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RESEARCH ARTICLE

Abscisic acid-responsive proteins induce salinity stress tolerance in finger millet (*Eleusine coracana* Gaertn.) seedlings

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Pretreatment of finger millet (*Eleusine coracana* Gaertn.) seedlings with 200 mM NaCl significantly enhanced the survival of the seedlings at a lethal stress of 400 mM NaCl and subsequent recovery growth when the stress was withdrawn. However, when the pretreatment included 10 μM abscisic acid (ABA) along with 200 mM NaCl, the seedlings could survive lethal stresses of 500 and 600 mM NaCl and recover remarkably upon withdrawal of stress. Pretreatments with ABA and NaCl resulted in the appearance of several new proteins of 18, 23, 31, 45, 48, 54, 66 and 68 kDa. The ABA responsive proteins were heat-stable and their accumulation increased with ABA concentration in the medium. This increase was also associated with an enhanced tolerance of the seedlings to lethal levels of salinity stress. ABA alone however was not effective either in the synthesis of ABA responsive proteins or in imparting tolerance to salinity stress. ^{35}S-methionine incorporation studies indicated the synthesis of 21, 26, 39, 45, 68, 70, 74 kDa proteins of which 21 kDa was the prominent polypeptide synthesized during induction.

Abiotic stresses such as high or low temperatures and salinity induce the synthesis of stress-shock proteins in plants and animals[1–8]. These proteins have been shown to impart tolerance against lethal levels of stresses[9,10]. The phytohormone, abscisic acid (ABA) that accumulates in tissues under abiotic stresses such as desiccation, salt and extreme temperatures[9–11] has been shown to be involved in the synthesis of the stress-shock proteins.

For correspondence

Exogenous application of ABA induces the expression of specific genes regulating the synthesis of stress shock-proteins[6,12-14]. Studies on wild type and ABA-deficient *flacca* mutant of tomato indicated the role of ABA in synthesis of specific ABA-responsive proteins under drought stress[15,16].

In this article we report the involvement of ABA in the synthesis of specific proteins by finger millet (*Eleusine coracana* Gaertn.) seedlings subjected to non-lethal salinity stress. Further, we show that these ABA-responsive proteins are heat-stable and impart tolerance to the seedlings under lethal levels of salinity stress.

Methods

Response of finger millet seedlings to pretreatment with ABA and NaCl

Finger millet seeds were germinated on moist filter paper in petri dishes at 30° C. After 36 h of germination, the seedlings were pretreated for 16 h with different concentrations of NaCl, ABA and ABA + NaCl. The seedlings were then subjected to lethal stress for 36 h by transferring them to higher concentrations of NaCl (400 to 600 mM). Subsequently the lethal stress was alleviated by transferring the seedlings to water and allowed to recover for 64 h. In all pretreatments, there was no or negligible root growth during the lethal stress. Hence, the recovery growth was obtained as the difference in root length of seedlings between that at the end of recovery and that at the end of pretreatments.

Root length of 30 seedlings was measured at each stage. The extent of response to various pretreatment was calculated as the ratio of recovery growth of pretreated seedlings to that of non-pretreated seedlings.

Protein preparation

To study the synthesis of ABA-responsive proteins, the seedlings (300 per treatment) were removed after the treatment, washed thoroughly and roots were separated and ground in 150 mM Tris-HCl (pH 8.0). The homogenate was spun at 10,000 *g* for 10 minutes and the supernatant was used for further studies.

Heat stability

The heat stability of proteins was determined following methods reported earlier[17-19]. The supernatant prepared above was subjected to 70° C and 80° C for 10 minutes in a water bath and those that did not precipitate were considered as heat-stable. The denatured proteins were removed by centrifugation at 12,000 *g* for 10 minutes. The resulting supernatant was collected and the proteins were precipitated with 5 volumes of cold acetone. The pellet was suspended in Laemmlli's SDS buffer (0.0625 M Tris HCl with 5% SDS, 2% MCE, 1 mM PMSF, 0.002% bromophenol blue and 10% sucrose) and separated on SDS-PAGE. The proteins were determined by Lowry's method[20].

Electrophoresis and fluorography

In order to determine the proteins synthesized during different pretreatments, seedlings were incubated with 200 μCi of ^{35}S-methionine (supplied by BARC, Bombay with specific activity 500 mCi/mM and with activity of 100 μCi/ml) during the last 4 h of different pretreatments.

After pretreatment, the seedlings were washed thoroughly with cold methionine followed by water and extraction buffer. The soluble protein and heat stable protein fractions were prepared as described earlier. The radioactivity in the protein fraction was determined by a liquid scintillation system (LSS-20 ECIL) using Brays scintillation solution[21].

Heat stable proteins were separated using 10% SDS-PAGE following Laemmlli[22] and were stained with silver stain[23]. For fluorography, gels were stained with coomassie brilliant blue for molecular weight markers and treated with PPO (2,5 diphenyloxazole); the gels were then dried and exposed to Kodak X-ray film at −70° C and developed following the manufacturer's instructions.

Western blots

Western blotting was done following the procedure of Winston *et al.*[24] and Reid and Walker-Simmons[19] using polyclonal antiserum containing antibodies for a group of six ABA-responsive proteins isolated from wheat embryos.

The proteins separated by SDS-PAGE using 12.5% gel were transfered to a polyvinylidene difluoride (PVDF) membrane by electroblotting. The ABA-responsive proteins were detected using the polyclonal antibodies for ABA-responsive proteins and secondary antibody (goat antirabbit IgG, Sigma) conjugated with alkaline phosphatase using BCIP (5-bromo-4-chloro-3-indolyl-phosphate) as the substrate.

Results

Recovery of root growth following pretreatment with ABA and NaCl

The survival and recovery growth of roots of finger millet seedlings following a lethal stress of 400 mM NaCl

were markedly enhanced where seedlings were pretreated either with 200 mM NaCl or with ≥ 100 μM ABA (Figure 1).

Presence of ABA even at low concentrations along with 200 mM NaCl during pretreatment, resulted in a significant increase in recovery growth following a lethal stress (400 mM NaCl; Figure 2). The response was linear up to 10 μM ABA beyond which reduction in growth was observed.

Pretreatment with either 100 mM NaCl or 10 μM ABA did not significantly improve the recovery growth of roots following 400 mM NaCl lethal stress. However, pretreatment with 10 μM ABA along with 100 mM NaCl markedly increased the recovery growth (Table 1). Seedlings pretreated with 200 mM NaCl and 10 μM ABA tolerated extreme lethal stresses of 500 and 600 mM NaCl (Table 2).

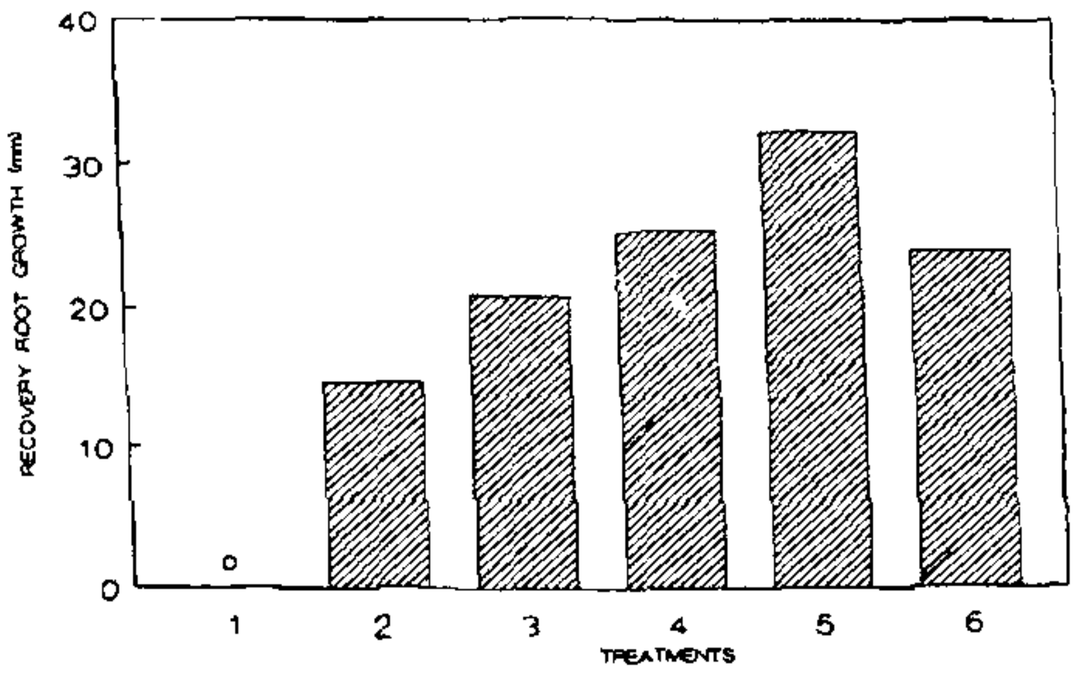

Figure 2. Recovery growth of finger millet seedlings following 400 mM NaCl stress. Seedlings were pretreated with different concentrations of ABA along with 200 mM NaCl and then subjected to 400 mM NaCl stress (CD = 1.44, $P < 0.05$). D, dead; 1, Water; 2, 200 mM NaCl; 3, 200 mM NaCl + 1 μM ABA; 4, 200 mM NaCl + 5 μM ABA; 5, 200 mM NaCl + 10 μM ABA; 6, 200 mM NaCl + 50 μM ABA.

Heat stable nature of ABA responsive proteins

Seedlings pretreated with either 200 mM NaCl alone or along with 10 μM ABA had higher amount of heat stable protein compared to the control (non-pretreated) both at 70 and 80° C (Table 3). Presence of ABA in the pretreatment medium resulted in relatively higher levels of heat stable proteins.

Similar trend was observed when the heat stability of the proteins synthesized during the induction period was studied. Incorporation of ^{35}S-methionine during the last 4 h of pretreatment in the heat stable fraction was nearly two-fold greater in seedlings pretreated with 200 mM NaCl and 10 μM ABA compared to the control (Table 4).

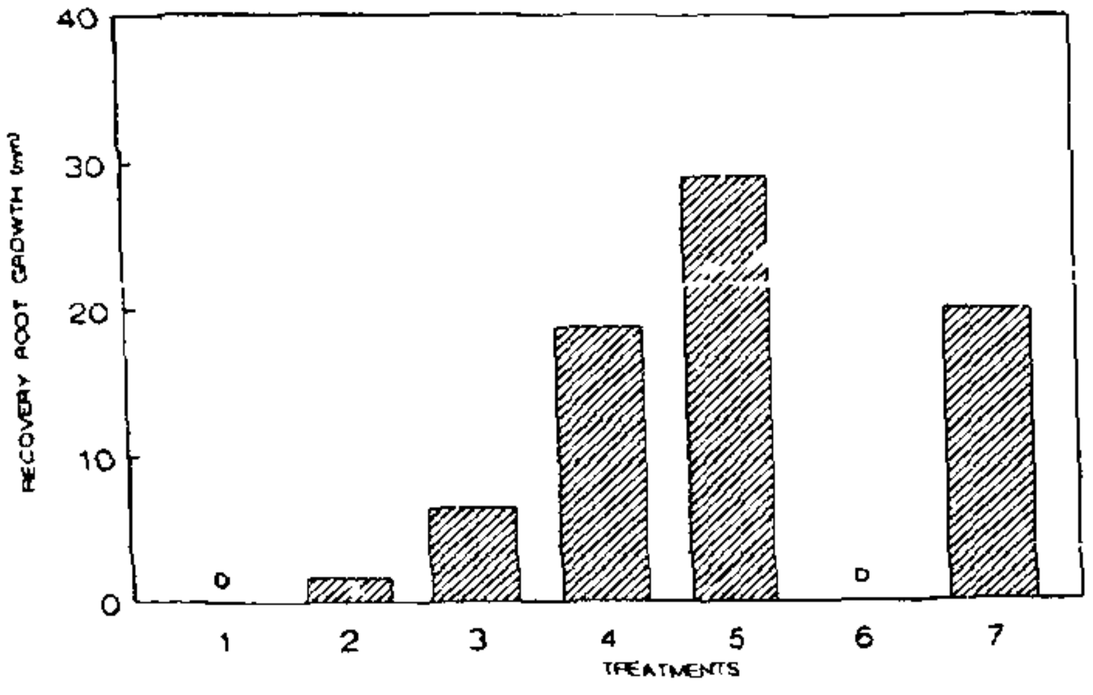

Figure 1. Effect of pretreatment with ABA and NaCl on finger millet seedlings following 400 mM NaCl lethal stress (CD = 0.886, $P < 0.05$), D = Dead. 1, Water; 2, 10 μM ABA; 3, 100 μM ABA; 4, 200 μM ABA; 5, 1 mM ABA; 6, 100 mM NaCl, 7, 200 mM NaCl.

Synthesis of ABA responsive proteins

The heat stable proteins were separated on 10% SDS polyacrylamide gels and stained with silver stain. A major polypeptide of 21 kDa was observed in the protein fraction of the seedlings pretreated with 200 mM NaCl + 10 μM ABA (Figure 3). The fluorography of such a heat stable fraction revealed synthesis

Table 1. Effect of pretreatment of finger millet seedlings with ABA and NaCl on root growth following a lethal stress of 400 mM NaCl

Treatment	Root length (mm)			
	Growth at the end of pretreatment (16 h after pretreatment)	Growth at the end of lethal stress (36 h after lethal stress)	Growth at the end of recovery (64 h after recovery)	Recovery growth (mm)
Control	22 6	22 0	22.0	0.00
100 mM NaCl	18.4	20 0	21 5	3 06
10 μM ABA	18 9	19 1	21 5	2 60
100 mM NaCl + 10 μM ABA	9 1	14 4	40 3	31 20
CD at 5%	2 08	1 84	2.58	1 09

Table 2. Effect of pretreatment with ABA and NaCl on root growth of seedlings (mm) during recovery period following different levels of lethal stress.

	NaCl (mM) medium		
Pretreatment	400	500	600
Water	2.0	D	D
NaCl 200 mM	18.6	1.2	D
ABA 10 μM	1.7	D	D
NaCl 200 mM + ABA 10 μM	26.5	22.0	21.4
CD at 5%	0.68		

D – Dead.

Table 3. Effect of pretreatment of finger millet seedlings with ABA and NaCl on the protein content in heat stable fraction

	Protein content (mg)	
Pretreatment medium	70° C	80° C
Water (control)	0.80 (28)	0.79 (27)
200 mM NaCl	1.46 (50)	0.83 (29)
200 mM NaCl + 10 μM ABA	1.64 (57)	1.05 (36)

Initial protein content before heat treatment was 2.9 mg ml^{-1}. Figures within bracket refer to proportion of heat stable protein expressed as per cent of the initial protein content.

Table 4. Radioactivity in the heat stable protein (70° C for 10 min) fraction of finger millet seedlings pretreated with NaCl and ABA

	Radioactivity in protein fraction (cpm × 10^5)/mg protein	
Pretreatment	Before heat treatment	After heat treatment
Water (control)	450	90 (20.0)
200 mM NaCl + 10 μM ABA	480	236 (49.2)

Values in parenthesis are per cent radioactivity in heat stable protein fraction.
Seedlings were incubated in ^{35}S-methionine during the last 4 hours of pretreatment. The soluble proteins were extracted, subjected to heat treatment and the radioactivity in the heat stable and denatured protein fractions was measured.

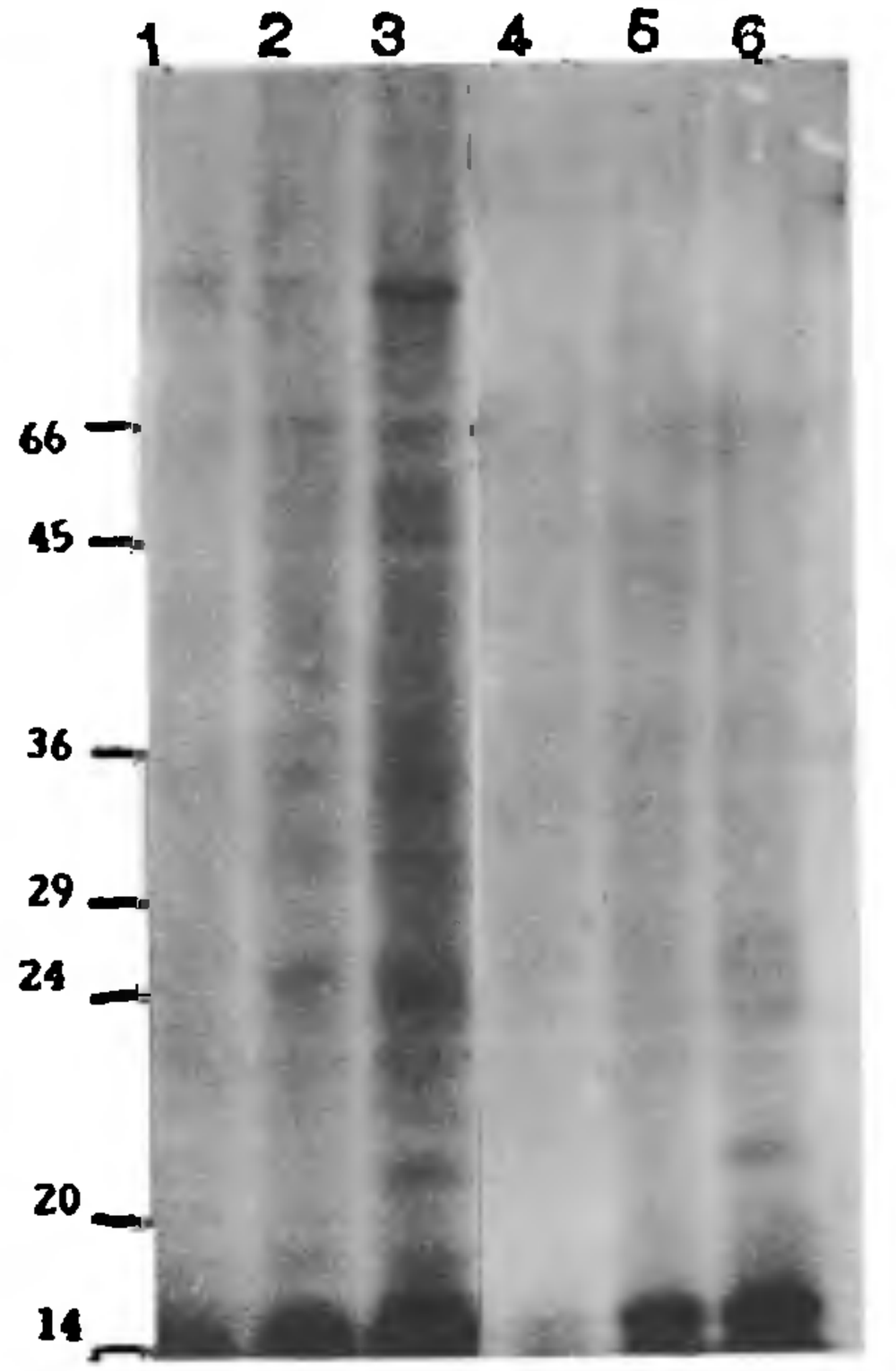

Figure 3. Pattern of proteins following SDS polyacrylamide gel electrophoresis and staining of heat stable fraction following various pretreatments (lanes 1 and 4, water; lanes 2 and 5, 200 mM NaCl; lanes 3 and 6, 200 mm NaCl + 10 µM ABA). Lanes 1 to 3 are profiles of protein incubated at 70°C for 10 minutes; lanes 4 to 6 are those from incubation at 80°C for 10 minutes. Quantity of protein loaded are: Lane 1, 70 µg; Lane 2, 130 µg; Lane 3, 150 µg; Lane 4, 70 µg; Lane 5, 75 µg; Lane 6, 96 µg. The numbers on left show the position of molecular weight markers (kDa).

of several proteins of molecular weight 21, 26, 39, 45, 68, 70 and 74 kDa. A major 21 kDa protein appeared, which was synthesized to a greater extent as revealed by the fluorography (Figure 4).

When blots were probed with antibodies against a group of ABA-responsive proteins from wheat embryos, the synthesis of a number of polypeptides (18 to 90 kDa) was observed during pretreatment with ABA. Significantly these polypeptides of molecular weight 18, 23, 31, 45, 48, 54, 66, 68 kDa were found only in seedlings of pretreated systems induced with ABA (10 μM) along with NaCl (200 mM) but not when the seedlings were pretreated with ABA alone (Figure 5). The 21-kDa protein which was synthesized in response to pretreatment with ABA and NaCl, did not cross-react with the antibodies.

There was also an increase in the synthesis of these proteins with increasing concentration of ABA along with 200 mM NaCl (Figure 6). However, even in 200 mM NaCl, a few ABA-responsive proteins were observed although their quantity was considerably reduced.

Discussion

Our results demonstrate that pretreatment of finger millet seedlings with ABA and non-lethal concentration of NaCl result in the accumulation of ABA-responsive proteins; further these seedlings exhibit tolerance to lethal salinity stress. Seedlings pretreated with either ABA or NaCl alone, however, neither accumulated the

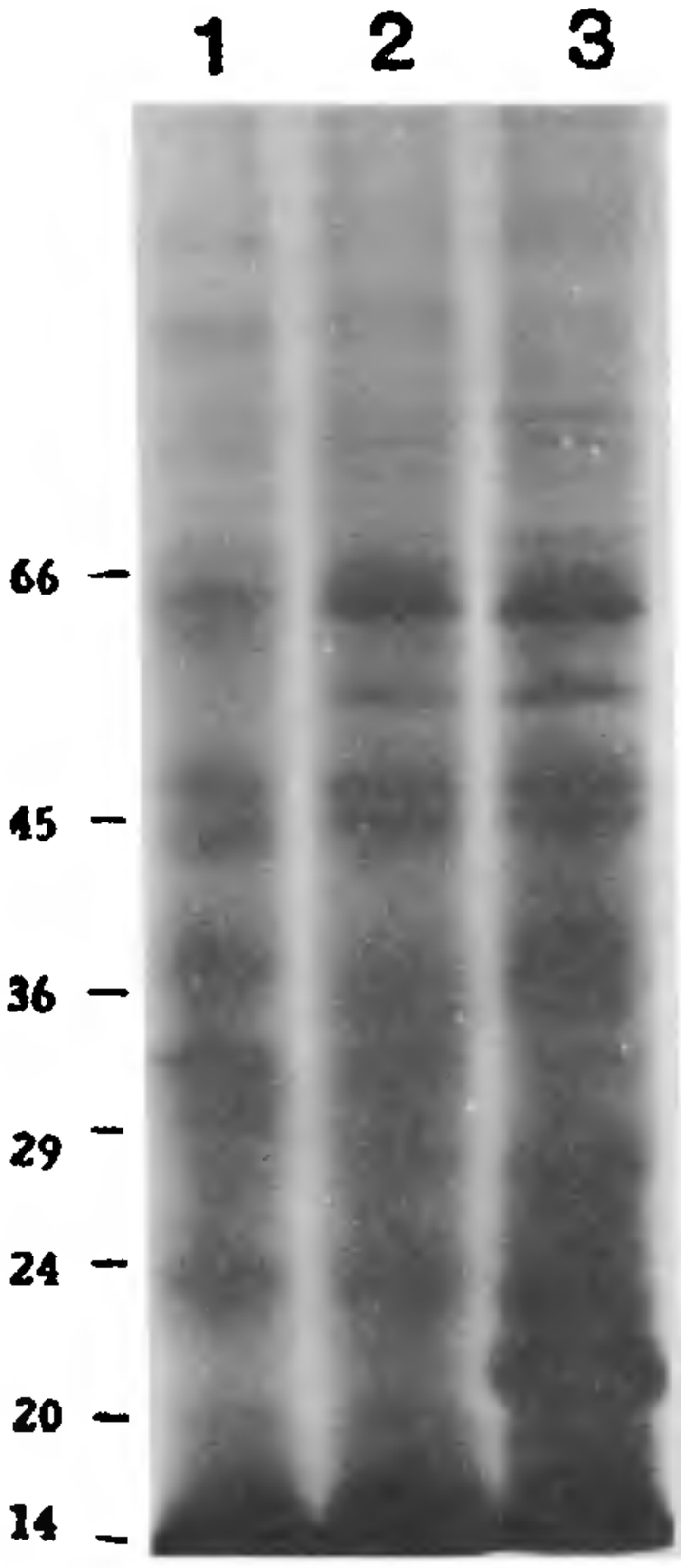

Figure 4. Fluorograph of a SDS polyacrylamide gel showing the ^{35}S-methionine labelled protein synthesized during the last four hours of various pretreatment (lane 1, water; lane 2, 200 mM NaCl; lane 3, 200 mM NaCl + 10 µM ABA). Protein sample (150 µg) was loaded in each of the lanes. The numbers on left show the position of molecular weight markers (kDa).

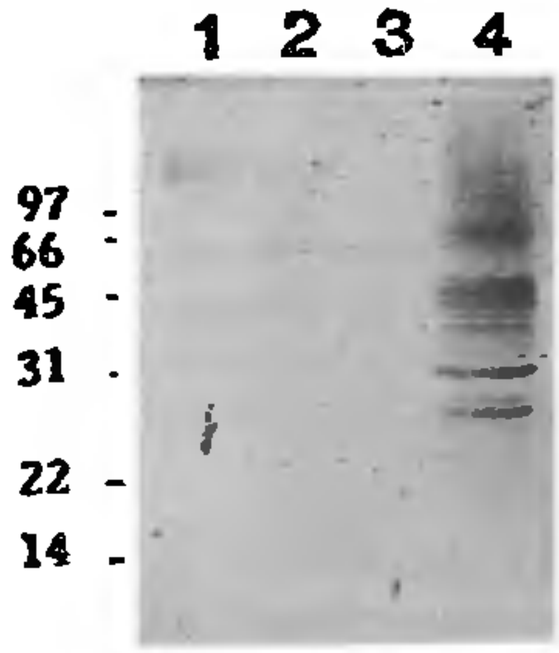

Figure 5. Western blot indicating ABA-responsive proteins with various pretreatments (lane 1, water, lane 2, 200 mM NaCl; lane 3, 10 µM ABA; lane 4, 200 mM NaCl + 10 µM ABA). Proteins were extracted at the end of 16 h of pretreatment from roots and separated on 12.5% SDS-PAGE. Quantification was done after transferring to PVDF membrane by using a combination antibodies for ABA-responsive proteins. The numbers on left show the position of molecular weight markers (kDa).

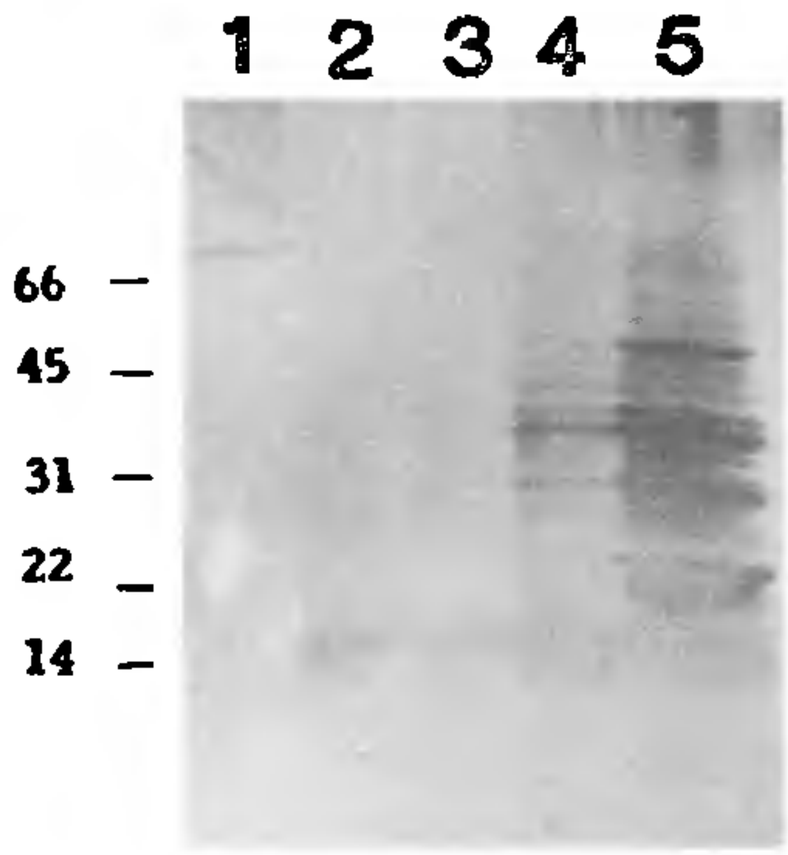

Figure 6. Western blot showing the ABA-responsive proteins following pretreatment with increasing concentrations of ABA along with NaCl (lane 1, water; lane 2, 200 mM NaCl, lane 3, 200 mM NaCl + 1 µM ABA; lane 4, 200 mM NaCl + 10 µM ABA, lane 5, 200 mM NaCl + 50 µM ABA). Proteins were extracted from roots at the end of 16 h of pretreatment and separated on 12.5% SDS-PAGE. The numbers on left show the position of molecular weight markers (kDa).

ABA-responsive proteins nor survived the lethal stress. These results corroborate the findings of Singh *et al.*[7] who showed that though ABA alone can induce the synthesis of mRNA for several proteins, their translation requires the presence of stress environment.

The relevance of the ABA-responsive proteins in imparting tolerance is also evident from the association between the accumulation of these proteins and tolerance of seedlings to lethal stresses. Pretreatment with increasing concentration of ABA along with 200 mM NaCl resulted in increased recovery growth of seedlings subjected to lethal salinity stress (Figure 2). However, survival at extremely high lethal stresses such as 600 to 700 mM NaCl was maximum with pretreatment of seedlings with 50 μM ABA and 200 mM NaCl at which the amount of ABA-responsive proteins synthesized was also high (Figure 6). It appears that higher levels of ABA-responsive proteins may be essential for survival at extremely high lethal stresses.

The reduction in recovery growth under 400 mM NaCl lethal stress after pretreatment with higher concentration of ABA (50 μM) and 200 mM NaCl is intriguing (Figure 2). This might be partly attributed to the fact that high concentration of ABA might have resulted in accumulation of ABA-responsive proteins, a few of which might be proteinase inhibitors. For instance, Robertsen *et al.*[25] reported the synthesis of α-amylase inhibitor in barley as a pretreatment response to ABA and dehydration stress. ABA responsive late embryogenesis abundant (LEA) proteins that accumulate during embryo maturity and degrade on rehydration of the seeds have been reported in several species[14,19,25]. In

these the embryo resumes growth only after a substantial decline in the LEA proteins. Similarly degradation of ABA-responsive proteins on stress alleviation might also be necessary for the recovery growth of seedlings.

Most of the ABA-responsive proteins were heat stable at 70° C and 80° C though the basis for their heat stability is not clear[18,26]. These proteins might have significance in protecting other polypeptides as proposed for LEA proteins of cotton[27] and ABA-responsive proteins in barley aleurone layers[26].

Among a host of protein species synthesized as a consequence of pretreatment with 10 μM ABA and 200 mM NaCl, a major fraction was the 21 kDa protein (Figure 6). This protein did not cross-react with the antibodies developed against the ABA-responsive proteins of wheat embryos. Therefore it appears that the 21 kDa protein is specific to fingermillet and might have a role in imparting tolerance against abiotic stresses.

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RESEARCH COMMUNICATIONS

p53 Mutation in human hepatocellular carcinoma in India

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We have analysed a number of primary hepatocellular carcinomas (HCC), both fresh and paraffin-embedded tissues, for the presence of a hotspot mutation in p53 at the 249th codon of exon 7. Of the samples analysed, ten were found to be hepatitis-B antigen-positive and one among them showed the hotspot mutation. We have shown that paraffin-embedded samples can easily be used for PCR analysis of mutations. Our results show that alteration in this mutational hotspot is not very frequent in Indian HCC cases similar to the situation obtained in Australia and Taiwan and that hepatitis B virus infection cannot be a major factor for alteration in the 249th codon of exon 7 in p53.

PRIMARY hepatocellular carcinoma (HCC) is a common, highly malignant and aggressive cancer of humans with high incidence in African and Asian subcontinents. There has been a recent interest in elucidating subtle genetic alterations that probably occur early on and accelerate the progression of hepatic carcinogenesis[1,2]. The tumour suppressor gene p53 is a candidate locus, where mutations occur at multiple hotspots with an