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COMPARISON OF SALT STRESS RESPONSIVE PROTEINS IN *ATRIPLEX AMNICOLA* ANTIBODIES AND TWO ECOTYPES OF *LEPTOCHLOA FUSCA* (L.) KUNTH.

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Abstract

Three salt tolerant plant types viz., *Atriplex amnicola, Leptochloa fusca* (L.) Kunth. (Pakistani origin) and *Leptochloa fusca* (L.) Kunth (Australian origin) were used for a comparative study of altered gene expression under salt stress. The plants were aseptically grown in hydroponic medium and exposed to increasing levels of salt starting from 50mM NaCl to 500mM NaCl. Under these stress treatments there was variable expression of a number of proteins as revealed by SDS-PAGE. Specific activities of plant GDH, GOGAT and GS in response to salt stress were also determined. *Klebsiella* sp., NIAB-1, a diazotroph isolated from roots of *Leptochloa fusca* (L.) Kunth was also exposed to 50mM NaCl to 1000mM NaCl treatments, to examine salt responsive protein profiles. Blots of protein profiles from the three plant types were probed with antibodies raised against p-20 of *Atriplex amnicola* and p-26 of *Klebsiella* sp., NIAB-1, to reveal common epitopes among the salt responsive proteins.

Introduction

Response to salt stress by variable expression of certain proteins have been reported both in halotolerant bacteria and plants (Csonka, 1989; Hurkman *et al.*, 1991). It has been established that the basic mechanism of salt tolerance is similar for both bacteria and plants, especially when osmolytes (compatible solutes) are accumulated in plant vacuole (Greenway & Munns, 1980; Gilmour, 1990). A similarity in physiological stress tolerance mechanism may be indicative of conserved genetic response to salt stress.

Plants differ in the level of tolerance towards salt stress. These differences are mainly due to varied ability in ion translocation to shoot and their compartmentalization in leaf cells (Greenway & Munns, 1980). The three salt tolerant plant types viz., *Atriplex amnicola* and two ecotypes of *Leptochloa fusca* (one from Punjab, Pakistan and other from Eastern states, Australia) used in the present studies differ in the degree of tolerance towards salinity. The observed salt tolerance levels for *A. amnicola*, *L. fusca* (Pakistani origin) and *L. fusca* (Australian origin) are 33.3 dSm-1, 22 dSm-1 and 14.6 dSm-1 respectively (Dr. Zahoor Aslam, personal communication).

Here we report a comparative account of the expression of salt responsive proteins, for the above mentioned three plant types. SDS-PAGE protein profile of *Klebsiella* sp. NIAB-1, a diazotroph isolated from the roots of Pakistani Kallar grass (Qureshi *et al.*, 1988) is also presented for comparison.

Materials and Methods

Plant Growth Conditions: Seeds of *Atriplex amnicola* and the two ecotypes of *Leptochloa fusca* (from Pakistan and Australia) were surface sterilized by washing in 10% NaOCl

solution with subsequent thorough rinsing in sterilized distilled water. Seeds were germinated on MS medium (Murashige & Skoog, 1962) having 1.5% agar. Seven day old seedlings were aseptically transferred into long tube assemblies containing 30 ml Hoagland's solution (Arnon & Hoagland, 1940). In these long tube assemblies, the seedlings were anchored on a perforated perspex disc supported over the nutrient medium with the help of a glass rod (Bilal., 1988). One month old seedlings were given successive 50mM NaCl treatments at 48 h intervals, to reach the final NaCl treatment of 500mM.

Extraction of Soluble Plant Proteins: Frozen leaf tissue (0.1 g) were homogenized in 250 μl of TND buffer (90 mM Tris-HCl [pH 8.3], 90 mM NaCl, 9 mM DTT, 2 mM leupeptin) at 4°C in a precooled mortar (Ostrem *et al.*, 1987). Cell debris were removed by centrifugation for 10 minutes at 10,000 rpm in an Eppendorf microfuge. Glycerol was added to a final concentration of 10% (v/v) in the supernatant and protein samples were frozen to store.

Determination of Activities of Enzymes and Protein Concentration: Aliquots of freshly prepared protein samples were assayed for the activities of glutamate dehydrogenase (GDH), glutamate synthase (GOGAT) and glutamine synthetase (GS) according to the methods reported by Farden & Robertson (1980). Protein concentration of the samples were determined using the method as suggested by Bradford (1976).

Gel Electrophoresis of Plant Proteins: Just before SDS-PAGE, the soluble protein samples were mixed in equal parts with preheated sample buffer (10% glycerol, 4% 2-mercaptoethanol, 4% SDS, 0.002% bromophenol blue in 62.5 mM Tris-HCl, pH 6.8). Equal protein contents were loaded into each sample well and these were resolved by SDS-PAGE using a discontinuous buffer system (Laemmli, 1970). Gels were stained in coomassie blue (Kee & Nobel, 1986).

Culture Maintenance of Klebsiella sp.NIAB-1 and Protein Analysis: Bacterial strain was grown aerobically in a shaking incubator at 37°C in glucose minimal medium (GMM) as described earlier (Qureshi *et al.*, 1988). Osmolarity of GM medium was increased by the addition of NaCl as required. The pH of the medium was adjusted to 8.0 with NaOH. Whole cell protein samples, each from a 250 ml equilibrated culture (O.D. 0.80 at 600 nm), were prepared in sonication buffer (100 mM Tris-HCl, pH 8.0, 2 mM 2- mercaptoethanol) through sonication as described by Qureshi *et al.*, (1988). SDS-PAGE was performed by the method of Laemmli (1970).

Production of Antibodies: From coomassie blue stained gels, 20 kD band of *Atriplex amnicola* and 26 kD band of *Klebsiella* sp., NIAB-1 were excised and washed three times with phosphate buffer saline. The gel slices in minimal wash buffer, were broken by passage through a series of syringes with decreasing diameter. An equal volume of incomplete Freunds' adjuvant was mixed with these two crushed slices to make suspensions by repeated passage through a syringe. These two suspensions were then injected subcutaneously into rabbits followed by booster injections after 12 days. Antisera were collected 12 days after the second injection.

Immunoblot Analysis: Followed by SDS-PAGE, the gels were vacuum blotted on nitrocellulose paper as described by Peferoen (1988). Non specific binding sites on the membrane were blocked by 2% Tween-80. Blots were then probed with antibodies. Immunolabelling of the blots was visualized by using protein-A peroxidase (Sigma Chemicals Co.) activity as outlined by Suresh *et al.*, (1986). Antiserum was used in 1:10 dilution.

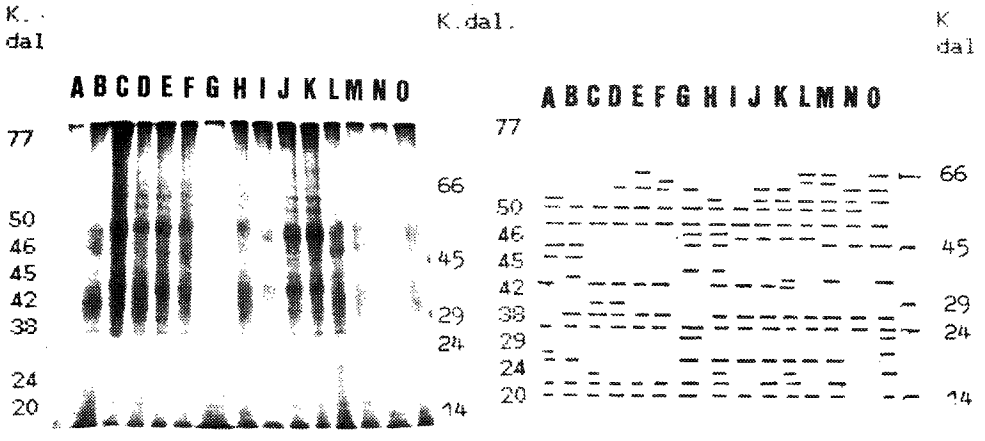

Fig.1. Pattern of SDS-extractable proteins of salt treated seedlings of *Leptochloa fusca* (L.) Kunth (Pakistani origin) and *Leptochloa fusca* (Australian origin). Lanes A to H: Seedlings of Pakistani kallar grass kept at control (B), 0.05M NaCl (C), 0.10M NaCl (A), 0.15M NaCl (D), 0.25M NaCl (E), 0.35M NaCl (F), 0.40M NaCl (G) and 0.50M NaCl (H). Lanes I to O; Seedlings of Australian kallar grass kept at control (I), 0.10M NaCl (J), 0.15M NaCl (K), 0.30M NaCl (L), 0.35M NaCl (M), 0.40M NaCl (N) and 0.50M NaCl (O). Figures on left represent molecular mass in KD of proteins discussed in the text. Molecular weights in KD of standards are given at right.

Results

Protein Profile of Leptochloa fusca (Pakistani origin) grown under Salt Stress: In the Pakistani Kallar grass, four polypeptides having apparent molecular sizes of 77 kD, 42 kD, 24 kD and 20 kD became pronounced under 50mM to 500mM NaCl treatments (Fig. 1, Lanes A to H). These bands were not exhibited in control. Under the salt treatments, level of expression of a 50 kD polypeptide is greatly reduced in comparison to control. There was also a reduction in the level of expression of 45 kD protein under all salt treatments as compared to control.

Salt Responsive Synthesis of Polypeptides in Leptochloa fusca (Australian origin): Under 100 mM to 500 mM NaCl treatments, there was enhancement of 4 polypeptides in Australian kallar grass. Apparent molecular weights of these bands were 76 kD, 38 kD, 24 kD and 20 kD (Fig. 1, Lanes I to O). Expression level of 46 kD polypeptide was enhanced under 50mM to 300mM NaCl treatments in comparison to control, while it's level seems to be reduced under higher concentrations of 300mM to 500mM NaCl. There was also a variable salt responsive expression of 50 kD protein which was enhanced in 50mM to 150mM NaCl treatments, but salt stress of 150mM to 500mM NaCl reduced the expression of this polypeptide.

Protein Synthesis in Atriplex amnicola in Response to Salt Treat ment: In the upper leaves of this halophytic plant, salt stress induced the synthesis of 64 kD, 48 kD, 34 kD, 24 kD and 20 kD polypeptides (Fig. 2, Lanes A to G). Other 3 polypeptides having apparent molecular weights of 83 kD, 74 kD and 65 kD showed reduced levels of expression as compared to control.

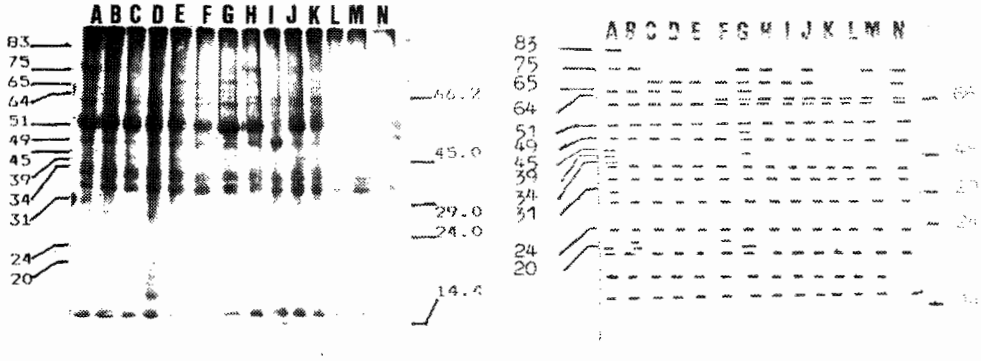

Fig.2. SDS-PAGE of total cellular proteins on 12.5% polyacrylamide gel of NaCl treated upper leaves (lanes A to G) and lower leaves (lanes H to N) of *Atriplex amnicola*: A, control ; B, 0.05M NaCl; C, 0.15M NaCl; D, 0.20M NaCl; E, 0.40M NaCl; F, 0.45M NaCl; G, 0.50M NaCl in upper leaves of *A. amnicola* respectively; H, control; I, 0.05M NaCl; J, 0.15M NaCl; K, 0.20M NaCl; L, 0.40M NaCl; M, 0.45M NaCl; N, 0.50M NaCl in lower leaves of *A. amnicola* respectively. Molecular weights (in KD) of salt responsive proteins are indicated at left margin and molecular weight standards (in KD) are indicated at right margin.

There was enhancement of 39 kD, 31 kD, 24 kD and 20 kD polypeptides in the lower leaves of *A. amnicola* under the stated salt treatments (Fig. 2, Lanes H to N). However, 49 kD bands seemed to be enhanced only at higher salt concentrations of 400 mM to 500 mM NaCl. The level of expression of 45 kD protein was slightly increased at salt levels of 50 mM to 400 mM NaCl and seemed to be reduced under salt treatments of higher concentrations (400 mM to 500 mM NaCl). There was reduction in the level of expression of 75 kD and 65 kD proteins in response to NaCl treatments, in comparison to control.

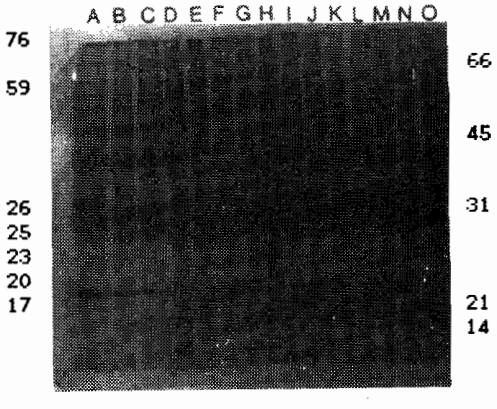

Fig.3. SDS-polyacrylamide slab gel of polypeptide banding pattern of *Klebsiella* sp. NIAB-I treated with NaCl at different concentrations: Lane A, control; Lane B, 0.05M NaCl; lane, C, 0.10M NaCl; lane D, 0.015 M NaCl; lane E, 0.20 M NaCl; lane F, 0.25M NaCl; lane G, 0.30M NaCl; lane H, 0.35M NaCl; lane I, 0.40M NaCl; lane J, 0.45M NaCl; lane K, 0.5M NaCl; lane L, 0.55M NaCl; lane M, 0.60M NaCl; lane N, 0.8M NaCl and lane O, 1.00M NaCl. Molecular masses (in KD) of salt responsive proteins are indicated at left margin and molecular weights (in KD) of standards are at right margin.

Polypeptide having apparent molecular weights of 51 kD which was not detected at 50 mM NaCl, reappeared at 200 mM NaCl and reduced under 200 mM to 500 mM NaCl treatments.

Salt Stress Response of Klebsiella sp. NIAB-I: In response to salt treatments ranging from 50 mM NaCl to 1 M NaCl, there was increase in the level of expression of seven proteins (Fig. 3) as compared to control. Apparent molecular sizes of these polypeptides were 76 kD, 59 kD, 26 kD, 23 kD, 20 kD, 17 kD and 14 kD. At higher salt levels i.e., from 450 mM to 1 M NaCl treatments, there was reduction in the level of expression of a 25 kD polypeptide as compared to control.

Activities of Enzymes involved in the Synthesis of Glutamate in Salt Stressed Plants: A specific decline in the activities of glutamate dehydrogenase (GDH), glutamate synthase (GOGAT) and glutamine synthetase (GS) under salt treatments (Fig. 4) in the three plant types have been observed, when a certain threshold salt stress level reached for individual plant types. Before reaching these threshold levels, the enzyme activities appear to be stimulated. These threshold levels were found to be the highest (400 mM NaCl) in upper leaves of *A. amnicola*, higher (150 to 400 mM NaCl) in lower leaves of *A. amnicola*, lower (150 to 350 mM NaCl) in *Leptochloa fusca* (Pakistani origin) and the lowest (100 to 300 mM NaCl) in *L. fusca* (Australian origin).

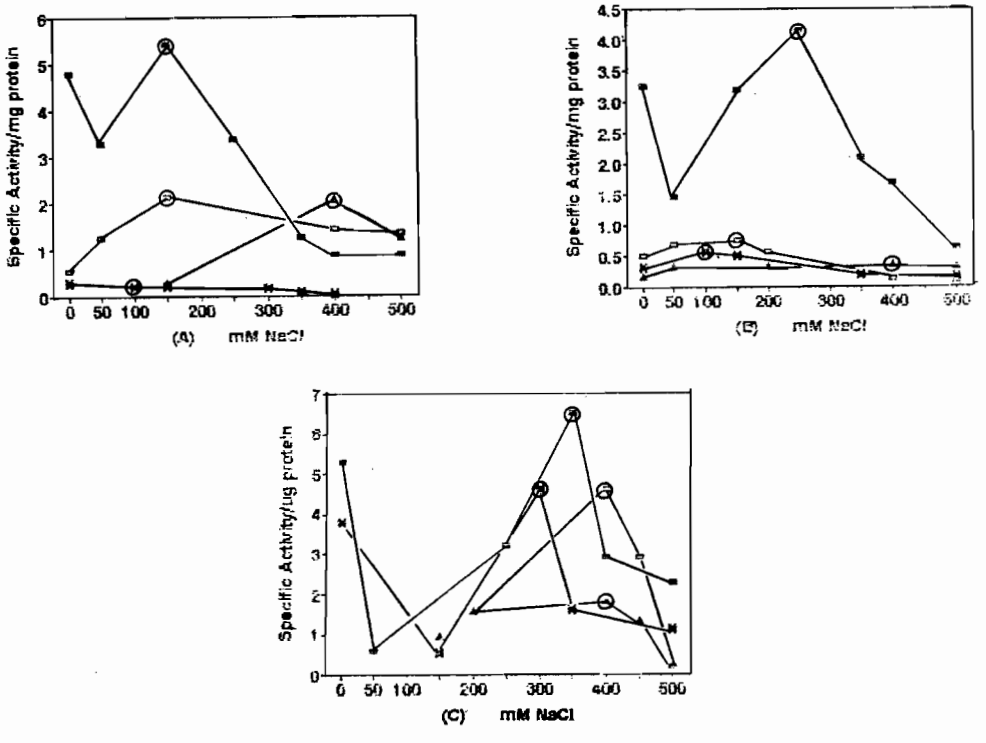

Fig.4. Enzyme activities of glutamate synthase (A), glutamate dehydrogenase (B) and glutamine synthetase (C) in Pakistani kallar grass ( ) Australian kallar grass (X), upper leaves of *A. amnicola* (Δ) and lower leaves of *A. amnicola* ( ) under vary concentrations of NaCl. Threshold levels for decline in the activities are encircled.

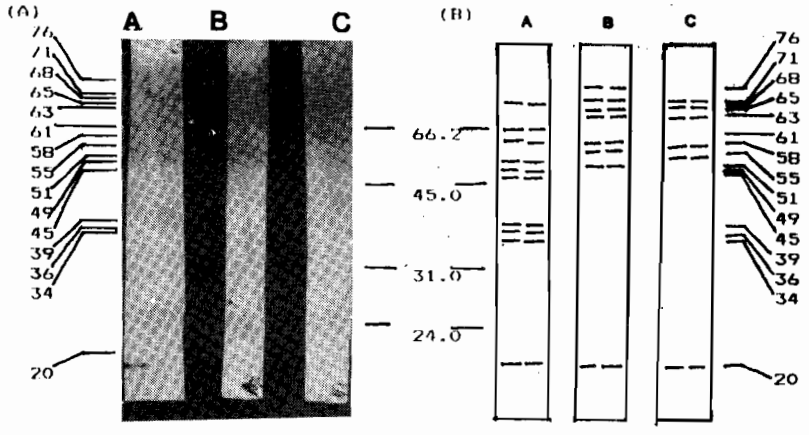

Fig.5. (A): Western analysis of crude extracts of *Leptochloa fusca* L. kunth (Pakistani origin), *Leptochloa fusca* L. (Australian origin) and *Atriplex amnicola* with anti p-20 antibodies of *Atriplex amnicola*. Cell free extracts were electrophoresed, blotted and probed as described in "Materials and Methods". Lane A, *Atriplex amnicola* lane B, Pakistani kallar grass; lane C, Australian kallar grass. (B): Line diagram of figure 5(A). Molecular sizes (in kD) of cross reacted bands are given at left and right margins. Molecular weight standards (in kD) are indicated in the middle.

Western Blotting with Antibodies to p-20 of A. amnicola: Antibodies against 20 kD polypeptide of *A. amnicola* cross reacted with 76 kD, 71 kD, 65 kD, 63 kD, 58 kD, 55 kD, 50 kD and 20 kD bands on the immunoblot of *L. fusca* (Pakistani origin). These antibodies recognized 71 kD, 68 kD, 63 kD, 58 kD , 55 kD and 20 kD proteins of *L. fusca* (Australian origin). In immunoblot of *A. amnicola* 68 kD, 61 kD, 58 kD, 51 kD, 49 kD, 45 kD, 39 kD, 36 kD, 34 kD and 20 kD polypeptides were discernible with above mentioned antibodies (Fig. 5).

Cross Reaction with Antibodies raised against 26 kD Antigen **of** *Klebsiella* sp., ***NIAB-I:*** Anti-p26 antibodies of *Klebsiella* sp., NIAB-I cross reacted with 68 kD, 58 kD and 50 kD bands on the immunoblot of Pakistani kallar grass. These antibodies recognized 61 kD, 58 kD and 50 kD polypeptides on immunoblot of Australian kallar grass. Five polypeptides of 58 kD, 51 kD, 49 kD, 45 kD and 39 kD molecular sizes were discernible on immunoblot of *A. amnicola* with above mentioned antibodies (Fig. 6).

Discussion

The results indicate that some proteins are enhanced and others are reduced in the plants treated with salt stress. Similar variable expression of the proteins have also been reported for other salt treated plants (Singh *et al.*, 1985; Hurkman *et al.*, 1989). Comparison of results summarized in Tables 1 and 2 reveal that 20 kD, 24 kD and 58 kD salt responsive proteins are ubiquitous in halotolerant bacteria and plants. This similarity

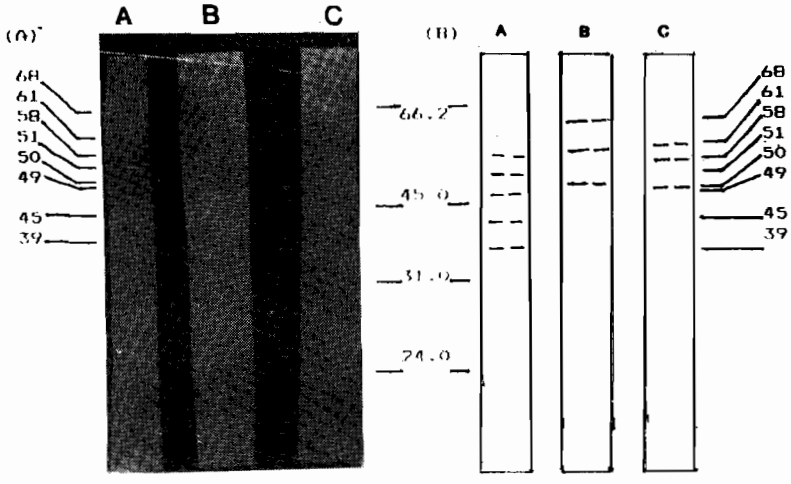

Fig.6. (A): Immunoblots of cell free extracts of *Atriplex amnicola* (lane A), Pakistani kallar grass (lane B) and Australian kallar grass (lane C) with anti p-26 antibodies of *Klebsiella* sp. NIAB-1. Crude extracts were electrophoresed, transferred and probed with antibodies as described in "Materials and Methods". (B): Line diagram of figure 6(a). Figures on leaf and right margins represent molecular weights (in kD) of cross reacted proteins. Molecular mass (in kD) of standards are given in the middle.

may imply a general importance of these proteins in salt tolerance. The 24 kD polypeptide has been reported as thaumatin like protein related to embryogenesis, pathogen resistance and osmotic stress resistance in plants (Rodrigo *et al.*, 1991). This suggests a general stress responsive role of p- 24. The results also reveal that 50 kD, 58 kD, 63 kD and 68 kD proteins are common among the three plant types, presumably indicating a conserved salt stress response. Results summarized in Tables 1 and 2 indicate that 76 kD and 55 kD proteins are specific to both the kallar grasses and are not found in *A. amnicola*. However, 61 kD, 46 kD and 38 kD proteins are shared by Australian kallar grass and *A. amnicola*. This similarity is probably presenting some association between these two plants in the evolutionary tree.

Variable salt responsive expression of 75 kD, 65 kD and 49 kD proteins, is common among lower and upper leaves of *A. amnicola*. However, lower leaves have altered expression of four additional proteins, having apparent molecular sizes of 51 kD, 45 kD, 39 kD and 31 kD (Table 1). Such extra protein expression in lower leaves support the previous findings that stress signals are translocated from mature leaves to young leaves (Davis *et al.*, 1991). As Pakistani kallar grass is more halotolerant than Australian kallar grass; the enhancement of 42 kD polypeptide in Pakistani kallar grass and that of 46 kD polypeptide in Australian kallar grass (Table 1) are in agreement with previous report, where 42 kD and 46 kD proteins enhancement were respectively specific to salt tolerant and salt sensitive barley lines (Hurk man *et al.*, 1989). The results of salt inducible protein

Table 1. Polypeptides in *Leptochloa fusca* (Pakistani kallar grass and Australian kallar grass), *Atriplex amnicola* (upper and lower leaves) and *Klebsiella* sp. NIAB-Iin response to salt stress.

Leptochloa fusca (Pak)	(Aust)	*Atriplex amnicola* (Upper 1)	(Lower I)	*Klebsiella* sp. NIAB-I
Polypeptides (sizes in KD) that increased in response to NaCl				
77	76	64	49	76
42	46	48	45	59
24	38	34	39	26
20	24	24	31	23
	20	20	24	20
			20	17
				14
Polypeptides (sizes in KD) that decreased in response to NaCl				
50	50	83	75	25
45		74	65	
		65	51	

These polypeptides are also indicated on the electrophoretic patterns depicted in Fig.1, 2 and 3.

pro files are in general agreement with Hurkman *et al.*, (1989), that salt treatment induces quantitative rather than qualitative changes in polypeptides and specific changes in the level of polypeptide for plant types is probably due to variability in response to salt by individual genotypes.

Activities of the three enzymes involved in glutamate synthesis (i.e. GDH, GOGAT and GS) were stimulated by the salt concentration below the declining threshold levels for individual plant types (Fig. 4). Such stimulation of enzyme activity by NaCl concentration below the tolerance level of an organism, have been reported earlier (Warr *et al.*, 1984; Sharma & Garg, 1985). *A. amnicola*, Pakistani kallar grass and Australian kallar grass exhibited the highest, higher and lower threshold levels for decline in enzyme activity, respectively (Fig. 4); corresponding to their salt tolerance levels. Expression levels of 46 kD and 50 kD bands for Australian kallar grass (Fig. 1, Lanes I to O) and that of 45 kD and 51 kD polypeptide for *A. amnicola* (Fig. 2, Lanes H to N) seems to be first enhanced and then reduced, under increasing levels of NaCl concentrations; which is in accordance to enzyme activities in response to salt stress (Fig. 4). The 46 kD subunit of glutamine synthetase (Chai & Wong, 1988) and 53 kD subunit of glutamate dehydrogenase (Prunkard *et al.*, 1986) may correspond to above mentioned polypeptide of Australian kallar grass and *A. amnicola*. However, due to the notion that salt tolerant plants do not use a large portion of their photo synthates as osmotic solutes (Greenway & Munns, 1980), recent emphasis is also on the study of membrane bound enzymes involved in ion compartmentalization (Narasimhan *et al.*, 1991).

It has been reported that an antibody may be multivalent i.e., have more than one specificity against a pure protein; corresponding to more than one epitopes on the antigen (Graber, 1958; Heibelberger, 1979; Harlow & Lane, 1988). High content of proline and hydroxyproline in salt stress proteins (Ericson & Alfinito, 1984; Sachs & Ho, 1986), and presence of conserved domains in osmotic stress proteins (Skriver & Mundy, 1990) also suggest common epitopes among these proteins. Similarly, immunoblots of *A. amnicola, L. fusca* (Pakistani origin) and *L. fusca* (Australian origin) with antibodies against p-20 of *A. amnicola* and p-26 of *Klebsiella* sp., NIAB-I cross in addition reacted with several proteins of larger molecular sizes (Table 2). These immunoblots results may indicate the presence of common epitopes among salt responsive proteins.

A 26 kD protein characterized as germin-like, have been found in salt treated tobacco cells (Singh *et al.*, 1985) and barley roots (Hurkman *et al.*, 1991). This protein was not detected in any of the shoot extracts from the three plant types, neither by coomassie blue staining (Table 1) nor by immunoblotting (Table 2). The absence of 26 kD protein in salt treated leaf tissue has been reported previously (Hurkman *et al.*, 1991). Presence of 26 kD protein in NaCl treated *Klebsiella* sp., NIAB-I cells (Fig.3), undifferentiated tobacco cells (Singh *et al.*, 1985) and barley roots (Hurkman *et al.*, 1991) suggests a function of this protein, specifically required for the cells which are in direct contact with environmental salt stress. Such function may be associated with cell wall expansion as proposed earlier (Jaikaran *et al.*, 1990). Besides this polypeptide immunocytochemical localization of ubiquitous salt responsive proteins would be advisable to reveal conserved molecular mechanism of salt tolerance among plants.

Table 2. Polypeptides recognized by anti-p20 antibodies of *Atriplex amnicola* and anti-p26 antibodies of *Klebsiella* sp. NIAB-I, on immunoblots of *Leptochoa fusca* (Pakistani kallar grass and Australian kallar grass) and *Atriplex amnicola*.

Bands (sizes in kD), recognized by anti-p20 antibodies of *Atriplex amicola*			Bands (sizes in kD), recognized by anti-p26 antibodies of *Klebsiella* sp. NIAB-I		
Leptochloa fusca (Pak)	*Leptochloa fusca* (Aust)	*A triplex amnicola*	*Leptochloa fusca* (Pak.)	*Leptochloa fusca* (Aust)	*Atriplex amnicola*
76	71	68	68	61	58
71	68	61	58	58	51
65	63	58	50	50	49
63	58	51			45
58	55	49			39
55	20	45			
50		39			
20		36			
		34			
		20			

These polypeptides are also indicated on the western blots depicted in figures 5 and 6.

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