Galvez *et al.,* 1993; Moons *et al.,* 1995) cold (Arora and Wisniewski, 1994) and freezing stress (Robertson *et al.,* 1994) conferred tolerance.

2.5.3 Early methionine (Em) labeled Proteins:

Em (Early methionine labelled) gene is a member of the *Lea* gene family and characterized from wheat embryos (Cumings, A.L. and Lane, B. G., 1979). Levels of*Em* gene expression increases dramatically during maturation of wheat (Quatrano *et al.,* 1992) and maize (McCarty *et al,* 1991) embryos, and it can be induced in vegetative

tissues by exogenous ABA and osmotic stress (Hetherington and Quatrano, 1991; Quatrano *et al,* 1992). The *Em* gene was molecularly characterized (Cumings, 1984; Litts *et al,* 1992) and its ABA response element (ABRE) was identified (Guiltinan *et al,* 1990; Quatrano *et al.,* 1992). Regulation of *Em* gene expression by ABA has been shown to occur at the transcriptional as well as at a post-transcriptional levels (Williamson and Quatrano, 1988).

Bostock and Quatrano (1992) characterized the *Em* gene expression in rice suspension cultures subjected to various concentrations of ABA, osmotic stress and salt stress. It was demonstrated that salt stress and ABA are synergistic in controlling the expression *of Em* mRNA levels. Further, during salt stress, ABA inhibitors significantly inhibited (50%) the expression of the *Em* genes. These results indicate that in rice *Em* gene expression is mediated by two pathways; one is ABA mediated and the other pathway is specific to salt response that includes an intermediate common to both ABA and salt response.

2.5.4 Salt responsive proteins:

Recently several salt responsive proteins have been characterized from plants species such as rice (Rani and Reddy 1994, Rani, 1996; Claes *et* or/., 1990), sugarcane (Ramgopal and Carr, 1991), tobacco (Singh *et al.*, 1985), tomato (Godoy *et al.*, 1990), and yeast (Gaxiola *et al.* 1992). The two salt responsive proteins SRP-15 and 26 were characterized from rice are reported to be boiling stable (Rani and Reddy, 1994). Cultured tobacco cells, on adaptation to NaCl, synthesize a 26-kDa protein, osmotin (Singh *et al.* 1989). This protein constitutes as much as 12% of total cellular protein in adapted cells. It is related to several proteins including pathogen-induced protein, fruit protein thaumatin and a maize trypsin inhibitor. Changes in protein profiles induced by salinity stress have been reported in roots of a salt-sensitive rice cultivar, Taichung native 1 (Claes *et al,* 1990). At least eight proteins were induced under salinity stress. One of such salinity induced. *salT* gene encodes a protein of mol.wt 14.5 kDa was located in rice roots, leaf sheath but not in leaf lamina. SalT was also induced by ABA, air-drying, PEG, sodium chloride (1%) and potassium chloride (1%) treatments. Godoy *et al,* (1990) have shown that a tomato gene *TAS14,* was inducible by salt stress and ABA and not by cold,

wounding. Its nucleotide sequence revealed an-open reading frame that encodes a highly hydrophilic and glycine-rich (23.8%) protein of 130 amino acids.

2.5.5 Cold responsive proteins:

Several cold induced proteins have been reported from *Arabidopsis*, wheat, barley, alfalfa etc.. (Houde *et al.*. 1992; Cattivelli and Bartels. 1990; Mohapatra *et al.,\9S9).* Of the several cold responsive genes characterized, Gilmour *et al.*. (1992), reported one cDNA belonging to the *Cor* gene family encoding a 47 kDa hydrophilic polypeptide from *Arabidopsis.* Cor 47 shows amino acid sequence homology with Group II Lea proteins (Dure *el al.*. 1989). Lin and Thomashow (1992) demonstrated that a cDNA clone, *Cor15*, responsive to cold stress encodes a boiling-stable polypeptide. A novel gene, Kinl was shown to be inducible during cold stress in *Arabidopsis* seedlings (Kurkela and Frank. 1990). Water stress and ABA also induced Kinl polypeptide. Sequence comparison revealed that the Kinl gene product has similarities to a fish antifreeze protein (Pickett *et al.*. 1984). In *Arabidopsis,* Lti 140 gene is induced by low temperature ABA and also by water stress (Nordin *et al.,* 1991). The expression of this gene during the stress conditions might be mediated by separate signal transduction pathways. In wheat cold induced Wcs120 protein was associated with freezing tolerance (Houde *et al.,* 1992).

2.6 The role of ABA in stress response:

Abscisic acid is a naturally occurring plant hormone having an important role in the control of diverse physiological processes such as seed development and plant adaptation to environmental stress. (King 1976; Suzuki *et al.,* 1981; Skriver and Mundy, 1990; Zeevart and Creelman, 1988). Studies on the seed maturation and physiological aspects of different stress responses revealed that the ABA regulates the transcriptional activation of specific genes and proteins. Though the ABA receptors have not yet been identified, the down-stream elements of ABA regulatory pathways is known The elucidation of the role of ABA in physiological processes of the plants has been done by

monitoring the endogenous ABA content and analyzing the effect of exogenous application of ABA or by the use of ABA inhibitors.

Role of mutants:

Mutants deficient in ABA biosynthesis provide a direct assessment of role of ABA in various plant responses. The analyses of such mutants reveal the role of ABA in seed development and response of vegetative tissues to various environmental stress conditions (Giraudat *et al.,* 1994). ABA deficient mutant have been identified in a variety of plant species, for instance, the maize *viviparous* (Neill *et al.*,1986), tomato *flacca, sitiens* and *notabilis* (Tal and Nevo, 1973), *Arabidopsis aba* (Koornneef *et al.,* 1982), potato *droopy,* (Quarrie, 1982), and tobacco *abal* (Parry *et al.,* 1991) mutants. Such mutants exhibited two important traits: viz. precocious germination (Neill *et al.,* 1986) and reduced dormancy (Rousselin *et al.,* 1992). These mutants which are deficient in the hormone can be restored to normal condition by the exogenous application of ABA.

Studies on gene expression during seed development in ABA deficient mutants of maize (Pla *et al,* 1991; Paiva and Kxiz, 1994) and *Arabidopsis* (Finkelstein, 1993) revealed that the mutation inhibits, though not completely, the accumulation of storage protein synthesis. These mutant seeds also showed reduced levels of ABA and *Lea mRNAs*. In most cases, the extent of induction of LEA proteins did not linearly correlate with the reduction in ABA content. The *LEA mRNAs* reach a peak at the end of seed maturation with a simultaneous decrease in ABA content (Hughes and Galau. 1989). The reversal of desiccation intolerance of *aba, abi3* digenic mutants to wild type by the application of exogenous ABA reveals a crucial role of ABA in seed development (Koornneef *et al.,* 1989; Meurs *et al.,* 1992).

In tomato, the accumulation of ABA has been shown to regulate gene expression which occur during water deficit (Bray, 1988). The ABA deficient mutant of tomato, *flacca,* accumulates low levels of ABA during water deficit compared to the wild type. This mutant was used to distinguish expression of cDNAs between ABA responsive and non-responsive types. Several cDNA clones that are expressed simultaneously in response to ABA and drought, have been isolated. Three such genes, pLE4, pLE16 and pLE25 expressed preferentially in wilted wild type leaves but not in wilted leaves of

flacca mutant. Expression of LE16 was induced in leaf tissue not only by water stress, ABA but also under salinity, cold and heat stress as well. None of these stresses or direct application of ABA induced the expression of LE16 in roots of these plants.

In *Arabidopsis*, the low temperature induced Lti 140 gene which is also induced by ABA and water stress. Such expression seems to be mediated by separate signal pathways (Nordin *et al.*. 1991). ABA as was shown by the treatment with fluridone. an ABA biosynthetic inhibitor, did not mediate the low temperature induction of Lti 140. The effects of low temperature and ABA are not cumulative, indicating that that these pathways are different. Induction of this gene by ABA is abolished in the ABA-insensitive mutant, *abi-1* indicating that the mutation defines a component in the ABA response pathway. On the contrary, accumulation of the Lti 140 mRNA in plants exposed to water stress was reduced by treatment with fluridone and also in the ABA insensitive mutant, *abi-1*, suggesting that the water stress induction of Lti 140 could be partly mediated by ABA. Three separate but converging signal transduction pathways regulate the expression of the Lti 140 gene. Several genes that are induced by desiccation apparently do not respond to ABA, further suggesting that there are ABA independent as well as ABA mediated signal transduction pathways (Guerrero *et al,* 1990).

Promoter analysis:

Changes in gene expression in plants under stress involve mainly transcriptional regulation. Many of the changes that occur due to drought are also induced by ABA application. Although not much progress has been made in elucidating the function of stress induced proteins in plants, significant advances have been made in understanding the ABA regulation of LEA proteins. Investigation were carried out to decipher the interactions between DNA binding proteins and ABA responsive elements (ABRE) such as regulator)' element of Em1 from wheat (Guiltinan *et al,* 1990) and Motif 1 isolated from rice *rab 16A* gene (Mundy *et al.*,1990)

The comparison of the promoter sequences of *rabl6* family (*rab16A to 16D*) of rice revealed two highly conserved motifs, motif I (5'-GTACGTGGC-3') and motif II (5'-CGG/CCGCGCT-3') (Yamaguchi-Shinozaki *et al.,* 1989). ABREs have an important role in the ABA induced gene expression, which has been proved by transient expression

experiments in barley aleurone protoplasts. These experiments revealed hexamer motif I of ABRE was responsible for the ABA induced gene expression and not motif II. In wheat, a protein showing affinity for an ABRE has been cloned and sequenced (Guiltinan *et al*., 1990).

Recent studies have revealed that other elements distinct from Motif-1 are also involved in ABA inducible expression of the *Craterostigma* CDeT27-45 sequences (Nelson *et al.* 1994). The analysis of the HVA22 promoter from barley revealed that ABRE alone is not sufficient for transcriptional activation: a coupling element CE1 is necessary for conferring high ABA induction (Shen and Ho, 1995)

The investigation of drought induced genes in *A. thaliana* has also revealed the presence ABA-independent signal transduction pathways (Yamaguchi-Shinozaki and Shinozaki. 1994). In *Arabidopsis,* Yamaguchi-Shinozaki *et al.* (1992), have isolated a group of Desiccation Responsive (RD) genes. Interestingly, ABA induces mRNA of the RD22 and RD29 but not the RD19, RD21 and RD28 indicating that there are several signal transduction pathways operating during water stress-mediated induction of RD genes.

2.7 Signal Transduction:

Broadly, the events involved the expression of stress induced genes can be divided into three steps. 1) Perception of stimulus, 2) Signal processing and 3) A response leading to gene expression. Todate, the exact molecular events of signal perception is not fully charecterised, however turgor change is implicated as a possible signal. Physiological studies on drought stress responsive genes in a number of plants suggests that endogenous ABA levels have been reported to increase during drought and thus ABA is thought to be involved in the signal transduction. Also, many drought stress induced genes were also induced by the application of ABA exogenously, although several ABA independent pathways exist in stress induced gene expression

There are reports of involvement of calcium ions in ABA induced *RAB* gene expression (Van der Meulen *et al*., 1996). In barley protoplasts, the application of Ca^{+2}

blockers and inhibitors did not induce the *RAB* gene expression even after the application of ABA. The stress induced HVAl promoter linked *Luc* reporter gene was expressed in maize protoplasts (Jensheen, 1996) suggesting role of two calcium dependent protein kinases in signal transduction during stress treatment.

In *Arabidopsis* the induction of *cas* (cold responsive) genes under cold acclimation was strongly correlated with Ca^{+2} ion influx. The Ca^{+2} influx was more with the decrease in temperature, influx of calcium started from $15^{o}C$ onwards and reached maximum at $4^{o}C$. The *cas* genes are expressed even at $25^{o}C$ with application of Ca^{+2} ionophores. which allow calcium influx. The involvement of Ca^{+2} dependent protein kinases in stress signal transduction has been reported (Monroy and Dhindsa, 1995).

Protein kinases:

In wheat, an ABA and water stress responsive gene, PKAB1 was isolated and its deduced amino acid sequence revealed homology to protein kinase (Anderberg and Walker-Simmons, 1992). The transcript was not detectable in growing seedlings, but it is induced when plants are subjected to dehydration stress and application of ABA.

ABA responsive protein, namely RAB-17 is found to be highly phosphorylated (Vilardell *et al.*, 1990). *In vitro* studies have shown that a serine cluster region of the RAB-17 protein can be phosphorylated by maize protein kinase II. In wheat, phosphorylated stress induced proteins have been identified as kinases based on their substrate specificity.

In maize seedlings, primary roots adapted to low water potentials by continuous elongation. whereas shoot growth is completely inhibited. A 45-kDa protein kinase was identified in the elongation zone of primary root within 30 min of stress exposure and was independent of ABA accumulation and protein synthesis (Conlay *et al.*, 1997). This protein kinase was thought to be responsible at an early step in signaling pathway involved in adaptation of root growth at low water potentials.

2.8 Desiccation tolerance:

The plants and specific plant parts, which are able to survive adverse environmental conditions were used as models to study the desiccation tolerance processes (Ingram and Bartels. 1996). Most of observations were derived from seeds (McCarty, 1995) and plants includes desiccation tolerant species like resurrection plants (Bartels *et al.*. 1990), mosses and ferns. Mature seeds and a resurrection plant are used as a model system for desiccation tolerance. *Mesembryanthemum crystalinum* a halophyte is a model plant for salinity, desiccation and cold tolerance. The elucidation of the biochemical, physiological and molecular changes involved in tolerance mechanisms of these plants and mature seeds can be exploited to manipulate and improve crop plants for stress tolerance.

2.8.1 Seed and its desiccation tolerance:

In higher plants, only seeds appear to have the capacity to withstand severe desiccation. In seed, during maturation processes. 90% of original water is removed to attain dormancy. Desiccation of the seed helps in withstanding extreme environmental conditions and facilitates wide dispersal. The embryo can not withstand desiccation uniformly at all developmental stages. Tolerance usually acquired specific stage of embryogenesis and is lost on the onset of germination. Desiccation tolerance mechanisms were studied in seeds of many plant species such as *Arabidopsis* (Parcy *et al.*. 1994). cotton (Baker *et al.* 1988), barley (Bartels *et al.* 1988). rice (Mundy and Chua, 1988) etc. Understanding the mechanisms of drought tolerance in seed is an important aspect of improving tolerance levels at the whole plant level.

Role of ABA in seed maturation:

Seed maturation involves a series of programmed events and ABA plays a major role in the regulation of such processes, including embryo morphogenesis (Quatrano, 1987). storage protein synthesis (Finkelstein *et al.*. 1985), desiccation tolerance (Bartels *et al,* 1988), onset and maintenance of dormancy (Koornneef, 1986) etc. In many plants endogenous ABA levels increase during embryogenesis and return to low levels in the mature seed. Precocious germination and intolerance to desiccation was shown by

immature embryos and vivipary mutants, deficient in ABA. can be prevented by the application of exogenous ABA. suggesting that increased seed ABA levels play a similar role *in vivo* (Skriver and Mundy, 1990; Neill *et a!.,* 1986).

Elegant experiments of Bartels *et al.,* (1988) revealed several steps involved in the acquisition of desiccation tolerance during barley embryo development. Premature embryos (8 DAP) were found to be capable of germination but are unable to withstand desiccation. The embryos from 16 DAP were able to germinate despite desiccation treatment. The protein profiles from 12 DAP embryos (desiccation-intolerant) were different from 16 DAP embryos (desiccation-tolerant). In 12 DAP embryos. desiccation tolerance can be induced *in vitro* by the application of ABA. It also prevented the precocious germination. In *in vitro* cultured 12 DAP embryos, ABA stimulated the appearance of a set of 30 proteins similar to that of 16 DAPs leading to the process of desiccation tolerance. In seeds. the application/rise in ABA levels is characterized by the accumulation of distinct sets of mRNAs and corresponding LEA proteins in embryo and endosperm (Dure *et al.,* 1989).

*Late **embryogenesis** abundant (LEA) proteins and their role in desiccation tolerance:*

The LEA proteins are divided into 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) are predicted to have an enhanced water-binding capacity. Total desiccation is probably lethal. and therefore such proteins could help maintain the minimum cellular water requirement. One member of this group, Em protein from wheat is considerably more hydrated than most globular polypeptides because of its 70% random coil, which provides high capacity for binding with water.

Group-2/Rab/dehydrin LEA (D11-family) proteins generally accumulates in plants in response to dehydration caused by desiccation, salts, or chilling. Dehydrin-encoding cDNA clones have been identified from rice, barley, maize (Mundy and Chua, 1988; Close *et al,* 1989; Vilardell *et al.,* 1990) and several dicots (Piatkowaski *et al.,* 1990; Baker *et al.,* 1988). These proteins resemble chaperones.

Group 3 LEA proteins (D-7 family) are predicted to play a role in the sequestration of ions that are concentrated during cellular dehydration. Such problems are

counteracted by the formation of salt bridges **with** amino acid residues of highly charged proteins. These proteins have an 11-mer motif with a consensus sequence (TAQAAKEKAGE) repeated 13 times (Dure, 1993a). This motif is predicted to form an amphiphilic α-helix thus helping in the sequestration of ions and counteracting the damaging effects of increasing ionic strength in the cytoplasm during desiccation. cDNA clones encoding group LEA 3 proteins have been isolated from cotton, barley, rape, and wheat, *Craterostigma* and shown to be induced during dehydration, cold, ABA application and salt stress (Curry and Walker-Simmons, 1993).

Group-4 LEA (D-113) proteins may replace water to preserve the structures. Group-5 of LEA proteins (D-29 family) is also predicted to have a similar role as the D-7 family, sequestered ions during water loss. A major effect of dehydration is the loss of water leading to crystallization of cellular components, which in turn damages cellular structures. LEA proteins may counteract this, and in fact some of the LEA proteins are essentially considered as compatible solutes, which acts like sugars in maintaining the structure of cytoplasm in the absence of water. LEA proteins appear to be accumulated in many cell types at different concentrations and are predominanth' cytosolic (Schneider *et al,* 1993; Mundy and Chua, 1988). Interestingly, in cotton embryo cells, the D7-LEA proteins represent almost 4% of non-organellar cytosolic proteins (Roberts *et al,* 1993). LEA proteins are hydrophilic and lack cysteine and tryptophan residues.

The LEA proteins in all the tested plant species disappear at the onset of germination but are induced in vegetative tissues under stress (Close *et al,* 1989; Piatkowaski *et al,* 1990). It is important to determine whether such plant responses have an adaptive role or are a consequence of stress responses that are triggered by environmental as well as developmental signals. A correlation was reported between the organ survival and LEA protein accumulation during dehydration of wheat seedlings. Shoots and scutellar tissue resume recovery' from 90% water loss, where as roots are killed. This is correlated with thew observation LEA proteins accumulate in scutellum and shoots but not in roots (Reid and Walker-Simmons, 1993). Interestingly, there was no

mRNA accumulation in both shoots and scutellum, indicating the transcriptional regulation of gene expression during stress.

LEA proteins from *Craterostigma plantagineum* arc independently expressed in transgenic tobacco plants. The leaf discs from the transgenic and the control plants did not differ in tolerance to mild stress (Iturriaga *et al.,* 1992). In contrast, recently the LEA proteins from barley, HVA 1, when expressed independently in transgenic rice led to an improved tolerance to salt and drought stress (Xu *et al.,* 1996). This contradiction is understandable. The over expression of a single protein alone is not sufficient to confer the tolerance. Probably such over expression of a protein should be supplemented with accumulation of osmolytes, sugars and other such compounds..

***2.8.2** Craterostigma plantagineum as a model plant to study desiccation tolerance:*

In higher plants, only seeds are capable of surviving the extended periods of dehydration. However, a small group of angiosperms termed as "resurrection plants" constitute a distinct class of plants, which exhibit the phenomenal ability of tolerating to severe desiccation. Further, these plants can recover from such drastic stress and resume their biological activities upon rehydration (Bartels *et al.,* 1990). Recently several desiccation related, ABA responsive transcripts and proteins have been isolated by differential hybridization (Bartels *et al.,* 1990; 1992) from *Craterostigma plantagineum (Scrophulariaceae).* The majority of the transcripts encode polypeptides closely related to proteins which are abundantly induced during maturation process of seeds of many higher plants or to some extent in vegetative tissues under stress (Bray, 1991; Bartels *et al.,* 1988). Five such cDNA clones encode proteins of mol.wts of 16-34 kDa and three of them were hydrophilic in nature (Piatkowski *et al.,* 1990). The clones *pcC 27-04* and *pcC 6-19* are homologous to desiccation related genes expressed in embryos or dehydrated seedlings. The *dsp-22* is one of the desiccation related genes of *Craterostigma* that is regulated by light. These proteins are assumed to serve as osmoprotectants (Dure *et al.,* 1989: 1993). When introduced into tobacco, the CDeT27-45 promoter construct with Gus reporter expressed only during embryo maturation and in pollen. However, these tissues are naturally desiccation tolerant in non-transformed tobacco itself. Interestingly, CDeT27-45 encoded protein accumulates in leaves, callus and seeds of *Craterostigma*

(Schneider *et al.* 1993) under normal conditions. The promoter analysis of *Cratestigma* genes (Micheal *et al.*. 1993) further suggests the differences in stress responsive regulation *of* gene expression between resurrection plants (desiccation-resistant) and others (non-resistant).

The transgenic tobacco plants earning a LEA type *Craterostigma* genes proteins did not show desiccation tolerance (Iturriaga *et al.*, 1992), indicating that the single protein over expression is not sufficient and other components such as carbohydrates are necessary to confer tolerance. Interestingly, seeds and fully-hydrated leaves of *Craterostigma* accumulate high levels of octulose, an unusual C8 sugar (Bianchi *et al.*, 1991). *In vitro* studies revealed that sugars play role in protecting the cellular components during dehydration processes (Crowe *et al.*. 1992) protected in association with osmolytes during dehydration. These compatible solutes act as molecular chaperones. The osmolytes accumulation during cellular dehydration varies from plant to plant. Free amino acids, glycine betaine, organic acids, reducing sugars, sugar alcohols and non-reducing sugars are reported as compatible osmolytes under environmental stress conditions (Ford 1984, Smirnoff and Cumbes, 1989).

2.9 Polvol biosynthesis in relation to desiccation tolerance:

Polyols arc straight chain metabolites such as mannitol and sorbitol (Bieleski, 1982) or cyclic such as myo-inositol and its methylated derivatives (Loewus and Dickinson. 1982). They are widely distributed among plants, animals, bacteria and algae. Polyols are reported to have a role in osmotic adjustment and osmoprotection. They act as osmolytes facilitating water retention in cytoplasm and allowing sodium sequestration into the vacuole or apoplast. Mannitol is a sugar alcohol and is accumulated in the vacuoles of tobacco cell adapted to salt stress (Binzel *et al.*, 1988). The role of mannitol in higher plants was analyzed through transgenic approach. Recently, tobacco plants have been transformed with *E.coli mtlD* gene, which encodes mannitol-phosphate dehydrogenase. under the control of a constitutive promoter (Tarczynski *et al.*. 1992). The overexpression of *mtlD* gene lead to the accumulation of mannitol upto 6 µmol/g

fresh weight. Such transgenics showed a better growth in comparison to the wild type tobacco plants under salt stress. Mannitol was reported as a scavenger of free radicals having wide implications in stabilizing the organellar function. For insatnce, the overexpression of mannitol in chloroplasts (Shen *et al.,* 1997) resulted in an improved tolerance to oxidative stress.

Sorbitol is a sugar alcohol of glucose. It is ubiquitous in all plant species, predominantly accumulating in seeds. It is also found as translocated carbohydrate in *Rosaceac* species and in vegetative tissues of halo-tolerant *Plantago maritima* (Ahmad *et al.*, 1979). Sorbitol concentrations were increased to an eight-fold in shoot tissues and a 100-fold in root tissues of *Plant ago* under salt stress. The concentration of sorbitol was 60-75 μmol/gr of dry weight, which is similar to the levels of mannitol observed in mtlD transgenic tobacco plants (Tarczynski *et al.*, 1992). Sorbitol accumulation in *Plantago* leaves and roots may serves as osmoregulatory function and its accumulation in plant seeds may contribute to the desiccation tolerance of the mature embryo. In animals, sorbitol was reported to be involved in the osmoregulatory processes. It accumulated in the epithelial cells of the inner medulla where urine is concentrated thus serving an osmoregulatory function (Garcia-Perez *et al.,* 1989). Sorbitol was reported to protect Pst I restriction enzyme from desiccation as shown by *In vitro* analysis (Colaco *et al.,* 1992). Bartels *et al.*,(1991) have identified a polyol biosynthetic enzyme, aldose reductase from barley embryos. This protein is responsive to ABA and other osmotic stresses. Plants transformed with this gene can be used to test the physiological effects of sorbitol in tolerance processes.

The cyclic alcohol's, pinitol and ononitol were involved in the osmoregulatory processes of the legumes, iceplant, etc.(Ford, 1984). In the iceplant, *Msembryanthemum crystallinum*, inositol pathway plays a crucial role in conferring stress tolerance. The inositol-O-methyltransferase (IMT1) is under environmental control at any developmental stage and is induced by salt stress and low temperature (Vernan and Bohnert; 1992). The activity of this enzyme leads to the production of D-ononitol, which further epimerized to cyclic sugar alcohol D-pinitol. Pinitol is low molecular weight

compound, which is present as high as 700mM in cytosol and Chloroplast (Adams *et al.,* 1992). Pinitol accumulates in salt and drought tolerant plants. A second mechanism that protects the young ice plant against water stress is the regulation of ion uptake and compartmentation. Under salinity stress most of the sodium ions are sequestered in the vacuoles of young tissues, which compensated by accumulation of pinitol in the cytoplasm (Adams *et al.,* 1992).Tobacco plants transformed with the D-myo-inositol methyl transferase (IMT1) cDNA from *Mesembryanthemum crystallinum* overproducing ononitol showed enhanced tolerance to salt and drought stress compared with non transformed tobacco plants (Sheveleva *et al.* 1997) .

Role of sugars in desiccation tolerance:

The involvement of soluble sugars in desiccation tolerance in plants varies from plant to plant and depends on the type of sugar accumulated during desiccation processes. Disaccharide trehalose though rare in plants, is an important carbohydrate reported in animals, fungi and bacteria aiding desiccation. In plants sucrose and other sugars help in conferring desiccation tolerance. Studies with seeds have shown the accumulation of soluble sugars during the acquisition of desiccation tolerance (Leprince *et al.,* 1993). The leaves of well-watered plant *Craterostigma* accumulates unusual C8-sugar. 2-octulose (90% of the hydrated leaves) which is converted into sucrose during dehydration, where it constitutes 40% of the total dry weight (Bianchi *et al.*, 1991).

Sugars protect the cellular structures during severe desiccation by glass formation preventing the cellular collapse thus restricting the molecular diffusion as demonstrated in viable maize seed (Williams and Leopold, 1989). Apart from conferring desiccation tolerance sugars play role in protein stabilization. Crowe *et al.,* (1992) demonstrated that trehalose protects the functional vesicles during in-vitro drying and rehydration of the sarcoplasmic reticulum which may result in the fusion of vesicles. It is hypothesized that sugars confer protection of the membranes *in vitro* by altering the properties of dry membranes so that they resemble those of fully hydrated biomolecules.

2.10 Functional aspects of stress induced proteins:

The functional roles of stress-induced polypeptides in plants remain to be established. It is known that genes upregulated by different stresses encode polypeptides of both known and unknown functions (Ingram and Bartels. 1996). One frequent way of identification of a function to such induced proteins is to deduce from their amino acid sequences. For instance. such proteins identified are storage polypeptides (Finklestein *et al.,* 1985; Bray and Beachy. 1985). lectins (Raikel and Wilkins. 1987) and enzyme inhibitors (Mundy *et al.*, 1986). Such proteins may be involved in protection from stress-mediated damage and/or maintenance of dormancy (Finklestein and Crouch. 1986; Ramagopal, 1987). One such salt-inducible protein has been isolated from tobacco and found to be homologous to a group of protease inhibitors (Singh *et al.,* 1987; Richardson *et al.,* 1987).

Bartels *et al.,* (1991) isolated a desiccation tolerant cDNA clone (PG22-69) from barley embryo encoding a 34 kDa protein, that shows homology to a mammalian gene encoding NADPH-dependent aldose reductase. This enzyme is known to be involved in the synthesis of osmolyte sorbitol. Vernon and Bohnert (1992) studied the molecular mechanisms of osmotic stress tolerance in *Mesembryanthemum crystallinum,* a facultative halophyte, capable of adjusting to and surviving under highly saline conditions has been a model plant to investigate stress response mechanisms. In this plant, myoinositol-0-methyl transferase (MIT), which catalyzes the first step in the biosynthesis of the cyclic sugar alcohol, pinitol and known to accumulate in salt and drought tolerant plants. Sugar alcohols play an important role in osmotic stress tolerance. The overexpression of the MIL in transgenic tobacco confers the salt and desiccation tolerance (Sheveleva *et al.,* 1997). The mannitol overproducing transgenic tobacco plants also showed an improved tolerance to salt stress (Tarczynski *et al.,* 1993).

Gaxiola *et al.*. (1992) have isolated a novel yeast gene. HAL1, which upon overexpression improves growth under salt stress. In addition, deletion of this gene decreases salt tolerance. Therefore HAL1 is reasoned to constitute a rate-limiting determinant for halo tolerance. In cells with over expression of HAL 1, sodium toxicity

seems to be counteracted by an increased accumulation of potassium. Cryoprotection assay of Lin and Thomashow (1992) showed that the cold responsive protein from *Arabidopsis* Cor15 was very effective in protecting the cold-labile enzyme lactate dehydrogenase against freeze-thaw inactivation. It was shown to be more effective as a cryoprotectant than sucrose and certain proteins including bovine serum albumin.

Sequence analysis of RD28 gene from *Arabidopsis* revealed that it encodes a membrane protein with sequence homology to the major intrinsic protein of bovine lens, soybean nodulin-26 and glycerol facilitator of *E.coli* indicating that it is a member of the family of the transmembrane channel proteins (Yamaguchi-Shinozaki *et al*., 1992).

The stress induced endogenous ABA levels and protein profiles were compared in tolerant and sensitive varieties of wheat (Walker-Simmons, 1987) and rice (Lee *et al*., 1993, Moons *et al.,* 1995) and analyzed the gene expression in genotypes differing in tolerance (Galvez *et al*., 1993). The expression level of a group of specific genes has been correlated with improved salt, desiccation and cold tolerance of cell lines, e.g. the chilling induction of a Gly-rich protein from alfalfa (Mohapatra *et al*., 1989) and a group 2 LEA protein of wheat (Houde *et al.,* 1992). Dehydration induced group 3 LEA mRNA levels were correlated with an increase in endogenous ABA levels in wheat (Curry *et al.,* 1991) and rice seedlings. Molecular basis of salt tolerance was analyzed in salt tolerant (Pokkali and Nona Bokra) and sensitive (Taichung N1) rice lines by Moons *et al.,* (1995). The effect of ABA on total soluble proteins in seedling roots revealed that tolerant line, Pokkali accumulated significantly higher amounts of stress induced proteins. The levels of dehydrins and group 3 LEA proteins were significantly higher in roots from tolerant varieties compared to sensitive ones. The induction of the proteins was correlated with the level of increase in endogenous ABA levels during salt stress. Higher endogenous ABA levels and ABA induced proteins were found to be a characteristic of the tolerant varieties. After the reversal of the stress conditions, the induced polypeptides and the corresponding mRNAs were found to disappear (Claes *et al.,* 1990; Ho and Mishkind, 1991).

Accumulation dehydrins also conferred desiccation tolerance in many plants such as in wheat, *Populus* and sunflower (Labhilili *et al.,* 1995; Pelah *et al.,* 1997; Cellier *et*

al, **1998). Accumulation** of **dehydrin related** proteins during **salt (Galvez *et al.,* 1993;** Moons *et al.,* 1995) cold (Arora and Wisniewski, 1994) and freezing stress (Robertson *et al,* 1994) also conferred tolerance. Recently the Group 3 LEA protein from barley, **HVA** 1 when expressed independently in transgenic rice showed an improved tolerance to salt and drought stress (Xu *et al,* 1996).

2.11 Stress mediated oxidative damage and antioxidant enzymes

Much of the injury to plants caused by stress exposure is associated with oxidative damage at cellular level. Wide spread loss of forests and crops due to ozone pollution provide a visible example of oxidative stress. Oxidative damage associated with periods of cold or drought also has a role in incremental setbacks during a growing season of many crop plants. In plants many of the degenerative reactions associated with several biotic, abiotic and xenobiotic stresses are mediated by the toxic, Reactive Oxygen Intermediates (ROIs) formed from the superoxide and hydroxyl radicals (Scandalios, 1993). These stresses include ozone (Van camp *et al,* 1994), anoxia (Monk *et al,* 1989), pathogens (Mehdy, 1994), desiccation (Senaratna *et al,* 1985a) and freezing (Kendall and Mckersie, 1989). During water depletion oxidative stress processes in plants are intensified and hydrogen peroxide levels increases. Plants with high level of antioxidants, either constitutive or induced, have reported to have a greater resistance to oxidative damage (Harper and Harvey, 1978; Madamanchi and Alcher, 1991). There are several reports on antioxidant enzymes in relation to the stress tolerance (Bowler *et al,* 1992; Allen, 1995) in crop plants. Recently many transgenic plants carrying antioxidant genes have been reported to exhibit improved stress tolerance (McKersie *et al,* 1996).

Oxidative stress arises from the deleterious reactions of reduced oxygen species, such as superoxide radicals and hydrogen peroxide which in Haber-Weiss reaction, can form hydroxy radicals that are toxic to cellular components (Bowler *et al,* 1992).

$$H_2O_2 + O_2^{\cdot} \xrightarrow{Fe^{2+} + Fe^{3+}} OH^{\cdot} + O_2 + OH^{\cdot}$$ (Haber-Weiss reaction)

Superoxide radicals, hydrogen peroxide and singlet oxygen are formed from many cellular reactions (Cadenas, 1989; Fridovich. 1986; Salin. 1987) which are highly toxic and react indiscriminately with other biomolecules causing lipid peroxidation, denaturation of proteins and damage of DNA. Lipid peroxidation levels are used as indicators of oxidative stress. Superoxides are generated, when electrons are misdirected and donated to oxygen and electron transport chain of mitochondria and chloroplasts. In chloroplasts excitation energy from chlorophyll transferred to oxygen also leads to generation of singlet oxygen (Bowler *et al.,* 1992). Efficient removal of ROIs from Chloroplast is very essential. since H_2O_2 concentrations as low as 10 μM can inhibit photosynthesis by as much as 50% (Kaiser. 1979). Therefore. immediate scavenging of H_2O_2 is indispensable to maintain photosynthetic activity of chloroplasts (Asada and Takahashi, 1987).

Plants have evolved protective mechanisms to keep these deleterious reactions to a minimum level. These involve both enzymatic and non-enzymatic mechanisms. Antioxidant systems of plants include enzymes such as superoxide dismutase (SOD) and ascorbate peroxidase (APX) and non-enzymatic components such as ascorbic acid and glutathione. Together with glutathione reductase (GR), dehydro ascorbate reductase (DHAR) removes H_2O_2 through a mechanism known as Halliwell-Asada pathway named after the discoverers (Foyer and Halliwell, 1976; Nakano and Asada, 1980). Superoxide radicals are produced by the reaction of molecular oxygen at PS1 via the Mehler reaction. This superoxide radical is rapidly dismutated to H_2O_2 by SOD associated with thylakoid membranes. The H_2O_2 produced is effectively scavenged by a thylakoid-bound ascorbate peroxidase (APX). Monodehydroascorbate radicals produced by APX can be quickly reduced to ascorbic acid via Fd or by stromal monodehydroascorbate reductase. Alternatively, they can spontaneously disproportionate into ascorbic acid and dehydroascorbic acid, which is converted to ascorbic acid by DHAR using reduced glutathione as an electron donor. Subsequent regeneration of reduced glutathione requires GR and NADPH (Bowler *et al.,* 1992). In addition, plant cells contain relatively high

levels of ascorbate, glutathione. and **α-tocopherol, which are efficient oxyradical scavengers** (Cadenas, 1989. Larson, 1988).

2.10.1 Superoxide Dismutase:

Superoxide dismutases react with superoxide radicals to produce hydrogen peroxide and catalyses the following reaction (Mc Cord and Fridovich. 1969) :

$$2O_2^{\cdot-} + 2H^+ \xrightarrow{\text{SOD}} H_2O_2 + O_2$$

SODs are a family of metalloenzymes and their activity determine the concentration of $O_2^{\cdot-}$ and H_2O_2 and have essential role in the defense mechanism of bacteria to eukaryotes. For example, SOD deficient mutants of *E.coli* and yeast (Van Loon *et al.,* 1986) are hypersensitive to oxygen and in *Drosophila* the null mutation of Cu/Zn SOD resulted in a reduced lifespan (Phillips *et al.,* 1989). Isoenzymes of SOD are found in various compartments of plant cells and contain Cu and Zn, Fe, or Mn as cofactors. The three forms of SODs (Cu/Zn, Mn, and Fe) can be identified by their differential sensitivities to KCN and H_2O_2. Cu/Zn SOD (cytosolic) localized in Chloroplast stroma, is sensitive to both H_2O_2 and KCN and is present only in eukaryotes and some prokaryotes. MnSOD is resistant to both inhibitors and found in mitochondria. FeSOD is present in Chloroplast and is sensitive to H_2O_2 (Kanematsu and Asada, 1990). The number of isozymes of each type of SOD varies greatly from plant to plant, as does the abundance of each enzyme.

2.10.2 Ascorbate peroxidase (APX):

Cytosolic and chloroplastic forms of APX are identified in plant cells and are the primary H_2O_2 scavenging enzymes with high substrate specificity for ascorbate (Asada, 1992). Although catalase is capable of scavenging large quantities of H_2O_2, it is not able to protect the Chloroplast from oxyradical damage due to its location in peroxisomes and **its** relatively low Km. The APX enzymes differ in substrate specificity, pH optimum, and sensitivity to ascorbate depletion (Asada, 1992). Two isozymes of APX have been reported from tea leaves (Chen and Asada, 1989). The cytosolic and chJoroplastic APXs

arc clearly distinguished from each other based on their substrate sensitivities. Chloroplastic isozyme have a short life in ascorbate depleted medium when compared to cytosolic APX and is more specific to ascorbate as electron donor than cytosolic isozyme (Chen and Asada, 1989).

*2.10.3 **Glutathione** Reductase:*

In plants GR is localized both in cytosol and Chloroplast (Foyer and Halliwell, 1976). Multiple forms of GR in pea (Edwards *et al,* 1990) and pine (Anderson *et al,* 1990) have been demonstrated. Individual isozymes may differ with respect to subcellular localization and their respective responses to stress. Not much information is available on GR in cereals, particularly rice.

*2.**10.4** Role of antioxidant enzymes in stress tolerance:*

Although plants have adapted to prevailing environmental conditions during evolution, the level of natural resistance to oxidants varies widely among plant species (Reinert *et al.*, 1982; Bennet *et al,* 1984). Differences in resistance can be related to modifications in the constitutive levels of antioxidativc enzymes and non-enzymatic antioxidants. These can be age dependent, species dependent or due to the modification of gene expression. Lee and Bennett, (1982) reported that the younger leaves show an elevated resistance than old leaves to ozone because of their higher endogenous SOD levels.

Plants survive during stress conditions by maintaining their defense mechanisms or amplify other protective mechanisms under prolonged stress. Antioxidant enzymes are induced during drought stress in maize, tobacco (Malan *et al.,* 1990; Van Rensburg and Kruger, 1994), ABA stress in maize (Zhu and Scandalios, 1994), Cold stress in red spruce, rice (Hausladen and Alscher, 1994; Saruyama and Tanida, 1995) and salt stress in pea (Olmos *et al*., 1994; Hernandez *et al,* 1995).

The levels of antioxidant enzymes increase along with lipid peroxidation as the water potential decrease in barley (Smirnoff and Colombe, 1988), tobacco (Van Rensburg and Kruger, 1994) and sunflower (Sgherri and Navari-Izzo,1995). When the stress is removed, these enzyme and lipid peroxidation levels revert to the control level relatively

faster in tolerant varieties than that of the sensitive lines (Van Rensburg and Kruger, **1994;** Burke *et al.*, 1985).

Studies on drought tolerant and non-tolerant maize plants by Malan *et al.* **(1990) show that** there is a cross-tolerance to various oxidative stresses. The correlation between **the** membrane leakage measurements and changes in enzyme activities during stress suggested that membrane leakage measurement is enough to determine oxidative damage without quantification of the enzymes. Interestingly, drought and photoxidative herbicide tolerances were both significantly correlated with high levels of Cu/Zn SOD and GR activities along with membrane integrity. Further, their study revealed that SOD or GR alone didn't correlate with any of the tolerances.

Van Rensburg and Kruger (1994) studied effect of drought stress on lipid peroxidation levels and antioxidant enzymes in four different tobacco cultivars of known drought tolerance. It was reported that GR and APX activities significantly increased; tolerant varieties accumulated high levels of GR, SOD and APX. Ascorbate peroxidase activity levels were 300-400% higher in the drought tolerant cultivars under stress and interestingly the increase in APX was more pronounced than that of catalase. This indicates that APX, not catalase,might be mainly responsible for scavenging drought stress produced H_2O_2.

The changes in antioxidant enzymes were analyzed in sunflower and pea subjected to various levels of water stress (Sgherri and Navari- Izzo, 1995; Iturbe-Ormaetxe *et al.,* 1998). In sunflower, enzymes related to the ascorbate/glutathione cycle were induced at moderate level of water stress. At severe water deficit, a discernable decrease in efficiency of defense mechanism was reported. In pea plants, increase in lipid peroxidation and the SOD activities of mitochondrial, cytosolic and Chloroplast were observed at moderate water deficit (-1.3 MPa). At severe water deficit (-1.9MPa), however, the antioxidant enzyme levels decrease. Moderate stress had little effect on APX and GR enzyme activity in maize (Brown *et al.,* 1995).

Saruyama and Tanida (1995) have analyzed chilling tolerance and antioxidant enzyme activities at germination and leaf stage in rice. Increase in APX activity at the germination stage showed chilling tolerance in both cultivars, but rewarming enhanced

the activities effectively in tolerant cultivar D.shali but not in sensitive K-sen4. Pea chloroplasts derived from salt tolerant lines undergoing salt stress showed an increase in Cu/Zn SOD II, APX, ascorbate content and more no of plastoglobuli. Olmos *et al.*,(1994) observed that in salt tolerant pea calli there was induction of Cu/Zn SOD isozymes which were not in sensitive calli. During cold hardening, a specific GR isozyme is induced in the tolerant red spruce plants (Hausladen and Alscher, 1994). Non-hardened plants have another specific GR isozyme that was absent in cold acclimated plants.

Zhu and Scandalios (1994) demonstrated the connection between the action of ABA and oxidative stress during seed maturation in maize. The Mn SOD gene family responded differentially to exogenous ABA and high osmoticum in developing maize embryos. The ABA is necessary for the accumulation of MnSOD transcript in response to a high osmoticum in wild type and mutant embryo of an ABA-deficient line. Although *Sod 3* transcripts increased in response to ABA in maize embryos, there is no corresponding increase in SOD-3 protein levels and enzyme activities remain same with or without the treatment of ABA. Interestingly, studies on the regulation of cytosolic Cu/Zn SOD promoters namely *Sod Cc1* and *Sod Cc2* with *GUS* gene expressed transiently in rice protoplasts showed differential response to ABA. These studies revealed that ABA has no effect on *SodCc1-GUS* expression while it induces the expression of the *SodCc2- GUS.* Further, these two promoters were stimulated by DTT. However, ABA and GA3 are applied simultaneously, induction effect of ABA was abolished (Sakamoto *et al.,* 1995). The differential expression of two similar genes indicates that they play different roles in the stress response and that different mechanisms are involved in *SodCc* expression in rice under environmental stress conditions.

Recently, manipulation of expression of the enzymes involved in scavenging ROIs by gene transfer technology has provided new insights into their relative contributions to oxidative stress tolerance (Foyer *et al.,* 1994). Transgenic tobacco plants with overexpressed Chloroplast targeted mitochondrial Mn SOD (Bowler *et al.,* 1991) and Chloroplast localized pea Cu/Zn SOD (Sengupta *et al.,* 1993), had greater resistance to photo-oxidative damage and to methyl viologen mediated oxidative stress than the

controls. In contrast transgenic tobacco plants that overexpressed high level of chloroplastic Cu/Zn SOD from *Petunia hybrida* did not show any detectable increase in resistance to methyl viologen (Tepperman and Dinsmuir, 1990) or ozone (Pitcher *et al.*, 1991). The constitutive overexpression of pea cytosolic Cu/Zn SOD in the cytosol of transgenic tobacco plants resulted in to an increased tolerance to ozone (Pitcher and Zilinskas, 1996). These discrepancies indicate that factors other than SOD overexpression might be involved in the establishment of an enhanced oxidative stress resistance in transgenic plants.

3. MATERIALS AND METHODS

Materials and Methods

3.1 Materials

3.1.1 Chemicals:

Tris, Acryiamidc, Bis-Acrylamide, P-mercapto ethanol, SDS, PMSF, ethanol, methanol, Glacial acetic acid, Coomassie brilliant bllue, TEMED, Ammonium per sulphate, Glycine, Sodium hypochlorite, hydrogen peroxide, L-Ascorbic acid, Isopropanol,Agar and Yeast extract are obtained from local companies. Radioactive $^{32}P\alpha dCTP$ is obtained from BARC, INDIA., Agarose, MOPS, EDTA, EGTA, Ethidium bromide, Riboflavin, PMSF, HRPO anti rabbit IgG conjugate, 4-Chloro-1-Napthol, Ampicillin, DL-Glyceraldehyde are obtaind from Sigma, USA, Nitroblue tetrazolium salt, NADPH, resriction enzymes, Alkaline phosphate anti rabbit IgG conjugate and BCIP were obtained from Boehringer Mannehim, Germany.

Random primer labelling kit is from Stratagene andHybond N+ membrane is purchased from Amersham,UK

3.1.2 Plant Material :

Rice lines used in the present study include both cultivars and land races. Annada, Hamsa, Tulsi, Prasanna are well known cultivars belonging to *indica* subspecies which were originally obtained from DRR, Hyderabad. The plants were grown under the green house and field conditions and selfed repeatedly. Selfed progeny seeds and seedlings were used in analysis. Hamsa is a known drought sensitive line while other lines exhibit a varying degree of tolerance. K39 is a cold tolerant cultivar grown in North Eastern regions of India. Other cereals used in the study include *Zea mays* (Ganga 10) Oats, *Eleucine coracana* (Var), *Hordeum vulgare, Sorghum bicolar, Pennisitum typhoidieum.*

3.2 Methods

3.2.1 Plant material and stress treatment:

Seeds were surface sterilized with 5% sodium hypochlorite (NaOCl) for 5 minutes and rinsed with water for several times. They were grown on rough filter papers in see-through germination boxes at room temperature in dark for protein analysis. The shoots and roots from treated and control samples were harvested separately and were quick frozen in liquid nitrogen and stored at -70^0C until use.

For enzymatic studies plants were grown in growth chambers with 12 hr light/ 12 hr dark cycle at 30 ± 2 °C. Stress treatment of the 8 day old seedlings were treated either with 20-40% poly ethylene glycol (PEG), 100 µM ABA or kept in cold at 4°C. Control seedlings received water only, at RT. The seedlings were harvested at regular intervals and quick frozen in liquid nitrogen or immediately processed.

3.2.2 Growth measurements:

For R/S growth analysis, the lengthy shoots and roots of both control and stressed seedlings of all the mentioned genotypes were measured in cms. The root/shoot ratios were calculated for all treatments and rice cultivars, using normal statistical methods.

3.2.3 Dehydration treatment of mature plants:

45 day old net house grown rice plants in pots were subjected to dehydration by withholding water supply. The Relative Water Content (RWC) of leaves was measured at the onset of leaf rolling and the tissue was collected at that stage for enzymatic analysis.

3.2.4 Biochemical methods:

3.2.4.1 Determination of proline :

Solutions : 1. 3 % sulphosalicyclic acid . 2. Toluene

3. Acid ninhydrin agent: 1.25 gms of ninhydrin in 50 ml of solution made of 30 ml glacial acetic acid, 8 ml of orthophosphoric acid and 12 ml distilled water. This solution was stored in a dark bottle.

Proline content was estimated following **the method** of Bates ***et al*** **(1973). One** gram of the plant tissue was extracted with 20 ml of 3 % sulphosalicyclic **acid in a** clean mortar. The homogenate was centrifuged and **supernatant** was collected. To 2 ml of **the** supernatant, 2 ml of acid ninhydrin reagent was added, mixed and kept in **a** boiling water bath for 60 min. The reaction was terminated by snap cooling the test tubes **on** ice bath. The pink color developed during heating was trapped **into 4** ml of toluene whose absorbance was measured at 520 nm using the Du-64 Beckman spectrophotometer. Proline concentrations in the samples were calculated from a standard curve calibrated with different concentrations of proline. Proline quantity was expressed in terms of microgram of proline per gram fresh weight tissue.

3.2.4.2 Total Protein extraction:

Total proteins were extracted according to Goday *et al*(1988) with few modifications. The tissue was finely ground in liquid nitrogen and extracted with extraction buffer (gm/2 ml) containing 0.25M Tris-HCl (pH 8.0), 0.4% SDS, 20 mM EDTA, 2 mM PMSF and 5% mercapto ethanol by mixing and boiling for 5 min. The extract was centrifuged at 12,000g for 10 min, and proteins in the supernatant were precipitated with 15% TCA at 4°C. The pellet was washed with 80% acetone thrice. The resulting pellet was dissolved in Tris buffer (pH.8.0) and used for protein estimation.

3.2.4.3 Protein estimation: The Lowry method (1951) and Bradfords method (1976) were used for the protein estimation

3.2.4.4 SDS-PAGE:

SDS-PAGE was performed according to Laemmeli *et al,* (1970). 50-100 μg of extracted proteins of control and treated samples were fractionated by preparative 15% SDS-PAGE. The separating and stacking gel compisitions is as follows. Separaring gel solution (30 ml) contains 16 ml of 28% acrylamide solution, 4.5 ml of 3M Tris buffer (pH 8.9), 3 ml of 1% SDS, 6.45 ml of distilled water, 100 μl of ammonium per sulphate

(10% APS) and 30 µl of TEMED. Stacking gel solution (5 ml) contains 0.9 ml acrylamide (28%), 2 ml of 0.5M Tris buffer (pH 6.7). 0.5 ml of 1% SDS, 0.6 ml of water, 40 µl of 10% APS and 10 µl of TEMED. Electrophoresis was carried at 150V after which the gels were stained with Coomassie blue. Gels were destained with a solution containing 7.5% methanol and 7 % glacial acetic acid. From the gels the induced 15 and 23 kDa (Rao *et al,* 1993) polypeptides were identified by comparison with that of the control sample, and the band was cut out and electroeluted, using BIORAD electro elution apparatus following the manufacture instructions.The purity of the eluted band was tested on SDS- PAGE followed by HPLC in Shim Pak Columns. Such purified 15 kDa protein was used to raise polyclonal antibodies in rabbit.The 15 kDa antibodies thus served to analyse rice seedlings.

3.2.4.5 Western Blotting:

Solutions: 1. Electroblotting buffer: 25 mM Tris-HCL (pH 8.3), 100 mM glycine and 20% methanol. 2. 0.2% Ponceau-S in -HC1 (pH.7.6) and 150 mM NaCl.

4. Blocking solution: 5% low fat milk 3% TCA.

3. Tris buffer saline (TBS) : 50 mM Tris powder in TBS.

5. Primary antibodies : Antibodies raised against the 15 and 23 kDa PEG-mediated water stress-induced proteins and anti aldose reductase antibodies were diluted to an appropriate dilution factor as it is required.

6. Secondary antibody: HRPO goat anti-rabbit IgG conjugate or AP goat anti rabbit IgG conjugate are purchased commercially and used according to the manufacturer's specifications.

7. 4-Chloro -1-naphthol (200mg/ 1ml) dissolved in methanol.

8. AP buffer : 16 mM Tris-HCl (pH.9.5), 4 mM NaCl and 0.2 mM $MgCl_2$

9. NBT: 50 mg of NBT was dissolved in 700 µl of diethyl formamide and 300 µl of AP buffer

10. BCIP: 50 mg of 5-Bromo- 4-Chloro-3- Indolyl Phosphate (BCIP) dissolved in 1 ml of diethyl formamide.

The SDS-PAGE gels were electrophoretically blotted onto nitro-cellulose filters with the electro blotting buffer. The protein transfer was performed overnight at a constant voltage of 30V at 4°C. The membranes were stained with Ponceau-S solution and the M.Wt. standards were marked. The nitro-cellulose blots were probed with the anti-15 and 23 kDa antibodies. Detection was done using either Horseraddish peroxidase (HRPO) or Alkaline Phosphate (AP) conjugates. Detection of HRPO anti IgG conjugate are achieved by incubating the filters in the presence of the precepitable substrate, 4-chloro-1-naphthol and hydrogen peroxide while AP anti-rabbit IgG was visualized using precipitable substrates NBT and BCIP.

3.2.4.5.1 Procedure:

1. The filter was incubated in blocking solution for 60 min to block non specific sites at room temp.
2. The filter was probed with primary antibodies suitably diluted with blocking solution based on their titer value for 60 min..
3. The non specifically bound primary antibodies were removed by washing with blocking solution for three times (10 min each).
4. The blot was treated with secondary antibodies, either HRPO or AP anti rabbit IgG conjugate (diluted according to the specifications) for 60 min.
5. The non specifically bound secondary antibodies were removed by washing with TBS solution for three times (10 min each). The bands were detected by using color reaction and photographed immediately.

When Alkaline phospatase conjugate was used as secondary antibody it was visualized by treating with a color developing solution containing 60 µl of NBT and 33 µl of BCIP in 10 ml of AP buffer. Reactive areas turn purple within 1 to 15 min. The reaction was stopped by rinsing the blot with water

For visualisation of bands in the membrane when horseradish peroixdase was used as secondary antibody, the blot was treated with 200 µl of 4-chlor-1-naphthol (200 mg/ml) diluted with 2 ml of methanol. 8 ml of electroblotting buffer. The colour reaction

was started by adding 25 µl H_2O_2. The primary and secondary antibody complex was visualized by the development of purple color, after which the reaction was terminated by rinsing with water.

3.2.4.6 Preparation of microsomal membranes:

All the operations for preparation of microsomal membrane fractions were carried at 4^{0}C according to the protocol of Basu *et al,* (1994). Fresh tissue (1gr) was ground with a mortar and pestle in an ice cold homogenization medium (1:1) consisting of 50 mM MOPS, 2 µM PMSF, 1 mM DTT, and 1 mM EDTA (pH 6.5). The homogenate was then filtered through two layers of cheesecloth at 20,000g for 15 min in a Hitachi centrifuge. The resulting supernatant was collected and centrifuged at l,20,000g for 45 min in a Beckman Ultracentrifüge with the SW 40 swing out rotor. The supernatant represents the cytoplasmic fraction. The microsomal fraction (pellet) was then suspended in 50 µl of suspension buffer containing 50 mM MOPS, pH 6.5, 1 mM EDTA, 1 mM DTT and 1 µM PMSF and thoroughly homogenized with glass homogeniser. Aliquots of membrane and cytoplasmic fractions were stored at -70°C for further analysis. Protein content was determined by Lowry *et al* (1951) with BSA as the standard. Microsomal membrane samples (80-100µg) were mixed with the sample buffer (0.125M Tris-HCl, 4% W/V SDS, 20% (V/V) glycerol, 10 V/V 2-mercapto ethanol) and incubated at 70°C for 20 min and loaded onto the 15 % polyacrylamide gel.

3.2.4.7 Extraction of boiling stable proteins:

Extraction of boiling stable proteins was done according to the protocol of Robertson *et al,* (1994)*:* Control and treated shoot tissues were taken and were ground to a fine powder in liquid nitrogen and extracted with the extraction buffer (3g /6 ml) containing 62.5 mM Tris, pH 6.8 and with PMSF (1 mM). The suspension was centrifuged at 13000 rpm for 20 min. The supernatant fractions were kept at 90^{0}C for 30 min and centrifuged to pellet the heat coagulated protein fraction. Proteins remaining in the supernatant were stored at -70^{0}C for electrophoretic analysis or precipitated with

acetone. The heat stable fractions were used in heat induced coagulation protection assays.

3.2.4.8 Heat induced coagulation protection assays:

Spectophotometric measurements for heat induced coagulation were initiated at **600** nm at 50^0C according to the protocol followed by Robertson *et* ***al.,*** 1994. **The temperatures** of the cuvetts was controlled by **a** circulating temperature bath connected to **a** peltier temperature controlling accessory in a Hitachi spectrophotometer. The sample cuvette with 62.5 mM Tris buffer (pH 6.8) were preequilibrated to the desired temperature (50°C). **The** increase in absorbance at 600 nm is used for turbidity measurement. In protection assay 400 μg of fractionated heat stable protein from control and stressed samples were added to 2 mg of total soluble proteins extracted from untreated shoots obtained from the 13000 rpm supernatant fraction and increase in the O.D values were measured continuously for 20 min.

3.2.4.9 Isolation of total proteins from immature seeds :

Rice plants were grown in pots and panicles were labelled at different stages. The panicles were collected on different days after pollination (DAP), quick frozen in liquid nitrogen and used for protein extraction. The total proteins from immature seeds, embryos and germinating seeds were isolated as previously mentioned.

3.2.4.10 Partial purification of Aldose Reductase activity:

Partial purification of AR was performed according the protocol described in Bartels *et al* (1991). The protocol is as follows. One gram of fresh shoot tissue was finely ground in liquid nitrogen and extracted with 10 ml of extraction buffer containing 20 mM potassium phosphate buffer, pH 7.0, 5 mM β-mercaptoethanol and 0.5 mM EDTA. The homogenate was subjected to centrifugation at 12000g for 20 min. The supernatant was collected and saturated with 40% ammonium sulphate and recentrifuged. The supernatant was further raised to 80% ammonium sulphate saturation. The proteins thus precipitated

were dissolved in the small fraction of extraction buffer, dialysed over night at 4°C in the same extraction buffer, Aliquots of which were used for enzyme activity.

3.2.5 Enzyme Assays

3.2.5.1 Aldose reductase activity:

Aldose reductase activity was photometrically determined by measuring the decrease in the concentration of NADPH at 340 nm for 5 min at room temperature (Bartels *et al*, 1991). Assay mixture contained 100 mM sodium phosphate buffer pH 6.9 and 0.15 mM NADPH with 25 mM DL- glyceraldehyde (Sigma) as substrate. One unit of enzyme activity was defined as the amount of enzyme catalysing the oxidation of 1 nmol of NADPH per min under the conditions mentioned above. The protein concentrations were determined by Bradford method (1976).

3.2.5.2 Antioxidant enzyme extraction:

Fresh leaves (200 mg) were homogenised with 2 ml of potassium phosphate buffer pH 7.0 containing, 1 mM EDTA and 2 % PVP. The homogenate was centrifuged for 10 min at 15,000 g (for Ascorbate peroxidase assay 1 mM of ascorbate was used in extraction buffer). The supernatant, after protein estimation is used as enzyme source for all enzymatic analysis, like superoxide dismutase, ascorbate peroxidase and glutathione reductase. All the steps involved in the preparation of the enzyme extract were carried out at 4^0C.

Native gel electrophoresis:

Non denaturing polyacrylamide gel electrophoresis was performed on 10 % acrylamide gels under non denaturing conditions, at 4° C, and then were used for activity staining of superoxide dismutase and ascorbate peroxidase. 75-125 ug of protein was loaded in each slot. The gels were washed with water and used for staining of enzymes superoxide dismutase and ascorbate peroxidase as required.

3.2.5.3 Ascorbate Peroxidase Assay:

Ascorbate peroxidase Activity was determined according to the protocol of Nakano and **Asada** (1981). The reaction mixture (1 ml) contained 50 mM potassium phosphate buffer, pH 7.0, 0.5 mM ascorbate and 0.1 mM hydrogen peroxide. Oxidation of ascorbate was followed by the decrease in the absorbance at 290 nm (2.8 mM-1 cm-1 molar extinction coefficient). One unit of ascorbate peroxidase activity is defined as the amount of enzyme that oxidises the one mole of ascorbate per minute at room temperature.

Activity staining of Ascorbate peroxidase enzyme :

The activity staining for APX enzyme was done based on the extent of inhibition of ascorbate dependent NBT reduction by the method of Mittler and Zilinskas (1993). 75-100 µg of the protein extract was loaded on to 10% native polyacrylamide gel with running buffer containing 2 mM ascorbate. The gel was incubated in the 50 mM sodium phosphate buffer,pH 7.0, containing 2 mM ascorbate for 30 min. The equilibration buffer was changed every 10 min. The gel was further incubated in phosphate buffer (pH 7.0) containing 4 mM ascorbate and 2 mM H_2O_2 for 20 min. (H_2O_2 was added just prior to incubation of the gel.) The gel was subsequently washed with phosphate buffer for 1 min and soaked in a sodium phosphate buffer (pH 7.8) containing 28 mM TEMED, and 2.45 mM of nitroblue tetrazolium salt (NBT) with gentle shaking. The APX protein bands observed as an achromatic bands on the purple blue back ground. The reaction was stopped by rinsing in water.

3.2.5.4 Superoxide Dismutase assay:

The activity of SOD was assayed based on the method of Beauchamp and Fridovich (1971), which was modified by Dhindsa and Matowc (1981) by measuring its ability to inhibit the photochemical reduction of nitroblue tetrazolium (NBT). The 3 ml reaction mixture contained 50 mM phosphate buffer pH 7.8, 13 mM methionine, 75µM NBT, 2µM riboflavin, 0.1 mM EDTA, and 0-50µl enzyme extract. Riboflavin was added last and the tubes were shaken and placed 30 cm below a light consisting of two 15W

fluorescent lamps. The reaction was started by switching on the lights and was allowed to run for 10 min, and was stopped by switching off the lights. The reaction mixture lacking enzyme developed the maximum color which decreases with increasing volume of enzyme extract added. The absorbance was read at 560 nm and activity was calculated according to the method of Ginnopolitis and Ries (1977).

Enzyme Quantification:

Beauchamp and Fridovich (1971) defined 1 unit of SOD as the amount that inhibits the NBT reduction by 50% and quantitated the enzyme on basis of the percent inhibition. Percent inhibition and SOD enzyme concentration are not linear. However, Asada *et al* (1974) using the crystalline spinach SOD and xanthine oxidase assay system established the relationship between SOD concentration and V/v ratio by following the equation:

$$V/v = 1 + K' [SOD]$$

Where V and v represents the rate of assay reaction in the absence and presence of the SOD enzyme respectively. Based on the above equation, the accurate determination of SOD activity as follows.

$$K'[SOD] = (V/v)-1$$

At 50% inhibition of the reaction the product K'[SOD] is equal to unity, then SOD activity can be determined directly from the V/v ratio according to the equation

SOD units/ml = [(V/v)-l] dilution factor

Activity staining of SOD enzyme: The activity staining was performed according to the procedure given by Beauchamp and Fridovich (1971) with minor modifications. The enzyme extract was applied to 1 mm and 10% acrylamide gels at concentrations of 50 to 100μg protein. Gels were first soaked in a staining solution consisting of 50 mM potasssium phosphate buffer, pH 7.8, 0.03 mM riboflavin, 0.326% (v/v) N-N-N'-N'-tetraethylene diamide (TEMED) and 1.25 mM of NBT in dark for 30 min. The stain was then removed and the gel was immersed in a solution containing potassium phosphate buffer (pH 7.8) and lmM EDTA and exposed to light source. SOD isozymes were

visualised as white bands on blue back ground. The stained gels were photographed using **a red** filter.

Identification of individual isozymes of Mn SOD and Cu/ZnSOD was done by **soaking the** gels in 5 mM H_2O_2 or 2 mM KCN prior to activity staining. **The** Mn SOD is resistent to both KCN and H_2O_2 while Cu/Zn SOD is sensitive to **the** both reagents. Fe SOD is sensitive to H_2O_2 and resistent to KCN (Fridovich, 1982).

3.2.5.5 Glutathione Reductase assay:

Glutathione Reductase assay was performed according to the protocol of Carlberg and Mannervik (1985) 3 ml of reaction mixture contained 100 mM phosphate buffer (pH 7.0), 1 mM GSSG, 1 mM EDTA, 0.1 mM NADPH and 25-50 µl of enzyme extract. The reaction was started by adding the enzyme extract. The oxidation of NADPH was followed by monitoring the decrease in absorbance at 340 nm with a spectrophotometer.

3.2.6 Molecular techniques:

3.2.6.1 Northern Blotting:

Isolation of Total RNA:

Total RNA was isolated from plant tissues according to the method of Weisshaar *et al* (1991). The harvested tissues were quick frozen in liquid nitrogen and stored at -70°C. The solution used for RNA preparation were autoclaved at 120 °C for 15 min. Non autoclavable solutions and equipment were made RNAase free by adding 0.1% diethyl pyrocarbonate (DEPC).

Lysis Buffer: 150 mM Tris (pH 9.0), 100 mM NaCl, 20 mM EGTA and 2% SDS.

1. The tissue was ground to a fine powder and transferred to a falcon tube.
2. 10 ml of lysis buffer and 5 ml phenol was added and mixed well for 10 min. To this 5 ml of chloroform was added, mixed for 10 min and subjected to centrifugation at 3000 rpm for 10 min at room temp.
3. The supernatant was re-extracted with phenol :chloroform for 2 times. Final extraction was carried only with chloroform.

4. The total nucleic acids are precipitated by adding 3M sodium acetate (1/10 volume) and ethanol (2.5 volumes) and kept at -70°C for 30 min and centrifuged at 10,000 rpm for 15 min at 4^0C.

5. The pellet was suspended in 5 ml of DEPC treated water. It was precipitated by adding the 5 ml of 5M LiCl. This was kept on ice overnight and centrifuged at 4500 rpm for 15 min at 4^0C. The pellet was resuspended in 1 ml of water and was precipitated with 3M sodium acetate and ethanol.

6. The RNA pellet was washed with 70% ethanol and air dried. The RNA pellet was dissolved in water and stored at -70^0C. The quality of RNA was checked on 1.2 % denatured agarose formaldehyde gels and quantity was estimated spectrophotometrically at 260 and 280 nm. The concentration of RNA was quantified as follows.

Conc. of RNA (µg/µl) = O.D at 260 x 40 x dilution factor. A solution of RNA absorbance at 260 nm that equals to 1.0 O.D contains approximately 40 µg/µl of RNA. A ratio of 2.0 for O.D 260/ O.D 280 is an indication of RNA purity.

Preparation of RNA sample for electrophoresis: The 30-40 µg of RNA (6 µl) was taken in 19 µ1 of denaturing buffer (containing 2.5 µl of 10 X MOPS, 4 µ1 of formamide and 12.5 µ1 of formaldehyde 37%) was added, kept at 65°C for 10 min and snap cooled on ice and 3 µ1 of loading dye was added.

Loading dye: 50% glycerol, 1 mM EDTA, 0.25% bromo phenol blue, 0.25% xylene cyanol

3.2.6.2 Northern Analysis:

Equal concentrations of total RNA was separated through 1.2 % agarose formaldehyde gel (each 100 ml solution containing 1.2 g of agarose, 10 ml of 10X MOPS, 73 ml of water and 17 ml of 37% of formaldehyde. Approximate molecular weights of RNA are determined from 18S (2366 bp) and 28S (6333 bp) rRNAs. After the electrophoresis the gel was incubated in the 0.05N NaOH for 15 min and washed with water for 5 min and was soaked in 2X SSC for 15 min. Total RNA was transferred to Hybond N+ membrane in the presence of 20X SSC. The filter was UV cross linked for 2

min and/or baked at 80^{0}C for 60 min. Prehybridization and hybridization were performed at 60°C for 3 hr and overnight respectively in 1M NaCl, 10% Denhardt's solution, *2%* SDS and 100 µg/ ml sheared denatured calf thymus DNA. Hybridization solution includes the radio labelled probe. The membranes were washed three times (10 min each) at 60 ^{0}C in 2X SSC, 0.1% SDS. Final wash was done with 1X SSC and 0.1% SDS. The membranes were exposed to X-ray films with intensifying screens and kept at -70°C.

Denhardt's solution 50X: 1% BSA, 1% ficoll, 1% PVP

10X MOPS solution: 200 mM MOPS, 100 mM sodium acetate, 10 mM EDTA (pH 8.0)

3.2.6.3 Random-primer labelling:

The APX cDNA fragment from *Arabidopsis* was used as primer for preparation of radiolabelled probe by random primer labelling using Stratagene kit. 24 µl of template cDNA (400 ng) denatured at 100^{0}C for 5 min and snap cooled on ice) with 10 µL of dNTPS for a-dCTP32 buffer, 50 µCi 32pα-dCTP (3000 Ci/mM) and 3 µl of Klenow were added and incubated at 37^{0}C for 60 min. Sephadex G-25 spin column was run to remove unincorporated a-dCTP32. The radioactively labelled cDNA was denatured and used for probing.

3.2.6.4 Transformation of plasmid DNA:

1-10 ng of plasmid DNA carrying APX cDNA was added to 50 µl of competent cells and given heat shock at 42°C for 2 min. The cells were recovered by incubating them at 37°C for 60 min without antibiotic. 100 µl of transformed cells were plated on LB agar plates ampicillin (50µg/ml) for the selection of tranformed cells (Sambrook *et al*, 1989).

3.2.6.5 Miniprep of plasmid DNA:

The overnight grown bacterial culture (1.5 ml) was harvested by centrifugation. of the bacterial pellet 200 µl of TELT buffer (50 mM Tris pH 8.0, 2.5 M LiCl, 62.5 mM EDTA pH 8.0 and 0.4% Triton X--100 added later) and 20 µl of freshly prepared

lysozyme (10 mg/ml) were added. After through mixing the suspension was incubated at 95^{0}C for 3 min. The bacterial debris was removed by centrifugation at 15000 rpm for 20 min at 4^{0}C. The pellet was discarded with tooth pick. The plasmid DNA was recovered by adding 100 µl of ice cold isopropanol and incubated on ice for 15 min. **And centrifuged** at 12000 rpm for 20 min at 4°C. The pellet was washed with 70% ethanol dried and dissolved in appropriate volumes of TE buffer (10 mM Tris, pH 8.0 and lmM EDTA, pH 8.0).

3.2.6.6 Maxipep of plasmid DNA :

1.The over night grown 500 ml bacterial culture was centrifuged at 5000 rpm at 4°C for 10 min. The pellet was resuspended thoroughly in 15 ml solution 1 (50mM glucose, 25 mM Tris, pH 8.0, and 10 mM EDTA)

2.This suspenpension was alkali lyzed with freshly prepared 20 ml of solution II (0.2N NaoH and 1% SDS) and incubated on ice for 10 min. This was neutralized with 15 ml of solution III (5M potasium acetate, pH 4.8), mixed gently and incubated on ice for 10 min.

3. The supernatant was recovered by centrifugation at 12000 rpm at 4^{0}C for 30 min. To the supernatant 0.6 volumes of isopropanol was added and incubated at RT for 20 min and centrifuged at 4°C for 30 min. The pellet was recovered and dried and dissolved in 5 ml of TE buffer.

4. The RNA contamination was removed form this preparetion by adding equal volume of 5M LiCl, incubated on ice for 15 min and centrifuged at 4°C for 10 min. To the supernatant one volume of isopropanol was added and incubated on ice for 10 min. Plasmid DNA was recovered by centrifugation at 10000 rpm for 10 min. The pellet was air dried and dissolved in TE.

5. After RNAase (10 µg/ ml) treatment at 37°C for 60 min, the total protein contamination was removed with phenol-chloroform extractions. The final aqueeous phase was precipitated with 3M sodium acetate (1/10 volume) and 2.5 volumes of ice cold ethanol. Plasmid DNA was recovered by centrifugation at 10000 rpm at 4^{0}C for 20

min. The pellet was washed with 70% ethanol and air dried and dissolved in TE (Sambrook *et al,* 1989)

4. RESULTS

Results

The effect of PEG-mediated water stress and exogenous application of ABA on rapidly growing young seedlings of different indica rice lines differing in their relative drought tolerance has been investigated. The parameters studied are plant growth, proline content, protein profiles, and enzyme activity. Experiments include partial purification of stress induced proteins, determination of functional identity of a few prominently induced proteins and finally documentation of qualitative and quantitative changes in the activities of antioxidant enzymes under stress.

4.1 Effect of PEG and ABA on Plant growth:

The root and shoot growth ratios were analyzed in Prasanna, Tulasi, Annada and Hamsa seedlings during PEG mediated water stress and exogenous application of ABA. In general, seedlings grown under normal conditions showed a higher rate of shoot growth than that of root. On the contrary', seedlings subjected to water stress showed reduced shoot growth and increased root growth. Both etiolated and light grown seedlings revealed similar pattern where root growth was promoted and shoot growth was inhibited under water limiting conditions and by exogenous ABA (Fig 4.1.1 and 4.1.2). A significant increase in root/shoot growth ratios was observed in all rice cultivars during water stress (Fig. 4.1.3). We have analyzed the root/shoot ratios of Annada and Hamsa cultivars during different concentrations of PEG (6000) treatment (Fig. 4.1.4A and 4.1.4B) which offer different osmotic potential (Table 4.1). The root lengths increased to a maximum in both cultivars during 20% PEG mediated water stress with the increase more in Annada than Hamsa. 40% PEG treatment drastically reduced plant shoot growth of both cultivars. During 30% and 40% PEG stress the root/shoot ratios increased though to a less extent. Upon rehydration, 20% PEG treated seedlings recovered fully compared to 30% and 40% PEG treated ones. The root and shoot growth of both cultivars were **not significantly** affected by 10% PEG. It is known that under water stress, **the root growth** is

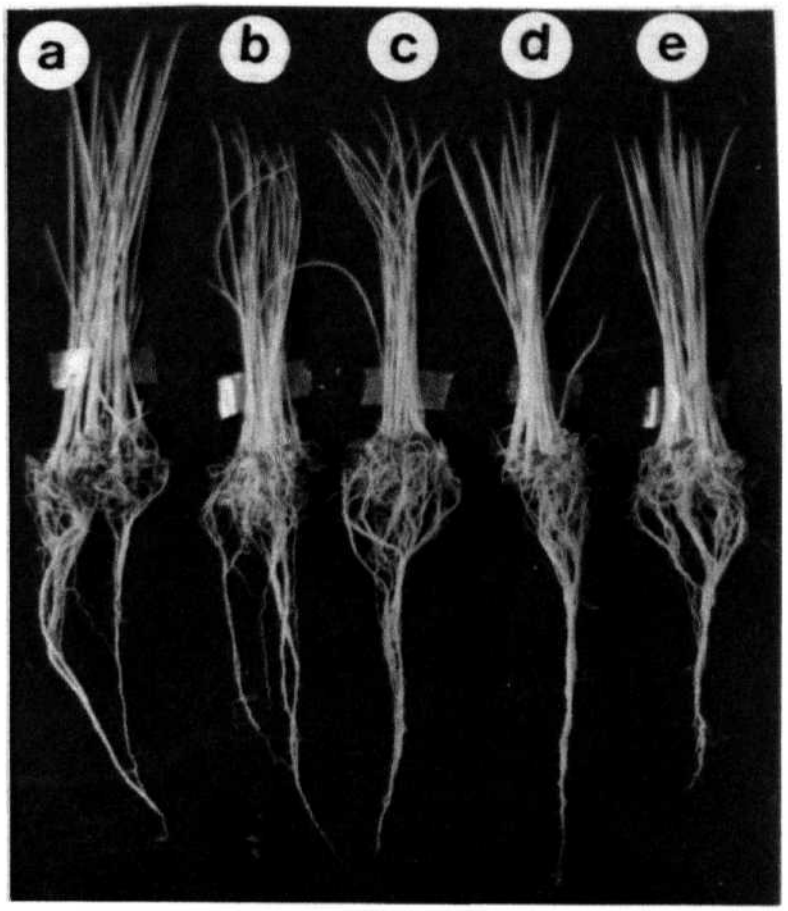


Fig. 4.1.1: Root and shoot growth of dark grown 7-day old seedlings exposed to different stress conditions for 3 days
a) Control b) PEG 40% c) PEG 20% d) ABA (100 μM) e) Cold

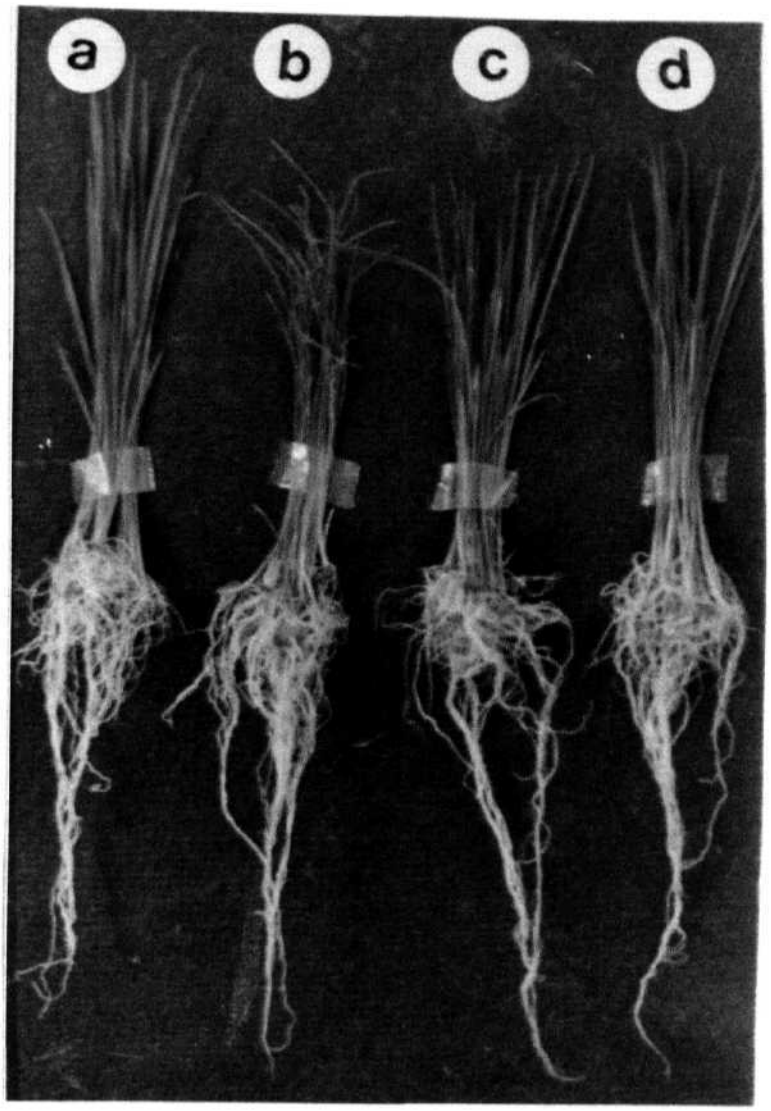


Fig. 4. 1. 2: Root and shoot growth of light grown 7-day old seedlings exposed to different stress conditions for 3 days
a) Control b) PEG 40% c) PEG **20%** d) ABA (100 μM)

Fig. 4.1.3 Root / Shoot growth ratios in seedlings in response to PEG and ABA stress

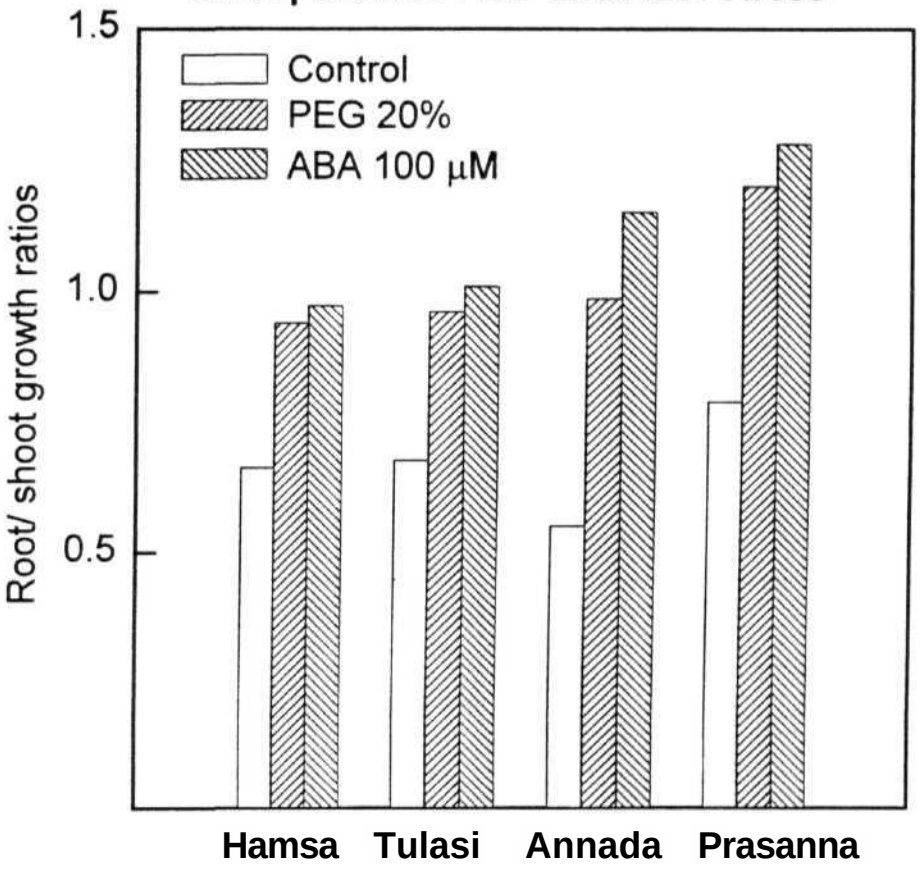


Fig. 4.1.4A Root/Shoot growth ratios of Annada seedlings during PEG mediated water stress

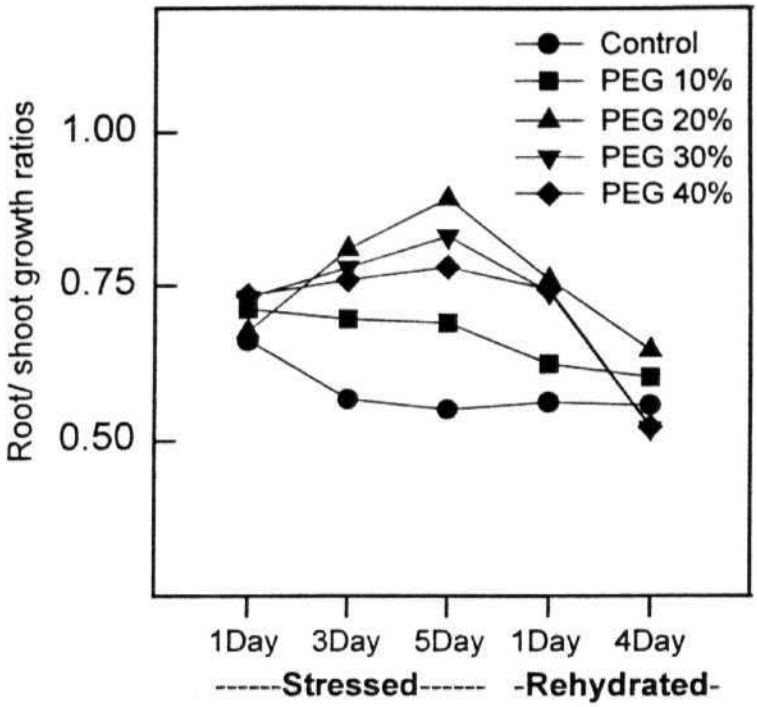


Fig. **4.1.4B** Root/Shoot growth ratios of Hamsa seedlings during PEG mediated water stress

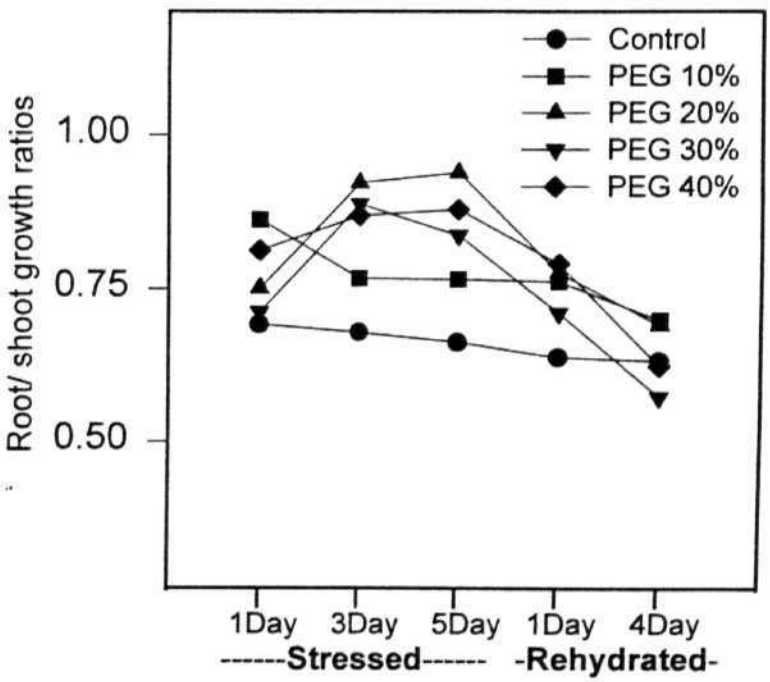


Table 4.1 The osmatic potential of different concentrations of Polyethylene Glycol (PEG) measured in a Vapour Pressure Osmometer

PEG (6000) Concentration	**Osmotic potential (M.Pa)**
5%	**-0.025**
10%	**-0.01**
15 %	**-0.02**
20%	**-0.55**
25%	**-0.82**
30%,	**-1.3**
35%	**-2.1**
40%	**-3.1**

higher than that of shoot (Meyer and Boyer, 1981; Creelman *et al.* 1990). The response of rice seedlings to PEG stress reflects the same. The tolerant rice lines have shown increased root growth than the sensitive cultivar. ABA treatment resulted in increased root/shoot lengths compared to PEG treatment. A significant reduction in the growth of vegetative tissues in mature rice plants was observed during dehydration processes (Fig. 4.1.5). Annada. a tolerant rice cultivar showed maximum relative water content when compared to other rice cultivars during dehydration while Hamsa showed least relative water content (Fig. 4.1.5).

4.2 Proline content of different rice seedlings during ABA and PEG stress:

Stress responsive accumulation of proline in rice seedlings is investigated and the results are given in Fig. 4.2.1 A and 4.2.1B. The basal levels of proline in tested cultivars did not show any significant difference. The seedlings stressed with PEG and ABA, however, showed significant increase in proline levels in general. Further, varieties differed in their proline levels under stress. Interestingly, the proline levels in ABA treated shoots are comparatively lower than that of roots. Moreover, tolerant rice lines accumulated higher levels of proline both in shoots and roots than the sensitive cultivar. Hamsa. Infact. tolerant cultivars showed about a two fold increase in roots and three fold increase in shoots. In general there is an increase in proline accumulation in seedlings under PEG-mediated water stress than ABA application.

4.3 Stress induced proteins in rice:

4.3. J Changes in seedling polypeptide profiles under water stress:

Total proteins were extracted from the etiolated rice seedlings of different genotypes grown under PEG stress and normal conditions. The changes in the protein profiles were analyzed on 15% SDS-PAGE. The protein patterns revealed both qualitative and quantitative changes under stress. A specific set of polypeptides of Mol. Wt. 45, 33, 23. 21, 18, 15 and 8 kDa were induced during PEG mediated water stress

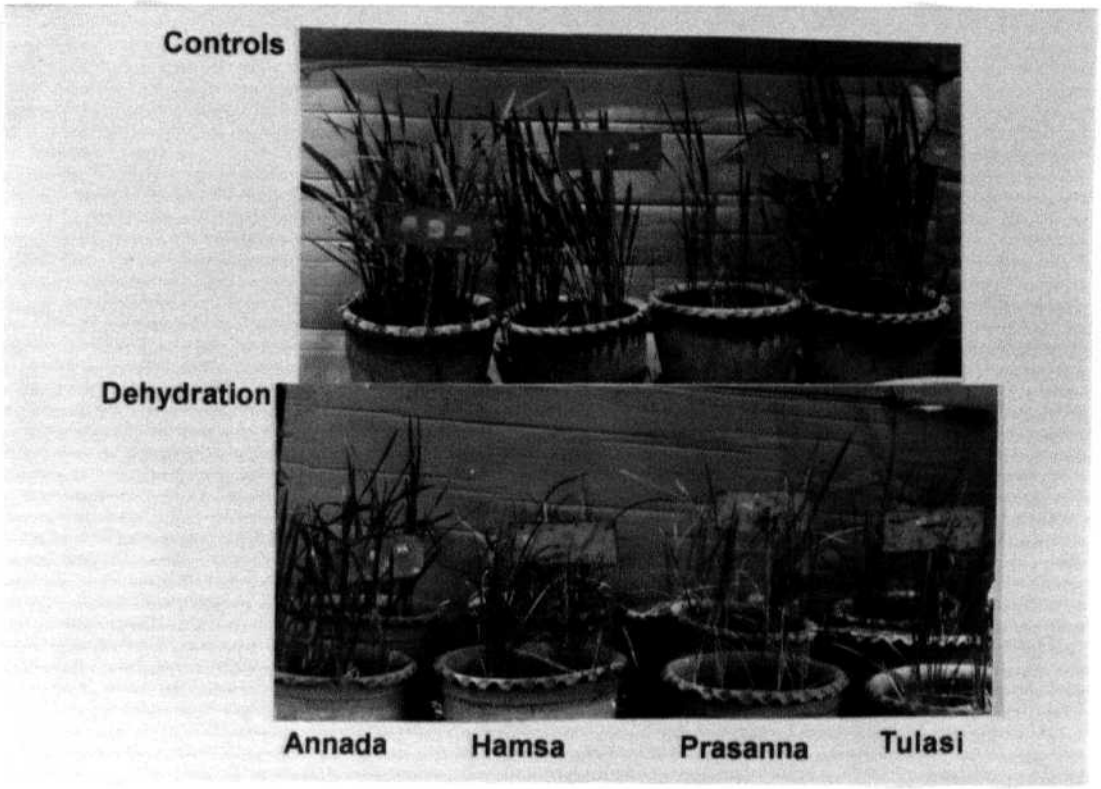


Fig. 4.1.5: Mature rice plants exposed to dehydration stress

Fig. 4.2.1A Root proline content in different seedlings in response to PEG and ABA stress

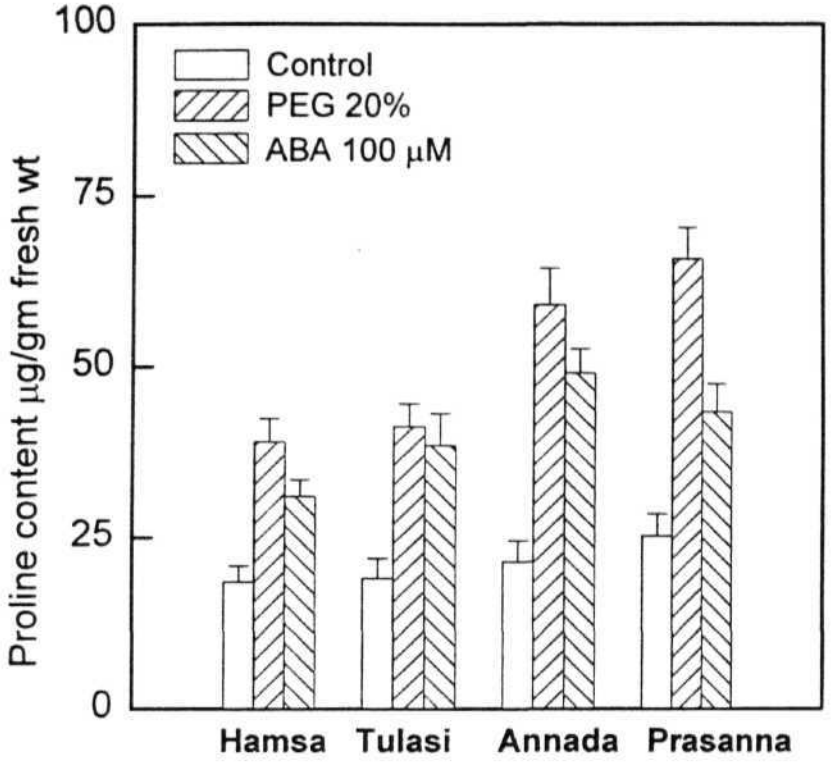
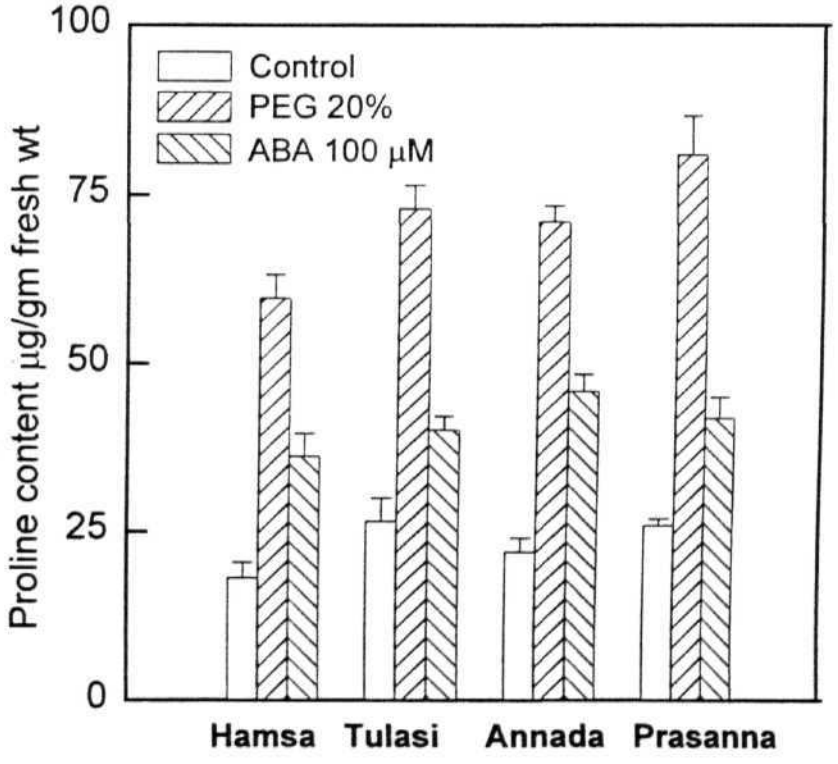


Fig. 4.2.1B Shoot proline content in different seedlings in response to PEG and ABA stress



(Fig. 4.3.1), while some proteins present in the control samples were either quantitatively reduced or disappeared on PEG stress. For instance, the 42-kDa protein disappeared upon stress (Fig. 4.3.1). The changes in protein profiles and nature of specific proteins induced by different stress factors were further analyzed.

4.3.2. Characterization of stress induced 15-kDa polypeptide:

Of the several stress-induced polypeptides, we have characterized the 15-kDa and 23-kDa and (Rao *et al,* 1993) prominently induced polypeptides during PEG mediated water stress and ABA application. The total proteins from control and stressed seedlings were separated on 15% preparative SDS-PAGE gels and the induced 15-kDa band was electro-eluted from acrylamide gel slices. The electro-eluted 15-kDa protein was tested for its purity on SDS-PAGE and HPLC. The results revealed a single band on SDS-PAGE and a single peak on HPLC (Fig. 4.3.2A and B). The purified 15-kDa protein was used to raise polyclonal antibodies in rabbit and furthur used for Western analysis.

4.3.3 Induction of 15-kDa protein by diverse stresses:

Several experiments were performed to check whether water stress or different stresses, such as ABA. cold and salt stress induce 15-kDa protein. The results from SDS-PAGE (Fig. 4.3.3A) show the induction of 15-kDa polypeptide by different stresses. To check the immunological identity of the induced 15-kDa polypeptide by different stresses, we have done the Western analysis using the antibodies raised against PEG induced 15-kDa polypeptide. The Western blot data (Fig. 4.3.3B) revealed that the stress induced 15-kDa polypeptide showed immunological cross reactivity with anti-15-kDa antibodies. This confirms that the 15-kDa induced by different stresses were immunologically similar.

The mature seed extracts were tested for the presence of the 15-kDa polypeptide and the results (lane 6 and 7 of the Fig.4.3.3B) reveal its total absence as determined by Western analysis. So this 15-kDa polypeptide is apparently different from the well-known LEA class of proteins. It is well known that LEA proteins accumulate during seed

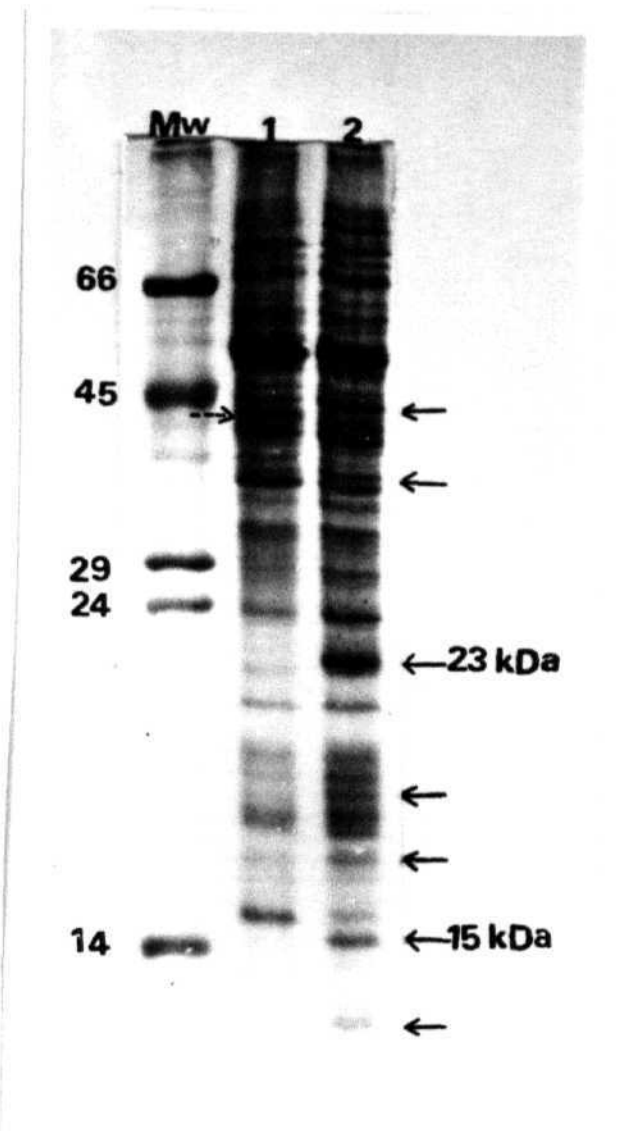


Fig. 4.3.1: SDS-PAGE of Hamsa shoot proteins

Lanes:Mw: mol.wt 1) Control 2) PEG treated
(75μg of protein was loaded onto each lane)
←Arrow represents the stress induced polypeptides
<---Arrow represents the disappearance of polypeptide

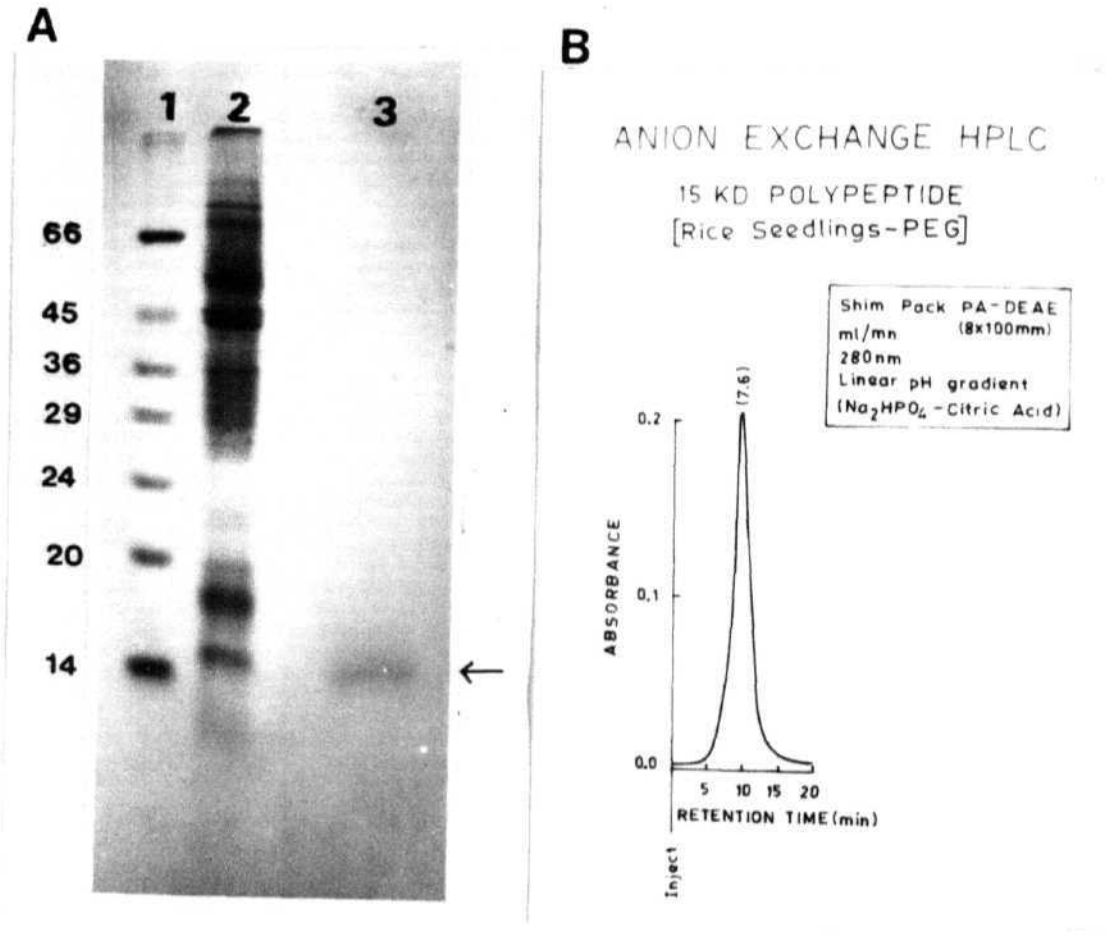


Fig. 4.3.2A: SDS-PAGE of purified PEG induced 15-kDa from Hama seedligs

Lanes: 1. Molecular weight markars; 2 Control total protein from shoot (50μg); 3. Purified polypeptide from the PEG treated shoot proteins (10μg)

Arrow represents the stress induced 15 kDa polypeptide

Fig. 4.3.2B: HPLC Profile of purified 15-kDa polypeptide

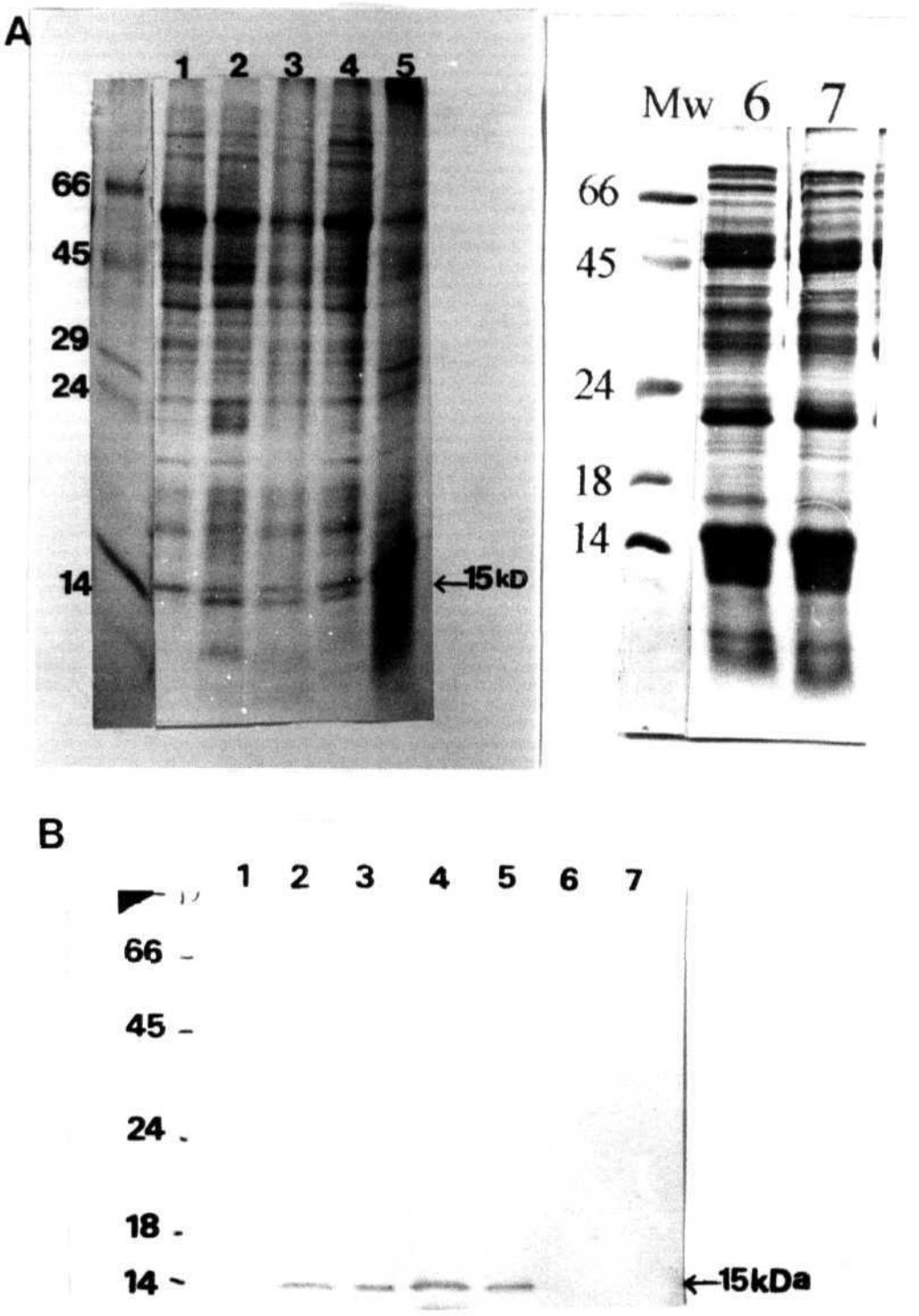


Fig. 4.3.3: SDS-PAGE (A) and Western blot (B) showing the induction of 15 kDa polypeptide by different stresses in Hamsa shoots

Lanes: 1) Control *2)* PEG 20% *3)* ABA (100 μM) *A)* Cold *5)* 200mM NaCl *6)* Hamsa seed *1)* K39 Seed (75μg of protein was loaded into each lane) Arrow represents the stress induced 15 kDa polypeptide

Note: Anti-15 kDa polyclonal antibodies raised against the purified PEG induced 15 kDa polypeptide were used for **Western analysis** (30μg of protein was loaded into each lane)

maturation in higher plants (Dure *et al,* 1989) and are induced in vegetative tissues of plants exposed to different stresses such as desiccation, salt, cold stress and application of exogenous ABA (Skriver and Mundy, 1990).

4.3.4 Accumulation of the 23-kDa polypeptide in mature seeds:

We have also tested for the presence of 23-kDa polypeptide both in mature seeds and embryos. The protein extracts from the mature rice seeds and embryos were fractionated on 15% gels, electro-blotted and probed with anti-23-kDa antibodies raised against PEG-induced 23-kDa polypeptide (Rao *et al,* 1993). PEG induced (lane 2 of Fig. 4.3.4) 23 and 45-kDa shoot proteins showed cross reactivity with anti-23kDa antibodies. The control shoot proteins (lane 1 of Fig. 4.3.4) did not cross react, thus indicating the induction of this polypeptide in rice shoots under PEG stress. The 23-kDa antibodies also showed cross reactivity with several seed and embryo ((lane 4 and 5) proteins, most prominent being 23 and 45-kDa proteins. Thus accumulation of this protein in mature seeds and embryos of rice confirms that this polypeptide belongs to the LEA group of proteins.

4.3.5 The induction pattern of 15-kDa polypeptide in germinating embryos under stress:

In order to test whether the 15-kDa polypeptide is accumulated, in response to stress, in other tissue as well, we analyzed the mature embryos subjected to different stresses-PEG (5%-10%), NaCl 100-200 mM, and ABA 50-100 uM and cold acclimation for various periods. The SDSPAGE profiles of seed extract samples are given in Fig. 4.3.5. The Western data from the gels probed with anti-15-kDa antibodies revealed immunological cross reactivity to 15-kDa from the germinating embryos under different stresses (Fig. 4.3.6). This induction pattern was same in stressed shoots as well. These results further suggest that the 15- kDa is a Generalized Stress Responsive Protein (GSRP)

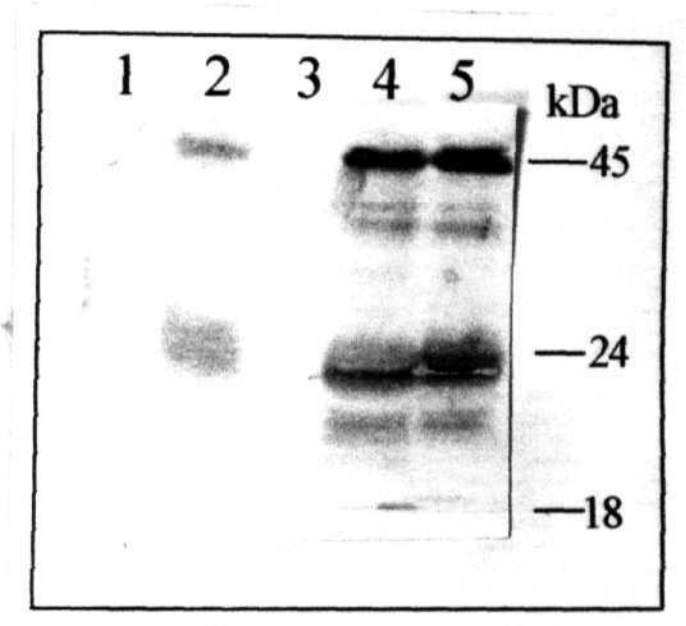


Fig. 4.3.4: Immnodetection of 23 kDa polypeptide in mature seeds and shoots of PEG treated Hamsa seedlings

Lanes: 1) Control shoot proteins *2)* PEG treated shoot proteins 3*)* Empty *A)* Mature Seed 5*)* Embryo (30μg of protein was loaded into each lane)

Note:Anti-23 kDa polyclonal antibodies raised against the purified PEG induced 23 kDa polypeptide were used for **western analysis**

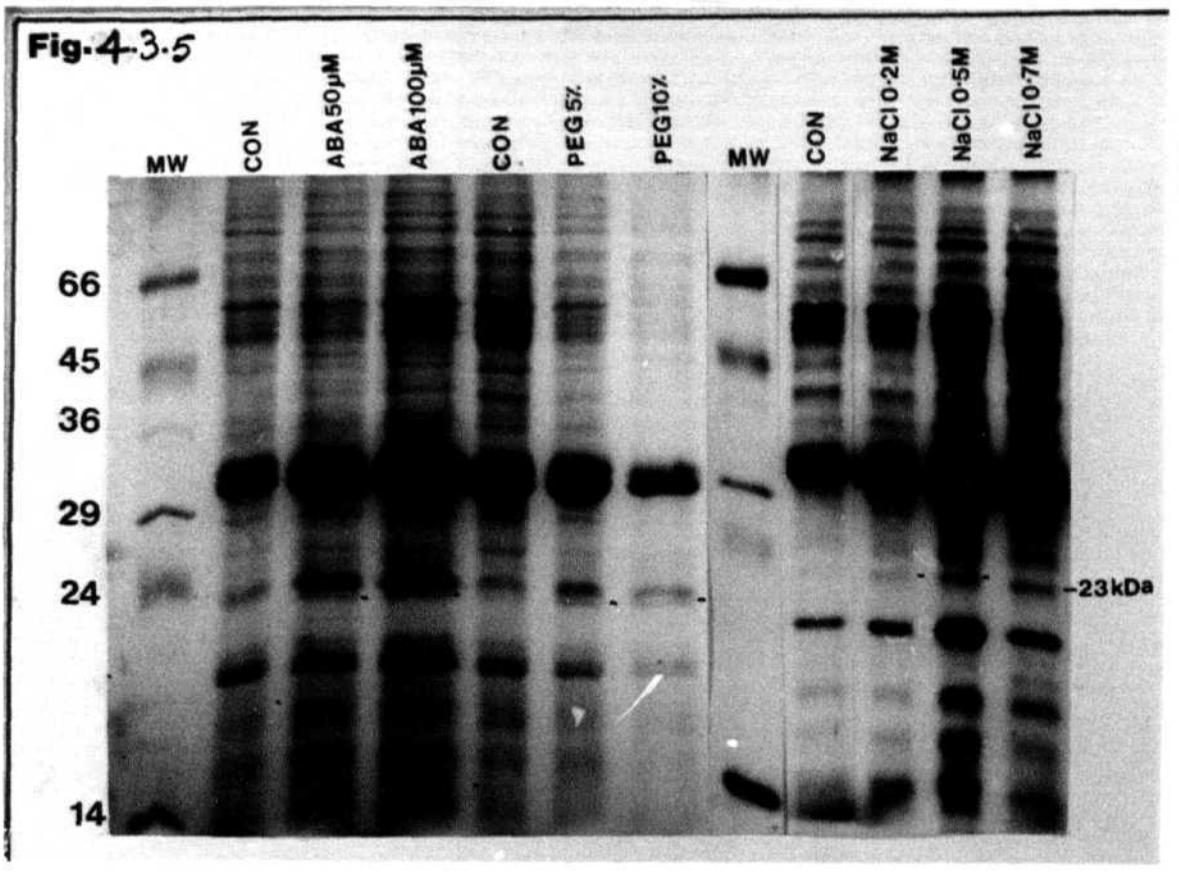


Fig. 4.3.5: SDS-PAGE of germinating embryos (72 hr imbibition) exposed different stress conditions
(75μg of protein was loaded into each lane)

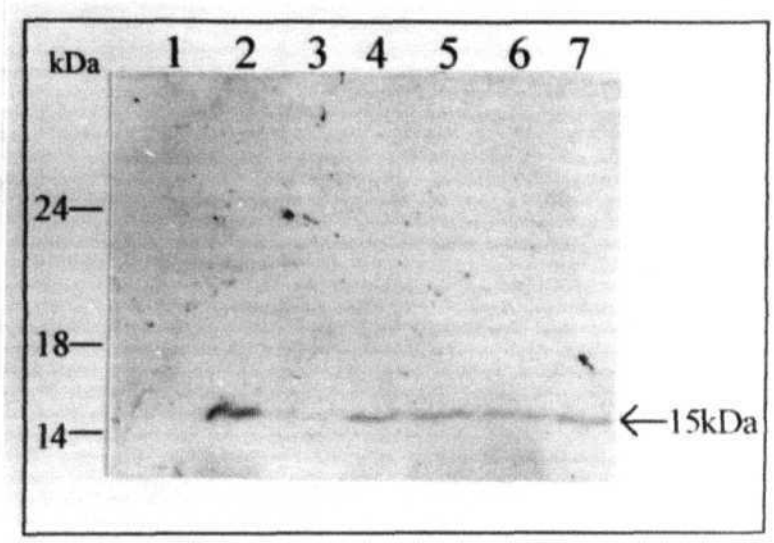


Fig.4.3.6: Immunodetection of the anti-15 kDa polypeptide in germinating embryos (72 hrs imbibition) exposed different stress conditions
Lanes: 1*)* Control shoot *2)* PEG shoots *3)* Control embryo *A)* PEG 10 % 5*)* ABA 50μM *6)* Cold *7)* NaCl (200mM) (30μg of protein was loaded into each lane). Arrow represents the stress induced polypeptide

*4.3.6 **Differential Induction of 23-kDa polypeptide** in germinating embryos under stress:*

Hamsa embryos were subjected to different stresses fractionated on SDS-PAGE (Fig. 4.3.5) and probed with anti-23-kDa antibodies. Cross reactivity of 23-kDa antibodies (Fig. 4.3.7) with protein extracts from germinating embryos under PEG. 200-700 mM ABA and salt stress was observed. However, there was no cross reactivity with proteins from cold stressed (Fig 4.3.7 lane 3 and 4) and control 3-day old germinating embryos grown under normal conditions (lane 2 and 5). We have already shown (Fig.4.3.4) the presence of 23-kDa polypeptide both in embryos and mature seeds, but not in 3-day old dark grown germinating embryos grown normally.

*4.3.7 **Immunological** cross-reactivity of rice anti-23-kDa antibodies with stress induced proteins from maize and Sorghum*

The protein extracts from maize and *Sorghum* seedlings exposed to ABA and PEG stress were probed with anti 23-kDa antibodies. The *Sorghum* proteins did not show any cross reactivity, while the 21-kDa maize protein cross-reacted with anti-23-kDa antibodies (Fig. 4.3.8). The PEG and ABA induced 23 and 45-kDa proteins from rice cross-reacted with anti-23 antisera (positive control).

*4.3.8 Localization of water stress induced **15-kDa** and 23-kDa polypeptides:*

Experiments were carried out to determine the intracellular distribution of stress induced 15 and 23-kDa polypeptides. The proteins from the membrane and cytosolic fractions from both control and PEG treated shoot tissues of Hamsa were separated on SDS-PAGE (Fig. 4.3.9). The 15 and 23-kDa polypeptides were identified in the PEG-treated total protein fraction as well as cytosolic fraction (lane 2 and 4 of Fig. 4.3.9). This cytosolic and membrane protein fractions were probed with anti 15 and 23-kDa antibodies independently which showed the presence of 15 and 23-kDa polypeptides in

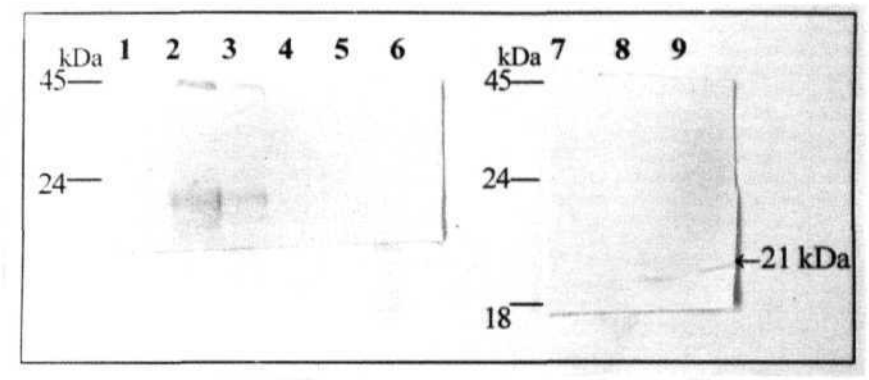


Fig. 4.3.8: Western blot showing the cross reactivty of anti-23 kDa antibodies with stress induced proteins in different cereal plants

Lanes: 1*)* Control rice *2)* ABA rice *3)* PEG rice 4) Control maize 5) ABA maize *6)* PEG maize *7)* Control *Sorghum 8)* ABA *Sorghum 9)* PEG *Sorghum* (30µg of protein was loaded onto each lane) Arrow represent the stress induced polypeptide

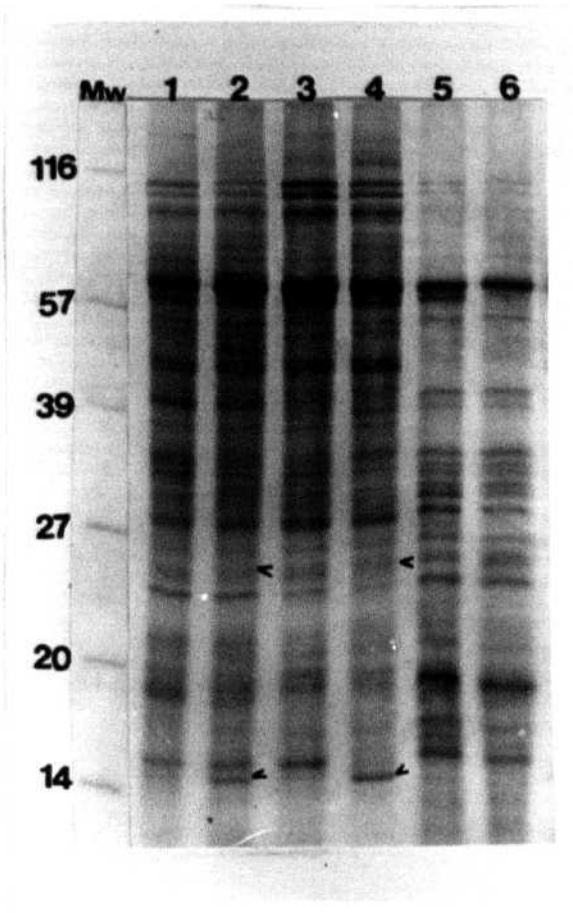


Fig.4.3.9: SDS-PAGE showing the localization of PEG induced polypeptides from Hamsa shoot extracts

Lanes: 1) Control total proteins *2)* PEG treated total proteins *3)* Control cytosolic fraction *A)* PEG treated cytosolic fraction *5)* Control membrane proteins *6)* PEG treated membrane proteins (75μg of protein was loaded onto each lane)

Arrow represent the stress induced polypeptides

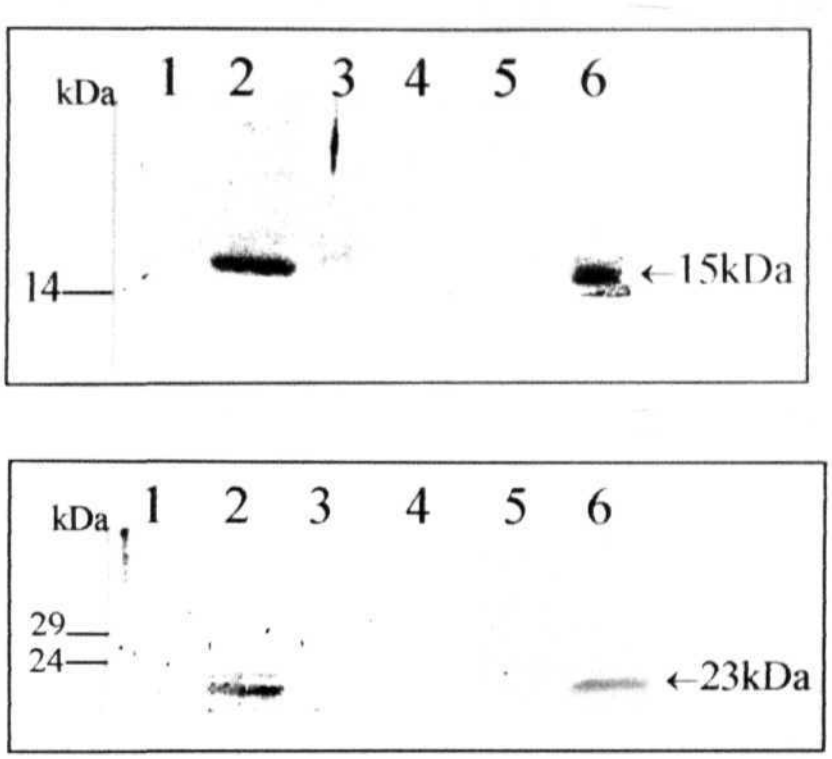


Fig: 4.3.10 Immunodetection of 15 and 23 kDa polypeptides in cytosolic fraction of PEG treated Hamsa shoots

Lanes: 1) Control cytosolic fraction 2) PEG treated cytosolic fraction 3) Control membranes proteins 4) PEG treated membrane proteins 5) Control total proteins 6) PEG treated total proteins

(50µg of protein was loaded onto each lane)

Arrow represents the stress induced polypeptide

the cytosol **but** not in the membranes fraction **(Fig. 4.3.10).**

4.3.9 Boiling stability of the stress induced proteins:

Earlier reports from our group showed that the PEG. ABA and salt stress **induced** 15 and 26 kDa polypeptides (Rao *et al.* 1993, Rani *et al,* 1994) are boiling stable. Such boiling stable or boiling soluble (physiologists* choice) proteins were isolated from the shoots of control and PEG treated seedlings (Fig. 4.3.11 A). Further, denaturation experiments were conducted to decipher the stabilizing function of stress induced proteins, particularly, the boiling stable protein fractions from stressed rice seedling.

Heat induced (50^{0}C for 20 min) coagulation and denaturation of crude protein samples from control seedlings were assayed spectrophotometrically at 6()0nm in presence of boiling stable protein fractions from control and PEG treated seedlings separately. The data reveal that the heat-induced coagulation of proteins in presence of boiling stable stress-induced proteins was considerably reduced and took longer time (about six minutes) to start denaturation in comparison with that of control fraction (Fig. 4.3.11B).

4.4 Search for stress-induced functionally important proteins/enzymes

Over the past few years, hundreds of stress responsive proteins were identified and characterized partially. Of these only few were fully characterized and sequenced. Further, only a small number of genes were identified each coding for such protein. Interestingly, the functional characterization of such protein was done only in rare cases. The functions were determined largely based on their deduced amino acid sequences. Our approach was to first identify the immunolgical similarities between induced proteins **and** functionally known plant proteins/enzymes.

4.4.1 An induced protein with immunological similarity to barley aldose reductase:

The partially purified protein extracts from roots, shoots and mature seeds were

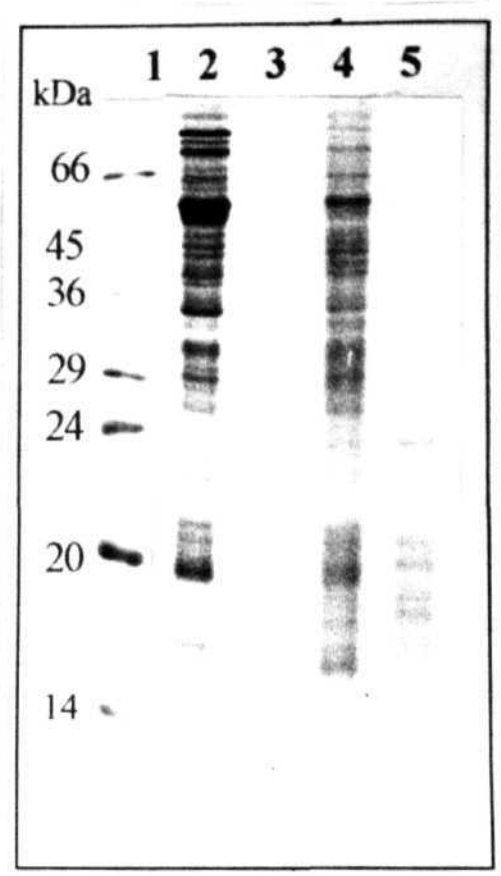
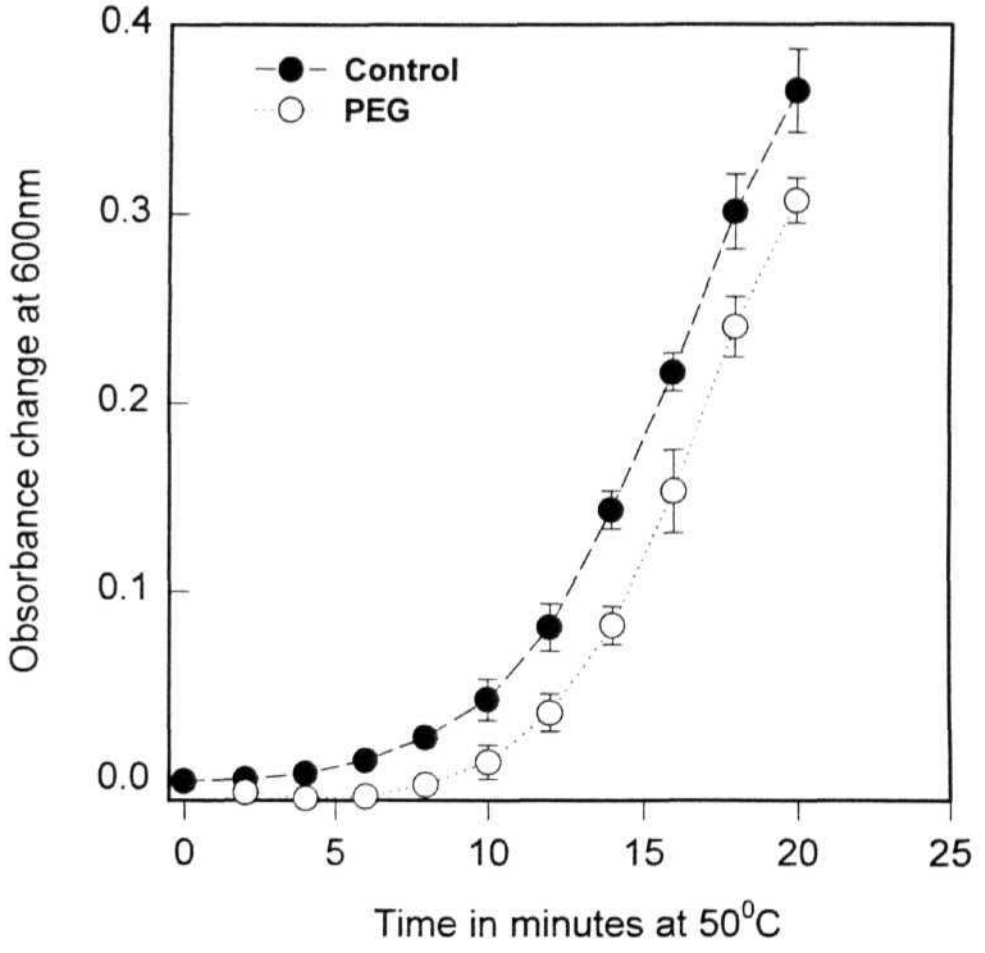


Fig:4.3.11A: SDS-PAGE showing boiling stable proteins from control and PEG treated Hamsa seedlings

Lanes: 1*)* M*)*Wt markers *2)* Control shoot protein 3*)* Control heat stable protein 4*)* PEG treated shoot protein *5)* PEG treated heat stable protein (30µg of protein was loaded into each lane)

Fig. 4.3.11B Protein coagulation in presence of boiling stable Control and PEG protein fractions



fractionated on SDS-PAGE and electro-blotted onto membranes. Membranes were probed with antibodies raised against aldose reductase like protein from barley embryos (Courtesy, Dorothea Bartels. Germany). The Western blot data clearly showed that the barley antibodies detected a 34-kDa protein only in the mature seeds of Hamsa and K39. In both cultivars, only one protein was detected. On the contrary, this protein could not be detected in shoots and roots of the seedlings grown under normal conditions (Fig. 4.4.1). These results suggest that, in rice, this protein accumulates during seed maturation and is probably associated with desiccation tolerance process.

4.4.2 Developmental profiles of aldose reductase related protein during embryogenesis and germination:

Since the AR-like protein was delected in mature seeds, an attempt was made to understand its developmental regulation during embryogenesis in rice. Proteins isolated from the immature seeds of K39 at different stages of maturation were fractionated on SDS-PAGE (Fig. 4.4.2A). The blots were probed with anti-AR antibodies. Western analysis revealed a stage specific accumulation of this protein, reminiscent of typical LEA pattern. It begins to appear at 15 DAP increases as the embryogenesis proceeds reaching the maximum at maturation (Fig 4.4.2A and B), coinciding with the rise in the internal concentrations of ABA and onset of desiccation tolerant phase during the embryogenesis (Skriver and Mundy, 1990).

We have performed, in parallel, aldosc reductase enzyme assays. The Aldose reductase activity in partially purified extracts from germinating seedlings was monitored using DL-gleceraldehyde as substrate, in the presence of NADPH and the results arc given in Fig. 4.4.3A. There was negligible activity when the fructose and glucose used as substrates. We could assay the activity only when DL-glyceraldehyde was used as substrate at pH. 6.9. It was reported in acidophilic and thermophilic red alga purified aldose reductase show a high affinity towards substrate DL-glyceraldehyde, followed by Xylose (Gross *et al,* 1997)

The AR like protein accumulated during late embryogenesis and was found to

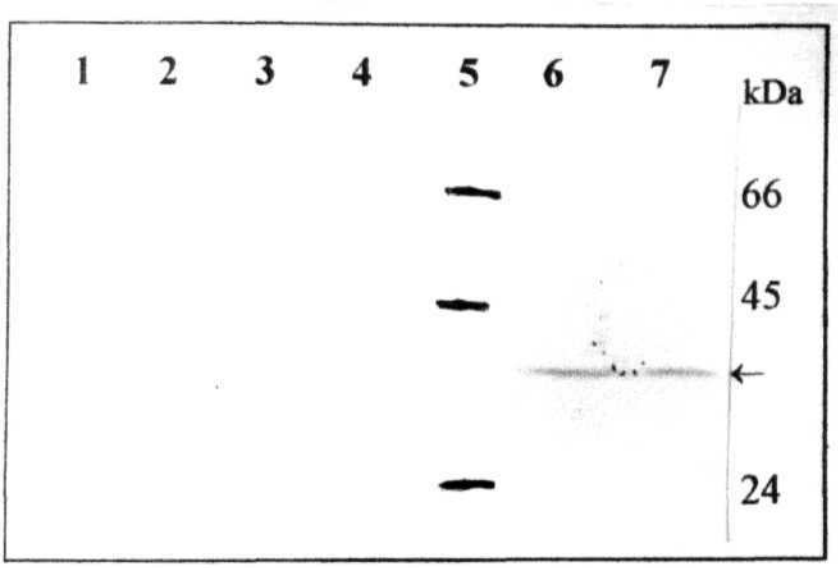


Fig. 4.4.1: Western blot showing aldose reductase like protein in mature rice seeds with pG22-69 antibodies raised against barley embryo protein

Lanes: 1) Hamsa shoot 2) K39 shoot 3) Hamsa root 4) K39 root 5) mol. wt markers 6) Hamsa seed 7) K39 seed

Arrow represents aldose reductase protein

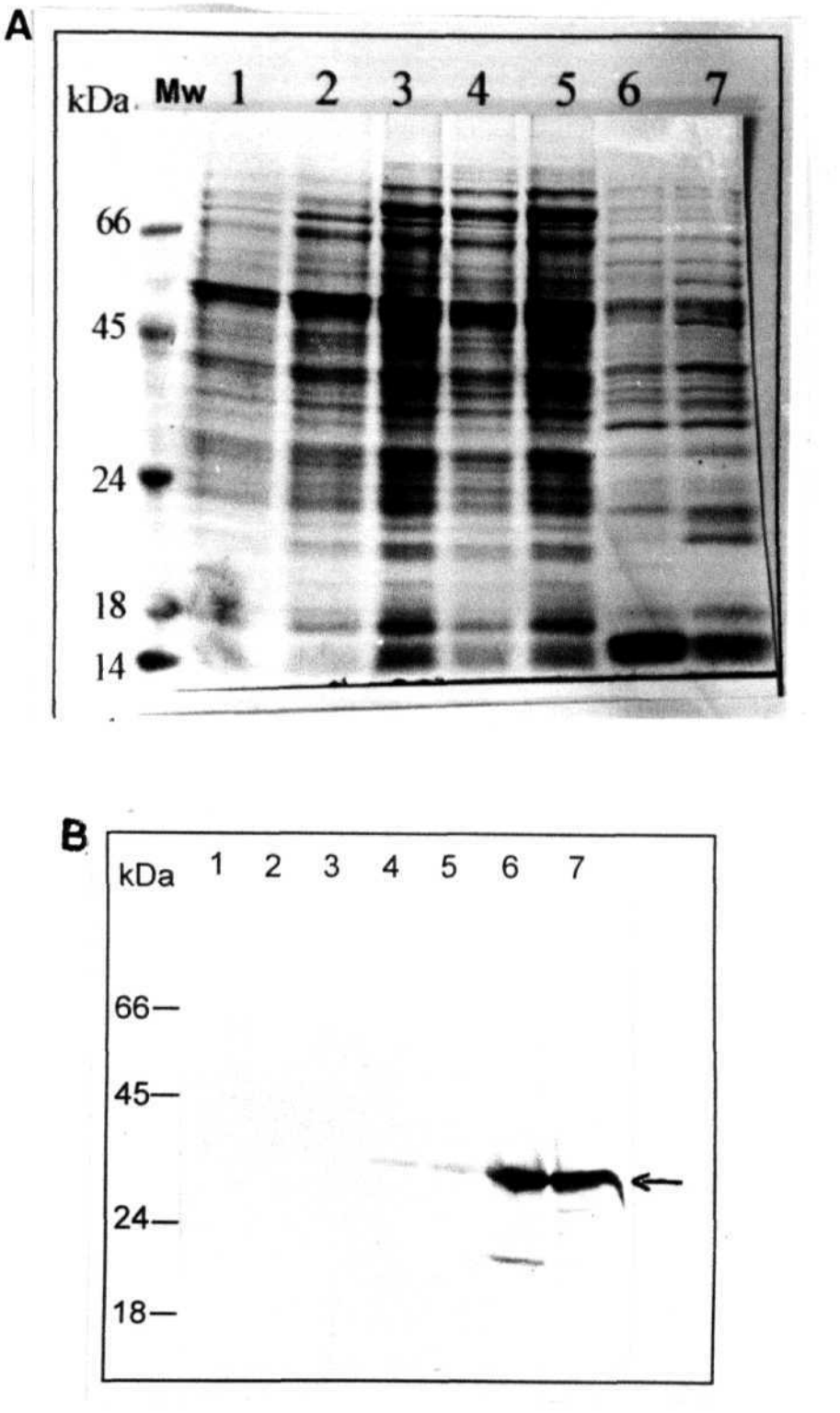
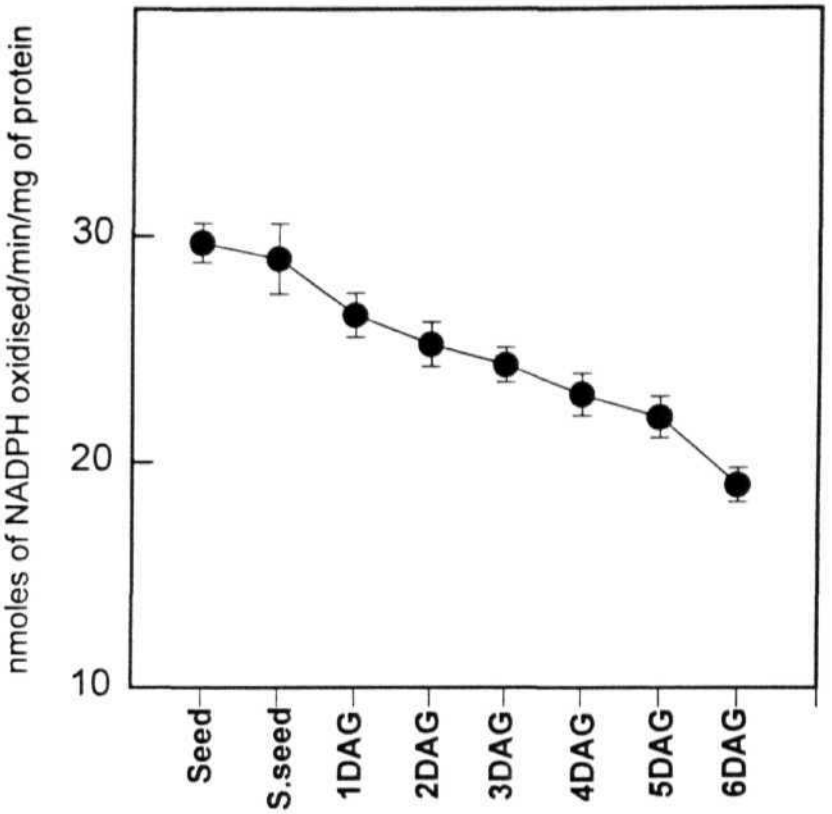


Fig. 4.4.2: SDS-PAGE (A) Western blot (B) of immature rice seed proteins probed with anti- pG22-69 antibodies raised against barley embryo protein

Lanes: Mw: mol.wt markers 1) 0 DAP 2) 4 DAP 3) 8 DAP 4) 15 DAP 5) 25 DAP 6) Seed 7) Embryo (30µg of protein was loaded onto each lane) Arrow represents the stress induced polypeptide (DAP:Days After Pollination)

Fig. 4.4.3A Aldose reductase activity in K39 seeds at different days of germination



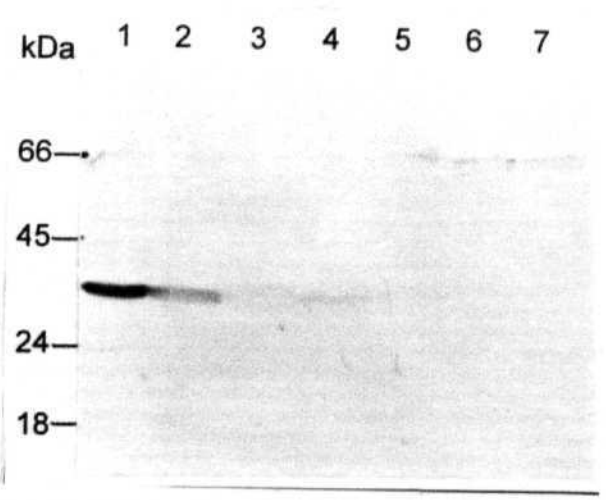


Fig. 4.4.3B: Immunodetection of 34 kDa protein in rice seeds of different days after germination (DAG) by using anti- pG22-69 antibodies
Lanes: 1) Seed 2)1 DAG 3) 2 DAG 4) 3 DAG 5) 4 DAG 6) 5 DAG 7) DAG (30μg of protein was loaded into each slot)
Arrow represents the stress induced polypeptide

disappear upon germination. As can be seen in Fig **4.4.3A.** the activity **gradually** decreases during germination. Enzyme activity profiles of germinating seeds by and large correlated with Western data (Fig.4.4.3B). This pattern is similar to LEA protein accumulation in various groups of plants which are known to selectively appear during the late embryogenesis and disappear upon germination (Baker *et al.,* 1988).

4.4.3 Induction of aldose reductase protein in rice shoots by ABA and other abiotic stresses:

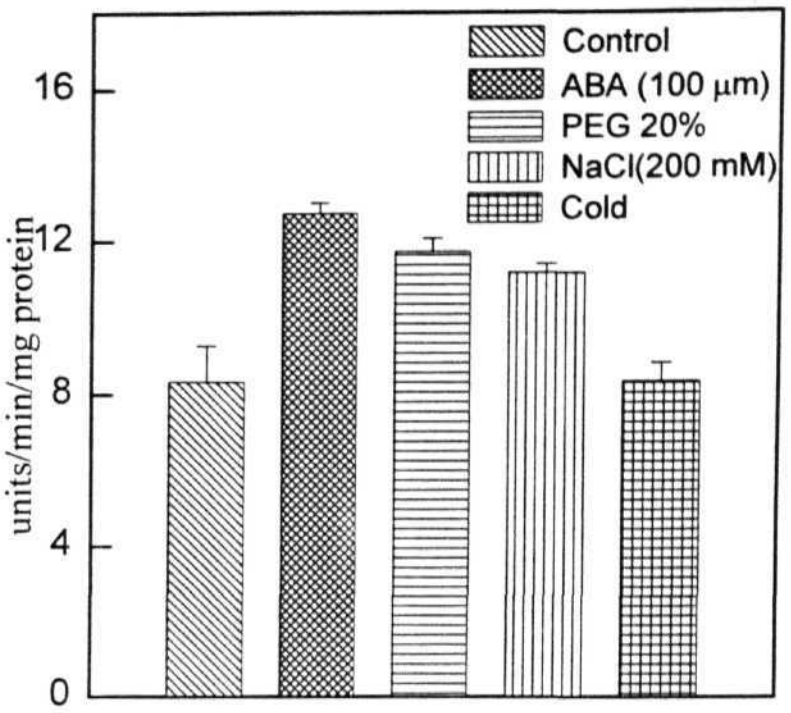
As majority of the LEA proteins from various crop plants are typically known to respond to ABA and abiotic stress conditions in vegetative tissues (Baker *et al.,* 1988; Close *et al.,* 1989). -an attempt was made to understand the stress responsive nature of this protein in young rice shoots. Aldose reductase activity was determined in ammonium sulphate protein fractionations (80%) from stressed K39 shoots. The results (Fig. 4.4.4A) revealed significant increase in AR activity in shoots exposed to ABA, PEG and Salt. The increase was maximum in response to ABA (50% increase over control) followed by PEG (40%) and salt (35%). On the contrary, cold acclimated shoots did not show any increase in AR activity. Such an increase in the enzyme activity was in correlation with the induction of this protein as measured by Western blot assays.

Western analysis revealed that this protein was induced in shoots of K39 seedlings treated with 100 μM of ABA. water stress (PEG 20%) and salinity (200mM) stresses (Fig. 4.4.4B). The protein fraction utilized for the Western analysis and enzymatic analysis was the same. Moreover, the quantitative induction of this protein in response to ABA and abiotic stresses was proportional to the increase of the enzyme activity (Fig. 4.4.4A and 4B).

4.4.4 Varietal differences in the ABA- inducibility of AR-related protein:

There were clear differences in the ABA inducibility of AR-like protein among the rice varieties of varying drought tolerance. Application of 100 μM of ABA was found to induce this protein in drought tolerant varieties Annada and Tulasi but not in the

Fig. 4.4.4A Aldose Reductase activity in K39 Variety under different stress conditions



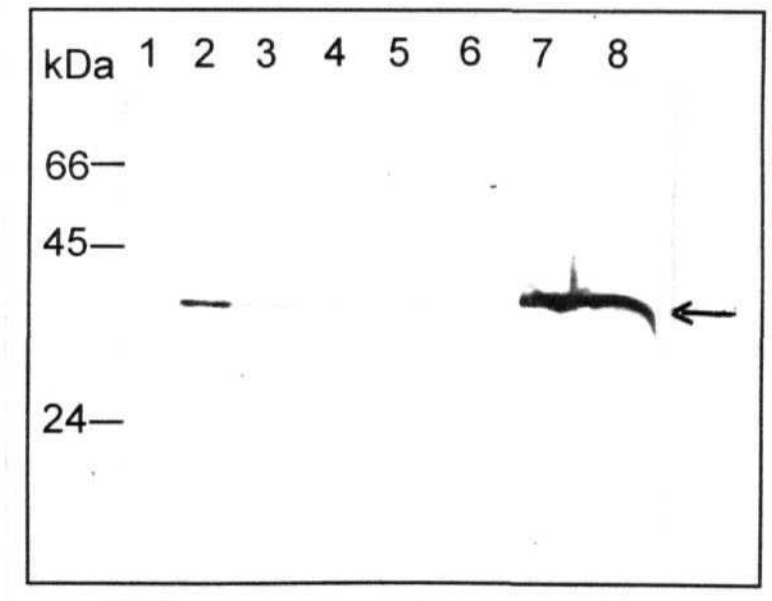


Fig. **4.4.4B: Western blot showing the cross reactivity of K39 shoot and seed proteins under different stresses with anti-pG22-69 antibodies**
Lanes: 1) Control 2) ABA (100 µM) 3) PEG 20% 4) 200mM NaCl 5) Cold 6) Empty 7) Seed 8) Embryo (30µg of protein was loaded into each slot) Arrow represents the stress induced polypeptide

sensitive line Hamsa as revealed by Western data (Fig. 4.4.5A). In shoots the induction of this protein was in positive correlation with the increased aldosc reductase activity in ammonium sulphate fraction (Fig. 4.4.5B). Further. ABA treated Hamsa seedlings did not accumulate AR-like protein nor they showed any increase in AR activity. Small increase of AR activity was detected in protein extracts of Annada seedlings alter 3 days of ABA treatment, while in Tulasi no such increase in AR activity was found. These results were in correlation with Western data. The protein extracts of Annada seedlings treated with ABA for five days have shown maximum increase in AR activity (about 55%) which was correlated with maximum induction of AR in Western analysis. In Tulasi. the increase in enzymatic activity was less in comparison with Annada (about 45%). In Hamsa we could not detect any increase in AR activity which was equal to the control seedlings grown in normal conditions.

4.4.5 AR-like protein in mature seeds of other Graminae members:

In view of the reported presence of sorbitol in many higher plants, we have looked for the existence of the aldose reductase related protein in mature seeds of cereals. SDS gels run from crude protein extracts from mature seeds of several cereals were blotted and probed with the same Anti-AR antibodies. A single protein was detected in mature seeds of all the tested members of the graminae family (Fig. 4.4.6). Interestingly, this protein in rice, barley, *Sorghum, Pennisietum*, maize, and wheat is of about the same molecular weight. The oat AR-like protein, however, seems to be slightly smaller than the others. Presence of this protein in all cereal seeds tested indicates that this protein is evolutionarily conserved. These results further confirm the premise that basic stress responsive mechanisms were conserved all through the evolution.

4.4.6 ABA-inducibility of AR related protein in Graminae members

Since the protein was found to be present in large quantities in mature seeds, an attempt was made to test their inducibility in the vegetative tissues of gramenaceous members. The results from the Western analysis of the shoot protein extracts made from

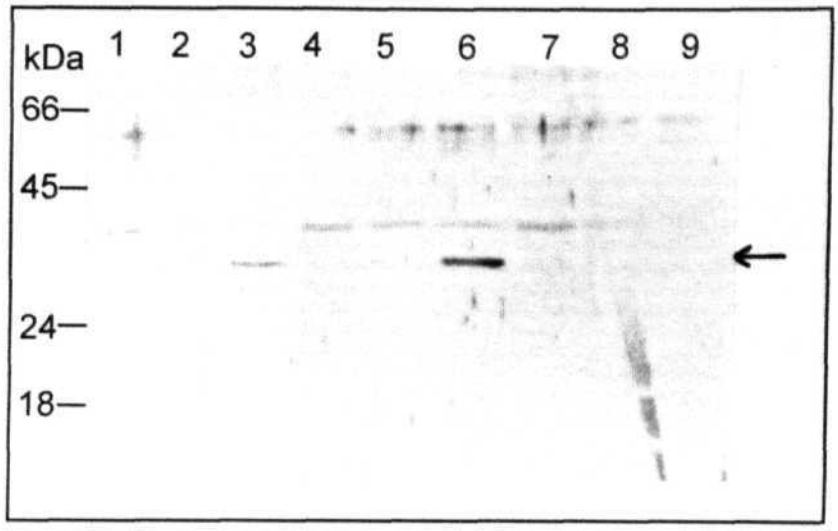


Fig. 4.4.5A: Cross reactivity of anti- pG22-69 antibodies with proteins from different rice cultivars treated with ABA for different days
Lanes: 1) Control Tulasi 2) ABA 3 days 3) ABA 5 days 4) Control Annada 5) ABA 3 days 6) ABA 5 days 7) Control Hamsa 8) ABA 3 days 9) ABA 5 days (30|ig of protein was loaded onto each lane) Arrows represent the stress induced polypeptides

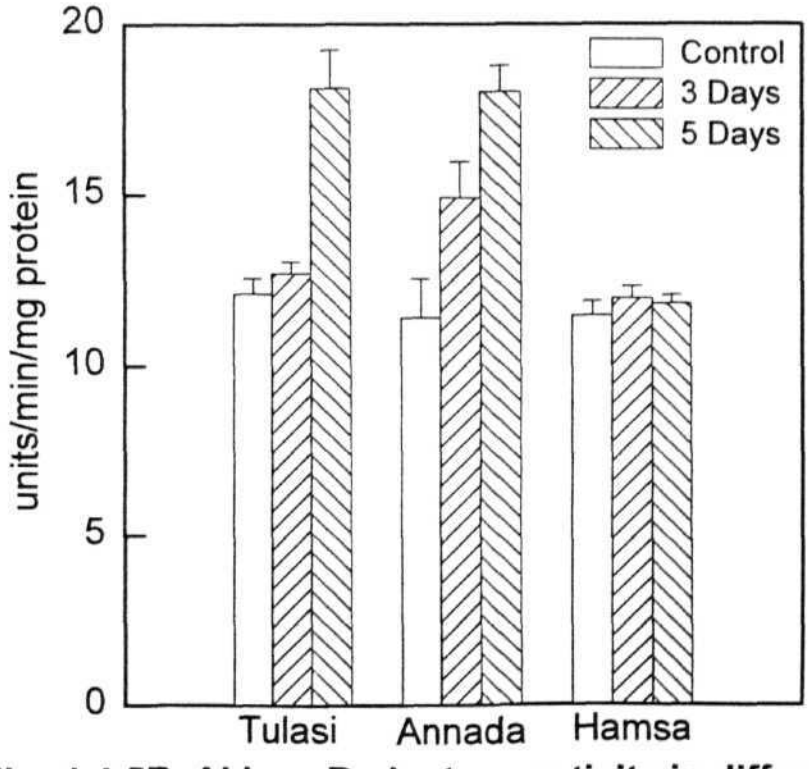


Fig. 4.4.5B Aldose Reductase activity in different seedling during ABA stress

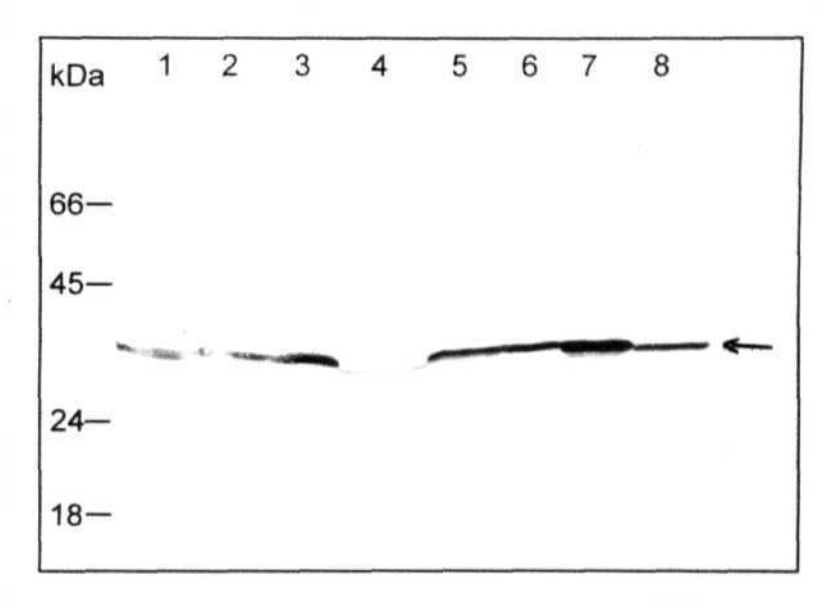


Fig. 4.4.6: Western blot showing the cross reactivity of seed proteins from different cereals with pG22-69 antibodies

Lanes: 1) Rice 2) Barley 3) Wheat 4) Oats 5) *Sorghum* 6) Pearl millet 7) Ragi 8) Maize (30µg of protein was loaded into each slot)
Arrow represents stress induced polypeptide

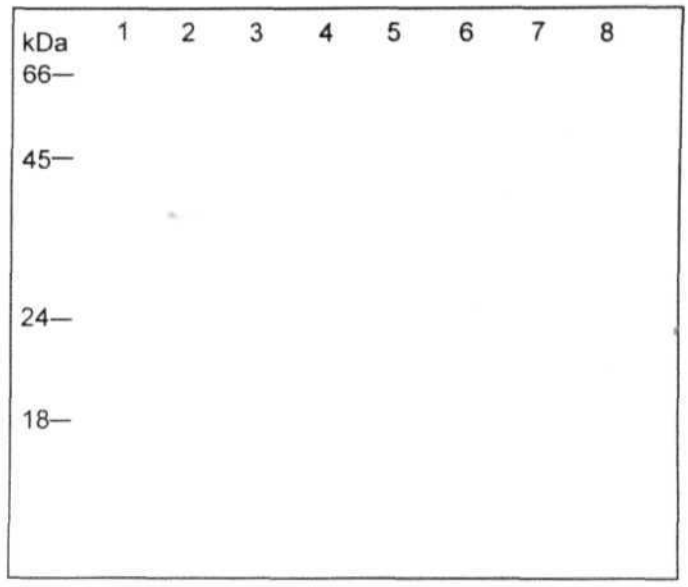


Fig. 4.4.7: **Immonodetection of Aldose reductase protein.**
Lanes: 1) Control Rice 2) Rice+ABA 3) Control **Maize 4) Maize** +ABA 5) Control Sorghum 6) Sorghum + ABA7) **Control Ragi 8)** Ragi +ABA (30µg of protein was **loaded** onto **each lane)** Arrow represents the stress induced polypeptide

seedlings stressed with 100 μM ABA revealed that the AR related protein is induced only in shoots of rice but not of maize. *Sorghum* and *Eleucine coracana* (Fig. 4.4.7).

4.5 Antioxidant enzyme levels and their isozyme profiles in young seedlings and mature plants under ABA and water stress:

Plants have evolved a diverse array of defense mechanisms to prevent oxidative injury to cellular components from exposure to various environmental stress factors. Plant defense enzymes including antioxidant enzymes arc known to increase in rice in response to stress conditions. However these response mechanisms, arc reported to be stage specific as well as cultivar specific. Changes in activity of the antioxidant enzymes. SOD. APX. and GR were determined in both seedlings and mature plants under stress. In parallel, isozyme profiles of SOD and APX enzymes were also determined in seedlings and mature plants under stress.

*4.5.1 SOD activity **and** isozymc profiles in young seedlings **and mature** plants:*

Crude enzyme extracts from seedlings and mature rice plants were analyzed by activity staining of SOD enzyme on native PAGE. Qualitative and quantitative differences in the isozyme profiles of mature plant (45day old) and seedling (8day old) have been given in Fig. 4.5.1. It is interesting to note that the mature plants contain only three isozymes where as the seedlings show about six on the zymogram. Further, enzyme activity in mature plant extracts is significantly lower than that of seedlings. For instance. basal levels of SOD activities were four times higher in seedlings than that of mature plants of Annada cultivar.

Further. we have characterized SOD isozyme based on their metal cofactors as Cu/Zn SOD. Mn SOD and Fe SOD using a sensitivity assay for detection of specific isozyme. Incubating gel in H_2O_2 or KCN and monitoring the activity on native gels enabled us to classify different types of SODs. Mn SOD is known to be resistant to KCN and H_2O_2 while Cu/Zn SOD is sensitive to both. The FeSOD is resistant to KCN and

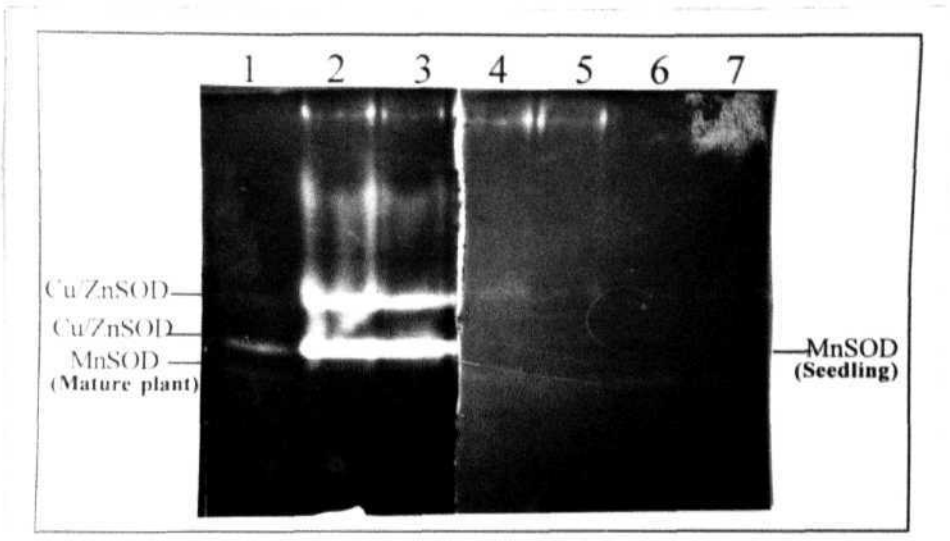


Fig. 4.5.1 A Native PAGE showing the difference in SOD isozymes in seedlings and mature plants

Lanes: 1, 6, 7) Mature plant 2-5) Seedlings 4 -7) treated with 3mM KCN (Cu/Zn SOD is sensitive to KCN, while MnSOD and FeSOD are resistant)

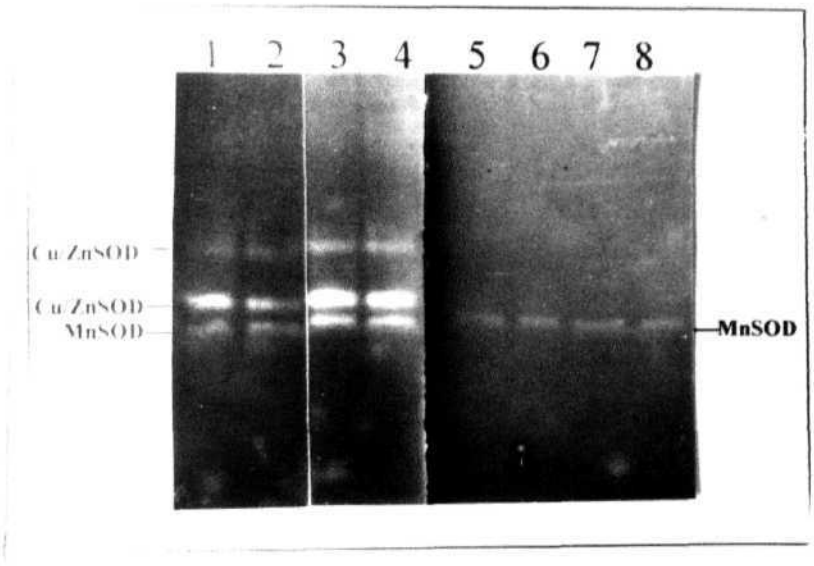


Fig. 4.5.1 B Native PAGE showing the SOD isozymes from mature plant

Lanes: 5-8 treated with H_2O_2 (showing H_2O_2 resistant MnSOD and inactivation of Cu/Zn SOD isozymes) Lanes: 1, 2, 5 & 6 (50 μg of protein) Lanes: 3, 4, 7 & 8 (100 μg of protein)

sensitive to H_2O_2. Based on the reaction, the two slow migrating bands were identified as Cu/Zn SOD and the fastest band as Mn SOD (Fig 4.5.1 A and B). Among the three, the Cu/Zn SOD seem to be the most predominant in seedlings and mature plants.

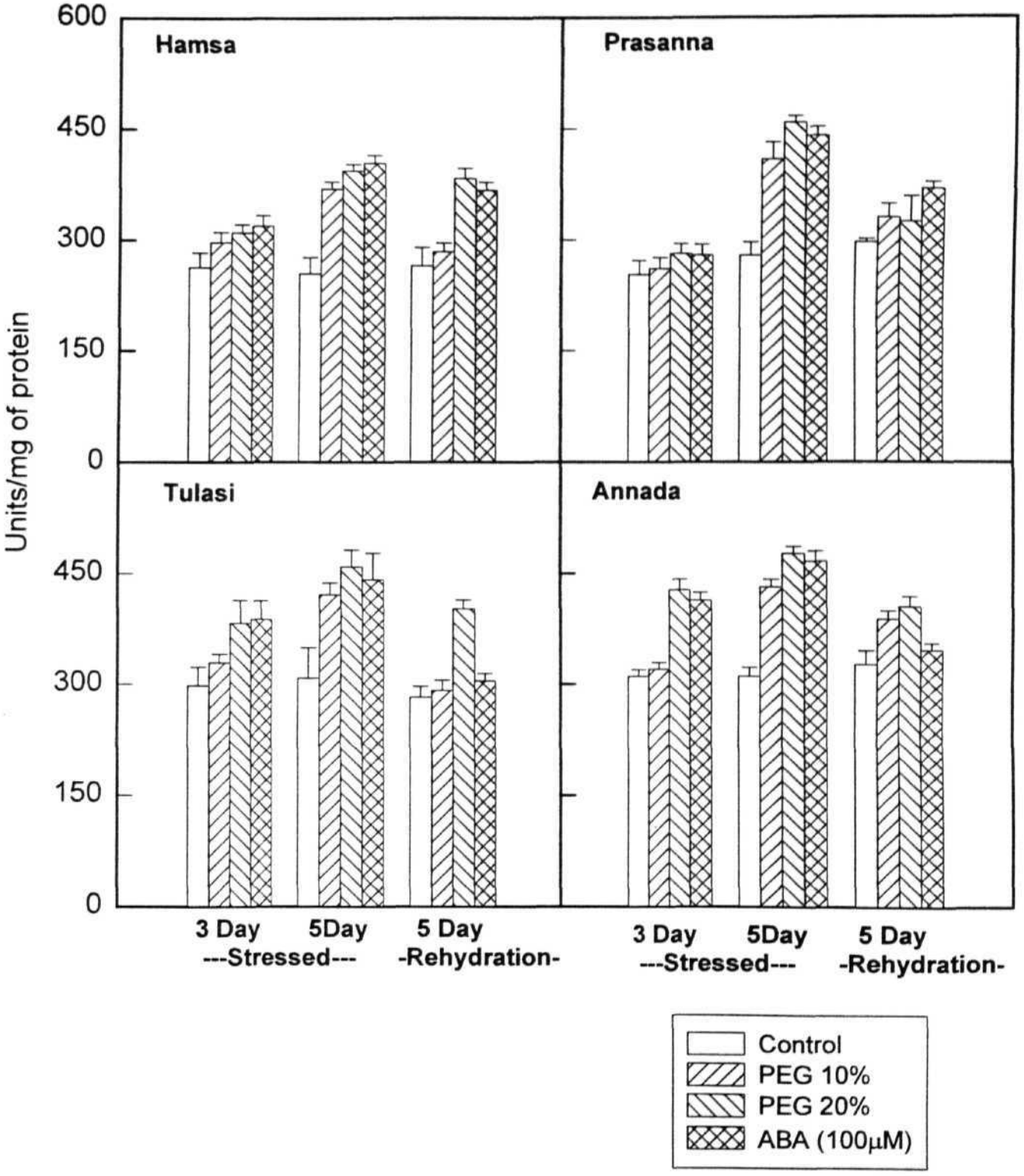
4. 5 .2 SOD activity and isozyme profiles in seedlings under PEG mediated water stress and upon rehydration

Superoxide dismutase activity levels were analyzed in seedlings of different cultivars under ABA and PEG mediated water stress and the results are given in (Fig. 4.5.2A). Three day-stressed seedlings did not show significant changes in SOD levels in Hamsa and Prasanna. On the contrary, both Tulasi and Annada seedlings showed a significant increase (about 30%) in SOD levels. Five day-stressed seedlings also showed significant increase in SOD activity under ABA and PEG stress. Prasanna showed the maximum increase (about 75%) of SOD activity under both stresses. The other three cultivars showed an average increase of about 50% in SOD activity. SOD levels were also increased in seedlings treated with 10% PEG, which is reported to be a mild stress.

Rehydration experiments were carried out in order to test reversal trends in SOD activity upon recovery from stress. After five days of rehydration. the seedling SOD levels indicated varietal differences. The results (Fig. 4.5.2A) revealed that the SOD activity falls significantly upon rehydration in all cultivars except Hamsa. It is to be noted that Hamsa is a stress sensitive cultivar compared to the other three. Further. SOD levels upon recovery reach almost to control levels in PEG and ABA stressed seedlings, again with one exception. that is, PEG 20% treated Tulasi seedlings continue to show high SOD activity.

Isozyme profiles of SOD of different rice seedlings subjected to water stress and ABA are displayed in Fig. 4.5.2B. All seedling extracts revealed three clear bands (arrows). There are quantitative differences between cultivars and under various stress treatments. The most prominent change is the increase in intensity of the slow migrating isozyme, upon stress. in all seedlings. The increase in SOD isozyme levels, under stress, appears to be maximum in Prasanna (lanes 1, 2, 3 of Fig 4.5.2B). Furthur, the enhanced isozyme belongs to the Cu/Zn SOD class in all the varieties tested.

Fig. 4.5.2 A Changes in superoxide dismutase levels in different rice seedlings under ABA and PEG stress



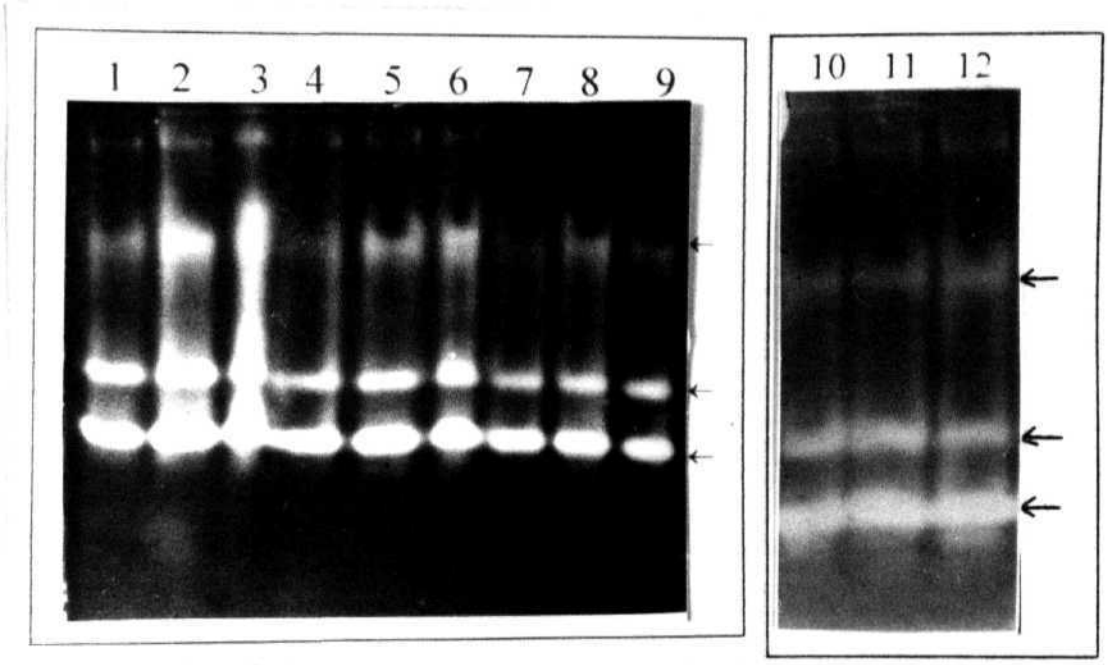


Fig. 4.5.2B Native PAGE showing the SOD isozymes in rice seedlings treated with ABA(100μM) and PEG (20%) for 5 days

Lanes:1) Prasanna Control 2) Prasanna PEG 20% 3) Prasanna ABA
4) Annada Control 5) Annada PEG 20% 6) Annada ABA
7) Tulasi Control 8) Tulasi PEG 20% 9) Tulasi ABA 10) Hamsa Control
11) Hamsa PEG 20% 12) Hamsa ABA
(100 μg of protein was loaded) in each slot)
Arrows represent the enhanced isozyme

4.5.3 SOD enzyme levels and isozyme profiles in mature rice plants during dehydration stress

Mature rice plants (45 day old) subjected to ten days of dehydration were analyzed for changes in SOD activity levels. The results in Fig. 4.5.3A indicate an increase in SOD activity in all cultivars. There were varietal differences in dehydration-induced SOD activity levels i.e. Annada. Tulasi. Prasanna. Hamsa in the descending order.

SOD isozyme profiles of mature plants after dehydration treatment for ten days were analyzed on native PAGE. SOD zymogram (Jig. 4.5.3B) reveals a detectable increase in isozyme levels in all cultivars upon dehydration, with a more prominent increase in Annada and Tulasi. Unlike in seedlings, no new SOD isozyme was detected in mature plants.

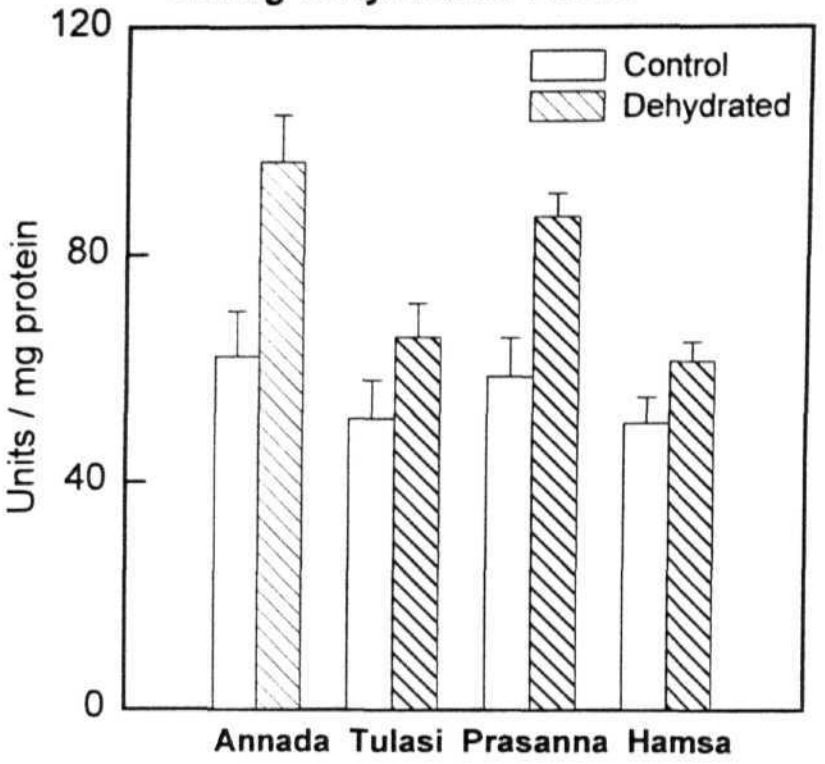
4.5.4 APX isozyme profiles of mature plant and young rice seedling:

The APX isozyme profiles of mature plants and seedlings of Annada are given in Fig. 4.5.4. As can be seen, seedlings clearly show two bands, one extremely slow migrating and the other fast. Interestingly, the slow band was not detectable in mature plants. No other differences were found on the zymogram. The fast band is actually a duplex apparently consisting of two closely migrating APX isozymes. We observed such a duplex consistently during the analysis. The seedlings also showed significantly higher levels (64%) of APX activity compared to the mature plants.

4.5.5 Quantitative and qualitative changes in APX enzyme levels and isozymes under PEG mediated water stress and ABA:

Ascorbate peroxidase levels were analyzed in rice seedlings under ABA and PEG mediated water stress. The Fig.4.5.5A reveals not only an increase in APX levels under stress. There was a consistent increase in the enzyme levels during the entire stress response period. Further, such an increase by and large correlated with severity of the

Fig. 4.5.3A SOD activity in diffferent cultivars during dehydration stress



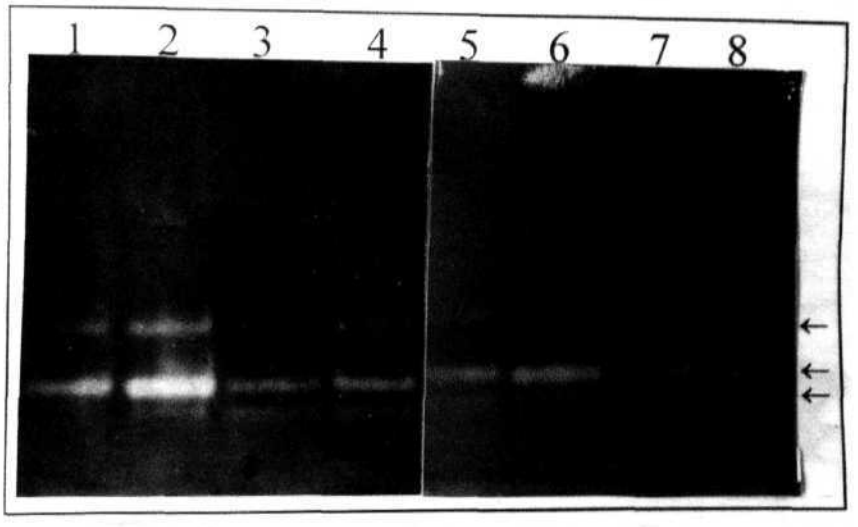


Fig. 4.5.3B Native PAGE showing the SOD isozymes in mature rice plants dehydrated for 10 days

Lanes: 1) Annada Control 2) Annada dehydrated 3) Prasanna Control 4) Prasanna dehydrated 5) Tulasi Control 6) Tulasi dehydrated 7) Hamsa Control 8) Hamsa dehydrated (100 μg of protein was loaded in each slot)

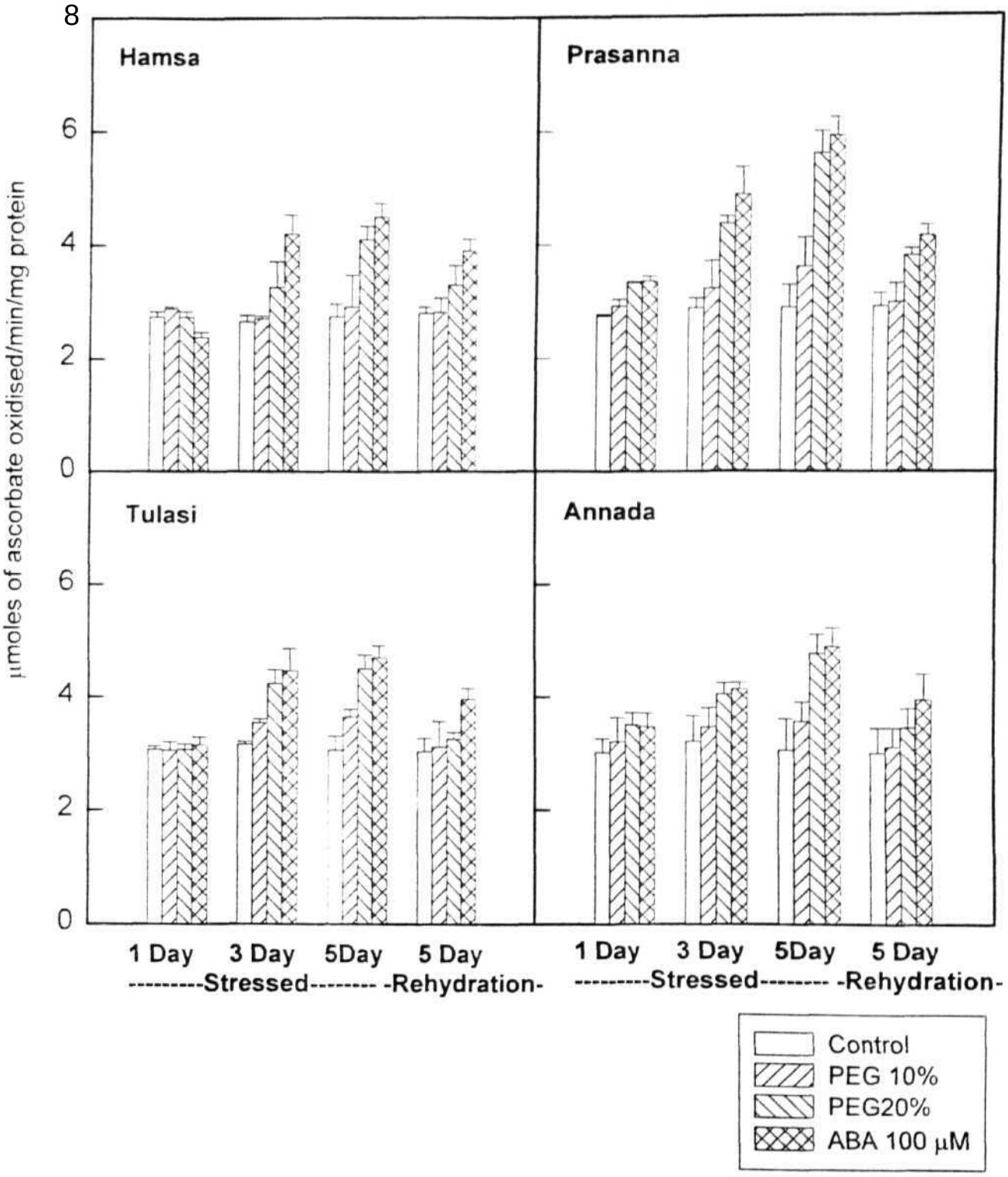
Fig. 4.5.4 Native PAGE showing variation in the ascorbate peroxidase isozymes of mature plant and seedling

Lanes: 1) Seedling 2) Mature plant

(100 µg of protein was loaded in each slot)

Arrows represents the APX isozymes

Fig. **4.5.5A Changes in ascorbate peroxidase levels in different** seedlings under **ABA and PEG stress**



stress.

Increase in APX activity was observed in three day stressed seedlings under PEG 20% and ABA. However, no increase was observed under PEG 10%. Among the tested cultivars, Prasanna seedlings showed the maximum increase (about 70%). APX activity in five day stressed rice seedlings increased progressively and reached the maximum level in all cultivars under PEG 20 % and ABA treatment. The increase in APX activity was maximum in Prasanna (about 100% increase) followed by other three cultivars (ranging between 50-60%). On the contrary, one day stressed seedlings did not show any detectable increase in APX activity. ABA treated seedlings, upon recovery, showed high APX activity levels even after five days. On the other hand, the PEG 20% stressed seedlings, upon recovery, showed decresed levels of APX activity, almost similar to controls.

APX isozymc profiles of different rice seedlings subjected to ABA and PEG stress were analyzed on native PAGE. Fig. 4.5.5B, C and D showed the qualitative, quantitative and varietal specific differences in APX isozymc patterns. The zymogram (Fig 4.5.5B) reveals a) three to four prominent isozyme bands, b) stress by PEG and ABA increases all three isozymes and most importantly c) Prasanna shows a new fast migrating isozyme upon PEG 20% and ABA. The zymogram (Fig 4.5.5C) further substantiates this observation. The slow migrating APX isozyme pattern in Prasanna was entirely different from the other three cultivars tested (Fig. 4.5.5B, C and D). Interestingly, in Annada. there was a reduction in one of the slow bands and increase in the fast band under stress. In Hamsa, the slow isozyme level increased both under ABA and PEG stress (Fig.4.5.5D). There was no significant difference in other isozyme levels.

4.5.6 APX activity and isozyme profiles in mature plants during dehydration stress:

Rice plants (45day old) were subjected to dehydration stress for ten days and analyzed for changes in ascorbate peroxidase activity levels. The Fig. 4.5.6A shows an increase in APX activity in all the cultivars tested during dehydration stress. Maximum increase (about 50% increase) was noted in Annada. Increase in enzymatic activity was

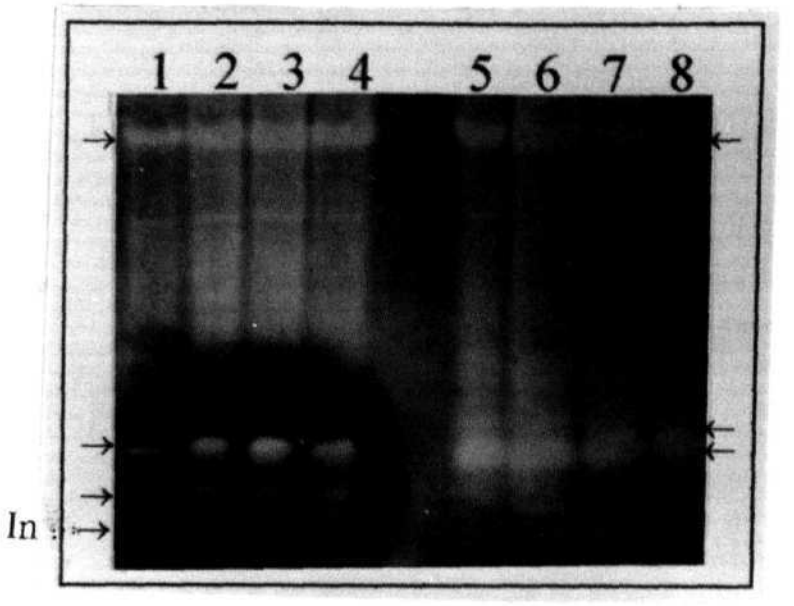


Fig. 4.5.5B Native PAGE showing the ascorbate peroxidase isozymes in seedlings treated for 5 days with PEG and ABA (100μM)

Lanes: 1) Control Prasanna 2) PEG 10% 3) PEG 20% 4) ABA 5) Control Tulasi 6) PEG 10% 7) PEG 20% 8) ABA (100 μg of protein was loaded) Arrows represents the enhanced isozymes → In. represents the Induced isozyme

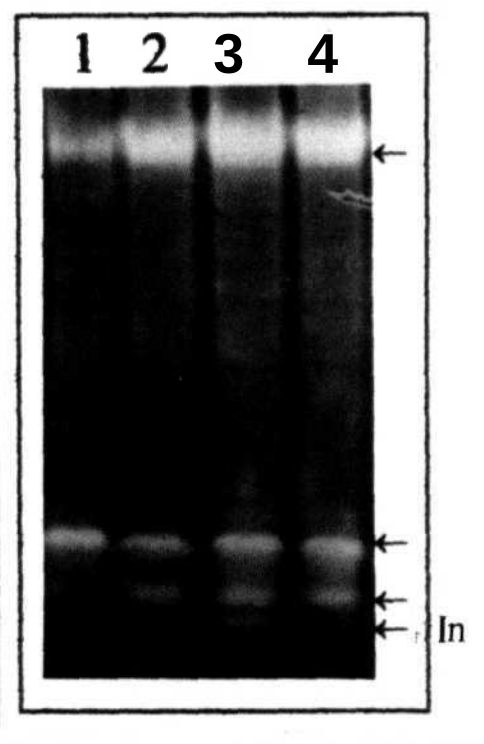


Fig. 4.5.5C Native PAGE showing variation in the ascorbate peroxidase isozymes in rice seedlings stressed for 5 days

Lanes: 1) Control Prasanna 2) PEG 20% 3) PEG 40% 4) ABA (100 μg of protein was loaded in each slot) Arrows represents the enhanced isozymes. → In. represents the Induced isozyme

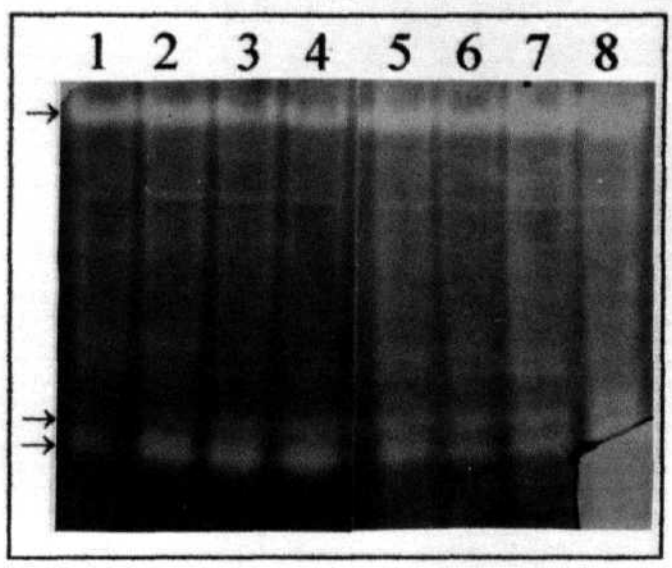
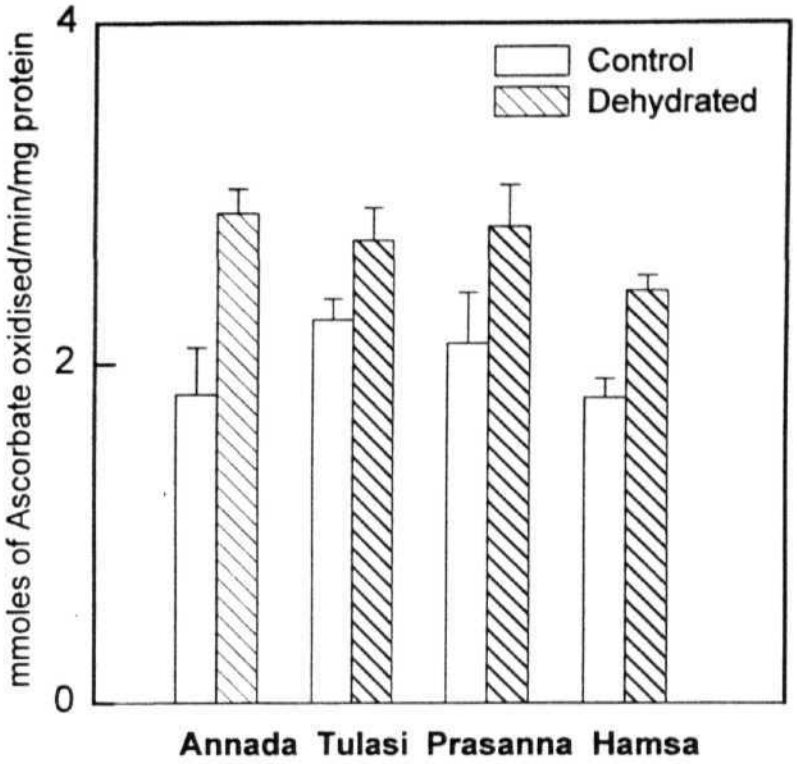


Fig. 4.5.5D Native PAGE showing the Ascorbate Peroxidase isozymes in rice seedlings treated with PEG(10-20%) and ABA(100μM)

Lanes: 1) Annada Control 2) PEG 10% 3) PEG 20% 4) ABA
5) Hamsa Control 6) PEG 10% 7) PEG 20% 8) ABA
Arrows represents the enhanced isozymes

Fig. 4.5.6A Ascorbate peroxidase activity in different cultivars during dehydration stress



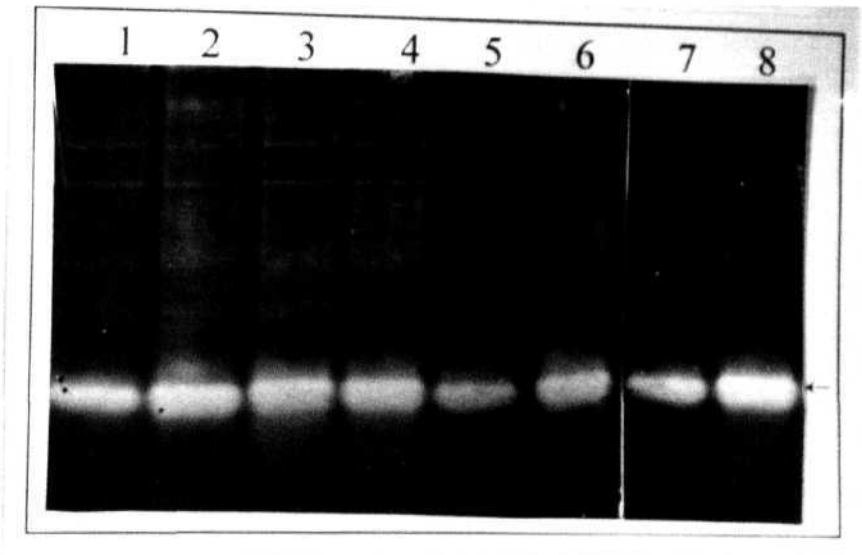


Fig. 4.5.6B Native PAGE showing the ascorbate peroxidase isozymes in mature rice plants dehydrated for 10 days

Lanes: 1) Control Annada 2) Dehydrated Annada 3) Control Prasanna 4) Dehydrated Prasanna 5) Control Tulasi 6) Dehydrated Tulasi 7) Control Hamsa 8) Dehydrated Hamsa (100 µg of protein was loaded into each slot)

more in seedlings under PEG and ABA treatment than in mature plants under dehydration stress.

Mature plants subjected to dehydration were analyzed for APX isozyme profiles and the results (Fig. 4.5.6B) showed that the profiles are different from that of seedlings. There were no qualitative differences between the tested cultivars. All except Prasanna showed an increase in the intensity *of* the fast band. Interestingly, APX profiles of Prasanna seedlings (Fig. 4.5.5C) were also different from that of the other cultivars tested.

4.5.7 Effect of ABA and PEG stress on glutathione reductase levels in seedlings and mature plants

Changes in glutathione reductase activity levels were analyzed from protein extracts of different rice seedlings stressed with ABA and PEG (l()%-20%). The GR activity levels increased progressively during stress and the extent of increase varied among the cultivars tested (Fig. 4.5.7). After three days of stress, the GR activity in seedlings reached the maximum (35%) in Prasanna and (36%) in Annada under PEG (20%) and ABA stress.

After 5 days of stress treatment. GR activities increased to maximum in all the cultivars tested. Prasanna and Annada showed the maximum increase of about 60% in GR activity under PEG 20% and ABA stress. Tulasi and Hamsa showed an increase of about 32-40% increase in GR activity under stress. The above results clearly reveal that GR activities increased under PEG and ABA stress. the extent of increase varies among the cultivars tested. The tolerant varieties showed relatively higher increases over the sensitive ones. Upon rehydration of the stressed seedlings, there was no significant change in GR levels. However. GR levels of 10% PEG treated seedlings, upon rehydration, reached almost control levels (Fig. 4.5.7).

Changes in glutathione reductase activity levels were analyzed in seedlings subjected to ten days of dehydration stress. Increase in GR activity was recorded under dehydration stress in all the cultivars tested (Fig. 4.5.8); with Annada showing the

Fig. 4.5.7 Changes in glutathione reductase levels in seedlings under ABA and PEG stress

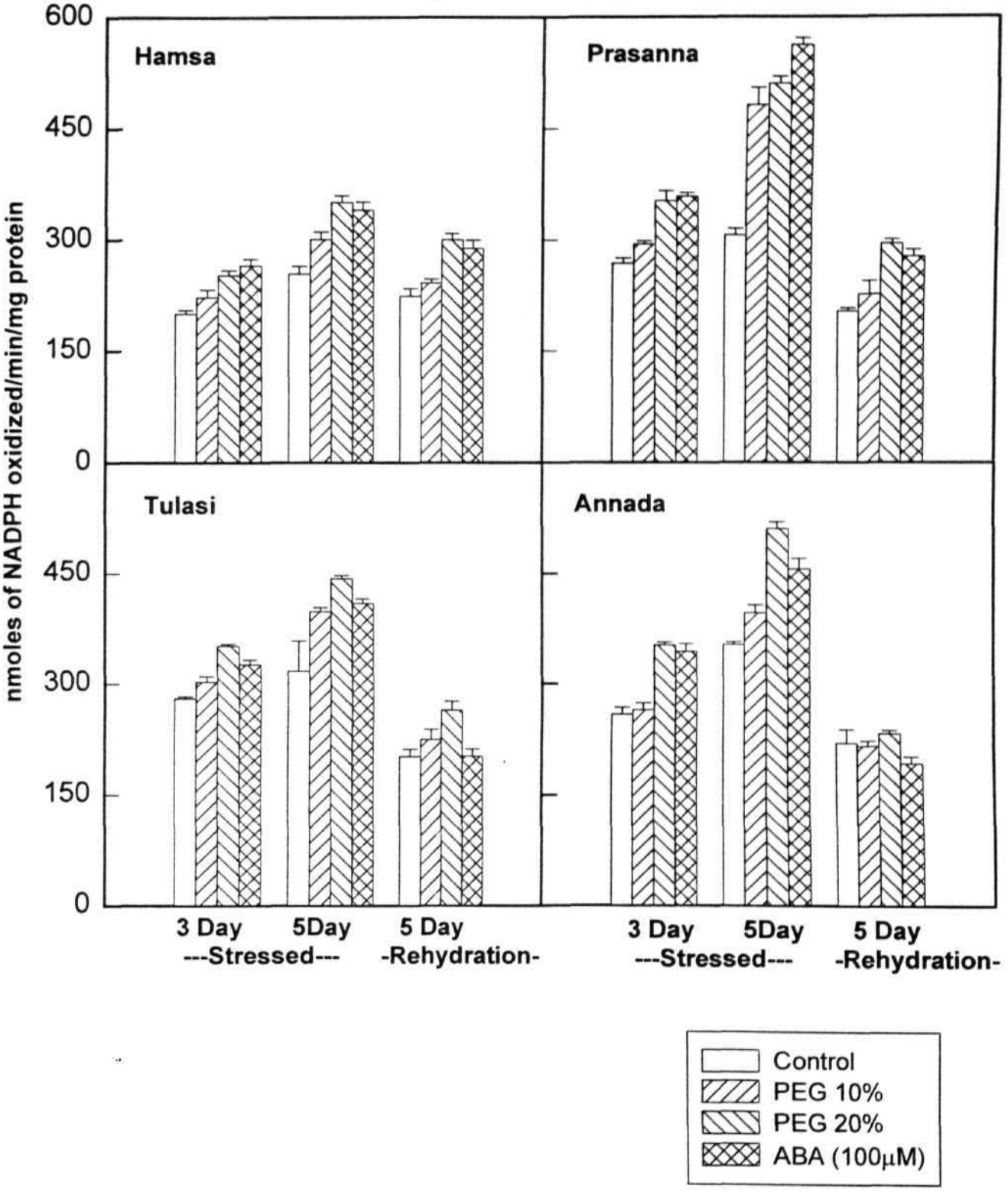
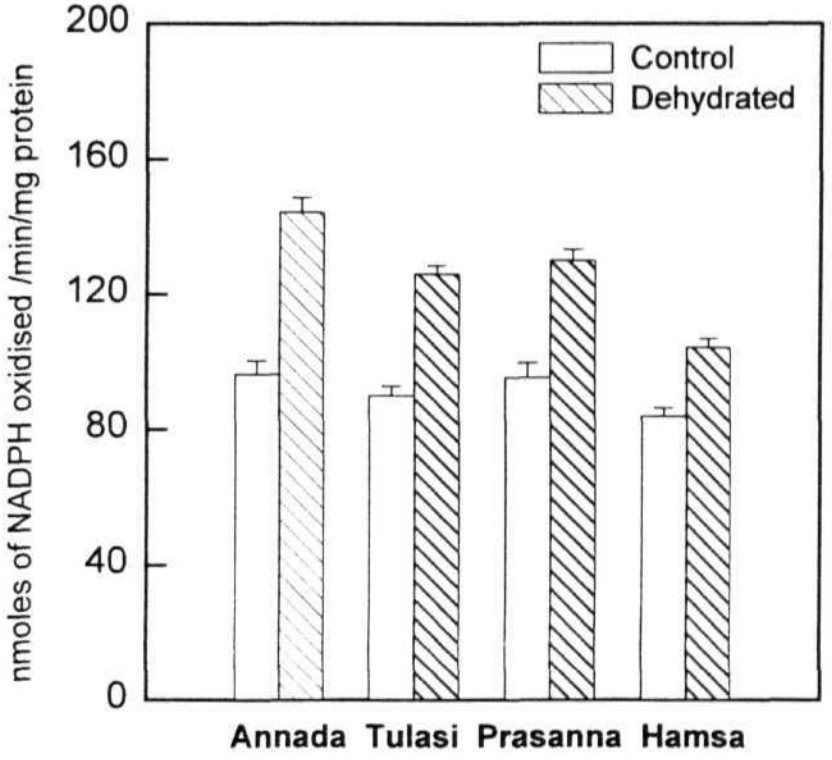


Fig. 4.5.8 Glutathione reductase activity in different cultivars during dehydration stress

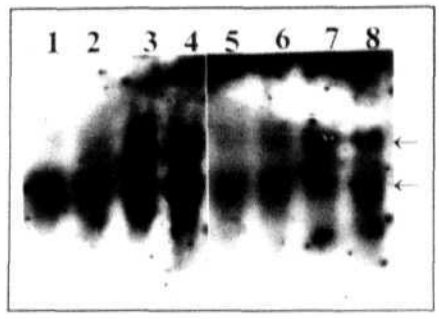


maximum increase (49%).

4.5.S APX mRNA levels under PEG and ABA stress in seedlings:

Our experiments were aimed at determining the changes in APX gene expression in rice seedlings exposed to 48 hr of PEG 20% and ABA application. Northern analysis (Fig. 4.5.9) data using the *Arabidopsis* APX probe (Courtesy. Van Montagu. Ghent) revealed two hybridizable bands. Further. ABA and PEG treatment both lead to an increase in the APX specific mRNA band (A). The lower panel (B) shows that equal amount of RNA was loaded in control and treated samples of each of the cultivars.

A. Northen blot



B.RNA gel

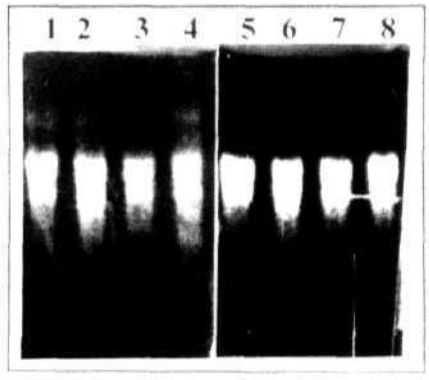


Fig. 4.5.9 Northern blots showing differential accumulation of APX transcripts by PEG and ABA stresses in seedlings stressed for 48hr

Lanes: 1) Control Tulasi 2) PEG 10% 3) PEG 20% 4) ABA 5) Control Hamsa 6) PEG 10% 7) PEG 20% 8) ABA
(30 μg of RNA was loaded into each slot)

5. DISCUSSION

Discussion

This study is primarily aimed at analyzing the stress responsive alterations in seedling growth, protein profiles and enzyme activities. Further, varietal differences in stress responsive changes in the above mentioned parameters have been documented. Attempts were made to determine the probable function of such stress-induced proteins in rice. The stress-induced changes in antioxidant enzymes have been studied in order to decipher their role in stress tolerance processes. The results of this study and implications are discussed below.

5.1 Increase in root/shoot growth ratio under PEG and ABA mediated stress is an adaptive feature

Our results showed an increase in root/shoot growth ratios in both etiolated and light grown seedlings of all the cultivars during PEG mediated water stress and exogenous application of ABA (Fig 4.1.1 and 4.1.2). Further, there are significant varietal differences in root/shoot growth under water stress (Fig 4.1.3). with tolerant lines showing relatively higher ratios. For instance, Annada. a tolerant line showed significantly higher increase than Hamsa, a sensitive line (Fig. 4.1.4A and 4.1.4B). It is clear that the 40% PEG treatment was a severe stress, which drastically inhibited shoot growth of both cultivars. Our observations on promotion of root growth and inhibition in shoot growth under ABA and PEG stress in tested cultivars are in agreement with previous reports. In higher plants, both shoot growth and tissue water potential decline as water deficit develops. However, at certain tissue water potentials where shoot growth is inhibited, roots continue to grow resulting in increased root/shoot ratios (Creelman *et al*, 1990; Kramer, 1983; Meyer and Boyer. 1981; Sharp and Davïes, 1979).

ABA treatment lead to increased root/shoot ratios compared to PEG mediated water stress. It is well known that under reduced water availability, leaf area decreases which in turn reduces water loss and thus provides an adaptive stress response mechanism. In addition to reducing the rate of water use, there is evidence for an

increased access to soil water utilization that subsequently lead to an increase in root/shoot growth ratios (Kramer. 1983; Meyer and Boyer. 1981). Root elongation is important for efficient water uptake, particularly for seedlings growing in dry soil. It was reported that accumulated solutes in shoots are translocated into roots thereby promoting root elongation (Sharp and Davies. 1979). This was suggested to be an adaptive feature and roots might be having a higher capacity for osmotic adjustment than shoots. The solutes invoked in osmotic adjustment were mostly free amino acids, glucose, fructose and sucrose, which account for most of the increased dry weight under stress (Meyer and Boyer. 1981). Root elongation during water deficit was reported to be mediated by altered levels of abscisic acid. ABA is a key molecule in promoting root growth and inhibiting shoot growth at low water potentials (Creelman *et al*, 1990; Saab *et al*., 1990). Our results showing a significant reduction in the growth of vegetative tissues during dehydration processes (Fig.4.1.5) in mature rice plants substantiate the above observation. The stress tolerant cultivar, Annada showed significant high relative water content when compared to other rice cultivars during dehydration. On the contrary, the sensitive cultivar. Hamsa showed the least relative water content. This pattern suggests that, in general, the stress tolerant cultivars seem to be better adapted with higher relative water content of leaves.

5.2 Proline is accumulated under ABA and PEG mediated water stress

Roots and shoots of different rice seedlings accumulated proline in response to water stress and ABA. There was a steady increase in proline in all the cultivars tested, with shoots showing higher levels compared to roots (Fig. 4.2.1 A and B). Interestingly, the accumulation of proline in shoots was less during ABA treatment compared to PEG stress. However, proline levels in roots did not show significant differences between PEG and ABA stress (Fig. 4.2.1 A). Increase in proline accumulation was more in roots and shoots of tolerant rice lines than that of the sensitive cultivar Hamsa.

Role of proline accumulation in stress response process in plants is still not clear. Accumulation of proline upon dehydration and under osmotic pressure has been recorded in many organisms ranging frombacteria to eukaryotes. Varietal differences

in accumulation of proline under water stress have been widely reported. Plants that could accumulate more proline showed a better survival under stress condition than those with low proline levels did. Examples include barley, rice and *Sorghum* (Singh *et a.l*, 1973; Mali and Mehta, 1977; Sivaramakrishnan *et al.*, 1988). Kishore *el al.*, (1995) demonstrated that transgenic tobacco plants that accumulate high proline levels are reported to show an improved salt tolerance On the contrary. Ilahi and Dorffling (1982) have reported a negative correlation between proline accumulation and drought resistance in barley. Ibarra-Caballero *et al.*, (1987), however. suggested that proline accumulation under drought stress is not necessarily a true indication of drought stress resistance but rather is a symptom of it.

Proline accumulates upon exogenous ABA application in many plant species (Stewart. 1980; Stewart and Voetberg, 1985; Stewart *et al.*, 1986). Our results also indicate an increased proline accumulation in tolerant varieties during PEG and ABA treatments. Proline is presumed to play a role as a compatible osmolyte in plants, though the evidence is not rigorous. Under stress conditions, proline is reported to be osmoregulatory and might prevent enzyme denaturation (Paleg *et al*, 1984; Rajendrakumar *et al*, 1994). More recently, data from our lab on *in vitro* studies on proline-DNA and proline-protein interactions revealed interesting possibilities of proline mediated protection of biomacromolecules from strong stress-mediated effects of salts in plant cells under stress (Rajendrakumar *et al*, 1997).

5.3 ABA and PEG-mediated water stress affects gene expression

5.5.7 *Induction of specific set of proteins*

The stress-mediated changes in gene expression were studied in terms of qualitative and quantitative changes in polypeptide profiles of seedlings. Specifically, the PEG mediated water stress induced several proteins with mol. wt 45, 33, 23, 21, 18. 15 and 8 kDa. Several other proteins showed either an increase or decrease under stress (Fig. 4.3.1). In fact the 42-kDa protein was completely missing in protein extracts of stressed seedlings. Changes in gene expression resulting either in the synthesis of new polypeptides or disappearance of already present polypeptides is well known in plants under stress conditions (Bray, 1988; Heikkila *et al*, 1984).

There is an increasing evidence for the stress responsive changes in gene expression mediated by a hierarchy of regulatory steps and signal transduction mechanisms (Giraudat *et al,* 1994). In rice, almost not much is known about signal transduction mechanisms associated with stress response.

5.3.2 The stress induced 15-kDa is a Generalized Stress Responsive Protein

We demonstrated the induction of the 15-kDa polypeptide in rice seedlings by PEG mediated water stress, ABA. salt and cold treatment (Fig. 4.3.3A). Further, an earlier report from our group showed the induction of another15-kDa polypeptide in rice cultivars by different concentrations of salt (Rani and Reddy, 1994). Western analysis confirmed the immunological identity of the 15-kDa induced by different stresses (Fig, 4.3.3B). Germinating embryos subjected to different stresses also show the induction of the 15-kDa polypeptide (Fig. 4.3.6). In the light of the above, it is reasonable to argue that the 15-kDa protein is associated with an yet unidentified facet of the general stress response process in rice. We therefore tentatively name this as Generalized Stress Responsive Protein 15 (GSRP15). On the contrary, ABA and PEG mediated water stress induced 23-kDa polypeptide was not induced by cold and salt stress (Rao *et al,* 1993). This suggests that while some stress-induced proteins are common to many types of stresses, certain others are specific to a given stress. The *RAB 16* (Mundy and Chua, 1988) and *SalT* (Claes *et al.* 1990) genes from rice were induced by various stresses such as ABA. water and salt stress. In rice, salt, ABA but not cold or heat shock induced *Em* gene (Bostock and Quatrano 1992).

5.3.3 The 23-kDa polypeptide belongs to the LEA family of proteins

The Late-Embryogenesis-Abundant proteins (LEA) are naturally expressed primarily during the desiccation phase of the seed development in plants and can be induced at other stages of plant development by desiccation stress or by the application of exogenous ABA (Dure *et al,* 1989). Our data from Western blot analysis of seed proteins with anti-23-kDa antibodies clearly revealed that the 23-kDa polypeptide was developmentally regulated in rice, as it is accumulated in seeds and

embryos and induced in vegetative tissues under different stresses (Fig. 4.3.4). Interestingly, anti-23-kDa antibodies not only cross-reacted with 23-kDa proteins but also with several other seed and embryo proteins (Fig 4.3.4). That the LEA proteins from dicots and monocots (Baker *et al,* 1988; Close *et al.* 1989) that share sequence homology with each other (Dure *et al.*, 1989) follow the general developmental norm of appearing in maturing embryo, disappearing during germination and inducible in vegetative parts is well documented. It follows then that the 23-kDa belongs to the LEA family of proteins and is developmentally regulated in embryos, disappears during germination and is induciblc in vegetative tissues by environmental stresses. Further, our results (Fig. 4.3.5 and 4.3.7) demonstrate that the induction of the 23-kDa polypeptide is tissue and stress specific.

5.3.4 Stress proteins are conserved among cereals

Interestingly, the rice 23-kDa antibodies were immunologically cross reacted with a PEG and ABA–induced 21-kDa protein of maize. On the contrary, stress responsive proteins from *Sorghum* did not show any cross reactivity (Fig. 4.3.8). These results suggest that atleast a few of the stress-induced proteins of maize and rice share amino acid sequence homology. Antibodies raised against a maize dehydrin cross-reacted with polypeptides in dehydrating barley and wheat. Barley dehydrins show an extensive sequence homology with dehydrins of wheat and other grasses (Close and Chandler, 1990). These results indicate the similarity among dehydrin proteins and mRNA sequences in different plants (Close and Chandler, 1990). Still *et al,* (1994) showed that rice seed proteins were immunologically cross-reacted with antibodies raised against a conserved dehydrin oligopeptide sequence. Rao *et al,* (1993) reported that the 23-kDa polypeptide was immunologically similar to the RAB 16 proteins from rice (Mundy and Chua, 1988). *Rab16* gene belongs to D-11 of LEA (Group 2) protein family. As the 23-kDa was shown to be immunologically related to the Rabl6, it might belong to the LEA Dl 1 family. Baker *et al,* (1988) suggested that Dl 1 LEA proteins could be involved in the solvation of cytosolic structures under stress. Based on the above information from biophysical and chemical studies on LEA proteins, it is speculated that the 23-kDa protein may have a role in stress response mechanisms, particularly stress mediated by osmotic disturbances.

The PEG induced 23 and 15-kDa are cytosolic in nature and arc not associated with membranes (Fig. 4.3.9 and 4.3.10). Previous reports had also indicated that LEA proteins located in many cell types at different concentrations and with in the cell appear to be predominantly cytosolic (Schneider *et al,* 1993; Mundy and Chua. 1988). The RAB 16 protein induced by ABA and water stress was localized in cytosol of rice tissues. The concentrations are so high that in cotton embryo cells. D7 LEA proteins represent about 4% of non-organellar cytosolic protein (Roberts *et al.*, 1993). Further studies are required to precisely localize these proteins and their intracellular distribution under stress.

5.3.6 Stress induced 15 and 23-kDa proteins are boiling stable and presumably play a protective role in protein denaturation

ABA and PEG- induced 15 and 23-kDa are proteins are boiling stable (Fig. 4.3.11 A). An earlier report from our lab showed that the salt stress induced 15 and 26 polypeptides (Rani *et al.*, 1994) are boiling stable. Cold responsive proteins from *Arabidopsis thaliana* (Lin *et al,* 1990; Gilmour *et al,* 1992). stress responsive proteins from barley (Jacobson and Shaw, 1989; Close and Chandler, 1990) and dehydrins from maize (Chandler *et al,* 1988) were also reported to be boiling stable. All these proteins share sequence homologies and are highly hydrophilic in nature. The boiling stability of the stress-induced proteins suggests they might have a role in protection of the cellular proteins and membrane structures during dehydration. Protein denaturation experiments clearly revealed that the stress-induced boiling stable proteins protect other proteins from denaturation (Fig. 4.3.11B). These observations suggest that the boiling stable PEG and ABA-induced proteins may play a unique role in conferring stability during heat induced coagulation of proteins and membranes.

In *Bromus inermis*, ABA induced heat stable proteins also shown to protect the proteins from denaturation and confer heat tolerance (Robertson *et al,* 1994). Such a protection was more effective in the presence of sucrose. Jinn *et al,* (1989) reported that low mol wt Hsps confer in vitro heat tolerance to soluble thermo-sensitive proteins. Similarly, Hincha *et al,* (1990) demonstrated that a boiling stable protein fraction, isolated from cold-acclimated cabbage, conferred stability during a

freeze-thaw cycle against membrane damage of nonhardy spinach thylakoid membranes. The cold induced a 15-kDa polypeptide. COR15. of *Arabidopsis* is reported to show cryoprotective activity *in vitro* (Lin and Thomashow, 1992). The COR15 was highly effective in protecting the cold labile enzyme, lactate dehydrogenase, against freeze-inactivation. It is concluded that the boiling stability of the stress responsive protein is indicative of a protective function in plants.

5.4 Aldose reductase related protein is identified in rice seeds

Our approach to determine the identity and function of the induced proteins in rice resulted in identification of an aldose reductase-like protein and the enzyme activity (Results 4.4). Bartels *et al,* (1991) first reported a desiccation tolerance-responsive protein from barley embryos showing aldose reductase activity. The identification of a protein with aldose reductase activity during late embryogenesis coinciding with the desiccation tolerance was the first ever indication on the probable utility of the sorbitol biosynthetic pathway in the desiccation tolerance processes in plants.

The identification of aldose reductase assumes importance in view of its role in the synthesis of the well-known osmolytes, sugar alcohols. The accumulation of different osmolytes in cytoplasm in response to various abiotic stress conditions is a well-established phenomenon in plants (Delauny and Verma, 1993). Among the osmolytes, proline. betaine and sugar alcohols have been implicated in osmoregulatory functions in plants under osmotic stress and have been thought to be of great adaptive value based on the biochemical and physiological evidences (Csonka. 1989). However, a direct molecular confirmation of the role of these osmolytes in the stress tolerance however, comes from the genetic manipulation of polyol biosynthetic pathways. The over production of osmolytes, proline, mannitol, and ononitol in cytoplasm of transgenic tobacco plants was proved to confer salinity tolerance (Tarczynski, *et al,* 1993; Kishore, *et al,* 1995; Sheveleva *et al,* 1997). It is believed that that the elaboration of the genetic, biochemical and regulatory elements of the osmolyte pathways would lead to the genetic manipulation of stress response in **rice.**

5.4.1 AR accumulation in seed is developmentally regulated

The accumulation of AR is demonstrated to begin at 15 days after pollination and continue into the maturity stage of rice seed development (Results 4.4.2). In fact. mature seeds show the maximum accumulation of AR and also enzyme activity. Such a developmental pattern of AR accumulation resembles closely to the pattern of accumulation of endogenous ABA reported in rice (Skriver and Mundy 1990). In higher plants, at a specific stage during embryogenesis. the desiccation intolerant embryos become desiccation tolerant, with a concomitant induction of a specific set of genes. Such a stage specific synthesis of proteins is associated with the acquisition of desiccation tolerance. One such gene (pG22-69) product following this pattern was AR, which was thought to be involved in attaining the desiccation tolerance in barley embryos (Bartels *et al,* 1988). AR protein accumulation studies in germinating seeds revealed that it begins to appear at 1-DAG. gradually decline thereafter and disappear by 5-DAG (Fig.4.4.3B). This pattern resembles that of LEA protein accumulation.

5.4.2 AR is responsive to exogenous ABA and other abiotic stresses

Aldosc reductase protein is induced in rice shoots by the application of exogenous ABA. water stress and salinity (Fig. 4.4.4B). Western data was correlated with an increase in enzymatic activities under those stresses. The increase in AR activity was the maximum in response to ABA (50% increase), followed by PEG (40%) and salt stress (35%) (Fig.4.4.4A). However, under cold acclimation, there was no increase either in protein levels or enzyme activity (lane 5, Fig. 4.4.4B). It is concluded that AR seems to be inducible primarily by osmotic stress. It is not clear whether endogenous ABA levels under osmotic stresses mediate AR accumulation in shoots. Bartels *et al.,* (1991) showed that AR is accumulated in desiccated leaves of *Craterostigma*, providing further evidence that it might play a role in osmoprotection. Ln bromegrass cell cultures, the ABA-induced aldose reductase protein accumulation as well as AR activity was correlated with freezing tolerance (Lee and Chen,1993).

The selective induction of this protein in rice seedlings under different stresses is presumably due to the existence of both the ABA-dependent and independent signal transduction pathways in rice. In Arabidopsis, the low temperature induced Lti 140

gene was also induced by ABA and water stress (Nordin *et al.,* 1991). Three separate-but converging signal pathways regulate the expression of the Lti 140 gene.

5.4.3 Induction of AR protein scents to be genotype-dependant

An important observation in the present study is the non-accumulation of AR in ABA–treated shoots of a drought sensitive cultivar. Hamsa (4.4.5A and B). All the other tested cultivars. which arc relatively drought tolerant. accumulate this protein under stress. This could be due to the inherent genetic differences that exist between the varieties These observations taken together. though not rigorously analyzed, suggest that aldosc reductase protein in rice may be associated with desiccation tolerance processes in rice seedlings. It is tempting to speculate that Hamsa plants are genetically deficient in a regulatory element that governs the expression of AR, particularly in vegetative tissues (It is to be noted that the Hamsa seeds do accumulate AR). This is contrary to the general observation that many of the stress-responsive proteins characterized in plants arc induced both in tolerant and sensitive plants. However. our results showed that the tolerant lines selectively accumulate a group of LEA proteins under stress. We believe the AR protein may serve as a marker for tolerance/sensitivity phenotype. Further work is needed to develop the AR marker system in rice. In any case. the data will be of considerable use in genetic characterization of AR locus in rice.

There are reports showing a correlation between organ survival and LEA protein accumulation during dehydration process in plants. In wheat, scuttellum and shoots subjected to dehydration resume normal growth after rehydration that was correlated with accumulation of LEA proteins (Reid and Walker-Simmons, 1993). However, under the same conditions, roots are killed presumably due to non inducibility of LEA proteins. The stress induced endogenous ABA levels and protein profiles were correlated with the level of tolerance of varieties of wheat (Walker-Simmons, 1987) and rice (Lee *et al.,* 1993, Moons *et al.,* 1995). Similarly, analysis of the gene expression in plants influenced by exogenous ABA in genotypes differing in tolerance (Galvez *et al.,* 1993) also indicated such a correlation. The expression level of a group of specific genes have been reported to be correlated with improved salt, desiccation and cold tolerance of cell lines, e.g. the chilling induction of a Gly-rich

protein from alfalfa (Mohapatra *et al,* 1989) and a group 2 LEA protein of wheat (Houde *et al.* 1992). Dehydration induced group 3 LEA mRNA levels were correlated with an increase in endogenous ABA levels in wheat (Curry *et al.,* 1991) and rice seedlings. The dehydrins and group 3 LEA protein levels were significantly higher in roots of tolerant rice varieties than the sensitive ones. The salt tolerant rice line. Pokkali. was shown to accumulate significantly higher amounts of stress induced proteins under a mild stress (Moons *et al,* 1995). Overexpression of barley LEA protein. HYA1 in transgenic rice imparted an improved tolerance to salt and drought stress (Xu *et al.*. 1996). On the other hand. over expression of a *Craterostigma* LEA protein in transgenic tobacco did not confer tolerance (Iturriaga *et al.*, 1992). Thus. overexpression of a single protein alone is not sufficient to confer the tolerance and probably need to be supplemented with expression of other genes controlling accumulation of osmolytes. sugars etc.

5.4.4 AR accumulation is evolutionarily conserved

We have detected the AR protein in mature seeds of other cereals. (Fig. 4.4.6). The results reveal the conservation of AR protein among cereals. This is a logical proposition since plants already have this machinery at their disposal that needs to be activated under stress. The AR protein is involved in the synthesis of sorbitol and its biosynthetic pathway was detected in different parts of plants, such as in developing maize kernels (Shaw and Dickinson. 1984) and in barley seeds (Gohl *et al,* 1978). These observations further strengthen the hypothesis that the recapitulation of some of the evolutionarily conserved embryogenic events in the vegetative tissue through ABA mediation is likely to hold the key to the stress tolerance process in plants. AR is known to function as carbon source and also plays an osmoregulatory role as reported in apples (Raese *et al,* 1978) and in a salt tolerant marsh plant, *Plantago maritima* (Ahmad *et al,* 1979). In bromegrass cell cultures, expression of an aldose reductase gene by application of ABA, which in turn conferred the freezing tolerance was reported (Lee and Chen. 1993). A significant increase in AR activity was detected in bromegrass cells after ABA treatment. These observations clearly indicate that increased AR activity during ABA treatment might be associated with induction of freezing tolerance in bromegrass cells.

Sugars and polyols arc well-known osmoregulators in plants and other organisms. For example, in mammalian renal medullary cells, an increase in AR activity was detected under salinity stress (Garcia-Perez *et al,* 1989; Moriyama *et al* 1989). This protein was homologue to the human NADPH dependent aldose reductase. The results suggest that the osmoregulatory processes were evolutionarily conserved at the level of genera. Our results further confirm that AR is associated with desiccation tolerance mechanisms not only during seed maturation but also in vegetative tissues subjected to ABA and other environmental stresses.

Interestingly, among the tested cereals, only rice accumulates AR under ABA stress in seedlings (Fig. 4.4.7). This is surprising in view of the close taxonomic relationship of these cereals with *Oryzae.* We have no explanation for this observation yet. However, Bartels *et al,* (1991) showed that this protein is induced in desiccated leases of *Craterostigma,* a resurrection plant having an elaborate machinery for withstanding 90% dehydration. Further studies are needed to characterize AR in different plant species clearly differing in their stress tolerance levels to a variety of stress conditions.

5. 5 Antioxidant enzyme levels increase under stress

Our analysis of alterations in antioxidant enzyme levels under stress in seedlings and mature plants of different rice cultivars revealed an increase of various antioxidant enzymes, namely SOD. APX and GR. This increase in enzyme levels is found to be age specific, cultivar specific and also stress specific. Qualitative and quantitative changes in SOD and APX isozyme profiles were also analyzed. This anah sis is done to establish a correlation between mature plants and seedlings under stress. The plants survive during stress conditions by maintaining their defense mechanisms or amplify other protective mechanisms under prolonged stress. There are several reports on increase in antioxidant enzyme levels during drought stress in maize and tobacco (Malan *et al,* 1990; Van Rensburg and Kruger, 1994), ABA stress in maize (Zhu and Scandalios, 1994), cold stress in red spruce, rice (Hausladen and Alscher, 1994; Saruyama and Tanida, 1995) and salt stress in pea (Olmos *et al,* 1994; Hernandez *et al,* 1995). Changes in the enzyme and isozyme profiles in relation to the tolerance of different rice cultivars will be discussed in the following sections.

5.5. J Younger plants accumulate higher levels of antioxidant enzymes than mature plants

As a first step the basal levels of antioxidant enzymes and isozyme profiles both quantitative and qualitative. in seedlings and mature plants were determined and the results (4.5) revealed that the seedling levels are higher than that of mature plants. For instance, the SOD enzyme activity was four times more in seedlings compared to mature plant (4.5.1). Our results are in agreement with an earlier report of Lee and Bennett (1982) which showed that the younger leaves contain higher levels of endogenous SOD than old ones and also exhibit a better resistance to ozone. Differences in resistance can be related to modifications in the constitutive levels of antioxidant enzymes and also non-enzymatic antioxidants. These can be age dependent, species dependent or due to the modification of gene expression. Casano *et al,* (1994) showed that young barley leaves responded to photooxidative stress with an increase in SOD transcripts and activities in comparison with mature plants where SOD decreases in senescing leaves. Further, mature plants showed three SOD isozymes, while seedlings have about six isozymes. However, the significance of this observation is not clear. The APX enzymatic activity is 64% more in seedlings than mature plants of a drought tolerant cultivar, Annada (Results 4.5.1). The GR activity is also higher (about two and half times) in seedlings. These results clearly indicate that there are stage specific differences in antioxidant enzyme activities among the cultivars. Such differences might reflect differences in resistance levels. particularly against oxidants. In any case. there is a wide variation in antioxidant activity levels among plant genera (Reinert *et al,* 1982; Bennet *et al.*, 1984).

5.5.2 Seedling SOD levels are enhanced under ABA and water stress

ABA and PEG mediated water stress lead to a significant increase in SOD activity (Fig. 4.5.2A). There are several reports on the enhanced SOD enzyme activity under various stresses such as water stress in tobacco, sun flower, pea plants (Van Rensburg and Kruger, 1994; Sgherri and Navari- Izzo, 1995; Iturbe-Ormaetxe *et al,* 1998) ABA in rice and maize (Sakamoto *et al,* 1995; Zhu and Scandalios, 1994) and salt stress in pea (Olmos *et al,* 1994).

Stress induced increase in SOD varied among cultivars. Prasanna showed the

maximum increase (about 75%) of SOD activity under both stresses. The other three cultivars showed an average increase of about 50%. SOD levels were also increased in seedlings treated with a mild stress like 10% PEG (Fig. 4.5.2A). In addition, analysis of SOD activity in mature rice plants subjected to ten days of dehydration also revealed varietal differences i.e. Annada. Tulasi. Prasanna. Hamsa in the descending order (Fig. 4.5.3A). In pea plants, an increase in activity of mitochondrial, cytosolic and chloroplastic SOD was observed at a moderate water deficit (Iturbe-Ormaetxe *et al,* 1998).

Rehydration experiments were carried out in order to test the reversal trends in SOD activity upon recovery from stress and the results (Fig. 4.5.2A) revealed that the SOD activity fell significantly upon rehydration in all cultivars except Hamsa. The recovery of seedlings from stress treatment clearly points out that the treatments are not lethal. Hamsa, a sensitive cultivar, did not recover from the stress.

In addition to the enzymatic alterations, the isozyme profiles of SOD of different rice seedlings subjected to water stress and ABA showed (Fig. 4.5.2B) quantitative differences. The most prominent change is the increase in intensity of the slow migrating isozyme, upon stress, in all seedlings, particularly Prasanna (lanes 1, 2, 3 of Fig 4.5.2B). The enhanced isozymes belong to the Cu/Zn SOD class in all the varieties tested. Mature plants after dehydration treatment for ten days (Fig. 4.5.3B) revealed a detectable increase in isozyme levels belonging to Cu/Zn SOD type in all cultivars tested, with Annada and Tulasi showing prominent increase. Olmos *et al,* (1994) reported that Cu/Zn SOD isozymes were induced only in salt tolerant pea calli but not in sensitive calli. Drought and photoxidative herbicide tolerances were both significantly correlated with high levels of Cu/Zn SOD and GR activities under drought stress in maize plants (Malan *et al,* 1990).

Our data on changes in SOD activity under stress revealed that the young seedlings have efficient oxyradical scavenging enzymes in comparison with that of mature plants. Under water stress, Cu/Zn SOD isozymes were more prominently induced in comparison to other isozymes. The genotype dependant differences were clear.

Transcription of the Sod multigene families of rice, SodCc2 (Sakamoto *et al,* 1995) and of maize Sod 3.2, 3.3 and 3.4 (Zhu and Scandalios, 1994) are induced by

ABA. On the other hand. Kupera *et al,* (1997) reported **that** in **pea,** SodCp (CVZn SOD) **and** SodB gene (FeSOD) expression in fact decreased in response to **ABA.** These reports indicate that both SOD and ABA have been implicated in stress tolerance in plants.

Transgenic alfalfa plants overexpressing MnSOD gene from *Nicotiana* are reported to exhibit an improved performance under water deficit at field level, indicating that the manipulation of genes associated with oxidative stress tolerance can improve potentially plant's survival and performance under water limiting conditions (McKersie *et al.,* 1996 and 1993). Transgenic alfalfa plants with overexpressing MnSOD are also reported to be tolerant to freezing stress (McKersie *et al.* 1993). Rigorous analysis of the oxidative stress responses in plants will be useful in genetic improvement of crop plants for stressful environments.

5.5.3 Increase in APX levels and change in isozyme profiles under ABA and water stress

Analysis of ascorbate pcroxidase levels in rice seedlings under ABA and PEG mediated water stress (Fig. 4.5.5A) revealed a consistent increase in the enzyme levels during the entire stress response period. Several reports indicated an increase in APX activity under drought stress in tobacco (Van Rensburg and Kruger, 1994); salt stress in pea (Olmos *et a!.,* 1994) and chilling stress in rice (Saruyama and Tanida, 1995).

APX activity in five day stressed rice seedlings increased progressively and reached the maximum in all cultivars under both stresses. The increase in APX activity was the maximum in Prasanna, followed by the other three cultivars. On the contrary, one day stressed seedlings did not show any detectable increase in APX activity. Recovery experiments showed that there are differences in APX activity levels during rehydration period between the cultivars. The seedling data indicate that the tolerant cultivars seem to accumulate higher levels of APX than the sensitive ones. The exact role of APX in stress tolerance in plants is yet to be elucidated.

Mature plants (45day old), when subjected to dehydration stress for ten days showed (Fig. 4.5.6A) an increase in APX activity in all the cultivars tested. Increase in enzymatic activity was more in seedlings under PEG and ABA treatment than in mature plants under dehydration stress. Van Rensburg and Kruger (1994) reported

that APX activities significantly increased (300-400%) in drought tolerant tobacco cultivars under stress. Interestingly, the increase in APX was more pronounced than that of catalase. This indicates that APX. rather than catalase. might be responsible for scavenging drought stress produced H_2O_2. Pea chloroplasts derived from salt tolerant lines undergoing salt stress showed an increase in APX, ascorbate content and an increased number of plastoglobuli (Hernandez et al. 1995). In sunflower, at moderate level of water stress. the enzymes related to the ascorbate/glutathione cycle were induced. Under severe water deficit. a decrease in efficiency of plant defense mechanisms was reported (Sgherri and Navari- Izzo. 1995). Saruyama and Tanida (1995) reported that APX has a role in chilling resistance al germination stage of cold sensitive and tolerant cultivars of rice.

Analysis APX isozyme profiles of different rice seedlings subjected to ABA and PEG stress (Fig. 4.5.5B. C and D) indicated both qualitative and quantitative differences. Under PEG and ABA, all three APX isozymes increase in all tested cultivars. Most importantly, Prasanna seedlings show induction of a new fast migrating isozyme upon PEG 20% and ABA stress. The slow migrating APX isozyme pattern in Prasanna was entirely different from that of the other three cultivars tested. The identification of stress specific isozyme in Prasanna is important as it may potentially be used as a marker in breeding. There arc very few such markers available for the seedling stage in selection and breeding for drought tolerance in rice. However, this system needs further analysis and refinement in order to be routinely used in lab and field.

Analysis of APX isozyme profiles of mature plants subjected to dehydration (Fig. 4.5.6) showed quantitative differences but no qualitative differences between the tested cultivars. All except Prasanna showed an increase in the intensity of the fast band. Interestingly, APX profiles of mature plants of Prasanna (Fig. 4.5.5C) were also different from that of the other cultivars tested.

Northern analysis (Fig. 4.5.9) of APX using the Arabidopsis APX probe (Courtesy Van Montague, Ghent) revealed two hybridizable bands in Tulasi and Hamsa exposed to PEG 20% and ABA. Further, ABA and PEG treatment both lead to an increase in the APX specific mRNA band. These results further substantiate our conclusion that APX is stress responsive in rice , both at the level of transcription and

translation. Such a stress responsive increase in cytosolic APX mRNA was reported in pea plants exposed to Paraquat (Donahue *et al.,* 1997). Sen Gupta *et al.*. (1993b) have reported a 3-fold increase in APX activity and mRNA expression in transgenic tobacco plants that overexpress chloroplastic Cu/Zn SOD. Transgenic tobacco plants that overexpress cytosolic APX have also showed an increased tolerance to MV damage (Pitcher *et al.*. 1994). but not the Chloroplast targeted isoform (Pitcher *et al.,* 1991). These results indicate that the regulatory mechanisms that control the expression of SOD and APX in tobacco remain intact in transgenic plants and result in changes in the activity of enzymes, including those not encoded by introduced genes. It is reasonable to argue that a combination of different enzymes are needed in a given tissue at a specific developmental stage for an increased protection from oxidative damage.

5.5.4 Increase in Glutathione reductase levels under ABA and water stress

The GR activity levels increased progressively during stress and the extent of increase varied among the cultivars tested (Fig. 4.5.7). GR levels have been reported to increase under drought stress in maize (Malan *et al.,* 1990; Brown *et al.,* 1995) and pea plants (Moran *et al.*. 1994). After five days of stress treatment, GR activities increased to the maximum in all the cultivars tested. The tolerant varieties showed relatively higher increases over the sensitive ones. Upon rehydration of the stressed seedlings, there was no significant change in GR levels. However, GR levels of 10% PEG treated seedlings, upon rehydration, reached almost control levels (Fig. 4.5.7). Increase in GR activity under dehydration stress was recorded in mature plants of all the cultivars tested(Fig 4.5.8) During cold hardening. a specific GR isozyme was found to be induced in the tolerant red spruce plants (Hausladen and Alscher, 1994). Non-hardened plants have other specific GR isozyme which are absent in cold acclimated plants. Our results and earlier reports suggest the role of GR in stress response mechanisms. Interestingly, drought and photoxidative herbicide tolerances were both significantly correlated with high levels of Cu/Zn SOD and GR activities along with membrane integrity. Further, their study revealed that SOD or GR alone didn't correlate with tolerance to both the stresses (Malan *et al.,* 1990).

Targeting the *E.coli* GR into the chloroplasts of transgenic tobacco plants resulted in an increase in tolerance to MV and sulphur dioxide but not to ozone (Aono *et al.* 1993). These observations indicate that exposure to environmental stress can stimulate the plants to enhance their scavenging systems, and this enhancement can apparently provide a generalized stress response. It supports the hypothesis that antioxidant enzymes are critical components in preventing oxidative stress in plants (Allen, 1995).

6. SUMMARY

Summary

Stress responsive changes in protein profiles, seedling growth pattern, and enzyme activities in different rice cultivars were investigated. The rice cultivars include both stress tolerant (Annada, Tulasi and Prasanna) and sensitive (Hamsa). Also, attempts were made to determine the possible function of stress induced proteins. Specific conclusions are given below:

- The root/shoot growth ratios under PEG and ABA stress is prominently increased in all cultivars; tolerant cultivars showing significantly high levels.
- Water and ABA stress involves alterations in gene expression leading to the induction enhancement/reduction of proteins levels. Disappearance of specific proteins was also recorded. In shoots there is a specific induction of 45, 33, 23, 21, 18, 15 and 8 kDa polypeptides.
- The 15-kDa polypeptide was purified and antibodies were raised against it.
- 15-kDa polypeptide is induced during various stresses in shoots and germinating embryos, hence it was coined as Generalized Stress Responsive Protein (GSRP). Its absence in seed indicates that it does not belong to the LEA class of protein.
- 23 kDa polypeptide is accumulated in mature seeds and embryos, thus suggesting that it belongs to LEA class of proteins.
- The induction of 23-kDa ploypeptide is found to be tissue and stress specific. In shoots it is induced by PEG and ABA but not by cold and salt. It is induced in germinating embryos during salt stress.
- 23-kDa protein is immunologically similar to PEG and ABA induced 21-kDa protein from maize, indicating the conserved nature of stress induced proteins.
- The 15 and 23-kDa polypeptides are localized in cytosol.
- Boiling stability of these polypeptides, and their behaviour in protein denaturing experiments revealed that these polypeptides might play a role in protection of cellular protein denaturation during stress.

- An aldose reductase like protein was detected in rice and shown to be induced in shoots by exogenous application of ABA.
- The induction of AR protein is varietal and stress specific. For instance, ABA induces protein in Annada, Tulasi and K39, but not in Hamsa. In K39, AR polypeptide is induced by ABA. PEG. and salt stress but not by a cold stress. The appearance of this polypeptide is correlated with the induction of aldose reductase activity. In seeds, this polypeptide accumulates during late embryogenesis and disappears on gemination. The seeds also show AR activity.
- The presence of this polypeptide in all cereal seeds confirms that this protein is evolutionarily conserved .
- The antioxidant pathway in rice is induced by water and ABA stress. Enzymatic activity of SOD. APX and GR are increased in seedlings and mature plants. This increase was found to be age and cultivar specific with tolerant cultivars showing increased levels.
- The enzymatic activities of APX, SOD and GR were more in seedlings compared to mature plants. The isozyme pattern of APX and SOD varies between seedling and mature plant.
- Of the three enzymes, the APX is induced to a maximum level by ABA and water stress. Prasanna seedlings which show maximum levels of stress induced APX activity also exhibit a distinct isozyme pattern. However, the mature plant extracts show no such pattern. Prasanna seedlings show an additional isozymes under stress. This suggests the utility of this isozyme as a marker in stress tolerance studies in rice.
- In Tulasi and Hamsa seedlings two stress induced APX specific transcripts are detected using *Arabidopsis* cDNA clone as a probe.
- The Cu/Zn SOD activity is found to be stress responsive both in seedlings and mature plants. Isozymes of each of the SOD class are identified .
- Stress enhances the activity of GR in seedlings and mature plants, with tolerant cultivars showing increased levels.

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8. LIST OF PUBLICATIONS

Research Papers published:

1. K.R.K.Reddy, A.H.K.Rao, **B.Karuna Sree**, and A.R Reddy (1993) Water Stress induced 23 kDa polypeptide in cell Suspension Cultures of Rice *(Oryza saliva L.)* is immunologically similar to that of Seedlings. ***J.Plant Physiol*** 141: 373-375

2. A.H.K.Rao, **B.Karuna** Sree and A.R.Reddy (1993) Water stress responsive 23-kda polypeptide from rice seedlings is boiling stable and is related to Rab 16 family of proteins. ***J.Plant*** *Physiol.* 142:88-93.

3. Chadalavada S.V. Rajendrakumar, **B. Karunasree,** & Arjula R. Reddy *(1996)* Molecular approaches to abiotic stress tolerance. pp 112-121. In the ***5th National Rice Biotechnology Network (The Rockefeller Foundation National Grantees Meet),*** ICGEB, New Delhi, Nov-13-16,

Research papers under communication/preparation :

- Identification Of A Generalized Stress Responsive Polypeptide In Rice Seedlings
  B.Karuna Sree, Abbaraju H.Rao and Arjula R. Reddy
  (Under preparation to *Plant Science)*

- Identification of a developmentally regulated and ABA responsive aldose reductase related protein in rice *(Oryza sativa L)*
  B.Karuna Sree, Chadalavada S.V. Rajendrakumar, & Arjula R Reddy
  (Under preparation to *Plant Physiol.)*

- Identification of new Ascorbate peroxidase isozymes induced by water and ABA stress in rice seedlings *(Oryza sativa L.)*
  B.Karuna Sree and Arjula R.Reddy
  (Under preparation to *Plant.Physiol.)*

- Abscisic acid mediated salinity and chilling tolerance in rice requires the induction of stress responsive polypeptides
  Chadalavada S.V. Rajendrakumar, B. **Karuna** Sree & Arjula R.Reddy
  (Under preparation to *Plant Science)*

Abstracts presented at the Conferences from the present investigation:

- PEG induced changes in water stress associated gene expression in rice seedlings. A.H.K.Rao, **B.Karuna Sree** and A.R.Reddy in 3rd international congress of Plant Molecular Biology. 6-11 October 1991, Tucson convention center Tucson, Arizona USA Ed. R.B.Hallick

- Rao AHK. **Karuna Sree B.** Reddy A.R (1992) Response pattern of rice cultivars to different abiotic stresses :Differential induction of specific polypeptides, 631. p 38 in First International Crop Science Congress 14-22 July lowa State Center Ames, lowa USA.

- Rao AHK. **Karuna** Sree B. Radha Rani U, Reddy KRK and Reddy AR (1993) Genetic and Molecular studies on stress in Rice: Identification and characterization of certain stress responsive mRNAs and proteins. 6th International symposium of Rockefeller Foundation Rice Biotechnology Programme, Chungamai, Thailand.

- **Karuna Sree** B, Rao AHK and A.R.Reddy (1994) Characterization of a 15 **kDa** polypeptide induced by PEG mediated water stress. Second Asia pacific confercnc on Agricultural Biotechnology, Madras, 6-10 March, 1994.

- Radharani, U., **Karunasree, B.,** Rajendrakumar, C.S.V., Madhuri, G., Joanny, K.,Padmavathi, M., Jayaseela, S., Reddy, K.R.K., and Reddy, A,.R. (1994) Studies on stress responses in rice. In Third Annual Meeting, National Rice Biotechnology Network, Madras, 3-5 March.

- Rajendra Kumar, C.S.V., **Karuna** Sree, **B.,** Reddy, A.R. (1996)The possible role of stress responsive proteins in the ABAmediated salinity and cold tolerance processes in rice,.In the II International Crop Science Congress, New Delhi, Nov-17-24.

- Reddy, A.R., Reddy, V.S., Madhuri, G., Padmavathy, M., **Karuna Sree, B.,** Rajendrakumar C.S.V., Parvathy, R., and Singh, D.T. Anthocyanin pathway in rice and disease resistance: Acorrelative study .Internatiuonal Programme on Rice Biotechnology September, 15-19. 1997. Malacca, Malaysia.

- **Karuna** Sree, B., Rajendrakumar, CSV., and Reddy, A.R. Identification of a evelopmentally regulated and ABA responsive aldose reductase related protein in rice (Oryza sativa L). 4th Asia Pacific Conference on Agricultural Biotechnology 13-16 July, 1998. Darwin, Australia.

Workshops Attended during doctoral program

"Theory and Methodology for Physiological and Molecular basis of Stress Resistance in Crop Plants" held at IARI, 3-24 April ,1995.

Symposia and Conferences attended :

1. Attended the Second Asia Pacific Conference on Agricultural Biotechnology held at Madras, India between 6-10 March-1994
2. II International Crop Science Congress, New Delhi, Nov. 17-23, 1996.
3. National symposium on current trends in Plant Physiology and Plant Biochemistry University of Hyderabad. Jan 29-31, 1998

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Short Communication

Water Stress-induced 23 kDa Polypeptide in Cell Suspension Cultures of Rice *(Oryza sativa* L) is Immunologically similar to that of Seedlings

K. R. K. REDDY, ABBARAJU H. RAO, B. KARUNA SREE, and ARJULA R. REDDY*

Plant Molecular Genetics Laboratory, School of Life Sciences, University of Hyderabad, Hyderabad - 500 134, India

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Summary

The response of cell cultures to PEG-mediated water stress and ABA treatment has been investigated in several rice cultivars. The SDS-PAGE protein profiles revealed a number of changes, notably the induction of the 23 kDa polypeptide by both the stresses. Western analysis revealed that this 23 kDa polypeptide is the same as the one induced in intact seedlings. These studies demonstrate that synchronised cell populations of rice can be used as a rapid and reproducible experimental test system for evaluating osmotic stress responses and for studying genetics and molecular biology of stress responses in rice.

Key words: Oryza sativa, suspension cultures, water stress, polypeptides.

Abbreviations: **ABA - abscisic acid; 2,4-D = 2,4-dichlorophenoxyacetic acid; PAGE = polyacrylamide gel electrophoresis; PEG = polyethylene glycol; SDS = sodium dodecylsulphate.**

Introduction

Suspension cultured undifferentiated plant cells offer a relatively homogeneous and experimentally controllable alternative for the study of cellular responses to different abiotic stresses (Fallon and Phillips, 1989). The effect of various stresses like water deficit, salinity and ABA in rice has been studied in callus cultures in terms of growth parameters (Kavi Kishore, 1989), accumulation of certain metabolites (Kavi Kishore, 1988), selection and isolation of stress adapted cells (Kavi Kishore and Reddy, 1989) and subsequent plant regeneration (Liu et al., 1985; Reddy and Vaidyanath, 1986). However qualitative and quantitative changes in polypeptides under stress conditions of rice cell cultures of specific genotypes have not been studied in detail. Borkird et al. (1991) reported that water-stressed rice cells as well as cultured organs and tissues show induction of two polypeptides encoded by hsp70 and ubiquitin genes.

Extensive work has been done on the response of cells and whole plants of rice to different stresses. Alteration of gene expression as a specific response of seedlings and mature plants to salt stress and drought have been reported (Mundy and Chua, 1988; Claes et al., 1990). Various rice genotypes have been screened for their stress response in terms of physiological characters contributing to salinity resistance and their relationship to overall performance (Yeo et al., 1990). Salinity and drought stress in rice were extensively reviewed (Caplan et al., 1990). These studies led to the concept of alterations in gene expression under stress conditions, especially those stresses that involve osmotic disturbances. Implicit in this analysis is the assumption that the response of cell cultures to a given stress is similar to that of the whole plant. Therefore it is necessary to test whether the *in vitro* and *in vivo* responses are the same. Borkird et al. (1991) have addressed this problem by comparing cell and tissue (of the cultured organ) responses to different stresses. However, only one genotype was tested. In the present study, cell suspension cultures of a number of different rice lines were tested for their responses to osmotic stress conditions. Our aim is to develop a suitable experimental cell culture system of rice to study the genetic and molecular aspects of stress response in rice. Further we attempt here to test the validity of

* For all correspondence.

using the cell culture responses to stress for interpreting the response of intact organisms, specifically the seedlings. We show here that the stress responses *in vitro* and *in vivo* (intact seedlings), at least in terms ot induction of a specific polypeptide, are similar.

Materials and Methods

Callus cultures of indica rices, *Oryza sativa* cvs. Hamsa, Akashi, Annada and N22 were initiated from mature embryos on a modified MS medium (Murashige and Skoog, 1962). The calli were subcultured every 15 days and the same were used for suspension cultures. The cell suspension cultures were raised on the same medium without agar. One week old suspension cultures were collected through nylon mesh filtration, allowed to drain and resuspended in the same volume of fresh medium supplemented with different concentrations of PEG (5 to 30%) and ABA (50 to 200 µM). Controls received medium only. After 48 hrs of incubation under constant shaking at 100 rpm, the cells were collected and soluble proteins were extracted. The protein extracts were subjected to one dimensional SDS-PAGE according to a modified Laemmli's procedure (Laemmli, 1970). Each lane was loaded with 30 to 50 µg of protein. The gels were stained with Coomassie brilliant blue. Antibodies raised against the PEG induced 23 kDa polypeptide from seedlings (Rau et al., under communication from our lab) were used for Western blotting experiments with HRPO-IgG conjugate-diamino benzidine system of detection.

Results and Discussion

The SDS-PAGE profiles of protein extracts from the control and treated cell suspension cultures of Hamsa revealed that ABA (200 uM) and PEG (10%) treatments lead to the induction of specific polypeptides as well as inhibition of some others. The most prominent effect of ABA and water stress is the induction of two major polypeptides with apparent molecular weights of 15 and 23 kDa (Fig. 1. Lanes 1-3). In order to see whether there are varietal differences in stress

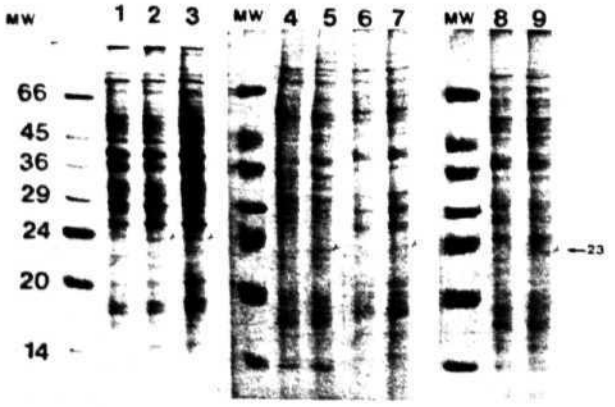

Fig. 1: SDS-PAGE of protein extracts of different rice lines. Lanes 1 and 2. Hamsa control and treated with ABA, Lane 3 Hamsa treated with PEG, Lanes 4 and 5. Akashi control and treated with PEG, Lanes 6 and 7. Annada control and treated with PEG, Lanes 8 and 9. N22 control and treated with PEG.

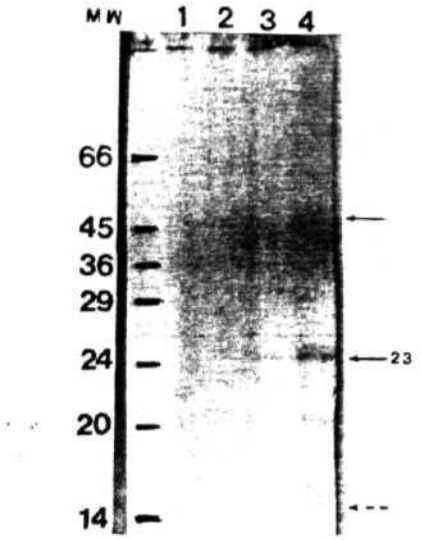

Fig. 2: SDS-PAGE of Hamsa proteins followed by Western blotting and immunostaining. Lanes 1 and 2. Controls loaded with 50 µg and 75 µg protein respectively, Lane 3. PEG, Lane 4. ABA.

response, particularly in the induction pattern, three different rice lines, namely, Akashi, Annada and N22 were tested by SDS-PAGE (Fig. 1. Lanes 4-9). The data clearly reveal that the induction pattern in these cultivars is similar to that of Hamsa.

Whether the PEG and ABA induced 23 kDa polypeptide in cell suspension cultures is the same as the one induced in seedlings was tested by Western blot analysis using antibodies raised against the PEG-induced 23 kDa polypeptide from Hamsa seedlings. The data in Fig. 2 clearly show that the 23 kDa polypeptides of cells and seedlings are immunologically identical. The same is found to be true with the 15 kDa polypeptide (data not shown). The data also demonstrate that the PEG and ABA-induced 23 kDa proteins in cell suspensions are identical.

The present observations show that ABA and PEG-mediated water stress lead to specific changes in polypeptide profiles of cell suspension cultures. It is clear that the response of different rice lines to each of the stress forms in terms of induction of these proteins is similar. Rice cell suspension cultures can thus be used as a rapid and reproducible experimental system to investigate stress response mechanisms in rice. The seedling assay normally takes about 10 days while the cell suspension culture assay takes only two days. Further, the synchronized cell populations provide a homogeneous sample and thus offer additional advantages. Western data further substantiate the above conclusion as there is cross reactivity of this induced protein with the anti-23 kDa antibodies in all the tested lines.

Interestingly, the tested rice lines differ in their relative tolerance to water stress. For instance, Annada and Akashi are relatively stress tolerant, N22 is a drought stress escaper and Hamsa is relatively stress sensitive. Since this protein is present in all the four tested lines under both PEG and ABA treatments, perhaps it represents a generalised component of

the response mechanism to osmotic stress, both *in vitro* and *in vivo*. We conclude that the cell suspension culture system can be effectively used to investigate the mechanisms of stress response in rice.

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Water Stress-Responsive 23 kDa Polypeptide from Rice Seedlings is Boiling stable and is Related to the *RAB16* Family of Proteins

ABBARAJU H. RAO, B. KARUNASREE, and ARJULA R. REDDY*

Plant Molecular Genetics Laboratory Department of Plant Sciences, School of Life sciences, University of Hyderabad, Hyderabad-500134, India

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Summary

The response of rapidly growing young seedlings of certain indica rice *(Oryza sativa* L. Cvs) cultivars to polyethylene glycol (PEG)-mcdiated water stress and abscisic acid (ABA) treatment was investigated. Several water stress-responsive proteins were identified by SDS-PAGE and one such protein, namely the 23 kDa polypeptide, was purified. Using antibodies raised against it, we proved that this protein was also responsive to air drying (desiccation) of seedlings and ABA treatment but not to NaCl and cold treatment. Further, Western analysis revealed that the 23 kDa protein is immunologically related to the *RAB16* family of proteins. Finally, we demonstrated that the 23 kDa polypeptide is boiling stable. In view of its appearance in response to a variety of water stress conditions in both sensitive and tolerant cultivars, its immunological identity with the *RAB16* protein and its novel feature of boiling stability, it is proposed that the 23 kDa protein is associated with a generalized water stress response mechanism and might play a role in water stress tolerance in seedlings.

Key words: Water stress, boiling stability, desiccation, Oryza sativa.

Abbreviations: ABA = abscisic acid; PEG - polyethylene glycol; HRPO IgG - horse radish peroxidase immunoglobulin conjugate; *RAB16* - ABA responsive 16 kDa protein; DAB = diamino benzidine tetrahydrochloride.

Introduction

Rice plants, when subjected to stress situations caused by drought (water deficit), desiccation, ABA and high or low temperatures, respond through an array of diverse morphological, physiological and biochemical adaptive alterations. Although the physiology and biochemistry of drought injury and resistance in rice, including indica rice, have been investigated in some detail, the results could not effectively be used in developing genetics and breeding programmes for drought resistance. The main reason for this gap is lack of precise information on stress response mechanisms and the role of specific genes in the resistance process. Recent experiments from a number of laboratories show that stress response in rice involves changes in the expression of specific gene sequences. Several novel genes associated with stress response have been molecularly cloned, sequenced and their protein products characterized in rice (Mundy and Chua, 1988; Claes et al., 1990; Bostock and Quatrano, 1992), maize (Gomez et al., 1988; Close et al., 1989) barley (Bartels et al., 1988; Close et al., 1989; Hong et al., 1992) tomato (Cuming, 1984; Bray, 1988; Marcotte et al., 1989; Plant et al., 1991) wheat (King et al., 1992) *Craterostigma plantagineum* (Bartels et al., 1990; Piatkowski et al., 1990) and *Arabidopsis thaliana* (Hajela et al., 1990; Gilmour et al., 1992; Shinozaki et al., 1992).

In rice, stress response was investigated by detailed analyses of some induced novel polypeptides under different types of stress conditions. Such stress-induced proteins in rice include the ABA responsive *RAB16* family (Mundy and

* For reprint requests.

Chua, 1988), which show sequence homologies to a late embryo abundant (*Lea*) protein family (Galau et al., 1986; Dure et al., 1989), salt tolerant protein (*sal T*) (Claes et al., 1990) and *Em* proteins in rice (Bostock and Quatrano, 1992) and wheat (Williamson and Quatrano, 1988). These proteins with distinct structural features are suggested to play a role in stress resistance (Close and Chandler, 1990). Such comparative studies on the water stress response of both stress sensitive and tolerant indica lines in terms of induction of polypeptides are rare, nor is there much information about properties of such stress-induced novel proteins. One way to test whether the accumulation of stress-induced proteins is correlated with the stress tolerance of the plant is to compare well defined rice lines distinctly differing in their stress tolerance.

The present report deals with a comparative analysis of water stress response in both sensitive and tolerant indica rice cultivars in terms of induction of specific proteins and analysis of their novel features. We show here that the PEG-mediated water stress leads to the induction of several proteins in both sensitive and tolerant cultivars. We purified one such induced protein from seedlings and demonstrated that it exhibits the unique property of boiling resistance. Further, we show here that this protein is related to the *RAB16* family of proteins in rice.

Materials and Methods

Rice Stocks

Five indica rice (*Oryza sativa* L.) cultivars differing in their relative tolerance to field drought were used. These lines are classified on the basis of their field performance (yield) in multi-location and multi-season yield trials by rice breeders. Hamsa is a relatively drought-sensitive, medium-maturing cultivar, whereas Annada, Tulasi and Akashi are relatively drought tolerant elite cultivars. N_{22} is an early-maturing, drought escaper. All these lines were obtained from the Directorate of Rice Research at Hyderabad, India. They were grown in the field or in a greenhouse (pot culture) and the mature dry seeds were harvested and used for seedling culture.

Seedling Culture and Stress Treatment

Seeds were imbibed in water in clean glass beakers and surface sterilized by treatment with 5% sodium hypochlorite (v/v) for 5 min and were thoroughly washed with sterile water. Germination was on moistened filter papers under dark conditions. The average temperature during seedling culture was 28 °C ± 2 °C. After 5 days, the seedlings were treated with 40% polyethylene glycol (PEG-6000, Sigma) for 48 hours (PEG osmotic pressure, −3.0 MPa). Control plants received only water. The stressed seedlings continued to grow at a significantly reduced rate. The slow and prolonged stress would presumably allow the seedlings to adjust to changes in osmotic potential. Shoots and roots were separately harvested and quick frozen in liquid nitrogen and stored at −80 °C until further analysis. The same protocol was used for abscisic acid (200 µM, Sigma) treatment. For air drying, the seedlings were left on the lab bench until 80% loss in fresh weight was achieved.

Protein Extraction and SDS-PAGE

Total proteins were extracted according to Goday et al. (1988) with minor modifications. Shoot tissue was finely ground and extracted with buffer (0.5 g/mL) containing 0.25 M Tris-HCl, pH 8, 0.4% sodium dodecyl sulfate, 20 mM EDTA, 2 mM phenylmethyl sulfonyl flouride and 5% β-mercaptoethanol. By mixing and boiling for 5 min. The extract was centrifuged at 12,000 × g for 10 min, and the proteins in the supernatant were precipitated with 15% (v/v) trichloroacetic acid at 4 °C. The pellet was washed 4 times with cold abolute methanol containing 0.4 M ammonium acetate and vacuum dried. The pellet was resuspended in a known quantity of sterile double-distilled water. Protein content was determined by a modified method of Lowry et al. (1951). SDS-PAGE was performed according to Laemmli (1970).

Purification of the 23 kDa Polypeptide

Both control and treated samples were subjected to preparative SDS-PAGE and the gels were stained with coomassie. The induced 23 kDa band was identified by comparison with that of the control, and the band was cut out and electroeluted. The eluted protein was precipitated in cold acetone at −80 °C over-night. The pellet was collected by centrifugation and resuspended in 10 mM sodium phosphate buffer (pH 7.4). The purity of the 23 kDa polypeptide was confirmed by SDS-PAGE.

Anion Exchange HPLC of the 23 kDa Polypeptide

The partially purified 23 kDa polypeptide was applied to a Shimpack PA-DEAE column (8 × 100 mm) equilibrated with sodium phosphate and critic acid. Protein was eluted with a linear gradient formed from sodium phosphate (100 mM, pH 7.4) and citric acid (100 mM at pH 7.4) at a flow rate of 1 mL/min for 20 min. The purified 23 kDa protein (about 200 µg) was used to raise anti- 23 kDa antibodies in a rabbit. The specificity of the antibodies was confirmed by single radial immunodiffusion using both purified 23 kDa protein as well as whole extracts of induced seedlings.

Western Blotting

The SDS-PAGE gels of control and treated samples were electrophoretically irreversibly blotted onto nitrocellulose filters (Hoeffer Scientific) according to the manufacturer's specification. The filters were stained with ponceaus-s solution and the molecular weight standards were marked. At this stage induced proteins were clearly visible. The nitrocellulose blots were probed with the anti 23 kDa antibodies. Detection using the Horse radish peroxidase (HRPO) anti-IgG conjugate was achieved by incubating the filters in the presence of the precipitable substrate, diaminobenzidine tetrahydrochloride and hydrogen peroxide.

Heat Stability of the 23 kDa Polypeptide

Boiling stable proteins were extracted by the method of Lin et al. (1990). Crude protein samples from control and treated seedlings were boilied in a water bath for 25 min and the insoluble material removed by centrifugation in Eppendorf microfuge (15 min 10,000 rpm). The soluble polypeptides remaining in the supernatant were precipitated with 7 volumes of chilled acetone overnight and collected by centrifugation. The pellet was resuspended in the SDS-PAGE loading sample buffer and electrophoresed.

Results

Water stress-Induced Polypeptides in Shoots

The SDS-PAGE profiles of PEG treated Hamsa shoot protein extracts (Fig. 1) revealed the induction of three poly-

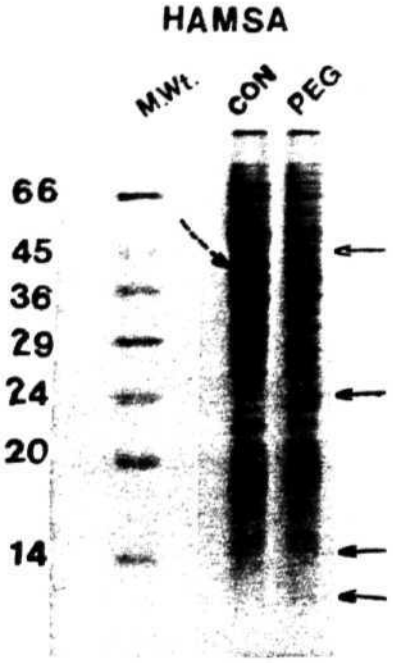

Fig. 1: SDS-PAGE (15%) of proteins extracted from shoots of the Hamsa cultivar. (50 µg of protein was loaded into each slot). Arrows indicate the PEG induced proteins. Broken arrow indicates the disappearance of a protein.

peptides with apparent molecular weights of 15, 23 and 46 kDa. In contrast, a major protein with an apparent molecular weight of 42 kDa totally disappeared under stress. In addition, the relative abundance of several polypeptides was also affected by PEG. These are, however, not considered here in detail. Whether these three polypeptides are also inducible by ABA in all cultivars was tested through SDS-PAGE. The profiles in Fig. 2 A and B clearly reveal that both PEG and ABA induce these proteins in all of the tested cultivars. It is to be noted here that the 42 kDa protein disappeared under stress in all of the cultivars. However, this protein was absent in N_{22} control itself. It appears that as far as these stress-induced proteins are concerned, both sensitive and tolerant cultivars exhibit qualitatively similar responses.

Purification of water stress-Responsive 23 kDa Protein

The 23 kDa protein was purified from PEG treated shoot extracts of Hamsa by SDS-PAGE and electroelution. The purified protein shows a single band on SDS-PAGE (Fig. 3) and a single peak on HPLC (Fig. 4). The purified 23 kDa protein (200 µg) was used to raise antisera in a rabbit. The specificity of the antibodies was confirmed by single radial immunodiffusion.

Western Blot Analysis

Using the polyclonal anti-23 kDa antibodies, we tested whether the PEG/ABA-responsive 23 kDa protein is also induced by other osmotic stress conditions (Fig. 5). The Western blots of crude extracts of shoots from seedlings treated

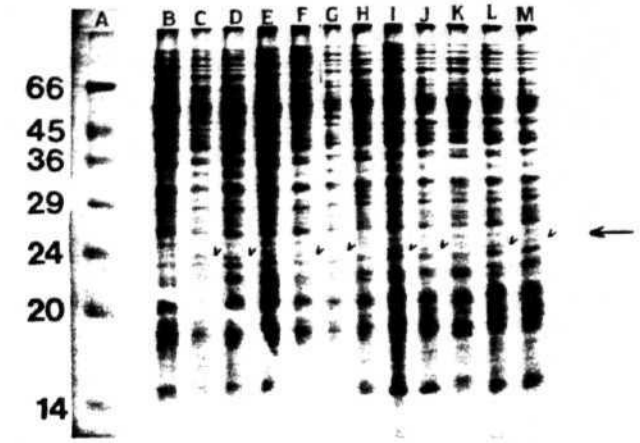

Fig. 2 A

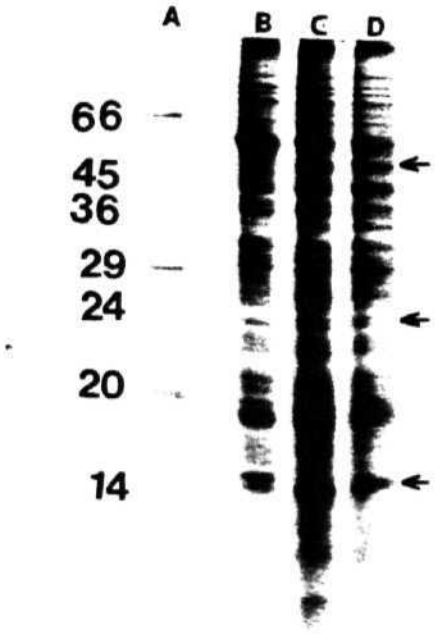

Fig. 2 B

Fig. 2 A: SDS-PAGE profiles of shoot extracts of different cultivars treated with PEG and ABA. Lane A Molecular wt markers; Lanes B, C and D Hamsa control, PEG and ABA treated; Lanes E, F and G Tulasi control, PEG and ABA treated; Lanes H, I and J Annada control, PEG and ABA treated; Lanes K, L and M Akashi control, PEG and ABA treated (each lane except G (20 µg) received 50 µg of protein). «V» indicates the induced protein.

Fig. 2 B: SDS-PAGE profiles of shoot extracts of N22. Lane A = mol. wt. markers; Lane B = control shoots; Lane C = PEG treated; Lane D = ABA treated (each lane was loaded with 50 µg of protein).

with PEG, ABA, cold acclimation, air drying and NaCl treatment revealed cross reactivity with anti 23 kDa antibodies in all except NaCl and cold samples. Further, another protein with an apparent mol. wt. of 46 kDa also shows up in all, except in NaCl treated samples. This protein, which is responsive to PEG, ABA and desiccation,

Fig. 3: SDS-PAGE of crude protein extracts of Hamsa. Lane A = mol. wt. markers; Lane B = Hamsa control (75 µg of protein); Lane C partially purified 23 kDa protein (10 µg of protein).

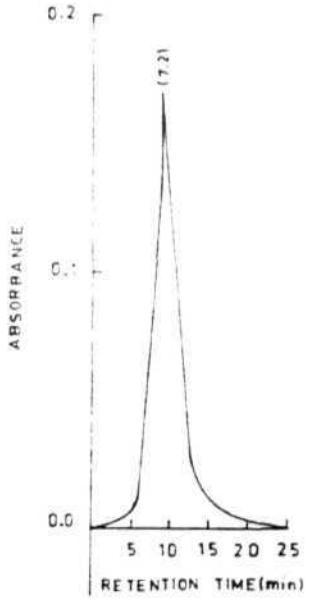

Fig. 4: HPLC profiles of the partially purified 23 kDa protein (details in material and methods).

might possibly share some sequence homology with that of the 23 kDa protein. If it is to be considered as a simple dimer of the 23 kDa protein, then we have no explanation for its intriguing presence in cold treated samples in which the 23 kDa protein is missing. Whether the 23 kDa protein shows any homology with that of the other stress-induced proteins like RAB16 protein (Mundy and Chua, 1988) was tested by Western blotting. Data in Fig. 6 show that the

Fig. 5: Immunodetection of the 23 kDa protein by anti-23 kDa antibody in shoot extracts of Hamsa seedling; A = control, B = PEG, C = ABA, D = NaCl (1 M for 2 days, 5 days after germination), E = air drying of seedlings and F = cold acclimation (4 °C for 5 days, 5 days after germination). 75 µg of protein was loaded into each slot; Note that the 23 kDa band is missing in lanes D and F.

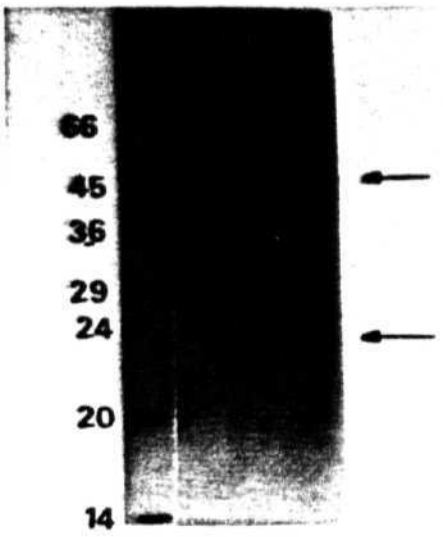

Fig. 6: Immunodetection of the 23 kDa protein by anti-*RAB16* antibodies in protein extracts of Hamsa. A = control, B = PEG, C = ABA. (75 µg of protein was loaded into each slot).

23 kDa protein shows cross reactivity with anti-RAB16 antibodies. Further, the 46 kDa protein was also detected.

Heat Stability of the 23 kDa Protein

In order to test whether the stress-induced proteins are heat stable, the crude protein extracts of shoots treated with PEG and ABA were boiled for 25 min in a boiling water bath and the protein fractions were subjected to SDS-PAGE. The protein profiles in Fig. 7 show that the 23 kDa protein in both PEG and ABA lanes remains detectable after boiling, while a majority of proteins disappeared.

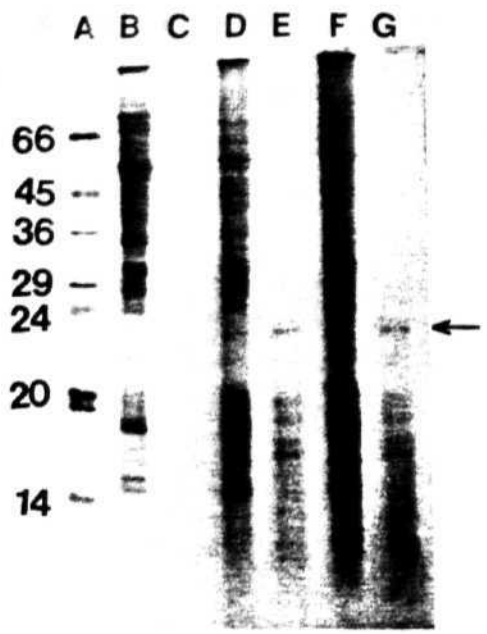

Fig. 7: SDS-PAGE of boiling stable proteins from shoot extracts of Hamsa. A = mol. wt. markers, B = control, C = control boiled, D = PEG treated, E = PEG treated-boiled, F = ABA treated, G = ABA treated-boiled (75 µg of protein was loaded in each lane).

Discussion

The water stress-Responsive 23 kDa Protein is also Responsive to several but not all Osmotic stresses

All of the tested cultivars show the induction of the 23 kDa polypeptide under water stress and ABA. Western analysis using the anti-23 kDa antibodies clearly revealed that the water stress induced 23 kDa protein was also induced by ABA and air dryng but not by cold acclimation and NaCl treatment (Radha Rani and Reddy 1991). These results suggest that the 23 kDa protein might belong to a class of proteins that are associated specifically with the water stress response pathway(s) rather than the cold and salt stress response pathways. In plants, there is more than one response pathway for cellular osmotic adjustment and different proteins may be associated with different pathways. For instance, a group of *Lea* proteins associated with seed maturation pathways (Baker et al., 1988; Dure et al., 1989; Close et al., 1989; Curry et al., 1992) is also associated with ABA as well as dehydration response pathways (Skriver and Mundy, 1990) but not with salt stress response. The *Em* (early methionine labelled) polypeptide that belongs to the *Lea* family is associated with ABA and salt stress response pathways but not heat shock or cold-responsive pathways in rice (Bostock and Quatrano, 1992). Interestingly, the last two stress conditions might also cause cellular dehydration. We conclude that the 23 kDa Protein is associated with a generalized water stress response mechanism in rice as it appears in both stress sensitive and tolerant cultivars. Infact, we demonstrated that the 23 kDa protein was also induced in cell suspension cultures of several indica rice cultivars under PEG and ABA stress (Reddy et al., 1993). It is proposed that this protein may be used as a water stress response marker in rapid mass screening of rice seedlings for stress responses. For instance, appearance of this protein in seedlings under a given stress, other than the water deficit, would mean either that particular stress also causes a water stress situation or the recovery process involves a typical water stress response mechanism.

The 23 kDa Protein is Immunologically Similar to RAB16 Protein

The Western blot data using anti *RAB16* antisera (Courtesy of John Mundy) clearly demonstrate that the 23 KDa protein is homologous to the *RAB16* protein at the immunological level (independenly confirmed by Dr. John Mundy, Carlsburg). Interestingly, however, the *RAB16* family of proteins is both cold and salt responsive (Mundy and Chua, 1988), whereas the 23 kDa is not. This suggests that the 23 kDa protein is only partially homologous but not identical to *RAB16* protein. In the absence of sequence data we cannot be definitive about this possibility. Although, proteins from diverse sources, such as *Lea* proteins from cotton (Galau et al., 1986; Baker et al., 1988; Dure et al., 1989) the *Em* proteins of rice (Bostock and Quatrano, 1992) wheat (Williamson and Quatrano, 1988) and dehydrins in barley, maize (Close et al., 1989; Close and Chandler, 1990) and rice (Bradford and Chandler, 1992), desiccation resistance proteins (deduced from the cDNA sequence) of *Craterostigma plantagineum* (Piatkowski et al., 1990) share sequence homologies with the *RAB16* proteins, they are not identical to it.

Boiling Stability and Possible Physiological Function of the 23 kDa Protein

Our results demonstrate that the 23 kDa protein is boiling resistant. In plants, several stress-induced novel proteins are found to be boiling stable. Prominant examples include the cold responsive (*cor*) proteins of *Arabidopsis thaliana* (Lin et al., 1990; Hajela et al., 1990; Gilmour et al., 1992) boiling stable proteins in barley (Jacobson and Shaw, 1989; Close and Chandler, 1990) and dehydrins of maize, (Chandler et al., 1988). All of these proteins share specific sequence domains and are highly hydrophilic in nature. Such proteins are presumably associated with osmotic adjustment mechanisms by their very nature of hydrophilicity. In the absence of sequence data we can only speculate that the 23 kDa protein might play a role in water stress tolerance. Experiments are underway to sequence the protein and also molecularly clone the gene.

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