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BRIEF COMMUNICATION

Differential expression of LEA proteins in two genotypes of mulberry under salinity

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Abstract

The relative water content (RWC), cell membrane integrity, protein pattern and the expression of late embryogenesis abundant proteins (LEA; group 1, 2, 3 and 4) under different levels of salt stress (0, 1.0, 1.5 and 2.0 % NaCl) were investigated in mulberry (*Morus alba* L.) cultivars (S1 and ATP) with contrasting salt tolerance. RWC and membrane integrity decreased with increase in NaCl concentration more in cv. ATP than in cv. S1. SDS-PAGE protein profile of mulberry leaves after the NaCl treatments showed a significant increase in 35, 41, 45 and 70 kDa proteins and significant decrease in 14.3, 18, 23, 28, 30, 42, 47 and 65 kDa proteins. Exposure of plants to NaCl resulted in higher accumulation of LEA proteins in S1 than ATP. The maximum content of LEA (group 3 and 4) was detected in S1 at 2.0 % NaCl, which correlates with its salt tolerance.

Additional key words: cell membrane stability, *Morus alba*, NaCl stress, RWC.

Plants have developed different mechanisms to withstand salt stress (Niknam *et al.* 2006, Sotiropoulos *et al.* 2007, Melgar *et al*. 2008). These include alterations in the content of numerous proteins and mRNAs. Exposure of plants to high salinity increases the gene expression for protective proteins such as osmotin, late embryogenesis abundant (LEA) proteins, pathogenesis related (PR) proteins, ion transporters and SALT proteins (Moons *et al*. 1997a,b, De Souza *et al*. 2003, Jyothsnakumari 2005, Rorat 2006).

LEA proteins were firstly observed in cotton and wheat during embryogenesis and germination (Dure *et al*. 1981). LEA proteins have been identified in many plant species, and at least six different groups of LEA proteins have been defined on the basis of expression pattern and amino acid sequence, among these the major categories are group 1, group 2 and group 3. Group 1 LEA proteins have been sub-divided into two super families and are found only in plants (Wise 2003). Group 2 LEA are an abundant and diverse family of proteins that are synthesized in response to salinity, drought and dehydration stress including low/freezing temperatures and are mainly found in plants (Svensson *et al.* 2002, Allagulova *et al.* 2003, Cherian *et al.* 2006, Wahid and Close 2006). Group 2 LEA (dehydrins, DHN, or responsive to abscisic acid, RAB proteins) have also been predicted to function as chaperones useful for maintaining protein structure and function. The group 3 LEA proteins, comprising of two super families are characterized by repeated 11-mer amino acid motif. LEA 3 is known to counteract the irreversible damaging effects of increased ionic strength in cytosol during desiccation by sequestration of ions (Dure 1993). The group 4 LEA proteins are suggested to bind water molecules and may also act as chaperones, whereby these would stabilize the surface of membranes and possibly proteins by binding water and functioning as solvation film. It has been reported that genes encoding group 4 LEA are expressed in vegetative tissues in

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Abbreviations: ABA - abscisic acid; DHN - dehydrin; LEA - late embryogenesis abundant; PR - pathogen related; RAB - responsive to ABA; SDS-PAGE - sodium dodecyl sulfate polyacrylamide gel electrophoresis.
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response to salinity, drought, ABA and low temperature (Hsing *et al*. 1995).

Protein synthesis is severely affected in response to salt stress and their alterations vary within plant species (Bishnoi *et al.* 2006). Changes in protein synthesis are attributed to changes in the gene expression induced in salt-stressed plant. The protein analysis provides a direct assessment of proteins involved in stress response pathways and consequently a valuable link between the classical physiological approaches like relative water content (RWC), membrane integrity and molecular tools. Measurement of cell membrane stability (CMS) has often been used for screening of drought and salinity tolerance in various plants such as sorghum, maize, rice, wheat and also in mulberry (Sudhakar *et al*. 2001, Farooq and Azam 2006). In this context, the dynamic comparison of physiological and protein expression changes in control and stressed plants, is of importance to understand the stress coping mechanisms in plants.

The present study aims to depict the interacting processes involved in salt application to mulberry plants through physiological and protein expression analyses.

Mulberry (*Morus alba* L.) cultivars (a salt tolerant cv. S1 and a salt susceptible cv. ATP) were procured from Regional Sericultural Research Station (CSB), Anantapur, India. The cuttings of approximately equal length and diameter, with three to four active buds were planted in pots containing red-loamy soil and farmyard manure (3:1) ratio, watered daily and kept in the botanical garden under natural photoperiod of 12 - 13 h and maximum temperature of 32 ± 4 °C. Three-month-old plants were subjected to salt stress induced by a range of NaCl concentrations 0.0 (control), 1.0, 1.5 and 2.0 % (m/v). The electrical conductivity of the soil saturation extract was 1.7, 4.0, 5.9 and 7.8 mS cm^{-2} respectively. Care was taken to avoid drainage of solution during the treatment by giving water slightly less than field capacity. The electrical conductivity of soil extract was monitored and adjusted using conductivity bridge (*Elico*, Hyderabad, India) on alternate days. After 7 d, leaf samples were collected and flash frozen. The third pair of leaves from top was selected as it showed the highest photosynthetic activity (Sudhakar *et al*. 2001).

The detached fresh leaves were weighed immediately to record fresh mass (FM). Then the leaves were immersed in distilled water and after 4 h they reweighed (TM). Then the leaves were kept at 80 °C for 48 h and dry mass (DM) was recorded. Relative water content (RWC) was calculated according to Turner (1981): RWC = (FM - DM)/(TM - DM) × 100. Cell membrane stability (CMS) was measured according to the method of Premachandra *et al*. (1991). Thirty leaf discs of 1.1 cm diameter were cut from control and salt treated plants, washed three times with deionized water, immersed in 30 cm^3 of deionized water for 24 h at 10 °C, and electrical conductivity was measured at 25 °C. Then the leaf tissues were killed by autoclaving at 121 °C for 15 min to release all electrolytes, and electrical conductivity (EC) was again measured at 25 °C. CMS was calculated: $CMS = 1 - [(1 - T_1/T_2) / (1 - C_1/C_2)] \times 100$, where T_1 and T_2 are the first and second conductivity measurements of the salt treated, and C_1 and C_2 are the first and second conductivity measurements of the control samples.

Plant material (1 g) was frozen and powdered by using liquid nitrogen and extracted in 50 mM Tris-HCl (pH 7.4) buffer contained 10 mM Na EDTA, 5 % β-mercaptoethanol and 10 % glycerol. The homogenate was centrifuged at 5 000 *g* for 10 min in a refrigerated high speed centrifuge to remove the cellular debris. The resulting supernatant was centrifuged at 10 000 *g* for 20 min to obtain clear supernatant. The concentration of proteins was estimated by the method of Lowry *et al*. (1951) using bovine serum albumin (BSA) as the standard. For one-dimensional separation, 12.5 % linear sodium dodecyl sulphate (SDS)-polyacrylamide slab gels (1 mm thickness) overlayed with stacking gel (Laemmli 1970) was used. The electrophoresis was carried out at 50 V for 1 h followed by 100 V for 2 h. Once tracking dye reached the anode, run was stopped and the gel was carefully removed and placed in 5 volumes of Coomassie briliant blue (CBB) for 1 h or overnight. The gel was washed with the distilled water and destained using methanol, acetic acid and distilled water in ratio 30:30:40. The electrophoregrams were photographed and analyzed using gel documentation system.

After SDS-PAGE, the proteins from the gel were transferred to polyvinylidene difluoride (PVDF) membrane by semi-dry blot method using (25 mM Tris, 40 mM amino caproic acid and 20 % methanol) at 60 mA for 1 h (Towbin *et al.* 1979). After rinsing the membrane in Tris-buffered saline (TBST - 100 mM Tris, 1500 mM NaCl, 0.05 % Tween-20, pH 7.5), the unoccupied sites on membrane were blocked with 1 % BSA in TBST. The membrane was washed in TBST and then incubated with 1:250 dilutions of anti-LEA 1, 2, 3 and 4 antibodies separately. Antiserum of LEA 2 and 3 were raised against the synthetic peptides corresponding to the conserved regions of LEA-2 (EEKKGIMKIKEKLPG), or LEA-3 (TAQAAKEAGE) proteins. Unlike the antisera of LEA 2 and LEA 3, the LEA 1 and LEA 4 were against *Eleusine coracana* (Gaertn.) under salt stress (antisera generously supplied by Dr. M. Udaykumar, GKVK, Bangalore). After incubation for 1 h on a rotary shaker, membrane was washed and incubated in 1:1000 dilution of goat-anti-rabbit IgG-peroxidase conjugate (*Sigma,* Singapore) for 45 min. After washing, colour was developed using diamino-benzidine as colour developing reagent and cobalt metal as catalyst. The reaction was stopped after the desired colour intensity was obtained by rinsing the membrane in distilled water. Images of the blots were scanned, analyzed and recorded using *Gel Documentation Image Analysis System* (*Alpha Innotech*, San Leandro, USA) computer software.

The data obtained in all cases were subjected to analysis of variance (*ANOVA*), and the mean values

Table 1. Relative water content and cell membrane injury induced by 1.0, 1.5 and 2.0 % NaCl in leaves of two mulberry cultivars. Means from 5 experiments ± SE. The mean values in a row followed by a different letter for each species are significantly different ($P \leq 0.05$) according to Duncan's multiple range test.

Parameter	Cultivar	Control	1.0 %	1.5 %	2.0 %
Relative water content [%]	S1	96.41 ± 0.396a	72.03 ± 0.278a	65.41 ± 0.391a	53.05 ± 0.230b
	ATP	96.13 ± 0.780a	61.74 ± 2.060b	55.24 ± 0.378b	41.43 ± 1.609b
Cell membrane injury [%]	S1	-	59.00 ± 0.360a	71.00 ± 0.550a	85.00 ± 0.611a
	ATP	-	31.00 ± 0.200a	88.00 ± 0.200b	97.00 ± 0.150c

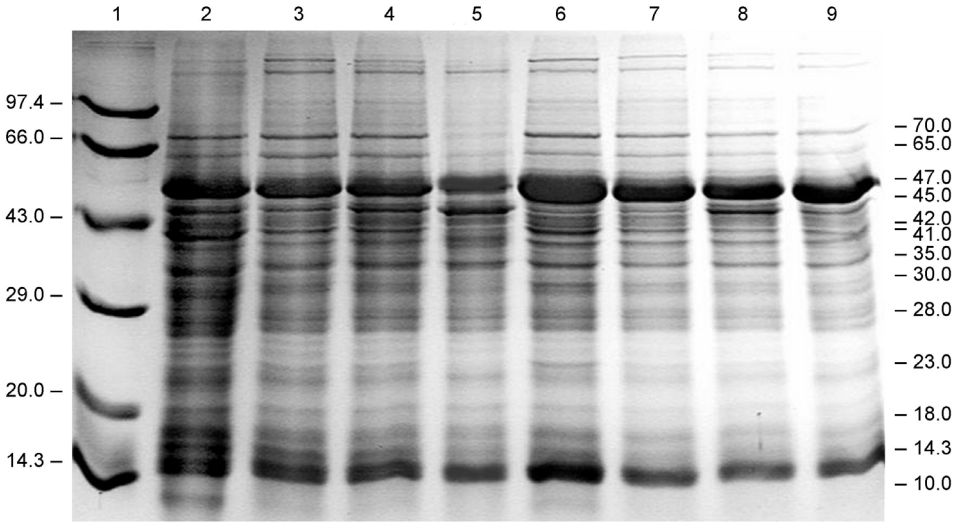

Fig. 1. SDS-PAGE protein profile of leaves from two genotypes of mulberry treated by different concentrations of NaCl. *Lane 1* - molecular mass marker; phosphorylase B - 97.4 kDa; bovine serum albumin - 66.0 kDa; ovalbumin - 43.0 kDa; carbonic anhydrase - 29.0 kDa; soybean trypsin inhibitor - 20.0 kDa; lysozyme - 14.3 kDa; l*ane 2* - cv. S1 control; *lane 3* - cv. S1 1.0 % NaCl; *lane 4 -:* cv. S1 1.5 % NaCl; *lane 5* - cv. S1 2.0 % NaCl; l*ane 6* - cv. ATP control; *lane 7* - cv. ATP 1.0 % NaCl; *lane 8* - cv. ATP 1.5 % NaCl; *lane 9* - cv. ATP 2.0 % NaCl.

(n = 5) were compared by Duncan's Multiple Range Test (DMRT) at 0.05 % level (Duncan 1955).

RWC was decreased and at higher NaCl concentrations the decrease was more significant. In S1 at 2.0 % NaCl the decrease of RWC was 42 % whereas in ATP 56 %. The extent of membrane damage was assessed by an indirect measurement of electrolyte leakage. The ion leakage increased with increasing NaCl concentrations. The increase was higher in the leaves of ATP than of S1, which means that S1 had better cell membrane stability (Table 1). The CMS technique is suitable for screening plant cultivars under high salinity and for detecting differences that may arise due to cumulative effects of salinity and reduced water content. Sairam *et al*. (2002) have noticed a salinity-induced reduction in CMS and relative water content (RWC) in genotypes with differing sensitivity. Similarly, in the present study we observed less reduction of RWC and membrane injury in tolerant cultivar S1 compared to susceptible cultivar ATP (Table 1). In support to our results, Sudhakar *et al*. (2001) reported a higher increase of membrane damage in sensitive cultivar than tolerant cultivar of mulberry under salinity stress.

The cell membrane plays an important role in maintaining cell integrity, being involved in signal transduction and ion homeostasis (Jyothsnakumari and Sudhakar 2003/4, Kumari *et al.* 2006). The signal induced by the membrane perturbation leads to expression of late embryogenesis abundant proteins (Hoekstra *et al*. 2001). Salinity stress induces the accumulation of a large number of stress proteins like LEA, dehydrins, Sal T, osmotin and others (Dure 1992, Singh *et al*. 1989). In the present study salt-treated plants showed the alteration of overall pattern of protein synthesis in both tolerant and sensitive cultivars. Salt stress induced the synthesis of few new polypeptides and enhances or represses synthesis of others. NaCl-treated plants showed the accumulation of 35, 41, 45 and 70 kDa proteins. The concentration of these proteins was higher in tolerant cultivar S1 than in sensitive cultivar ATP. In ATP, content of 45 kDa protein was higher under 1.5 % NaCl when compared to 1.0 and 2.0 % NaCl. The amount of 14.3, 18, 23, 28, 30, 35, 41, 42, 43, 47 and 65 kDa proteins decreased under salt stress compared to control in both cultivars, the decrease of 70 kDa protein was observed only in ATP (Fig. 1).

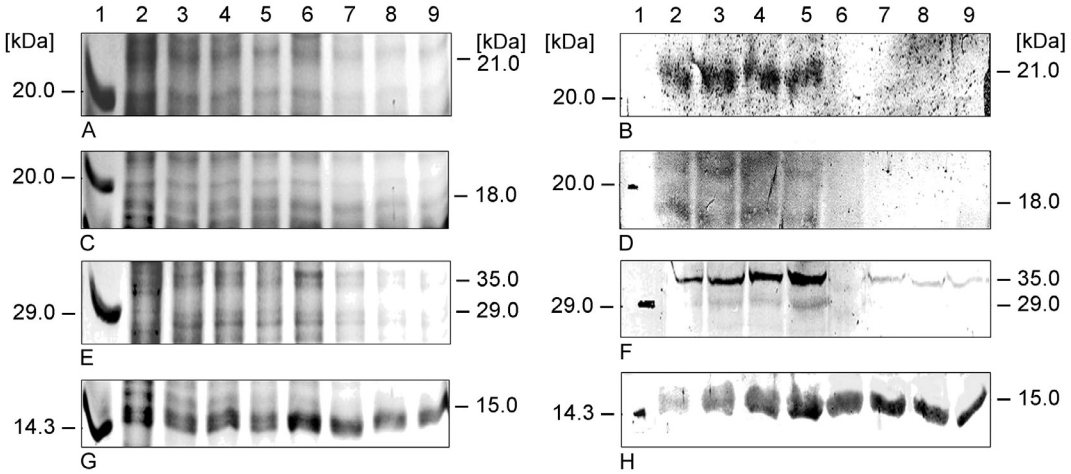

Fig. 2. Expression of LEA 1, 2, 3 and 4 proteins in leaves from two cultivars of mulberry exposed to different salinity stress. Equal amount of proteins (20 µg) was loaded on each lane separated by SDS-PAGE (*on the left*), electro transferred to PVDF membranes, probed with antibodies and immunologically related proteins were visualized using DAB (*on the right*). *A* - SDS portion showing the accumulation of 21 kDa protein; *B* - Immunoblot showing a cross reaction of anti-LEA 1 antibodies with mulberry leaf protein (21 kDa); *C* - SDS portion showing the accumulation of 18 kDa protein; *D* - Immunoblot showing a cross reaction of anti-LEA 2 antibodies with mulberry leaf protein (18 kDa); *E* - SDS portion showing the accumulation of 35 and 29 kDa proteins; *F* - Immunoblot showing a cross reaction of anti-LEA 3 antibodies with mulberry leaf proteins (35 and 29 kDa). *G* - SDS portion showing the accumulation of 15 kDa protein; *H.* - Immunoblot showing a cross reaction of anti-LEA 4 antibodies with mulberry leaf protein (15 kDa). *Lane 1* - molecular mass protein marker; l*ane 2* - cv. S1 control; *lane 3* - cv. S1 1.0 % NaCl; *lane 4* - cv. S1 1.5 % NaCl; *lane 5* - cv. S1 2.0 % NaCl; l*ane 6* - cv. ATP control; *lane 7* - cv. ATP 1.0 % NaCl; *lane 8* - cv. ATP 1.5 % NaCl; *lane 9* - cv. ATP 2.0 % NaCl.

Gel blot analysis indicated not significant expression of LEA proteins in S1 and no expression in ATP (Fig. 2*A*). Similarly we found no apparent cross-reaction of anti-group 2 LEA antibodies with the mulberry proteins isolated from both salt stressed S1 and ATP cultivars. However, there was a faint signal of cross reaction of antibodies with 18 kDa protein of salt stressed S1 as evi-denced from bands on the immunoblot and no cross reaction with proteins of ATP cultivar (Fig. 2*B*).

The expression of 35 and 29 kDa proteins was increased with salt treatments and the expression was highest at 2.0 % NaCl in S1. The expression of 29 kDa protein was found only in S1, whereas in ATP the expression of 35 kDa protein was relatively high at 1.0 and 1.5 % NaCl and it was declined at 2.0 % NaCl (Fig. 2*C*).

The cross-reaction of anti-group 4 LEA antibodies was specific with 15 kDa protein induced by NaCl treatments in both the cultivars. In S1, the expression of this protein was concentration dependent. In contrast, the expression of this protein was decreased with the NaCl concentration in ATP (Fig. 2*D*).

The salt responses of plants depends on the duration and concentration of NaCl (Tripathy *et al.* 2007). Many studies have reported a higher accumulation of soluble proteins in salt tolerant than in salt sensitive cultivars of barley (Hurkman *et al*. 1989), rice (Pareek *et al*. 1997) finger millet (Uma *et al*. 1995) and wheat (Majoul *et al*. 2000, Ouerghi *et al*. 2000). In concurrence with these results in our study, a higher accumulation of proteins (35, 41, 45 and 70 kDa) were noticed in salt tolerant S1 cultivar than salt sensitive ATP cultivar. Besides that the reduction of proteins (14.3, 18, 23, 28, 30, 35, 41, 42, 43, 47 and 65 kDa) was lesser in tolerant cultivar S1 than ATP susceptible cultivar. In conflict, Ashraf and O'Leary (1999) reported that the increase of total soluble proteins was higher in salt sensitive cultivar (Potohar), and low in a salt tolerant line (S24) of wheat.

In connection with the membrane perturbation and expression of LEA proteins under salinity, we checked the cross reactivity of anti group LEA 1, 2, 3 and 4 proteins with mulberry leaf proteins induced with or without salinity in both species. The expression of LEA 1 and 2 was observed only in S1 cultivar and was completely absent in ATP cultivar (Fig. 2*A*, 2*B*). Cheng *et al.* (2002) showed the expression of wheat LEA group 1 and 2 protein genes in two transgenic rice plants under salt stress. Similarly, Moons *et al*. (1997a,b,c) have identified group 2 LEA and DHN proteins in the roots of rice under salinity.

The genotypic variability in differential expression of *lea* genes and proteins in response to salinity stress was noticed by Jayaprakash *et al*. (1998) in finger millet (*Eleusine coracana*) and rice (*Oryza sativa* L.) seedlings. In analogy, in our present study the LEA 3 expression was found higher in both mulberry cultivars subjected to salinity stress treatments, besides there is a marked difference in the expression levels of LEA 3 in the two genotypes with higher level of expression in S1 than in ATP (Fig. 2*C*). Correspondingly, higher levels of LEA 3 proteins were observed in roots of salt tolerant rice genotypes than salt sensitive genotypes (Moons *et al.*

1995). Interestingly the levels of LEA 3 and 4 were decreased with increase of stress in sensitive cultivar ATP, whereas the level of LEA 4 protein was increased with severity of salinity treatments in tolerant cultivar S1. This observation would strengthen our results that the higher cell membrane injury in susceptible cultivar was due to the decreased expression levels of LEA 4, which are involved in the membrane protection (Fig. 2*C*, 2*D*).

In the present investigation we examined genotypic variability in membrane injury and RWC, protein pattern and expression of LEA 1, 2, 3 and 4 proteins under different salinity treatments in two mulberry genotypes. From these results we conclude that the lesser reduction in membrane injury and RWC in S1 cultivar when compared to ATP susceptible cultivar could perhaps be due to higher expression of LEA proteins (especially LEA 3 and 4) in S1 since these proteins are thought to play an important protective role during cell dehydration and membrane stabilization. Further, it might be suggested that LEA proteins acts as a special form of molecular chaperones to prevent the formation of damaging protein aggregates during stress.

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