STUDIES ON RESPONSE TO ABIOTIC STRESS IN RICE *(Oryza sativa L):* IDENTIFICATION OF CERTAIN mRNAs AND PROTEINS

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DECLARATION

I here by declare that the work presented in this thesis has been carried out by me under the supervision of Prof. A. Ramachandra Reddy and this has not been submitted for a degree or diploma of any other University.

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CERTIFICATE

This is to certify that Mr. A. Harikishan Rao, has carried out the research work embodied in the present thesis under my supervision and guidance for the full period prescribed under the Ph.D. ordinance of this University. I recommend his thesis entitled "Studies on Response to Abiotic Stress in Rice (Oryza sativa, L.) : Identification of certain mRNAs and proteins" for submission for the degree of Doctor of Philosphy of this University.

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TO MY MOTHER

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A. Harikishan Rao

CONTENTS

ABBREVIATIONS

ABA	Abscisic acid
ABRE	Abscisic acid responsive **element**
BSA	Bovine serum albumin
cDNA	**Complementry** deoxyribonucleic acid
dCTP	deoxy cytidine triphosphate
DEPC	Diethyl pyrocarbonate
EDTA	Ethylene **diamine** tetra acetic acid
g	Centrifugal force
HPLC	High performance liquid **chromatography**
kDa	Kilodalton
M.W.	Molecular Weight
PAGE	**Polyacrylamide** gel electrophoresis
PMSF	Phenyl methyl sulfonyl **flouride**
PBS	Phosphate buffered saline
RAB	Responsive to Abscisic acid
rpm	revolutions per minute
SDS	Sodium dodecyl sulphate
SSC	Sodium citrate buffer
TEMED	**N,N,N,N-tetramethyl** ethylene diamine
Tris	Tris (hydroxy methyl) **amino** methane
TCA	Trichloro acetic acid
µgm	**microgram**
µl	**microlitre**

INTRODUCTION

Rice is one of the most important cereal crops of the world. About 60 percent of the world's population use rice as the staple food, Mostly in the third world countries with a total production amounting to 407 million tonnes. Although in terms of area and production, rice crop is second to wheat, it is the foremost cereal crop of the world, providing 26 percent of the world's supply of calories and about 17 percent of the protein.

More than 95 percent of the whole World's rice is produced as well as consumed in South and South East-Asia. The monsoon areas of Asia have the widest surface area under paddy cultivation, about 130 million hectares. India is the largest rice growing country in the World with approximately 40 million hectares under cultivation which accounts for 28 percent of the total area and 16 percent of the total world rice production. Although India has the maximum area under rice cultivation, it is one of the lowest in the world in terms of yield per hectare. The world average rice yield is 2615 kg/ha whereas India average yield is only about half of that. The low yield in India is attributed to several factors like socio-economic conditions, lack of inputs, disease, pests, soil, drought etc. Among these factors, drought is the most important constraints which influence rice yields in India.

Approximately 600 million people live in semi-arid areas of the World. More than 80% of them live in the semi-arid tropics and of them, 50% live in semi arid tropical India. Thus, nearly 40% of the total world's population is affected by drought. Of the 146 million hectares of land on which rice is grown, only one half is irrigated, and the rest, which accounts for one quarter of grain production, suffers from significant yield losses due to frequent drought periods. Moreover, in many irrigated rice soils, hard pans considerably affect root growth and thereby impede water extraction leading to water stress conditions. Concerted efforts were made by a number of countries to reduce the chance of crop failure due to drought by improving crop and soil management.

Evolving new drought tolerant cultivars by breeding was the main approach for crop improvement in semi-arid tropics. A basic and thorough understanding of the effects of drought stress on plant and crop performance is required for the succesful development of drought resistant lines. Conventional plant breeding and genetics have significantly contributed to the development of rice lines which are relatively tolerant to water stress. However, these advances were made largely on the basis of emperical understanding of the response patterns of rice plants to water stress. The approach was to measure the water stress response of the whole plant and crop in terms of yield and other agronomical attributes to select drought resistant varieties for breeding programmes.

The physiology and **biochemistry** of drought injury and resistance in rice, including indica rice, have been investigated in some detail. However, the results could not effectively be used in developing genetic 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. On the contrary, comparative studies on stress response patterns of sensitive and tolerant cultivars of rice are rather sporadic.

Our present work deals with the analysis of certain stress - induced biochemical and physiological changes in rice seedlings, and a comparative analysis of stress response in both sensitive and tolerant indica rice cultivars. A number of rice cultivars were analysed for their response to water stress, ABA and cold acclimation **interms** of their proline **accumalation,** protein and **mRNA** profiles. Of the several stress responsive polypeptides a water stress induced 23kDa protein was detected, isolated, partially purified and antibodies were raised against it. The induction of the

23kDa protein was tested in a number of cultivars under different stress conditions using Western blotting technique. We showed that the 23kDa protein exhibits the unique property of heat stability. Further, this protein is demonstrated to be related to the RAB16 family of proteins in rice. The induction of several stress response associated gene specific mRNA were demonstrated by Northern gel blots and Dot-blot studies. These include the transcripts detected by the desiccation resistance specific cDNA sequence pcC 27-04 of Craterostigma plantagineum and salt tolerane associated SalT cDNA sequence of rice. We have attempted to develop specific marker proteins for both water stress response and general stress response pathways in rice.

LITERATURE REVIEW

Effect of water deficit on cereal crop performance has been extensively investigated using agronomical, physiological and often biochemical methodologies. Various aspects of drought response and osmoregulation in a number of crop plants have been extensively reviewed (Baker, 1989)). In the present context literature pertaining to three selected parameters of stress response, namely (a) proline accumulation (b) changes in specific protein profiles and (c) mRNA accumulation in cereal plants is reviewd.

Abscisic acid Response During Stress Conditions:

A wide range of physiological responses observed during plant development has been attributed to a relatively few and structurally simple phytohormone molecules. Each hormone can elicit a number of quite varied responses in differet tissues.

Abscisic acid (ABA) is a phytohormone which has been implicated in the control of a wide range of physiological processes in higher plants (Addicott, 1983). Chemically, ABA is a sesquiterpene synthesized from mevalonic acid through the carotenoid pathway (Taylor *et al.*, 1988; Zeeraart & Creelman, 1988; Parry & Horgan, 1991) and exists in higher plants in both free and conjugated forms (Li amd Walton, 1987) .

Much of the research over the past several decades in higher plants has demonstrated that physiological responses to ABA, such as **stomatal** closure, inhibition of precocious **germination** of embryos and tolerance to stress are dependent upon the levels of ABA and the sensitivity of the competent tissue to ABA.

Endogenous concentration of ABA increases in leaves during the **drought-induced** reduction, inavailability of water associated with soil dehydration. This has been correlated with a decrease in stomatal conductance, which might be expected to reduce leaf water loss (Kramer, 1988). Similarly, it was possible to demonstrate the increase in endogenous concentration of ABA during other environmental **stresses** e.g. wounding (Pena-cortes et ***al.***, 1990) and cold acclimation (Guy, 1990). In addition to it's role as a local signal in these stresses, there are data which suggest that ABA participates in long distance signalling within the plant (Pena-cortes et *al.,* 1990; Davis & Zhang, 1991). In this case, it was proposed that ABA is transported from the site of stimulus perception to another location where it brings about a characteristic response. However, in most cases the role of various processes (e.g. transport, synthesis, compartmentalization) in the modulation of ABA levels has not been elucidated. Mechanism of action of abscisic acid at the cellular level in plants was reviewed by Hetherington and Quatrano, (1991).

Cold Stress:

Temperature is one of the major environmental constraints, governing the distribution of wild and cultivated plants. Many tropical and subtropical **crops,** for example **rice,** maize, sorghum, citrus and tomatoes, as well as frost **resistant** plants such as barley are grown close to the climate limits of their cold tolerance in some parts of the world. These plants are seriously injured by low temperatures below 12°C but above the freezing point (Lyons, 1973).

Perhaps the most dramatic manifestation of cold acclimation, or cold hardening, is the increased freezing tolerance that occurs in many plant species (Levitt, 1980; **Sakai** and Larcher, 1987). The woody perennials, Birch and Dogwood offer two extreme examples. Whereas non-acclimated trees are severely injured or killed by temperature of about -10°C, trees that are fully cold acclimated can survive experimental freezing temperature upto -19°C in their natural environments. These trees can survive even at temperatures of -40 to -50°C. Other plants including a number of **important** crop species, can attain intermediate levels of frost hardiness. For examples, non acclimated wheat and rye are killed at temperature between -5 and -10°C, but after hardening, wheat can survive a temperature of about -15 to **-20°C** and hardened rye can survive **from** -25 to -30 C.

A number of biochemical changes have been shown to occur in plants during cold acclimation. One example is the alteration in lipid composition. There is a general increase in lipids, especially phospholipids, and in the degree of unsaturation of fatty acid chains during growth of most plants at low temperature (Clarkson et *al.*, 1980, Quinu et *al.*, *1978,* Sikorska et *al.*, 1979, Smolenska and Kuiper, 1977). These alterations are in accordance with the need to increase the fluidity of membranes at cold temperature (Berry and Raison, 1982). The importance of linolenic acid has been emphasized by the decrease in chilling resistance of cotton seedlings and of cold hardiness in cereals when these plants were treated with an inhibitor of linolenic acid synthesis (St. John et *al.*, 1979).

During low temperatures, plants were shown to accumulate new isozymes, increased level of sugar, soluble proteins, proline and certain other organic acids (Levitt 1980;Sakai and Larcher, 1987; Steponkus, 1984). Some of these changes, such as the alterations in lipid composition, appears to have a role in the increased frost tolerance of acclimated plants (Steponkus and Lynch, 1989). These and other reported changes potentially comprise or mediate modifications that increase the overall fitness of the plant for low temperature survival. In most cases, however, the precise role of each of such changes in the cold acclimation process is not yet certain.

The biochemical, biophysical and physiological changes that occur in plant cells during cold acclimation could be brought about by preexisting macromolecules such as enzymes, structural proteins, lipids and membranes that undergo changes in their physical properties at low temperature.

Much of the research on cold acclimation has been aimed at understanding the mechanism(s) of frost tolerance of acclimated plants. Initial studies primarily on determining the critical forms of injury that occur in plants during a freeze-thaw cycle. Further it was suggested that membrane damage results primarily from severe dehydration during the freeze-thaw cycle. It is clear that the tolerance to freezing must also include tolerance to dehydration stress.

" Expansion-induced lysis" is one form of cell and membrane injury in response to relatively high freezing temperatures, about -3 to 7°C (Steponkus 1984). As temperature drops below 0°C, the extracellular water of the plant begins to freeze, resulting in a lowered water activity and an increased solute concentration in the extracellular spaces. In response to these changes in chemical and osmotic potentials, water moves out of the cells, causing a severe dehydration and subsequent cell shrinkage. When the extracellular ice melts, the cells rehydrate and expand. In order to survive, the plasma membrane of cells must be able to withstand efflux and influx of water. This occurs in frost hardernd cells. The non-acclimated cells simply lyse.

Weiser (1970) suggested that cold acclimation involves changes in gene expression. Indeed, there is now evidence that alteration of gene expression occurs durng cold acclimation in a wide variety of plant species; Arabidopsis (Guy et *al.*, 1985; Kurkela and Franck, 1990; Hajela *et al.*, 1990; Gilmour et *al.*, 1992; Lin et *al.*, 1990); Barley, (Cattivelli and Bartels, 1990; 1989). Rice, (Hahn and Walbot, 1989), Spinch (Guy *et al.*, 1985), Brarsicanapus (Meza-Basso et *al.*, 1986), Alfalfa (Mohapatra et *al.*, 1987), Bromegrass (Robertson et *al.*, 1987) and Potato (Tseng et *al.*, 1991). The role of such gene alteration in the acclimation and tolerance response mechanism in plants is yet to be clarified.

Proline Accumulation:

Among the amino acids which accumulate during water deficit in plants, proline turned out to be the most prominant. Accumulation of this amino acid upon dehydration due to water deficit or increasing osmotic pressure has been recorded in several organisms ranging from bacteria to eukaryotes. Measures (1975) suggested that the proline accumulation is a primitive response in all living organisms under a variety of environmental stress conditions.

In barley, the proline accumulation is rapid upon exposure of the leaves to dehydration and the free proline increase in leaves primarily depends on the length of exposure to water deficit (Singh *et al.*, 1973;

Sivarainakrishnan *et al.*, 1987). The rate of proline accumulation during water deficit changes according to the leaf water status. Rajagopal (1977) showed that, the proline content of unirrigated field grown wheat leaves reaches the maximum level at mid day, which correlates closely with the time of minimum relative water content of the leaves. Increased proline accumulation not only occurs during water deficit but also during cold (Charest and Phan, 1990) and salt stress (Chu et *al.*, 1976) conditions. Some halophytes like Triglochin maritima accumulate free proline upto 123 µg/mg fresh weight (Stewart and Lee, 1974) .

ABA and proline accumulation are two of the widely known metabolic responses to stress in higher plants (Hanson and Hitz, 1982). Proline also accumulates upon application of exogenous ABA 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 (Stewart & Voetberg, 1985). Proline synthesis and utilization in plants appears to be associated with stress response. Several reports indicate that proline is associated with osmoregulation. Although there is extensive information on stress induced proline accumulation in a range of higher plants, it's exact correlation with stress response and tolerance of the plant still remains debatable.

Stress responsive proteins;

Stress responsive changes in gene expression in general and protein profiles in particular have been targeted for intensive investigation. It was clear that plants under stress situations under go a series of adaptive alterations in cell metabolism. These adaptive changes originate at molecular level. Oneway to analyse such stress induced cellular and molecular events is to study induction or inhibition of synthesis of specific proteins and transcriptional status of the genes encoding these proteins. A growing body of evidence suggests that stress response involves synthesis of one set of proteins and degradation of the other. Different classes of proteins accumulated under stress have been identified, isolated and characterized in a number of plants. The following is a description of such sets of proteins and genes encoding these proteins,

RAB (Responsive to **abscisic** acid) Genes and Proteins:

The well known phytohormone ABA mediates a number of important physiological processes in plants, especially during seed development and in response to osmotic stress (review Skriver and Mundy, 1990; Hetherington and Quantrano, 1991). In rice, Mundy and Chua (1988) isolated a cDNA clone, pRAB21, which encodes a **glycine-rich,** cytosolic 16.5 kDa protein from rice (RAB21) that accumulates in embryos, leaves, roots and suspension cultures in response to ABA.

The primary sequence of RAB21 (now refered to as RAB16) contains a duplicated domain structure, each containing an A and B peptide repeat. Such repeats are conserved (Dure et *al.*, 1989). The RAB16 proteins are encoded by at least four tightly linked genes spanning about a 30 kb region on the rice chromosome (Mundy and Chua 1988, Shinozaki et *al.*, 1989). The four proteins encoded by these genes are highly homologous (65-92%) and share several conserved peptides, including the B peptide, serine cluster and TGGAYG sequence. The mRNAs of all four RAB genes, except Rab D are expressed in mature embryos.

The sequence comparison of promoter regions of the known Rab genes revealed two conserved sequence motifs. While one such motif was homologous (80%) to the transcription-modulating SP1 binding site (Briggs *et al.*, 1986), the other (PuTACGTGGCPu) was similar to the cAMP-responsive element (Deutsch et *al.*, 1988). In a transgenic tobacco plant, the expression of a chimeric gene containing 5' sequence of the rice rab-16B gene fused to the β-glucuronidase (GUS) reporter gene (Shinozaki et *al.*, 1990) was tested. In zygotic embryos, GUS activity begins to accumulate 10 days after flowering and increases until seed maturation at 25 Days after flowering. Immunological measurements of endogenous abscisic acid revealed a close parallel between ABA levels and GUS activity. It was demonstrated that a 482 bp sequence at the 5' end of the rice rab-16B promoter is responsible for the developmentally

regulated expression in embryos but not ABA responsive expression in vegetative tissue of transgenic tobacco. This elegent analysis reveals the basis of tissue specific response to stress.

Gomez et *al.* (1988) have reported a 15 kDa glycine-rich protein from maize containing an RNA binding domain (Mortonson and Dreyfuss, 1989) is inducible in embryos and seedlings by ABA and desiccation. The 15 kD protein was localized in scutellar epidermal cells of the embryo and accumulates in water-stressed seedlings (when endogenous ABA levels are elevated) and in redponse to leaf wounding.

Pla et *al.* (1991) have isolated a new maize gene, rab 28 that responds to ABA treatment. This gene has been characterized by determining the sequence of the cDNA and corresponding genomic copy. The rab 28 gene encodes a protein of predicted molecular weight 27,713 kDa which shows strong homology with the Lea D-34 protein identified in cotton (Baker et *al.,* 1988). The promoter proximal region contains the conserved ABA response element, CACGTGG, reported in other plant genes to be responsible for ABA induction. The rab 28 mRNA has been shown to be ABA inducible in embryos as well as in young leaves. It was also induced by water stress in leaves of wild type maize plants. It was shown that the rab 28 transcripts do not accumulate to a significant level during embryogenesis. Induction of rab 28 mRNA can be achieved in young embryo by exogenous ABA

treatment. Regulation of the rab 28 gene was studied in excised young embryos of ABA dificient VP2 mutants, in which influence of the maternal environment is absent.

Vilardell et *al.* (1990) reported that the ABA-induced MA12 cDNA in maize encodes a set of highy phosphorylated embryo proteins. This gene, called RAB 17 encodes a basic glycine rich protein (mol.wt 17, 164 kDa) containing a cluster of 8 serine residues, seven of them contiguous. It is a homologue of the rice Rab-16 (Mundy and Chua 1988) - Phosphoamino acid analysis of the protein indicates that only the serine residues are phosphorylated and a putative casein type kinase phosphorylatable sequence was identified. The pattern of expression and *in vivo* phosphorylation of the RAB-17 protein was studied during the germination of maize embryo and also in calli of the both meristamatic or embryonic origin. ABA treatment was shown to induce the synthesis of RAB-17 mRNA and protein in calli. Interestingly the RAB-17 proteins were found to be highly phosphorylated only in embryos.

King et *al.* (1992) reported the DNA sequence of an ABA responsive gene, rab 15, expressed in water stressed wheat roots. The predicted amino acid sequence of rab 15 showed significant regions of homology with the reported amino acid sequence of other member of RAB family, DHN and LEA proteins from both monocots and dicots. The highly conserved domains are present between amino acids 52 to 88 and 130 to 150 of wheat RAB-15 sequence in comparison with other RAB, DHN and

LEA proteins from rice (Mundy & Chua, 1988) maize (Vilardell et *al.,* 1990) barley (Close et *al,* 1989) tomato (Godoy et *al.,* 1990) Craterostigma plantagineum (Piatkowski et *al.,* 1990) and cotton (Baker et *al.,* 1988; Dure et *al.,* 1989).

Late Embryogenesis Abundant (Lea) Genes and Proteins:

In angiosperms, seeds are developmentally programmed to go through a period of dehydration during the maturation process. The embryonic cells of a developing seed are capable of tolerating severe desiccation. The biochemical and genetic basis of this desiccation tolerance has been extensively investigated. Recently, much attention has been focused on a group of genes, called Lea (late embryogenesis abundant) in a number of cereal plants. Based on the regulation and their amino acid sequences, these proteins are suggested to play an important role in desiccation tolerance as osmoprotective proteins or as desiccation-damage repair proteins (Dure et *al.*, 1989; Galau et *al.*, 1987). The Lea proteins represent a diverse group of distinct proteins and are encoded by small multigene families. They are strongly hydrophilic (Baker et *al.,* 1988; Close et *al.,* 1989; Dure et *al.,* 1989). The Lea gene specific mRNA levels were found to be most abundant just before the terminal desiccation of the embryo (Galau et *al.,* 1987; Hughes and Galau, 1987, 1989). When excised immature embryos were treated with ABA or subjected to an osmotic stress, or simply desiccated, many of the Lea mRNAs will accumulate to dry, mature seed levels

(Galau *et al.*, 1986; Hughes and Galau, 1991; Morris et *al.*, 1990; Mundy and Chua, 1988). In dicot embryos, the increased accumulation of Lea mRNAs is simply an effect of embryo desiccation (Galau et *al.*, 1991; Hughes and Galau, 1991). The stress induced increase in mRNA levels is primarily due to the enhanced transcription rates (Comai and Harada, 1990; Williamson and Quatrano, 1988). However, post-transcriptional regulation is probably also involved, at least during exogenous ABA treatment (Marcotte et *al.*, 1988, 1989; Williamson and Quatrano, 1988).

Although not much progress has been made in elucidating Lea protein function in plants, significant advances have been made in understanding the ABA regulation of Lea gene expression in monocots. Investigations were carried out to look for interaction between ABA response elements (ABRE) in specific sets of genes and DNA binding proteins. In rice and wheat, several putative ABREs have been identified (Guiltinan et *al.*, 1990; Mundy et *al.*, 1990), and in wheat a protein with affinity for an ABRE has been cloned and sequenced (Guiltinan et *al.*, 1990). An activity in nuclear extracts that interacts with the response ABRE from the 5' regulatory region of the wheat Era gene was identified. A complementry DNA clone was subsequently isolated whose product was shown be a DNA binding protein (EMBP-1) that interacts specifically with an 8-base pair (bp) sequence (CACGTGGC) in the ABRE,

Recently, the gene product of the *viviparous 1* (*Vp1*) gene in maize was shown to activate transcription from a wheat ABRE promoter of the Em gene in transformed protoplasts (McCarty *et al.*, 1991). However, the transduction pathway of ABA and it's suggested connection to other Lea-inducing stress factors are still unknown. For instance, in cotton embryos during normal development and in cultured wheat embryos there was no clear correlation between endogenous ABA levels and Lea mRNA abundance (Galau et *al.*, 1987, 1991; Morris et *al.*, 1990). Even more ambiguous is the role of ABA in the vp mutant embryos of maize which are capable of synthesizing at least some Lea or other ABA-responsive mRNAs (Kriz et *al.*, 1990; Pla et *al.*, 1989; Williams and Tsang, 1991). Pla et *al.*, (1991) showed that the Lea mRNA (rab 28) were induced by exogenous ABA or water stress in embryos and vegetative tissues of the ABA-insensitive mutant *vp1*. Further, the mRNA level of the VP1 mutant embryos was found to be unaffected by ABA (McCarty et *al.*, 1991). Thus, the question whether ABA is involved in the induction of Lea genes in plants during normal development remains to be answered.

Since some of the Lea proteins in dicots and monocots were found to be extremely conserved (Dure et *al.*, 1989), their regulatory functions might also be conserved. Using the cotton Lea cDNAs as probes (Espelund *et al.*, 1992), several Lea genes are found to be conserved at the mRNA level in dicots and monocots. By screening a barley cDNA library

with a cotton lea D19 cDNA, a family of three Lea members were isolated. The putative B19 proteins have strong similarities to the Em protein in wheat and to Lea proteins from several dicots. However, the middle part of the B19 proteins consists of a 20-amino acid motif repeated, three and four times in B19.3 and B19.4 respectively, but only once in B19.1. Their gene products were found to be strongly hydrophilic and the internal 20 aminoacid motif is the most hydrophilic part. This motif was found to be present once in cotton lea D19 but repeated twice in cotton lea D132, suggesting that the repeats are universal among B19 like genes of monocot and dicot plants. During embryo development, B19 family of proteins are differentially regulated by ABA and various types of osmotic stress. In immature embryos, all three genes are responsive to ABA and mannitol. The B19.1 gene is responsive to salt but not to cold.

Dehydrins:

Dehydrins represent a group of closely related proteins whose abundance in cereal seedlings increases during a gradual non-lethal dehydration conditons (Close *et al.*, 1989). Several cDNAs related to an ABA-induced gene from barley aleurone were isolated from barley and corn seedlings that were undergoing dehydration. From the nucleotide sequences of the cDNAs, four different barley polypeptides namely 22.6, 16.2, 14.4 and 14.2 kDa and a single corn

polypeptide with an apparent mol. wt. 17.0kDa were predicted. These dehydration-induced proteins, called dehydrins were found to be very similar to each other and to a previously identified rice protein induced by ABA and salt (Mundy & Chua 1988). Further, they were found to have at least some similarity to a cotton embryo protein (Baker et *al.*, 1988). Dehydrins are hydrophilic, glycine-rich, cysteine and tryptophan-free and also contain repeated units in a conserved linear order. A lysine-rich repeating unit occurs twice in each protein, once at the carboxyltenninus and once half way through the polypeptide, adjacent to a sucession of serines. A less conserved repeating unit was found to be branched by two copies of the lyine-rich unit.

In dehydrating seedlings, the mRNAs corresponding to each of the dehydrin cDNA are abundantly produced. The amino acid sequence of tryptic peptides from purified dehydration induced proteins revealed that they correspond to a protein that is produced in abundance during the response of corn seedlings to dehydration. Antibodies raised against a maize dehydrin cross-react with polypeptides in dehydrating barley and wheat seedlings (Close & Chandler, 1990). Barley dehydrin cDNA hybridises to RNA present in dehydrating wheat and other grass seedlings. These results indicate the similarity of dehydrin proteins and mRNA sequences, in different plants including wheat and barley. Two barley dehydrin genes were assigned to chromosome 6 (dhn3 and dhn4) and to chromosome 7 (dhn1 and dhn2) using barley dehydrin

cDNA clones and DNA from wheat, barley and additional lines as probes. Genes on the same chromosome are found to exhibit a greater similarity in sequence than those found on different chromosomes.

Bradford and Chandler (1992) showed a North American wild rice (Zizania palustris var interior [Fassett] Dore) tolerant to dehydration upto < 10% moisture content (fresh weight basis). In comparison, seeds of paddy rice (Oryza sativa L.) readily tolerate desiccation to < 5% water content. Expression of "dehydrin-like" proteins in Zizania and Oryza seedlings and embryos was examined to understand the relationship between these proteins and desiccation tolerance. Both Zizania and Oryza synthesize a novel heat stable protein of apparent mol. wt 20 kDa when dehydrated to < 70% of their initial fresh weight. ABA (100 µM) was shown to induce the synthesis of a protein with similar molecular weight in both species. Western blots using anti-dehydrin antibodies of maize (Zea mays L) detected a protein band from dehydrated Zizania shoots and mature embryonic axes that comigrated with the labelled 20 kDa polypeptide. Northern hybridization with rab 16a showed that both seedligs and excised embryonic axes of Zizania accumulated RNA of similar sequence to rab16a in response to water loss. Zizania seedlings and embryonic axes were also capable of ABA accumulation during dehydration, indicating that the intolerence of Zizania seeds to dehydration is apparently not due to an absence of dehydrin-like proteins or an inability

to accumulate ABA.

An antiserum raised against dehydrin from Zea mays recognised several polypeptides in extracts of pea (pisum sativum) cotyledons. Roberton and Chandler (1992) prepared a cDNA expression library from mRNA of developing cotyledons and screened with the anti-dehydrin antiserum and several positive clones were isolated, and characterised. The sequence, both DNA and amino acid analysis of one such clone pPsB12 revealed regions of significant amino acid sequence similarity to dehydrins from other plant species. The deduced amino acid sequence of the pea dehydrin encoded by B12 is 197 amino acids in length, has a high glycine content (25.9%), lacks a tryptophan and is highly hydrophilic. This polypeptide has an estimated molecular mass of 20.4 kD a and pI=6.4. An *in vitro* synthesised product from this clone comigrates with one of the in *vivo* proteins recognised by the antiserum.

A comparison of the pea dehydrin sequence with that of the other species revealed conserved amino acid regions. Unexpectedly, pea dehydrin lacks a stretch of serine residues which is conserved in other dehydrins. B12 mRNA and dehydrin protiens accumulated in dehydration stressed seedlings are associated with elevated levels of endogenous ABA. Exogenous ABA induced expression of dehydrins in unstressed seedlings. Dehydrin expression was found to be reversible. During pea cotyledon development, dehydrin mRNA and proteins accumulated

in mid to late embryogenesis. Dehydrin proteins were some of the most actively synthesised at about the time of maximum fresh weight and represent about 2% of protein in mature cotyledons.

Early methionine labelled (Em) Genes and Proteins:

The Em protein was first identified as a major product of the *in vitro* translation of poly (A) RNA obtained from dry mature wheat embryos (Cuming and Lane, 1979; Grzelezak et *al.*, 1982). It was later shown that Em protein accumulation begins late in the eiabryogenesis and the level of accumulation can be changed in culture by exogenously applied ABA (Williamson et *al.*, 1985). The Em gene encodes a hydrophilic protein of the late-embryo abundant Lea class (Dure et *al.*, 1989) which is one of the most abundant proteins in embryos of dry seeds of cereals such as wheat and rice (cuming 1984; Grzelezak et *al.*, 1982; Quantrano et *al.*, 1992; Williamson and Quatrano, 1988). Levels of Em mRNA were shown to increase dramatically during maturation process of wheat (Quatrano et *al.*, 1992) and maize embryos (McCarty et *al.*, 1991). The expression of the Em gene was also observed in vegetative tissue exposed to exogenous ABA or osmotic stress (Hetherington & Quatrano 1991, Quatrano et *al.*, 1992). The Em gene was molecularly characterized (Cuming 1984; Litts et *al.*, 1987, 1992) and the ABA response element (ABRE) has been identified (Guiltinan et *al.*, 1990; Marcotte et *al.*, 1988; Marcotte et *al.*, 1989 and Quatrano et *al.*, 1992). Regulation of the Em gene expression by ABA has been shown to

occur at the transcriptional as well as at a posttranscriptional levels (Williamson & Quatrano, 1988). Bostock and Quatrano (1992) characterized the Em expression in rice suspension cultures derived from immature rice embryos under treatment with various concentrations of ABA, a high osmoticum and NaCl. It was demonstated that NaCl and ABA are synergistic in controlling Em mRNA levels. Further, they showed that NaCl operates not only through changes in ABA levels but also through an independent pathway that changes the sensitivity of rice cells to ABA.

Desiccation Responsive Genes and Proteins:

Resurrection plants constitute a distinct class of plants which exhibit the remarkable property of tolerance to severe desiccation (Bartels *et al.*, 1990; Galf 1971). These plants can survive in a dry state resuming their physiological activities immediately upon rehydration. Recently several desiccation-related, ABA-responsive cDNAs have been cloned (Bartels et al., 1990) from a resurrection plant Craterostigma plantagineum - (Scrophulariaceae). Five of such cDNA clones were further characterized in detail (Piatkowski et *al.,* 1990). Their nucleotide sequences were determined and proteins were predicted with a molecular mass between 16 and 34 kDa. Three of these proteins were reported to have unusual amino acid composition and exhibited extreme hydrophilic characters. Putative protein deduced from the cDNA sequence of two such clones pcC 27-04 and pcC 6-19

revealed contiguous serine residues and lysine-rich repeats. These sequence motifs display partial homologies to desiccation-related genes expressed in embryos or dehydrated seedlings of several plants. The pcC 3-06 cDNA clone shows partial sequence homology to that of cotton Lea gene (Baker *et al.,* 1988). Secondary structure predictions suggest that the deduced proteins could play a role in protecting core cell structure in dehydrated cells. The expression patterns of all five transcripts were studied in desiccated leaves, dehydrated roots, wound stressed leaves and salt-stressed callus.

Another desiccation related nuclear gene, (dsp-22 desiccation stress protein) from Craterostigma plantagineum encoding a mature 21 kDa protein which accumulates in the chloroplast (Bartels et *al.*, 1992) was described. Sequence analysis indicates that dsp-22 is closely related to early light inducible genes (ELIP) of higher plants and to a carotene biosynthesis related gene (cbr) from a green alga (Lers *et al.,* 1991a). In contrast to other desiccation-related genes, the dsp-22 gene expression is positively regulated by light. Further, it was shown that this interesting interaction of light and stress responsive gene expression opens up new vistas in stress research in cereals.

Borkird et *al* (1991) have isolated polyethylene glycol (PEG) adapted rice cells that can proliferate in a medium

with 20% PEG as well as in a medium with 1% NaCl. The adapted-cells over produce a set of proteins. A cDNA Library constructed and differentrally screened and 5 cDNA clones which showed preferential hybridization to mRNA of adapted cells were isolated. Analysis of cDNA sequences reveled that the pOS4.1 (one of the cDNA clone) is identical (83.3%) to that of the maize heatshock protein (hsp 70). pOS2.3 is 88.2% identical to the barley ubiquitin. However the identities of the other 3 cDNA sequences are not known. The expression of all 5 genes fluctuate slightly during the growth cycle of the PEG adapted cells grown in control or in PEG-containing medium. In contrast, gene expression in parental cells fluctuates to a much greater extent and always begins with an enhanced expression during the first day after subculture.

In tomato, the accumulation of ABA has been shown to regulate some of the changes in gene expression which occur during water-deficit (Bray, 1988; Cohen and Bray, 1990). Several cDNA clones that represent genes which are expressed in response to ABA during drought stress, have been isolated. The ABA deficient mutant of tomato, flacca, synthesizes low levels of ABA during water deficit compared to the wild type (Lycopersicon esculentum Mill C.V. Ailsa Craig). The mutant flacca was used to distinguish cDNAs corresponding to response to elevated levels of ABA from those mRNAs which do not respond to ABA. A differential screening of a cDNA library yielded three cDNAs identified as pLE4, pLE16 and

pLE25. The corresponding mRNAs were preferentially expressed in wilted wild type leaves and were not expressed in wilted ABA deficient mutant leaves. The cDNA clone pLE16 is expressed specially in arial vegetative tissue (Plant *et al.,* 1991). The aminoterminus of the deduced protein is found to be hydrophobic and characteristic of a signal sequence that target polypeptides for export from the cytoplasm. There is a partial sequence homology (47.2% identity) between the amino terminus of the LE 16 polypeptide and the corresponding amino terminal domain of the maize phospholipid transfer protein. Lel6 was shown to express in drought stressed leaf, pet'ole, and stem tissue and to a much lower extent in the pericarp of mature green tomato fruits and developing seeds. In contrast, no expression was detected in the pericarp of red fruit or in drought stressed roots. Expression of le16 was also induced in leaf tissue by a variety of other abiotic stresses including PEG mediated water stress, salinity, cold and heat stress. None of these stresses or direct application of ABA induced the expression of le16 in the roots of same plants. The unique expression characteristics of this gene suggests a novel regulatory stress response mechanism.

Organ-specific and environmentally regulated expression of two ABA induced genes of tomato was demonstrated (Cohen *et al.,* 1991). DNA sequence analysis of these genes showed that the deduced polypeptides were 13.9 and 9.3 kD respectively. Each polypeptide was hydrophilic, cysteine and tryptophan

free, and found to be similar to that of the Lea proteins (Baker et *al.,* 1988). The mRNAs of these two genes accumulated in a similar **organ-specific** pattern in response to specific abiotic stresses. The endogenous ABA is an important regulator of this gene in response to drought. This gene is similar to the cotton Lea gene **D113** (Cohen and **Bray,** 1992) . The **mRNA** of the other gene accumulated predominantly in drought stressed leaves. The reported similarities and differences in the accumulation characterstics of these two **mRNAs** indicate that more than one mechanism exists for the regulation of their corresponding **genes.**

Transfer of soybean seedlings to a low-water-potential vermiculite (ψ - -0.3 MPa) resulted in a reversible decrease in hypocotyl growth and modulation of several **polysomal** mRNAs **(Creelman** et *al.,* 1990). **Creelman** and Mullet (1991) reported the isolation of two cDNA clones (pGE16 and pGE95) which correspond to genes whose **mRNA** levels are increased, and one cDNA clone (pGE 23) which corresponds to a gene whose mRNA levels is decreased in the hypocotyl zone of cell elongation under water deficit condition. In well-watered seedlings, mRNAs hybridizing to two such genes (pGE 16 and pGE 95) are found to be the most abundant in mature regions of the seedling. However, in water-deficient seedlings, mRNA levels are reduced in mature regions and enhanced in elongating regions. The mRNA corresponding to soybean **proline-rich** protein 1 **(sbPRP1)** shows a similar tissue distribution and response to water deficit. In contrast, in

well-watered seedlings, the gene corresponding to pGE23 was highly expressed in hypocotyl and root growing zones. Transfer of seedlings to low-water-potential vermiculite caused a rapid decrease in mRNA hybridizing to pGE23. Sequence analysis revealed that pGE23 shows significant homology with β-tubulin. Water deficit also reduced the level of mRNA hybridizing to JCW1, an auxin-modulated gene, although with a different kinetics. Furthremore, mRNA encoding actin, glycine-rich proteins (GRPs), and hydroxyproline-rich glycoproteins (HRGPs) were down-regulated in the hypocotyl zone of elongation of seedlings exposed to water deficit. No effect of water deficit was observed on the expression of chalcone synthase, an enzyme associated with flavonoid biosynthesis. Decreased expression of β-tubulin, actin, JCW1, HRGP and GRP and increased expression of sbPRP1, pGE95 and pGE16 in the hypocotyl zone of cell elongation could participate in the reversible growth inhibition observed in water-deficient soybean seedlings.

Salinity Responsive Genes and Proteins:

Protein changes induced by salinity stress have been evaluated in roots of the salt-sensitive rice cultivar, Taichung native 1 (Claes et *al.*, 1990). At least eight proteins were shown to be specifically induced by salinity stress as analysed by two-dimensional PAGE. Using an oligonucleotide probe based on partial sequence of a salt induced 15 kDa polypeptide, a cDNA clone, called salT, was isolated. The salT sequence encodes a slightly acidic (pI

5.5) protein, 145 residuces in length, containing small glycine rich repeats reminiscent of RAB16, but apparently does not share any direct homology with that of RAB21 or any known ABA responsive protein. Interestingly, the extent and tissue specificity of salT expression was consistent with the pattern of sodium ion accumulation in various tissues of salt grown plants, the salT mRNA levels were found to be highest in sheath tissue, lowest in leaves, and intermediate in roots. The salT gene was shown to be activated by ABA, air drying, polyethylene glycol, sodium chloride (1%) and potassium chloride (1%).

Godoy et *al.*, (1990) reported a tomato cDNA, TAS14, whose expression was inducible by salt stress and ABA. It's nucleotide sequence predicts an open reading frame encoding a highly hydrophilic and glycine-rich (23.8%) protein of 130 amino acids. TAS14 mRNA accumulates in tomato seedlings upon treatment with NaCl, ABA or manitol. It is also inducible in roots, stems and leaves in hydroponically grown tomato plants. However it is not induced by cold and wounding. The sequence of the predicted TAS14 protein shows five structural domains similar to the coding sequence of PLE4 (Cohen et *al* 1991), rice RAB16 (Mundy and Chua, 1988) Cotton LEA D11 (Baker et *al.*, 1988), and barley and maize dehydrin genes (Close et *al.*, 1989).

Singh et *al.* (1989) reported that cultured tobacco cells, (Nicotiana tabacum Cv Wisconsin 38) on adaption to

NaCl, synthesize a 26 kDa protein which has been named **osmotin** due to it's induction by low water potential. This protein constiutes as much as 12% of total cellular protein in adapted cells. A cDNA clone of osmotin has been subsequently isolated. Abscisic acid induces osmotin **mRNA** in adapted cells to approximately 15 fold higher than in unadapted cells. Osmotin **mRNA** levels remain constant through the growth cycle of adapted cells. While in unadapted cells, the level decreases during exponential phase of growth and increases again when the cells approach the stationary phase. While ABA induces the osmotin mRNA, a low water potential environment appears to be required for accumulation of the protein. The increased expression of osmotin in adapted cells is not correlated with an increase in osmotin gene copy number. Osmotin is homologous to a 24 kDa NaCl induced protein in tomato (King et *al.,* 1988) as well as to thaumatin (Edens et ***al.,*** 1982), maize **α-amylase/trypsin** inhibitor (Richardson et ***al.,*** 1987) and a tobacco mosaic virus-induced pathogenesis related protein (Cornelissen et ***al.,*** 1986).

A **chimeric** gene fusion of the osmotin promoter and **β-glucuronidase** was transferred to tobacco by **Agrobacterium** mediated transformation (Kononowicz et *al.,* 1992). A specific pattern of temporal and spatial regulation of the osmotin promoter during normal transgenic plant development and after adaptation **to** NaCl was demonstrated. The osmotin promoter was found to have a very high natural level of

activity in mature pollen grains during anther dehiscence and in pericarp tissue at the final desiccating stages of fruit development. GUS activity was rapidly lost after pollen germination. The osmotin promoter thus appears to be unique among active pollen promoters described to date in that it is active only in dehydrated pollen. The osmotin promoter was also active in corolla tissue at the onset of senescence. Adaptation of plants to NaCl highly stimulated osmotin promoter activity in epidermal and cortex parenchyma cells in the root elongation zone; in epidermis and xylem parenchyma cells in stem internode; and in epidermis, mesophyll, and xylem parenchyma cells in developed leaves. The spatial and temporal expression patterns of the osmotin gene appears consistent with both osmotic and pathogen defense functions of the gene.

Gaxiola et *al* (1992) have isolated a novel yeast gene, HAL1, which upon over expression improves growth under salt stress. In addition, disruption of this gene decrease salt tolerance. Therefore HAL1 is reasoned to constitute a rate-limiting determinant for halotolerance. It encodes a polar protein of 32 kDa located in the yeast cytoplasm and unrelated to published sequences in data banks. The expression of this gene is increased by high concentrations of either NaCl, KC1 or sorbitol. The growth advantage obtained by over expression of HAL1 was found to be specific for NaCl stress. In cells with overexpression of HAL1, sodium toxicity seems to be counteracted by an increased

accumulation of potassium. The HAL1 protein could interact with the transport systems which determine intracellular K^+ homeostasis. The HAL1 gene and the encoded protein are conserved in plants (Arabidopsis, Maize, Tomato) being induced in these organisms by salt stress and abscisic acid. These results suggest that yeast serves as a convenient model system for the molecular biology of salt tolerance in plants.

Cold Stress Responsive Genes and Proteins:

In Arabidopsis, Hajela *et al.* (1990) reported the isolation of a number of cDNA clones carrying four cold-regulated (cor) genes and examined their expression in response to low temperature, ABA, water stesss and heat shock. Northern data revealed that the transcript levels for all four cor genes, increased markedly between 1 and 4 hours of cold treatment, reaching a maximum at about 8 to 12 hours, and remained at elevated levels for as long as the plants were under cold stress. Cold acclimated plants on returning to normal temperature resulted into a rapid fall in transcript level. Nuclear run-of transcription assays indicated that the temperature-regulated expression of all the cor genes, except one was controlled primarily at the posttranscriptional level. Northern analysis also indicated that the levels of cor gene transcripts increased in response to both ABA treatment and water stress but not to heat shock.

Of several cold regulated cDNA sequence determined by Gilmour *et al.*, (1992), one cDNA corresponds to a cor gene designated cor 47, that encodes a 47 kDa hydrophilic polypetide. Cor47 shows amino acid sequence homology with Group II Lea proteins (Dure *et al.*, 1989). Another cDNA sequence designated cor 66, encodes an alanine-rich 66 kDa hydrophilic polypeptide. Lin and Thomashow (1992) showed a cDNA clone, Corl5, for a cold regulated gene encoding one of the boiling-stable polypeptide. DNA sequence and deduced amino acid sequence indicated that the N-terminus of Corl5 protein closely resembles transit peptides that target proteins to the stomatal compartaments of chloroplasts. Immunological studies indicated that Corl5 is processed *in vivo.* Cryoprotection assay of Lin and Thomashow (1992) showed that the Corl5 was very effective in protecting the cold-labile enzyme lactate dehydrogenase aganist freeze - thaw inactivation. It was shown to be more effective as a cryoprotectant than sucrose and certain proteins including bovine serum albumin.

A novel gene, **Kinl** was shown to be inducible at +4°C in Arabidopsis seedlings (Kurkela and Frank, 1990). The nucleotide sequences of both the genomic clone and the corresponding cDNA were determined and the deduced 6.5 kDa polypeptide shows an unsual amino acid composition rich in alanine, glycine and lysine. Northern blot analysis revelaed that the level of kinl mRNA was increased 20 fold in cold treated plants. In addition to being expressed in cold, Kinl

was also induced by water stress and the plant hormone abscisic acid which has been shown to be a common mediator for osmotic stress responses and cold acclimation in higher plants. Sequence comparison showed that the Kin1 gene product has similarities to a fish antifreeze protein (Pickett et *al.,* 1984). Orr et *al.,* (1992) reported that the cDNA and deduced amino acid sequence of BN 28, a low temperature regulated mRNA in Arabidopsis is homologous to kinl gene.

Houdes (1992) reported the isolation of a gene in wheat, called Wcs 120. The Wcs 120 cDNA contains a long open reading frame encoding a protein of 390 amino acids. The encoded 50 kDa protein is boiling stable, highly hydrophilic, shows a pI value of 7.3 (Houdes et *al.,* 1992). It was not inducible by heat shock, drought, or abscisic acid. It was strongly induced during cold acclimation of wheat seedlings. Immunoblotting experiments with the anti-Wcs 120 antibody identified several cold-induced proteins named freezing tolerance markers (FTMS) since they are associated with the development of freezing tolerance. This protein family was found to be coordinately regulated specifically by low temperature. The accumulation kinetics during the acclimation period indicated a positive correlation with the freezing tolerance of each genotype. Accumulation of these proteins was higher in the freezing tolerant genotypes than in the less tolerant one. In addition, their accumulation was more pronounced in the crown and leaf tissues compared

with roots. Analysis of different cereal species indicated that this protein family is specific for freezing tolerant cereals because the antibodies did not cross react with any of the non-cereal species. The anti-FTMs antibody thus represents a potential tool for improving freeze tolerance through breeding programmes of cereals.

In Arabidopsis separate signal pathways regulate the expression of low-temperature induced gene, Lti 140 (Nordin *et al.*, 1991). This single copy Lti 140 gene encodes a 140 **kDa** cold acclimation related polypeptide. The Lti 140 **mRNA** accumulates rapidly in both leaves and roots when plants were subjected to a low temperature or water stress but not by **heat-shock.** The low-temperature induction of Lti 140 is not mediated by ABA, as shown by treatment with the ABA biosynthesis inhibitor fluridone. The effects of low temperature and exogenously added ABA are not cumulative, suggesting that these two pathways are different. The induction by ABA is abolished in the ABA-insenstive mutant, **abi-1,** indicating that the **abi-1** mutation defines a component in the ABA response pathway. Accumulation of the Lti 140 **mRNA** in plants exposed to water stress was some what reduced by treatment with fluridone and in the ABA insenstive **mutant** abi-1 suggesting that the water stress inductin of Lti **140** could be partly mediated by ABA. Three separate but converging signal pathways regulate the expression of the Lti **140 gene.**

Cattivelli and Bartels (1990) isolated five different cDNA clones from barley and their further analysis indicated that the transcripts accumulate to differnt levels during cold treatment. Hybridization experiments using RNAs isolated from different tissues indicate that several cold regulated genes are expressed in a tissue specific manner. These expression studies suggest that in barley several different genes are involved in the cold hardening process depending on developmental stages and tissue. Homology has been found between the isolated cDNAs and cold induced transcripts of related cereals. DNA sequence analysis of the clone pT 59 and PAO 86 and the deduced amino acid sequence reveals that the longest open reading frame contains arginine rich basic domains.

Cold-acclimation specific (CAS) gene expression has been examined by screening a cDNA library prepared from poly $(A)^+$ RNA of cold-acclimated seedlings of a freezing tolerant variety alfalfa (Medicago falcata Cv Anik), (Mohapatra et *al.*, 1989). Three CAS cDNA clones representing differnt sequence species have been used to investigate the relative abundance and time-course of accumulation of corresponding transcripts. The expression of these CAS genes is found to be regulated in a coordinated manner, most likely, at the level of transcription. The expression of these genes is not inducible by heat shock, water stress, abscisic acid, or wounding. A positive correlation was observed between the expression of these cloned sequences and the degree of freezing tolerance in four alfalfa cultivars.

Enzymology:

Crespi et *al.* (1991) showed that when wheat seedlings are exposed to a cold temperature (2-4°C) above 0°C, sucrose accumulates and sucrose synthase activity increases. The effect of a cold period on the level of sucrose synthase (SS) was investigated. Using the antibodies aganist wheat germ sucrose synthase, Western blot studies showed that the amount of the sucrose synthase peptide increased during 14 days in the cold, when plants were moved from 23 C to 4°C. The levels of sucrose synthase diminished when plants were moved back to 23°C. Northern blots confirmed a five to six fold induction of sucrose synthase in wheat leaves during cold acclimation.

Christie et *al.* (1991) reported that the low temperature stress causes a rapid increase in steady-state levels of alcohol dehydrogenase - 1 message (Adh1) and protein activity (ADH1) in maize and rice seedlings. Maize roots and rice shoots from 7 day old seedlings shifted to low temperature (10°C) accumulate as much as 15 fold more Adhl mRNA and 8-fold more ADH1 protein activity than the corresponding tissues from untreated seedlings. Time-course studies showed that these tissues accumulated Adhl mRNA and ADH1 activity increases several fold within 4 to 8 hours. With in 24 hours of returning cold stressed seedlings to ambient temprature, Adhl mRNA and ADH1 activity decreased to pretreatment levels. Short term cold stress induced Adhl

gene expression. Tissue imprints showed that ADH activity was enhanced along the lengths of cold-stressed maize primary roots and rice roots.

In plants, many enzyme activites are affected during water stress. Increased activity of α-amylase was reported during water stress in Barley (Jacobson et *al.,* 1986). Mali and Mehtha (1977) in their studies on water stress response of drought tolerant and sensitive varieties of rice observed that peroxidase, protease, nitrate reductase and proline activities were increased in tolerant lines compared that of sensitive lines. A reduced nitrate reductase activity was observed in barley during water stress (Rajagopal et *al.,* 1977). Reduced nitrate reductase activity coincided with the increased proline concentration during water stress in sorghum (Sivaramakrishnan et *al.,* 1988).

The functional aspects of stress induced polypeptides shown by Bartels et *al.,* (1991) suggested that the desiccation tolerance protein cloned from barley embryo (PG22-69) which codes for a 34kDa protein, shows homology with the mamalian gene encoding an NADPH dependent aldose reductase which is involved in the synthesis of osmolyte sorbitol. Like other polyols, sorbitol is a common cell osmolyte associated with balancing osmotic strength of the cytoplasm with that of the environment without affecting the function of important macromolecules. Shinozaki et *al.* (1992) reported a cDNA clone (RD 28) from Arabidopsis

thaliana, a desiccation tolerant, that encodes a membrane protein with sequence homology to the major intrinsic protein of bovine lens fiber gap junction, soybean nodulin-26 and the glycerol facilitator. This indicates that the RD28 protein forms intracellular junctions between plant cells and functions in the intercellular transport of small molecules. RD28 protein is involved in the transport of small molecules across intracellular membrane, such as the tonoplast membrane.

Vernon and Bohnert (1992) studied the molecular mechanism of osmotic stress tolerance in Mesembryanthemum crystallinum, a facultative halophyte, capable of adjusting to and surviving in highly saline conditons. They found a novel protein (Mol. wt 40 kDa) myoinositol O-methyl transferase, which catalyzes the first step in the biosyntheis of the cyclic sugar alcohol, pinitol. Pinitol accumulates in salt-stressed M.crystallinum and is abundant in a number of salt and drought tolerant plants. Sugar alcohols play an important role in osmotic stress tolerance. Whether the exact function of these compounds is to serve as non-disruptive cytoplasmic osmolytes or to act as 'osmoprotectant' of protein and membrane structure remains to be elucidated.

MATERIALS AND METHODS

CHEMICALS: Complete adjuvant, Incomplete adjuvant, Horse-Radish Peroxidase anti-immunoglobulin conjugate (HRPO), Sodium lauryl sulphate, Protein molecular weight standards, Albumin Bovine, Albumin egg, Glyceraldehyde-3-phophate dehydrogenase, carbonic anhydrase, trypsinogen, trypsin inhibitor, soybean α-lactalbumin, Formaldehyde, Calf Thymus DNA, Ficoll,PVP, Formamide, Sodium chloride, Sodium phosphate, EDTA, Hydroxyquinoline, Lithium chloride, MOPS buffer, Sodium acetate, Ethidium bromide, Diethyl pyrocorbonate, Abscisic acid and Agarose were obtained from Sigma Chemicals, USA. Nitrocellulose paper was obtained from Hoeffer Scientifics, USA and Hybond N was form Amersham, U.K. Random Primer labelling kit including Klenow, were obtained from Amersham. α^{32}P dCTP was procured from BARC, Bombay, India.

Acrylamide, Bromophenol blue, Trichloroacetic acid (TCA), Bovine serum albumin (BSA), EDTA, Polyethylene glycol (6000), Methanol, Acetone, Glacial acetic acid, NaCl, NaH_2PO_4, Na_2HPO_4, Na_2CO_3, KH_2PO_4, Tris (hydroxymethyl) aminoethane, Ninhydrin, Coomassie Brilliant blue, β-Mercaptoethanol, Sulphosalicylic acid, Glycine, Ponceaus, Orthophosphoric acid, N,-N'-Methylene-bis-Acrylamide, N,N,N',N'-Tetramethyl-ethylenediamine (TEMED), chloroform, phenol and ethanol were obtained from standard companies of INDIA.

Table 3.1

Genetic Stocks

Cultivar	Stress Response	Source
Hamsa	Drought sensitive & Cold sensitive	DRR
IR8	Not clear	DRR
HR12	Not clear	DRR
TN1	Not clear	DRR
Tulasi	Drought tolerant	DRR
Annada	Drought tolerant	DRR
Akashi	Drought tolerant	DRR
N22	Drought escaper	DRR
Jaya	Cold sensitive	DRR
K39	Cold tolerant	DRR
Khonorullo	Cold tolerant	
RCPL-1-1C	Advanced selection for cold tolerance	ICAR complex Shillong
RCPL-1-2C		
RCPL-1-3C		
RCPL-1-6C		
RCPL-1-9C		

DRR - Directorate of Rice Research, Hyderabad, India.

RCPL - Research Complex Paddy Lines.

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 five minutes and were thoroughly washed with sterile water. Germination was upon moistened filter papers under dark conditions. Average temperature during seedling culture ranged from 28°C ± 2°C. After 5 days, these seedlings were treated with 40% polyethylene glycol (PEG-6000 Sigma) for 48 hours. Control plants received only water. Shoots and roots were separately harvested and quick frozen in liquid nitrogen and stored at $-80^{o}C$ until further analysis. Same protocols were used for abscisic acid (200μM) treatment. Seedlings were left on the lab bench until 80% loss in fresh weight was achieved. This was termed as desiccation. Cold temperature stress was given by growing 5 day old seedlings at 4 C for 5 days. Salt stress response was studied by treating the 5 day old seedlings with 0.5M NaCl for two days (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 polonged stress would presumably allow the seedlings to adjust the changes in osmotic potential. These treatments are not lethal to plants as they were found to grow normally after removing from the stress. The control and stressed seedings are shown in Fig.3.1.

Determination of Proline content:

Proline content was estimated by following the method of Bates et *al.*, (1973). One gram of fresh shoot or root

Fig. 3.1. Appearance of stressed **Hamsa** rice cultivar seedlings A) control B) PEG treated C) ABA treated D) cold treated.


A
B
C
D

tissue was ground with 20ml of 3% sulphosalicyclic acid (w/v) in a clean mortar. The homogenate was centrifuged to get a clean supernatant. To 2ml of the supernatant, 2ml of acid ninhydrin reagent and 2ml of glacial acetic acid were added. The mixture was boiled for 1 hr in a water bath. The reaction was terminated by dipping the test tubes in an ice bath. The reaction mixture was extracted with 4 ml of toluene after mixing vigorously on a cyclo-mixer for 30 seconds. The chromatophore, was aspirated from acqueous phase and absorbance at 520 nM was recorded using toluene as the blank, on a Hitachi Spectrophotometer. Proline concentration in the sample was determined by a standard curve calibrated with different concentrations of proline standard. Proline quantity was expressed interms of microgram proline per gram fresh weight tissue.

Acid Ninhydrin Reagent:

1.25 gms Ninhydrin in 50 ml solution containing 30 ml of Galcial acetic acid, 8 ml of Orthophosphoric acid and 12 ml of water. It was stored in an amber colored bottle until use.

Extraction of Rice Proteins:

Total proteins were extracted as per (Goday *et, al.,* 1989) with minor modifications. Shoot tissue were finely ground and extracted with buffer (0.5g/ml) containing 0.25 M Tris-HCl pH 8, 0.4% sodium dodecyl sulphate, 20mM EDTA, 2mM phenyl methyl sulfonyl flouride and 5% β-mercaptoethanol by

mixing and boiling for 5 minutes. The extract was centrifuged at 12,000g for 10 minutes, 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 absolute methanol containing 0.4M ammonium acetate and vaccum dried. The pellet was resuspended in a known quantity of sterile double distilled water.

Protein Estimation:

Protein content was estimated following (Lowry *et al.,* 1951) · method with minor modifications. Reagent A is 4% sodium carbonate in 0.2N sodium hydroxide. B is 1% cupric sulphate, C is 2% sodium potassium tartarate and D is 1N folin reagent. The working reagent is a mixture of A, B and C in a ratio 23:1:1. 1 ml of working reagent was added to 1 ml of protein sample and allowed to stand for 15 min. Then 0.2ml of reagent D was added and mixed thoroughly on a cyclomixer. After incubating for 30 min, absorbance was recorded at 750 nm. Bovine serum albumin (fraction-V) was used as the protein standard.

Two-dimentional Gel Electrophoresis:

Isoelectric focusing in glass tubes (100 mm x 2.5 mm) was done according to the method of O'Farrel (1975). The 1.2 ml gel mixture [55% urea w/v, 4.% acrylamide (w/v) 25% NP-40 (w/v), 2% ampholines pH range 3.8 - 7.5 (v/v); 0.01% ammonium persulfate (w/v) and 0.07% TEMED (v/v)] was degassed under vaccum and immediately poured into the tubes taking care not

to entrap air bubbles. The gel was over layed with 8M urea solution and allowed to polymerize for 2 hours. The gel overlay solution was removed and replaced with lysis buffer (9.5 M urea; 2% NP-40 w/v); 2% ampholines 1.6% of pH range 5 - 7 and 0.4% (v/v) of pH range 3 - 10, 5% β-mercaptoethanol (v/v). The gels were placed in a standard tube gel apparatus and prerun at 200 volts for 15 minutes - 300 volts for 30 minutes and 400 volts for 30 min. The upper reservoir buffer was 0.02 M NaOH and lower reservoir buffer was 0.01M H_3PO_4. The buffers were extensively degassed before use.

At the end of the pre-run as per the above schedule the uppper reservoir buffer was emptied, NaOH and lysis buffer was carefully removed with a syringe and replaced with samples that were prepared in sample lysis buffer. One gel was run under the same conditions but with out sample to calculate the pH gradient of the gels. The sample was overlayed with 9M urea; 2% ampholines (v/v) 1.6% of pH 5 - 7 range 0.4% (v/v) of pH 3-10 range, followed by 0.02M NaOH. The upper reservoir was refilled with 0.02M NaOH and the gels were run at 400 V for 12 hrs and then at 800 V for an hour.

At the end of the run the gels were carefully removed from the tubes, the gels were fixed in 10% TCA (w/v) for 2 hrs. The fixed gels were stained with Coomassie brilliant blue 0.02% (w/v) in methanol; isoproponol [27:50 (v/v)] mixture, and copper sulfate for an hour. The gels were extensively destained in a mixture of methanol : acetic acid : water (50:7:43) till the blue back ground is clear.

Measurement of pH gradient and calculatin of isoelectric point:

The isoelectric focusing gel which was run with out sample was cut into 0.5cm sections and incubated in a freshly prepared solution of 9.2M urea in degassed water for 15 min. The pH of the solution was measured on a pH meter. A standard graph of measured pH versus length of the gel was plotted. The isoelectric point of samples was estimated by measuring the length from the anodal end and the corresponding pH was taken as isoelectric point of the protein.

SDS-Polyacrylamide gel Electrophoresis:

SDS-PAGE was performed according to Sigma technical bulletin No. MWS-877L based on **Laemmli** (1970). The following solutions and buffers were prepared to polymerize the gels and also to perform the SDS-PAGE.

(A) **Acrylamide** solution: 28g **acrylamide** and 0.74g of methylene bis acrylamide were dissolved in double distilled water and the volume was adjusted to 100ml.

(B) Separting gel buffer: **36.3g** Tris in double distilled water pH 8.9 adjusted with **HCl.**

(C) Stacking gel buffer 5.98g Tris in 100ml double distilled water pH 6.8 adjusted with **HCl.**

(D) 1% SDS solution: **1g** SDS in 100 ml double distilled water.

(E) APS **souliton:** 10% ammonium per sulphate solution (prepared **freshly).**

(F) Reservoir buffer: **6.025g** Tris, **14.4g** glycine and lg of

SDS in 1000ml distilled water (pH 8.3).

Sample buffer: 0.062M Tris (pH 6.7), 10% glycerol, 2% SDS, 5% β-mercaptoethanol and 0.001%, Bromophenol blue. 15% separating gels (total volume 30 ml) were prepared by mixing 16ml of solution A, 4.5ml of solutions, 3ml of solution D and 6.450 ml of double distilled water and polymerised by adding 30 µl of TEMED and 150µl of solution E. Stacking gel 5% (total volume 5ml) were prepared by mixing 0.9 ml of solution A, 2ml of solution C, 2ml of solution D and 1.5 ml distilled water and polymerised by adding 10 µl TEMED and 40 µl of solution E.

Silver staining for the detection of proteins separated by electrophoresis:

Silver staining of the electrophoresed gels was done as per Blum et *al.*, (1987). The gels, following electrophoresis, were fixed for one hour with 50% methanol containing 12% glacial acetic acid and 0.5ml 37% formaldehyde per liter. The fixed gels were washed thrice, each time with 50% ethanol for 20 minutes and treated with 0.02% sodium thiosulphate for 1 minute. Excess thiosulphate was removed from the gel surface by rinsing thrice with distilled water for 20 seconds. Silver nitrate impregnation was carried out by shaking the gel in 200ml of 0.2% silver nitrate solution containing 150 *ul* of 37% formaldehyde for 20 minutes. Upon washing with excess of water to remove unimpreganted silver nitrate, the gels wre developed with 6% sodium carbonate solution containing 0.5ml of 37% formaldehyde and sodium

thiosulphate (4mg/litre) for 10 minutes. After the bands were fully developed, the gels were washed in water for 2 minutes with 2 changes of water and the reaction was stopped in 50% methanol (u/v) 2% acetic acid (v/v) mixture for 10 minutes. Finally the gels were washed in 50% methanol (v/v) and were stored in 7% acetic acid (v/v) at 4°C.

Extraction of Boiling stable proteins:

Boiling stable proteins were extracted by the method of (Lin et *al.*,1990). Crude protein samples from control and treated seedlings were boiled in a water bath for 25 minutes and the insoluble material removed by contrifugation in an Eppendorf microfuge (15 min. 10,000 rpm). The soluble polypeptides remained 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 buffer and electrophoresed.

Purification of PEG induced 23 and 46kDa proteins by electroelution:

A number of prepartaive SDS gels were made and both control and treated samples were run. The induced 23 and 46 kDa polypeptides were identified after coomassie staining. The induced 23 and 46 kDa bands were cut out by comparison with that of the control. The long pieces containing 23 and 46 kDa proteins were kept in dialysis bags containing sodium phosphate buffer (10 mM at pH 7.4) and placed in a horizontal electrophoreis chamber containing phosphate buffer and

electroeluted at 100 V. The eluted proteins were precipitated with cold acetone at -80°C. The pellet was dissolved in buffer. The purity of 23 and 46 kDa polypeptides was tested on SDS-PAGE gel.

Anion exchange HPLC of 23 kDa polypeptide:

The purified 23 kDa polypeptide was applied to a Shimpack PA-DEAE column (80 x 100 mM) equilibrated with sodium phosphate and citric acid. Protein was eluted with a linear gradient formed from sodium phosphate (100 mM) and citric acid (100 mM) at a flow rate of 1ml/min for 25 minutes.

Raising Antibody to purified 23 kDa polypeptide:

A healthy rabbit was injected with the protein antigen solution containing 200 *uq* of 23 kDa protein in sodium chloride (0.14M, pH7.3) and sodium phosphate (0.02 M) (phosphate-buffer saline) . It was emulsified with an equal volume of Freund's adjuvant to give a total of 2ml of emulsion. To emulsify complete Freund's adjuvant with protein solution, the constituents were pumped in and out of the glass hypodermic syringe until a thick white emulsion was formed. The emulsion (0.3 to 0.5 ml) was injected intradermally into the rabbit on either side of the thigh.

A booster injection of protein (150 *uq* of antigen) was administered after 28 days. Incomplete Freund's adjuvant instead of complete Freund's adjuvant was used and the

injection site was just above that of previous injection. A week later, the rabbit was given a second booster of 100 µg of protein as described above. After 5 days, the blood was collected from the rabbit. Antisera was obtained from the collected blood.

Assay of Antibody activity by **Immunodiffusion:**

Antibody activity was tested by simple immunodiffusion on 1% agar solution on glass slides. Serially diluted antiserum was placed in a succession of peripheral wells and the antigen, at a concentration of (1mg/ml) was placed in the centre. The plates were left in a humid environment for 24 to 48 hours. The maximum dilution of antiserum which gives a visible white precipitin line after 48 hours was taken as the titer of the antiserum.

Western blotting:

The SDS-PAGE gels of control and treated samples were electrophoretically irreversibly blotted on to a nitrocellulose papers. The nitrocellulose blots were probed with the antibody. The antigen antibody complex was detected by horse radish peroxidase (HRPO) anti-immunoglobulin (IgG) conjugate and visualized by incubating the filters in the presence of precipitable substrate, diaminobenzidine tetrahydrochloride in the presence of hydrogen peroxide as per the procedure in Current Protocols in Molecular Biology manual (Winston et *al.,* 1987).

Solutions.

1. Electroblotting buffer: 20mM Tris/150 mM glycine pH 8.0 in 4 litres of water to which 1200ml methanol was added to bring to 6 liters with H_2O.

2. Ponceaus S solution: 0.5% ponceaus-S 1% glacial acetic acid in 100 ml of water.

3. Blocking buffer: 5% BSA in PBS buffer.

4. Primary antibody: antibodies raised against 23 kDa protein.

5. Secondary antibody: Horse radish peroxidase (HRPO) anti-IgG conjugate, (1:5000 dilution).

6. Diamino benzidine (DAB) solution: 50 mg of Diamino benzidine tetrahydro chloride, 2 ml of 1% CoCl in water, 98ml of phosphate buffer saline and 0.1ml 30% hydrogen peroxide was added immediately prior to use.

Assembling the Western blotting sandwich: 2 pieces of 2mm filter papers and nitrocellulose paper were taken and cut into the same size as the gel prewetted with electroblotting buffer. First 3mm filter paper was kept on the scotch brite pad and nitrocellulose papers was placed towards the anodal side taking care to avoid air bubbles. The gel and a whatman 3mm papers were placed in a sequential manner towards cathode. Air bubbles were removed. Another scotch brite pad was placed on the top of the whatman paper. This sandwcih was placed in Western blot apparatus in correct orientation.

Transfering proteins from gel to nitrocellulose:

The proteins were electrophoretically transfered from the gel to the nitrocellulose paper at a constant voltage (30V) overnight at 4°C. Transfer of the protein onto nitrocellulose paper was confirmed by using Ponceaus-S solution and the molecular weight standards were marked. The Nitrocellulose papers were blocked with 5% BSA. The nitrocellulose paper was soaked in the pre diluted (using blocking buffer) primary antibody for 1 hour. Nonspecifically bound primary antibody was removed by washing thrice with PBS. The HRPO conjugate was diluted with blocking buffer. The filter was placed in HRPO conjugate for 1 hour at room temperature with constant agitation. The nonspecifically bound HRPO conjugate was removed by washing with PBS.

The filter was placed in 25ml of freshly prepared DAB substrate solution. The colour was observed within 2 minutes. The colour reaction was stopped by rinsing the filter paper in water. Photograph for permanent record was taken immediately as the bands on original blot fade when exposed to continuous light.

Extraction of total RNA:

Extraction of total RNA was done according to the procedure mentioned in Plant Molecuar Biology Manual 1988 (De Vries et *al.*,1988).

The plant material was harvested in liquid nitrogen and it's fresh weight was determined. The tissue was ground in liquid nitrogen in a mortar until a fine homogenous powder was obtained. The powder was transferred to a flask and was mixed with phenol/extraction buffer at 90°C, in the ratio of 2ml per gram tissue. The flask was swirled vigorously and was kept in the water bath (90°C) occasionally until a milky suspension was obtained. One ml of chloroform was added per gram of tissue and shaking was continued for 15-30 min at room temperature. The milky suspension was subjected to centrifugation at 20,000g for 30 min at 25°C. Upper aqueous phase was collected and equal volume of chloroform was added (1ml/gr) and kept on gyratory shaker for 15 min at 100 rpm. The aqueous phase was collected and treated with 1/3 volume of 8M LiCl. The contents were mixed well and RNA was precipitated for 16-48 hr at 0-4°C. The resultant RNA pellet was washed once with 2M LiCl at 0-4°C and twice with 80% ethanol. The resultant pellet was dissolved in distilled water and stored at -20°C.

RNA Extraction Buffer: 100 mM LiCl, 1% SDS, 100mM Tris-NaOH at pH 9.0, lOOmM EDTA.

RNA DOT-BLOT:

Blots were prepared according to the procedure mentioned in Molecualr Cloning by Sambrook *et al.,* 1989. volume I. To 20-25 µg of RNA (in 20 µl), 20 µl of denaturing buffer was added, and incubated for 15 min at 65°C and snap cooled on ice. Nitrocellulose membrane and 3mm whatman

filters were placed in DEPC treated water for 2 minutes.

The whatman 3mm filter was placed on to DOT-BLOT unit, followed by nitrocellulose membrane and the unit was closed tightly. Denatured RNA was added into the wells of the unit and allowed to stand for 30 min. Vaccum was applied to the Dot-Blot unit for 30 seconds to entrap the RNA samples to nitrocellulose. The filter was removed from Dot-Blot unit and air dried for 30 minutes at room temperature. The filters were baked at 80°C in an air oven.

Denaturing Buffer:

1ml formamide. 200µl 0,1M phosphate buffer (pH 6.5) 455 µl of sterile DEPC treated water, 350 µl of 37% formaldehyde.

Hybridization:

The baked nitrocellulose filters were incubated in 25 ml of prehybrization solution for one hour at 65°C. The filters was changed to 25ml of hybridization solution which contains denatured radiolabelled probe and was kept at 65°C (for homologous cDNA probes) and 55°C (for heterologous) probes for 16 to 20 hours. The filters were washed twice with a solution containing 2 x SSPE + 0.1% SDS at 65°C and once again washed with the same solution at room temperature. The filters were dried between Whatman papers and exposed to X-ray film at -80°C for specified periods.

Random Primer Labelling:

To 3 µl of the probe (100 to 200 ng of cDNA), 27 µl of distilled water was added and heated at 95°C for 10 min, and

cooled immediately on ice for 2 min. To this mixture 5 µl of oligobuffer, (Amersham) 2 µl BSA, 3 µl of of [^{32}P]dCTP solution, 50 µCi, (specific activity 2500 Ci/m mole). 2 µl of klenow were added and left at room temperature for 2 to 3 hours. To this mixture, 2 µl of 0.5 M EDTA, 5 µl of calf thymus DNA, 5 µl of 4 M ammonium acetate and 125 µl of ice cold ethanol were added, and incubated at room temperature for one hour. After keeping on ice for 30 min. the reaction mixure was spun at 12000 rpm for 30 minutes at 0-4°C. The pellet was dried and 500 µl of 0.1% SDS was added. Before adding to the hybridization solution the above mixture was heated at 100°C for 10 min.

Prehybridizatin solution: 6x SSPE; 0.02% Ficoll; 0.02% PVP, 0.1% SDS an Calf thymus DNA (1mg/100ml).

Hybridization solution: 3x SSPE, 0.02% Ficoll, 0.02% PVP and 0.1% SDS.

Northern-blot analysis:

Preparation of the sample: To 30 µg of RNA, 2 µl of 5x formaldehyde gel running buffer, 3.5 µl of formaldehyde and 10 µl of formamide were added. These samples were incubated at 65°C for 15 min and were chilled in ice. Samples were centrifuged for 5 seconds. To the sample, 2 µl of the formaldehyde gel loading buffer was added. The gel was casted in a chemical hood and was allowed to set at room temperature for atleast 30 minutes. Before loading the samples, the gel was prerun for 5 min at 5V/cm. Then the samples were loaded and run at 60V for 3 hours and at the end

of electrophoresis the gel was stained with ethidium bromide. The samples were transferred from gel to hybond N^+ filter and rest of the experiment was performed as in RNA DOT-Blot.

Formaldehyde gel loading buffer: 50% of glycerol, 1mM EDTA pH 8.0, 0.25% Bromophenol Blue and 0.25% Xylene cynol.

Formaldehyde gel running buffer: 0.1M MOPS at pH 7.0, 40mM sodium acetate, 10mM EDTA at pH 8.0 and stored in dark.

Developing and fixing of **X-ray films:**

The film developer was prepared by dissolving 90gm of developer and 16gm of the hardner in 1 litre of double distilled water. The fixer was prepared by dissolving 264 grams of X-ray acid fixing salt with harder (Kodak) in one liter of double distilled water. X-ray films were developed for 1 to 2 minutes and washed in water to remove any traces of developer. The developed films were fixed for 10 minutes before washing them under water. The washed films were dried and photographed. Kodak **XAR-5** film (high contrast) were used.

RESULTS

The response of various rice lines to different stresses has been investigated using rapidly growing seedlings under controlled conditions. These rice lines can be broadly grouped into 3 groups on the basis of stress response evaluation. As summarised in 4.1 Table (1), some lines were tested for their response to a number of stresses, and some were tested only for one, two or three different stresses. Group 1 lines were mainly used for PEG and ABA stress, group 2 lines were for cold acclimation and group 3 was essentially, **Hamsa,** additionally tested for salt and desiccation stress.

4.1 EFFECT OF PEG MEDIATED WATER STRESS, **ABA AND** COLD ON PROLINE CONTENT.

Proline accumulation in response to water stress and ABA was studied in seedlings of different rice cultivars. On the basis of the data presented in Fig. 4.1 A & B stress induced shoot proline accumulation in tested rice lines can be broadly classified in two classes. Class 1 rice lines, Hamsa, Tulasi, Annada and Akashi, showed an increased shoot proline content (3.5 fold) only in response to water stress, whereas class 2 rice lines N22, IRS, HR12 and **TN1** showed increased proline content (2 to 5 fold) in response to both water stress and ABA. Further, there are significant quantitative differences between cultivars. The tested cultivars do not show dramatic differences in their **basal** (control) proline levels in shoots. The shoot **proline**

4.1 Table I

Stress treatment

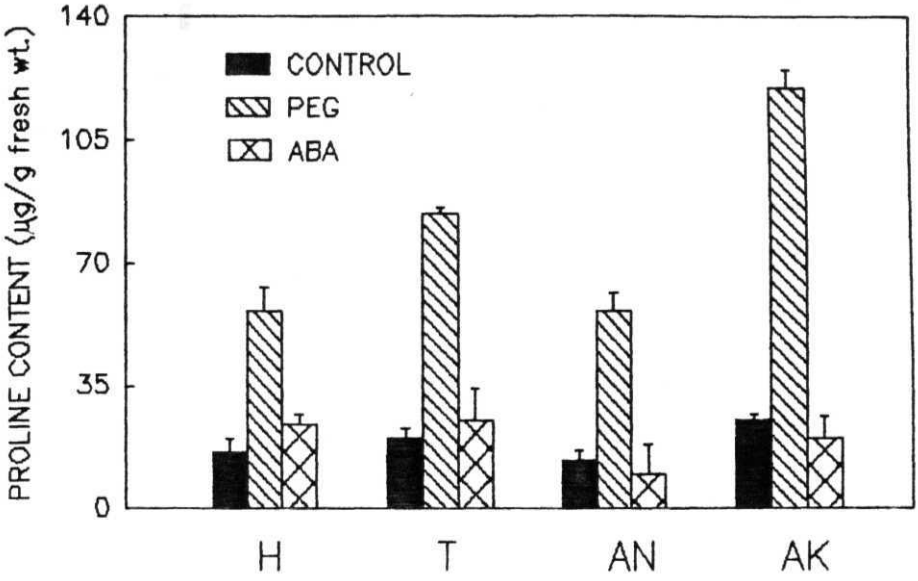
Cutivar	PEG	ABA	Cold	Salt	Desiccation
Group I					
Tulasi	+	+	-	-	-
Annada	+	+	-	-	-
Akashi	+	+	-	-	-
N22	+	+	-	-	-
IR8	+	+	-	-	-
TN1	+	+	-	-	-
HR12	+	+	-	-	-
Group II					
K39	-	+	+	-	-
Khonorullo	-	-	+	-	-
RCPL-1-1C	-	-	+	-	-
RCPL-1-2C	-	-	+	-	-
RCPL-1-3C	-	-	+	-	-
RCPL-1-6C	-	-	+	-	-
RCPL-1-9C	-	-	+	-	-
Jaya	-	-	+	-	-
Group III					
Hamsa	+	+	+	+	+

\+ = Work done - = Not done

Table II Rice cultivars and stress treatments.

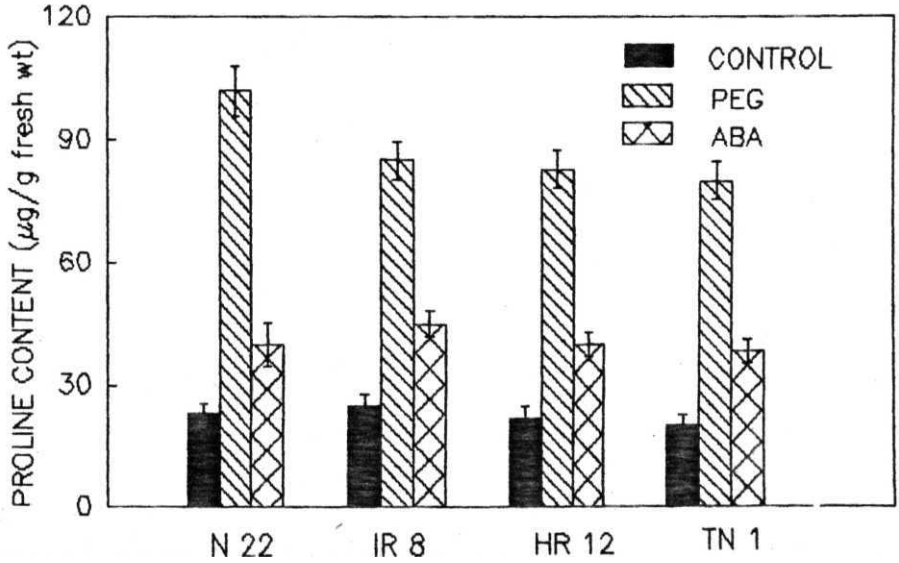
Fig. 4.1. (A & B) Proline accumulation in shoots in response to PEG and ABA treatment. Values represent mean ± SE (n=10); H : Hamsa; T : Tulasi; An : Annada; Ak : Akashi; N22, IR8; HR12 and TN1.

Fig.4.1 A SHOOT PROLINE CONTENT IN RESPONSE TO PEG AND ABA STRESSES



B

SHOOT PROLINE CONTENT IN RESPONSE TO PEG AND ABA STRESSES



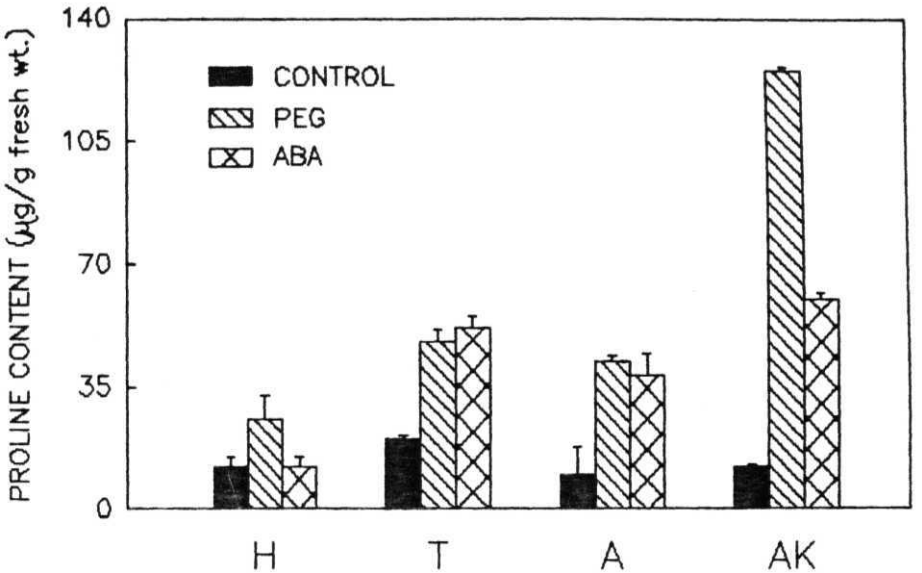
content under water stress is highest in Akashi (5 fold) and N22 (5 fold) and lowest in Hamsa (3 fold) and Annada (4 fold). The exogenous ABA also induces the increased accumulation of shoot proline in class 2 seedlings but the levels are significantly lower than those induced under water stress. For instance, the maximum increase in shoot proline content by ABA treatment is 50µg/g fresh wt, whereas it is as high as 125 µg/g under water stress. The observation that class 1 plants do not respond to ABA, atleast in terms of proline accumulation in shoots, is interesting and this property can perhaps be used to discriminate cultivars for their specific stress responses.

Root proline content was observed to increase in response to both PEG and ABA with two exceptions, Hamsa and TN1, which show either a marginal increase or not at all under ABA stress. The increase in root proline content among class 1 cultivars ranges from a minimum of 2 fold in Hamsa to 10 fold in Akashi.

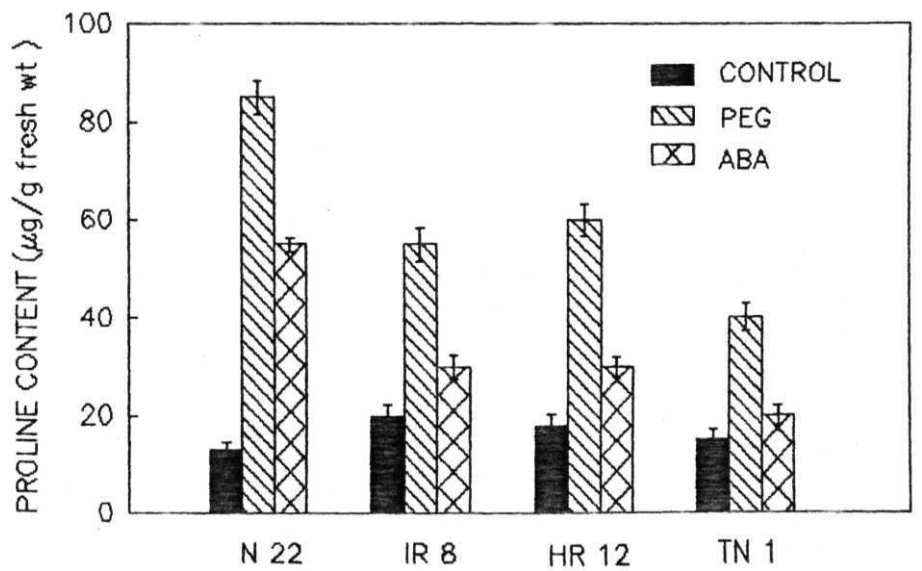
The data in Fig. 4.2 A and B reveal that the root proline content under stress can be as high as that of shoot. For instance Akashi showed about 125 µg/g both in roots and shoots under water stress. Again, the tested cultivars do not show drastic difference in their basal (control) proline levels in roots.

Fig. 4.2. (A & B) Proline accumulation in roots in response to PEG and ABA treatment. Values represent mean ± SE (n=10); H : Hamsa; T : Tulasi; An : Annada; Ak : Akashi; N22, IR8; HR12 and TN1.

Fig.4.2A.R00T PROLINE CONTENT IN RESPONSE TO PEG AND ABA STRESSES



B. ROOT PROLINE CONTENT IN RESPONSE TO PEG AND ABA STRESSES



Seedlings exposed to cold temperatures were also shown to accumulate significantly increased levels of proline. The data on shoot proline content (Fig. 4.3a&b) reveal that the rice lines do not exhibit dramatic difference (the maximum is about 20 *uq/q* fresh weight and minimum is about 16 µg/g fresh weight). It can be seen that there are significant differences between basal proline levels of these lines (the range is from 8 µg/g fr. wt in RCPL-1-2C to 13 µg in RCPL-1-1C). On the contrary, root proline content shows significant difference between lines (Fig. 4.3b). The maximum is about 10 µg/g fr. wt. in K39 and the minimum of about 4 µg/g fr. wt in RCPL-1-2C. Further, these rice lines also differ significantly in their basal proline level (the range is about 2 *uq/q* fr. wt .in RCPL-1-2C to about 5 *uq/q* fr. wt in K39) in roots. It was observed that the roots seem to accumulate significantly less proline than the shoots in all tested rice lines.

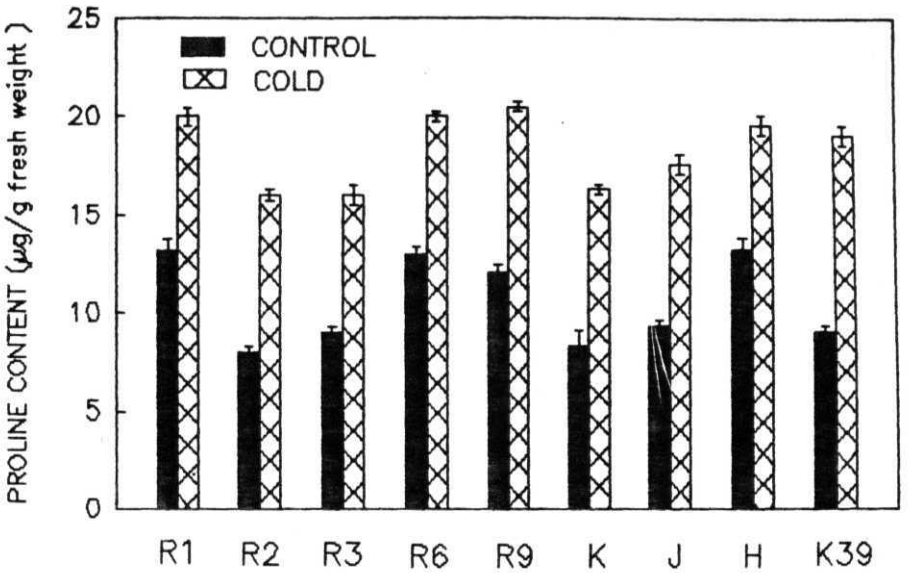
4.2 EFFECT OF PEG-MEDIATED WATER STRESS AND EXOGENOUS ABA ON POLYPEPTIDE PROFILES.

The stress responsive changes in protein profiles were broadly classified into (1) induction of new polypeptides (2) inhibition of polypeptides and (3) changes in the relative abundance of polypeptides. First, the induction of new polypeptides was detected by one dimensional SDS-PAGE. Second, 2D-gel analysis was employed for confirmation. Third, the induced polypeptides were purified and fourth they are tested by Western analysis.

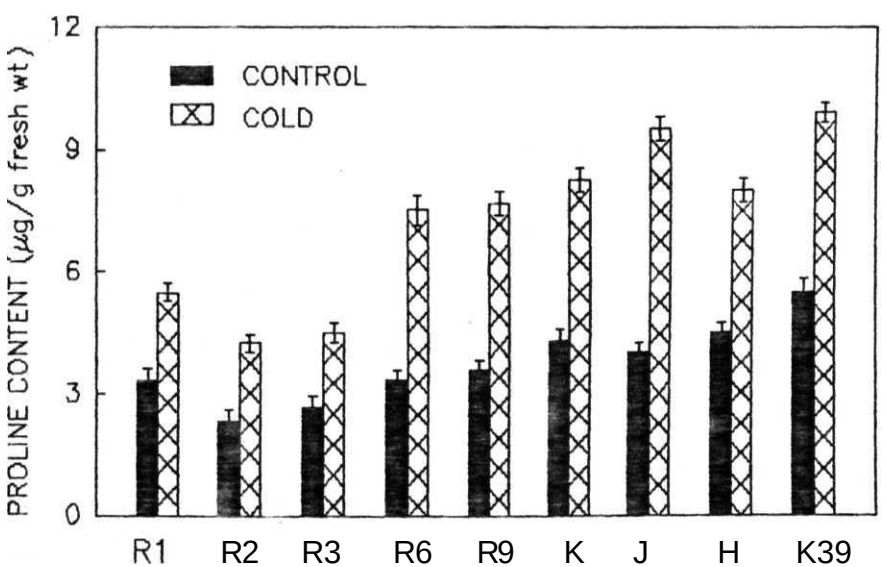
Fig. 4.3 Proline accumulation in (A) Shoots (B) Roots in response to cold treatment. Values represent mean ± SE (n = 10); R1 : RCPL-1-1C; R2 : RCPL-1-2C; R3 : RCPL-1-3; R6 : RCPL-1-6; R9 : RCPL-1-9C; K : Khonorullo; J : Jaya; H : Hamsa and K39.

Fig.4.3A

SHOOT PROLINE CONTENT IN RESPONSE TO COLD STRESS



B. ROOT PROLINE CONTENT IN RESPONSE TO COLD STREES



The induction of specific polypeptides by PEG-mediated water stress in Hamsa seedlings was displayed in Fig. 4.4. As can be seen, three polypeptides with apparent molecular weight 46, 23 and 15 kDa are induced by dehydration caused by PEG. Also, it is clear that a polypeptide with an apparent molecular weight of 42 kDa disapeared on stress. Although there were several differences between the protein profiles of control and induced seedlings, we concentrated only on these 3 proteins for further studies.

Whether other rice lines, including drought tolerant ones also show the induction of these proteins upon water stress was investigated. The data in Fig. 4.5 clearly reveal that all the tested lines, namely, Hamsa, Tulasi, Annada and Akashi show the induction of these three proteins under water stress. Interestingly the 42 kDa was missing in all the tested lines under the stress.

Does exogenous ABA also induce these proteins? This was investigated by treating the seedlings with ABA and the data in Fig. 4.6 demonstrate that all the three polypeptides are also inducible by ABA. Again the 42 kDa polypeptide disappeared after ABA treatment.

The effect of water stress on protein profiles of N22, an early maturing drought escaper with good deep root system was studied under identical test conditions. The data in Fig. 4.7 reveal that all the three polypeptides namely 46, 23

Fig. 4.4 SDS-PAGE (15%) of proteins extracted from shoots of **Hamsa** cultivar (50 **µg** of protein was loaded into each slot). Solid arrows indicate the PEG induced proteins and the broken arrow indicate the disappreance of a protein.

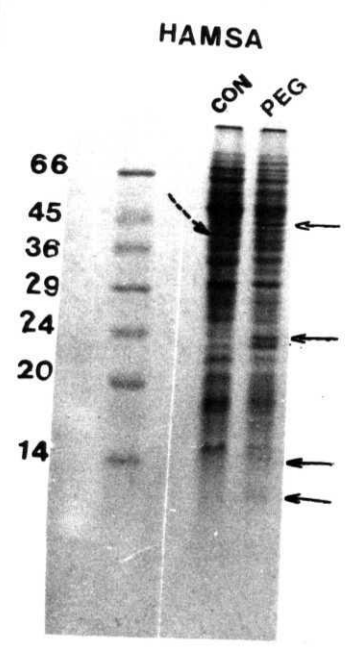

HAMSA
CON
PEG
66
45
36
29
24
20
14

Fig. 4.5 SDS-PAGE (15%) profiles of shoot extracts of different cultivars treated with PEG lane (A) Mol. wt. markers; (66 kDa Albumin Bovine, 45 kDa Albumin egg, 36 kDa Glyceraldehyde-3-phosphate dehydrogenase, 29 kDa carbonic anhydrase, 24 kDa Trypsinogen, 20 kDa trypsin inhibitor, Soybean and 14 kDa α-lactalbumin. Lanes B, C Hamsa control and PEG treated, lanes D, E Tulasi control and PEG treated, lanes F, G Annada control and PEG treated; lanes H, I Akashi control PEG treated. Each lane except 'G' (25 μg) received 50 μg of protein. Induced proteins were marked.

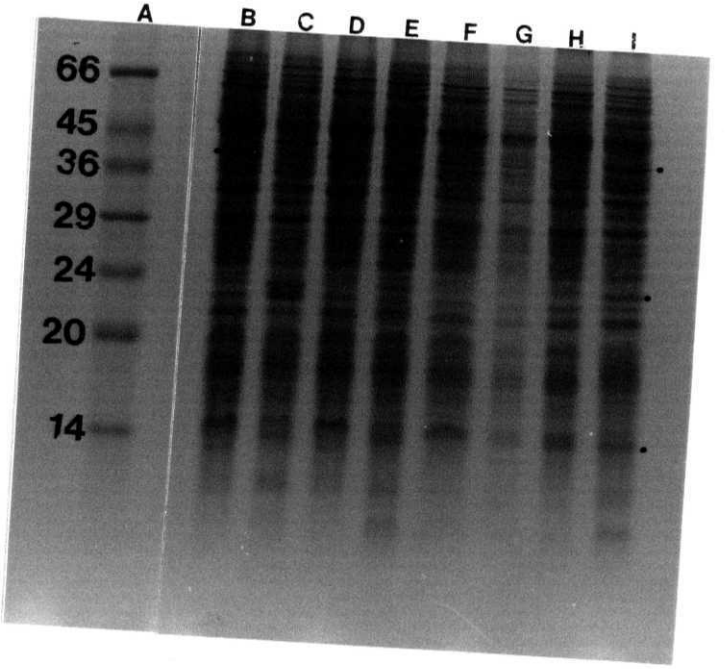
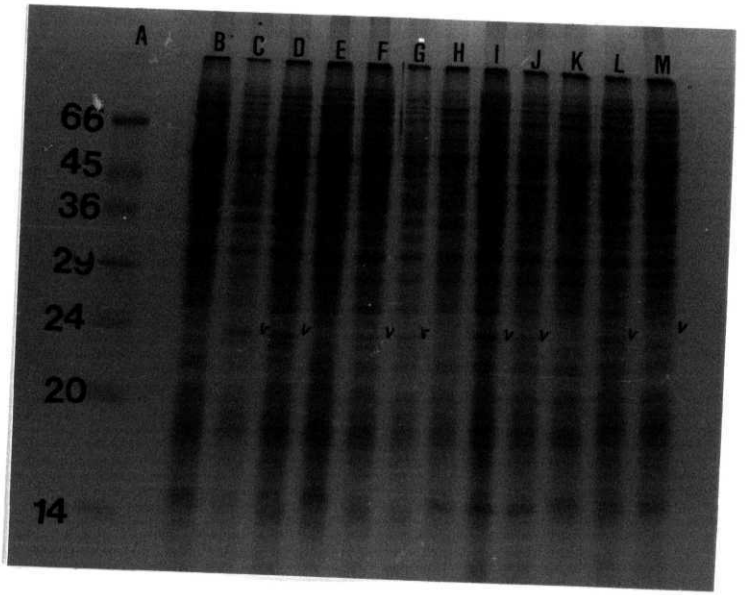

A
B
C
D
E
F
G
H
I
66
45
36
29
24
20
14

Fig. 4.6 SDS-PAGE profiles of shoot extracts of different cultivars treated with PEG and ABA lane A, Mol. 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 (25 µg) received 50 µg of protein. The induced protein were marked.


A
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C
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E
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G
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I
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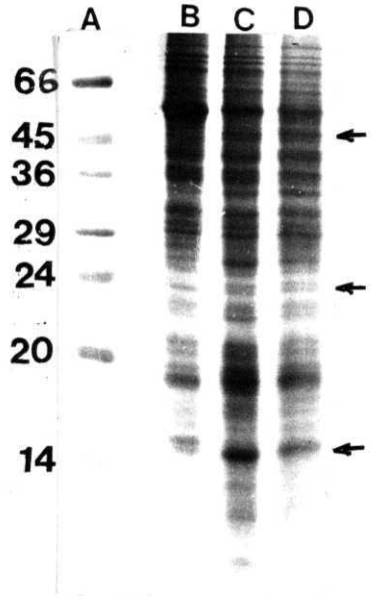
K
L
M
66
45
36
29
24
20
14

Fig. 4.7 SDS-PAGE profiles of shoot extracts of N22, lanes A Mol. wt. markers; B, control shoots; C, PEG treated (D) ABA treated (each lane was loaded with 50 µg of protein) Arrow marks indicate the induced proteins.

Fig. 4.8 SDS-PAGE profiles of shoot extract of different rice cultivars treated with PEG and ABA; lane A, Mol. wt. markers, lanes B, C and D IR8 control, PEG and ABA treated; lanes E, F and G HR12 control, PEG and ABA treated; lanes H, I and J TN1 control, PEG and ABA treated (Each lane was loaded with 50 µg protein).


A
B
C
D
66
45
36
29
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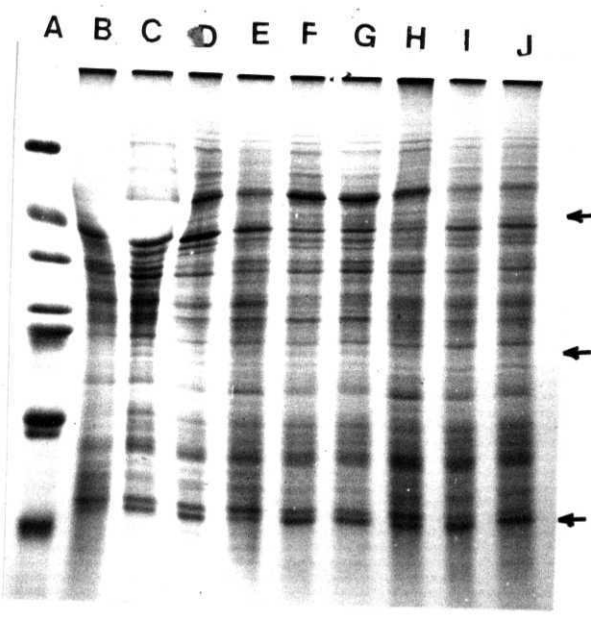
20
14


A
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J

and 15 kD are induced by both water stress and ABA. Interestingly, the 42 kDa polypeptide is missing in both control as well as induced samples. This is contrary to what was observed with the other lines.

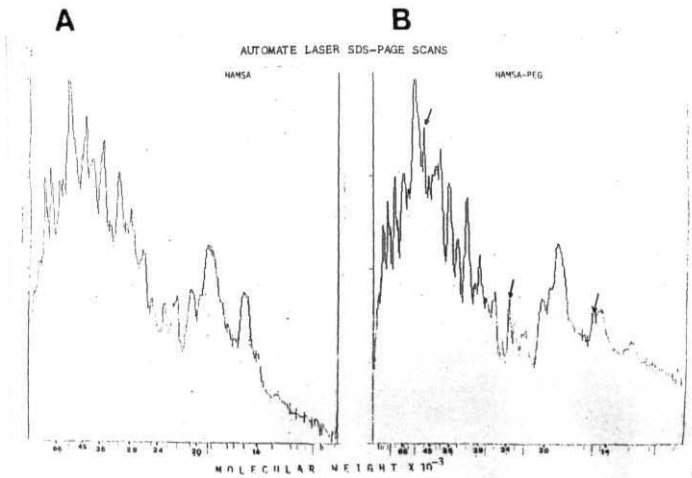
Attempts were made to find out whether these three induced polypeptides also appear in parental lines. For this purpose, the parental lines IR8, HR12 and TN1 were subjected to a similar analysis and their stress responsive polypeptide profiles are given in Fig. 4.8. It is to be noted that N22 and IR8 are parental lines for Akashi whereas HR12 and TN1 are parental lines for Hamsa. The data revealed that both the parental lines and their advanced progeny (F10) show the induction of all three polypeptides. With respect to comparision of Akashi protein profiles with that of IR8 and N22, it was observed that the 42 kDa polypeptide does not appear at all in N22 (in control as well as treated). However, it was present in IR8 and Akashi controls but disappear under stress. This is the single most apparent difference between parental lines and it is suggested that 42 kDa polypeptide has it's origin in IR8 parent.

4.3 ANALYSIS OF THE STRESS RESPONSIVE POLYPEPTIDES :

One dimentional SDS polyacrylamide gels were further analysed by a soft laser scanner to detect any minor changes in the relative abundance of these stress induced proteins. Although laser scans of samples of all the tested lines were obtained, only the representative scan data for Hamsa are

Fig. 4.9 Automated laser scans of Hamsa. A, control; B, PEG treated. Arrow indicate the induced proteins.

Fig. 4.10 Automated laser scans of Hamsa; A, control; B, ABA treated. Arrow indicate the induced proteins.


A
B
AUTOMATE LASER SDS-PAGE SCANS
HAMSA
HAMSA-PEG
MOLECULAR WEIGHT X 10⁻³

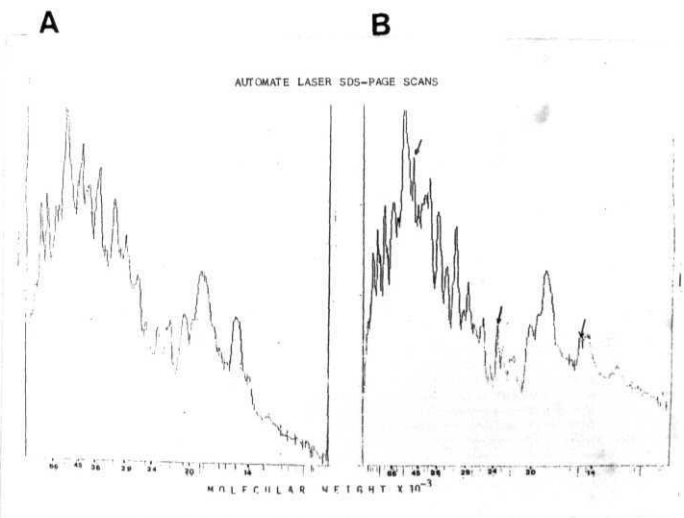

A
B
AUTOMATE LASER SDS-PAGE SCANS
MOLECULAR WEIGHT X 10⁻³

Fig. 4.11 Two-Dimensional gel electrophoresis of Hamsa shoot extracts A, control; B, PEG treated. Arrow mark indicate induced proteins. These gels were silver stained.

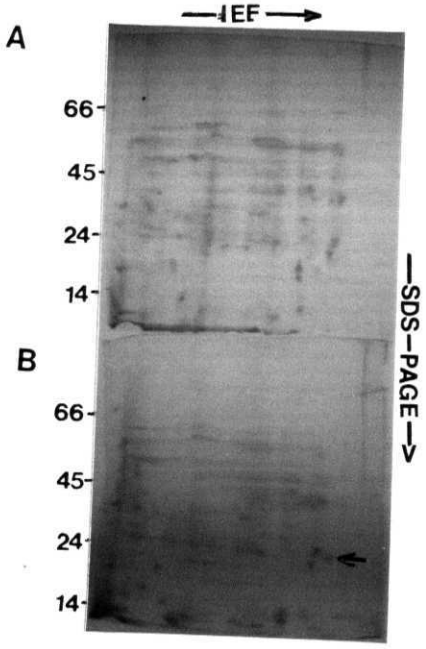

IEF
A
66
45
24
14
B
66
45
24
14
SDS-PAGE

Fig. 4.12 Two-Dimensional gel electrophoresis of Hamsa shoot extracts after silver staining A, control; B, ABA treated. Arrow marks indicate induced proteins.

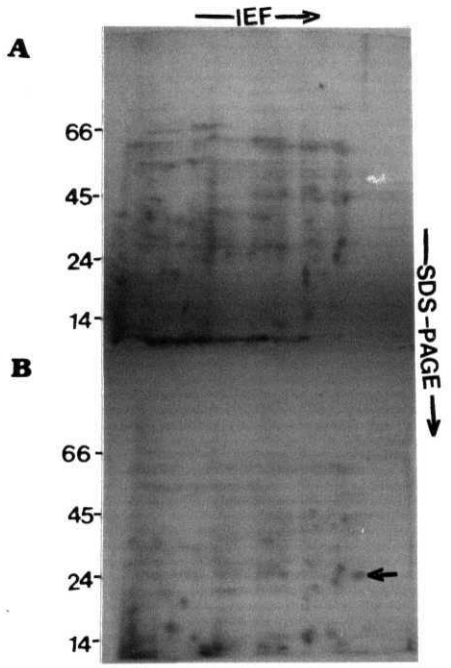

IEF
A
66
45
24
14
B
66
45
24
14
SDS-PAGE

Fig. 4.13 **Two-Dimensional** gel electrophoresis of **Tulasi** shoot extracts A, control; B, PEG treated. Arrow marks indicate induced proteins. These gels were silver stained.

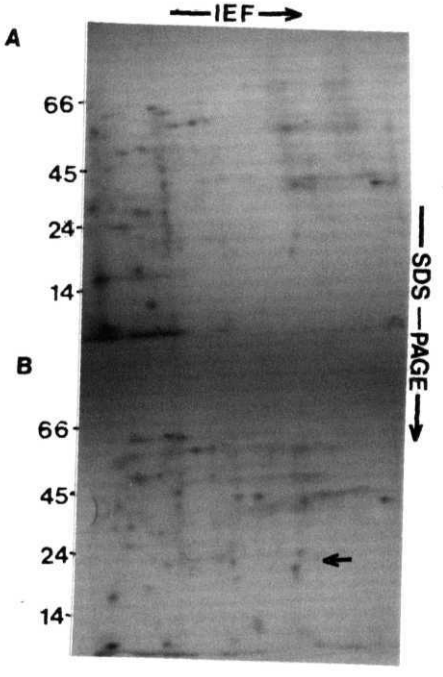

IEF
A
66
45
24
14
SDS — PAGE
B
66
45
24
14

Fig. 4.14 Two-Dimensional gel electrophoresis of **Tulasi** shoot extracts **A,** control; B, ABA treated. Arrow mark indicate induced proteins. These gels were silver stained.

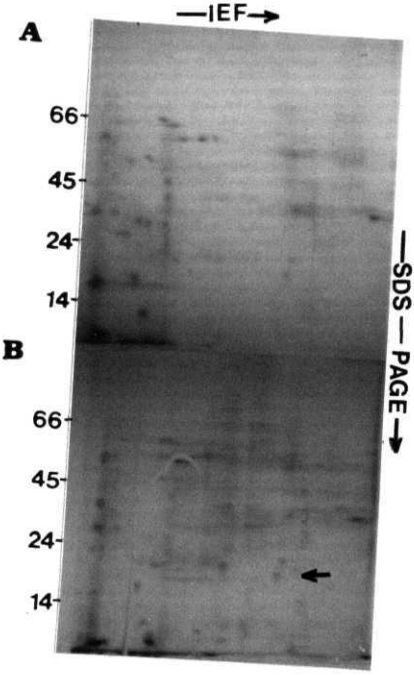

—IEF→
A
66
45
24
14
B
66
45
24
14
—SDS—PAGE→

Fig. 4.15 Two-Dimensional gel electrophoresis of Akashi shoot extracts A, control; B, PEG treated. Arrow marks indicate induced proteins. These gels were silver stained.

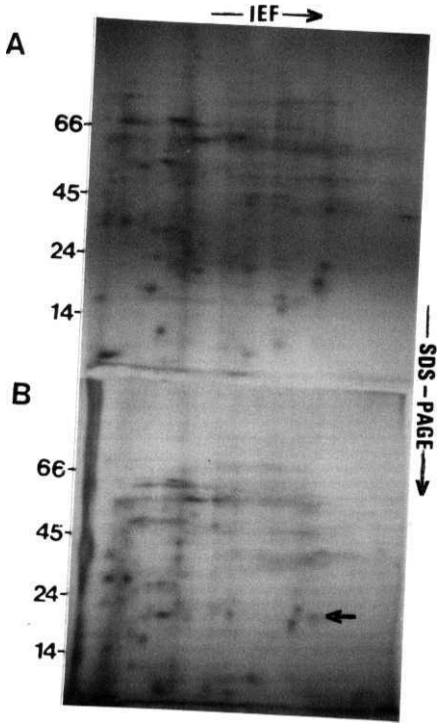
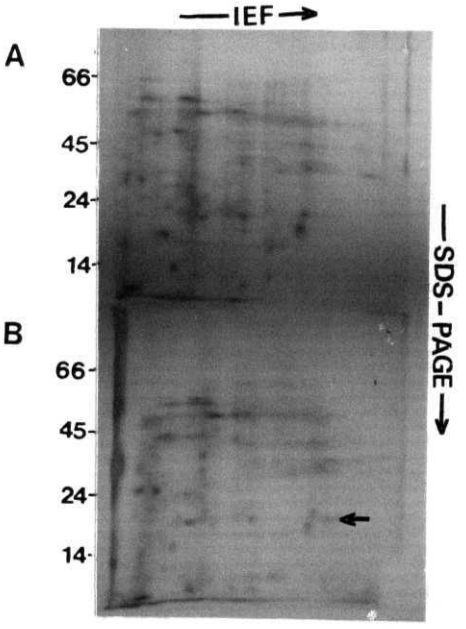

IEF
A
66
45
24
14
SDS - PAGE
B
66
45
24
14

Fig. 4.16 Two-Dimensional gel electrophoresis of **Akashi** shoot extracts A, control; B, ABA treated. Arrow marks indicate induced proteins. These gels were silver stained.


IEF
A
66
45
24
14
B
66
45
24
14
SDS-PAGE

given in Fig. 4.9 & 4.10 with respect to PEG and ABA stress. It is clear that the induction of 15, 23 and 46 kDa polypeptides in response to PEG and ABA confirms the One-D SDS-PAGE data.

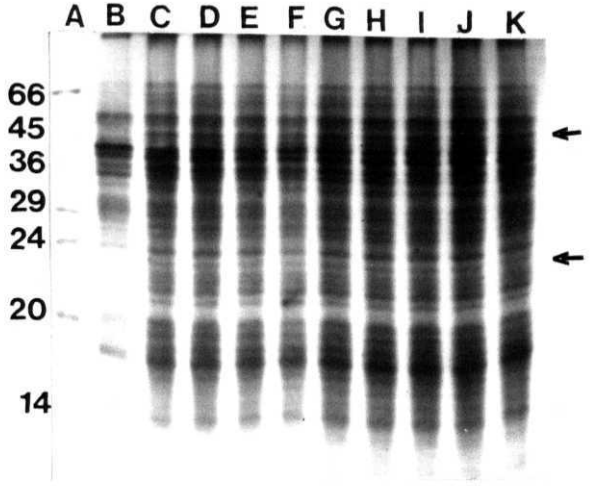
Two-dimensional electrophoresis (Isoelectric focusing followed by SDS-PAGE) was done for each of the tested lines. The 2-D data (Fig. 4.11 & 4.12) reveal the clear seperation of 46, 23 and 15 kDa polypeptides, in Hamsa extracts. Similarly, the 2-D gel profiles of Tulasi (Fig. 4.13 & 4.14) and Akashi (Fig. 4.15 & 4.16) reveal that these lines respond to both water stress and ABA in the induction of these three polypeptides. The pI value of 46, 23 and 15 kD polypeptides are calculated to be 5.7, 7.2 and 5.7 respectively.

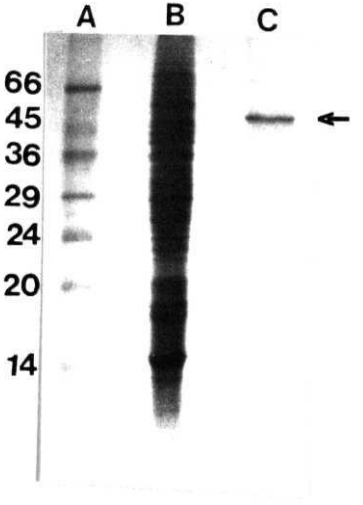
4.4 PURIFICATION OF STRESS RESPONSIVE PROTEINS :

The preparative gel electrophoresis of water stress induced polypeptides, specially 46 and 23 KD, from Hamsa seedlings extract were performed as a first step in the purification scheme. These two polypeptides were identified after coomassie staining the gel (Fig. 4.17) and cut out. These cut bands from a number of such preparative gels were electroeluted at high voltage and dialysed against buffer. The concentrated samples were re-electrophoresed to checked for their the purity. The SDS-PAGE profiles of control and purified samples of 46 KDa polypeptide are shown in Fig. 4.18. The appearance of a single band on SDS-PAGE demonstrated the purity of the sample. Similarly, the 23 kDa

Fig. 4.17 SDS-PAGE crude protein extracts of **Hamsa** shoots. Lanes A, **Mol.** wt. markers, **B,** control (C to K) PEG treated samples. (Each lane was loaded with 50 **μg** of protein).

Fig. 4.18 Purification of stress responsive proteins: SDS-PAGE of Hamsa shoot protein extracts and purified proteins. Lanes A, Mol. wt. markers; B. Hamsa control (50 μg of protein); C, partially purified 46 kDa protein (5 μg of protein).


A B C D E F G H I J K
66
45
36
29
24
20
14


A B C
66
45
36
29
24
20
14

polypeptide was also purified using the same strategy and it's purity was shown in Fig. 4.19.

The partially purified 23 kDa polypeptide was further purified by HPLC and the appearance of this protein as a single peak (Fig. 4.20) revealed it's purity.

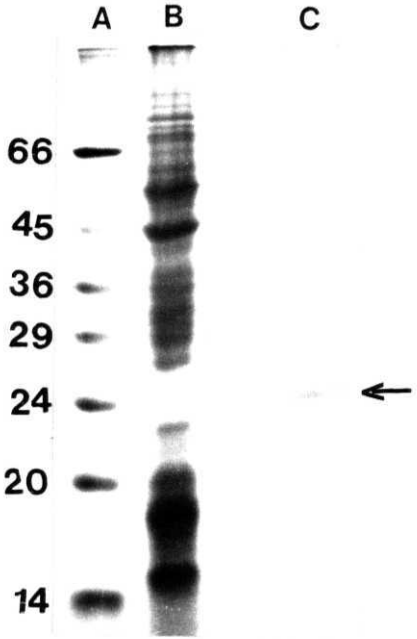
4.5 WESTERN BLOTTING ANALYSIS :

Antibodies were raised against the HPLC pure 23 kDa polypeptide and the same were used in Western analysis. The antibody specificity was tested against 46, 23 and 15 kDa purified polypeptides. The Western blot in Fig. 4.21 showed that the anti-23 kD antibody is highly specific to the 23 kDa polypeptide. A very faint band at 46 kDa mol. wt. was also detected.

Are the 23 kDa polypeptides induced by other kinds of stresses identical to the 23 kDa polypeptide induced by water stress? This was investigated by using anti-23 kDa antibodies in Western blots prepared from ABA, desiccation, salinity and cold treated seedling extracts. Data in Fig. 4.22 clearly reveal that the 23 kD protein induced by PEG, ABA, desiccation are immunologically similar. However, anti-23 kD antibodies do not detect any such protein in cold and salt treated seedling extracts. However a faintly visiable band of an apparent Mol. wt. of 46 kD was observed in cold treated samples (lane F).

Fig. 4.19 Purification of stress responsive proteins: SDS-PAGE of Hamsa shoot protein extracts and purified protein. Lanes (A) Mol. wt. markers (B) Hamsa control (50 µg of protein) (C) partially purified 23 kDa protein (5 µg of protein) .

Fig. 4.20 HPLC profiles of the partially purified 23 kDa protein (details in materials and methods)


A
B
C
66
45
36
29
24
20
14

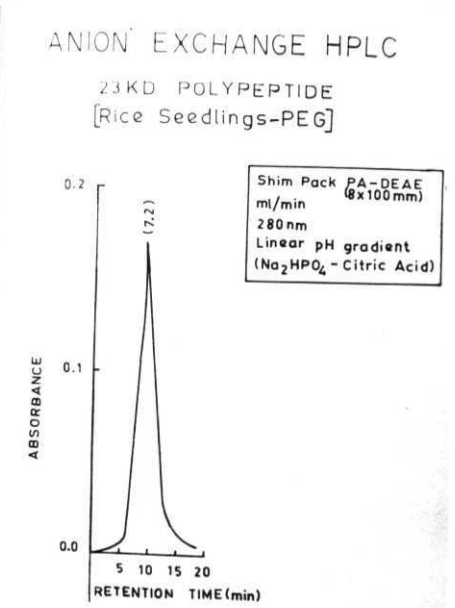
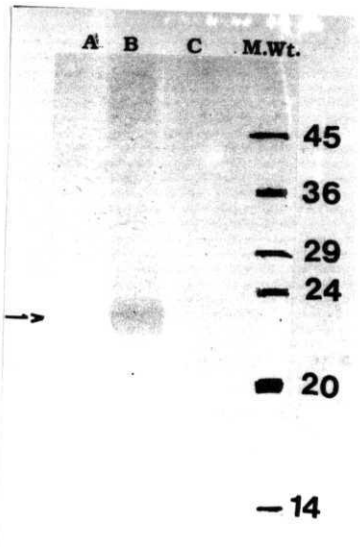
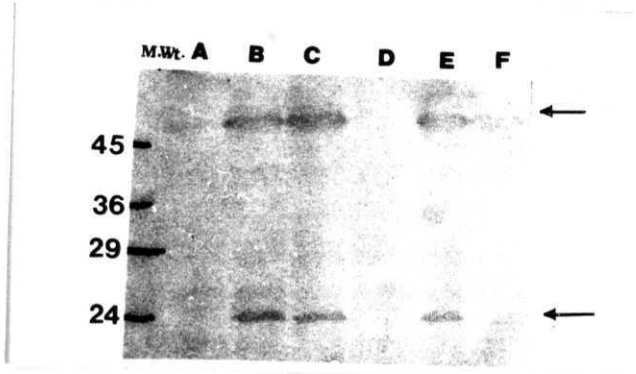

ANION EXCHANGE HPLC
23KD POLYPEPTIDE
[Rice Seedlings-PEG]
Shim Pack PA-DEAE (8x100mm)
ml/min
280nm
Linear pH gradient
(Na_2HPO_4 - Citric Acid)
(7.2)
0.2
0.1
0.0
ABSORBANCE
5 10 15 20
RETENTION TIME(min)

Fig. 4.21 Western blot analysis; (A) purified 15 kDa protein (B) 23 kDa protein (C) 46 kDa protein. Probed with anti-23kDa antibody (Each lane was loaded with 10 μg of purified protein).

Fig. 4.22 Immuno detection of 23 kDa protein by anti-23 kDa antibody in shoot extracts of Hamsa seedlings; A : control; B : PEG; C : ABA; D : 0.5M NaCl; E : air drying of seedlings and F : cold acclimation. (75 μg of protein was loaded into each lane.
Note: 23 kDa band is missing in lanes D and F.


A
B
C
M.Wt.
45
36
29
24
20
14


M.Wt.
A
B
C
D
E
F
45
36
29
24

Is the 23 kra polypeptide related to the ABA responsive proteins (RAB family) of rice. This was investigated by using anti-RAB 16 antibodies in Western blots of purified 15, 23 and 46 kD proteins The Western data in Fig. 4.23 show crossreactivity between 23 kDa protein and anti-RAB16 antibody. Further a faint band with an apparent mol. wt. 46 kDa was also detectable (lane b).

Western blots of crude extracts from PEG and ABA treated Hamsa seedlings were probed with anti-RAB16 antibodies and data in Fig. 4.24 demonstrate the crossreactivity with a 23 and 46 KDa polypeptides. However, the control lane did not show any proteins showing cross reactivity.

4.2 Table II

Characterization and analysis of the three stress responsive proteins.

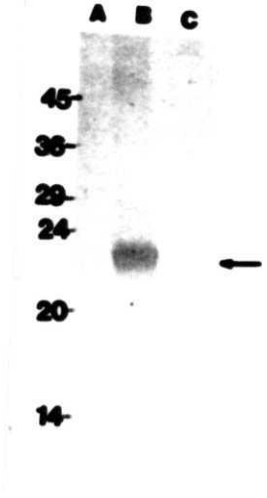
Summary Table

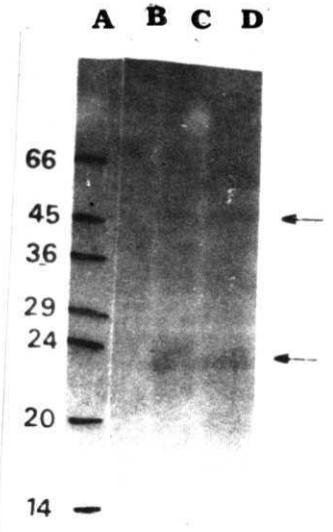
Polypeptides	1D	2D	Purification	Antibodies
46	+	+	+	-
23	+	+	+	+
15	+	+	-	-

1D : One dimentional SDS-PAGE, 2-D : Two-dimentional Electrophoresis (IEF/SDS-PAGE)

Fig. 4.23 Western blot analysis of purified proteins (A) 15 kDa (B) 23 kD (C) 46 kDa protein probed with anti RAB16 antibody. (Each lane was loaded with 10 µg of purified protein).

Fig. 4.24 Immuno detection of the 23 kDa protein by anti-RAB16 antibodies in crude protein extracts of Hamsa. (A) Mol. wt. markers; (B) Control (C) PEG; (D) ABA (75 µg of protein was loaded in each lane.


A
B
C
45
36
29
24
20
14


A
B
C
D
66
45
36
29
24
20
14

4.6 HEAT STABILITY STUDIES OF STRESS RESPONSIVE PROTEINS :

The stress induced crude extracts were exposed to boiling temperature (as in Materials & Methods) and protein samples were run on SDS-PAGE (Fig. 4.25). As can be seen, the 23 kDa polypeptide is clearly heat stable. Further, several other polypeptides were also found to be heat stable. These heat stable bands appear prominantly in the treated lane compared to that of the control.

4.7 COLD STRESS RESPO SIVE CHANGES IN POLYPEPTIDES PROFILES:

The possible genetic differences in cold response between the cold sensitive and tolerant rice cultivars in terms of their protein profiles were investigated. In addition, the relative abundance of several polypeptides was also affected by cold stress. The response of rice seedlings to cold stress (4° C) wan studied with an objective of discriminating between water stress and cold stress response pathways. The effect of cold temperature on the polypeptide profiles of a number of rice lines (Group II, 4.1 Table 1) was investigated mainly by SDS-PAGE analysis.

The cold and ABA stress induced proteins in K39, a cold tolerant indica rice cultivar, was studied by SDS-PAGE. The shoot/root protein profiles (Fig. 4.26) revealed the induction of certain proteins during ABA stress conditions and with apparent molecular weight 15 kD and 46 kD. One of the prominent polypeptide with a mol. wt. of 42 kDa disappears during the cold and ABA stress. We also

Fig. 4.25 SDS-PAGE of Boiling Stable proteins from shoot extracts of Hamsa. Lanes (A) Mol. wt. markers; (B) control (C) control boiled (D) PEG treated; (E) PEG treated - boiled; (F) ABA treated; (G) ABA treated - boiled.

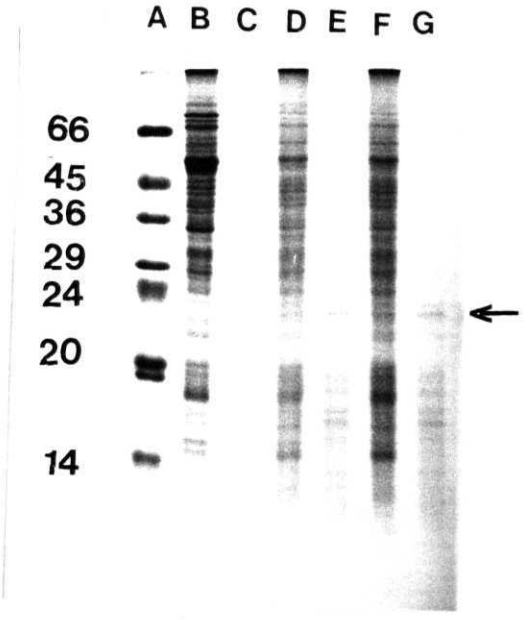
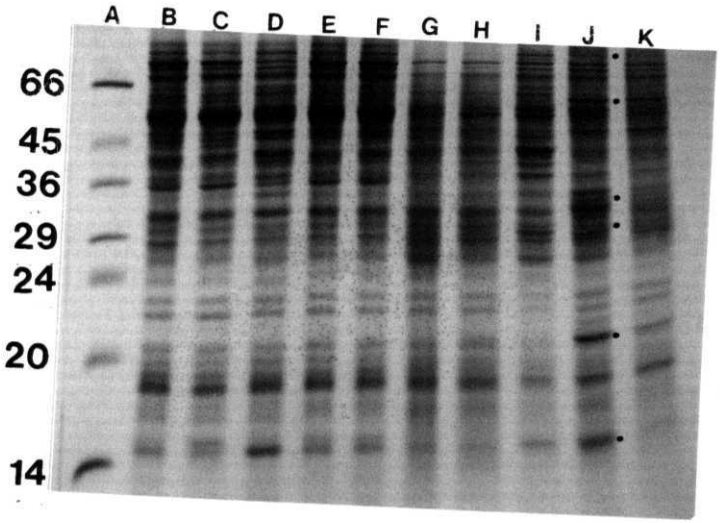

A
B
C
D
E
F
G
66
45
36
29
24
20
14

Fig. 4.26 **SDS-PAGE** of K39 rice seedlings. Lanes (A) **Mol.** wt. **markes.** (B) 5 day old control shoots (C) 10 day old control shoots (D) 10 day old ABA treated shoots (E) 10 day old cold treated shoots (F) 10 day old cold + ABA treated shoots (G) 5 day old control roots (H) 10 day old control roots (I) 10 day old ABA roots (J) 10 day old cold roots (K) 10 day old cold + ABA roots (Each lane was loaded with 50 µg of protein).


A
B
C
D
E
F
G
H
I
J
K
66
45
36
29
24
20
14

maintained two controls. One is before treatment and another one at harvesting time to see that any developmental stage specific induced proteins are also induced during the stress conditions. However, we did not find any difference between the two controls. Same practice was followed for roots also. In roots, ABA stress induced the 80, 66, 45 and 3 3 KD polypeptides. Cold stress leads to induction of at least 7 polypeptides with apparent molecular weights 80, 60, 50, 33, 29, 21 and 15 kD. 4.3 Table 3 shows the changes in relative abundance of polypeptides under cold stress.

We also investigated the cold stress response of a sensitive line Jaya and a tolerant line Khonorullo and also a few advanced (F10) progeny lines RCPL-1-1C, 2C, 3C, 6C and 9C. The SDS-PAGE data (Fig. 4.27) clearly demonstrated the induction of specific polypeptides with an apparent mol. wt 79, 70, 46, 38 and 15 KD in shoots of Jaya and Khonorullo. The SDS-PAGE of RCPL-1-1C, 2C, 3C, 6C and 9C revealed (Fig. 4.28) the induction of the same polypeptides. Interestingly, one of the major polypeptide with an apparent molecular weight of 42 kDa disappears during the cold stress conditions in all of above tested rice cultivars.

4.8 HEAT STABILITY OF THE COLD STRESS INDUCED PROTEINS :

In order to test whether the cold stress induced polypeptides are heat stable, the crude protein extracts of Khonorullo shoots treated with cold were boiled for 25 minutes in a boiling water bath and the protein fractions

4.3 Table 3

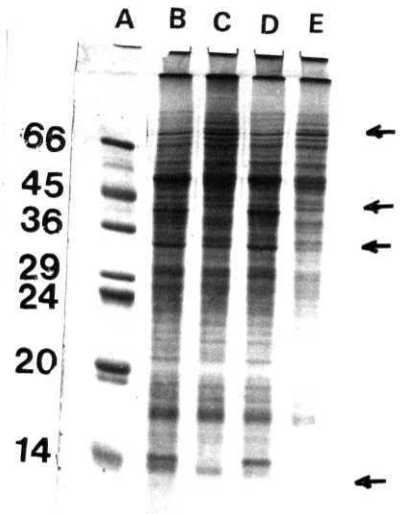
Relative abundance of the cold stress responsive polypeptides: K39

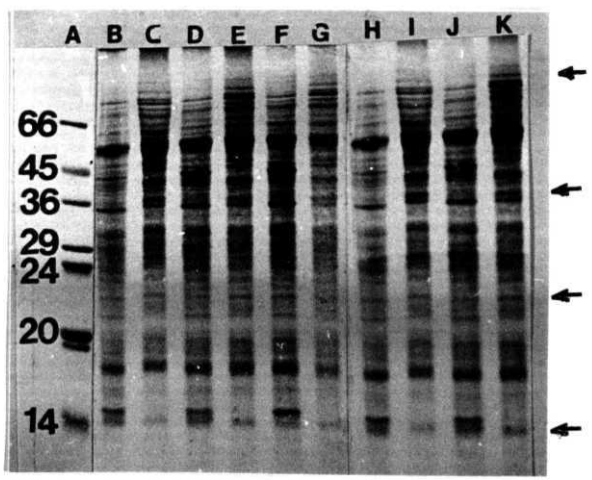
Apparent Mol. Wt. polypeptides (kD)	Control H / L	ABA H / L	Cold H / L	Cold + ABA H / L
Shoots				
85	-	-	+	+
80	+	-	+	+
75	+	-	+	+
49	-	+	+	+
35	+	-	+	+
15	-	+	-	-
Roots				
80	-	+	+	-
65	-	-	+	+
48	-	+	-	-
33	-	-	+	-
30	-	+	+	+
21	-	-	+	+

H - High L - Low

Fig. 4.27 SDS-PAGE profiles of shoot extracts of different rice cultivars after cold treatment. lane. A, Mol. wt. markes, lanes B and C khonorullo control and cold treated; lanes D and E Jaya control and cold treated. (Each lane was loaded with 50 µg protein).

Fig. 4.28 SDS-PAGE profiles of shoot extracts of differnt rice cultivars after cold treatment. Lane A, Mol. wt. markers; lanes B and C RCPL-1-1C control, cold treated; lanes D and E RCPL-1-2C control, cold. treated; lanes F and G RCPL-1-3C control, cold treated; lanes H and I RCPL-1-6C control, cold treated; lanes J and K RCPL-1-9C control, cold treated. (Each lane was loaded with 50 µg of protein).


A
B
C
D
E
66
45
36
29
24
20
14


A
B
C
D
E
F
G
H
I
J
K
66
45
36
29
24
20
14

were subjected to SDS-PAGE. The protein profiles in (Fig. 4.29) clearly show that 15 and 46 KDa proteins remain detectable even after boiling while a majority of polypeptides disappeared.

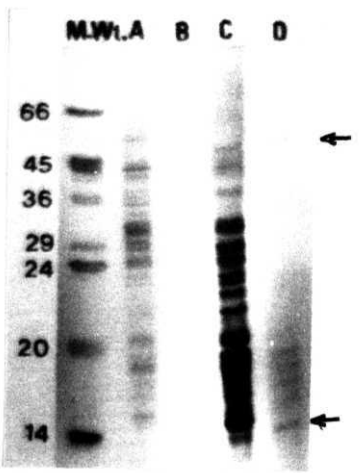
4.9 STUDIES ON STRESS RESPONSIVE RNAs IN SEEDLINGS:

Effect of various stresses on certain gene specific mRNAs have been carried out. For this purpose we have used two different cDNA probes. These are pcC 27-04 and SalT. Of these, the former is a cDNA clone from a cDNA library of a resurrection plant Craterostigma plantagineum (D. Bartels Max-Planck Inst.). The later clone, salT cDNA, is from rice (A. Caplan, Ghnt). The Craterostigma probe represents one of the desiccation resistance specific genes (as these are found to be fully expressed during desiccation). The SalT gene was found to be active in salt stressed roots and also certain other tissues.

The purpose of using these clones was to see whether there are any sequences homologous to that of Craterostigma existing in rice genome and also to find out whether they are expressed in response to dehydration and other stresses. Similarly, we have also attempted to find out whether the salT responsive genes are expressed under dehydration and temperature stress.

Total RNA preparations obtained from the control and stressed shoots were blotted on to nitrocellulose filters and

Fig. 4.29 SDS-PAGE of boiling stable proteins from Khonorullo shoot protein extracts. (A) control (B) Boiled control (C) Cold (E) cold-boiled. (Each lane was loaded with 75 μg of protein) .


M.Wt.
A
B
C
D
66
45
36
29
24
20
14

Hybridized with individual probes.

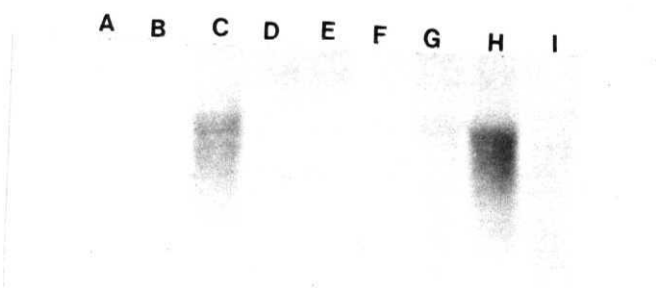
The Northern data with pcC 27-04 were shown in Fig. 4.30 and 4.31. The data reveal that Hamsa, N22 and K39 accumulate pcC 27-04 specific transcript under salinity stress. Interestingly they do not accumulate the same RNA under dehydration and ABA stress. On the other hand, the cultivar Akashi accumulates pcC 27-04 specific mRNA under cold stress. None of the other three cultivars show this kind of response. Controls did not show any hybridization.

RNA Dot-blot analysis was also carried out in dark grown seedlings using the salT cDNA gene probe. The results (Fig. 4.32) demonstrate the accumulation of SalT specific mRNA in PEG and ABA seedlings, whereas Tulasi and Akashi show only under ABA stress. Both these cultivars failed to show any hybridization under cold stress. What is interesting, however, is the observation that the K39 gave intense signal in non-stressed control itself and failed to show any signals under cold stress. This interesting aspect needs further study.

In parallel, experiments were also carried out with light grown seedlings using the SalT probe. The results (Fig: 4.33) are some what different from dark experiments. Tulasi

Fig. 4.30 Northern blot analysis of the expression of Craterostigma plantagineum cDNA clone pcC 27-04, in response to various stresses in different rice cultivars (shoots).
A) Ilamsa control. B) PEG. C) Salt. D) Cold.
F) N22 control. F) PEG. (1) AHA. II) Salt. I) Cold.
Each lane is loaded with 30μg of total UNA.

Fig. 4.31 Northern blot analysis of the expression of Craterostigma plantagineum cDNA clone pcC 27-04, in response to various stresses in different rice cultivars (shoots).
A) K39 Control. B) PEG. C) ABA. D) Salt. E) Cold.
F) Akashi control. G) PEG. H) ABA. I) Salt. J) Cold.
Each lane is loaded with 30μg of total UNA.


A B C D E F G H I

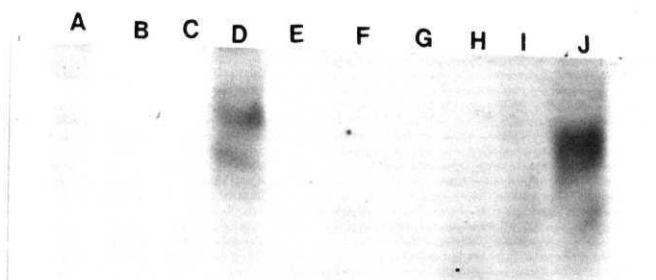

A B C D E F G H I J

Fig. 4.32 RNA Dot-BLOTS of dark grown shoot extracts of differnt rice cultivars. 20 µg of total RNA loaded in each dot and hybridized with SalT cDNA probe (A) Hamsa control (B) PEG (C) ABA (D) Cold (E) Tulasi control (F) PEG (G) ABA (H) Akashi control (I) PEG (J) ABA (K) K39 control and (L) cold.

Fig. 4.33 RNA DOT-BLOTS of light grown shoot extracts of different rice cultivars. 20 µg of total RNA loaded in each dot and hybridized with SalT cDNA probe. (A) Hamsa control (B) PEG (C) ABA (D) Tulasi control (E) PEG (F) ABA (G) Akashi control (H) PEG (I) ABA.


A B C D E F G H I J K L


A B C D E F G H I

did not show any signals under PEG stress unlike under dark conditons. Moreover, ABA does not seem to induce SalT specific mRNA in Tulasi. However, light grown Akashi shows good hybridization signal under ABA stress as in the case of dark grown seedlings.

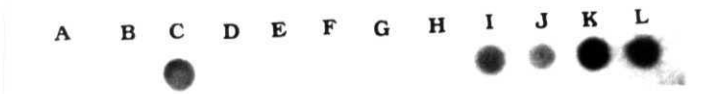
Accumulation of SalT gene specific RNA was tested in the total root RNA extracts of the these line under various stresses. Results (Fig. 4.34) show that the SalT specific RNAs were detectable in PEG treated samples of Hamsa, Akashi and the cold treated samples of K39. It is interesting that while K39 shoots did not accumulate SalT gene specific RNA under cold stress the root extracts accumulate significant amount of same RNA.

In order to confirm the above, we have done Northern gel blot of different rice lines using the SalT cDNA probe. The results are given (Fig. 4.35). The data clearly show that the dot-blot and Northern blots agree very well. Further, the SalT gene specific mRNA shows abundant accumulation in seedlings of Hamsa, Tulasi and Akashi under PEG as well as ABA stress. Further, K39 interestingly accumulates SalT RNA only in controls.

In summary the RNA dot-blot and Northern blot experiments clearly demonstrated the presence of pCC 27-04 and SalT specific gene homologues in rice.

Fig. 4.34 **RNA** DOT-BLOTS of root extracts of different rice cultivars. 20 μg of total RNA loaded in each dot and hybridized with SalT cDNA probe. (A) **Hamsa** control (B) PEG (C) ABA (D) Cold (E) Tulasi control (F) PEG (G) ABA (H) Akashi control (I) PEG (J) ABA (K) K39 control (L) cold.

Fig. 4.35 Northern blot analysis of the expression of SalT cDNA clone in response to various stresses in different rice cultivars. (A) Hamsa control (B) PEG (C) ABA (D) cold (E) Tulasi control (F) PEG (G) ABA (H) Akashi control (I) PEG (J) ABA (K) K39 control (L) cold.


A B C D E F G H I J K L


A B C D E F G H I J K L

PEG-Mediated Water Strss and ABA Causes Differential Increase in Proline Levels in Shoots and Roots

Present results show that shoots respond differently to PEG and ABA. PEG causes a moderate to dramatic increase **in** proline levels in all the eight tested cultivars **(Fig. 4.1** A & B and **4.2** A & B). In contrast to PEG, ABA causes significant **increase** only in N22, **IR8,** HR12 and **TN1 (Fig.4.1B)** and a marginal increase or not at all in **Hamsa,** Tulasi, Annada and **Akashi** (4.1A). It is interesting that both the parents of Akashi, i.e, IR8 and N22 show response of shoots to ABA, whereas Akashi does not, indicating that the stress tolerance is not directly associated with proline accumulation. Alternatively this response pattern is only specific to seedling stages. The tolerance response of Akashi however, was defined routinely on the basis of the yield and other agronomical attributes. The lack of increase in proline content in Akashi can also be explained on the basis that Akashi, represents an advanced selection from the progeny (F10) of the cross of IR8 X N22, and **therfore** may be a segregant.

It is well known in plants that shoots accumulate increased amounts of proline under water stress conditions **(Stewart,** 1980; Aspinall and **Paleg,** 1981) and rice is no exception. However, the intriguing lack of response of shoots to ABA in accumulation of proline in certain rice

lines needs further analysis. Several factors like exogenous ABA concentration and uptake, method of application, physiological status of the shoot, seedling age etc., may contribute to the non-response of shoots to ABA. Roots, however, showed an increased proline accumulation under both PEG and ABA stress.

Drought Tolerant Rice Cultivars Accumulate more Proline under Water Stress than Drought Sensitive Ones

It is clear from the results that under stress, the tolerant cultivars Akashi, N22, Tulasi and Annada in the descending order, show greatly increased proline levels in shoots and roots than that of the sensitive cultivars like **Hamsa.** Further, Akashi, which is used as a check entry in breeding programmes for drought tolerant rice lines, showed the maximum proline accumulation under stress. In **contrast,** the sensitive line, Hamsa, did not show any such typical increase in root proline content under ABA stress.

In cereals, significant differences exist between sensitive and tolerant cultivars in terms of proline accumulation under stress (Hanson et ***al.,*** 1977; **Stewart,** 1980). We conclude that in rice, the stress induced proline accumulation in shoots in general and roots in particular is a useful parameter of stress response and may be used in screening **germplasm** and also in allied breeding methodologies for developing drought responsive lines. We caution, however, that our results do not necessarily mean **that** the increased proline accumulation is positively correlated with

stress resistance. Although the rapid proline accumulation under water stress conditions is suggested to be associated with cellular osmotic adjustment (Aspinall and Paleg, 1981), it's exact role in stress resistance mechanism in rice is yet to be clarified.

Proline accumulation was reported to be rapid in cereal leaves subjected to dehydration and the extent of increase was primarily correlated with the length of the water stress period (Singh et *al.*, 1973; Sivarama Krishnan, 1988). In the present study, the water deficit caused by PEG is a prolonged one and the seedlings have ample time to respond through osmotic adjustment by accumulation of proline and other solutes. Thus the dehydration responsive increase in proline content may serve as an indicator of stress response of the seedlings.

Cold acclimation causes increase in Proline content:

A variety of abiotic stresses like cold acclimation and salt treatment were reported to cause a significant increase in proline content in several plant species (Charest and Phan, 1990, Chu et *al.*, 1976). A halophyte like Triglochin maritima was reported to accumulate as much as 123 µg/mg fresh wt. of free proline under stress (Stewart and Lu, 1974). Our results also showed that cold acclimation leads to increase in proline accumulation in rice seedlings.

In contrast to dehydration and ABA stress which showed differential action, cold stress was found to cause a significant increase in the proline content of shoots and roots in all the tested rice lines. These include both cold sensitive ones, i.e. Jaya and Hamsa and cold tolerant cultivars K39, Khonorullo, and RCPL-series. It is to be noted that the later lines are advanced selections from the progeny of the cross Jaya x Khonorullo or Pusa-33 X Khonorullo cold tolerance in Himalayan and Subhimalayan regions.

Interestingly the extent of average increase in proline content by cold is significantly small compared to that of dehydration and ABA. It is not clear whether proline accumulation is directly correlated to cold acclimation or it is simply a primitive response (Measures, 1975) to stress injury. Further, the response of seedlings to cold is not through death or lethal reaction since these seedlings are viable and can be recovered by transfering them to normal temperature subsequent to the treatment.

Dehydration and ABA induce changes in Polypeptide Profiles: Appearance of new Polypeptides:

SDS-PAGE profiles followed by 2-Dimentional electrophoresis revealed distinct changes in polypeptide profiles under dehydration and ABA stress. As can be seen (Fig. 4.4 to 4.8 and 4.11 to 4.16) the relative abundance of

a number of polypeptides was affected and specific polypeptides, namely the 15 kDa, 23 kDa and 46 kDa appeared under stress. The 2-D data confirm the induction of the 23 kDa polypeptide under dehydration and ABA. It is interesting that a 23 kDa protein was also induced by ABA and desiccation (air drying) but not by cold acclimation and NaCl, as revealed by the Western blot experiments.

The PEG-Responsive 23kDa Protein is also Responsive to Several but not all Osmotic Stress:

All the tested cultivars show the induction of 23 kDa polypeptide under water stress and ABA. The western analysis using the anti-23kDa antibodies clearly revealed that the water stress induced 23kDa protein was also induced by ABA and air drying but not by cold acclimation and NaCl treatment. 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 (Barker et.al., 1988;Dure et.al,1989; Close et.al., 1989; Curry et.al., 1992) are also associated with ABA as well as

dehydration response pathways (Skriver & Mundy, 1990) but not with salt stress response. The Em (early methionind lebelled) polypeptides which belong to the Lea family are 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 a 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 **23kDa** protein was also induced in cell suspension cultures of several indica rice cultivars under PEG and ABA stress (Reddy **et.al.,** 1992). It is proposed that this protein may be used as a water stress response marker in rapid mass screening of rice seedlings for their 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 water stress situation or the recovery process involves a typical water stress response mechanism.

The exact role of the **2 3** kDa polypeptide in stress response remains to be ascertained. One way to ascribe a functional role to this protein is to analyse it's structural similarities to the known stress responsive proteins in rice. Such proteins include lea proteins, **(Galau,** et *al.,* 1986; Baker et *al.,* 1988; Dure et al., 1989)) RAB proteins (Mundy and Chua, 1988), Em (Bostock and Quatrano, 1992; Williamson

and Quartrno, 1988) and dehydrins (Close et *al* , 1989; Close and Chandler, 1990; Bradford and Chandler, 1992). To this end we have tested whether 23 kDa polypeptide is same as RAB family proteins by using the Western blotting methodologies.

The 23kDa Protein is Immunologically Similar to RAB16 Protein:

The Western blot data using anti RAB16 antisera (Courtesy of John Mundy) clearly demonstrated that the 23kDa protein is homologous to the RAB 16 protein at the immunologial level (independenly confirmed by Dr. John Mundy, Carlsburg). Interestingly,however,the RAB 16 family of proteins are both cold and salt responsive (Mundy and Chua, 1988), whereas the 23kDa polypeptide is not. This suggests that the 23kDa protein is only partially homologous but not identical to the 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) and wheat (Williamson and Quartrno, 1988), dehydrins from barley, maize (Close et.al., 1989; Close and Chandler, 1990) and rice (Bradford and Chandler, 1992) and desiccation resistance proteins (deduced from the cDNA sequence) of Craterostigma plantagineum (Piatkowski et.al., 1990) share some sequence homologies with the RAB16 proteins, they are not identical to it.

Footnote: The observed differences can also be due to differences in the genetic background of the rice cultivars culture regimes or specific organs of the plants used by us and the earlier workers.

Boiling Stability and Possible Physiological Function of the 23kDa Protein:

Our results demonstrate that the stress responsive 23kDa protein is boiling resistant (Fig. 4.25). 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 these proteins share among themselves 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 23kDa protein might play a role in water stress tolerance.

Intrestingly, several cold stress induced polypeptides were also found to be boiling resistant (Fig. 4.29). There were a number of instances of cold acclimation leading to the accumulation of boiling stable proteins (Lin et *al.,* 1990; Hajela et *al.,* 1990; Gilmour et *al.,* 1992). It was demonstrated that some of these cold induced boiling resistant proteins like 'cor' proteins of Arabidopsis thaliana were found to have both thermoprotec tive as well as cryoprotective activities (Lin and Thomashow, 1992). Recently it was reported that there is a partial sequence

homology between cor genes of Arabidopis and certain stress responsive genes of Rice (Mundy and Chua, 1988), Cotton D11 (Dure et *al.*, 1987), Barley dehydrin (Close et *al.*, 1989), Maize M3 (Close et *al.*, 1989), Tomato TAS14 (Godoy et *al.*, 1990) Craterostigma plantagineum pcC 27-04 (Patkowski et *al.*, 1990). Our observations suggest that, these cold induced polypeptides may have a role to play in stress response mechanisms in rice seedlings.

Cold stress Responsive polypeptides:

Exposure of rice cultivars to cold temperature lead to significant changes in protein profiles including both qualitative and quantitative type. The data on cold induced polypeptides in K39 seedlings substantiate the above (Fig. 4.26). It appears that roots respond dramatically compared to shoots to the cold stress. The K39 cultivar is one of the well known cold tolerant variety and used in breeding programmes for developing cold tolerant indica rice lines. Similarly Khonorullo is another cold tolerant rice line which is also used extensively in breeding for cold tolerance. A comparison of these two rice lines between themselves and a cold sensitive line Jaya revealed interesting deference. While cold sensitive cultivar Jaya does not show significant changes in protein profiles, both the tolerant lines K39 and Khonorullo respond by accumulation of specific induced proteins in shoots (Fig. 4.26 and 4.27). These results lead to the conclusion that roots show more profound changes in

protein profiles than that of shoots under cold stress. This observation is perhaps the first of it's kind in rice and other cereals.

The results on the advanced progeny selections from the cross between Jaya X Khonorullo or pusa 33 X Khonorullo suggest no clear cut information on the inheritance of induced proteins. We could only report that all the RCPL lines used in the present study respond to cold stress in more or less a similar way. The most prominantly induced proteins in parents are those with apparent mol. wt. about 39, 36 and 29 kDa respectively. These proteins also appared in RCPL series. Another notable proein is the 15 kDa which was induced by cold stress in all the tested cultivars. Our results suggest that cold stress induces a different set of proteins the ı those induced by other stresses, with one exception, the 15 kDa protein. We are now using the proteins of apparent mol. wt. of 39, 36 and 29 kDa as markers of cold stress response in rice seedlings.

Dehydration stress induced transcripts hybridizable a desiccation resistance associated gene sequence of an Afric resurrection plant, Craterostigma plantagineum:

Craterostigma plantagineum is an African resurrection plant, which can tolerate upto 90% of water loss (Bartels et *al.,* 1990; Gaff, 1971). A number of cDNA clones were isolated from a cDNA library constructed from poly-A^+ RNA from desiccated tissue (Bartels et *al.,* 1990). We have

tested one such clone namely, pcC 27-04, whether it can detect any hybridizable transcripts of stressed seedlings. The data from Dot-Blot (Fig. 4.30) analysis clearly revealed that the pcC 27-04 homologus sequences can be detected in stressed seedlings of several cultivars.

It is interesting that cold stress also leads to the accumulation of pcC 27-04 homologous transcripts in K39. This is true for both shoots and roots. It is interesting that since pcC 27-04 sequence was reported (Piatkowski et *al.*, 1990) to be partialy homologus to Rab (Munday and Chua, 1988), dehydrins from Barley (Close et *al.*, 1989), Maize (M3) (Close et *al.*, 1989) and Cotton D11 (Baker et *al.*, 1988). We propose that this transcript in rice seedlings is associated with stress response mechanism. This is, perhaps the first report to show the accumulation of water stress and cold stress induced transcripts homologous to a desiccation resistance associated gene of Craterostigma. It is still not clear whether the product of pcC 27-04 sequence is an enzyme or a structural protein having specific attributes of other known stress responsive proteins.

Dehydration and ABA stress induce Salt responsive Transcripts:

The Dot-blot (Fig.4.32) experiments using a salt responsive cDNA probe (SalT of rice) obtained from (Dr. Caplan, (Gent, Belgium) revealed interesting information. Dehydration and ABA cause induction of SalT specific mRNA in Hamsa, Tulasi and Akashi cold acclimation does not.

What was more interesting was the accumulation of SalT specific mRNA in K39 control shoots but not under cold treated samples. Further the dot blot data showed that the roots of Hamsa and Akashi accumulate SalT specific mRNA under PEG ADA stress. Similarly K39 accumulates control and cold stressed roots and other hand Tulasi did not show any hybridization. Northern data on shoots revealed that while Hamsa and Akashi accumulated salt specific RNA under both PEG and ABA, Tulasi shoots accumulate only under ABA stress. This indicates that the room temperature it self may be a stress for the K39 cultivar which is a cold adapted line. This needs to bo further analysed.

Data from Northern experiments (Fig: 4.35) are in complete? agreement with that of the Dot-blot experiments of dark grown seedlings under stress conditions. (Fig:4.32) In summary, we conclude that SalT mRNA is inducible not only by salt and dehydration as reported earlier in rice (Claes et al., 1990) but also by cold stress in roots. The role of theSaltT specific mRNA and proteins in stress response mechanisms in rice seedlings is yet to be clarified.

The data presented here would allow us to speculate a possible correlation between prolinc levels and stress tolerance. The most drought tolerant cultivars tested here, Akashi and N22, accumulate the highest levels of proline in shoots and roots under stress condition. Similarly, the least tolerant line, Hamsa accumulate significantly low levels of proline. Such a positive correlation suggests an

adaptive role of proline **in** adjusting to stress induced osmotic disturbances. This is in agreement with the suggested role of proline in osmoregulation under water stress environment.

The fact that both sensitive as well as tolerant lines show same patterns of stress responsive proteins, suggests these proteins are associated with basic stress response pathways in rice seedlings.

These proteins therefore presumed to be encoded by basic genes of stress response pathways. An immediate approach for analysing the genetic basis of such a response pathway would be to look for mutations at any of these genes encoding such proteins. Availability of such mutants will be helpful in unraveling the adaptive role of stress induced proteins and their possible interactions with other gene products, including small molecular entities. However, to date no such mutants have been discovered in rice.

The presence of pcC 27-04 specific mRNA and thereby the gene homologue is demonstrated here for the first time in rice. This paves the way for an interesting set of studies on desiccation resistance genes in rice. The very presence of this mRNA in rice seedling under a variety of stress conditions is an indication that probably similar basic mechanisms operates in Craterostigma and Oryza. These two genera are far a part in the evolutionary history, the rice homologue probably represents a modified version of that of Craterostigma. The sequence data at both DNA and protein level would ultimately unravel the relationship.

SUMMARY AND CONCLUSIONS

Response of rice seedlings to a variety of abiotic stresses like PEG mediated dehydration, ABA, desiccation and cold temperature has been investigated. The experimental genetic stocks include both sensitive as well as tolerant indica rice cultivars. PEG-mediated water stress and ABA cause a differential increase in proline levels in shoots and roots. Drought tolerant rice cultivars accumulate more proline under water stress than drought sensitive ones. Cold accumulation also caused increased levels of proline in both sensitive as well as tolerant lines. Several polypeptides were induced by dehydration and ABA stresses, specifically 15 kDa, 23 kDa and 46 kDa polypeptides. These were further charecterized. Water stress induced 23 kDa and 46 kDa polypeptides were partially purified and antibodies were raised against 23 kDa polypeptide. Using the anti-23 kDa antibodies we demonstrated that this protein is induced by dehydration ABA and airdrying but not by salt and cold. The 23 kDa protein belongs to the RAB16 family of rice as shown by Western blot analysis. The PEG and ABA induced 15 kDa and 23 kDa polypeptides were found to be boiling stable. Further, we demonstrated that the cold stress induced 15 kDa and 46 kDa polypeptides were also boiling stable. Several cold stress responsive proteins have been identified and their relationship with water stress and ABA responsive ones was investigated, in cold sensitive as well as cold tolerant rice cultivars.

We demonstrated that the induction of a transcript in stressed seedlings detectable by te desiccation resistance gene probe (pcC 27-04) of Craterostigma plantagineum, an African resurrection plant. Further DOT-BLOT and Norther blot data revealed the induction of SalT gene-specific mRNA under dehydration, ABA and cold treated roots

It is proposed that the 23 kDa polypeptide is a generalized water stress responsive protein and this can be used as a marker protein to descriminate water stress response from other types of stress responses in seedlings. The unique property of heat stability of these protein suggest some important role in stress response/ resistance of rice seedlings.

REFERENCES

Addicott, F.T. (1983). Ed. Abscisic acid, Praeger, New York.

Aspinall, D. and Paleg, L.G. (1981). Proline accumulation physiological aspects. In paleg, L.G., Aspinall D. eds. The physiology and Biochemistry of drought resistance in plants. Academic Press, Sideney, Australia, pp. 215-226.

Baker, J.C, Steel. D., and Dure, III L (1988). Plant. Mol. Biol. 11: 277-291.

Baker, F.W.G. (1989). Ed. Drought Resistance in Cereals. CAB, International, U.K.

Bates, L.S., Waldren, R.P. and Teare I.D. (1973). Plant Soil. 39: 205-208.

Bartels, D., Hanke, C., Schneider, K., Michel, D. and Salamini, F. (1992). EMBO J 11: 2771-2778.

Bartels, D., Engelhardt, K., Roncarati, R., Schneider, K., Rotter, M. and Salamini, F. (1991). EMBO J 10: 1037-1043.

Bartels, D., Schenider, K., Terstappen, G., Piatkowski, D. and Salamini, F. (1990). Planta 181: 27-34.

Berry, J. and Rasion, J.K. (1982). In Encyclopedia of Plant Physiology ed. 0. Lange, C.B. Osmand. P.S. Nobel 129: 277-338, Berlin, Springer.

Blum, H., Beier, H. and Gross, H.J (1987). Electrophoresis 8: 93-99.

Borkird, C., Simoens, C, Villarroel, R. and Van Montagu, M. (1991). Physiol. Plant. 82: 449-457.

Bostock, R.M. and Quatrano, R.S. (1992). Plant Physiol. 98: 1356-1368.

Bradford, K.J. and Chandler, P.M. (1992). Plant Physiol. 99: 488-494.

Bray, E.A. (1988). Plant Physiol. 88: 1210-1214.

Briggs, M.R., Kadonaga, J.T., Bell, S.P. and Tjian, R. (1986). Spl. Science 234: 24-52.

Cattivelli, L. and Bartels, D. (1990). Plant Physiol. 93: 1504-1510.

Cattivelli, L. and Bartels, D. (1989). Planta, 178: 184-188.

Charest, C. and Phan, C.T. (1990). Physiol. Plant. 80: 159-168.

Chandler, P.M., Walker-Simons, K. , King, R.W., Crouch, M. J. and Close, T.J. (1988). J. Cell. Biochem. (Suppl). 12C : 143.

Chu, T.M., Aspinall, D. and Paleg, L.G. (1976). Aust. J. Plant Physiol. 3: 219-228.

Christie, P.J., Hann, M. and Walbot, V. (1991). Plant Physiol. 95: 699-706.

Claes, B., Dekeyser, R,, Villarroel, R., Van den Bulcke, M., Bauw. G. Van Montagu, M. and Caplan, A. (1990) . Plant Cell. 2: 19-27.

Clarkson, D.T., Hall, K.C. and Roberts, J.K.M. (1980). Planta 149: 464-71.

Close, T.J., Kortt, A.A. and Chandler, P.M. (1989). Plant Mol. Biol. 13: 95-108.

Close, T.J. and Chandler, P.M. (1990). Aust. J. Plant Physiol. 17: 333-344.

Cohen, A., Plant, A.L., Moses, M.S. and Bray, E A. 91991). Plant Physiol. 97: 1367-1374.

Cohen, A. and Bray, E.A. (1990). Planta 182: 27-33.

Cohen, A. and Bray, E.A. (1992). Plant Mol. Biol. 18: 411-413.

Comai, L. and Harada, J.J. (1990). Proc. Natl. Acad. Sci. U.S.A. 87: 2671-2674.

Cornelissen, B.J.C., Hooftvan, Huijsduijnen, R.A.M. and Bol, J.R. (1986). Nature 321: 531-532.

Creelman, R.A. and Mullet, J.E. (1991). Plant Mol. Biol. 17: 591-608.

Creelman, R.A., Mason, H.S., Bensen, R.J., Boyer, J.S. and Mullet, J.E. (1990). Plant Physiol. 92: 205-214.

Crespi, M.D., Zabaleta, E.J., Pontis, H.G. and Salerno, G.L. (1991). Plant Physiol. 96: 887-891.

Cuming, A.L. and Lane, B.G. (1979). Eur. J. Biochem. 99: 217-224.

Cuming, A.C. (1984). Eur. J. Biochem. 145: 351-357.

Curry, J., Craigmorris, F. and Walker-Simmons, M.K. (1992). Plant Mol. Biol. 18: 1073-1076.

Davies, W.J. and Zhang, J. (1991). Annul. Review of Plant Physiol. and Plnat Molecular Biol. 42: 55-76.

Deutsch, P.J., Hoeffler, J.P., Jameson, J.L. and Jabenser, J.F. (1988). Proc. Natl. Acad. Sci. U.S.A. 85: 7922-7926.

Devries, S., Hoge, H. and Bisseling, T., (1988). Plant Molecular Biology Manual B6: 1-13. Kluwer Academic Publishers.

Dure, III.L., Crouch. M., Harada, J. HO, T-H.D., Mundy, J., Quatrano, R.S., Thomas, T. and Sung, Z.R. (1989). Plant Mol. Biol. 12: 475-486.

Edens, L., Heslinga, L., Klok, R., Ledeboer, A.M., Maat, J., Toonen, M.Y., Visserc. and Verrips, C.T. (1982). Gene 18: 1-12.

Espelund, M., Saeboe-larssen, S., Wayne Hughes, Galau, G.A., Larsen, F. and Jakobsen, K.S. (1992). The Plant Journal 2(2): 241-252.

Gaff, D.F. (1971). Science 174: 1033-1034.

Galau, G.A., Hughes, D.W. and Dure, L. (1986). Plnat Mol. Biol. 7: 155-170.

Galau, G.A., Bijaisoradat, N. and Hughes, D.W. (1987). Dev. Biol. 123: 198-212.

Galau, G.A., Jakobsen, K.S. and Hughes, D.W. (1991). Physiol. Plant. 81: 280-288.

Gaxiola, R., Decarrinoa, I.F., Villalba, J.M. and Serrano, R. (1992). EMBO J. 11: 3157-3164.

Gomez, J., Sanchez-Martinez, D., Stieftel, V., Rigau, J., Puigdomenech, P. and Page, M. (1988). Nature 334: 262-264.

Gilmour, S.J., Artus, N.N. and Thomashow, M.F. (1992). Plant Mol. Biol. 18: 13-21.

Goday, A., Torrent, M., Ludevid, M.D. and Puigdomench, P. (1988). Electrophoresis 9: 738-741.

Godoy, J.A., Pardo, J.M. and Pintor-Toro J.A. (1990). Plant Mol. Biol. 15: 695-705.

Grzelezak, Z.F., Sattolo, M.H., Hanerly, Bowdoni L.K., Kennedy, T.D. and Lane B.G. (19P2). Can. J. Biochem. 60: 389-397.

Guiltinan, M.J., Marcotte, W.R. and Quatrano, R.S. (1990). Science 250: 267-271.

Guy, C.L. (1990). Annul Rev. of Plant Physiol. and Plant Mol. Biol. 41: 187-223.

Guy, C.L., Niemi, K.J. and Brambl. R. (1985). Proc. Natl. Acad. Sci. U.S.A. 82: 2673-3677.

Hahn, M. and Walbot, V. (1989). Plant Physiol. 91: 930-938.

Hajela, R.K., Horvath. D.P., Gilmour, S.J. and Thomashow, M.F. (1990). Plant Physiol. 93: 1246-1252.

Hanson, A.D., Nelson, C.E. and Everson, E.H. (1977). Crop. Sci. 17: 720-726.

Hanson, A.D. and Hitz, W.D. (1982). Annual Review of Plant Physiol. 33: 163-203.

Hetherington, A.M. and Quatrano, R.S. (1991). New Phytol. 119: 9-32.

Houde, M., Dhindsa, R.S. and Sarhan, F. (1992). Mol. Gen. Genet. 234: 43-48.

Houde, M., Danyluk, J., Laliberte, J.F., Rassart, E., Dhindsa, R.S., and Sarhan, F. (1992). Plant Physiol. 99: 1381-1387.

Hughes, D.W. and Galau, G.A. (1991). Plant Cell. 3: 605-618,

Hughes, D.W. and Galau, G.A. (1989). Genes Devel. 3: 358-369.

Hughes, D.W. and Galau, G.A. (1987). Plant Mol. Biol. 9: 301-313.

Jacobson, J.V. Hanson, A.D. and Chandler, P.C. (1986'. Plant Physiol. 80: 350-359.

Jacobsen, J.V. and Shaw, D.S. (1989). Plant Physiol. 91: 1520-1526.

King, S.W., Joshi, C.P. Nguyen, H.T. (1992). Plant Mol. Biol. 18: 119-121.

King, G.J., Turner, V.A., Hussey, C.E., Wurtele, E.S. and Lee, S.M. (1988). Plnat Mol. Biol. 10: 401-412.

Kononowicz, A.K., Nelson, D.E., Singh, N.K., Hasegawa, P.M. and Bressan, R.A. (1992). Plant Cell 4: 513-524.

Kramer, P.J. (1988). Plant Cell Environ 11: 565-568.

Kriz, A.R., Wallace, M.S. and Raiva, R. (1990). Plant Physiol. 92: 538-542.

Kurkela, S., Franck, M. (1990). Plant Mol. Biol. 15: 137-144.

Laemmli, U.K. (1970). Nature 227: 680-685.

Levitt., J. (1980). Responses of Plants to Environmental Stress. Vol. I Chilling Freezing and High Temperature stresses and High temperature stresses. New York, Academic.

Lers, A., Levy, H., and Zamir, A. (1991a). J. Biol. Chem. 226: 13698-13705.

Litts, J.C., Colwell, G.C., Chakerian, R.L. and Quantrano, R.S. (1987). Nucl. Acids Res. 15: 3607-3618.

Litts, J.C., Erdman, M.B., Huang, N., Karrer, E.E., Noueiry, A. Quatrano, R.S. and Rodriguez, R.L. (1992). Plant Mol. Biol. 19: 335-337.

Li, Y. and Walton, D.C. (1987). Plant Physiol. 85: 1155-1158.

Lin, C, Guo, W.W., Everson, E. and Thomashow, M.F. (1990). Plant Physiol. 94: 1078-1083.

Lin, C. and Thomshow, M.F. (1992). Biochem. Biophys. Res. Commun. 183: 1103-1108.

Lin, C. and Thomshow, M.F. (3992). Plant Physiol. 99: 519-525.

Lowry, O.H., Rosebrough, N.J., Farr A.L., and Randall, R.J. (1951). J. Biol. Chem. 193: 265-275.

Lyons, J.M. (1973). Annu. Rev. Plant. Physiol. 24: 445-466.

Marcotte, W.R., Bayley, C.C. and Quantrano, R.S. (1988). Nature, 335: 454-457.

Marcotte, W.R., Russel, S.H. and Quantrano, R.S. (1989). Plant Cell. 1: 969-976.

Mali, P.C. and Metha, S.L. (1977). Phytochemistry, 16: 1355-1357.

McCartry, D.R., Hattori, T., Carson, C.B., Vasil, V., Lazar, M. and Vasil, I.K. (1991). Cell, 66: 895-907.

Measures, J.C. (1975). Nature, 275: 398-400.

Meza-Bass, O.L., Alberdi, M., Raynal, M., Ferrero-Cadinanos, M.L. and Delseny, M. (1986). Plant Physiol. 82: 733-38.

Mohapatra, S.S., Poole, R.J. and Dhindsa, R.S. (1987). Plant Physiol. 84: 1172-1176.

Mohapatra, S.S. Wolfraim, L., Poole, R.J. and Dhindsa, R.S. (1989). Plant Physiol. 83: 375-380.

Morris, C.F., Anderberg, R.J., Goldmark, P.J. and Walker-Simmons, M.K. (1991). Plant Physiol. 95: 814-824.

Morris, P.C., Kumar, A., Bowles, D.J. and Cuming, A.C. (1990). Eur. J. Biochem. 190: 625-630.

Mortenson, E. and Dreyeuss, G. (1989). Nature, 337: 312.

Mundy, J. and Chua, N-H. (1988). EMBO J. 7: 2279-2286.

Mundy, J., Yamaguchi-Shinozaki, K. and Chua, N-H (1990). Proc. Natl. Acad. Sci. U.S.A. 87: 1406-1410.

Nordin, K., Heino, P. and Palva, E.T. (1991). Plant Mol. Biol. 16: 1061-1071.

O'Farrell, P.H. (1975). J. Biol. Chem. 250: 4007-4021.

Orr, W., Lu, B, White, T.C., Robert, L.S., and Singh, J. (1992). Plant Physiol. 98: 1532-1534.

Parry, D.D., Horgan, R. (1991). Physiologia, Plant 82: 320-326.

Pena-Cortes, H., Sanchez-Serrano, J.J. Willmitzer, L. and Part, S. (1990). Proc. Natl. Acad. Sci. U.S.A. 86: 9851-9855.

Piatkowski, D., Schneider, K., Salamini, F.and Bartels, D. (1990). Plant Physiol. 94: 1682-1688.

Pickett, M., Scott 1, F., Davies, P., Wang, N., Joshi, S. and Few, C. (1984). Eur. J. Biochem. 143: 35-38.

Pla, M., Gomez, J., Goday, A. and Pages, M. (1991). Mol. Gen. Genet. 230: 394-400.

Pla, M., Goday, A., Vilardell, J., Gomez, J. and Pages, M. (1989). Plant Mol. Biol. 13: 385-394.

Plant, A.L., Cohen, A., Moses, M.S. and Bray, E.A. (1991). Plant Physiol. 97: 900-906.

Quatrano, R.S., Guiltinan, M.J. and Marcotte, W.R. (1992). The Telford Press, Caldwell (in press).

Quinn, P.J. and Williams, W.P. (1978). Prog. Biophys. Mol. Biol. 34: 109-73.

Rajagopal, V., Balasubramanian, V. and Sinha, S.K, (1977). Physiol. Plant 401: 69-71.

Ricardson, M., Valde-Podriguez, B. and Blanco-labra, A. (1987). Nature 327: 432-434.

Reddy, K.R.K., Rao, A.H., Karuna Sree, B. Reddy, A.R. (1992). J. Plant Physiol. 140:

Richardson, M., Rodriguez, S.V. and Labia, B.A. (1987). Nature, 327: 432-434.

Robertson, A.J., Gusta, L.V. Reaney, M.J.T. and Ishikawa, M. (1987). Plant Physiol. 84: 1331-1336.

Roberton, M. and Chandler, P.M. (1992). Plant Mol. Biol. 19: 1031-1044.

Sakai, A. and Larcher, W. (1987). "Frost Surival of Plants Responses and Adaptions to freezing stress" Springer-verlay, Berlin and New York.

Sambrook, J., Fritsch, E.F. and Maniatis, T. (1989). Molecular cloning: A Laboratory Manual Ed.2, Cold Spring Harbor Laboratory, Cold Spring Harbor, New York.

Shinozaki, Y.K., Mundy, J. and Chua, N-H (1989). Plant Mol. Biol. 14: 29-39.

Shinozaki, Y.K., Mino, M., Mundy, J. ana Chua, N-H. (1990). Plant Mol. Biol. 15: 905-912.

Shinozaki, Y. K., Koizumi, M., Urao, S. and Shinozaki, K. (1992). Plant Cell Physiol. 33: 217-224.

Sikorska, E., Kacperska-Palacz, A. (1979). Physiol. Plant. 47: 144-150.

Singh, N.K., Nelson, D.E., Kuhn, D., Hasegawa, P.M. and Bressan, R.A. (1989). Plant Physiol. 90: 1096-1101.

Singh, T.N., Paleg, L.G., Aspinall, D. (1973). Aust. J. Biol. Sci. 26: 45-56.

Skriver, K. and Mundy, J. (1990). Plant Cell. 2: 503-512.

Smolenska, G. and Kuiper, P.J. (1977). Physiol. Plant 41: 29-35.

St. John, J.B., Christiansen, M.N., Ashworth, F.N. and Genther, W.A. (1979). Crop. Sci. 19: 65-69.

Steponkus, P.L. (1984). Annu, Rev. Plant Physiol. 35: 543-581.

Steponkus, P.L. and Lynch, D.V. (1989). J. Bioenerg. Biomembr. 21: 21-41.

Stewart, G.R. and Lee, J.A. (1974). Planta, 120: 279-289.

Stewart, C.R. (1980). Plant Physiol. 66: 230-233.

Stewart, C.R. and Voetberg, G. (1985). Plant Physiol. 79: 24-27.

Stewart, C.R., Voetberg, G. and Rayapati, P.J. (1986). Plant Physiol. 82: 703-707.

Stewart, C.R. and Hanson, A.D. (1980). In adaption of plants to water and high temperature stress. N.C. Turner, P.J. Kramer, eds. John Wiley and Sons, New York. pp.173-187.

Sivaramakrishnan, S. Patell, V.Z., Flower, D.J. and Peacock, J.M. (1988). Physiol. Plant. 74: 418-426.

Taylor, I.R., Linfortn, R.S.T., Al-Naibb, R.J.,Bowman, W.R. and Marples, B.A. (1988). Plant Cell and Environment 11: 739-745.

Tseng, M.J. and Li, P.H. (1991). Physiol. Plant 81: 349-358.

Vilardell, J., Goday, A., Freire, M.A., Torrent, M., Martinez, M.C., Torne, J.M. and Pages. M. (1990). Plant Mol. Biol. 14: 423-432.

Vernon, D.M. and Bohnert, H.J. (1992). EMBO J. 11: 2077-2085.

Weiser, C.J. (1970). Science 169: 1269-1278.

Williamson, J.D., Quatrano, R.S. and Cuming, A.C. (1985). Eur. J. Biochem. 152: 501-507.

Williamson, J.D. and Quatrano, R.S. (1988). Plant Physiol. 86: 208-215.

Williams, B. and Tsang, A. (1991). Plant Mol. Biol. 16: 919-923.

Winston, S.E. (1987). Western blotting in current protocols in Molecular Biology (Supplement) 2 pp.10-8.1 to 8.5.

Zeeraart, J.A.D. and Creelman, R.A. (1988). Annu. Rev. Plant Physiol and Plant Mol. Biol. 39: 439-473.