Annals of Botany **82**: 513–522, 1998
Article No. bo980712

Genotypic Variability in Differential Expression of lea2 and lea3 Genes and Proteins in Response to Salinity Stress in Fingermillet (*Eleusine coracana* Gaertn) and Rice (*Oryza sativa* L.) Seedlings

T. L. JAYAPRAKASH*, G. RAMAMOHAN, B. T. KRISHNAPRASAD, GANESHKUMAR, T. G. PRASAD, M. K. MATHEW† and M. UDAYAKUMAR‡

Department of Crop Physiology, University of Agricultural Sciences, GKVK, Bangalore-65, India

Received: 15 December 1997 Returned for revision: 11 February 1998 Accepted: 15 June 1998

Some late embryogeny abundant (LEA) proteins, which are developmentally regulated in embryos, are also known to be expressed in meophytic tissues in response to osmotic stress. Here we report the extent of genetic variability in the level of expression of lea2 and lea3, under stress, in fingermillet and rice seedlings. In both species, the expression of lea genes was seen in the mesophytic tissue in response to salinity, partial dehydration and abscisic acid. Tolerant genotypes exhibited higher expression of rab16A and M3 that code for LEA2 proteins, than susceptible genotypes. A novel approach, that of raising antibodies against the conserved peptides of these proteins was used to study genetic variability in LEA protein levels. Since stress proteins are known to be expressed in response to mild, non-lethal induction-stress (Uma, Prasad and Udayakumar, *Annals of Botany* **76**: 43–49, 1995), we developed an optimum induction protocol for salinity stress in rice and fingermillet. We studied the quantitative differences in expression of these proteins by western blot and ELISA techniques in different genotypes. A positive correlation was found between LEA2 and LEA3 protein levels and the growth of seedlings during stress and recovery in both rice and fingermillet, indicating a possible relevance of these proteins in stress tolerance.

Key words: LEA proteins, ABA responsive proteins, induction response, ELISA, fingermillet, rice, salinity-stress.

INTRODUCTION

Plants have developed different adaptive mechanisms to withstand abiotic stress conditions such as high or low temperature, salinity and drought. Many of these adaptive mechanisms are a consequence of stress perception and are likely to be mediated through stress induced expression of specific genes. This stress induced gene expression leads to the synthesis of specific stress responsive proteins, which may impart tolerance. Stress induced genes are many and diverse. In response to desiccation stress alone, as many as 74 genes are expressed (Ingram and Bartels, 1996). An important group of stress responsive genes are late embryogeny abundant (lea) genes which were first identified in maturation and desiccation phases of seed development in cotton. Several lea genes have been characterized and, based on deduced amino acid sequences, different LEA groups of proteins have been identified and their functions postulated (Baker, Steele and Dure, 1988).

Amongst the different groups of LEA proteins the LEA2 and LEA3 groups have been extensively characterized. The LEA2 group of proteins, also referred to as dehydrins, are characterized by a highly conserved sequence, KIKEKLPG in the carboxy terminus (Baker *et al*., 1988). The consensus regions among the LEA3 group of proteins contains tandem repeats of an 11-amino acid motif that may form an amphiphilic α-helical structure (Dure *et al*., 1989). LEA2 proteins have been predicted to function as chaperones useful for maintaining protein structure and function (Bray, 1993). LEA3 could counteract the irreversible damaging effects of increasing ionic strength in the cytosol during desiccation by sequestration of ions (Dure, 1993). Some of the characterized lea genes are known to be expressed in vegetative tissues in response to osmotic stress and are ABA responsive (Bray, 1993; Bracale *et al*., 1997). Given that LEA proteins are expressed under osmotic stress in mesophytic tissues and since they have a unique structure and function, these proteins can be expected to play important roles in imparting stress tolerance (Skriver and Mundy, 1990; Chandler and Robertson, 1994).

In recent years attention has focused on the relevance of LEA proteins. Reid and Walker-Simmons (1993) demonstrated that higher levels of LEA3 proteins accumulate in severely dehydrated wheat seedlings and this was correlated with high levels of desiccation tolerance. Levels of the LEA2 and LEA3 groups of proteins were significantly higher in roots of salt tolerant rice genotypes than salt sensitive genotypes (Moons *et al*., 1995). Recently, Xu *et al*. (1996) have shown the relevance of LEA3 proteins in imparting tolerance by over-expressing HVA1 gene, coding for LEA3 protein, which conferred a small increase in tolerance to water deficit and in salt tolerance in transgenic rice. Expression of the LE25 protein, belonging to LEA4 group,

* Present address: Section of Biochemistry, Molecular and Cell Biology, Cornell University, Ithaca, NY 14853, USA.
† National Center for Biological Sciences, TIFR, Bangalore, India.
‡ For correspondence.

0305-7364/98/100513+10 $30.00/0

led to increased resistance to high salinity and freezing-stress in yeast (Imai *et al*., 1996). Although these reports indicate the relevance of LEA in stress tolerance, very few studies actually quantify LEA proteins. The possible relationship between genetic variation in LEA proteins and stress response has not been clearly elucidated to date.

It is evident that genetic variability exists for stress responses and this could be due to the differential expression and regulation of stress responsive genes when the plants are exposed to mild, non-lethal stress, often referred to as induction-stress (Krishnan, Nguen and Burke, 1989; Uma *et al*., 1993). In a previous study we showed that stress-responsive proteins are expressed only when seedlings are exposed to mild, non-lethal stress, and genetic variability in stress response was seen only upon induction (Uma *et al*., 1995). However, information on differential expression of stress-responsive proteins in genotypes differing in stress-tolerance is inconclusive (Krishnan *et al*., 1989; Vierling and Nguyen, 1992).

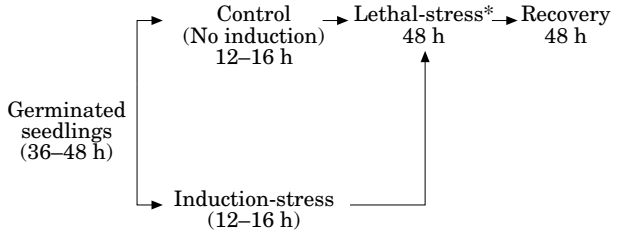
In this investigation, using optimum induction protocols, we report on genetic variability in the expression of some lea genes. Adopting a novel approach to quantify LEA2 and LEA3 proteins through ELISA, we also show the existence of genetic variability in the LEA2 and LEA3 protein content in fingermillet and rice genotypes. The observed correlation between LEA protein content and stress response indicates the possible relevance of LEA2 and LEA3 proteins in stress tolerance.

MATERIALS AND METHODS

Induction responses

Stress-responsive proteins are known to be synthesized in plants under mild, non-lethal stress conditions, often referred to as 'induction-stress'. The general protocol followed to study the induction responses in fingermillet and rice seedlings is shown in Fig. 1.

Seeds of fingermillet genotypes were obtained from the Project Coordinator, Small Millets, Bangalore, India and those of rice genotypes were obtained from the Research Station, VC Farm, Mandya, India. The response of these genotypes with respect to seedling growth and accumulation of LEA proteins was examined under NaCl-stress either with or without prior induction-stress treatment.



*Lethal stress, stress level at which non-induced seedlings show more than 80 % reduction in growth after recovery

FIG. 1. General protocol followed to study the induction response in fingermillet and rice seedlings.

Seeds were germinated on moist filter paper at 28 °C for 36 h and seedlings of uniform size were subjected to induction stress for 16 h at 28 °C and 75 % relative humidity (RH) by transferring them to Petri dishes containing either 150 (rice) or 200 mM NaCl (fingermillet). Seedlings were then subjected to lethal levels of stress of 300 (rice) or 400 mM NaCl (fingermillet) for 48 h at 28 °C and 75 % RH. Following this, seedlings were transferred to Petri dishes containing water and allowed to recover for 48 h at 28 °C and 75 % RH. The growth of the roots and shoots was recorded at the end of induction-stress, lethal-stress and recovery. Variation between genotypes was determined by comparing the extent of response to induction-stress.

For northern and western analysis, different stress treatments such as NaCl-stress and dehydration-stress were imposed on seedlings in the presence or absence of ABA. To induce salt stress, germinated seedlings were treated with 150 (rice) or 200 mM NaCl (fingermillet) in the presence or absence of 20 μM ABA for 16 h at 28 °C and 75 % RH. A set of seedlings was also subjected to induction by ABA alone by transferring them to Petri dishes containing only 20 μM ABA. For desiccation-stress, germinated seedlings of known weight [100 % relative water content (RWC)] were air dried on dry filter paper at 28 °C and 50 % RH. The weight of seedlings was constantly monitored and relative water content was determined in a subset of seedlings. When seedlings reached 80 % RWC, they were maintained at 100 % RH and 28 °C for 16 h and then analysed for RNA and protein content. Under these conditions the RWC showed a decline of 5 % at the end of incubation.

Expression of lea2 and lea3 genes

The levels of expression of lea genes were examined by Northern analysis in seedlings subjected to different stress treatments and also in seedlings treated with ABA. Total RNA was isolated from the induced and non-induced seedlings following the guanidine isothiocyanate method described by Chomczynksi and Sacchi (1987), with small modifications. Twenty-five micrograms of total RNA were separated on a formaldehyde-agarose gel and transferred to a nitrocellulose membrane according to Sambrook, Fritsch and Maniatis (1989). Appropriate cDNA inserts, rab 16A (donated by Dr John Mundy, Rockefeller University, USA) and M_3 (donated by Dr Peter Chandler, CSIRO, Australia) both belonging to the lea2 gene, and PMA 2005 (donated by Dr Walker-Simmons) belonging to the lea3 gene were eluted, purified and used for radio-labelling reactions. A random primed DNA labelling kit (Boehringer) was used to radiolabel cDNAs with ^{32}P-dCTP. The probe thus obtained was further purified by passing through a Sephadex G-50 Column and was then used for Northern analysis. Pre-hybridization was performed at 42 °C for 3 h (in 5 × SSC containing 50 % formamide, 2 % Denhardt's reagent, 0·1 % SDS and 100 μg ml^{-1} of denatured salmon sperm DNA). Denatured probe was added to the pre-hybridization solution and hybridization was carried out for 12–16 h at 42 °C in an Amersham hybridization chamber with gentle agitation. The membrane was washed and exposed to X-ray film following standard procedures (Sambrook *et al*., 1989).

Qualitative and quantitative studies of LEA proteins

By using antibodies raised against the synthetic peptides of the conserved amino acid sequences of LEA2 and LEA3 proteins, the qualitative and quantitative differences between LEA proteins of fingermillet and rice seedlings were studied in: (*a*) induced and non-induced seedling systems; and (*b*) genotypes differing in stress tolerance. To quantify LEA proteins the total soluble protein was extracted at the end of induction-stress. As LEA proteins are known to remain soluble even at high temperatures we used the heat-stable-protein fraction prepared from the total soluble proteins (see below).

Peptide conjugation and development of antibody

Conserved peptide sequences EEKKGIMDKDIKELPG of LEA2 proteins and TAQAAKEKAGE of LEA3 proteins were chemically synthesized and purified at ICGEB, New Delhi, India. These peptides were further conjugated to BSA, a carrier molecule, using EDC [1-ethyl-3-(diethyl aminopropyl) carbodimide hydrochloride] (Harlow and Lane, 1988). After confirming successful conjugation by SDS-PAGE, the product was used to immunize rabbits and the antiserum obtained was further assessed for cross reactivity with BSA-peptide conjugates by dot blot experiments. The antibodies raised were used as a probe for western analysis and for ELISA to quantify LEA2 and LEA3 protein content in the heat-stable-protein fraction of both induced and non-induced systems.

Preparation of heat stable proteins

Heat-stable proteins are those which do not coagulate upon boiling and remain in solution (Jacobsen and Shaw, 1989). These proteins were isolated from rice and fingermillet seedlings as described earlier by Close, Krott and Chandler (1989) and Uma *et al.* (1993). After induction, seedlings were washed thoroughly with water and ground in 100 mM Tris-HCl buffer, pH 7·8 containing 1 mM PMSF (1:4 w/v) at 4 °C. The supernatant, obtained after centrifugation at 10000 *g* for 10 min at 4 °C, was used for further studies as soluble-protein fraction. This fraction was incubated at 70 °C for 10 min in a water bath. The denatured proteins were removed by centrifugation at 12000 *g* for 10 min and the supernatant containing heat-stable proteins was collected and precipitated once again with five volumes of chilled acetone. The pellet was dried and re-suspended in PBS (phosphate buffered saline) for ELISA or in 1 × loading buffer (0·0625 M Tris Cl pH 6·8, 5% dithiothrietol, 2% SDS, 0·001% bromophenol blue, 10% glycerol) for western analysis.

Western analysis

Western analysis was performed in order to elucidate the expression of LEA2 and LEA3 proteins in mesophytic tissues in: (*a*) induced and non-induced seedling systems; and (*b*) contrasting genotypes of fingermillet differing in stress tolerance. LEA proteins were examined in the heat-stable-protein fraction at the end of different induction-stress treatments. Equal amounts of heat-stable-proteins were resolved on a 12% SDS-PAGE and western analysis was done using antibodies raised against LEA2/LEA3 peptides. The antiserum was incubated with 0·1% BSA in order to specifically remove the antibodies against BSA.

ELISA

To quantify LEA2 and LEA3 proteins in the heat-stable-protein fraction of (*a*) induced and non-induced seedling systems and (*b*) genotypes differing in stress tolerance, standard indirect ELISA procedures were followed (Hall, Deschamps and McDermott, 1990). Two micrograms of protein from the heat-stable-protein fraction were added to each well in a volume of 100 μl and incubated overnight at 4 °C to coat the wells with protein. Each well was washed with PBS containing 0·1% Tween-20. Following this, the uncoated sites in the wells were blocked by incubating with 100 μl 1% gelatin in PBS at 37 °C for 2 h to avoid non-specific binding of the antibodies. Primary antibodies, raised in rabbits against LEA2 and LEA3 and incubated with 0·1% BSA at 1:1200 dilution, were added to the wells and incubated overnight at 4 °C. Wells were then washed with PBS + Tween-20 and PBS. Secondary antibody (alkaline phosphatase linked anti-rabbit-IgG), diluted in 0·1% BSA at 1:1500 dilution was added and the plate was incubated at 37 °C for 2 h, followed by three washes with PBS + Tween-20 and three washes with only PBS. The wells were incubated with 100 μl of para-nitrophenyl phosphate (PNPP) solution prepared in carbonate buffer pH 7·5 (1 mg ml^{-1}) at 37 °C until colour developed. The reaction was stopped by the addition of 20 μl 5 N KOH and the absorbance of the coloured product was measured at 405 nm using an ELISA reader. Standard curves for quantification of LEA2 and LEA3 proteins were developed by using different concentrations of BSA-peptide conjugates. The quantity of LEA2 and LEA3 proteins in the heat-stable fraction was determined by extrapolating the ELISA reading in the standard curves developed.

Genetic variability studies

Based on our previous studies, genotypes of fingermillet (VL-146, GN-3, VL-149, DM-1, L-10, PR-2614, PR-202) and rice (Rasi, IR-64, IR-30864, Jaya, Prakash) differing in tolerance levels for salinity-stress, were selected for this study. Genotypic variations in stress responses in induced and non-induced control seedlings were assessed. The growth of seedlings was recorded at the end of induction, end of lethal-stress and also at the end of recovery. LEA2 and LEA3 protein levels were quantified at the end of induction-stress. For LEA protein quantification, heat-stable proteins were isolated from a known weight of the seedlings and 2 μg of protein from the heat-stable-protein fraction was used for ELISA. The contents of these two groups of proteins were expressed as μg per gram fresh weight of seedling and also as μg per mg of heat-stable proteins. The percent increase in LEA2 and LEA3 protein content (individually and together) in the induced seedlings

over non-induced control seedlings was determined. Relevance of LEA proteins in salinity-stress was established by correlation studies between LEA content and seedling recovery growth under stress in both rice and fingermillet.

RESULTS

Induction response

Induction responses of fingermillet genotypes (PR202, VL146, VL149) and rice genotypes (Rasi, IR64 and Prakash) to NaCl-stress were studied after subjecting the seedlings to induction (16 h), lethal-stress (48 h) and recovery (48 h). Seedlings without induction served as controls. Both induced and non-induced seedlings were subjected to lethal-stress and then allowed to recover. In both crop species, induced seedlings performed better than non-induced control seedlings. In the case of non-induced seedlings, the variation in recovery growth was not significant, while induced seedlings showed genetic variability in recovery growth (Table 1). This observation is consistent with Uma *et al*. (1995), that mild sub-lethal induction-stress is necessary to determine genetic variation in the response of seedlings to severe stress. In the case of fingermillet genotypes, the recovery growth of induced seedlings was seven to nine times greater than non-induced seedlings, while in rice genotypes it was three to five-fold higher (Table 1).

Expression of lea genes

In order to study the transcript levels of lea2 genes in rice, cDNA probes rab16A and M_3 which code for LEA2 proteins were used for Northern analysis. The pattern of expression of lea2 genes was similar in both the genotypes tested and the levels of transcripts increased substantially under induction-stress conditions. However, in case of the tolerant genotype Rasi, levels of transcripts were much higher than those in IR-64, a susceptible genotype (Figs 2 and 3). Northern analysis in fingermillet also indicated the enhanced expression of lea2 (rab16A) and lea3 (PMA2005) transcripts in response to different stress treatments (data not shown). Results of Northern analysis, which revealed quantitative differences in lea transcript levels in different genotypes, led us to further examine the quantitative and qualitative differences in LEA protein levels.

TABLE 1. *Variation in recovery growth* (*cm*) *of fingermillet and rice seedlings following lethal levels of NaCl stress with or without a prior induction-stress*

Genotypes	Non-induced	Induced
Fingermillet		
PR 202	0·21	1·280
VL 146	0·12	1·010
VL 149	0·16	1·180
Rice		
Rasi	0·25	1·880
IR 64	0·22	1·320
Prakash	0·18	1·560

l.s.d. = 0·1027 for fingermillet ($P < 0·05$).
l.s.d. = 0·1013 for rice ($P < 0·05$).
Induction-stress treatment was 16 h in 200 (fingermillet), or 150 mM NaCl (rice). The lethal-stress treatment was 48 h in 400 (fingermillet) or 300 mM NaCl (rice). Seedlings which were kept in water and directly exposed to lethal-stress served as non-induced controls. Recovery growth is the difference in growth between that at the end of recovery and that at the end of lethal-stress. Root and shoot lengths of 20 seedlings were measured at each stage and l.s.d. was calculated at the 5% level.

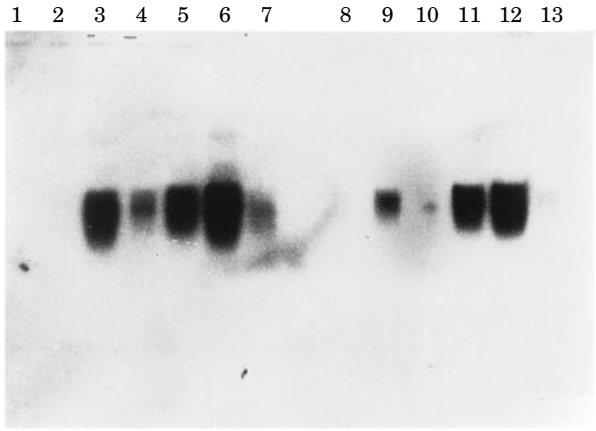


FIG. 2. Expression of lea2, under different induction-stress treatments, in two contrasting genotypes of rice (probed with rab 16A). Lanes 2–7, Tolerant genotype Rasi. Lanes 8–13, Susceptible genotype IR-64. Lanes 2 and 8, Non-induced control. Lanes 3 and 9, Induced with 150 mM NaCl for 16 h. Lanes 4 and 10, Maintained at 80% RWC for 16 h. Lanes 5 and 11, Induced with 20 μM ABA for 16 h. Lanes 6 and 12, Induced with 150 mM NaCl + 20 μM ABA. Lanes 7 and 13, Treated with ABA (20 μM) for 3 h, then maintained at 80% RWC for 16 h.

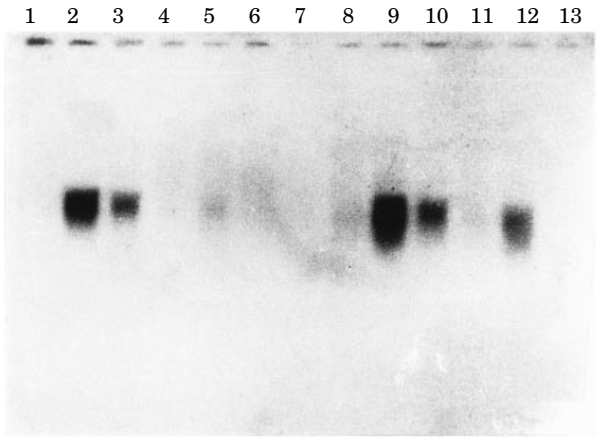


FIG. 3. Expression of lea2, under different induction-stress treatments, in two contrasting genotypes of rice (probed with M_3). Lanes 1–6, Susceptible genotype IR 64. Lanes 8–12, Tolerant genotype Rasi. Lanes 1 and 8, Treated with ABA (20 μM) for 3 h, then maintained at 80% RWC for 16 h. Lanes 2 and 9, Induced with 150 mM NaCl + 20 μM ABA. Lanes 3 and 10, Induced with 20 μM ABA for 16 h. Lanes 4 and 11, Maintained at 80% RWC for 16 h. Lanes 5 and 12, Induced with 150 mM NaCl for 16 h. Lanes 6 and 13, Non-induced control.

Western analysis

Antibodies raised against the conserved amino acid sequences of LEA2 and LEA3 proteins were used for

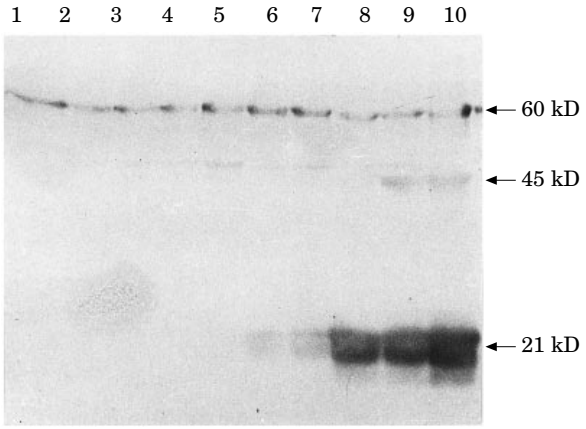


FIG. 4. Synthesis of LEA2 proteins as affected by different durations of NaCl induction (150 mM) in rice seedlings (Rasi). Lane 1, Non-induced control. 1 (Lane 2), 2 (Lane 3), 4 (Lane 4), 6 (Lane 5), 8 (Lane 6), 10 (Lane 7), 12 (Lane 8), 14 (Lane 9) and 16 h (Lane 10) after induction.

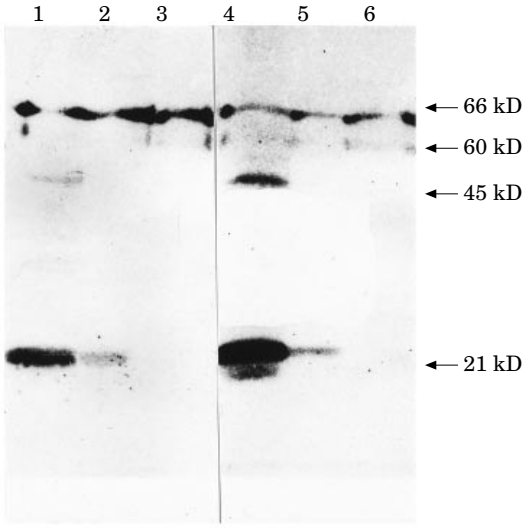


FIG. 5. LEA2 group of proteins in seedlings treated with different stress treatments in two contrasting genotypes of fingermillet. Lanes 1–3, Stress sensitive genotype Hullubele, Lanes 4–6, Stress-tolerant genotype PR202. Lanes 1 and 4, Induced with 200 mM NaCl + 20 μM ABA. Lanes 2 and 5, Induced with 20 μM ABA. Lanes 3 and 6, Non-induced control.

western analysis to study the expression of LEA2 and LEA3 proteins. Cross reactivity of the antibodies obtained was assessed by dot blot analysis. Antibodies raised against BSA were removed by incubating the antiserum with 0·1 % BSA.

To study the synthesis of LEA2 proteins throughout induction-stress, western analysis of heat-stable proteins from rice seedlings (Rasi) was performed at different stages of induction-stress. Two proteins were synthesized during induction with 150 mM NaCl: a 45 kD protein appeared after 6 h of induction and maintained the same level while a 21 kD protein appeared after 8 h of induction, reaching a maximum level after 14 h of induction (Fig. 4). Another protein of 60 kD was expressed with or without induction, indicating that the antibodies cross reacted with three polypeptides. Western analysis of LEA2 proteins in two genotypes of fingermillet (Hullubele and PR202) indicated that proteins of 66 kD and 60 kD cross reacted with LEA2 antibodies in all the treatments and their levels did not change much upon induction (Fig. 5). Two other proteins of 45 kD and 21 kD were expressed in response to ABA and NaCl + ABA treatment in both genotypes. However, there were quantitative differences in the level of expression (Fig. 5).

In the case of fingermillet seedlings exposed to dehydration or NaCl stress, LEA3 antibodies cross reacted with four proteins of size 60, 45, 28 and 20 kD of which the 60 and 45 kD proteins were also expressed in control non-stressed tissues. The levels of the low molecular weight proteins were higher in seedlings exposed to salinity-stress (NaCl) with ABA or dehydration-stress along with ABA (data not shown).

Western analysis indicated that the antibodies raised against the conserved stretches of LEA2 and LEA3 cross reacted with a few proteins which are synthesized in seedlings subjected to different stress treatments. The relevance of these proteins which appeared upon stress treatments was examined by quantifying these proteins by ELISA in induced and non-induced systems and also in genotypes differing in their inherent stress response.

Quantification of LEA2 and LEA3 proteins by ELISA and relevance of LEA2 and LEA3 proteins

BSA-peptide conjugate was used in different concentrations to develop standard curves to quantify LEA2 and LEA3 antibodies. Standard indirect ELISA procedure as described in the methodology, was used to quantify LEA2 and LEA3 proteins. The relevance of LEA2 and LEA3 proteins was examined by quantifying LEA2 and LEA3 proteins by ELISA and comparing results with growth parameters in seedling systems differing in stress tolerance.

Induced and non-induced seedlings. In induced seedlings the percentage of heat-stable-proteins was higher than non-induced control seedlings (Table 2). Induced systems which recorded higher growth rates during stress and recovery also accumulated higher amounts of LEA2 and LEA3 proteins. In the case of fingermillet, the LEA2 protein content increased three-fold in induced seedlings while LEA3 protein content increased five-fold with a corresponding increase in growth in induced seedlings compared to non-induced control seedlings (Table 2). A similar trend was also observed in rice seedlings but the percent increase was less than that in fingermillet.

Genetic variability in stress responses and LEA protein levels. Two genotypes of fingermillet differing in their stress response were selected to study the LEA protein content and stress response in induced and non-induced control seedlings subjected to stress. The tolerant genotype, PR-202, showed higher growth during stress and recovery (1089 %) while DM-1, a susceptible genotype, recorded only a 656 % increase in growth over the control during stress and recovery. The tolerant genotype also recorded higher increases in LEA2 (321 %) and LEA3 (386 %) than the

TABLE 2. *LEA2 and LEA3 protein content in control and induced seedlings of fingermillet and rice*

	Growth during S+R (cm)	HSP mg g^{-1} f.wt	HSPF %	LEA2		LEA3	
				μg g^{-1} f.wt	μg mg^{-1} HSP	μg g^{-1} f.wt	μg mg^{-1} HSP
Fingermillet							
Control	0·2	0·59	8·24	2·06	3·59	1·06	1·69
Induced	3·49	1·36	13·8	6·60	5·16	5·15	3·79
l.s.d.	0·236	0·12	1·94	0·54	0·90	0·51	0·43
Rice							
Control	2·53	0·73	18·12	0·98	1·35	0·61	0·84
Induced	3·90	0·94	24·20	1·82	1·93	1·22	1·32
l.s.d.	0·398	0·15	2·52	0·22	0·30	0·23	0·18

S, Stress; R, recovery; HSP, heat-stable proteins; HSPF, heat-stable protein fraction.
After 36 h of germination seedlings were subjected to induction-stress.
Induction-stress: 200 mM NaCl for 16 h for fingermillet; 150 mM NaCl for 16 h for rice.
Lethal-stress: 400 mM NaCl for 48 h for fingermillet; 300 mM NaCl for 48 h for rice.
Recovery: 48 h in water.
The seedlings which were kept in water and directly exposed to lethal-stress served as non-induced controls. Growth of 20 seedlings was measured at the end of stress and recovery. The LEA proteins were quantified at the end of induction-stress using the heat-stable-protein fraction (HSPF) by ELISA. Values given are the mean of seven genotypes of fingermillet (VL-146, GN-3, VL-149, DM-1, L-10, PR-2614, PR-202) and five genotypes of rice (Rasi, IR-64, IR-30864, Jaya, Prakash).

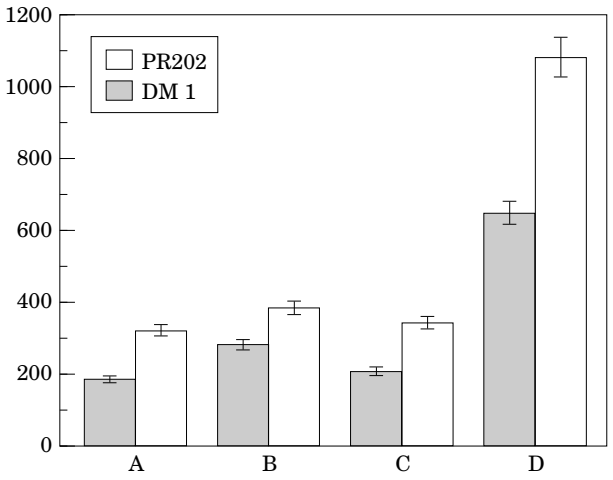


FIG. 6. Induction response in recovery growth and LEA protein content in tolerant (PR202) and susceptible (DM 1) fingermillet genotypes. A, Percent increase in LEA2; B, percent increase in LEA3; C, percent increase in LEA2+3; D, percent increase in growth during stress and recovery.

control, while in DM-1 the increase in LEA2 over the control was 222% and in LEA3 it was 121%, clearly indicating that growth during stress and recovery of these genotypes is related to their LEA2 and LEA3 protein content (Fig. 5). This result emphasized that the stress response of a particular genotype is related to the relative accumulation of LEA2 and LEA3 proteins during the stress period.

In another experiment, seven genotypes of fingermillet and five genotypes of rice were used to study the stress response and LEA protein levels in seedlings (Table 3). The growth, achieved during stress and during recovery, of induced seedlings of fingermillet was significantly higher than non-induced seedlings ultimately leading to higher absolute growth at the end of recovery (Table 3). Similar results were obtained in the case of rice genotypes, but even non-induced seedlings achieved a certain degree of growth during stress and recovery. However, induced seedlings always recorded higher growth during stress and recovery than non-induced control seedlings. The variation among genotypes in the growth at the end of recovery was highly significant only upon induction, confirming the earlier findings of Uma *et al.* (1995). All the genotypes of both fingermillet and rice accumulated higher amounts of LEA2 and LEA3 proteins upon induction, but showed a significant amount of variability in their response (Figs 7 and 8).

Correlative studies

Growth during stress and recovery of rice (Fig. 7) and fingermillet genotypes (Fig. 8) showed significant positive correlation with the LEA2+3 content. The correlative studies between the LEA2 and LEA3 protein content and growth during stress and during recovery indicated the increased stress tolerance in induced seedlings is associated with enhanced levels of LEA protein content (Table 4). A marked genetic variation was seen in this study in LEA protein levels and recovery growth upon induction. A significant relationship between percent increase in growth during recovery over control and percent increase in LEA2, LEA3 and LEA2+3 content over control suggests the relevance of LEA2 and LEA3 proteins in imparting stress tolerance (Fig. 9). These results indicate that the differential stress response of genotypes may be due to variation in LEA2 and LEA3 protein levels.

DISCUSSION

Apart from characterizing stress responsive genes, a major focus has been to examine the function of these stress responsive gene products and assess their relevance in stress tolerance (Dure, 1993; Bray, 1993). One class of stress responsive genes implicated in imparting stress tolerance are lea genes. Amongst the several LEA groups of proteins,

TABLE 3. *Variability of seedling recovery growth in different fingermillet genotypes in response to induction-stress with 200 mM NaCl*

Genotype	Growth at the end of induction (cm)		Growth during stress (cm)		Growth during recovery (cm)		Absolute growth at the end of recovery (cm)		% Increase in absolute growth after recovery
	Con	Ind	Con	Ind	Con	Ind	Con	Ind	
VL146	3·63	2·13	0·37	2·74	0·08	0·92	4·08	5·79	41·9
GN3	4·39	2·40	0·06	1·61	0·05	1·43	4·11	5·88	41·1
VL149	4·16	2·47	0·08	2·91	0·15	1·14	3·98	6·52	66·0
DM-1	3·73	2·18	0·24	1·65	0·20	1·68	3·75	5·51	46·9
L-10	3·65	2·52	0·01	1·59	0·02	1·69	3·41	5·40	58·6
PR2614	3·58	3·12	0·18	2·05	0·14	0·89	3·84	6·06	52·9
PR202	3·94	2·71	0·37	2·99	0·00	1·41	4·31	7·11	64·9
l.s.d. ($P < 0·05$)	0·46		0·84		0·20		0·70		5·01

After 36 h of germination seedlings were transferred to induction stress (200 mM NaCl). Induction-stress, lethal stress and recovery treatments as per Table 2. Seedlings grown continuously in water and directly exposed to lethal stress served as non-induced controls. Root and shoot growth of the seedlings was measured at the end of induction-stress, lethal-stress and recovery.

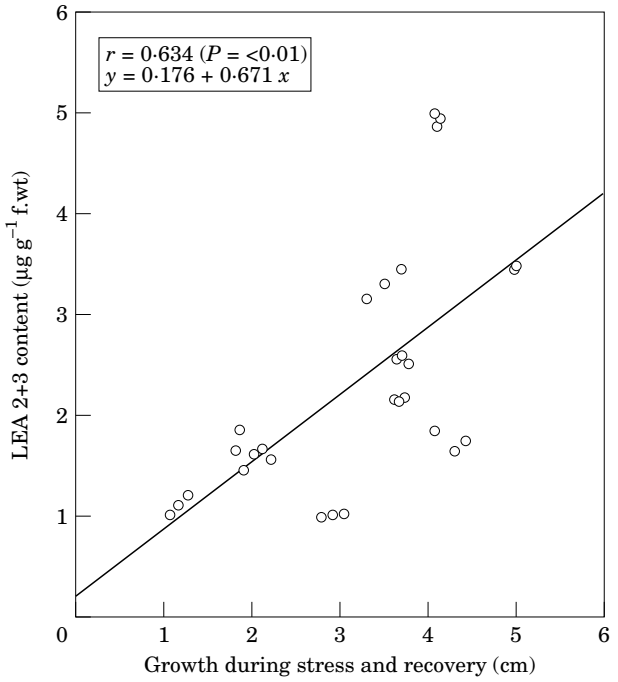


FIG. 7. Relationship between growth during stress and recovery and LEA2+3 content in control and induced seedlings of rice genotypes.

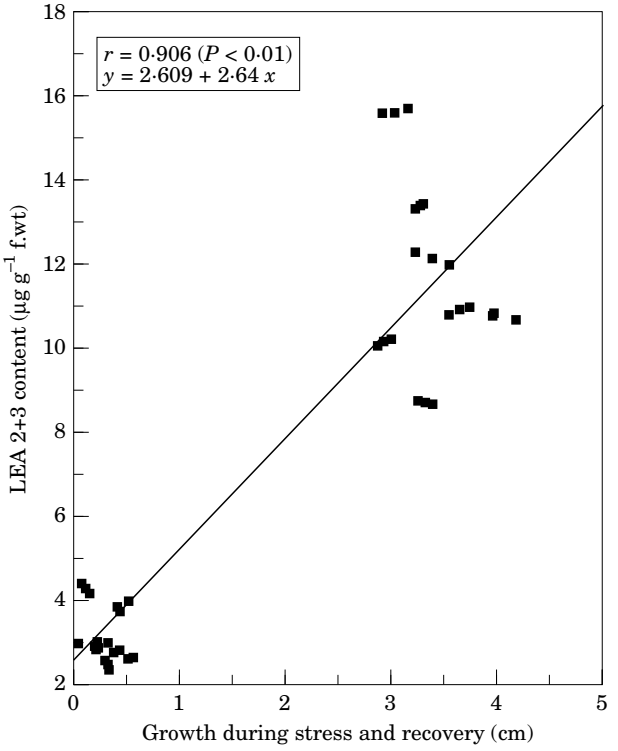


FIG. 8. Relationship between growth during stress+recovery and LEA2+3 content in control and induced seedlings of fingermillet genotypes.

LEA2 and LEA3 are characterized extensively. LEA2 and LEA3 have been described in a number of monocot and dicot species and are characterized by a highly conserved amino acid sequence (Ingram and Bartels, 1996). Predicted functions of these proteins implicate their role in desiccation tolerance (Bray, 1993). In view of this, mesophytic expression of these lea genes, which are otherwise programmed for expression during the maturation and desiccation phases of embryo development, assumes great importance. The expression of the genes coding for LEA2 and LEA3 proteins has been shown to occur upon partial dehydration and other osmotic stresses in vegetative tisues (Close *et al*., 1993; Curry and Walker-Simmons, 1993; Moons *et al*., 1995) and many of them are ABA-responsive (Mundy and Chua, 1988). Our studies also showed enhanced levels of lea2 and lea3 expression in fingermillet and rice seedlings subjected to different osmotic stress treatments (Figs 2 and 3). The transcript level was high when abscisic acid was given along with osmotic stress suggesting that they are ABA-responsive, as reported by Mundy and Chua (1988). Similar results were obtained for the expression of lea3 transcripts also (data not

TABLE 4. *Correlation between growth and LEA proteins content in fingermillet and rice*

	LEA2	LEA3	LEA2+3
Fingermillet			
Growth during stress	0·856**	0·693*	0·833**
Growth during recovery	0·816**	0·871**	0·906**
Growth during stress+recovery	0·869**	0·795**	0·906**
Rice			
Growth during stress	0·633**	0·461*	0·573**
Growth during recovery	0·577**	0·43*	0·573**
Growth during stress+recovery	0·662**	0·494*	0·634**

** $P < 0{\cdot}01$, * $P < 0{\cdot}05$.

Seven genotypes of fingermillet and five genotypes of rice differing in their stress response were subjected to salinity-stress treatment following the optimum induction protocols explained in the methodology. Growth during stress, growth during recovery and growth during stress+recovery were determined. The proteins were extracted from the seedlings at the end of induction and LEA2 and LEA3 protein content was quantified in the heat-stable protein fraction by ELISA.

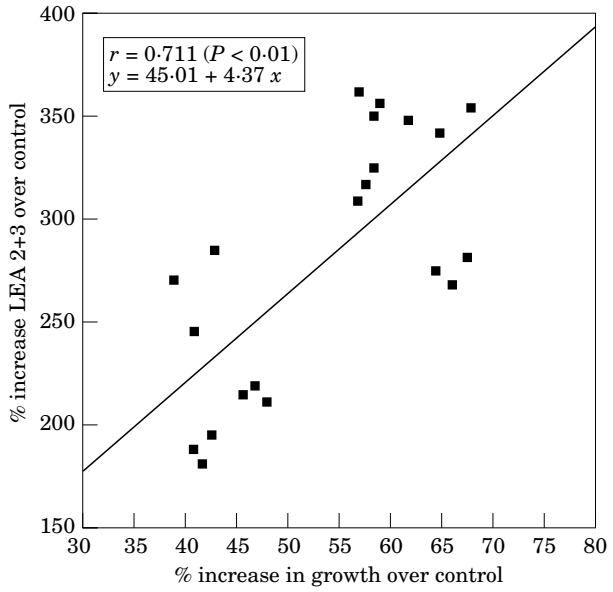


FIG. 9. Relationship between percent increase in LEA2+3 and percent increase in growth at the end of recovery in fingermillet genotypes.

shown) indicating that lea genes are expressed in mesophytic tissues in response to osmotic stress and, therefore, it is relevant to study their importance in stress tolerance.

In the present investigation we examined genetic variability in the expression of lea2 genes under different stress treatments. Two genotypes of rice differing in stress tolerance were used to study the expression of lea2 genes in seedlings exposed to desiccation and salinity stress and also when exposed to ABA. Northern analysis using two cDNA probes, Rab16A and M_3, showed that the known tolerant variety, Rasi, accumulated higher levels of transcripts under all stress treatments compared to a susceptible genotype IR64 (Figs 2 and 3), indicating genetic variability in lea gene expression in mesophytic tissues. There are many reports indicating enhanced expression of lea2 (Curry and Walker-Simmons, 1993) and lea3 (Moons *et al.*, 1995) in mesophytic tissues in response to stress only, and Moons *et al.* (1995) showed genetic variability in the lea2/lea3 gene expression in roots of rice seedlings. However, there are few reports of genetic variability in the levels of LEA proteins although it has been well documented that LEA levels increase in response to abiotic stress.

Since genetic variability exists in the expression of lea genes we examined the variability in LEA protein levels. Patterns of LEA2 and LEA3 protein expression and their quantitative differences were studied in (*a*) induced and non-induced seedling systems and (*b*) genotypes differing in recovery response. Following a novel approach we raised antibodies for LEA2 and LEA3 proteins. Peptides representing the known conserved amino acid sequences of the LEA2 and LEA3 group of proteins were synthesized, coupled to BSA, and antibodies were raised against the conjugated product. A similar approach was followed by Close *et al.* (1993) and more recently by Bracale *et al.* (1997) to raise antibodies against the LEA2 proteins. Western analysis indicated the accumulation of LEA2 and LEA3 groups in stressed tisues. In each group a few polypeptides cross reacted with these antibodies. Since the antibodies are raised against the conserved sequence stretches, all the proteins belonging to the LEA2/LEA3 group of proteins cross reacted. Using this approach, Close *et al.* (1993) and Bracale *et al.* (1997) demonstrated the expression of a few LEA2/dehydrin types of proteins in mesophytic tissues subjected to stress. Further, tolerant (PR202) and susceptible (Hullubele) genotypes of fingermillet accumulated the same polypeptides for the LEA2 group of proteins; however, the tolerant genotype showed more intense bands on the blots (Fig. 5). This indicates that there is only a quantitative difference in LEA2 proteins between tolerant and susceptible genotypes.

In view of the above results we concluded that genetic variability in stress tolerance could be associated with quantitative differences in LEA proteins accumulated under stress. By developing an ELISA protocol we studied the quantitative differences in LEA proteins in seedlings differing in stress tolerance. Stress responsive genes are known to be expressed during induction and might confer resistance to the adverse effects of subsequent severe stress (Krishnan *et al.*, 1989; Uma *et al.*, 1993). Genetic variability in stress response upon induction observed in our previous study (Uma *et al.*, 1995) could also be attributed to the differential expression of the stress responsive proteins. To

examine whether such genetic variability exists in the levels of LEA proteins upon induction, we initially developed an optimum induction protocol for salinity stress in fingermillet and rice. Induced seedlings of both crop species performed better than the non-induced seedlings in terms of growth under stress and recovery. Seedlings subjected to induction-stress also accumulated high levels of LEA2 and LEA3 proteins (Table 2). Only seedlings subjected to induction-stress showed higher levels of LEA2 and LEA3 proteins and also better recovery growth. Further we studied two contrasting genotypes, PR202 and DM-1, classified as tolerant and susceptible based on their induction response. The tolerant genotype achieved higher growth during stress and recovery and also higher amounts of LEA2 and LEA3 proteins compared to the susceptible genotype (Fig. 6) indicating the existence of genetic variation in LEA protein levels and stress responses.

Genetic variability in stress responses was also examined in a few genotypes of fingermillet and rice. The difference in recovery growth in non-induced control seedlings of fingermillet was not significant. In all genotypes, growth during stress and recovery was significantly higher in induced seedlings compared to non-induced control seedlings. Genetic variability in recovery growth was seen only upon induction-stress treatment (Table 3). A positive significant correlation exists between growth during stress and recovery and the LEA protein content (Table 4). In both crops studied, levels of LEA2 and LEA3 proteins were very low in non-induced seedlings and the genetic variability was minimal. However, the levels of these proteins not only increased in induced seedlings, but significant genetic variability was also seen (Figs 7 and 8).

The absolute growth of induced and non-induced control seedlings during stress and recovery varies amongst genotypes. Therefore the percent increase in growth in induced over non-induced controls is an accurate estimate of the stress response of the genotypes. Similarly genetic variability in the levels of LEA2 and LEA3 proteins can also be best expressed as percent increase in induced seedlings over controls. To assess the importance of LEA2 and LEA3 proteins in stress tolerance, the percent increase in growth over controls and percent increase in LEA2 + LEA3 content over controls were compared. The positive significant relationship between the percent increase in growth after recovery over controls and the percent increase in LEA2 + LEA3 over controls across the genotypes in both fingermillet ($r = 0{\cdot}711$) and rice ($r = 0{\cdot}55$) suggests the possible association of LEA2 and LEA3 proteins with stress tolerance. Very few reports show direct evidence of the involvement of LEA protein levels in stress responses. Xu *et al*. (1996) have only recently shown the relevance of LEA3 proteins in stress tolerance by over-expressing the HVA1 gene coding for LEA3 proteins. The transgenic plants, expressing HVA1 genes accumulated higher amounts of lea3 transcripts and also performed better under salinity stress.

Our investigations clearly demonstrate that a few polypeptides belonging to the LEA2 and LEA3 group of proteins are expressed in mesophytic tissues under stress. In the seedling system, however, we did not observe qualitative differences between tolerant and susceptible genotypes. The marked quantitative differences in LEA2 and LEA3 were highly correlated with the observed genetic variation in stress tolerance, signifying the possible role of these proteins in stress tolerance.

ACKNOWLEDGEMENTS

We thank Dr Sashidhar, CCMB, Hyderabad, India and Dr Anjali Karande, IISc, Bangalore, India for help in peptide conjugation and standardization of ELISA technique. This investigation is a part of a research project funded by Department of Science and Technology, Government of India (SP/SO/A-48/94).

LITERATURE CITED

Baker J, Steele C, Dure L III. 1988. Sequence and characterization of LEA proteins and their genes from cotton. *Plant Molecular Biology* **11**: 277–291.

Bracale M, Levi M, Savini C, Dicorato W, Galli MG. 1997. Water deficits in pea root tips: effects on the cell cycle and on the production of dehydrin like proteins. *Annals of Botany* **79**: 593–600.

Bray EA. 1993. Molecular responses to water deficits. *Plant Physiology* **103**: 1035–1040.

Chandler PM, Robertson M. 1994. Gene expression regulated by abscisic acid and its relation to stress tolerance. *Annual Review of Plant Physiology and Plant Molecular Biology* **45**: 113–141.

Chomczynksi P, Sacchi N. 1987. Single step method of RNA isolation by acid Guanidinium thiocyanate-phenol-chloroform extraction. *Analytical Biochemistry* **162**: 156–159.

Close TJ, Krott AA, Chandler PM. 1989. A c-DNA based comparison of dehydration induced proteins (dehydrins) in barley and corn. *Plant Molecular Biology* **13**: 95–108.

Close TJ, Fenton RD, Young A, Asghar R, Demanson DA, Crone DE, Meyer NC, Moonan F. 1993. Dehydrin: the protein. In: Close TJ, Bray EA, eds. *Plant responses to cellular dehydration during environmental stress*. Rockville, MD: American Society of Plant Physiologists.

Curry J, Walker-Simmons MK. 1993. Unusual sequence of group-3-LEA (II) messenger RNA inducible by dehydration stress in wheat. *Plant Molecular Biology* **21**: 907–912.

Dure L III. 1993. A repeating 11-mer amino acid motif and plant desiccation. *The Plant Journal* **3**: 363–369.

Dure L III, Crouch M, Harada J, Ho T-HD, Mundy J, Quatrano R, Thomas T, Sung ZR. 1989. Common amino acid sequence domains among the LEA proteins of higher plants. *Plant Molecular Biology* **12**: 475–486.

Hall CJ, Deschamps RJA, McDermott MR. 1990. Immunoassays to detect and quantiate herbicides in the environment. *Weed Technology* **4**: 226–234.

Harlow E, Lane D. 1988. *Antibodies – A laboratory manual. New York*: *Cold Spring Harbor Laboratory*.

Imai R, Chang L, Ohta A, Bray EA, Takagi MC. 1996. A *lea*-class gene of tomato confers salt and freezing tolerance when over-expressed in *Saccharomyces cerevisiae*. *Gene* **170**: 243–248.

Ingram J, Bartels D. 1996. The molecular basis of dehydration tolerance in plants. *Annual Review of Plant Physiology and Plant Molecular Biology* **47**: 377–403.

Jacobsen JV, Shaw DC. 1989. Heat stable proteins and abscisic acid action in barley aleurone cells. *Plant Physiology* **91**: 1520–1526.

Krishnan M, Nguen HT, Burke JJ. 1989. Heat shock protein synthesis and thermal tolerance in wheat. *Plant Physiology* **90**: 140–145.

Moons A, Bauw G, Dekeyser R, Von Montagu M, Van Der Straeten D. 1995. Novel ABA responsive proteins in vegetative rice tissue. *Current Topics in Plant Physiology* **10**: 288–289.

Mundy J, Chua NH. 1988. Abscisic acid and water stress induce the expression of a novel rice gene. *EMBO Journal* **7**: 2279–2286.

Reid JL, Walker-Simmons MK. 1993. Group 3 late embryogenesis abundant proteins in desiccation tolerant seedlings of wheat (*Triticum aestivum* L.). *Plant Physiology* **102**: 125–131.

Sambrook J, Fritsch EF, Maniatis T. 1989. *Molecular cloning – A laboratory manual*. New York: Cold Spring Harbor Laboratory Press.

Skriver K, Mundy J. 1990. Gene expression in response to abscisic acid and osmotic stress. *The Plant Cell* **2**: 503–512.

Uma S, Ravishankar KV, Prasad TG, Reid JL, Udayakumar M. 1993. Abscisic acid responsive proteins induce salinity stress tolerance in fingermillet (*Eleusine coracana* Gaertn.). *Current Science* **65**: 549–554.

Uma S, Prasad TG, Udayakumar M. 1995. Genetic variability in recovery growth and synthesis of stress proteins in response to polyethylene glycol and salt stress in fingermillet. *Annals of Botany* **76**: 43–49.

Vierling E, Nguyen HT. 1992. Heat-shock protein gene expression in diploid wheat genotypes differing in thermal tolerance. *Crop Science* **32**: 370–377.

Xu D, Duan X, Wang B, Hong B, Ho T-HD, Wu R. 1996. Expression of a late embryogenesis abundant protein gene, HVA1, from barley confers tolerance to water deficit and salt stress in transgenic rice. *Plant Physiology* **110**: 249–257.