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Full Length Article

Cross Tolerance Mechanisms of Osmotic and Ionic Stress Adapted Cell Lines of Rice Towards Salinity

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Abstract

Present study investigated the cross tolerance mechanisms of osmotic [25% poly ethylene glycol (PEG)] and ionic (25 mM LiCl) stress tolerant cell lines of rice (*Oryza sativa* L.) cv. Swat-1. The adapted lines showed tolerance up to six generation on stress free medium. When adapted and un-adapted cell lines were subjected to 200 mM NaCl, there was 98% reduction in relative growth rate (RGR) of un-adapted line and the adapted (PEG and LiCl) cell lines showed significantly higher tolerance. Albeit there were no qualitative differences among adapted and un-adapted cell lines under salt stress. However, responding mechanisms of adapted and un-adapted cell lines to osmotic and ionic homeostasis to tolerate NaCl stress were highly different. PEG adapted cells line appeared to acquire halophytic behavior of a salt accumulator, while LiCl adapted line performed like salt tolerant glycophyte. The tolerance strategies of adapted lines were not confined to the same type of stress but showed cross tolerance towards other abiotic stresses. © 2015 Friends Science Publishers

Keywords: Cross tolerance; Osmotic and ionic stress; Rice

Introduction

Salinity is one of the major abiotic stresses, which limit plant performance and production all over the world, while sodium chloride is the most abundant source of salinity in soil. About 7% of the world's land surface and 5% of the cultivated land is salt-affected. Almost 20% of irrigated land has suffered from secondary salinization i.e., poor quality of irrigation and deicing salts from roads etc. (Wainwright, 1984; Flowers and Yeo, 1995; Ghassemi *et al*., 1995). Salinization is a key issue in agriculture of Pakistan with about quarter of the salinized irrigated land and area increasing annually due to poor irrigation and drainage system (Ahmad, 1990; Ghassemi *et al*., 1995; Shah, 2009).

There are two possible strategies to use salt effected lands and brackish water. One is reclamation of salts by improved drainage system and by the application of gypsum, which is highly expensive. Biological approach involves growing of natural halophytes with a commercial potential, or to induce salt tolerance, using conventional breeding and biotechnological methodologies to improve salinity tolerance in existing crop plants (Wainwright, 1984; Saleh and Mafton, 2008). For exploitation of biological strategy to develop salt-tolerant crop species comprehensive knowledge of mechanism(s) of salt tolerance in different species is basic requirement (Zhu, 2002; Xu *et al.*, 2014).

Salinity exerts manifold and different types of osmotic, toxic and ionic imbalance effects to plant/cell at different stages of growth and development (e.g. germination, seedling, flowering etc.), while severity and intensity of stress also varies according to the growth stage. Salinity also interact with environmental factors such as, humidity, temperature, light, irrigation and soil fertility which alter effect of salinity making it more complicated (Nilsen and Orcutt, 1996; Shah *et al.,* 2012). Thus tolerant plant/cell must have the ability to cope with complex, manifold and intricate phenomenon of stress, which obviously requires an array of mechanisms. Therefore, despite of several attempts little commercial success has been achieved in development of salt tolerant crops all over the world. For success of biological strategy, the proposed logical approach is to dissect the complexity of the stress into components parts and complexity of tolerance mechanism into determinants of tolerance (Flowers and Yeo, 1995).

Tissue culture technology simplifies the complexity of salt stress by growing plant/cell in *in vitro* to minimize interaction with environment and by growing/selecting cell lines/calli lines, which bypasses differentiation and structural integrity of plants (Hasegawa *et al.,* 1994). The only possible option to dissect the stress tolerance mechanism into component part is to distinguish toxicity component of stress from osmotic and ionic component of salt stress i.e., tolerance to osmotic, toxic and ionic

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imbalance by exploitation of phenomenon of cross tolerance (Munns, 1993; Shah *et al*., 1993). Cross tolerance refers to the phenomenon where tolerance to a highly toxic alkali metal (LiCl) at low concentration confers tolerance to less toxic alkali metal (NaCl), at high concentration. The study of cross tolerance mechanism can improve our efficiency to target the genes that affect tolerance in plants (Shah *et al*., 2002). With this prospective an attempt has been made to investigate the cross-adaptation mechanism for salinity tolerance in adapted and unadapted cell lines of *Oryza sativa* .cv. swat-1.

Materials and Methods

Rice seeds were collected from Agricultural Research Station North (Swat). The research was conducted at the Institute of Biotechnology and Genetic Engineering (IBGE), NWFP, Agricultural University Peshawar. The design of the experiment was completely randomized design (CRD) with three calli/cell lines (unadapted, 25% PEG adapted and 25mM LiCl adapted) using five replications. Calli were induced from mature seed of rice (*Oryza sativa* L.) cv. Swat-1, using Murashige and Skoog (MS) medium (1962) supplemented with 2 mg L^{-1}, 2, 4-D, 0.25 mg kinetin, 2 g casein hydrolysate, 30 g sucrose and pH was adjusted to 5.8 and solidified with 9 g^{-1} agar. All the cultures were incubated in the dark at 27 ± 2°C.

Selection Procedure

A multi-step procedure (Shah *et al*., 2002) was used to raise adapted lines. Cell lines were subjected to an incremental increase of PEG and LiCl stresses. The sequence of increasing PEG and LiCl concentrations were 5% PEG and 5 mM LiCl (3 Passages), 10% PEG and 10 mM LiCl (8 passages), 15% PEG and 15 mM LiCl (12 Passages), 20% PEG and 20 mM LiCl for 15 passages and 25% PEG and 25 mM LiCl for 20 passages. Concurrently, control lines were maintained in absence of PEG and LiCl.

Determination of Proline and Inorganic Ions

The tissue mass from each flask was collected as a single callus culture. Care was taken to remove the solid media particles including the callus cells adhering to medium from base of callus tissue. The callus growth was estimated on fresh weight basis, and the fresh sample was fractionated into two parts for proline estimation and organic ions measurement. Proline was determined by the method of Bates *et al*. (1973). For which weighed calli was homogenized in 3% sulphosalicylic acid. Homogenate was filtered through Whatman No. 2 filter paper. The filtrate was reacted with 2 cm^3 acid ninhydrin in a test tube in boiling water bath for one hour. Reaction was terminated in an ice bath. Reaction mixture was extracted with 4 cm^3 toluene. Tubes were cool down to room temperature. Absorbance was measured at 520 nm against a toluene blank. While other inorganic ions (Na^+, K^+ and Ca^{2+}) were determined by the method of Hodson *et al.* (1981). Calli were oven dried to constant weight in boiling tubes. 10 mL of nitric acid was poured in boiling tubes. Then the tubes were heated in a sand bath for overnight, till the volume reduced to 2.5 mL. After cooling, the volume of extract was made up to 25 mL with double distilled water (10% nitric acid). The standards were prepared for atomic absorption Spectrometer Analyst 700 for Na^+, K^+ and Ca^{2+}.

Measurement of Growth

Growth of calli was measured by method of Shah *et al*. (1990). Pre-weighed wide necked conical flask containing 30 cm^3 of culture medium was inoculated with similar quantities of callus, and the inoculated flask was re-weighed to obtain the initial fresh weight of callus inoculums. The culture was incubated at 25°C for 28 d in dark. The relative growth rate (RGR) of the callus was calculated as:

$$RGR/Week = [\ln(\text{final weight}) - \ln(\text{initial weight})]/4$$

Statistical Analysis

For analysis of variance, "Analysis ToolPak" of MS Excel and Statistix 8.1 were used.

Results

Relative Growth Rate (RGR)

The analysis of variance showed highly significant effects of stress on cell lines and their interaction (>0.05). At respective medium, no significant difference was found in RGR of adapted and unadapted calli lines. But, when these lines were subjected to 200 mM NaCl stress RGR of unadapted lines reduced significantly (i.e. 98%). On the other hand reduction in RGR in PEG and LiCl adapted line against 200 mM NaCl stress was non-significant (i.e. 4 and 5% respectively) (Fig. 1).

Sodium (Na^+) Ions

Analysis of variance showed overall significant effect (>0.05) of NaCl stress on increase in Na^+ contents and significant difference in response of both calli. The Na^+ contents from dry matter of unadapted and adapted calli lines were almost similar at their respective medium (Fig. 2). At 200 mM of NaCl stress, contents of Na^+ increased significantly in unadapted and PEG adapted calli lines compared to their respective medium and LiCl adapted cell line. But there was non-significant increase in Na^+ contents of LiCl adapted cell line.

Potassium Ion (K^+)

Analysis of variance showed a significant effect (>0.05) of NaCl stress on K^+ accumulation and significant difference

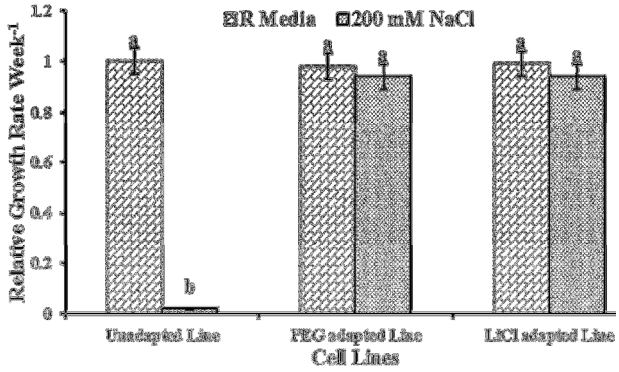

Fig. 1: The effect of NaCl on RGR of unadapted, PEG and LiCl adapted cell lines of *Oryza sativa* L. cv. Swat-1. The bars represent mean values ± SE

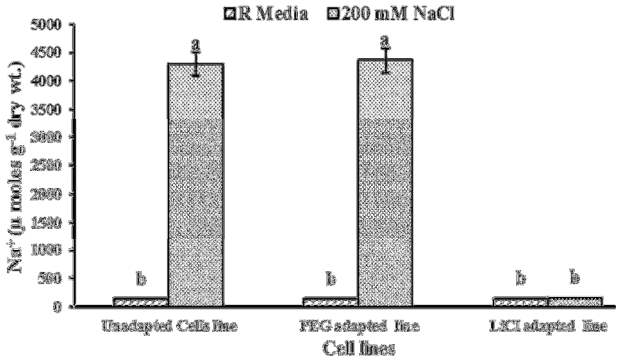

Fig. 2: The effect of NaCl on Na^+ contents of unadapted, PEG and LiCl adapted cell lines of *Oryza sativa* L. cv. Swat-. The bars represent mean values ± SE

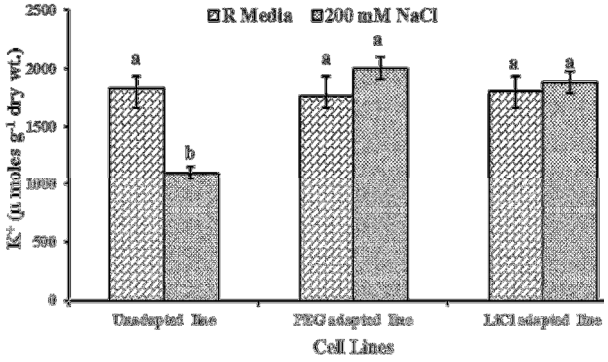

Fig. 3: The effect of NaCl on K^+ contents of unadapted, PEG and LiCl adapted cell lines of *Oryza sativa* L. cv. Swat-1. The bars represent mean values ± SE

in response of calli lines. At respective medium, K^+ contents of adapted and unadapted line were almost similar (Fig. 3). When these lines were exposed to 200 mM NaCl stress K^+ contents of unadapted line decreased significantly and in PEG adapted line an increase was observed. On the other hand LiCl adapted line maintained its K^+ contents at 200 mM NaCl stress.

Calcium (Ca^{2+}) Ions

The analysis of variance showed significant effect of stress on lines and their interaction (>0.05). PEG and LiCl adapted lines had significantly higher content of Ca^{2+} at their respective medium than unadapted one (Fig. 4). No change was observed in Ca^{2+} contents of un-adapted lines on exposure to NaCl stress. On the other hand, a significant increase in concentration of Ca^{2+} was noted in PEG and LiCl adapted lines at 200 mM NaCl stress.

Proline Contents

The NaCl effect on proline content was highly significant for cell lines and their interaction (>0.05). PEG adapted lines accumulated significantly higher amount of proline than un-adapted and LiCl adapted cell lines at their respective medium (Fig. 5). At 200 mM NaCl stress, there was a significant increase in proline level of PEG and LiCl adapted lines and a significant decrease in proline level of un-adapted cell line was observed.

Discussion

Understanding the fundamentals how the effects and response of osmotic and ionic stress differ from each other is essential for development of salt tolerant plants. In the present study we have developed cell lines of *Oryza sativa* L. cv. Swat-1 tolerant to osmotic (PEG) and ionic (LiCl) component of stress and successfully distinguished the characters related with osmotic tolerance from ion toxicity. As PEG has no ionic and LiCl possess no osmotic component of stress, therefore difference in adaptation to PEG and LiCl involved to cope with osmotic and ion specific toxicity stresses respectively. Moreover, the cross tolerance mechanism(s) of these lines towards NaCl stress were investigated, because adaptation to one specific stress allows plants/cells to adapt to a range of different stresses. The study was conducted at the cellular level, therefore, growth was considered as the best parameter to evaluate the tolerance of adapted lines (Bowler and Fluhr, 2000).

Our results showed that compared to respective medium, significant reduction in growth was observed in un-adapted callus/cell line on exposure to 200 mM NaCl stress and reduced to 98%. However, reduction at 200 mM NaCl stress in PEG and LiCl adapted line was only 4 and 5% respectively. High concentration of NaCl stress inhibits growth by disturbing normal metabolic processes of cells, leading to visible injuries and physiological disorder as found in present study and in *E. maculata* and *E. urophylla* after addition of 400-1600 mM $ZnSO_4$ for 5 weeks (Luo *et al.,* 2010; Tesoney and Lidon, 2012). Though growth of plants/cells is inhibited by majority of stresses but each species and even each variety of plants have specific

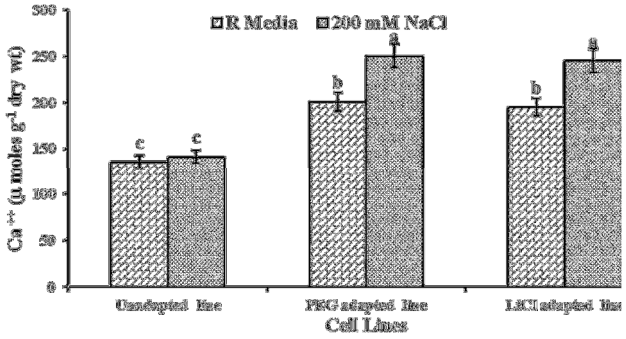

Fig. 4: The effect of NaCl on Ca^{2+} contents of unadapted, PEG and LiCl adapted cell lines of *Oryza sativa* L. cv. Swat-1. The bars represent mean values ± SE

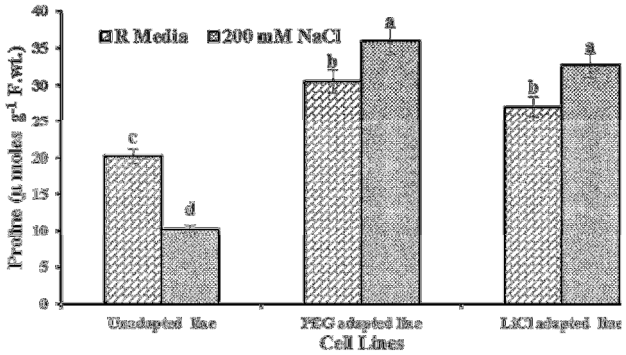

Fig. 5: The effect of NaCl on proline contents of unadapted, PEG and LiCl adapted cell lines of *Oryza sativa* L. cv. Swat-1. The bars represent mean values ± SE

limits for tolerance against stress (Broadley *et al.,* 2007). Our results showed that PEG and LiCl adapted lines are significantly more tolerant to NaCl stress than unadapted line. Alexieva *et al.* (2003) reported that; a mild treatment of short duration with one stress agent may counteract the toxic effects of succeeding stress possibly because of induction of defensive mechanism (s) by earlier stress which increased resistance against the later one.

Significantly higher contents of Na^+ were accumulated in un-adapted and PEG adapted lines at 200 mM NaCl stress as compared to respective medium. Despite of considerably higher accumulation of Na^+ contents, higher RGR of PEG adapted line than unadapted one revealed that adaptive mechanism(s) acquired by PEG adapted line seems to be effective compartmentalization/sequestration of toxic ions into vacuoles, away from metabolically active site, which otherwise had caused 98% reduction in RGR of unadapted line. Mathys (1977) reported that tolerance against excessive zinc could be the result of increased ability of transporting Zn into the vacuole. Stoyanova and Doncheva (2002) proposed more efficient transport of Zn into the vacuole by malate shuttle. On the other hand, LiCl adapted lines maintained significantly lower levels of Na^+ than un-adapted and PEG adapted lines, indicating the

Table 1: Analyses of variance for relative growth rates (RGR), sodium (Na^+), potassium (K^+), calcium (Ca^{2+}) and proline contents of adapted and un-adapted cell lines of *Oryza sativa* L. cv. Swat-1

Parameters	Source	DF	MS	P
	Replication	4	0.00873	
	Cell lines	2	0.26912	0.000
RGR	Treatment	1	1.56820	0.0000
	Cell Lines × Treatment	2	0.17428	0.0000
	Replication	4	35130.5	
	Cell lines	2	1.47800	0.0000
Na^+	Treatment	1	5.93900	0.0000
	Cell Lines × Treatment	2	1.49000	0.0000
	Replication	4	345270	
	Cell lines	2	549390	0.0013
K^+	Treatment	1	278604	0.0362
	Cell Lines × Treatment	2	830524	0.0004
	Replication	4	1058.80	
	Cell lines	2	25123.9	0.0038
Ca^{2+}	Treatment	1	7654.70	0.0157
	Cell Lines × Treatment	2	1430.90	0.2670
	Replication	4	11.3160	
	Cell lines	2	987.357	0.0000
Proline	Treatment	1	13.8720	0.3722
	Cell Lines × Treatment	2	264.717	0.0004

DF = degree of freedom, MS = Mean square, P = Probability

mechanisms to restrict the Na^+ ions at plasmalemma. This could be the result of unusual adaptation to LiCl, as Li is a conventional blocker of K^+ channels (Demidchik and Maathuis, 2007).

Overall, LiCl adapted line had significantly less K^+ contents as compared to unadapted and PEG adapted lines. On exposure to 200 mM NaCl stress, LiCl adapted lines maintained their K^+ contents and a significant increase was observed in K^+ contents of PEG adapted line. On contrary, there was a significant decrease in K^+ concentration of unadapted line at 200 mM NaCl stress. These results showed that accumulation/ increase (as in PEG adapted line) and maintenance (as LiCl adapted line) of K^+ concentration in adapted lines at NaCl stress is directly proportional to relative growth rates that seems to be the adaptive strategy of these lines and K^+ play a most important role in osmotic adjustment of ions in the cell (Hasegawa *et al*., 2000; Shah *et al*., 2002).

Calcium contents were significantly higher in adapted line at their respective media as compared to unadapted line. When these lines were subjected to 200 mM NaCl stress unadapted lines maintained their Ca^{2+} contents, while adapted lines showed significant increase in Ca^{2+} contents compared to respective medium. This may be the adaptive strategy of adapted lines against NaCl stress. Legge *et al.* (1982) reported that under stress, concentration of calcium in cell increases to maintain integrity and stability of membranes by bridging carboxylate and phosphate groups of phospholipids and proteins at the surface of membrane. Generally, it is accepted that by making complexes with the polysaccharides of matrix Ca^{2+} add to rigidity of cell wall in plants. Calcium also play role as secondary messenger as it

pairs a wide range of extracellular stimuli to intracellular responses (Snedden and Fromm, 1998).

At respective media the proline contents of PEG and LiCl adapted cell lines were significantly higher than unadapted cell line. At 200 mM NaCl stress the proline contents of both adapted (PEG and LiCl) lines further increased by contrast proline level of unadapted line decreased considerably representing probable death of callus/cells. The variation in rate of proline accumulation in PEG and LiCl adapted callus/cell with respect to NaCl stress indicated that accumulation and physiological role of proline varies according to the type of defensive mechanisms. Increased production and accumulation of proline is one of the plant's strategies for tolerance against abiotic stress especially for drought and salt stress (Siripornadulsil *et al.,* 2002). Because accumulation of proline in cell provides help to chelate metal ions, sustain the structural integrity of cytoplasmic proteins, maintain pH of cytosole, NAD(P) +/NAD(P)H ratio, protects enzymes from denaturation and also serves as nitrogen and carbon source (Siripornadulsil *et al.,* 2002; D'souza and Devaraj, 2012).

Higher growth rate of PEG adapted line at the highest tissue Na^+ contents with increased proline level is a halophytic behavior. Martinez *et al.* (2005) reported that in salt selected cell cultures of *Brassica napus, B. juncea, Vigna radiata* and *Arabidopsis thaliana,* tolerance to NaCl selected cell lines was considered as a general shift towards halophytic characteristics. On the other hand, restricted uptake of Na^+ and maintenance of K^+ level with enhanced growth under NaCl stress in LiCl adapted line could be the result of enhanced capacity of membranes for ionic discrimination like salt tolerance in glycophytes observed in NaCl tolerant cell lines of citrus, tobacco *Medicago sativa*, rice and *M. sativa* (Ben-Hayyim and Kochba, 1983; Shah *et al*., 1993), where the cells maintain a lower amount of Na^+ content under saline conditions.

As cell lines were developed precisely tolerant to osmotic and ionic stresses, PEG stress has no ionic and LiCl no osmotic component of stress. Therefore, induction of regulatory mechanisms under osmotic stress (PEG adaptation) and ionic toxicity stress (LiCl adaptation) empowered the cells to deal with toxic and osmotic components of salt stress rapidly and effectively. It is concluded that PEG adapted and LiCl adapted lines had to cope with single aspect of NaCl stress either toxicity or osmotic, while tolerance for associated stress was already present, resultantly adapted lines showed more tolerance and less reduction (4 and 5%) in the relative growth rate. In contrast unadapted line had to face both components (osmotic + toxic) of NaCl stress simultaneously that ultimately resulted in 98% reduction of relative growth rate. These results showed the effectiveness of cross tolerance mechanisms for salt tolerance that works in both directions i.e., cells/callus line adapted to toxicity component (LiCl) confers tolerance to osmotic components of stress and cells line adapted to osmotic components (PEG) reveals tolerance to toxicity component of stress exerted by NaCl. Based on our findings it can be concluded that though rice cell lines adapted to osmotic and ionic stresses showed parallel tolerance to 200 mM NaCl stress however physiological mechanisms of tolerance were greatly different. Thus present study helped in understanding the underlying mechanism(s) of cross tolerance in plants and the evidence engendered offer the basis for developing strategies for salinity and other abiotic (heat, cold and drought) stress tolerance in crops

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