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Genetic Variability in Recovery Growth and Synthesis of Stress Proteins in Response to Polyethylene Glycol and Salt Stress in Finger Millet

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Marked differences were found among 28 finger millet genotypes (*Eleusine coracana* Gaertn.) in acquired tolerance to osmotic stress as assessed by the recovery of root growth from severe stress [−1·2 MPa polyethylene glycol, (PEG) or 400 mM NaCl]. However, these differences in tolerance were observed only when the seedlings were subjected to a preceding mild induction stress (−0·6 MPa PEG or 200 mM NaCl). In two contrasting genotypes, synthesis of stress-induced proteins was studied. Proteins with apparent molecular weight of 70–72, 52, 37, 34 and 23 kDa were synthesized in the highly responsive genotype (GE 415) and poorly responsive (VL 481) genotype following a mild induction stress (200 mM NaCl). However, GE-415 synthesized a 54 kDa protein that was not observed in VL-481. Addition of abscisic acid (ABA) to the induction medium containing 200 mM NaCl enhanced the acquired tolerance of finger millet seedlings over those without ABA in association with the appearance of several ABA-responsive proteins. GE-415 required much less ABA than VL-481 to obtain the same response. With 10 μM ABA + 200 mM NaCl induction stress, GE-415 had significantly higher endogenous ABA. In association with higher levels of ABA, GE-415 had greater recovery root growth following severe stress from 600 mM NaCl. Pretreatment with 10 μM ABA + 200 mM NaCl induced several proteins with apparent molecular weights of 70–72, 54, 45, 36, 29 and 21 kDa in both genotypes. Qualitatively, GE-415 synthesized a unique 23–24 kDa protein and quantitatively there was significantly more of the 21 kDa protein in GE-415 compared to VL-481. The results indicate that the synthesis of stress proteins is correlated with the observed variation in acquired tolerance of the two genotypes.

Key words: *Eleusine coracana* Gaertn., salinity, polyethylene glycol, stress proteins, ABA, ABA-responsive proteins, finger millet seedlings.

INTRODUCTION

At the molecular level, one of the most extensively characterized stress responses in higher plants is the synthesis of stress shock proteins (SSPs). These proteins are synthesized under a variety of stresses such as high temperature (Key, Lin and Chen, 1981; Sachs and Ho, 1986; Lindquist and Craig, 1988), desiccation (Chandler *et al.*, 1988; Bray, 1988), salinity (Singh *et al.*, 1985; Ramagopal, 1987; Esaka *et al.*, 1992), heavy metals (Lin, Roberts and Key, 1984; Howarth, 1990), chilling (Tseng and Li, 1991) and anoxia (Czarnecka *et al.*, 1984). Many of these proteins are suggested to protect the cell against the adverse effects of stress. The significance and relevance of these stress proteins has been well characterized in several studies (Lin *et al.*, 1984; Bray, 1988; Krishnan, Nguyen and Burke, 1989). These proteins are shown to be synthesized when the organism is exposed to a mild non-lethal level of stress often referred to as an induction stress. The ability of induced systems to tolerate severe levels of stress signifies the importance of stress proteins (Lin *et al.*, 1984; Krishnan *et al.*, 1989; Vierling, 1991). Thermosensitive mutants that do not synthesize stress proteins when subjected to mild stress do not survive severe stress (McAlister and Finkelstein, 1980). Information on differential synthesis of stress proteins in genotypes differing in stress tolerance is however inconclusive (Fender and O'Connell, 1989; Krishnan *et al.*, 1989; Ristic, Gifford and Cass, 1991; Vierling and Nguyen, 1992).

In recent years, several workers have addressed the underlying mechanism of induction of these proteins by various stresses (Marcotte, Russel and Quatrano, 1989; Guiltinan, Marcotte and Quatrano, 1990; Skriver and Mundy, 1990; Gurley and Key, 1991; Hetherington and Quatrano, 1991; Bray, 1993). In contrast to those induced by heat stress, the stress proteins synthesized due to desiccation, salinity and cold stress have been shown to be mediated by turgor-dependent gene expression (Bray, 1993). The desiccation-induced proteins are often referred to as late embryogeny abundance proteins (LEA) (Bray and Zeevaart, 1986; Dure *et al.*, 1989; Curry, Morris and Walker-Simmons, 1991; Dure, 1993). These LEA proteins which are synthesized during the maturation and desiccation phases of seed development are also known to be synthesized in vegetative tissues due to desiccation (Close *et al.*, 1993; Close and Lammers, 1993; Curry and Walker-Simmons, 1993). Many of these genes are responsive to ABA, a phytohormone which also increases during desiccation and salinity stress (Singh *et al.*, 1987; Chandler *et al.*, 1988; Mundy and Chua, 1988). These proteins that are induced by

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elevated levels of ABA and those that are directly induced by stress have been distinguished by using ABA-deficient mutants of tomato (Bray, 1988) and maize (Chandler *et al.*, 1988). Several studies, based on correlative data, have indicated the role of certain ABA-induced stress proteins in imparting tolerance to otherwise lethal levels of stress (Singh *et al.*, 1987; Bartels *et al.*, 1990; Uma *et al.*, 1993).

In an earlier study we demonstrated the involvement of ABA responsive proteins in imparting tolerance of finger millet seedlings to severe levels of salinity stress. Interestingly when ABA was included during the induction stress with NaCl, seedling tolerance to severe stress was remarkably enhanced with the synthesis of several ABA responsive proteins of which a 21 kDa protein was most prominent (Uma *et al.*, 1993).

In this paper, we examined the variability in stress response among finger millet genotypes. Both growth and accumulation of stress proteins in response to a challenge by severe levels of polyethylene glycol (PEG) and NaCl stress following induction with mild levels of these stresses were studied. We also demonstrate the significance of exposing seedlings to an induction stress in determining the genetic variability in stress tolerance. We suggest that the genotypic response to severe levels of stress could be best achieved by evaluating the response after subjecting the seedlings to an induction stress. We relate the differential response of genotypes to (*a*) quantitative and qualitative differences in the production of stress shock proteins and (*b*) to the levels of ABA accumulated in them and their differential sensitivity to ABA.

MATERIALS AND METHODS

Stress response of finger millet genotypes with or without induction

Seeds of 28 finger millet (*Eleusine coracana* Gaertn.) genotypes were obtained from the Project Coordinator, Small Millets, Bangalore, India. The response of these genotypes with respect to their seedling growth and accumulation of stress proteins were examined under severe stress levels of PEG and NaCl following either with or without induction with the respective stresses.

Induction response

Seeds were germinated on moist filter paper at 30 °C for 36 h and uniform seedlings were subjected to induction stress by transferring them into Petri dishes containing either −0·6 MPa PEG, 6000 mw and/or 200 mM NaCl (analytical grade). The osmolarity of PEG solutions was confirmed by using a vapour pressure osmometer, Wescor Model 5100 C. The seedlings were then transferred to severe levels of stress of −1·2 MPa PEG or 400 mM NaCl for 36 h. Following this the seedlings were transferred to Petri dishes containing water and allowed to recover for 64 h in an incubator at 30 °C and 75% relative humidity in the dark. Root length was determined at every step. In all treatments there was little or no root growth during severe stress. Hence, recovery root growth was calculated as the difference in root length of the primary roots of the seedlings at the end of recovery and at the end of induction treatment. Root length of 30 seedlings was measured on each ocassion. Variation among genotypes was determined by comparing the extent of response to induction.

Response without induction

Seeds, germinated on moist filter paper at 20 °C for 36 h, were transferred directly to severe stress (−1·8, −1·2 MPa PEG or 300, 400 mM NaCl) for 48 h. Following this, the seedlings were allowed to recover in water. Non-stressed control seedlings were continuously grown in water (36+48 h). Root length was determined before the imposition of severe stress and at the end of recovery. The difference between the two observations was used to arrive at the per cent reduction in root growth due to stress. Root length of 30 seedlings was measured at each stage and the mean computed.

In vivo *labelling and protein extraction*

To study the synthesis of stress proteins during the induction stress, seedlings during the last 4 h of the induction period (see experimental details on induction response) were incubated with 200 μCi ^{35}S-methionine (BARC, Bombay with specific activity 500 mCi ml^{-1} and with final activity of 100 μCi ml^{-1}) along with their respective induction medium. At the end of the pretreatment the seedlings were washed thoroughly with 1 mM methionine (non-radioactive) followed by water and then with extraction buffer (150 mM Tris-HCl, pH 8·0) and homogenized in the same. The homogenate was centrifuged at 10000 *g* for 10 min and the supernatant used for further studies.

Preparation of heat stable proteins

Heat stable proteins were separated and diluted following Close, Kortt and Chandler (1989) and Uma *et al.* (1993). Briefly, the supernatant prepared above 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. The supernatant containing heat stable proteins was collected. The proteins were precipitated with 5 volumes of cold acetone. The pellet was collected and suspended in Laemmli's SDS buffer [0·0625 M Tris-HCl, 5% (w/v) sodium dodecyl sulphate, 2% (v/v) mercaptoethanol, 1 mM phenyl methyl sulphonyl fluoride and 0·002% (v/v) bromophenol blue] and used immediately or stored at −20 °C. The radioactivity in the protein fraction was determined by a liquid scintillation system (LSS-20 ECIL) using Bray's scintillation solution and the protein content was determined following Lowry *et al.* (1951).

SDS-PAGE and fluorography

Heat stable proteins were separated on 10% SDS-PAGE following Laemmli (1970). The 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 for 2 weeks and developed following the manufacturer's instructions.

ABA analysis

The seedlings were thoroughly washed three times with 1% (w/v) sucrose and lyophilized prior to ABA extraction. Preparation of the samples for ABA analysis and the quantifications of ABA by immunoassay using a monoclonal antibody for ABA follow Walker Simmons (1987) and Reid and Walker-Simmons (1993).

RESULTS

Genotypic variation in root recovery growth in response to induction

Marked variation in root recovery growth of 28 finger millet genotypes following induction was observed both in PEG and NaCl (data not shown). Few of the genotypes exhibited a very high recovery growth (highly responsive) compared to others such as VL 481 and VL 148 where recovery growth was very poor (low responsive) (Table 1).

Induction stress is necessary in determining genotypic variation

There were no significant differences in stress response among the genotypes when the seedlings were exposed to severe levels of stress without a prior induction treatment (Table 1). To confirm this observation, five contrasting genotypes (with marked variation in induction response) were directly subjected to −0·8 and −1·2 MPa PEG after 48 h germination. When the per cent reduction in root recovery growth (growth during 48 h stress + 48 h recovery) over water grown non-stressed seedlings was determined no significant difference was observed among the genotypes (Fig. 1). Similarly, in 300 and 400 mM NaCl stress without induction, none of the genotypes could survive. But with prior induction, there was an increase in recovery root growth over those not induced, both under severe levels of PEG (Fig. 2A) and NaCl stress (Fig. 2B). Furthermore, genotypes significantly differed in their response following induction treatment.

TABLE 1. *Variation in recovery root growth (mm) of finger millet genotypes following severe levels of stress with or without a prior induction period of mild stress*

Genotypes	PEG		NaCl	
	Non-induced	Induced	Non-induced	Induced
High response:				
GE-415	14·2 ± 1·8	45·3 ± 4·7	D*	26·8 ± 1·9
Budha local	15·8 ± 1·8	27·2 ± 3·1	D	31·2 ± 2·6
SRSDC-6·82	13·9 ± 1·6	33·7 ± 3·4	D	19·9 ± 1·8
GE-418	13·7 ± 1·7	30·1 ± 3·2	D	16·1 ± 2·1
PR-202	12·0 ± 1·2	24·9 ± 3·0	D	15·7 ± 1·8
HR-374	13·1 ± 1·4	25·0 ± 2·8	D	23·4 ± 2·0
Low response:				
VL-481	20·9 ± 2·3	22·9 ± 2·1	D	10·0 ± 1·3
VL-148	15·1 ± 1·7	21·1 ± 2·0	D	5·8 ± 1·1

* D, Dead. l.s.d. = 1·44 (PEG); 1·29 (NaCl). $P < 0·05$.

Induction treatment was 16 h in PEG (−0·6 MPa) or 200 mM NaCl. The severe stress treatment was 36 h in PEG (−1·2 MPa) or 400 mM NaCl. The seedlings were recovered for 64 h. The recovery root growth is the difference in root length between that at the end of recovery and that at the end of pretreatment. Root length of 30 seedlings was measured at each stage and least significant difference (l.s.d.) was calculated at less than 5% probability.

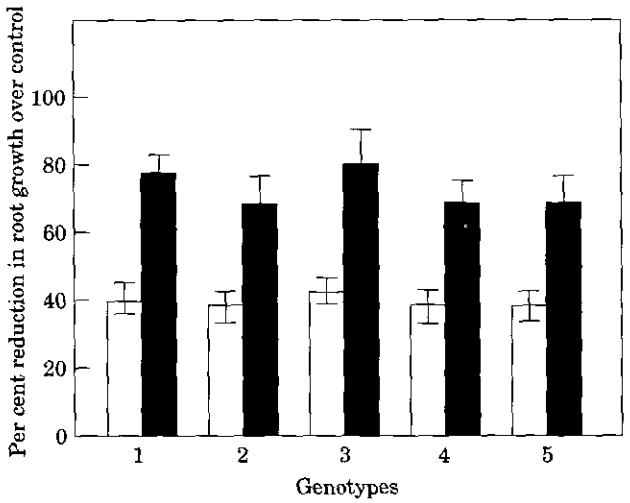

FIG. 1. Response of contrasting finger millet genotypes to severe stress without induction. After 48 h germinating seedlings were subjected to −0·8 and −1·2 MPa PEG stress directly for 48 h followed by 48 h recovery. Recovery root growth (calculated as explained in Materials and Methods) was compared with the root growth of seedlings continuously grown in water during the same period. Root length of 30 seedlings was measured at every stage. The per cent reduction in recovery root growth over non-stressed control is presented. Genotypes. High responsive: 1, GE-415; 2, PR-202; 3, HR-374. Low responsive: 4, VL-481; 5, VL-148. □, −0·8 MPa; ■, −1·2 MPa.

Response of the two selected genotypes to different levels of induction stress

In the light of the responses to both PEG and NaCl stresses, the genotypes GE-415 (high responsive) and VL-481 (low responsive) were selected for further studies. To determine if they differ in optimum induction stress requirement, several induction treatments were tried. At both 200 and 300 mM NaCl induction treatments, recovery root growth following 400 mM NaCl stress was always two- to three-fold larger in GE-415 (Fig. 3). However, the optimum induction stress for both the genotypes was 200 mM NaCl.

Variation in stress proteins in two genotypes differing in induction response

Quantitatively, the highly responsive genotype, GE-415 had a higher fraction of heat stable proteins (25%) compared to the low responsive type VL-481 (19%). Several stress

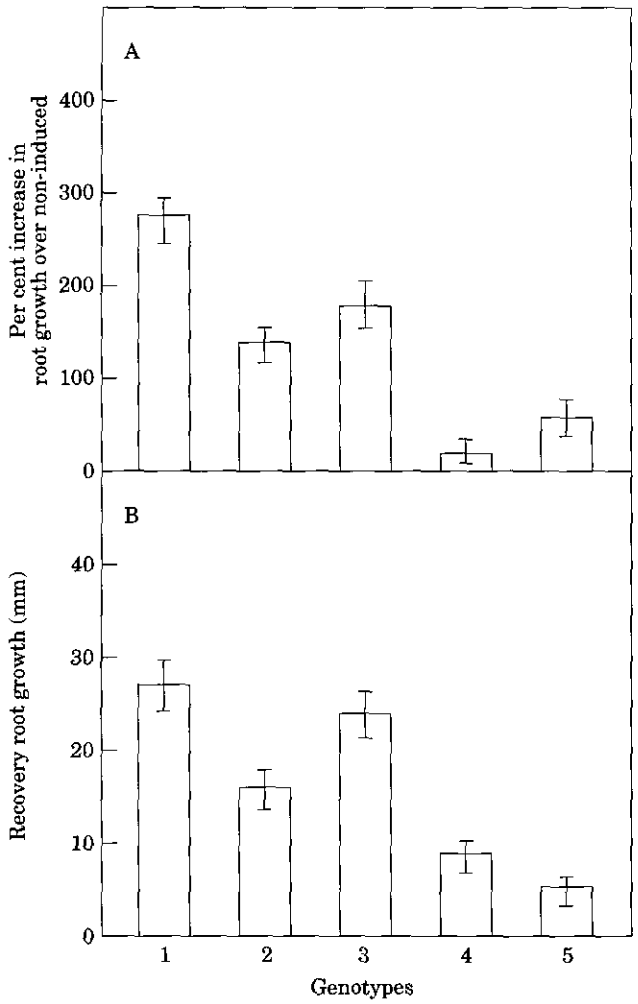

FIG. 2. Response of contrasting finger millet genotypes to severe PEG (A) and NaCl (B) stress with prior induction by mild stress. Genotypes are as in Fig. 1. The recovery root growth was determined as explained in Table 1. A, The recovery root growth following −1·2 MPa (severe) stress imposed with PEG preceded by −0·6 MPa PEG induction. The per cent increase in recovery root growth in induced seedlings over the non-induced seedlings is presented. B, Root recovery growth following 400 mM NaCl (severe) stress with 200 mM NaCl induction. The seedlings without induction did not survive the 400 mM NaCl stress.

proteins with apparent molecular weights of 70–72, 52, 37, 34 and 23 kDa were synthesized in the roots of both genotypes (Fig. 4). GE-415 synthesized a 54 kDa protein that was not observed in VL-481.

Recovery root growth of GE-415 and VL-481 when ABA was included in the induction medium (200 mM NaCl)

Addition of ABA to the 200 mM NaCl induction medium significantly enhanced root recovery growth in both genotypes following 400 mM NaCl stress (Table 2). The maximum recovery root growth was obtained with as little as 1 μM ABA for GE-415 whereas VL-481 requires at least 10 μM ABA. But, in both genotypes a reduction in recovery growth was observed with 50 μM ABA. Recovery root growth of GE-415 and VL-481 was the same with 200 mM NaCl + 10 μM ABA induction following 400 mM NaCl stress

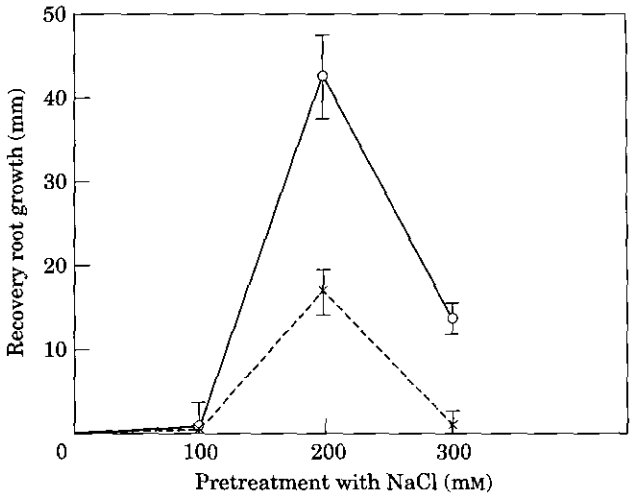

FIG. 3. Recovery root growth of GE-415 (highly responsive) and VL-481 (low response) following 400 mM NaCl stress with different induction treatments. The recovery root growth was calculated as explained in Table 1. Coefficient of difference = 1·29, $P < 0·05$. ○, GE-415; ×, VL-481.

(Table 2). At higher stress (600 mM NaCl) there was no seedling survival in the absence of ABA in the induction medium in either genotype (Table 3). However, with ABA, GE-415 had significantly more recovery root growth than VL-481.

Endogenous ABA

In neither genotype did the root ABA content increase with 200 mM NaCl induction treatment (Table 4). However, on addition of exogenous ABA to the induction medium, GE-415 contained more ABA than VL-481. There was no clear difference in the ABA concentration between the two genotypes.

Synthesis of stress proteins when ABA was included in 200 mM NaCl induction medium in GE-415 and VL-481

Synthesis of a unique 21 kDa protein with 30–40% label in the heat stable fraction in response to ABA (Fig. 5) was observed in finger millet seedling roots. With 200 mM NaCl + 10 μM ABA as the induction medium, several proteins with apparent molecular weights 70–72, 54, 45, 36, 29, 21 kDa were observed in whole seedlings of both the genotypes (Fig. 6). The number of proteins in whole seedlings was more than that in roots alone. The stress protein with apparent molecular weight of 54 kDa, which was observed only in GE-415 with 200 mM NaCl induction, was synthesized to the same extent in both genotypes when ABA was also given. GE-415 synthesized a unique protein of 23–24 kDa, which was not present in VL-481. GE-415 synthesized significantly more 21 kDa protein than VL-481.

DISCUSSION

Our results demonstrate a marked variation in acquired tolerance of finger millet genotypes (Table 1). It is important to note that the genetic variability was evident only after a

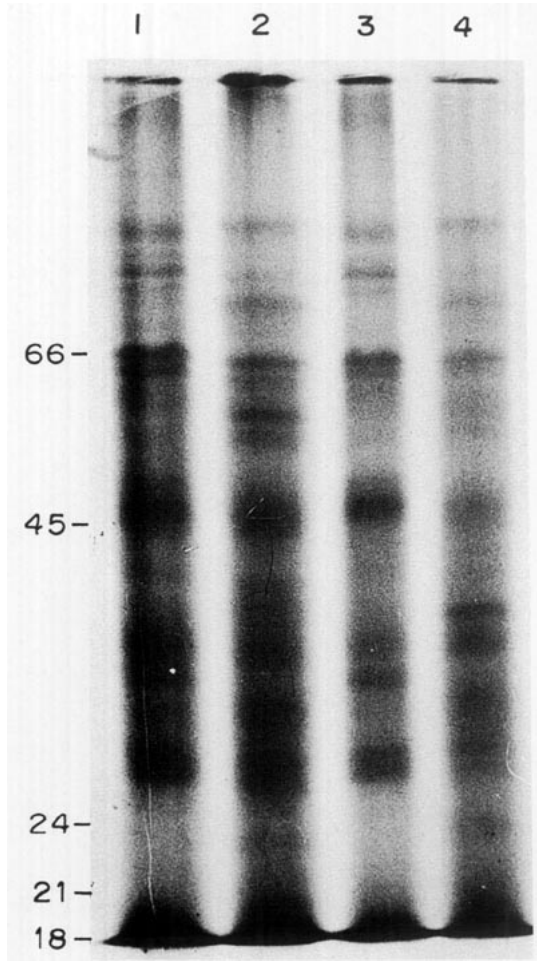

FIG. 4. Fluorograph showing the heat-stable proteins of GE-415 (lanes 1, 2) and VL-481 (lanes 3, 4). ^{35}S methionine was given during the last 4 h of induction treatment. The root heat stable proteins were prepared at the end of 16 h induction and separated on 10% SDS-PAGE. Equal counts were loaded for each genotype. The standard molecular weight markers (kDa) are indicated at the left. Lanes 1, 3: without induction; lanes 2, 4: induction with 200 mM NaCl.

TABLE 2. *Recovery root growth of GE-415 and VL-481 following 400 mM NaCl stress*

Induction for 16 h in:	Recovery root growth (mm)	
	GE-415	VL-481
Water	D*	D
200 mM NaCl	22·8±3·1	10·4±1·5
200 mM NaCl+1 μM ABA	36·4±4·0	19·4±3·1
200 mM NaCl+5 μM ABA	33·2±3·2	24·6±3·0
200 mM NaCl+10 μM ABA	34·7±3·6	34·2±4·0
200 mM NaCl+50 μM ABA	25·2±3·1	23·7±2·8

* D, Dead. l.s.d. = 2·04; $P < 0·05$.

Various concentrations of ABA were added along with 200 mM NaCl during the 16 h pretreatment. Seedling treatment and calculation of recovery root growth was as explained in Table 1.

mild induction stress given prior to the imposition of severe osmotic or salt stress (Fig. 2). This simulates natural conditions. A similar trend was observed in heat resistant

TABLE 3. *Recovery root growth of GE-415 and VL-481 following 600 mM NaCl severe stress*

Induction in (16 h):	Recovery root growth	
	GE-415	VL-481
Water	D*	D
200 mM NaCl	D	D
200 mM NaCl+10 μM ABA	26·1±3·0	14·4±1·6

* D, Dead. l.s.d. = 2·34, $P < 0·05$.

Seedling treatment and recovery root growth was determined as explained in Table 1.

TABLE 4. *Endogenous ABA (pg mg^{-1} d. wt.) in roots of two genotypes of finger millet exposed to 16 h induction stress in the presence and absence of 10 μM exogenous ABA*

	Water	200 mM NaCl	200 mM NaCl+ 10 μM ABA
GE-415	79±21	44±13	1094±120
VL-481	55±11	63±16	342±41

Each value is a mean of three replications±s.e.

and susceptible wheat genotypes (Krishnan *et al.*, 1989). The extent of cell viability as assessed by per cent triphenyl triazolium (TTC) reduction was the same in both genotypes without a prior heat shock at 50 °C. But with a 37 °C heat shock (induction stress), a marked variation was observed between genotypes. Under natural conditions any abiotic stress is always imposed gradually and plants experience a sublethal stress before the severe stress. Variation in the genotype response to the sublethal stress might be responsible for the observed genetic variability under severe stress. Further, because several physiological and biochemical parameters relevant for stress tolerance (stress proteins, proline and compatible solute accumulation, etc.) are induced during sublethal stress it is essential that genotypes are exposed to mild levels of stress before they are evaluated for their tolerance under severe levels of stress. It could also be one of the reasons for the lack of relation between these parameters and genetic variability when studied by exposing the plants directly to severe stress.

No marked differences were observed in the various stress proteins synthesized in relation to marked variation in acquired tolerance of two contrasting genotypes. However, a unique protein with apparent molecular weight of 54 kDa was observed only in GE-415 with 200 mM NaCl induction (Fig. 4). Similar qualitative differences were reported in maize (Ristic *et al.*, 1991) and wheat varieties (Krishnan *et al.*, 1989). But, we assume that the variation in acquired tolerance of these two genotypes is predominantly due to quantitative difference in stress protein synthesis. A positive correlation between the synthesis of heat stable proteins upon desiccation and ability to limit desiccation damage was reported in two rape seed species (LeBlanc and Dhindsa, 1993).

The observed variation in the stress response of the two

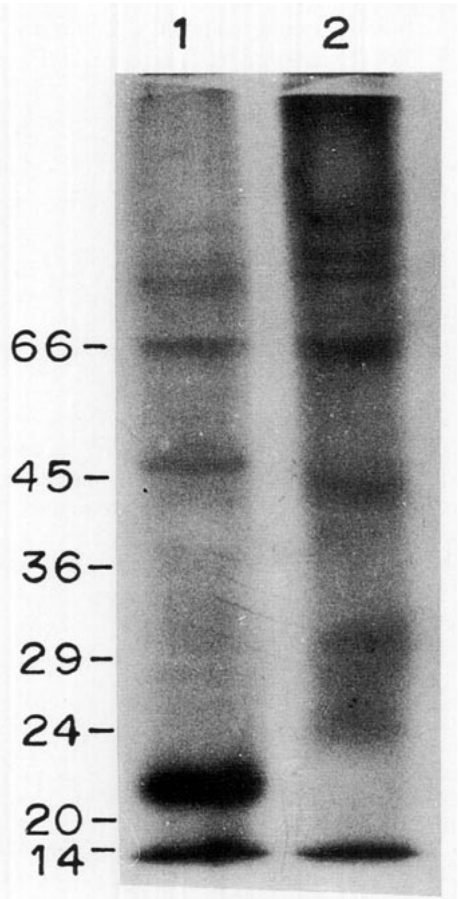

FIG. 5. Fluorograph showing prominent 21 kDa protein induced by ABA treatment. The heat stable labelled proteins from roots of non-induced (lane 2) and roots of induced with 200 mM NaCl + 10 μM ABA (lane 1) seedlings of GE-415 was prepared as explained in Fig. 4. Equal counts were loaded. The numbers on the left indicate the standard molecular weight in kDa.

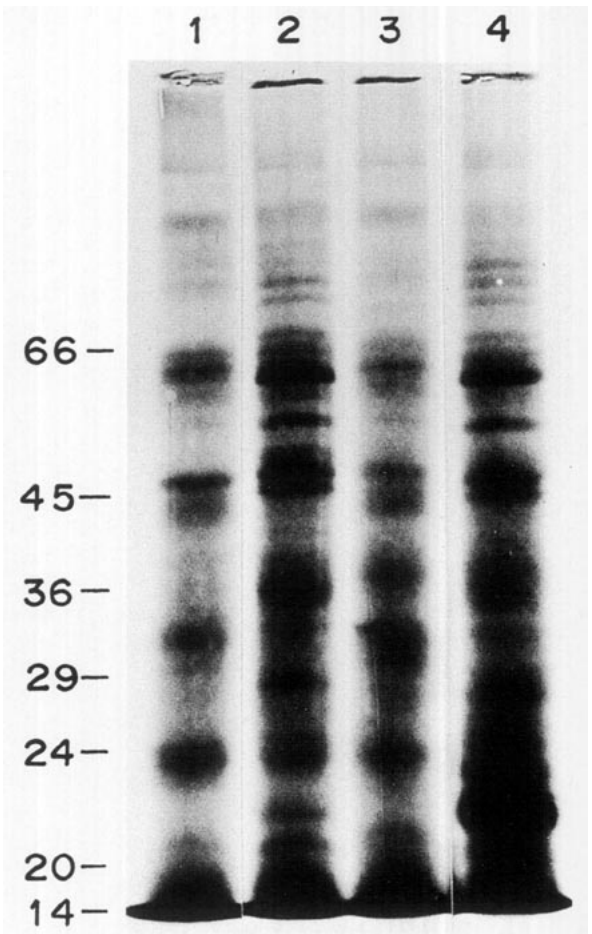

FIG. 6. ABA responsive proteins of GE-415 (lanes 3, 4) and VL-481 (lanes 1, 2). The heat stable proteins from whole seedlings were prepared as explained (Fig. 4) and separated on 10% SDS-PAGE. Equal counts were loaded. Lanes 1, 3: non-induced; lanes 2, 4: induced with 200 mM NaCl + 10 μM ABA. The molecular weight markers are indicated on the left in kDa.

genotypes could also be due to differential accumulation and sensitivity to ABA. The involvement of ABA and ABA responsive proteins in adaptation of plants to stress is well established (Bartels *et al.*, 1988; Skriver and Mundy, 1990; Hetherington and Quatrano, 1991). In both the genotypes, an increase in recovery root growth with addition of ABA in the NaCl induction medium (Table 2) associated with synthesis of several ABA responsive proteins (Fig. 6) corroborates several reports (Singh *et al.*, 1987; Reid and Walker-Simmons, 1993; Uma *et al.*, 1993). However, GE-415 had maximum recovery root growth with 1 μM ABA, while VL-481 required 10 μM ABA. Also the high responsive GE-415 had a higher endogenous ABA (Table 4) than VL-481 in 200 mM NaCl and when given exogenous ABA. This could be due to either less uptake by VL-481 or higher metabolism. Associated with higher ABA content, GE-415 had greater recovery root growth following an elevated level of stress (600 mM NaCl; Table 3). Significant quantitative difference in 21 kDa protein and synthesis of 23–24 kDa protein was observed only in GE-415. Interestingly the 54 kDa protein which was observed only in GE-415 with 200 mM NaCl induction was observed in both the genotypes with addition of 10 μM ABA. Using ABA deficient, insensitive and double mutants of *Arabidopsis*, Koornneef *et al.* (1989) demonstrated that both ABA content and sensitivity of the responding tissue to ABA were involved in the inhibition of seed development and reserve protein accumulation. This would explain the synthesis of 54 kDa protein only in GE-415 at 200 mM NaCl induction.

We conclude that genetic variability in salt and osmotic stress tolerance can be observed only when the genotypes are subjected to an optimum induction pretreatment involving mild stress. In finger millet, stress-induced 21 and 54 kDa proteins were associated with tolerance suggesting a causal role. The 54 kDa protein appeared to be induced by ABA. The 21 kDa protein may have an important role in stress response of finger millet genotypes.

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