

Full Length Research Paper

Sub-cellular distribution of two salt-induced peptides in roots of *Oryza sativa* L. var Nonabokra

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Accepted 23 April, 2009

Living systems respond to changing environment by changing gene expression. In addition to changes in the level of expression of existing proteins, some new proteins may be synthesized. Such proteins not only can serve as markers, but may also play important role in adjusting to that specific environment. Targeting of these proteins to specific location within or outside the cell may also yield important clues to their function. In such a pursuit, ten days old seedlings of a relatively salt tolerant rice variety grown on MS medium were treated with 2% sodium chloride for three days. Root were harvested, ground, and fractionated in to sub-cellular components. Microsomes were further fractionated into constituent membranes. Proteins from these fractions were analyzed by SDS-PAGE and/or two dimensional separations. Results show induction of two new polypeptides which were present in total protein extract and soluble fractions. All other fractions failed to reveal induction of any new peptide under experimental conditions used.

Key words: 2-D electrophoresis, fractionation, salt induced proteins, sub-cellular localization.

INTRODUCTION

Plants in their natural environment are subject to a number of environmental factors. Lack of ability to move away from adverse environment has resulted in evolution of different resistance/tolerance mechanisms against some such stresses while remaining vulnerable to others. Most widespread among a biotic stresses are drought and salinity, which constitute the most stringent factor in limiting plant distribution and productivity (Boyer, 1982).

High salinity leads not only to toxic Na⁺ effect but also decreases water availability to plant, thus mimicking the drought conditions (Majoul et al., 2000). Salinity has remained a subject of physiological investigation for considerable time, hardly yielding any reliable method for screening/improving salt tolerance in plants. These investigations have resulted in elucidating different cellular sites which undergo rapid changes due to exposure to high salt environment (Majoul et al., 2000; Ramani and

Afte, 1997).

In recent years, plant responses to salinity and other water deficit stresses such as drought have been investigated using proteomic/genomic based approaches (Wang et al., 2003; Yildiz, 2007). It is reported that salt stress responsive proteins are involved in regulation of carbohydrate, nitrogen and energy metabolism, reactive oxygen species scavenging, mRNA and protein processing, and cytoskeleton stability (Yan et al., 2005). Kav et al. (2004) suggested the possible existence of a novel signal transduction pathway involving superoxide dismutase (SOD), nucleoside diphosphate kinase (NDPK) and pathogenesis related (PR)10 protein with a potentially crucial role in a biotic stresses responses. Hashimoto et al. (2004) investigated stress response protein RO-292 from rice root which was induced specifically in roots when subjective to salinity and drought, showed high similarity to known PR10 protein.

Many of the identified polypeptides are induced in glycophytic plants. Being induced, these polypeptides are expected to play some role in mitigating the adverse effect of salinity. Determining the role of these proteins in salt tolerance is the next very important but difficult step.

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Table 1. Effect of different concentrations of NaCl on rice shoot.

Salt treatments (mM)	Length ¹ (cm)	Fresh weight (g)	Dry weight(g)	Dry weight ² %
0	17.80 ± 3.01 ^a	0.072 ± 0.01 ^a	0.015 ± 1.7 × 10 ⁻⁴ ^a	20.83
100	14.18 ± 1.146 ^b	0.060 ± 0.01 ^{ab}	0.015 ± 2 × 10 ⁻⁴ ^a	25.00
200	13.22 ± 1.70 ^b	0.051 ± 0.01 ^{bc}	0.013 ± 2 × 10 ⁻⁴ ^b	25.49
300	12.30 ± 1.311 ^b	0.043 ± 0.01 ^c	0.012 ± 1.7 × 10 ⁻⁴ ^c	27.91

¹: The length of all shoots per plant was first determined and then the mean and SD were calculated for 10 plants.

² (Dry weight)/ (fresh weight) × 100%.

A number of different approaches are being employed to obtain information about possible function of these proteins. These include study of the chemical nature, sequence homologies, conserved motif/domains, hydrophathy profiles, putative structure and sub-cellular distribution (Majoul et al., 2000; Kawasaki et al., 2001). Here, we report a pair of peptides, similar in molecular weight to the peptides previously reported in barley (Hurkman et al., 1988) and discuss its cellular distribution, a step towards understanding their possible function.

MATERIALS AND METHODS

Plant material

Seeds of relatively salt tolerant rice (*Oryza sativa* L) variety Nona-bokra were cultured on MS medium after surface sterilization with commercial bleach. Seeds were allowed to germinate for three days in dark and grown for seven days under 14 h / day, photoperiod at 26 ± 1°C.

Salt treatment

After 10 days, seedlings were shifted for 3 days into MS medium containing (0–300 mM) NaCl. Ten plants were harvested for each treatment for measuring shoot and root length, fresh and dry weights. For dry weight 10 shoots/roots were placed in 5 ml shell vials and lyophilized over night. Their weight was measured to the nearest 0.1 mg. The data were analyzed by ANOVA using Mini-tab.

Protein extraction

For protein analysis, seedlings similarly grown in 200 mM were harvested. Roots from salt treated and controlled plants were used for further studies. Total proteins were prepared from root tissues, ground under liquid nitrogen using the method of Damerval et al. (1986).

Cellular fractionation

For preparation of cellular fractionation, roots were excised into ice-cold homogenization buffer. Immediately before grinding medium was adjusted to 4 mM in β/ mercaptoethanol and polyvinyl pyrrolidone was added at a rate of 2 mg/g tissue. Homogenization was performed in a seven speed warring blender; 2 × 10 s at speed of 13250 rpm and 2 × 10 s at top speed of 2300 rpm with 10 s interval between two successive operations. Homogenates was filtered

through cheesecloth and residual material was considered to be crude cell wall fraction. Filtrate was centrifuged at 3000 × g for 5 min and pellet was designated as nuclear fraction. Supernatant was centrifuged at 13000 × g for 15 min. Pellet was considered to be crude mitochondrial fraction. Supernatant from this step was centrifuged at 8000 × g for 60 min. Supernatant contained soluble matrix while pellet consisting of microsomes was sub-fractionated on density gradient by the method of Hodges and Mills (1998), employing 20, 30, 34 and 45% sucrose (w/w) gradient at 95000 × g for 120 min in a swing-out rotor. Four bands were observed at different interfaces which were collected separately. Proteins from all fractions were precipitated by the method of Damerval et al. (1986) and estimated by the dye binding method (Bradford et al., 1976).

Protein separations

Proteins separation was performed by SDS-PAGE and/or by two dimensional electrophoresis (Naqvi et al., 1994). For SDS-PAGE, 10 µg protein was loaded per lane, while for two dimensional analyses, 150 µg protein was loaded per gel.

RESULTS

The effects of increasing concentrations of NaCl on length, fresh and dry weights of shoots and roots were measured to determine an appropriate concentration of NaCl to be used for protein analysis study. Shoot length, fresh weight and dry weight significantly decreased with increasing NaCl concentrations. Shoot length was similarly affected in all salt treatments, while fresh weight decreased gradually with increase in NaCl concentration. Dry weight remained unaffected at 100 mM salt but declined with higher concentration of salt. Nevertheless decrease in dry and fresh weight correlated in such a way that changes in dry weight as a percent of fresh weight become insignificant. Changes in length and fresh weight of roots were statistically insignificant under these experimental conditions. However, Dry weight, as percent of fresh weight showed significant differences within increasing NaCl concentrations.

The data in Tables 1 and 2 indicated that NaCl concentration greater than 200 mM caused substantial changes in rice plants. Therefore experiments for protein profiling were done using plants treated with 200 mM NaCl.

Protein were extracted from crude cellular fractions and separated by SDS-PAGE. Results are shown in Figure 1.

Table 2. Effect of different concentrations of NaCl on rice root.

Salt treatments (mM)	Length ¹ (cm)	Fresh weight (g)	Dry weight(g)	Dry weight ² %
0	12.67 ± 2.575	0.058 ± 0.010	0.007 ± 9.1x10 ⁻⁴	12.06
100	12.02 ± 1.16	0.050 ± 0.009	0.008 ± 9.1x10 ⁻⁴	16.00
200	11.35 ± 0.59	0.050 ± 0.009	0.008 ± 9.1x10 ⁻⁴	16.00
300	9.70 ± 1.473	0.045 ± 0.011	0.008 ± 9.5x10 ⁻⁴	17.78

¹: The length of all roots per plant was first determined and then the mean and SD were calculated for 10 plants.

²: (Dry weight)/(fresh weight) x 100%.

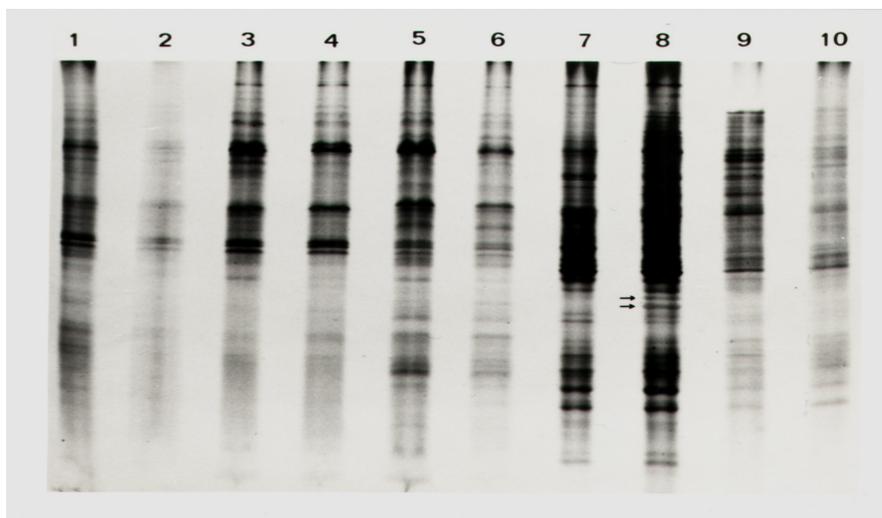


Figure 1. SDS-PAGE pattern of protein extracted from cellular fraction of root. Each pair of lane shows preparation from control and treated fractions respectively. Lanes 1, 2 represent cell wall proteins, lanes 3, 4 nuclear proteins, lanes 5, 6 mitochondrial proteins, lanes 7, 8 cytosolic proteins and lanes 9, 10 microsomal proteins. Two new peptide bands, arrow marked can be seen in lane 8.

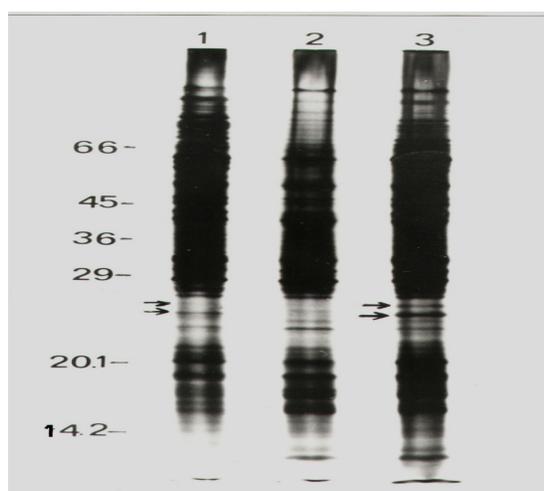


Figure 2. SDS-PAGE of whole root proteins from salt stressed tissue (lane 1), cytosolic proteins from control (lane 2) and cytosolic protein from salt stressed roots (lane 3). Stress proteins can be seen arrow marked in lanes 1 and 3.

Comparison between control and salt treated samples revealed no induction in any of the fraction except in cytosol, where two new bands of low molecular weight appeared in sample prepared from salt stressed roots. This indicates the peptides synthesized under salt stress are located in soluble matrix of the root cells.

Total proteins extracted from salt stressed roots were also run in parallels with soluble fraction protein extract from control and stressed tissues (Figure 2). Whole protein extract from stressed roots is shown in lane 1, while lanes 2 and 3 represent cytosolic proteins from control and stressed roots. Couplets of peptides arrow marked in lanes 1 and 3 are missing in lane 2. The separation of cytosolic proteins by two-dimensional SDS-PAGE confirmed the presence of two peptides of apparently low molecular weight. These peptides are arrow marked in Figure 3.

Microsomal membranes were sub-fractionated into endoplasmic reticulum (ER), tonoplast/golgi (TG) and plasma membrane (PM) components using the method of Hodges and Mills (1998). After centrifugation four bands

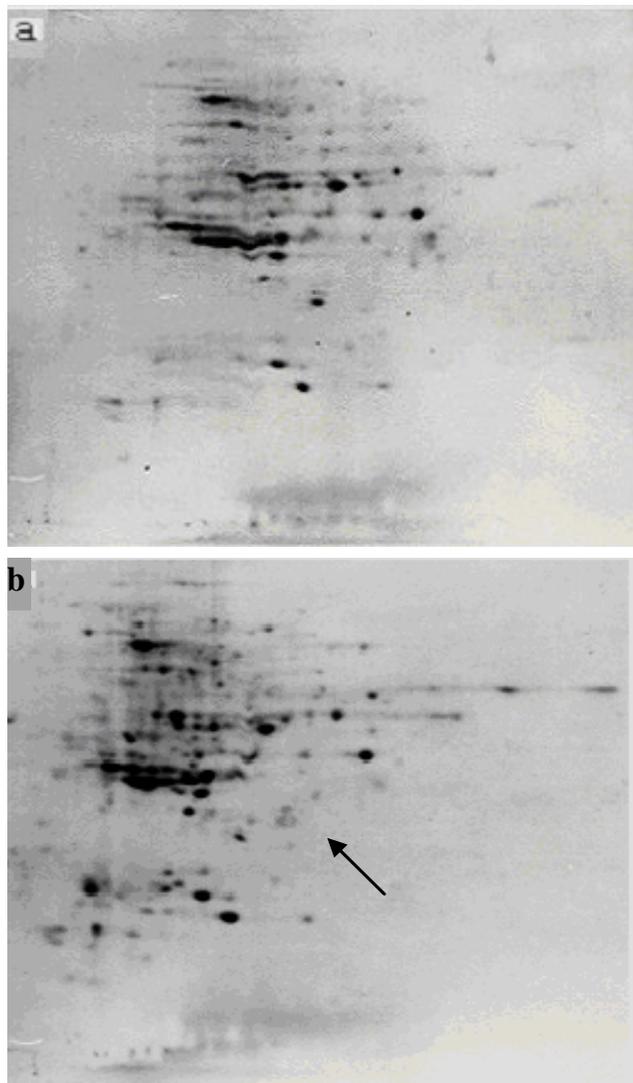


Figure 3. Two dimensional electrophoresis analyses of root cytosolic proteins from control (a) and from salt stressed tissue (b), Newly synthesized peptides are marked by arrows.

were obtained, first appearing at the sample/20% sucrose interface representing ER, second at 20/30% interface that contains mainly TG, third at 30/34% sucrose interface representing a mixture of TG and PM (discarded) and the fourth at 34/45% sucrose interface reporting mainly yields, PM.

Results of protein separation by two dimensional analyses from different microsomal fractions are shown in Figure 4. In all the Figures (1 to 4), bands/spot marked by arrow indicate the 27 and 25.5 kd peptides resulting from salt treatments.

DISCUSSION

Growth of glycophytes in saline environment is severely

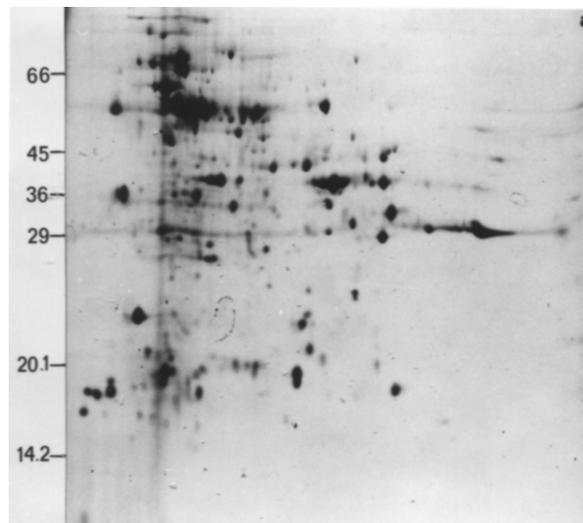


Figure 4. Two-dimensional separation of membrane fractions protein from salt stressed roots. Panel a, b and c are protein extracts from ER, TG and PM fractions. There are no spots corresponding to induced peptides arrow marked in Figure 3(b).

affected by reduction in metabolic activity due to elevated concentration of cytoplasmic NaCl (Flower, 1991). Within the plant cell there are various compartments, to which ions may be confined by one means or another. This discontinuous distribution between compartments may explain differences in plant responses to salinity at the same average tissue concentration of salt (Flower et al., 1991). It is therefore important to study the distribution of cellular protein in different compartment when studying mechanism operational during exposure to saline environment. Results of this study show that during short-term exposure, there is least, if any, effect on roots. However, the fact that shoot was severely affected, lies in transport of salt with sap to shoot, where a lot of water is transpired depositing the salt in the shoots.

Elevated levels of salts beyond species specific barriers become stressing factor. Living systems have evolved mechanisms to try to cope with such environmental onslaughts. As all these adverse conditions are not a generalized phenomenon, defense mechanisms are not constitutive. Thus qualitative changes in protein synthesis during exposure to a stress are widely considered as components of stress tolerant mechanisms (Ozalp et al., 2000).

Identification of induced proteins necessitates exploring their function, which is not an easy task. Transport of ions to various compartments is a function of bio-membranes; hence peptides induced during salinity may play some role in transport across these barriers by acting as components of ion exchange pumps/channels embedded in these membranes. Finding sub-cellular localization is therefore an important aspect. During this study an effort was made to track down the sub-cellular localization of

two salt induced peptides (25.5 and 27 kd) from a salt tolerant rice cultivar. The results show the presence of these peptides of rice root origin predominantly in the soluble fraction. These peptides are either a member of soluble protein family from the cytosol, extracellular matrix or of the peripheral proteins loosely associated with some membrane.

Barely, another cereal has also been extensively studied from the view point of induced proteins (Hurkman and Tanaka, 1987). These investigations have resulted in identification of two pairs of peptide with molecular ratio of 26 and 27 kd and pI 6.3 and 6.5 and are located in TG, PM and 40% of the total in soluble fraction (Hurkman et al., 1988). Proteins reported in barely are nearly similar in molecular weight as reported by Naqvi et al. (1995) in rice but differ in pI value and localization. Further, rice root proteins are synthesized *de novo* during salt stress while barely protein are synthesized constitutively (Hurkman et al., 1988). Interestingly, peptides similar to the ones being reported and found in cereals are apparently missing in protein profile of halotolerant algae *Dunaliella salina* (Katz et al., 2009), a, which might indicate that these peptides originated after the evolution of higher plants root system. Further, these preliminary investigations in cereal studied so far, indicate that these crops may have evolved similar strategies to cope with adverse changes in their ionic environment.

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